Behavioural Pharmacology Of Novel Kappa Opioid Compounds

By

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CONTENTS

Abstract

Rationale Kappa opioid receptor (KOPr) activation by traditional agonists has been shown to produce anti-addiction behaviours. However, adverse effects such as sedation, aversion and depression have limited their clinical development. Recently, salvinorin A (Sal A), an active component of the plant *Salvia divinorum* was shown to be a potent and selective KOPr agonist. Sal A has a short duration of effect and quick onset of action. It also produces similar behavioural pharmacology to traditional KOPr agonists. However, little is known about the anti-addiction profile of Sal A. If Sal A and its structural analogues produce anti-addiction properties with fewer adverse effects compared to traditional KOPr agonists, they have potential to be developed into anti-addiction pharmacotherapies. Therefore, Sal A and its structural analogues (DS1, MOM Sal B, EOM Sal B, herkinorin) and Mu opioid receptor (MOPr) antagonist/partial KOPr agonist, nalmefene were tested for their behavioural anti-addiction and adverse effect profiles in rats.

Methods To test the anti-addiction profile, a within session cocaine prime induced reinstatement paradigm was used. The selectivity of KOPr agonists in attenuating cocaine seeking behaviours was tested using sucrose reinforcement (anhedonia) and cocaine induced hyperactivity in self-administering rats (sedation during reinstatement test). Furthermore, behavioural adverse effects were screened using spontaneous open field activity (motor suppression), conditioned taste aversion (aversion) and forced swim test (depression) in rats. To further quantify the anti-addiction behaviours, the effect of KOPr agonists which attenuated drug seeking selectively without producing motor suppression by themselves were tested for cocaine produced motor function (hyperactivity and behavioural sensitization) in rats. The effect of serotonin transporter blockade on KOPr agonist induced depressive behaviour was also tested. The effects of KOPr activation on *in vitro* serotonin transporter function were also determined.

Results Sal A, DS1 and nalmefene attenuated cocaine prime induced drug-seeking, in a selective manner, via KOPr activation. MOM Sal B, a more potent and long acting Sal A analogue attenuated cocaine seeking in a non-selective manner. Sal A, DS1 and nalmefene did not induce aversion, however nalmefene suppressed motor function, which was not seen with Sal A and DS1. Furthermore, Sal A and DS1 suppressed cocaine behavioural sensitization. All three compounds (Sal A, DS1, nalmefene) produced depression. The depressive effects produced by Sal A and DS1 were diminished by blocking the serotonin transporter. Live-cell serotonin transporter assays showed potential differences between traditional (U50488H) and novel (Sal A, DS1) KOPr agonists in their ability to modulate serotonin transporter function.

Conclusion Out of six KOPr compounds tested, Sal A, DS1, MOM Sal B and nalmefene produced anti-addiction behaviours. However, MOM Sal B exposure also suppressed natural reward seeking behaviour. Sal A and DS1 had a better adverse effect profile than nalmefene. Thus, the order of efficacy for the compounds tested were DS1 ≥ Sal A > nalmefene > MOM Sal B. However depression was noted with all three compounds tested (Sal A, DS1, nalmefene) and our study provides evidence to suggest the involvement of the serotonin system in Sal A and DS1 induced depression. Moreover, a difference in modulation of serotonin transporter function by novel and traditional KOPr agonists was observed.

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List of abbreviations

5HT	- serotonin
ASP^+	- 4-(4-(dimethylamino)-styryl)-N-methylpyridinium
cAMP	- cyclic adenosine mono-phosphate
CPP	- conditioned place preference.
CPU	- caudate putamen
CREB	- cyclic AMP response element binding protein
CTA	- conditioned taste aversion
DA	- dopamine
DAT	- dopamine transporter
DS1	- 2-mesylate salvinorin B
DYN	- dynorphin
EOM Sal B	- ethoxymethyl salvinorin B
FLX	- fluoxetine
FR-1	- fixed ratio 1 schedule of reinforcement
FR-5	- fixed ratio 5 schedule of reinforcement
FST	- forced swim test
GFP-hSERT	- green fluorescence protein tagged human serotonin transporter.
HEK-293	- human embryonic kidney cells
ICSS	- intracranial self-stimulation
icv	- intracerebroventricular
ip	- intraperitoneal
iv	- intraveneous
KOPr	- Kappa opioid receptors
KRH	- Krebs Ringer Herpes buffer
MDS	- mesocorticolimbic dopamine system
MOM Sal B	- methoxy-methyl salvinorin B
MOPr	- Mu opioid receptors
myc-rKOPr	- myc tagged rat kappa opioid receptor.
NAc	- nucleus accumbens
NET	- nor-epinephrine transporter
Nor-BNI	- norbinaltorphimine
PFC	- pre-frontal cortex
RDEV	- rotating disc electrode voltammetry
Sal A	- Salvinorin A
Sal B	- salvinorin B
sc	- subcutaneous
SERT	- serotonin transporter
SSRI	- selective serotonin reuptake inhibitor
VTA	- ventral tegmental area

Chapter 1. General Introduction

Addiction is defined as "compulsive physiological need for and use of a habit-forming substance (as heroin, nicotine, or alcohol) characterized by tolerance and by well-defined physiological symptoms upon withdrawal; *broadly* **:** persistent compulsive use of a substance known by the user to be physically, psychologically, or socially harmful" (addiction. 2011. In *Merriam-Webster.com*). Drugs of abuse, such as psychostimulants produce a general sense of euphoria or hedonism which determines their abuse potential (Eddy et al., 1965; Koob and Le Moal, 1997). Initial exposures to these compounds produce strong reinforcing properties which have been characterised as a sense of well-being (Koob and Bloom, 1988; Gawin, 1991). The reinforcing properties change the complexion of normal brain function which is evident by the alteration in normal brain anatomy, biochemistry and underlying molecular and cell biology (Verdejo-Garcia et al., 2007; Nestler and Aghajanian, 1997; Wexler et al., 2001; Chang and Chronicle, 2007; Koob and Le Moal, 2005). These alterations are responsible for withdrawal effects such as anxiety, depression and stress, which predispose the addict to relapse back to the use of these drugs of abuse (Eddy et al., 1965; Sinha, 2001; Van Bockstaele et al., 2010; Valentino et al., 2010).

Apart from alcohol and nicotine use, commonly abused illegal drugs include psychostimulants (cocaine, amphetamine, methamphetamine), opioids (heroin, morphine) and recreational hallucinogens (lysergic acid diethylamide, psilocybin, cannabis) (Substance Abuse and Mental Health Services Administration 2008; World Drug Report, 2007). Among these illegal drugs of abuse, cannabis is most abused in The United States, Europe, Australia and New Zealand (World Drug Report, 2007; Substance Abuse and Mental Health Services Administration 2008; Australian Drug and Alcohol Use Survey 2007; New Zealand Alcohol and Drug Use Survey, 2010). The next most abused illegal drug in Europe and the United States is cocaine (Substance Abuse and Mental Health Services Administration 2008; The state of drug problem in

Europe, Annual report 2009). In New Zealand, around half of the population between 16-64 years of age had abused illegal drugs (excluding alcohol, nicotine and party pills) at least once in their lifetime. Moreover, the prevalence of psychostimulant use was comparatively lower than cannabis use, with fewer than 4% of the surveyed population abusing psychostimulants (cocaine, methamphetamine and prescription stimulants) as compared to 46.4% using cannabis (New Zealand Alcohol and Drug Use Survey, 2010). These surveys indicate that use of illegal drugs is widespread.

Substance abuse is associated with social, economic and civic disabilities (New Zealand Alcohol and Drug Use Survey, 2010; World Drug Report, 2007). The societal effects of substance use include loss in school hours, work hours, and unemployment (New Zealand Alcohol and Drug Use Survey, 2010). Moreover, increased crime and violence burdens healthcare and justice resources and add to the social and economic cost associated with substance abuse (World Drug Report, 2007). Furthermore, drug abuse contributes to an increase in co-morbid mental illnesses and infectious diseases such as acquired immune deficiency syndrome and hepatitis C, which are mainly caused by drug users sharing needles (Morton, 1999; Murrill et al., 2001; Sorenson et al., 2002). In 2008, the financial loss associated with substance abuse in the United Kingdom was over 20 billion pounds (The state of drug problem in Europe, Annual report 2009). Due to the large social and economic cost of addiction many strategies, such as strict law enforcement have been undertaken to control this menace (World Drug report 2007). From a medical perspective, one of the ways to tackle this problem is to develop pharmacological approaches to reduce or prevent addiction.

Numerous studies have been conducted to determine how drugs of abuse bring about the long lasting changes in brain physiology (Weiss et al., 1992; Pierce and Kalivas, 1997; Koob et al., 2004; Koob and Le Moal, 2008; Koob and Volkow, 2009). It is well accepted that drugs of abuse function by modulating the mesocorticolimbic

dopamine system (MDS; Di Chiara and Imparato, 1988a; Wise and Bozarth, 1984; Wise and Rompre, 1998; Kuhar et al., 1991; Spanagel et al., 1992; Koob, 1992). The MDS comprises of dopamine (DA) cell bodies located in the ventral tegmental area (VTA) and projections to the nucleus accumbens (NAc), amygdala and the pre-frontal cortex (PFC) (Olds and Milner, 1954; Olds, 1956, 1977; Bozarth, 1987). Almost all drugs of abuse act by increasing the DA levels in the MDS (Wise and Rompre, 1989). This finding formed the basis of the 'dopamine theory of addiction', which links the increase in reinforcing properties of the drugs of abuse to the elevated DA levels in the MDS (Wise and Bozarth, 1984; Wise and Rompre, 1989; Kuhar et al., 1991; Spanagel and Weiss, 1999). However, recent evidence also suggests that DA plays a role in craving, incentive learning for drug induced enhancement in reward experience and its sensitization (Robinson and Berridge, 1993; 2003; Di Chiara, 1995; Lambert et al., 2006). Long term use of drugs of abuse alters the normal reward and motivational behaviour by bringing alterations in normal reward circuitry (Weiss et al., 1992; Spanagel et al., 1994; Nestler et al., 2001). These long term neuronal adaptations can lead to withdrawal related behaviours such as depression and stress, which are implicated in relapsing back into substance use (Bruchas et al., 2010; Nestler et al., 2001). In addition to their effect on the reward pathway, drugs of abuse also bring about changes in brain regions involved in memory processing, decision making and learning novel tasks (Grant et al., 2000; Schneider et al., 2008; Schenk et al., 2010). The following section will describe various therapeutic strategies applied to tackle addiction at the clinical level.

1.1. Current pharmacotherapies for addiction

Addiction is a chronic relapsing disorder; therefore treatment strategies involve multiple behavioural and/or pharmacological interventions with intensive patient monitoring (DeRubeis and Crits-Christoph, 1998; Carrol and Enken, 2005). This makes for long term patient management rather than short term therapy (McLellan et al., 2000). Although, treatment choice varies between individuals, most treatment strategies include cognitive behavioural therapy in combination with pharmacological agents (Jupp and Lawrence, 2010). Behavioural therapy includes group counselling, social involvement (family and friends) and motivational therapy to enhance self-abstinence from drug taking (Carrol and Enken, 2005; Carrol et al., 2006). The role of pharmacological treatment is to tackle the patho-physiological alterations caused by the abused drugs, and to prevent the addict from relapsing back to drug use (Jupp and Lawrence, 2010; Lobmaier et al., 2010).

This section discusses some currently available pharmacotherapeutic strategies for drug of abuse including opioids, alcohol, nicotine and psychostimulant abuse. Opiate maintenance or replacement therapy for heroin addiction aims to reduce withdrawal related adverse effects such as depression, craving and preventing the abuser from relapsing back to drug use (Kreek et al., 2000; 2005). This therapeutic approach uses MOPr agonists such as methadone and methadone derivatives which reduce the craving effect of heroin addiction (Stimmel and Kreek, 2000). Because methadone is given via an oral route, the "high" produced by methadone is much less than heroin (Hickman et al., 2003; Lobmaier et al., 2010). This may account for its overuse and potentially fatal toxicity (Perret et al., 2000; Reingardiene et al., 2009). Recently, buprenorphine, a mixed opioid compound (MOPr agonist/antagonist and partial KOPr antagonist) has also been used in the opioid maintenance therapy (Soyka et al., 2008).

However, in clinical settings, methadone is still more widely used over buprenorphine (Mattick et al., 2008).

Similar to opiates, nicotine replacement therapy has been applied to manage the withdrawal effects associated with smoking cessation (Silagy et al., 2004). The principle behind this is to make nicotine available via delivery systems other than cigarette smoke such as nicotine patches, inhalers, chewing gums and lozenges (Silagy et al., 2004). However, high rates in relapse back to smoking have been noted within a short period with nicotine replacement therapy (Borgne et al., 2004). Thus, nicotine replacement therapy seems to fail in providing long term abstinence from quitting smoking. Other pharmacological approaches include the use of partial α4β2 nicotinic agonists, varenicline and bupropion (nicotinic receptor blocker and DA/nor-epinephrine (NE) neurotransmission modulator) (Jorenby et al., 2006). Both of these agents increase the period of abstinence (Jorenby et al., 2006) by decreasing craving and withdrawal effects (Coe et al., 2005). However, recent reports suggest the development of depression and suicidal tendencies with these pharmacotherapies (Gunnel et al., 2009; Wrightman et al., 2010).

Modulation of drug metabolism is often used in the treatment of alcohol addiction and disulfiram, via inhibiting enzyme acetaldehyde dehydrogenase has been used to prevent alcohol intake (Kitson, 1977). It produces acute aversive symptoms such as flushing, irritability, tachycardia, nausea and vomiting if alcohol is ingested, thus, preventing the abuser from consuming alcohol (Kitson et al., 1977). Although there are compliance issues, this treatment has been successfully applied in clinical settings under medical supervision (Ehrenreich and Krampe, 2004).

There are currently no successful pharmacotherapies to treat psychostimulant addiction (Taylor and Gold, 1990; Gawin, 1991). The cocaine analogue, cocaethylene

has been tested for use in cocaine withdrawal and maintenance therapy. Cocaethylene was effective in decreasing behavioural effects of cocaine in humans (Baker et al., 2007). However, due to its toxic liability, it is likely to fail as anti-cocaine agents (Tacker and Okorodudu, 2004). Monoamine oxidase inhibitors such as selegiline have shown promising anti-cocaine effects in pre-clinical studies but have failed to produce similar effects in humans (Elkashef et al., 2006; Gatch et al., 2006). Additionally, Negus and Mello, (2004) showed that methadone infusions (0.032-1.0 mg/kg/hr) produced non-specific decrease in operant responding without affecting cocaine reinforcements in rhesus monkeys.

These reports show that most of the currently available pharmacotherapies have shown limited success. In some cases drug-addicts have a high incidence of relapse and risk fatal drug induced toxicity. Furthermore, some of these medications can themselves cause addiction (methadone for opioid maintenance therapy), or have safety and compliance issues warranting caution while being used clinically (disulfiram, varenicline, bupropion). The development of more effective anti-addiction pharmacotherapies are therefore needed to help break the addiction cycle and to reduce relapse. Understanding the eiteology of addiction cycle can help in designing new treatment strategies which will be useful to combat the progression and development of substance abuse (Markou et al., 1993; Le Moal and Koob, 2005).

1.2. The addiction cycle

The development of addiction cycle has been extensively reviewed and has been studied both pre-clinically and clinically (Gawin and Kleber, 1988; Koob, 2008, Koob and Le Moal, 2008; Koob and Volkov, 2009). Briefly, the addiction cycle consists of three different stages, the initial binging stage, followed by drug abstinence and relapse (Fig 1.1; Koob and La Moal, 2008). The binging phase is characterized by the hedonic properties of the drugs of abuse (Gawin, 1991). During this stage the drug-taker experiences positive reinforcement (A in Fig. 1.1; Eddy et al., 1965; Gawin, 1991) and this leads to a gradual escalation in drug use (B in Fig. 1.1). During the abstinence from drug use (forced or self-imposed; shown as C in Fig 1.1), neuronal adaptations occurring especially in the brain reward circuits results into negative mood states such as dysphoria, stress and depression (Koob and Volkov, 2009; Carlezon et al., 1998; Nestler, 2001; D in Fig. 1.1). These negative mood states during abstinence can enhance drug craving (E in Fig. 1.1) and re-exposure to a drug prime or an environmental cue associated with drug use (such as party settings where drugs were abused) or stress can predispose relapse (Hunt et al., 1971; Goeders, 2002a; 2002b). Pre-clinical models of addiction have been shown to mimic the behavioural conditions observed in humans at different stages of the addiction cycle (Markau et al., 1993; Koob, 2000). The following section will discuss various animal models commonly applied in addiction research and anti-addiction drug discovery.

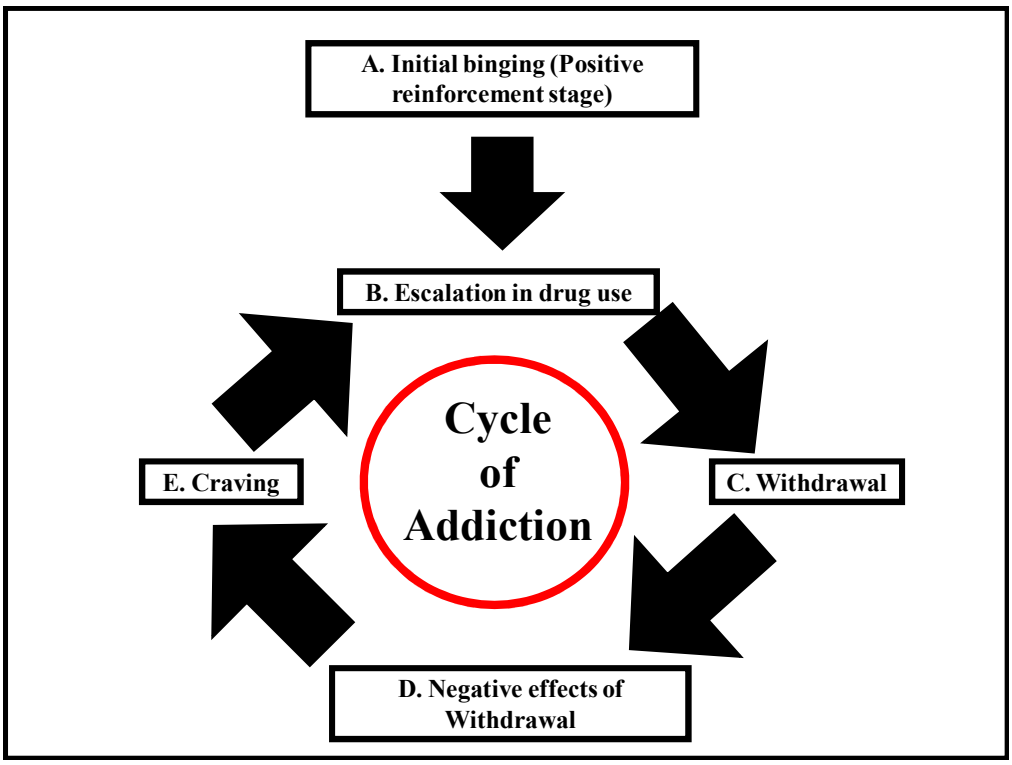


Fig 1.1 Stages in the cycle of addiction

(Adapted from Koob and Le Moal, 2008). After initial 'binges' of drug exposure (A), escalation in the drug administration develops (B). This is due to the 'positive reinforcing or hedonic effects of drugs of abuse. Later on during abstinence from the drug use (C), negative mood states such as depression and stress are observed (D). These negative states are due to the neuronal adaptations associated with drug use and can lead to craving (E). Exposure to drug, drug associated environmental cues and stressors can predispose the addict to relapse back to the drug use. This marks the establishment of the addiction cycle.

1.3. Animal models of addiction

Animal models are an important tool which have been utilized to study the underlying pathophysiology of addiction and also to test novel therapeutic strategies (Spanagel, 2003; Self, 2004; Kalivas et al., 2006). The use of various animal models in understanding the stages of the addiction cycle has been reviewed extensively (Auriacombe et al, 1997; Koob et al, 1999; Stewart, 2000; Sanchis-Segura and Spanagel, 2006; and Spanagel and Holter, 2000; Shalev et al., 2002; O'Brian and Gardner, 2005; Kalivas et al, 2006; Koob and Le Moal, 2008; Koob and Le Moal, 2005). Animal models commonly applied to study various stages in addiction cycle are shown in Tab. 1.1.

The positive reinforcement stage has been studied using models which measure intravenous or oral drug self-administration, conditioned place preference (CPP), or intracranial self-stimulation (ICSS). The craving stage can be depicted by models measuring the reinstatement of extinguished drug-seeking and behavioural sensitization. The negative reinforcement stage of the addiction cycle can be depicted in models measuring drug self-administration, ICSS, conditioned place aversion and drug discrimination. (Koob and La Moal, 2005; Sanches-Segura and Spanagel, 2006).

1.3.1. Operant conditioning in studying addictive behaviours.

Most of these paradigms are based on the rationale of 'Skinner's box' which applies the principle of 'operant reinforcer induced reward' (Skinner, 1938). Briefly, a reinforcer is any stimulus which is used to learn a task (operant lever pressing) and will lead to a response (delivery of reward such as palatable food or drugs of abuse) (Skinner, 1938; Shalev et al., 2002). The hedonic nature of response will lead to an increased lever pressing, thus leading to positive reinforcement (Skinner, 1938; Dinsmoor, 2004; Shalev et al., 2002). Once the animal learns to obtain reward, the effect of exposure to a pharmacological/ external stimulus can be determined on the positive reinforcement

(Shaham et al., 2003). Such conditioning is called 'instrumental conditioning' and the pharmacological/external stimuli applied are known as 'unconditioned stimulus' (Thorndike, 1911; Pavlov, 1927; 1941; Dinsmoor, 2004). Drugs of abuse such as cocaine, amphetamine and morphine act as positive reinforcers and have been shown to increase operant responding in laboratory animals (Deaneu et al., 1969; Devine and Wise, 1994). Furthermore, the principle of instrumental conditioning has been applied to understand the craving for abused drug by using the reinstatement model (Stewart, 2000; Shalev et al., 2002; Shaham et al., 2003). By using this model, presentation of drug primes (De Wit and Stewart, 1981; 1983), environmental cues (Wissenborn et al., 1995; Koob et al., 1996) and stressors (Erb et al., 1996) have been shown to reinstate extinguished drug-seeking behaviours in rats. This paradigm has been used extensively to determine the pathophysiology of drug addiction as well as anti-addiction drug discovery (Kalivas et al., 2006; Stewart, 2000; Shalev et al., 2002; Self, 2004).

1.3.2. Non-operant conditioning in studying aversive behaviours.

In addition to operant conditioning, laboratory animals can also be trained using non-operant conditioning methods to study the aversive behaviours (Sanchez Segura and Spanagel, 2006). One of the paradigms based on this principle is conditioned taste aversion (CTA). In this test, water deprived animals are presented with a novel tasting saccharin solution. The novel taste of saccharin (conditioned stimuli) is paired with the unconditioned stimuli (x-ray irradiation or pharmacological agents; Garcia et al., 1955; Davis et al., 2009). A reduction in the amount of saccharin consumed when presented next shows the aversive properties produced by the unconditioned stimuli (Sanchez Segura and Spanagel, 2006; Davis and Riley, 2010).

Tab. 1.1. Stage of addiction and corresponding pre-clinical paradigms.

Addiction stage	**Source of reinforcement**	**Preclinical models**
Binging	Positive reinforcement	Increased self-administration and CPP, decreased ICSS thresholds
Craving/ anticipation	Conditioned positive reinforcement	Drug prime, stress and cue induced reinstatement, behavioural sensitization
Withdrawal stage	Negative reinforcement	Conditioned place aversion, increased self-administration (dependence stage), increased ICSS thresholds

(Modified from Sanchez-Segura and Spanagel, 2006; Koob and La Moal, 2008).

1.4. Cocaine addiction in pre-clinical models.

Cocaine has been widely used by the indigenous communities in South America for over 3000 years (Siegel, 1982; Gawin and Kleber, 1988). Its addictive potential has been extensively studied in pre-clinical as well as clinical settings (Shaham et al., 2000; Sughondhabirom et al., 2005). Cocaine acts as a positive reinforcer and has been shown to produce self-administration in rats, mice and monkeys (Pickins and Thompson, 1968; Deneau et al., 1969; Hill and Powell, 1976; Papasava et al., 1981). It also produces discriminative stimulus effects (Spealman et al., 1991), CPP (Shippenberg et al., 1995) and decreases ICSS thresholds (Markou and Koob, 1991; Kornetsky and Esposito, 1981) in laboratory animals. A single injection of cocaine has also been shown to enhance motor function (Bhattacharya and Pradhan, 1979; Kalivas and Duffy, 1990). When cocaine is either experimenter- (Kalivas and Duffy, 1990; Kalivas and Stewart 1991; Heidbreder et al., 1996; Kalivas et al., 1998) or self-administered (Hooks et al., 1994; Philips and Di Ciano, 1996), it produces sensitization to its motor stimulant effects. Cocaine has been shown to reinstate extinguished drug-seeking behaviour following drug primes (Downs and Woods, 1974; de Wit and Stewart, 1981; 1983; Worley et al., 1994; Stewart, 2000; McFarland and Kalivas, 2001). Additionally, presentation of environmental cues associated with cocaine self-administration (Koob et al., 1995; Weissenborn et al., 1996) and environmental stressors have also been shown to induce reinstatement of cocaine seeking behaviour (Erb et al., 1996; Kupferschmidt et al., 2011). The following sections will briefly describe the mechanism by which cocaine induces addictive behaviours.

1.4.1. Effect of cocaine on DA transporter function

Synaptic DA neurotransmission following cocaine use is shown in Fig 1.2A. On activation, the pre-synaptic neurons release DA which binds to DA receptors and depending on the receptor subtypes it activates, can produce neuronal excitation (Fig

1.2B). Later, the released DA from the receptors gets transported back to the pre-synaptic neurons via the DA transporter (DAT). There it is either repackaged into vesicles for re-release or degraded by enzymes (Carlsson, 1969; Vogt, 1969; White, 1990).

The transportation of DA into the pre-synaptic neuron requires sodium and chloride ions to bind to DAT (Zhanisar and Dolen, 2001). This is followed by DA binding which leads to the structural conformation change in DAT, resulting in DA uptake into the neuron (Torres et al., 2003). This process requires de-phosphorylation of adenosine tri-phosphate to adenosine di-phosphate and is facilitated in the presence of the enzyme Na^+/K^+ ATPase (Fig 1.2C; Sonders et al., 1997). Along with DAT, cocaine is also a substrate for the nor-epinephrine transporter (NET) and serotonin transporter (SERT) (Ravna et al., 2003). Its action on the reward circuit is mediated mainly via its interaction with DAT (Kuhar et al., 1991). Recent studies have shown that cocaine and DA bind at the same site on DAT (Beuming et al., 2008). The increased binding of cocaine to DAT results into decreased DA uptake and increased availability of DA in the synapse (Fig 1.2D; Zhaniser et al., 1999). The rewarding effects of cocaine are due to its modulation of DA neurotransmission, thereby acting as an indirect DA receptor agonist (Kreek et al., 1999; Zhaniser and Dolen, 2001; Torres et al., 2003).

Due to this, pharmacological agents which modulate DA receptors have been studied as anti-cocaine pharmacotherapeutics (Jupp and Lawrence, 2010; Heidbreder, 2008). However, studies have indicated that DA receptor antagonists produce adverse effects such as suppression of reward reinforcements and extra-pyramidal motor effects which have discouraged their development as anti-cocaine agents (Woolverton et al., 1986; Wise and Schwartz, 1981; Mello and Negus, 1996). Recent studies have shown that the DA 3 receptor preferring compound ropinirole has an anti-addictive effect in humans (Meini et al., 2008). The partial DA 2 receptor agonist, aripiprazole has also

been shown to decrease cocaine craving (Vorspan et al., 2008). On the other hand, the DA 1/DA 2 receptor agonist amantadine showed no significant effect in treating cocaine dependence (Kampman et al., 2006). Furthermore, recent *in vitro* studies have shown that DA 2 receptor activation has shown to increase DAT function (Bolan et al., 2007). Taken together, these results indicate that modulating DA 2 receptor tone could be useful in maintaining cocaine withdrawal effects. However, serious adverse effects such as potentiating other drug use (aripiprazole potentiating methamphetamine and amphetamine use) can be a limiting factor in developing these agents as anti-cocaine pharmacotherapies (Tiihonen et al., 2007). Recently, benztropane DAT inhibitors such as JHW 007 (Velazquez-Sanchez et al., 2010) and AHN 1055 (Velazquez-Sanchez et al., 2009; Ferragud et al., 2009) have been tested as cocaine replacement agents in animals. Results from these studies showed that DAT antagonism may prevent cocaine relapse. This, however, needs to be tested in humans to determine its clinical application. Collectively taken, these results strongly suggest developing agents which indirectly modulate DA neurotransmission, ideally with fewer adverse effects may be an effective strategy to tackle cocaine addiction, (Sonders et al., 1997; Mello and Negus, 2000).

It is well documented that KOPr activation decreases DA levels in NAc (Di Chiara and Imparato, 1988a; 1988b; Spanagel et al., 1992). This effect produced by KOPr agonists might be due to an increase in DAT function (Thompson et al., 2000) as both KOPr and DAT are co-localized in NAc (Svingos et al., 2001). Furthermore, pre-clinical studies have shown that KOPr activation opposes cocaine produced behaviours (Heidbreder et al., 1993; Shippenberg et al., 1998). Therefore, KOPr activation can indirectly modulate cocaine produced behavioural and neurochemical alterations (Thompson et al., 2000). Thus, making them an ideal candidate to be developed as anti-cocaine agents (Prisinzano et al., 2005; Shippenberg et al., 2001).

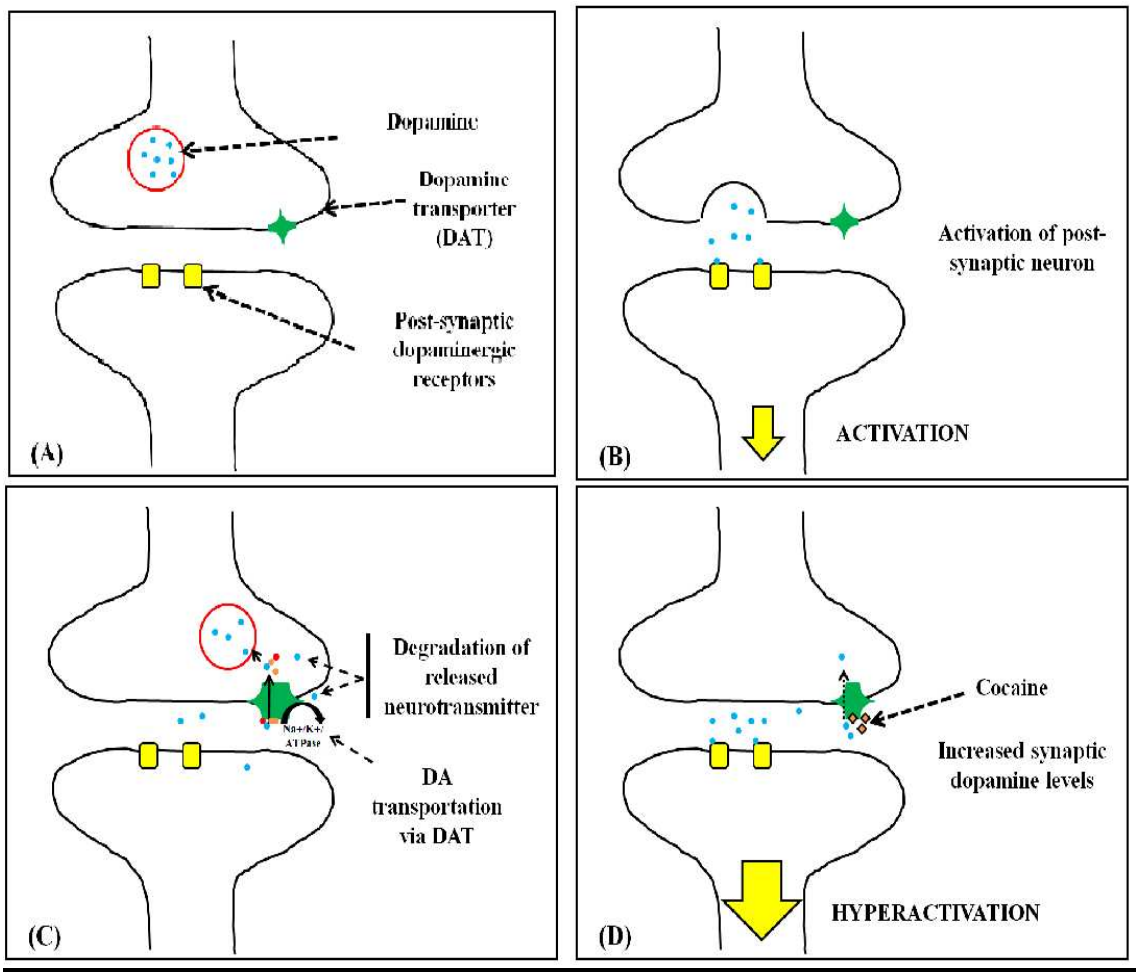


Fig.1.2 Effect of cocaine on dopamine transporter function

Schematic representation of the effect of cocaine on DA neurotransmission. ***A****. Pre-synaptic neuron and a post- synaptic neuron. The pre-synaptic neuron has stored dopamine (DA) in the vesicles and DAT is present on the surface of the neuron. The DA receptors are present on the post-synaptic neuron.* ***B.*** *The activated neuron releases DA in synapse which binds to the DA receptors and activates it.* ***C.*** *DA then dissociates from the receptors and is either repackaged back to the neurons by DAT or is destroyed by the enzymatic degradation. The transportation of DA is facilitated by* Na^{+}/K^{+} *ATPase.* ***D.*** *Cocaine competes with DA and binds to DAT leading to an increase in DA levels in the synapse. The freely available DA binds to the DA receptors and hyper-activates the post synaptic neuron. blue dots- DA; Orange dots- sodium ions; Red dot- chloride ions; Dotted lines indicates decreased activity; Straight lines indicates increased activity; Red circle- storage vesicles for DA.*

1.5. The kappa opioid receptors

The KOPr is a member of 7 transmembrane G-protein coupled receptor protein (Eguchi, 2004) and the endogenous neuropeptide dynorphin (DYN; Goldstein et al., 1979) binds with high affinity to the KOPr (Corbett et al., 1982; Chavkin et al., 1982). High levels of KOPr's are found in the NAc, caudate putamen (CPU), claustrum, amygdala, endoperiform nucleus, whereas low levels of KOPr's were observed in the VTA and substantia nigra (Mansour et al., 1995; 1996; Quirion et al., 1987). Correspondingly, high levels of DYN were detected in the forebrain, hypothalamus and substantia nigra, whereas low levels of DYN have been reported in the dorsal and ventral striatum, amygdala and hippocampus (Goldstein and Ghazarossian, 1980; Webber et al., 1982). Although, pharmacological studies have indicated three subtypes of KOPr's, to date only one subclass of KOPr, KOPr1, has been cloned (Connor and Kitchen, 2006).

The mechanism by which the KOPr activates is shown in Fig 1.3. The inhibitory G-protein coupled receptors are associated with the intracellular G-protein heterodimer (Eguchi, 2004). The G-protein heterodimer comprises of G_{α}, G_{β} and G_{γ} subunits (Connor et al., 1999). Upon activation of the KOPr, the G-protein trimer undergoes conformational changes followed by the dissociation of the G_{α} and $G_{\beta\gamma}$ subunits (Salamon et al., 2002). The dissociated subunits further modulate other signal transduction systems for example; the G_{α} subunit reduces adenylyl cyclise activity which decreases cyclic adenosine monophosphate (cAMP) induced protein kinase A signalling (Watts and Neve, 2005). The $G_{\beta\gamma}$ subunit directly activates the inwardly rectifying potassium channel and inhibits voltage gated calcium channels (Holtz et al., 1998). Overall, this stabilizes the neuron by maintaining its resting state potential (Lu, 2004). The following section describes the activation of the KOPr/DYN negative feedback loop in response to repeated exposures to cocaine.

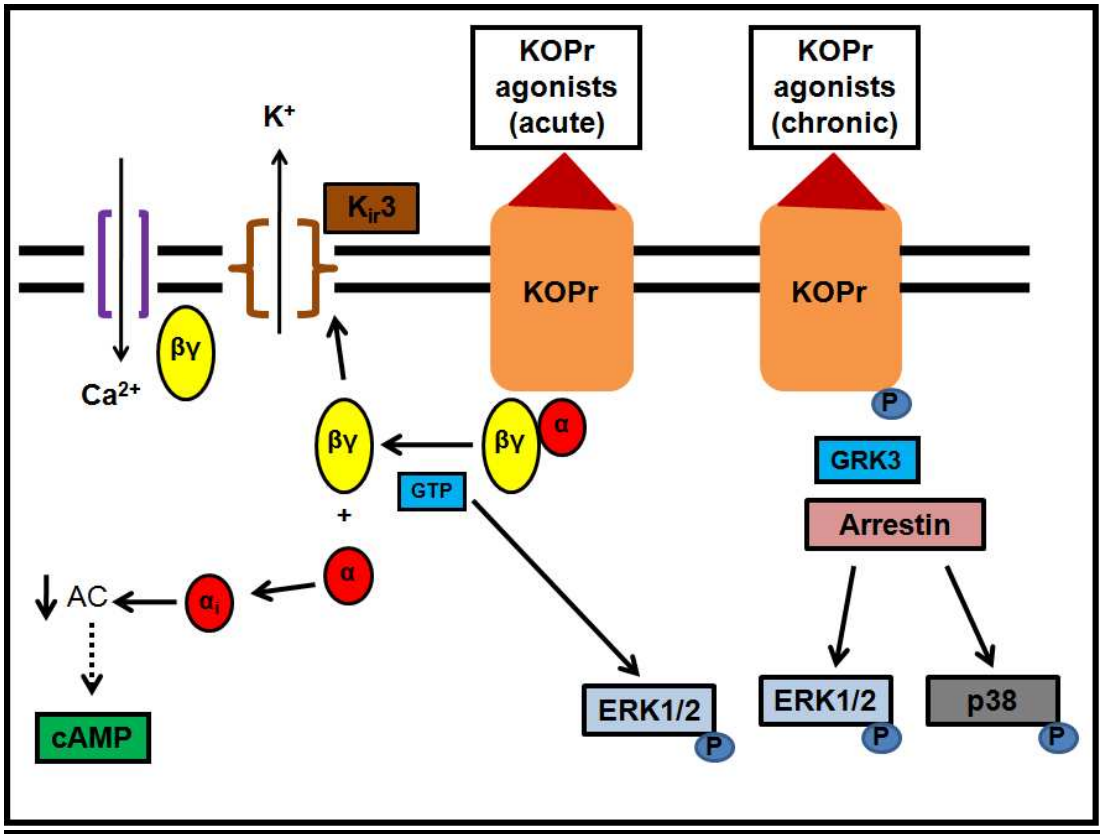


Fig.1.3. Signal transduction mechanism for kappa opioid receptor activation

(Adapted from Bruchas and Chavkin, 2010). The signal transduction mechanism following acute and chronic KOPr activation. KOPr is an inhibitory GPCR which has $G_{\alpha\beta\gamma}$ subunits. On KOPr activated, these subunits dissociates into G_{α} and $G_{\beta\gamma}$ subunits. The G_{α} subunit decreases the levels of adenylyl cyclase and the $G_{\beta\gamma}$ subunit modulates intracellular potassium and calcium ion levels. KOPr activation also leads to activation of protein kinases ERK1/2 and p38. However, long term activation of KOPr leads to receptor phosphorylation and kinase activation via GRK3-Arrestin dependent mechanism. KOPr- kappa opioid receptor; K^{+}- potassium ion; $K_{ir}3$- G-protein gated inward rectifying K^{+} channel; Ca^{2+}- calcium ion; α- G protein alpha subunit; βγ- G protein beta-gamma subunit; GTP- Guanosine tri-phosphate; AC- adenylyl cyclise; cAMP- cyclic adenosine mono-phosphate; P- phosphorylation; GRK3- G-protein coupled receptor kinase 3; ERK1/2- extra-cellular signal-regulated kinase; p38 – p38 mitogen activated protein kinase.

1.5.1. Negative feedback loop and DYN/kappa opioid system

The DYN/KOPr mediated negative feedback loop is presented in Fig 1.4. The gamma amino butyric acid - medium spiny neurons located in the NAc receives DAergic inputs from the VTA (Van Bockstaele and Pickel, 1995). This neuron expresses DA 1 receptors, which are coupled to stimulatory G-protiens (Carlezon et al., 1998) and functions by increasing the adenylyl cyclise activity. The increased cAMP activity enhances the transcription of CREB resulting in release of the endogeneous KOPr neuropeptide, DYN (Carlezon et al., 1998; Nestler, 2001). The spiny projections from NAc releases DYN at the pre-synaptic DA neurons where it binds to the pre-synaptic KOPr (Carlezon et al., 1998). The activated KOPr's has shown to oppose the hedonic effects produced by the drugs of abuse. (Nestler 2001; Shippenberg et al., 2007; Mysels and Sullivan, 2009). An increase in KOPr expression has been observed during chronic cocaine administration in NAc shell, CPU, claustrum and endopiriform nucleus (Collins et al., 2002). This is also accompanied by an elevation in striatal DYN levels and pro-DYN gene expression levels (Sivam, 1989; Daunais et al., 1995; Ramouldi et al., 1996). Agonists at KOPr's have shown to suppress elevation in DA levels in the midbrain dopaminergic systems (Di Chiara and Imparato 1988a; 1988b; Shippenberg et al., 1996). Therefore, activation of KOPr during the initial stages of the addiction cycle or prior to the drug withdrawal might help in curbing the development and progression of cocaine addiction (Shippenberg et al., 2007; Hasabe et al., 2004; Mysels, 2009; Prisinzano et al., 2005). This hypothesis has led to the idea of developing KOPr agonists as anti-addiction agents (Mysels and Sulliva, 2009; Shippenberg et al., 2007). The following section gives information on the endogeneous, synthetic and naturally derived KOPr agonists.

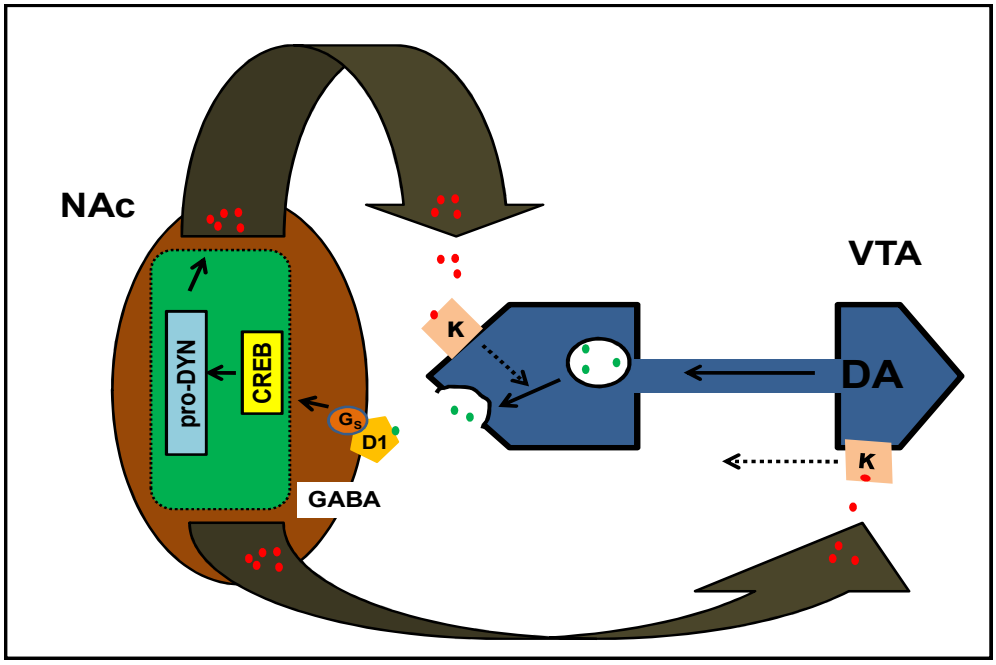


Fig.1.4.Negative feedback loop

(Adapted from Carlezon et al., 1998; Nestler, 2001). Opposing effects to the initial rewarding behaviours produced by drugs of abuse via the negative feedback loop. The NAc receives DA inputs from the VTA which activates the DA 1receptor present on the gamma amino butyric acid medium spiny neurons. Upon activation, these neurons activate CREB, which releases the neuropeptide DYN at the presynaptic neurons. DYN binds to the KOPr and opposes the hedonic properties of cocaine via modulating DA tone in NAc. NAc- nucleus accumbens; GABA- gamma amino butyric medium spiny neuron; VTA- ventral tegmental area; CREB- cAMP response element binding protein; pro-DYN- prodynorphin gene; DA- dopaminergic neuron commencing from VTA; D1- DA 1 receptor; G_s- Stimulatory G-protein coupled receptors; green dots- dopamine; κ- kappa opioid receptor; red dots- DYN.

1.5.2. Kappa opioid receptor agonists

The endogeneous and synthetic KOPr agonists are shown in Fig 1.5. The KOPr agonists consist of endogeneous peptide DYN (1-17) (Goldstein et al., 1980; Chavkin et al., 1982), arylacetamide analogues (U50488H; Vonvoigtlander et al., 1982; 1983, U69593; Lahti et al., 1985, spiradoline; Lahti et al., 1985); benzomorphans (bremazocine, Romer et al., 1980; cyclazocine, pentazocine, Harris and Pierson, 1964); epoxymorphinans (nalfurafine; Nagase et al., 1988); iboga alkaloids (ibogaine and its structural derivatives; Maisonneuve and Glick, 2003) and neoclerodane diterpenes derived from the plant *Salvia divinorum* (Sal A and its structural derivatives; Roth et al., 2002). The coming sections shall give an account of the behavioural anti-addiction as well as the adverse effect profile of KOPr agonists.

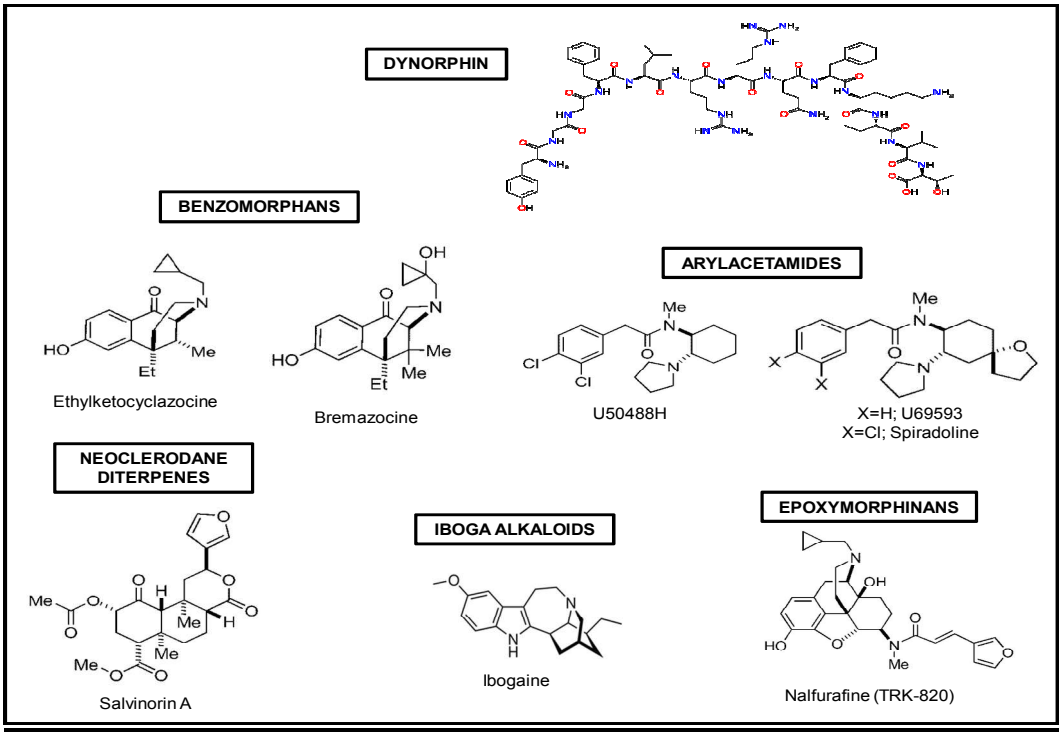


Fig. 1.5. Kappa opioid receptor agonists

(Modified from Eguchi, 2004; Baumann et al., 2001; Tachibana et al., 1982) Kappa opioid receptor agonists consist of the naturally occurring endogenous ligand DYN. Synthetic classes of agonists includes benzomorphans (ethylketazocine, bremazocine, cyclazocine); arylacetamides (U50488H, U69593, spiradoline); epoxymorphinans (nalfurafine); neoclerodane diterpenes (Sal A and its derivatives); Iboga alkaloids (ibogaine and its derivatives)

1.5.3. Kappa opioid agonists in pre-clinical models of addiction

The effect of KOPr activation on cocaine produced behaviours has been studied extensively in laboratory animals and summarised in Tab 1.2.

1.5.3.1. Arylacetamides

Acute KOPr activation by the arylacetamide KOPr agonist U50488H and U69593 attenuated nicotine induced behaviours in rats (Hahn et al., 2000; Ismailova and Shoaib, 2010). U69593 has been shown to attenuate self administration of heroin (Xi et al., 1998), and suppress amphetamine induced behaviours (Gray et al., 1999) in laboratory animals. U50488H and spiradoline both dose-dependently attenuated self-administration of cocaine and morphine in rats, which was reversed by KOPr antagonist pre-treatment (Glick et al., 1995; Kuzmin et al., 1997). The same study also showed that at a higher dose, both arylaceamides (5, 10 mg/kg) decreased water consumption in rats (Glick et al., 1995). Thus, suggesting a non-selective attenuation at higher dose. U50488H has also been shown to attenuate ethanol self-administration in rats (Lorgip et al., 2008). In a drug discrimination model, U50488H either had no effect (Broadbent et al., 2002) or attenuated rewarding effects produced by a low training dose of cocaine (3 mg/kg vs. 10 mg/kg; Kantak et al., 1999). U50488H, U69593 and spiradoline pre-treatment attenuated cocaine-induced hyperactivity (Vanderschuren et al., 2000) and -behavioural sensitization (Heidbreder et al., 1996; Puig-Ramos et al., 2008) in rats. Additionally, spiradoline attenuated morphine induced behavioural sensitization (Smith et al., 2003) and cross sensitization to cocaine (Smith et al., 2009). Also, U50488H, U69593, and spiradoline all attenuate cocaine prime induced reinstatement in rats (See et al., 2010; Morani et al., 2009; Schenk et al., 2000). These findings indicate that arylacetamide KOPr agonists produce anti-addiction like effects in laboratory animals which might be non-selective (Glick et al., 1996).

1.5.3.2. Benzomorphans

The benzomorphan analogue, cyclazocine has also been shown to attenuate cocaine self-administration without affecting water consumption in rats (Glick et al., 1998). However, in rhesus monkeys, bremazocine but not cyclazocine attenuated cocaine maintained behaviours (Negus and Mello, 2000). Bremazocine also attenuated self-administration of cocaine and oral ethanol with decreased saccharin consumption in rhesus monkeys (Cosgrove and Carrol, 2002). However, in another study, bremazocine induced attenuation of freely available ethanol was without any effect on sucrose consumption in rats (Nestby et al., 1999). Also, bremazocine pre-treatment attenuated cocaine and amphetamine induced enhancement in motor function, and amphetamine behavioural sensitization in laboratory animals (Vanderschuren et al., 2000). Collectively, these findings imply the role of benzomorphan compounds in producing anti-addiction effects.

1.5.3.3. Epoxymorphinans

The epoxymorphinan analogue, nalfurafine has also been shown to attenuate cocaine discriminative stimulus effects in a KOPr specific manner (Mori et al., 2002). Furthermore, nalfurafine attenuated rewarding and locomotor effects of cocaine in mice (Hesebe et al., 2004). Also, low doses of nalfurafine (10, 20 μg/kg) did not produce place preference or aversion (Mori et al., 2002; Hesebe et al., 2004). However, at high dose (80 μg/kg) it induces conditioned place aversion in laboratory animals. Thus suggesting that place aversion produced by nalfurafine is dose related.

1.5.3.4. Phytochemical KOPr agonists

Naturally derived KOPr agonists such as iboga alkaloids and neoclerodane diterpenes have been tested for their anti-addiction effects using animal models. Pre-

treatment with ibogaine, an alkaloid derived from plant *Tabermanthe iboga* (Family: Apocynaceae) led to decreased cocaine induced hyperactivity and cocaine self-administration in laboratory animals (Cappendijk and Dzoljic, 1993; Sershen et al., 1992). Synthetic derivatives of ibogaine have also been shown to be effective in suppressing cocaine produced behaviours (Maissaneuve and Glick, 2003). In addition to its cocaine antagonist actions, ibogaine also attenuates DA sensitization to cocaine treatment (Szumlnski et al., 2000), thus explaining its anti-addiction effects.

Taken together, these results suggests that KOPr activation by arylacetamides, benzomorphans, epoxymorphinans and phytochemicals such as ibogaine and Sal A produce anti-addiction behaviours in laboratory animals. Because of the behavioural pharmacology of KOPr agonists, they have been proposed for development as anti-addiction pharmacotherapeutics (Neumeyer et al., 2000; Mello and Negus, 2000; Shippenberg et al., 2001; Prisinzano et al., 2005; Shippenberg et al., 2007). KOPr agonists oppose the DA sensitizing effects produced by cocaine and this has been suggested as a possible mechanism by which KOPr activation produces anti-addiction effects. The following section briefly describes the mechanism of the anti-addiction effects produced by KOPr activation.

1.5.4. Mechanism of KOPr agonist induced anti-addiction effects

KOPr agonist pre-treatment has been shown to attenuate extracellular DA levels in the reward circuits (Shippenberg et al., 1996). Direct infusion of DYN in the NAc has been shown to decrease DA levels in the MDS (Zhang et al., 2004). Additionally, intracerebroventricular (icv) or systemic injections of U50488H and U69593 decreases DA levels in NAc (Spanagel et al., 1992; Devine et al., 1993; Di Chiara and Imparato, 1988a; 1988b; Maisonneuve et al., 1994; Shippenberg et al., 1996; Heidbreder et al., 1996; Thompson et al., 2000). This effect was reversed by nor-BNI pre-treatment (Spanagel et al., 1990; 1992). Furthermore, when the VTA was directly pre-treated with KOPr agonists a decrease in DA levels in the PFC was seen (Margolis et al., 2003; 2006). Also, pre-treatment with another arylacetamide analogue R-84760 and the novel KOPr agonist Sal A has been shown to dose dependently attenuate DA levels in CPU (Zhang et al., 2004; Zhang et al., 2005; Gherke et al., 2008). Systemic treatment with Sal A has also been shown to decrease DA levels in the NAc (Carlezon et al., 2006), and cocaine produced cFOS expression in the CPU was also reduced (Chartoff et al., 2008). KOPr activation by U69593 has also been shown to decrease cocaine induced dopamine- and adenosine 3′,5′-monophosphate-regulated protein with phosphorylation, which seems to play an important role in cocaine produced locomotor sensitization (D'Addario et al., 2007). Collectively, these findings show that KOPr activation opposes cocaine induced behaviours by decreasing extracellular DA levels in the NAc, thus indirectly acting as cocaine antagonists (Shippenberg et al., 2001; Prisinzano et al., 2005). The adverse effects however, associated with KOPr activation (depression, aversion, sedation) have limited their further clinical development. The following section describes the behavioural adverse effects associated with KOPr activation and the mechanisms that may mediate these behaviours.

Tab1.2. KOPr agonists in pre-clinical studies of addiction

Pharmacological effect	KOPr agonists tested	Behaviour produced	References
Cocaine self-administration	U50488H	Attenuate	Glick et al., 1995; Kuzmin et al., 1997; Negus et al., 1997
	Spiradoline	Attenuate	Wadenberg, 2003, Glick et al., 1995
	Bremazocine	Attenuate	Cosgrove and Carrol, 2002;
	Iboga alkaloids	Attenuate	Coppendijk and Dzoljic, 1993
Cocaine motor sensitization	U50488H, U69593	Attenuate	Heidbreder et al., 1993; 1995; Collins et al., 2001a; 2001b; Puig-Ramos et al., 2008
Cocaine induced brain stimulation reward	U69593	Attenuate	Tomasaiwicz et al., 2008
Cocaine induced discriminative stimulus effects	U50488H	No effect	Broadbent et al., 2002
	U50488H, Cl-977	Attenuates at low training dose of cocaine (3 mg/kg)	Kantak et al., 1999; Spealman and Bergman; 1992
	Nalfurafine	Attenuate	Mori et al., 2002
Cocaine induced reinstatement (self-administering rats)	U50488H, U69593, spiradoline, Sal A	Attenuate	Schenk et al., 1999b; 2000a; Morani et al., 2009
Cocaine induced enhancement in CPP	U69593, U50488H	Attenuate	Shippenberg et al., 1996
Cocaine induced hyperactivity	U69593	Attenuate	Collins et al., 2001a, 2001b; Vanderschuren et al., 2000
	U50488H	Attenuate	Vanderschuren et al., 2000; Heidbreder et al., 1995
	Nalfurafine	Attenuate	Hasabe et al., 2004
	Sal A	Attenuate	Chartoff et al., 2008
	R-84760	Attenuate	Zhang et al., 2004
	Bremazocine	Attenuates	Vanderschuren et al., 2000
Effect on midbrain dopamine release	U50488H,	Decreases in NAc	Di Chiara and Imparato, 1988a,1988b; Maisonneuve et al., 1994;
	U69593	Decreases in NAc	Shippenberg et al., 1996; Thompson et al., 2000
	R-84760	Decreases in CPU	Zhang et al., 2004
	Sal A	Decrease in NAc and CPU	Carlezon et al., 2006; Gherke et al., 2008, Zhang et al., 2005.
	Ibogaine	Decrease DA sensitization	Szumlinski et al., 2000
Amphetamine induced behaviours	U69593	Attenuate	Gray et al., 1999
Morphine self-administration	U50488H	Attenuate	Glick et al., 1995; Kuzmin et al., 1997
Morphine induced rewarding behaviours	Spiradoline	Attenuate	Glick et al., 1995; Smith et al.,2003; 2009
Heroin self-administration	U50488H	Attenuates	Xi et al., 1998
Nicotine self-administration	U69593, U50488H, CI-977	Attenuate	Hahn et al., 2000; Ismailova and Shoaib, 2010
Ethanol self-administration	Bremazocine	Attenuate	Nestby et al., 1999
	U50488H	Attenuate	Lorgip et al., 2008

1.5.5. Behavioural adverse effects of KOPr agonists

1.5.5.1. Sedation and psychotomimic effects

KOPr agonists U50488H, bremazocine, spiradoline and U69593 have been shown to decrease spontaneous open field activity in laboratory animals (Von Voigtlander et al., 1983; Lahti et al., 1982; 1985; Wadenberg, 2003). Furthermore, KOPr activation by Sal A and U69593 induces hallucinations in non-human primates (Butelman et al., 2007; 2009; 2010). Clinical studies showed that acute administration of enadoline, a selective KOPr agonist produces sedation, emesis, hallucinations, dizziness, excessive urination and psychotomimesis in humans (Walsh et al., 2001a). Moreover, KOPr activation by the benzomorphan analogue, Mr2033 also produces psychotomimetic actions in humans (Pfeiffer et al., 1996). These adverse behaviours produced by KOPr agonists have discouraged the development of KOPr agonists as potential anti-addiction agents (Wee and Koob, 2010).

1.5.5.2. Depression and aversion.

The KOPr agonists have been shown to produce depressive behaviours in laboratory animals. Both U69593 and Sal A dose dependently increase immobility time in the forced swim test (FST; Mague et al., 2003; Carlezon et al., 2006). Additionally, pre-treatment with U50488H, U69593 and Sal A increases the ICSS threshold in rats (Todtenkopff et al., 2004; Ebner et al., 2010; Dinieri et al., 2009). These data indicate that KOPr activation produces depressive effects in laboratory animals. In addition to its depressive effects, KOPr activation also produces aversive behaviours as pre-treatment with U50488H, bremazocine, ethylketocyclazocine or U69593 has been shown to induce conditioned -place and -taste aversion in laboratory animals (Shippenberg and Herz, 1986; Mucha and Herz, 1985). The following section gives an account of the mechanisms underlying KOPr activation mediated depressive and aversive behaviours.

1.5.5.3. Nucleus accumbens and KOPr agonists induced depression and aversion.

Direct or systemic injection of U69593 into the NAc has been shown to induce aversive behaviours in laboratory animals, which were reversed by pre-treatment with the selective KOPr antagonist, nor-binaltorphimine (nor-BNI; Bals Kubik et al., 1989; 1993). The aversion induced by KOPr activation is believed to involve DA neurotransmission in the NAc, as direct application of the DYN peptide E2078 to the NAc reduces release of phasic DA. This effect was reversed by KOPr antagonist pre-treatment (Spanagel et al., 1990).

Another reason for the depressive effects produced by KOPr agonists might be due to the phosphorylation of CREB in the NAc (Carlezon et al., 1998; Nestler et al., 2001; Hyman and Malenka, 2001; Carlezon et al., 2005; Dinieri et al., 2009). Recent results suggests that enhancement in levels of phosphorylated CREB in NAc is associated with increased immobility time in the forced swim test (FST; Plaikas et al., 2001). KOPr agonists have shown to increase time spent immobile in FST and also increase CREB levels in NAc (Mague et al., 2003; Carlezon et al., 2006). On the other hand blocking central KOPr's opposes depressive effects in FST and also reduces CREB phosphorylation in the NAc (Mague et al., 2003; Chartoff et al., 2009).

Collectively, this data strongly suggests that NAc is one of the major sites for KOPr activation induced depression (Nestler et al., 2001; Carlezon et al., 2009) and alterations in DA neurotransmission and CREB in NAc plays a major role in the pathophysiology of KOPr mediated depressive behaviours (Knoll and Carlezon, 2010; Carlezon et al., 2009).

1.5.5.4. Central 5HT systems and KOPr activation induced depression and aversion.

KOPr have been located in brain regions such as raphae nucleus, NAc, hippocampus which are rich in 5HT neurotransmission (Battaglia et al., 1991; Pinnock, 1992). Depletion of 5HT by parachlorophenylalanine (a synthetic amino acid which depletes serotonin from cell body) attenuated the analgesic effects of U50488H (Nemmani and Mogil, 2003). Whereas prior treatment with selective serotonin reuptake inhibitor; fluoxetine (SSRI; FLX) potentiated U50488H induced analgesic effects in mice (Nemmani et al., 2001). Additionally, 5HT is also believed to play a key role in controlling DYN levels. The depletion of 5HT has also been shown to decrease pro-DYN mRNA expression by approximately 60% in CPU, hippocampus and hypothalamus (Di Addario et al., 2007). These findings suggest the importance of 5HT systems in KOPr agonist induced behaviours. Moreover, modulation of 5HT systems by KOPr agonists have been implicated in cocaine induced behaviours. A recent study by Zakharova et al., (2008) suggested that depletion of 5HT from the brain decreased the ability of U69593 to attenuate cocaine induced motor stimulation. Additionally, selective SERT inhibitors FLX (5.6 mg/kg) and citalopram (10 mg/kg) attenuated cocaine induced drug-seeking behaviour in squirrel monkeys (Ruedi-Bettschen et al., 2010). Moreover, the same study showed that this effect produced by KOPr agonist enadoline and FLX was reversed by the pre-treatment with 5HT 2A receptor agonist 8-OH-DPAT (Ruedi-Bettschen et al., 2010). Thus, KOPr activation may modulate 5HT systems which, in turn also modulate cocaine induced behaviours (Ruedi-Bettschen et al., 2010).

The 5HT systems might be implicated in the KOPr agonist's induced adverse effects as KOPr activation has been shown to modulate the 5HT neurotransmission in various brain regions. Yilmaz and colleagues, (2006) reported that icv administration of U50488H significantly decreased the tissue levels of 5HT and its metabolite, 5-

hydroxyindole acetic acid in hippocampus and hypothalamus. These effects were prevented by nor-BNI pre-treatment. Also, a significant reduction (approx 30%) in 5HT concentrations using *in vivo* microdialysis following local infusions of U50488H (300 μM) was observed in the dorsal raphae nucleus (Tao and Auerbach, 2005). A, recently published report by Land et al., (2009) implicate 5HT projections from the dorsal raphae nucleus to NAc in KOPr activation and repeated stress induced aversion. These results strongly suggest that central KOPr activation alters 5HT neurotransmission. This may account for the KOPr mediated adverse effects and manipulation of 5HT systems might help in alleviating these adverse effects.

1.5.6. Effect of acute vs. chronic KOPr activation.

Previous studies have shown that intravenous (iv) infusions (23 hr/day for 10 consecutive days) of the KOPr agonist bremazocine (0.0032 mg/kg/hr); enadoline (0.001, 0.0032 mg/kg/hr); ethylketazocine (0.01, 0.0032 mg/kg/hr); Mr2033 (0.01, 0.0032 mg/kg/hr) and U50488H (0.1 mg/kg/hr) decreased cocaine self-administration in rhesus monkeys (Negus et al., 1997; Mello and Negus, 1998; 2000). This attenuation was also accompanied by a significant reduction in food reinforcement, sedation and emesis, which subsided after 2-3 days of treatment (Mello and Negus, 2000). Acute exposure to these compounds also suppressed food reinforcement in rhesus monkeys (Mello and Negus, 2000). The enadoline and Mr2033 induced reduction in cocaine self-administration reverted to baseline levels of cocaine self-administration during last week of treatment, when these compounds were administered for 28 subsequent days (Mello and Negus, 2000). This implies that chronic KOPr activation had no effect on cocaine's reinforcing effects in rhesus monkeys. Another study by Negus, (2004), however showed that monkeys trained to discriminate between food and cocaine using concurrent choice paradigm, when continuously infused with U50488H (0.032 mg/kg/hr, iv infusion) showed cocaine preference. The same study also showd that this effect was KOPr dependent as nor-BNI pre-treatment reversed it (Negus, 2004). This result suggests that continuous KOPr activation might enhance cocaine's reinforcing properties in comparison to natural reward such as food.

On the other hand, a study by Preston and colleagues (2004) showed that acute oral administration of cyclazocine (0.2-0.8 mg) for 4 consecutive days significantly decreased the acute subjective effects of cocaine in humans. Moreover, acute administration of KOPr agonists attenuated cocaine self-administration and reinstatement in rats (Glick et al., 1995; Kuzmin et al., 1997; Schenk et al., 1999; 2000; Morani et al., 2009). These findings show differences in acute and chronic KOPr

treatment on cocaine induced behaviour and support the short term use of KOPr agonists in attenuating the effects of cocaine.

The effects produced by KOPr agonists on DA levels in the NAc may depend upon the frequency of KOPr activation, as acute administration of U69593 inhibits the potassium ion evoked DA release from rat striatal synaptosomes which was reversed by nor-BNI pre-treatment (Ronken et al., 1993). In contrast, repeated administration of U69593 significantly increased potassium ion evoked DA levels in the NAc (Fuentelaba et al., 2006) and in cultured mesencephalonic DA neurons (Ronken et al., 1993). Moreover, acute administration of U69593 directly into the NAc of mice has been shown to attenuate DA release (Chefer et al., 2005). Whereas, repeated administration of U69593 decreases DA uptake without affecting extracellular DA levels in the NAc (Thompson et al., 2000). These data further strengthen the role of acute KOPr activation in producing desired anti-addiction effects at cell and tissue levels.

Chronic KOPr activation leads to receptor deactivation via KOPr phosphorylation and may cause KOPr internalization as decreased KOPr binding was observed after chronic treatment with U50488H *in vitro* (Bhargava et al., 1989). Also, chronic KOPr treatment activate protein kinase (p38 mitogen activated protein kinase and extracellular signal-regulated kinases 1 and 2; Bruchas et al., 2006; Belcheva et al., 2005). However, differences in acute and chronic KOPr activation mediated phosphorylation of protein kinases have been reported (McLennan et al., 2009; Fig 1.3). The KOPr mediated signal transduction mechanism has been extensively reviewed by Carr and Mague, (2008); Bruchas and Chavkin, (2010) and is shown in Fig 1.3. Taken together, these findings suggest that acute but not chronic KOPr activation might be beneficial in producing anti-addiction effects.

A study by Walsh and colleagues, (2001b) showed that, acute administration of selective KOPr agonist enadoline attenuated subjective effects of cocaine without

having any effect on cocaine self-administration in humans. Moreover, enadoline produced compliance problems and psychotomimesis when tested in healthy subjects (Walsh et al., 2001a). Thus, despite having anti-addiction properties, these adverse effects have limited the development of KOPr agonists as anti-addiction pharmacotherpies. Recently, Sal A, was reported to be a novel, naturally occurring, potent and selective KOPr agonist. One of the aims of this thesis was to determine behavioural anti-addiction and side effect profile of Sal A. The following section gives a detailed account on the behavioural pharmacology of Sal A.

1.6. Salvinorin A

Sal A is the selective KOPr agonist found in the plant *Salvia divinorum* (family:- Lamiaceae; Roth et al., 2002). This plant has been used for spiritual and divination purposes by the Mazatecs from Oaxaca, Mexico for centuries (Ott, 1995; Siebert, 1994; Valdez et al., 1984; Valdez, 94). Smoking dried *Salvia divinorum* leaves is associated with intense hallucinations of short duration (Valdez, 1994; Siebert, 1994). Sal A has a novel neoclerodane diterpene structure and has been shown to bind to the inhibitory $G_{\alpha i}$ protein coupled KOPr (Roth et al., 2002). Sal A is selective in binding and activating KOPr expressing in the human embryonic kidney (HEK -293) cells and at native KOPr expressed in guinea pig brain (Roth et al., 2002). Furthermore, Sal A has no affinity for MOPr or DOPr (Roth et al., 2002; Yan and Roth 2004; Chavkin et al., 2004: Vorthermes and Roth 2006). Also, Sal A showed no affinity for DA, serotonin (5HT), muscarinic acetylcholine, adrenergic, cannabinoid or sigma receptors, which was measured using functional binding assays (Roth et al., 2002). Thus, Sal A is a unique hallucinogen without any affinity for 5HT 2A receptors, which are the primary target for classic hallucinogens (Roth et al., 2002; Vorthermes and Roth, 2006). Taken together, this data suggests that hallucinations produced by Sal A are via KOPr activation (Roth et al., 2002; Killinger et al., 2010).

In vitro binding studies have shown that Sal A binds with greater affinity to KOPr than the arylacetamide KOPr agonists U50588H and U69593, and with equal affinity to the endogeneous KOPr ligand DYN (Chavkin et al., 2004). The incubation of Chinese hamster ovary cells stably expressing human KOPr with Sal A for 4 hr induced a receptor down-regulation which was similar to other KOPr agonists tested (Wang et al., 2005). However, another study by Wang and colleagues (2008) showed that Sal A pre-treatment had no effect, whereas U50488H pre-treatment induced 40 times more KOPr internalization *in vitro.* As Sal A has a different structure compared to

arylacetamides (Fig 1.5), these observed differences might, at least be related to the structural dissimilarities noted between these two class of KOPr agonists.

1.6.1. Behavioural pharmacology of Sal A

The behavioural pharmacology of Sal A is summarised in Tab 1.3.

1.6.1.1. Sedation, hallucinations and motor suppression.

Sal A has shown to produce hallucinations in humans (Siebert et al., 1994; Valdes et al., 1983; Johnson et al., 2010) and induce sedative effects in non-human primates (Butelman et al., 2009; 2010). At doses of 1 and 3.2 mg/kg it produces a KOPr mediated, nor-BNI reversible decrease in locomotor activity in mice (Zhang et al., 2005). Furthermore, Sal A decreases climbing behaviour in mice in an inverted screen model (Fantagrossi et al., 2005). However, another study indicated that Sal A pre-treatment (1 mg/kg, 0.25 – 2 mg/kg) had no effect on locomotor activity in rats (Hooker et al., 2008; Carlezon et al., 2006). Additionally, 2-methoxy-methyl salvinorin B (MOM Sal B), a more potent and longer acting Sal A analogue, produced a rapid, long lasting (about 3 hr) immobility in mice which was dose dependent and nor-BNI reversible (Wang et al., 2008). However, the same study showed that MOM Sal B increased locomotion in the Y-maze test in rats. Thus, indicating an effect which might be species specific.

1.6.1.2. Antinociception

Sal A has been shown to produce rapid anti-nociception (commencing within 10 min) with short duration of effects (30 min) in laboratory animals (McCurdy et al., 2006; John et al., 2006). This effect produced by Sal A was KOPr mediated, as nor-BNI pre-treatment completely reversed it (McCurdy et al., 2006; Ansonoff et al., 2006).

Furthermore, 2-propionate Sal A and MOM Sal B have been shown to produce anti-nociception in laboratory animals (Ansonoff et al., 2006; Wang et al., 2008).

1.6.1.3. Drug Discrimination

In drug discrimination studies, Sal A pre-treatment completely substituted for U69593 in rats paired with U69593 and conditioned to obtain food reinforcements (Willmore-Fordham et al., 2007). This effect was reversed by nor-BNI pre-treatment (Willmore-Fordham et al., 2007). Furthermore, the Sal A induced discriminative stimulus effects of U69593 in rats were also observed in non-human primates (Butelman et al., 2004). A recent study by Baker and colleagues, (2009) showed that the longer acting, more potent, Sal A analogues, 2-ethoxymethyl salvinorin B (EOM Sal B) and MOM Sal B also substituted for U69593 at doses lower than Sal A. These findings indicates a similar pharmacological effects induced by U69593, Sal A and its analogues in activating central KOPr's.

1.6.1.4. Anti-cocaine effects

Acute intraperitoneal (ip) administration of Sal A (2 mg/kg) has been shown to decrease cocaine induced hyperactivity in rats (Chartoff et al., 2008). This effect was dependent on context learning as Sal A, when injected in the activity boxes potentiated locomotor activity; whereas, administration of Sal A in the home cages did not potentiate motor function induced by the cocaine challenge (Chartoff et al., 2008). Furthermore, acute administration of Sal A, dose dependently (0.3, 1.0 mg/kg) attenuated cocaine prime induced reinstatement of cocaine seeking without suppressing sucrose reinforcements or causing sedation in rats (Morani et al., 2009). These findings indicate that anti-cocaine behaviours are produced by Sal A, just like traditional KOPr

agonists in laboratory animals. More work is necessary to characterize the therapeutic anti-addiction potential of Sal A-like compounds.

1.6.1.5. Depression and Aversion

Recent studies have shown that Sal A (2 mg/kg) dose dependently increases ICSS thresholds, decreases swimming time and increases immobility time in the FST paradigm in rats (0.25- 2 mg/kg; Carlezon et al., 2006). Furthermore, Sal A (2 mg/kg) has been shown to suppress sucrose reinforcement in laboratory animals (Ebner et al., 2010). Sal A (1.0, 3.2 mg/kg) pre-treatment has also been shown to induce conditioned place aversion in mice (Zhang et al., 2005). Additionally, the N-methylacetamide derivative of Sal A has also shown to produce long lasting increases in ICSS thresholds in rats (Beguin et al., 2008). However, in zebrafish, Sal A at high doses (80 µg/kg) induces place aversion, whereas at lower doses (1 µg/kg) induces place preference (Braida et al., 2007). Recent reports by Braida et al., (2009) suggests that Sal A pre-treatment produces anti-depressant and anxiolytic effects using FST, tail suspension and elevated plus maze tests in laboratory animals. Also, lower dose of Sal A (0.25, 0.3 mg/kg) did not suppress sucrose reinforcements in rats (Ebnar et al., 2010; Morani et al., 2010). Thus the aversive effects induced by Sal A are likely to be dose related.

1.6.1.6. Toxicity studies

Pre-clinical toxicological studies with Sal A show that Sal A does not produce any toxicity in mice, even at high dose (1000 mg/kg) (Mowry et al., 2003). These findings also show that chronic treatment (once every 14 days) of Sal A (dose range 0.4 – 6.4 mg/kg) did not significantly alter the histology of liver, spleen, kidney, brain and bone marrow, or change cardiac function, heart rate or pulse pressure in laboratory animals (Mowry et al., 2003). Recent studies have also shown that Sal A administration

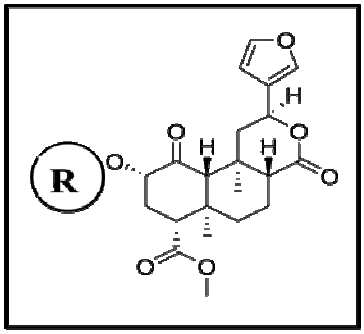
in healthy volunteers did not produce any cardiovascular adverse effects, however, hallucinations were observed (Johnson et al., 2010; Siebert, 1994). Also, some incidences of Sal A induced psychosis have been reported clinically (Singh, 2007; Przekop and Lee, 2009; Paulzen and Grunder, 2008). Therefore, further detailed studies are warranted to establish the psychological safety profile for Sal A at pharmacological doses.

Tab.1.3. Salvinorin A: Pre-clinical studies

Pharmacological effect	**Reference**
Anti-nociception	John et al., 2006; McCurdy et al., 2006; Ansenoff et al., 2006.
Hallucination	Butelman et al., 2007; 2009; 2010.
Sedation	Zhang et al., 2005; Fantagrossi et al., 2005; Butelman et al., 2009; 2010.
Discriminative stimulus	Willmore-Fordham et al., 2007; Butelman et al., 2004; Baker et al., 2009.
Depression	Carlezon et al., 2006; Ebnar et al., 2010.
Conditioned place aversion	Zhang et al., 2005.
Anti-depression/ Anti-anxiety	Braida et al., 2009; Braida et al., 2008.
Attenuate cocaine induced behaviours	Chartoff et al., 2008; Morani et al., 2009.
Decreased dopamine levels in MDS	Carlezon et al., 2006; Gherke et al., 2008, Zhang et al., 2005.

1.6.2. C-2 derived Sal A analogues

Sal A has a quick onset of action which is attributed to its rapid crossing of the blood brain barrier (approximately 1 min; Butelman et al., 2007; Schmidt et al., 2005; Hooker et al., 2008). Furthermore, Sal A has an elimination half life of 56.6 ± 24.8 min in non-human primates (Schmidt et al., 2005). Sal A gets hydrolysed at C-2 position via enzyme esterases to Sal B, which is pharmacologically inactive (Chavkin et al., 2004 Ansenoff et al., 2006; Wang et al., 2008; Fig 1.6). The position C-2 is considered as an important site for the binding and activation of KOPr by Sal A (Bikbulatov et al., 2007; Yan and Roth, 2004). Additionally, this position is also implicated in improving the pharmacokinetic profile of Sal A (Prisinzano and Rothman, 2008; Munro et al., 2008). Substitution of methoxymethyl and ethoxymethyl groups at C-2 position yields compounds with increased duration of effect and greater affinity for KOPr as compared to Sal A (MOM Sal B, EOM Sal B; Munro et al., 2005; Beguin et al., 2008; Fig 1.6). Additionally, aromatic substitution at the C-2 position produces compounds with a greater affinity for MOPr than KOPr (Herkinorin; Harding et al., 2005; Tidgewell et al., 2006; Fig 1.6). The substitution of a mesylate group at this position yielded a compound which had a similar affinity but slightly higher potency for KOPr as compared to Sal A (DS1; Harding et al., 2005, Fig 1.6). One of the aims of this study was to determine behavioural anti-addiction and side-effect profiles of novel Sal A analogues. The following section describes the pharmacological profile of Sal A and selected analogues..



-R	Compound	Binding affinity at KOPr *Ki* (nM)	Binding affinity at MOPr Ki (nM)	Potency at KOPr EC_{50} (nM)	Potency at MOPr (nM)	Reference
-CO-CH_3	Salvinorin A	1.9 ± 0.2[a]	>10000	1.8 ± 0.5[c] 40 ± 10[d]		Harding et al., 2005; Tidgewell et al., 2008; Munro et al., 2008
-H	Salvinorin B	>10000	>10000	-		Chavkin et al., 2004
-CH_2-O-CH_3	2-methoxymethyl Salvinorin B	0.6 ± 0.07[b]	ND	0.40 ± 0.04[c]		Munro et al., 2008
-CH_2-O-C_2H_5	2-ethoxymethyl Salvinorin B	0.32 ± 0.02[b]	ND	0.14 ± 0.01[c]		Munro et al., 2008
-SO_2-CH_3	DS-1	2.3 ± 0.1[a]	6820 ± 660[a]	30 ± 5[d]		Harding et al., 2005
-CO-C_6H_5	Herkinorin	90 ± 2[a]	12.1 ± 1[a]	1320±150[d]		Harding et al., 2005; Tidgewell et al., 2008
	Nalmefene	0.083 ± 0.0008[b]	0.24±0.006[b]	EC_{50} = 4.2±1.6[c] IC_{50} = 18±1.0[c]	IC_{50} = 13±2.3[c]	Bart et al., 2005

Fig.1.6. Salvinorin A, its structural analogues and nalmefene

(Adapted from Prisinzano and Rothman, 2008) Binding affinity of Sal A, C-2 derived analogues of Sal A and nalmefene for KOPr and MOPr.[a]Data expressed as ± SD; [b]Data expressed as ± SEM; ND= not defined.[c]Enhancement of [35S]GTPcS binding to CHO-hKOPr; EC50 ± SEM (nM). [d][35S]GTP-γS functional assay carried out in stably transfected CHO cells containing DNA for hKOPr;EC50 ± SD nM. ND= not defined.

1.7. Mixed opioid compounds as anti-cocaine agents.

MOPr and KOPr activation have been shown to produce opposite reward related behaviours (Pan, 1998; Smith et al., 2003; Glick et al., 1995). MOPr activation produces self-administration (Bozarth and Wise, 1981; Devine and Wise, 1994), decreases ICSS thresholds (Esposito and Kornetsky, 1977), enhances motor function (Oka and Hosoya, 1976), produces sensitization (Smith et al., 2003) and cross sensitization to the rewarding effects of cocaine (Kim et al., 2004; Smith et al., 2009). Thus, activation of MOPr produces reward related behaviours with the potential to cause addiction (Wang et al., 2004). Moreover, MOPr agonists have shown to produce place and taste preference in laboratory animals (Mucha and Herz, 1985; 1986). Activation of MOPr increases DA levels in the mesolimbic brain regions when measured using microdialysis techniques (Di Chiara and Imperato, 1988a; 1988b: Spanagel et al., 1992; Devine et al., 1993). On the other hand, KOPr activation produces opposite reward related effects (Mucha and Herz, 1985; Shippenberg and Herz, 1986; Di Chiara and Imperato, 1988a; Spanagel et al., 1992). Rats produced aversive behaviour when either MOPr was inhibited or KOPr was activated in the dorsal periaquiductal grey region (Nobre et al., 2000; Sante et al., 2000). Moreover, morphine induced place preference was blocked by KOPr agonists (Funada et al., 1993; Liang et al., 2006). Additionally, KOPr activation attenuated morphine self-administration in rats (Glick et al., 1995; Kuzmin et al., 1997). Taken together, these findings suggest the opposing role of KOPr and MOPr activation in reward related behaviours.

Nalmefene (REVEX; Ohmeda Pharmaceutical Products, Liberty Corner, NJ) is a longer acting structural analogue of the non-selective opioid receptor antagonist naloxone and naltrexone (June et al., 1999; Barson et al., 1989). It has a slow onset of action and has prolonged effects on morphine induced behaviours such as antinociception, locomotion and withdrawal effects in mice (Osborn et al., 2010).

Clinical studies with nalmefene indicate that it is effective in reducing symptoms of pathological gambling (Grant et al., 2006; 2010), attenuate ethanol self-administration in laboratory animals (June et al., 1998; 2002; 2004) and humans (Mason et al., 1999; Karhuvara et al., 2007). Because of these reasons, it has been proposed for clinical use in ethanol abuse (Jupp and Lawrence 2010; Soyka and Rosner, 2010). Moreover, nalmefene is used clinically in opioid overdose (Wang et al., 1998; Barson et al., 1989). Recent opioid receptor affinity and selectivity studies show that nalmefene has a greater affinity for KOPr and MOPr than delta opioid receptors (Bart et al., 2005). The same study showed that nalmefene is a MOPr antagonist and partial KOPr agonist (Bart et al., 2005).

The reported anti-addiction properties of nalmefene (Kreek et al., 2002; 2005; Bart et al., 2005) on cocaine are required to be evaluated before any conclusions can be made on its therapeutic potential. Therefore, one of the aims of this thesis was to study the anti-cocaine effects and behavioural adverse effect profile of nalmefene in rats.

1.8. Aims

KOPr agonists have been implicated in anti-addiction behaviours but have not been developed clinically because of the adverse effects associated with them (Walsh et al., 2001a; Mello and Negus, 2000). The emergence of Sal A as a novel KOPr ligand has opened a new avenue in kappa opioid research. Sal A is structurally dissimilar to the traditional KOPr agonists, yet it produces similar pharmacology at KOPr. Therefore, if Sal A produces anti-addiction behaviour like traditional KOPr agonists with fewer adverse effects, then Sal A (and its structural derivatives) could open a novel class of KOPr agonists with potential anti-addiction effects (Prisinzano and Rothman, 2008; Munro et al., 2008; Beguin et al., 2008; Wee and Koob, 2010; Prisinzano, 2008; Prevatt-Smith and Prisinzano, 2010).

With this as the central hypothesis of this thesis, Sal A, its novel analogues (MOM Sal B, EOM Sal B, herkinorin, DS1) and MOPr antagonist/ partial KOPr agonist nalmefene were pharmacologically screened for their anti-addiction effects using the cocaine prime induced reinstatement model. Furthermore, the anti-cocaine profile of those KOPr compounds were determined which attenuated drug-seeking in a selective manner without producing motor suppression by themselves. This was performed by using cocaine induced enhancement and sensitization to motor function in rats.

The next aim of this thesis was to test for the selectivity of the attenuation of cocaine induced reinstatement. This was done by measuring sucrose reinforcement (attenuation in operant reinforcement) and cocaine induced hyperactivity in self-administering animals (motor suppression during reinstatement test). Another aim of this thesis was to evaluate the behavioural adverse effects of the KOPr agonists which were successful at attenuating cocaine reinstatement. This was achieved by using spontaneous open field activity (motor suppression), conditioned taste aversion (aversion) and forced swim test (depression) in rats.

The last part of this thesis applies behavioural and *in vitro* models to better understand the mechanism of KOPr mediated depression. This was achieved by investigating the effects of serotonin transporter (SERT) blockade on KOPr agonists induced depression and effect of KOPr activation on *in vitro* SERT function.

Chapter 2. Anti-addiction profile of KOPr agonists.

2.1. Introduction

2.1.1. Cocaine prime induced reinstatement model

The phenomenon of reinstatement of drug seeking in animals has been shown to mimic human conditions (Sinha et al., 1999; Littleton, 2000; Shalev et al., 2002; Shaham et al., 2003). Presentation of stimuli such as drug primes, environmental cues and stressors have all been shown to initiate drug-seeking in both animals and humans (Ludwig et al., 1974; De Wit and Stewart, 1981; 1983; Jaffe et al., 1989; Koob et al., 1996; McFarland and Ettenberg, 1997; Erb et al., 1996; Sinha et al., 1999; 2001; Weiss et al., 2001). Due to these reasons, pre-clinical models have been extensively used to study the neurobiology of drug addiction (Markau et al., 1993; Koob and La Moal, 2001, 2005; Koob, 2006; Olmstead, 2006). The reinstatement of cocaine self-administration model mimics the potential to relapse back to drug use following a period of extinction in laboratory animals (Stewart, 2000; Epstein et al., 2006; Shalev et al., 2002; Fuchs et al., 1998; Shaham et al., 2003). Therefore, this paradigm has been used extensively for the pharmacological evaluation of potential anti-addiction compounds (Schenk et al., 1999a; 1999b; Beardsley et al., 2005; 2010).

Reinstatement of drug-seeking via self-administration training has been performed pre-clinically using between session (Stretch et al., 1971), within session (de Wit and Stewart, 1981; 1983) and between-within session models (Tran-Nguyen et al., 1998). In between session reinstatement, animals undergo self-administration, extinction training sessions and reinstatement test on separate days (Stretch et al., 1971; Stewart, 2000). The within session reinstatement model animals self-administer cocaine for 1-2 hr followed by the extinction training (for 3-4 hr) and then testing for reinstatement with all the procedures performed in a single day (de Wit and Stewart, 1981; 1984). In between-within reinstatement test, animals are trained to self-administer

cocaine on different days, whereas extinction and reinstatement tests are performed on the same day (Tran-Nguyen et al., 1998; Shaham et al., 2003).

In the current study, the within session reinstatement paradigm was used (Worley et al., 1994; Schenk et al., 1999b; 2000a). This paradigm mimics the potential of priming injection of cocaine to reinstate cocaine self-administration in rats which were previously self-administering cocaine and were subjected to short period of extinction training (Stewart, 2000; Epstein et al., 2006). An advantage of this model is that it can be used to screen the effects of acute pharmacological exposure on cocaine seeking behaviour (Schenk et al., 1999b; 2000a; 2001b; Schenk, 2000; Schenk and Partridge, 2001; Morani et al., 2009). Furthermore, traditional KOPr agonists, U69593, U50488H and spiradoline have all been shown to attenuate reinstatement of drug-seeking using this paradigm (Schenk et al. 1999b, 2000a; Morani et al., 2009). Therefore, in order to characterize the effects of KOPr compounds on cocaine seeking behaviour, we performed an initial preliminary screening using the within session reinstatement paradigm in rats.

2.1.2. Cocaine induced enhancement in motor activity

Experimenter administered cocaine has been shown to produce increases in motor function in laboratory animals (Post and Contel, 1983; Kalivas and Stewart, 1991). A robust enhancement in motor function is also observed in animals with prior cocaine experience in which an acute challenge of injected cocaine reveals motor sensitization (Post and Contel, 1983; Pierce and Kalivas, 1997; Vanderschuren and Kalivas, 2000). This sensitized response persists even after a period of prolonged abstinence (Robinson and Berridge, 1993; 2001; 2003), thus indicating the long lasting alterations in central neuronal circuits following intermittent cocaine exposures (Robinson and Berridge, 1993; 2000; 2003; 2008; Pierce and Kalivas, 1997). Expression and development of behavioral sensitization is a paradigm that highlights

cocaine's ability to alter neural circuits underlying its psychomotor effects (Robinson and Berridge, 1993; 2001; 2003; 2008; Kalivas *et al.,* 1998; O'Brian et al., 1992; Todtenkopff et al., 2002; Lambert et al., 2006). Therefore, increasing our understanding of the factors which attenuate the expression and development of cocaine induced behavioural sensitization could prove useful in designing therapeutics for cocaine addiction (Pierce and Kalivas, 1997; Robinson and Berridge, 2003). Recently, Chartoff and colleagues (2008) showed that acute exposure to Sal A attenuates hyperactivity to a single injection of cocaine in a similar way to traditional KOPr agonists. In order to extend the findings of Chartoff et al. (2008), this study characterizes the anti-cocaine profile of Sal A and another novel KOPr agonist (DS1) on cocaine induced hyperactivity and expression of behavioural sensitization in rats. As forward locomotion during sensitization experiments does not augment with cocaine produced stereotypy (Ushijima *et al.,* 1995; Post *et al.,* 1987), we further analysed the effect of Sal A and DS1 on cocaine produced stereotypic counts in sensitized rats.

2.1.3. Aims

The novel non-nitrogenous structure of Sal A has led to the synthesis of many KOPr agonists with unknown pharmacology (Prisinzano and Rothman, 2008; Munro et al., 2008; Section 1.6; Chapter 1). This chapter aims to determine if these novel compounds have anti-addiction pharmacology. To do this we will:

1. Screen the effect of novel KOPr compounds Sal A, DS1, MOM Sal B, EOM Sal B, herkinorin and MOPr antagonist/partial KOPr agonist, nalmefene on cocaine prime induced drug-seeking in rats.

2. Determine if the effects seen are in fact KOPr mediated. This will be performed by measuring the behavioural effect of pre-treatment with selective KOPr antagonist (nor-BNI; Portoghese et al., 1987) on KOPr agonist mediated attenuation in drug-seeking.

3. Compounds shown to attenuate drug-seeking in a specific manner without suppressing motor function by themselves (Chapter 3) will be further characterized behaviourally to determine their effects on cocaine induced enhancement in motor function (Heidbreder et al., 1993; Collins et al., 2001a; 2001b). This will be determined experimentally by measuring changes in cocaine induced hyperactivity, expression of behavioural sensitization and cocaine produced stereotypy in rats.

2.2. Methods

2.2.1. Subjects

Male Sprague-Dawley rats weighing 325-350 g (for reinstatement test; one per cage) and 200-250 g (for cocaine induced hyperactivity and behavioural sensitization tests; two per cage) were housed in a temperature (19-21°C) and humidity (55% relative humidity) controlled room at the animal facility in the School of Psychology, Victoria University of Wellington. Lights were maintained on a 12:12 hr cycle with lights on at 0700. Animals had free access to food and water except during experimental sessions. All rats were housed in the animal facility five days prior the commencement of experiments and were handled daily to reduce handling stress. All experimental procedures were reviewed and approved by the Animal Ethics Committee of Victoria University of Wellington.

2.2.2 Drugs

Cocaine HCl (Merck Pharmaceuticals, Palmerston North, New Zealand), nor-BNI and nalmefene (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in physiological saline. Sal A, MOM Sal B, EOM Sal B, DS1 and herkinorin (gifts from Dr. Thomas E. Prisinzano, University of Kansas) were suspended in 75% DMSO.

Subcutaneous (sc) or intraperitoneal (ip) injections were in a volume of 1 ml/kg. IV infusions were given in the volume of 100 µl. All drug weights refer to the salt.

2.2.3. Cocaine prime induced reinstatement test

2.2.3.1. Surgery

The surgery was performed by Mr. Caleb Carati, Mr. Alex Howard and Mr. Alex Crowther. Under deep anaesthesia produced by ketamine/xylazine (90/9 mg/kg, ip; Provet, Palmerston North, New Zealand), the right jugular vein was isolated and the catheter was inserted. The distal end (22 gauge stainless steel tubing) was passed sc to an exposed portion of the skull where it was fixed to embedded jeweller's screws with dental acrylic. The catheters were infused daily with 0.1 mL of a sterile saline solution containing heparin (30.0 U/mL; Provet, Palmerston North), penicillin G Potassium (250,000 U/mL; Provet, Palmerston North) and streptokinase (8000 U/mL; Health Care Logistics, Auckland) to prevent infection and the formation of clots and fibroids. The rats were allowed five days post surgery to recover.

2.2.3.2. Apparatus

Self-administration training and reinstatement testing were carried out in humidity (55%) and temperature (19-21°C) controlled environment in standard operant chambers (Med Associates, ENV-001) equipped with 2 levers. Depression of one lever (the active lever) resulted in a 0.l mL iv infusion of cocaine HCl dissolved in sterile physiological saline containing heparin (3.0 U/mL). Infusions were of 12 sec duration. Coincident with drug delivery was the illumination of a stimulus light located directly above the active lever. This stimulus light remained illuminated throughout each 12 sec infusion. Depression of the other lever (the inactive lever) was without programmed consequence.

Rats were maintained in the animal colony except during testing. Immediately prior to each daily self-administration session, the catheter lines were infused with 0.1 mL of heparin-penicillin-streptokinase solution. The stainless steel catheter was connected to a length of microbore tubing and connected to the syringe. At the end of each session, the lines were again infused with 0.1 mL of the heparin-penicillin-streptokinase solution, the stainless steel tubing was plugged and the animal was returned to its home cage. Drug delivery and data acquisition were controlled using Med Associates software (St. Albans, VA, USA). Cocaine deliveries were made via mechanical pumps (Razel, Model A with 1.0 rpm motor equipped with 20 mL syringe).

2.2.3.3. Cocaine self-administration training

The cocaine self-administration protocols we followed have been described by Worley et al, (1994), Schenk et al, (1999b) and Morani et al., (2009). Rats were initially trained to self-administer cocaine using a FR-1 schedule of reinforcement (pressing the active lever once delivered a single infusion of cocaine, 0.5 mg/kg/infusion) for 2 hr. The self-administration training was performed in the dark using light proof boxes. Once stable responding was achieved (at least 20 infusions per 2 hr session for 3 consecutive days), they were upgraded to FR-5 schedule of reinforcement, where in pressing the active lever five times resulted in a single infusion of cocaine (0.5 mg/kg/infusion). Self-administration was considered to be acquired when there was less than 20% variation in responding for three consecutive days.

2.2.3.4. Cocaine prime induced reinstatement test.

Once responding on the FR-5 schedule was stable, the effect of KOPr agonist (Sal A, MOM Sal B, EOM Sal B, DS1, herkinorin and nalmefene) administration on drug-seeking produced by a priming injection of cocaine was measured. This test was conducted in a single day and consisted of three phases. The first phase comprised a one hr period of cocaine self-administration (0.5 mg/kg/infusion, FR-5 schedule of

reinforcement) in which the light stimulus was paired with cocaine infusions. After the one hr self-administration period, the cocaine solution was replaced with heparinised saline and responding was reinforced with the vehicle infusion (FR-5 schedule of reinforcement) coupled with illumination of the light stimulus. Saline reinforced responding was measured during the second phase which was of 3 hr duration. By the end of the 3rd hr, animals which showed less than 20% of responding as compared to phase 1 were subjected to the reinstatement test. At the beginning of this third phase, separate groups of rats (n=5-10) received an injection of either vehicle (0.9% saline or 75% DMSO) or KOPr agonists Sal A (0.1, 0.3 and 1.0 mg/kg, ip); MOM Sal B (0.03, 0.1 and 0.3 mg/kg, ip); EOM Sal B (0.03, 0.1 and 0.3 mg/kg, ip); DS1 (0.1, 0.3 and 1.0 mg/kg, ip); herkinorin (0.1, 0.3 and 1.0 mg/kg, ip) or nalmefene (0.1, 0.3 and 1.0 mg/kg, sc). These injections were administered either 5 (Sal A, MOM Sal B, EOM Sal B, herkinorin), 15 (nalmefene) or 45 (DS1) min prior to an injection of cocaine (20 mg/kg, ip). This was followed by measuring saline reinforced responding at FR-5 schedule of reinforcement with an infusion of vehicle solution (heparinised saline) and illumination of the light stimulus. Responding was measured for 1 hr following the cocaine injection. Operant lever press responding observed during the three phases of the reinstatement test is shown in Fig 2.1.

The vehicle used to suspend Sal A and its derivatives was selected based on previous studies showing that 75% DMSO was well tolerated by rats (Willmore-Fordham et al., 2007; Baker et al., 2009; Chartoff et al., 2008; Morani et al., 2009). Sal A, MOM Sal B and EOM Sal B have a quick onset of action via the ip route of administration (Hooker et al., 2009a; Baker et al., 2009; Wang et al., 2008). Also, previous studies with herkinorin suggest a quick onset of action *in vivo* (Butelman et al., 2008). Therefore, the pre-treatment time was chosen as 5 min for Sal A, MOM Sal B, EOM Sal B and herkinorin. The route of administration (sc) and pre-treatment time (15

min) for nalmefene was selected based on previously published reports (June et al., 1998).

2.2.3.5. KOPr antagonist reversal experiment

The effect of 30 min pre-treatment with selective KOPr antagonist, nor-BNI (2 mg/kg, ip) on attenuation of cocaine prime induced drug-seeking by the KOPr agonists was also performed. This experiment was performed in order to verify that attenuation of reinstatement behaviour by the novel KOPr compounds was mediated via KOPr activation. Also, this experiment was performed on KOPr compounds using a minimum effective dose (0.3 mg/kg) at which they attenuated drug-seeking behaviour in a specific manner (Sal A, DS1, nalmefene; Chapter 3). The experimental conditions were similar to the reinstatement test, except that rats (n = 5-10 for each group) received an injection of nor-BNI (0, 2 mg/kg, ip) 30 min prior to the exposure to Sal A (0.3 mg/kg, ip), DS1 (0.3 mg/kg, ip) or nalmefene (0.3 mg/kg, sc). Saline-reinforced responding was recorded following the priming injection of cocaine (20 mg/kg, ip). The dose (2 mg/kg), route of administration (ip) and pre-treatment time (30 min) for nor-BNI was also selected based on previous reports (Xu et al., 2006).

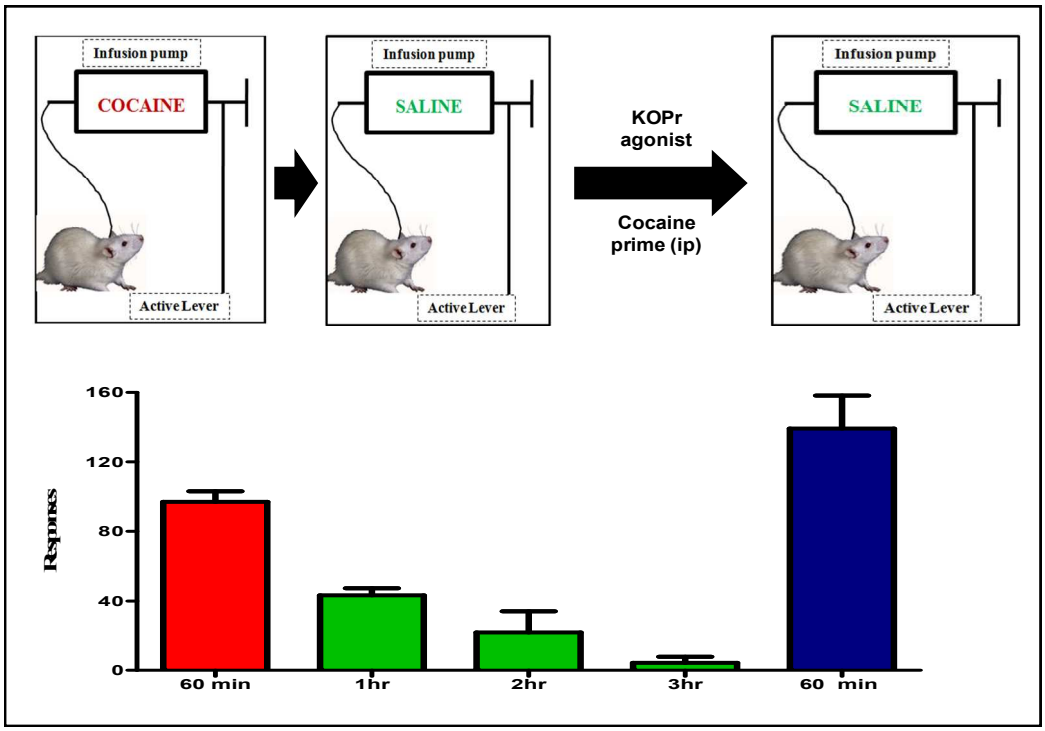


Fig 2.1. Cocaine prime induced reinstatement test.

Animals previously self-administering cocaine stably at a FR-5 schedule of reinforcement were subjected to the reinstatement test. During the first phase, rats were allowed to self administer cocaine (0.5 mg/ml/infusion) for 1 hr. Corresponding active lever presses were observed to be high. During the second phase, cocaine was replaced by saline in the infusion pump. This resulted in extinguished lever press responding by the end of the 3rd hr. Prior to the beginning of the third phase, animals received either KOPr agonist or corresponding vehicle and a single priming injection of cocaine (20 mg/kg, ip) and saline reinforced active lever responding was measured for 1 hr. In the above figure, the high rate of responding during the third phase is due to the priming injection of cocaine (20 mg/kg) as the infusion pump contained only heparinised saline. Therefore, this reinstatement is called cocaine prime induced reinstatement of cocaine seeking following a short period of extinction (n=8).

2.2.4. Cocaine induced hyperactivity and behavioural sensitization

Cocaine induced hyperactivity and behavioural sensitization tests were performed to further explore the anti-cocaine effects of novel KOPr agonists which showed attenuation of drug-seeking behaviour in a specific manner without suppressing motor function (Chapter 3).

2.2.4.1. Apparatus

Eight open field chambers (Med Associates, ENV-520) equipped with two banks of sixteen photocells on each wall were used to measure horizontal and vertical locomotion. The open field boxes were interfaced with an adjacent microcomputer. Stereotypic counts during the sensitization experiments were determined by measuring repetitive beam breaks obtained from the activity monitoring software (Med Associates). Testing was conducted in the dark between 1000 and 1600 hr. White noise was continually present to mask extraneous disturbances. The floor and the sides of the test chamber were cleaned before and after testing each animal using Virkon "S" disinfectant (Southern Veterinary Supplies, Palmerston North, New Zealand).

2.2.4.2. Procedure

A. Cocaine induced hyperactivity in drug naive rats.

For this study we modified the methods of Frankowska et al., (2009) and Xu et al., (2010). On the test day, drug naive rats received an injection of either vehicle (75% DMSO) or KOPr compound (Sal A, 0.3 mg/kg; DS1, 0.3 mg/kg). Five minutes later (or 45 min following DS1 injection) this was followed by an injection of either 0.9% saline (1 mL/kg) or cocaine (20 mg/kg, ip). Immediately following the second injection, total activity, a compilation of horizontal and vertical activity was measured every 5 min for 60 min.

B. Expression of cocaine sensitization and stereotypy.

For this study we modified the methods described by Frankowska et al., (2009) and Xu et al., (2010). Rats received a single injection of either 0.9% saline (1 mL/kg) or cocaine (20 mg/kg, ip) for 5 consecutive days (day 1-5) in their home cages. On days 6-9 the animals remained drug-free in their home cages. On the test day (day 10), animals first received KOPr compounds (Sal A, DS1) or vehicle (75% DMSO) followed by a single injection of cocaine (20 mg/kg, ip). Locomotor activity (total ambulatory counts) and cocaine produced stereotypy was measured at 5 min intervals for 60 min.

Tab 2.1 Experimental groups for cocaine induced hyperactivity.

Acute Pre-treatment (ip)	Challenge injection (ip)
Vehicle	Saline (1 mL/kg)
Sal A	Saline (1 mL/kg)
DS1	Saline (1 mL/kg)
Vehicle	Cocaine (20 mg/kg)
Sal A	Cocaine (20 mg/kg)
DS1	Cocaine (20 mg/kg)

Vehicle (75% DMSO; 1 mL/kg); Sal A (0.3 mg/kg), DS1 (0.3 mg/kg)

Tab 2.2 Experimental groups for cocaine behavioural sensitization.

Pre-treatment (day 1-5; ip)	Acute treatment (day 10; ip)	Challenge injection (day 10; ip)
Saline (1 mL/kg)	Vehicle	Cocaine (20 mg/kg)
Saline (1 mL/kg)	Sal A	Cocaine (20 mg/kg)
Saline (1 mL/kg)	DS1	Cocaine (20 mg/kg)
Cocaine (20 mg/kg)	Vehicle	Cocaine (20 mg/kg)
Cocaine (20 mg/kg)	Sal A	Cocaine (20 mg/kg)
Cocaine (20 mg/kg)	DS1	Cocaine (20 mg/kg)

Vehicle (75% DMSO; 1 mL/kg); Sal A (0.3 mg/kg), DS1 (0.3 mg/kg)

2.2.5. Statistical analysis

Data analyses were performed by using Prism Graphpad software (Version 5.0, San Diego, CA). Statistical analysis for cocaine induced locomotion and cocaine sensitization studies (for total ambulatory counts) were performed using one-way ANOVA followed by Tukey post-hoc tests. Further statistical analysis was performed using two-way ANOVA (treatment x time) with repeated measures on time for cocaine induced hyperactivity, expression of sensitization and cocaine induced stereotypic counts. A Bonferroni post hoc test was also performed. The number of active lever responses during phase 3 of the reinstatement phase is shown as mean +SEM. For cocaine induced reinstatement tests separate one-way ANOVA was performed followed by Tukey post-hoc test (Morani et al., 2009). Student t-tests were performed on the data from nor-BNI reversal experiments.

2.3. Results

2.3.1. Effect of Sal A on cocaine prime induced reinstatement.

The effect of Sal A (0, 0.1, 0.3, 1.0 mg/kg, ip) pre-treatment on cocaine prime (20 mg/kg, ip) induced reinstatement in rats is shown in Fig 2.2. Statistical analysis showed that pre-treatment with Sal A (0.3, 1.0 mg/kg) significantly attenuated cocaine prime induced reinstatement [$F(3,23) = 79.33$, $p<0.0001$; Fig 2.2a] in a dose dependent manner. This attenuation was not seen in animals pre-treated with the KOPr antagonist nor-BNI (0, 2.0 mg/kg, ip) ($p<0.001$; Fig. 2.2b).

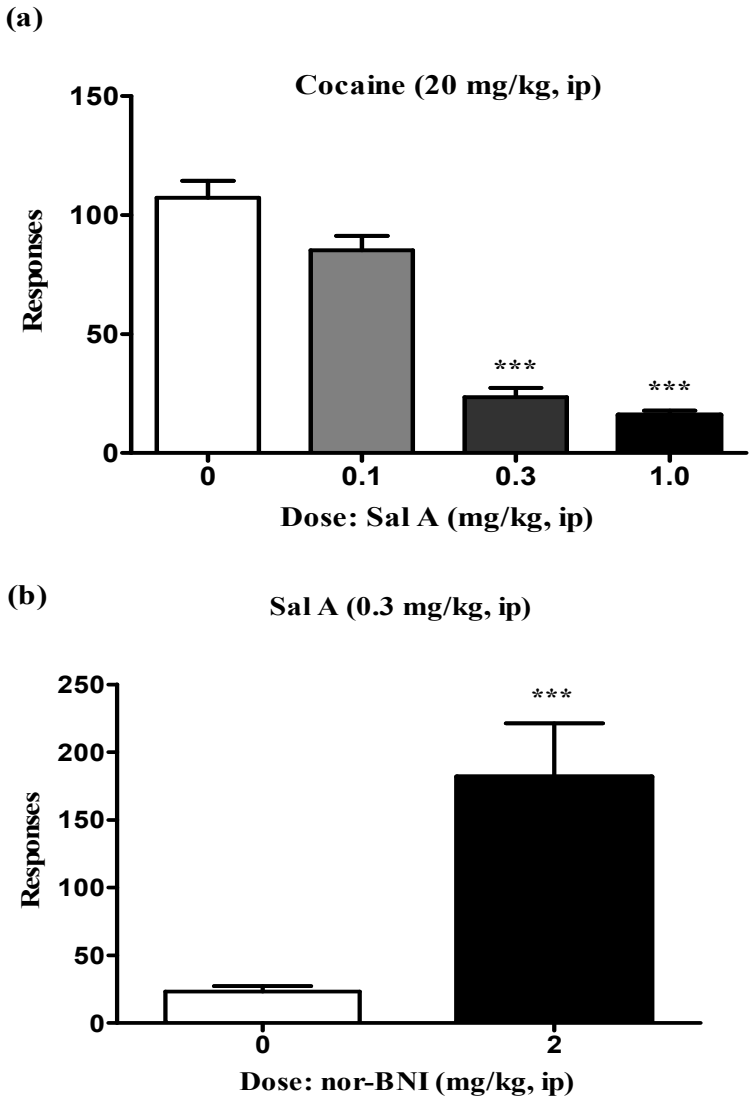


Fig. 2.2 Effect of Sal A on cocaine prime induced reinstatement

*Symbols indicate active lever press responses +SEM produced during phase 3 of the reinstatement test. (a) Rats which had shown extinguished saline reinforcement during the extinction phase were initially treated with different doses of Sal A (0, 0.1, 0.3 and 1.0 mg/kg) followed by a priming injection of cocaine (20 mg/kg) and saline reinforced responses were recorded for a period of 60 min. ***p<0.001, data were compared with vehicle treated group (0 mg/kg). One-way ANOVA followed by Tukey post hoc test. n=6-7 for each group (b) For nor-BNI reversal experiments, animals were first treated with nor-BNI (0, 2.0 mg/kg) followed by Sal A (0.3 mg/kg) and cocaine (20 mg/kg) injections.***p<0.001, Student t-tests. n=5-7 for each group.*

2.3.2. Effect of Sal A on cocaine induced hyperactivity (drug naïve animals)

The effect of Sal A (0, 0.3 mg/kg, ip) pre-treatment on cocaine (0, 20 mg/kg, ip) induced hyperactivity in rats is shown in Fig 2.3. A significant increase in the locomotion activity was observed in animals pre-treated with vehicle/cocaine as compared to those which were treated with vehicle/saline ($p<0.01$) demonstrating cocaine induced hyperactivity in rats [$F (3, 22) = 22.2$, $P < 0.0001$; Fig. 2.3a]. Post hoc analysis indicated no significant difference between Sal A and vehicle pre-treatment on the locomotor activity produced following the saline ($P > 0.05$) or cocaine ($P > 0.05$) injection (Fig 2.3). A non-significant trend towards an increase in total activity was observed in Sal A/cocaine treated rats as compared to vehicle/cocaine treated rats ($P > 0.05$). Further time course comparison revealed a significant treatment [$F (3, 364) = 101.4$, $P < 0.0001$] and interaction [$F (33,364) = 3.28$, $P < 0.0001$] effect (Fig 2.3b). Post-hoc tests showed a significant increase in activity for Sal A/Coc treated group vs. Veh/Coc treated group of animals 5, 10 and 15 min following cocaine injection ($P < 0.05$).

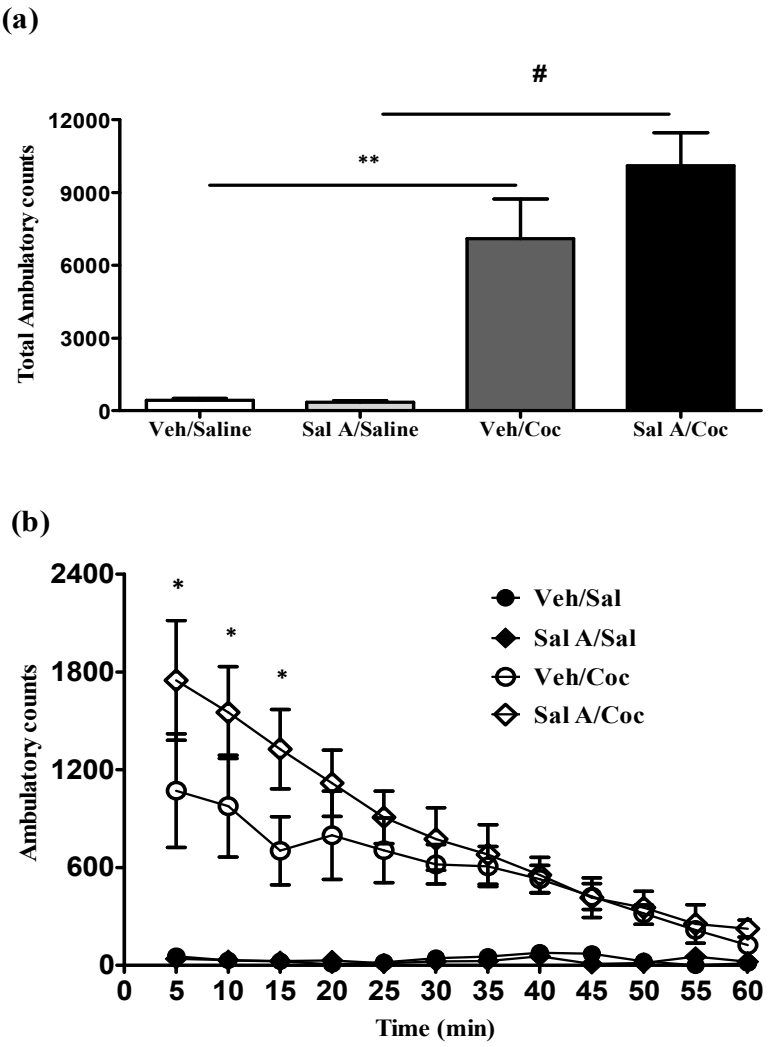


Fig. 2.3 Effect of Sal A on cocaine induced hyperactivity (drug naive animals).

*Drug naive rats were initially injected with either vehicle (75% DMSO) or Sal A (0.3 mg/kg) followed by saline (1 mL/kg) or cocaine (Coc; 20 mg/kg) and locomotion activity was measured for 60 min. (a) Figures represents mean total activity +SEM. **p<0.01, data compared with Vehicle/Saline treated group, #p<0.01, data compared with Sal A/Saline treated group. One-way ANOVA followed by Tukey post hoc test. (b) Activity counts (± SEM) measured after 5 min interval. *p<0.05, data compared with Veh/Coc treated group. Repeated measures two-way ANOVA followed by Bonferroni post-hoc test. n= 6-7 for each group.*

2.3.3. Effect of Sal A on expression of cocaine sensitization and stereotypy.

The effect of Sal A (0, 0.3 mg/kg, ip) pre-treatment on expression of cocaine behavioural sensitization in rats is shown in Fig 2.4. Animals were pre-treated with cocaine on days 1-5 and then on day 10 exposed to vehicle followed by a cocaine injection, showed a significant increase in locomotion as compared to the group of animals which received saline from day 1-5, demonstrating the expression of cocaine-induced sensitization [$F(3,23) = 4.45$, $P = 0.013$; Fig. 2.4a]. Post-hoc tests revealed no measurable difference in activity between animals exposed to Sal A (0.3 mg/kg) on the test day than those receiving either saline or cocaine from day 1-5 ($P > 0.05$; Fig. 2.4a). However, a further time course analysis showed a significant treatment [$F(3,276) = 23.18$, $P < 0.0001$] and interaction [$F(33,276) = 3.61$, $P < 0.0001$] effect (Fig. 2.4b). Post hoc tests showed a significant reduction in locomotion activity in Sal A pre-treated rats vs. vehicle treated rats at 5, 10, 15 and 20 min following a single cocaine injection ($P<0.05$; Fig.2.4b). Time course analysis on cocaine induced stereotypic counts showed significant treatment effect [$F(3, 276) = 3.99$, $P < 0.01$] but no interaction effect [$F(33, 276) = 1.2$, $P = 0.24$]. Bonferroni post-hoc tests showed no significant effect on cocaine induced stereotypic counts following Sal A pre-treatment in cocaine sensitized animals ($P > 0.05$; Fig 2.4c). Sal A pre-treatment however showed a trend towards an increase in cocaine produced stereotypic counts in sensitized animals 5, 10 and 15 min post cocaine treatment compared to vehicle treated animals ($P > 0.05$; Fig 2.4c).

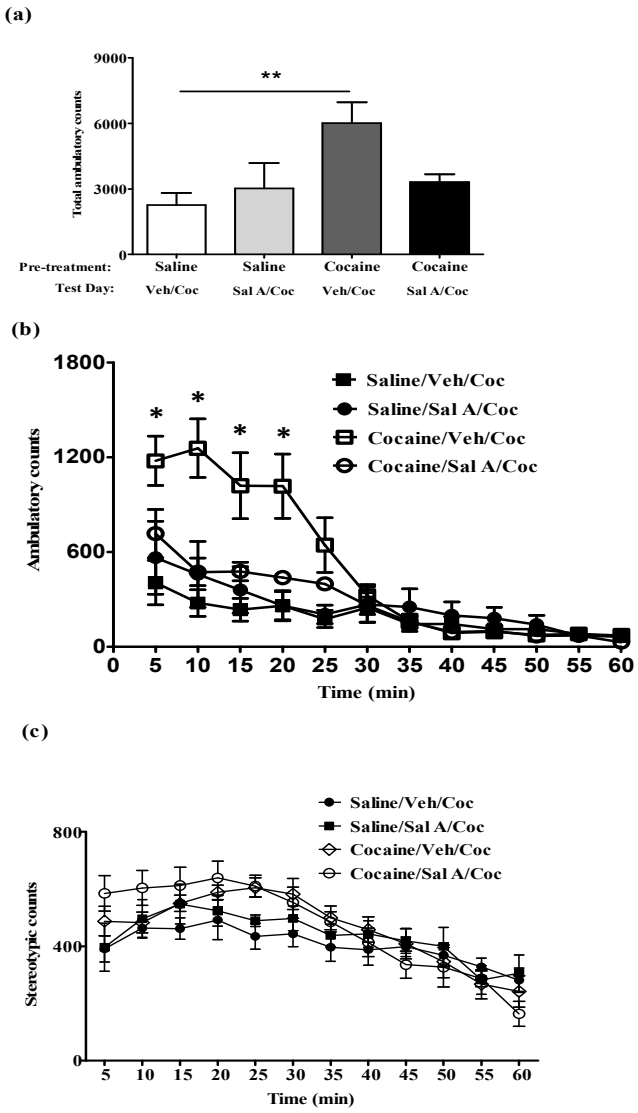


Fig. 2.4 Effect of Sal A on expression of cocaine sensitization and stereotypy.

*Rats were injected with saline (1 mL/kg) or cocaine (20 mg/kg) for 5 consecutive days. Animals remained drug free from day 6-9. On day 10, rats were injected with either vehicle (Veh, 75% DMSO) or Sal A (0.3 mg/kg) and 5 min later were injected with cocaine (Coc, 20 mg/kg) and activity was measured for 60 min. (a) Data expressed as mean total ambulations (+SEM).**p<0.01, data compared with Saline/Veh/Coc treated group. One-way ANOVA followed by Tukey test. (b) Time course measurement of mean (±SEM) of locomotion activity over a period of 5 min interval. *p<0.05, vs. Cocaine/Sal A/Coc treated group. Repeated measures two-way ANOVA followed by Bonferroni post hoc test. (c) Time course measurement of mean (±SEM) stereotypic counts every 5 min for 60 min. Repeated measures two-way ANOVA followed by Bonferroni post hoc test. n= 6-8 for each group.*

2.3.4. Preliminary study to determine pre-treatment time for DS1

Initial studies were carried out to determine the time taken for DS1 to have an effect on laboratory rat behaviour. This was ascertained by measuring the effect of DS1 on cocaine induced hyperactivity. Three separate groups of drug naive rats were pre-treated with DS1 (0.3 mg/kg, ip) for 5, 30 or 45 min prior to the injection of cocaine (20 mg/kg) and total ambulatory counts as well as activity after every 5 min interval was measured for 1 hr (Fig. 2.5). The results indicate that DS1, when injected 5 min or 30 min prior to cocaine, had no significant effect on total activity compared to the vehicle controls (75% DMSO; 1 mL/kg, ip). However, a significant suppression in cocaine induced hyperactivity was observed when DS1 was injected 45 min prior to cocaine exposure [$F(3,14) = 5.28$, $p<0.012$; Fig. 2.5a]. A further time course analysis showed that DS1 (administered 45 min prior to cocaine) significantly attenuated locomotor activity at 5, 10 and 20 min following cocaine ($p<0.05$) when compared to vehicle pre-treated controls [$F(3,168) = 23.64$, $P < 0.0001$; Fig.2.5b].

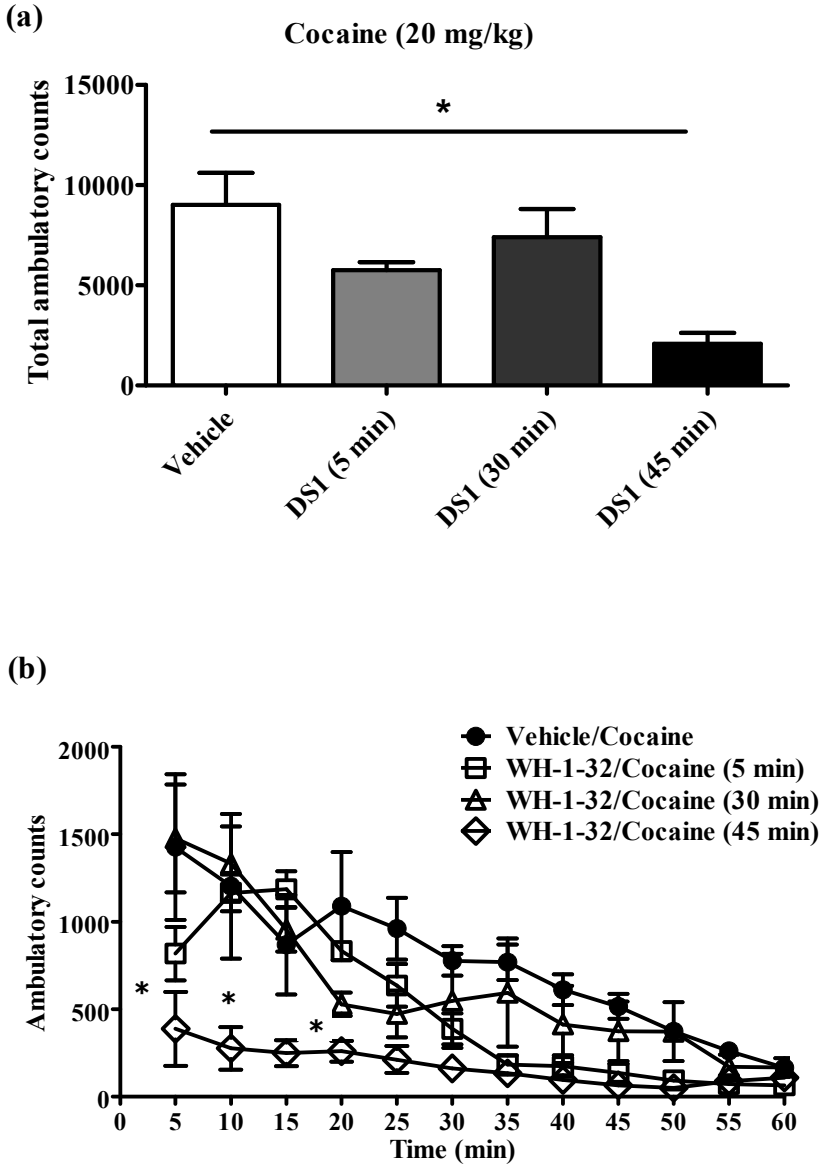


Fig. 2.5. Preliminary study to determine pre-treatment time for DS1.

*Rats initially received vehicle (75% DMSO) or DS1 (0.3 mg/kg) 5 min, 30 min or 45 min prior to an injection of cocaine (20 mg/kg) and locomotion was monitored for 60 min. a) Data are expressed as mean total activity (+SEM). *p<0.05, data compared with Vehicle/Cocaine treated group, One-way ANOVA followed by Tukey test. b) Data expressed as mean (± SEM) for locomotor activity measured at 5 min intervals. *p<0.05, for Vehicle/Cocaine vs. DS1/Cocaine (45 min) treated groups. Repeated measures Two-way ANOVA followed by Bonferroni post hoc test. n= 4-6 for each group.*

2.3.5. Effect of DS1 on cocaine prime induced reinstatement.

The effect of various dose of DS1 (0, 0.1, 0.3 and 1.0 mg/kg, ip) on cocaine (20 mg/kg, ip) induced drug-seeking is shown in Fig 2.6. Statistical analysis showed that pre-treatment with DS1 (0.3, 1.0 mg/kg) attenuated cocaine prime induced reinstatement in a dose dependent manner [$F(3,26) = 36.82$, $p<0.0001$; Fig 2.6a]. However, a trend towards increased active lever press responses was noted in animals treated with low dose DS1 (0.1 mg/kg), which was non-significant. The KOPr antagonist pre-treatment significantly reversed DS1 induced attenuation of cocaine reinstatement ($p<0.001$; Fig. 2.6b).

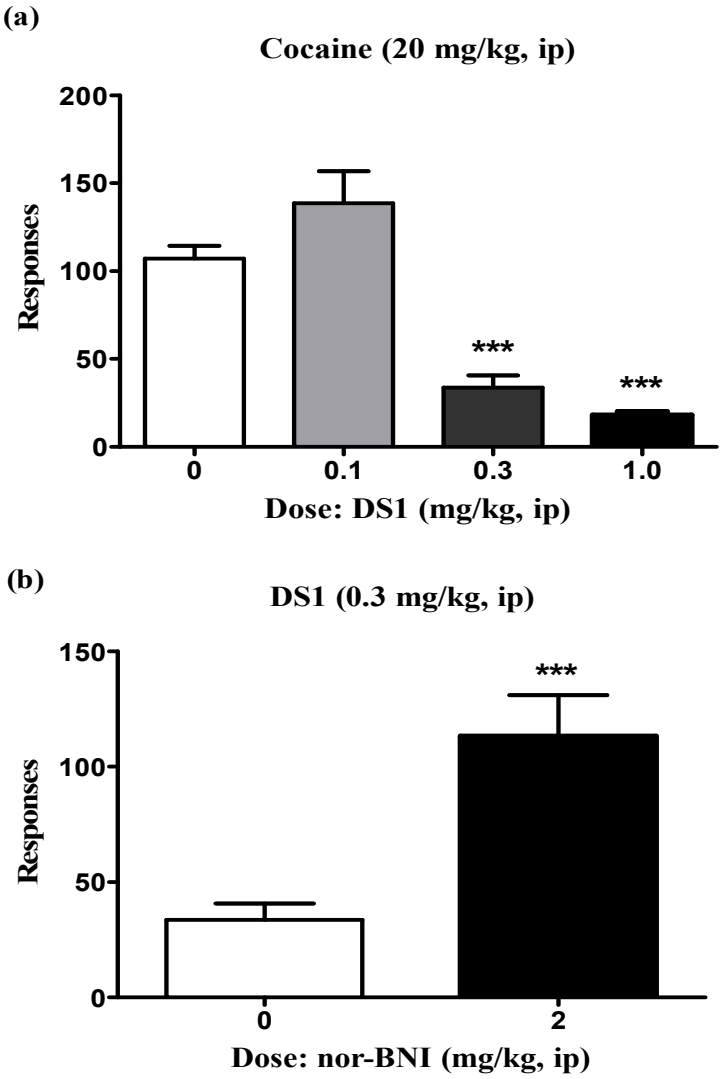


Fig. 2.6. Effect of DS1 on cocaine prime induced reinstatement.

*Bars indicate active lever press responses +SEM produced during phase 3 of the reinstatement test. (a) Rats which had shown extinguished responding during the extinction phase were initially treated with different doses of DS1 (0, 0.1, 0.3 and 1.0 mg/kg) followed by a priming injection of cocaine (20 mg/kg) and saline reinforced responses were recorded for a period of 60 min. ***p<0.001, data compared with vehicle treated group (0 mg/kg). One-way ANOVA followed by Tukey post hoc test. n=6-10 for each group. (b) For nor-BNI reversal experiments, animals were first treated with nor-BNI (0, 2.0 mg/kg) followed by DS1 (0.3 mg/kg) and cocaine (20 mg/kg) injections. ***p<0.001, Student t-tests. n=6-10 for each group.*

2.3.6. Effect of DS1 on cocaine induced hyperactivity (drug naïve animals)

The effect of acute exposure to DS1 (0, 0.3 mg/kg) on cocaine (0, 20 mg/kg) induced hyperactivity is shown in Fig 2.7. A significant increase in the locomotion activity was observed in animals pre-treated with vehicle/cocaine as compared to those which were treated with vehicle/saline ($p<0.001$) indicating cocaine induced hyperactivity in rats [$F(3,26) = 14.68$, $P<0.0001$; Fig. 2.7a]. Post hoc analysis indicated no significant difference between DS1 and vehicle pre-treatment on the locomotor activity produced following saline treatment ($P>0.05$; Fig 2.7a). However, an increase in total activity was observed in rats pre-treated with vehicle followed by an injection of cocaine (20 mg/kg) which was significantly attenuated by DS1 pre-treatment ($p<0.01$; Fig 2.9a). Also, a further time course comparison between DS1 and vehicle treated groups showed that DS1 pre-treatment significantly suppressed locomotion 10, 20, 25, 30, 35 and 40 min ($p<0.05$) following cocaine injection vs. vehicle treated group [treatment effect: $F(3,276) = 88.3$, $P<0.0001$; interaction effect: $F(33,276) = 2.2$, $P = 0.0003$; Fig. 2.7b).

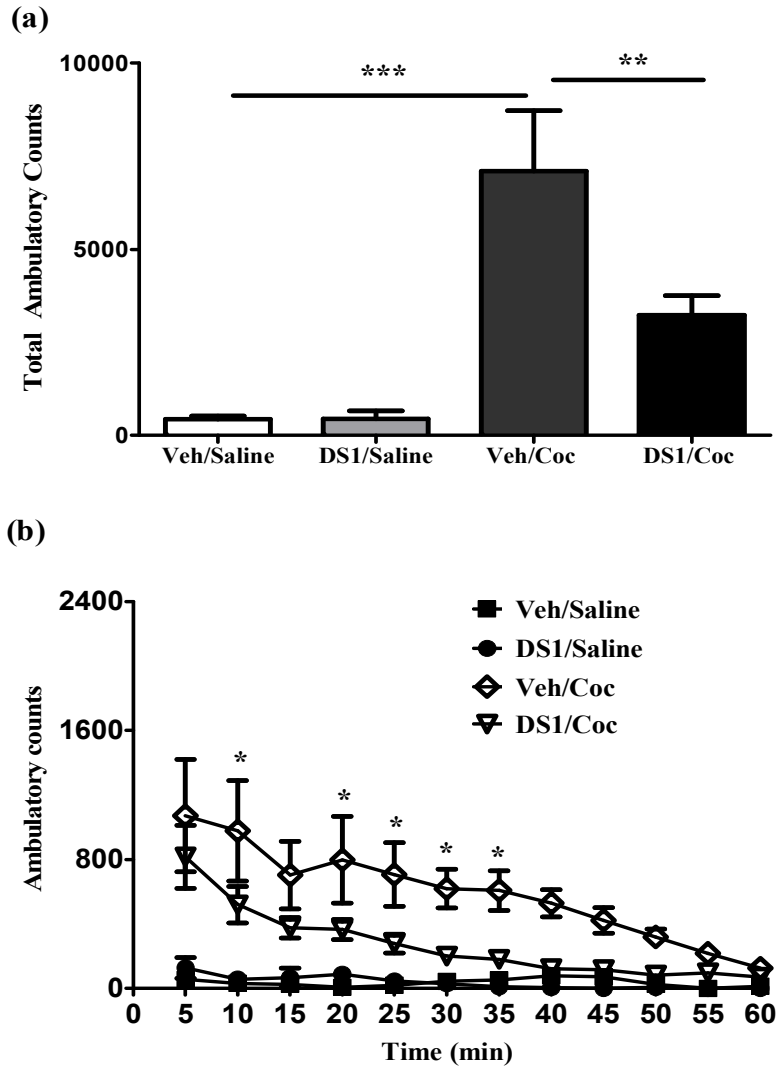


Fig. 2.7. Effect of DS1 on cocaine induced hyperactivity (drug naïve rats).

*Drug naive rats were initially injected with either vehicle (Veh; 75% DMSO) or DS1 (0.3 mg/kg) followed by saline (1 mL/kg) or cocaine (Coc; 20 mg/kg) and total locomotion activity was monitored for 60 min. a) Figures represents mean total activity +SEM. ***p<0.001, Veh/Saline vs. Veh/Coc treated group, **p<0.01, Veh/Coc vs. DS1/Coc treated group. One-way ANOVA followed by Tukey post hoc test. b) Data expressed as mean ±SEM for locomotor activity measured at 5 min intervals. *p<0.05, for Veh/Coc vs. DS1/Coc treated groups. Repeated measures Two-way ANOVA followed by Bonferroni post hoc test. n= 6-8 for each group.*

2.3.7. Effect of DS1 on expression of cocaine sensitization.

The effect of DS1 (0, 0.3 mg/kg, ip) exposure on the expression of cocaine sensitization in rats is shown in Fig 2.8. Animals that were pre-treated with cocaine on days 1-5 and exposed to vehicle followed by a cocaine injection on day 10, showed a significant increase in locomotion compared to the group of animals which received saline from day 1-5, indicating the expression of cocaine sensitization [$F(3,26) = 6.2$, $P=0.0031$; Fig 2.8a]. Post-hoc tests showed no measurable difference in activity between animals exposed to DS1 on the test day after recieving either saline or cocaine on days 1-5 ($P>0.05$; Fig. 2.8a). However, a significant reduction in cocaine induced hyperactivity was observed in DS1 pre-treated animals vs. vehicle treated animals, all of which received cocaine on days 1-5 ($p<0.05$; Fig 2.8a). Analysis of time course data also showed a significant treatment [$F(3,276) = 25.39$, $P < 0.0001$] and interaction [$F(33,276) = 3.84$, $P < 0.0001$] effect (Fig. 2.8b). Post hoc tests showed a significant reduction in locomotion activity in DS1 pre-treated rats vs. the vehicle treated group at 5, 10, 15 and 20 min following a single cocaine injection ($P < 0.05$). Statistical analysis for cocaine induced stereotypy showed significant treatment effect [$F(3, 276) = 3.6$, $P < 0.05$] but no interaction effect [$F(33, 276) = 1.37$, $P = 0.24$]. Post-hoc tests showed no significant effect on cocaine induced stereotypic counts following DS1 pre-treatment vs. vehicle pre-treatment in cocaine sensitized animals ($P > 0.05$; Fig 2.8c). However, DS1 pre-treatment showed a trend towards an increase in cocaine produced stereotypic counts in sensitized animals 5, 10 and 15 min post cocaine treatment (Fig 2.8c).

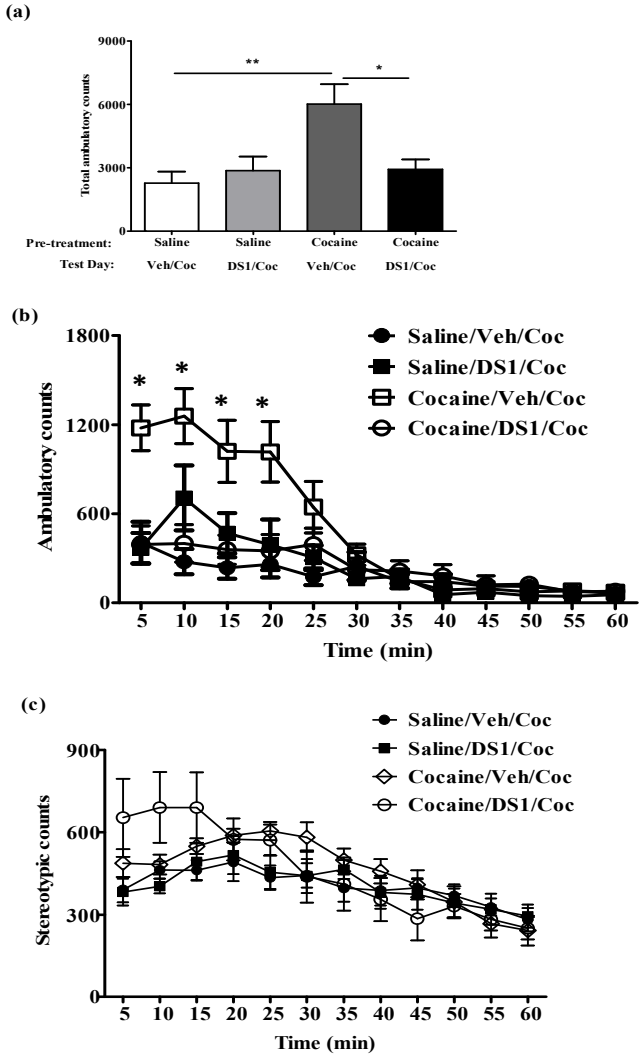


Fig. 2.8. Effect of DS1 on expression of cocaine sensitization.

*Rats were injected with saline (1 mL/kg) or cocaine (20 mg/kg) for 5 consecutive days. Animals remained drug free from day 6-9. On day 10, rats were injected with either vehicle (Veh, 75% DMSO) or DS1 (0.3 mg/kg) and 45 min later were injected with cocaine (Coc, 20 mg/kg) and activity was measured for 60 min. a) Data are expressed as mean total activity +SEM. **p<0.01 Saline/Veh/Coc vs. Cocaine/Veh/Coc treated group, *p<0.05 Cocaine/Veh/Coc vs. Cocaine/DS1/Coc treated group. One-way ANOVA followed by Tukey post hoc test. b) Time course measurement of mean ±SEM of locomotion activity over 5 min intervals. *p<0.05 Cocaine/Veh/Coc vs. Cocaine/DS1/Coc treated group. Two-way ANOVA followed by Bonferroni post hoc test. n= 6-8 for each group. (c) Time course measurement of mean (±SEM) stereotypic counts for a period of 60 min. Repeated measures two-way ANOVA followed by Bonferroni post hoc test. n= 6-8 for each group.*

2.3.8. Effect of MOM Sal B and EOM Sal B on cocaine prime induced reinstatement

Effect of MOM Sal B and EOM Sal B on cocaine prime induced drug-seeking is shown in Fig 2.9. Statistical analysis showed that MOM Sal B (0.3 mg/kg) significantly attenuated cocaine prime induced reinstatement [$F(3,17) = 4.71$, $p<0.05$; Fig 2.9a]. However, no significant decrease in cocaine prime induced lever press responding was noted following EOM Sal B exposure [$F(3,17) = 2.427$, $p = 0.989$; Fig 2.9b]. A non-significant trend towards increased active lever press responding was noted with MOM Sal B (0.03, 0.1 mg/kg) and EOM Sal B (0.1, 0.3 mg/kg).

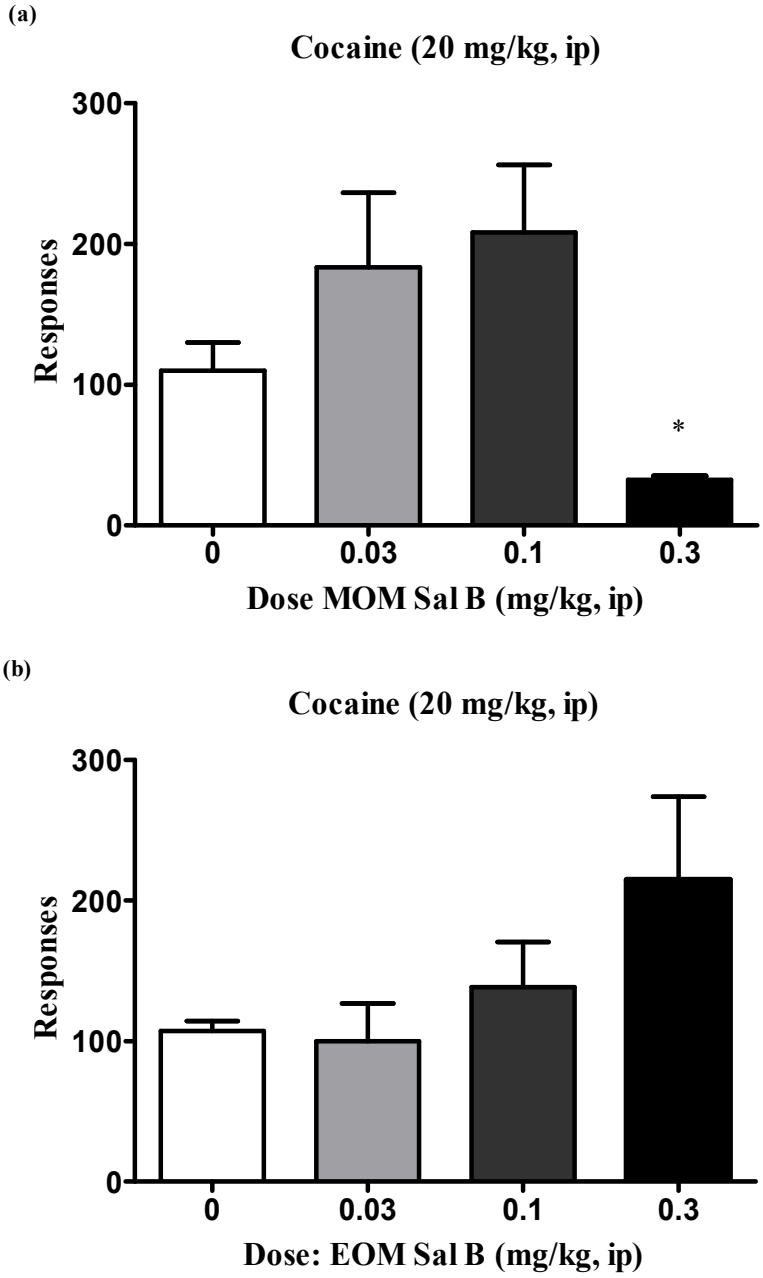


Fig.2.9. Effect of MOM Sal B and EOM Sal B on cocaine prime induced reinstatement.

*Symbols indicate active lever press responses +SEM produced during phase 3 of the reinstatement test. Rats which had shown extinguished saline reinforcements during the extinction phase were initially treated with different doses of (a) MOM Sal B (0, 0.03, 0.1 and 0.3 mg/kg) and (b) EOM Sal B (0, 0.03, 0.1 and 0.3 mg/kg) followed by a priming injection of cocaine (20 mg/kg) and saline reinforced responses were recorded for a period of 60 min. *p<0.05, data compared with vehicle treated group (0 mg/kg). One-way ANOVA followed by Tukey post hoc test. n=5-7 for each group.*

2.3.9. Effect of herkinorin on cocaine prime induced reinstatement.

The effect of herkinorin pre-treatment on cocaine prime induced reinstatement is shown in Fig. 2.10. No significant change in responding was observed in rats pre-treated with a high dose of herkinorin [0.3, 1.0 mg/kg; $F(3,24) = 1.48$, $p=0.245$]. However, a non-significant ($p>0.05$) trend towards an increase in lever press responding was observed in animals treated with a low dose of herkinorin (0.1 mg/kg). Also, a trend in decreasing responses was noted in rats treated with the higher dose of herkinorin (0.3, 1.0 mg/kg), which was non-significant.

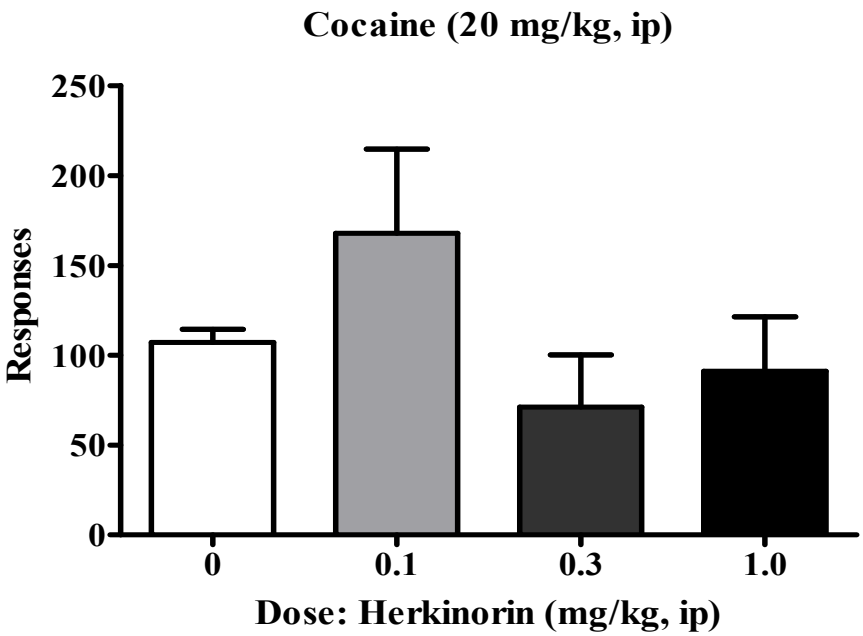


Fig.2.10. Effect of herkinorin on cocaine prime induced reinstatement.

Symbols indicate active lever press responses +SEM produced during phase 3 of the reinstatement test. Rats which had shown extinguished saline reinforcements during the extinction phase were initially treated with different doses of herkinorin (0, 0.1, 0.3 and 1.0 mg/kg) followed by a priming injection of cocaine (20 mg/kg) and saline reinforced responses were recorded for a period of 60 min. One-way ANOVA followed by Tukey post hoc test. n= 5-10 for each group.

2.3.10. Effect of nalmefene on cocaine prime induced reinstatement.

The effect of a single injection of nalmefene (0, 0.1, 0.3 and 1.0 mg/kg) on cocaine prime-induced reinstatement is shown in Fig. 2.11. Statistical analysis showed a significant attenuation in cocaine prime-induced drug-seeking in nalmefene pre-treated groups when compared to the vehicle treated group of rats [$F(3,17) = 6.48$, $p<0.01$]. This effect produced by nalmefene (0.3, 1.0 mg/kg) was reversed by nor-BNI (0, 2.0 mg/kg) pre-treatment ($p<0.001$; Fig 2.11b).

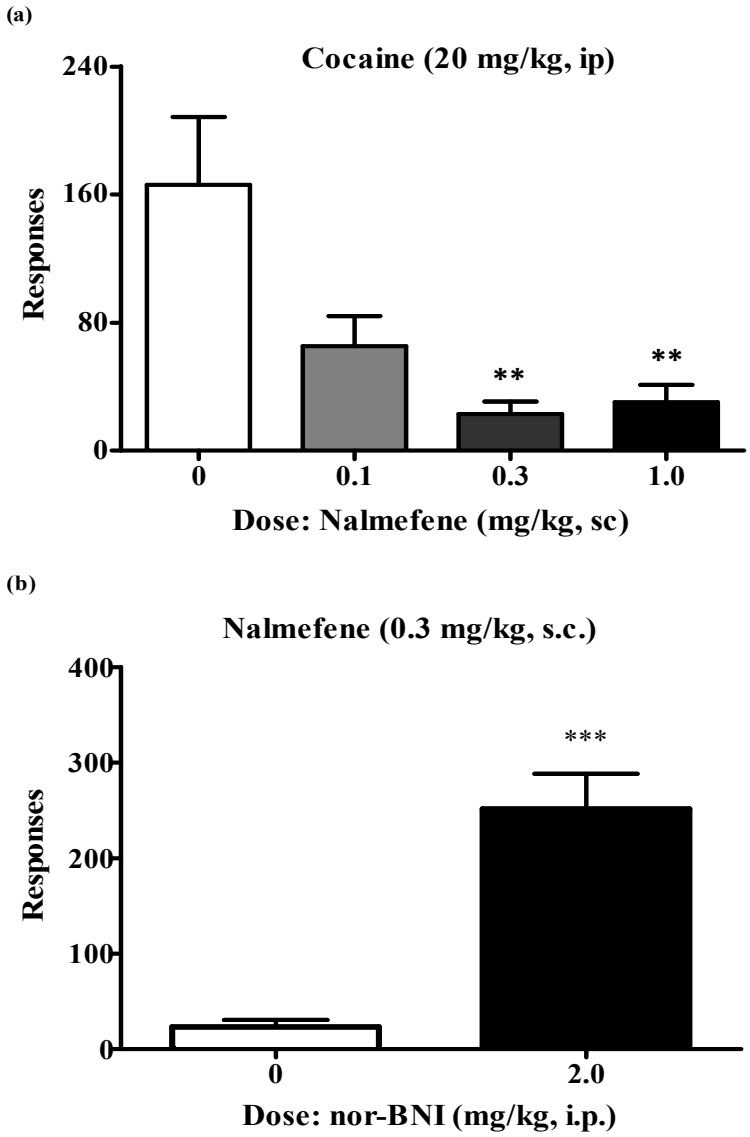


Fig. 2.11. Effect of nalmefene on cocaine prime induced reinstatement.

*Symbols indicate active lever press responses +SEM produced during phase 3 of the reinstatement test. (a) Rats which had shown extinguished saline responding during the extinction phase were initially treated with different doses of nalmefene (0, 0.1, 0.3 and 1.0 mg/kg) followed by a priming injection of cocaine (20 mg/kg) and saline reinforced responses were recorded for a period of 60 min. **p<0.01, data compared with vehicle treated group (0 mg/kg). One-way ANOVA followed by Tukey post hoc test. n=5-6 for each group. (b) For nor-BNI reversal experiments, animals were first treated with nor-BNI (0, 2.0 mg/kg) followed by nalmefene (0.3 mg/kg) and cocaine (20 mg/kg) treatment. ***p<0.001, Student t-tests. n=5-6 for each group.*

2.4. Summary of results

Tab. 2.3. Effect of KOPr agonists on cocaine prime induced drug-seeking

Compound tested	Cocaine prime induced reinstatement	KOPr antagonist induced reversal
Sal A	Attenuated (selective)	Reversed
DS1	Attenuated (selective)	Reversed
MOM Sal B	Attenuated (non-selective)	-
EOM Sal B	No effect	-
Herkinorin	No effect	-
Nalmefene	Attenuated (selective)	Reversed

Tab 2.4. Effect of KOPr agonists on cocaine induced motor activity

Compound tested	Cocaine induced hyperactivity	Expression of cocaine behavioural sensitization	
		Motor sensitization	Stereotypic counts
Sal A	Potentiated	Attenuated	No effect
DS1	Attenuated	Attenuated	No effect

2.5. Discussion

In the present study, the novel neoclerodane diterpene KOPr agonist Sal A and its structural analogues DS1, MOM Sal B, EOM Sal B, herkinorin and partial KOPr agonist/MOPr antagonist, nalmefene, were tested for their anti-addiction behaviours using cocaine prime induced reinstatement in rats. To determine if their behavioural effects were mediated via KOPr activation, self-administration animals were also pre-treated with the selective KOPr antagonist nor-BNI. The anti-cocaine reinstatement effects produced by novel KOPr agonists that attenuated cocaine seeking behaviours selectively without producing sedation by themselves (Chapter 3) were subjected to further behavioural tests. These tests included the measurement of cocaine induced behavioural sensitization and stereotypic counts. Effect of acute cocaine induced hyperactivity was also measured in rats.

Previous studies have shown that a single systemic injection of cocaine (20 mg/kg, ip) reinstates extinguished cocaine reinforced responding behaviour (Worley et al., 1994; Schenk and Partridge, 1999a; Schenk et al., 2000a). Injecting 20 mg/kg cocaine for 5 consecutive days has been shown to produce a sensitized response to cocaine. This has been shown by a decreased latency in self-administration acquisition (Schenk and Partridge, 2000b). Furthermore, cocaine (20 mg/kg, ip) when injected for 5 consecutive days also produces sensitization to cocaine's motor stimulating effects (Heidbreder et al., 1995; 1996). The results from this study support these previous findings as shown by cocaine (20 mg/kg, ip) initiated prime induced reinstatement (Fig 2.1) and expression of motor sensitization in rats (Fig 2.4; Fig 2.8).

Previous studies have shown that conditioning stimuli can play an important role in the expression of motor sensitized responses in laboratory animals (Post et al., 1981; Beninger and Herz, 1986). In the present study, rats which received cocaine or saline injections for 5 consecutive days were returned to their home cage after drug/vehicle

exposures. Therefore, the difference in ambulation which was measured in the locomotion chamber after cocaine was administered on the test day (day 10) was specifically due to cocaine exposures during pre-treatment days 1-5 (Fig 2.3; Fig 2.8). The presence of the house light above the active lever has been considered to be conditioned stimuli and has been used for training animals to self-administer cocaine (Schenk and Partridge, 2001a). Therefore, a light stimulus was presented just above the active lever and was lit whenever the animal pressed the active lever during this cocaine self-administration study. Consistent with this, presentation of such drug associated cues have been shown to initiate the reinstatement of extinguished drug-seeking (Stewart et al., 1984). In order to avoid any cue-induced effects while testing during the reinstatement phase, the light stimulus was presented to the animals above the active lever throughout all the phases of the reinstatement test. Thus, increase in the lever press responses observed during the self-administration phase followed by a decrease in lever press responding during the extinction phase may be attributed only to the presence of cocaine and saline in the infusion pump during phase 1 and 2 respectively (Fig. 2.1).

No previous *in vivo* work has been reported on the Sal A analogue, DS1. Therefore the onset of action time for DS1 (0.3 mg/kg) on cocaine induced hyperactivity was performed at various pre-treatment times. DS1 showed a significant suppression in hyperactivity produced when injected 45 min, but not 5 or 30 min prior to receiving cocaine injections (Fig. 2.5). This finding suggests that DS1 has a slower, more prolonged onset of action *in vivo* compared to Sal A (2-5 min; Schmidt et al., 2005). Behavioural tests measuring KOPr agonist induced analgesia have been previously used to determine the duration of action of the compound tested (Wang et al., 2008; John et al., 2005). Such tests apply the hot plate, tail flick and acetic acid induced writhing assays in laboratory animals (John et al., 2005; McCurdy et al., 2005; Wang et

al., 2008). As the duration of action of DS1 still remains unanswered, detailed studies using these behavioural paradigms are therefore warranted.

Results from this study indicate that Sal A and DS1 both attenuated cocaine prime induced drug-seeking behaviour (0.3, 1.0 mg/kg; Fig 2.2a; Morani et al., 2009; Fig 2.6a; Tab 2.3). In order to further characterise their anti-addiction potential, the effect of acute exposure to Sal A and DS1 (0.3 mg/kg) was tested on cocaine induced stimulation of motor function. Sal A and DS1, at the minimum effective dose which attenuated drug-seeking behaviour (0.3 mg/kg), significantly suppressed the expression of cocaine behavioural sensitization in rats (Fig 2.4; 2.8; Tab 2.4). In addition to its effect on cocaine sensitization, DS1 (0.3 mg/kg) pre-treatment significantly suppressed cocaine induced hyperactivity (Fig 2.7; Tab 2.4). In contrast, Sal A (0.3 mg/kg) exposure significantly increased motor function produced by a single cocaine injection (Fig. 2.3; Tab 2.4). Contrary to this present finding, a recent report by Chartoff and colleagues (2008) suggested that Sal A (2.0 mg/kg) pre-treatment decreases cocaine induced hyperactivity in rats (Chartoff et al., 2008). One of the reasons for the differences observed might be due to the relatively low dose of Sal A (0.3 mg/kg vs. 2.0 mg/kg; Chartoff et al., 2008) and relatively high dose of cocaine (20 mg/kg vs. 10 mg/kg; Chartoff et al., 2008) used in this study. In contrast to its effect on behavioural sensitization, Sal A and DS1 pre-treatment produced opposite effects on acute cocaine induced activity. Sal A significantly enhanced whereas DS1 significantly suppressed cocaine motor function. This observed discrepancy might reflect the difference in the pharmacokinetic properties of these compounds as Sal A has a quick onset of action (5 min; Schmidt et al., 2005b) compared to DS1 (45 min; Fig 2.5).

An increase in total activity was noted in animals treated with cocaine on the test day following no- (Fig 2.3, 2.5, 2.7) or prior-cocaine exposures (Fig 2.4, 2.8) when compared to saline injections. Thus indicating the presence of cocaine induced

hyperactivity and expression of cocaine sensitization. On close observation, a greater enhancement in motor function was noted in animals receiving vehicle followed by acute cocaine challenge (Fig 2.3, 2.7) compared to animals pre-treated with saline for 5 days followed by cocaine treatment on test day Fig 2.4, 2.8). Additionally, the sensitized animals (i.e. those receiving 5 days of cocaine pre-treatment; Fig 2.4, 2.8) had a cocaine response that was similar to that observed in naïve animals treated with acute cocaine (Fig 2.3, 2.7). A possible explanation for these discrepancies may include batch differences as both of these experiments were performed at different times.

An increase in stereotypy behaviour is generally not favoured by forward locomotion (Post et al., 1987). Repeated cocaine treatment or high dose of cocaine (20 mg/kg) has been shown to initially enhance locomotion activity followed by an increase in stereotypy behaviours in laboratory rats (Post et al., 1987; Ushijima *et al.,* 1995). Therefore, we analyzed the number of stereotypic counts in cocaine sensitized animals treated with either Sal A or DS1. There was no significant effect on cocaine produced stereotypy with Sal A or DS1 pre-treatment (Fig 2.4; 2.8). However, a non-significant enhancement in cocaine produced stereotypy was noted in both Sal A and DS1 treated animals which coincided with the suppression of motor sensitization (5-15 min post cocaine treatment; Fig 2.4, 2.8). Thus, the decrease in cocaine-induced behavioural sensitization produced by both the KOPr compounds might reflect either a decrease in the ability of cocaine to produce horizontal activity or an increase in the ability of cocaine to produce the competing behaviour of stereotypy following the administration of higher doses of psychostimulants (Ushijima *et al.,* 1995; Post *et al.,* 1987). Further studies are warranted to define the effects of Sal A and DS1 on cocaine induced stereotypic behaviours. Such experiments have been described in details in Chapter 4.

Ultrastructural studies by Svingos and colleagues (2001) have shown that KOPr and DAT are co-localized in the NAc and therefore activation of KOPr can modulate

cocaine induced behaviours via DAT. Because of the interaction between KOPr and dopaminergic mechanisms, a focus on the effects of Sal A on dopamine-mediated behaviours has been of interest (Gherke et al., 2008; Chartoff et al., 2008). In drug discrimination tests, Sal A and its synthetic derivatives, MOM Sal B and EOM Sal B, substituted completely for U69593 (Baker et al., 2009), suggesting they share effects mediated via the KOPr. Cocaine-seeking has been attributed to dopaminergic mechanisms (Schmidt et al., 2005a; Section 1.4; Chapter 1), which is supported by data showing that Sal A modulates the effects of cocaine through interactions with D1 receptor mediated signalling pathways in the dorsal striatum (Chartoff et al., 2008). Also, Sal A pre-treatment has been shown to decrease DA levels in the NAc (Carlezon et al., 2006) and CPU (Zhang et al., 2005; Gherke et al., 2008). However, the effects produced by Sal A might be bi-phasic as a lower dose of Sal A (40 μg/kg) increased DA levels in NAc (Braida et al., 2008) whereas higher doses (1.0 – 3.2 mg/kg) decreased DA levels in dorsal (Zhang et. al., 2005) and ventral (Carlezon et al., 2006) striatum. Therefore, the ability of moderate to high doses of Sal A to decrease cocaine-produced drug-seeking might be due to its effect on the dopaminergic system. However, further work is required to link the role of Sal A in modulating DA neurotransmission with its attenuation of cocaine seeking behaviour.

Sal A analogues, MOM Sal B (Wang et al., 2008) and EOM Sal B (Hooker et al., 2009), have a longer duration of action than Sal A. These compounds were tested for their effects on cocaine prime-induced drug-seeking behaviour. EOM Sal B, which is 10 times more potent than Sal A at KOPr (Beguin et al., 2005; Munro et al., 2008), had no effect on cocaine prime induced drug-seeking behaviour. However, a trend in enhancement of active lever press responding was observed when rats were pre-treated with the higher doses of EOM Sal B (0.1, 0.3 mg/kg; Fig 2.9b; Tab 2.3). On the other hand, Sal A at the same dose (0.3 mg/kg) significantly attenuated cocaine-seeking (Fig

2.2; Tab 2.3). A recent study by Hooker et al., (2009) showed that EOM Sal B was metabolically more stable than Sal A. Additionally, EOM Sal B when injected ip showed a gradual decline in the whole brain concentrations in the rats (Hooker et al., 2009). Also, EOM Sal B showed less affinity for plasma proteins in baboons reflecting more bioavailability (Hooker et al., 2009). These data are suggestive that differences between EOM Sal B and Sal A with respect to cocaine seeking might reflect their different pharmacokinetic profiles. MOM Sal B, is approximately 7 times more potent than Sal A at KOPr (Beguin et al., 2005; Munro et al., 2008), at low doses (0.03, 0.1 mg/kg) MOM Sal B also showed a non-significant trend towards an increase in active lever press responding. However at a higher dose (0.3 mg/kg), MOM Sal B significantly attenuated drug-seeking behaviour (Fig 2.9a; Tab 2.3). It was interesting to observe that although EOM Sal B and MOM Sal B are both potent and long acting Sal Aanalogues, they produce dissimilar effects on cocaine-seeking behaviour at the high dose tested (0.3 mg/kg; Fig 2.9). Further studies are required to explain these dissimilarities. As, KOPr activation has been shown to decrease natural reward seeking behaviour (Mello and Negus, 2000), it would be interesting to observe the effect of MOM Sal B (0.3 mg/kg) pre-treatment in animals trained to obtain natural reward related reinforcements, like sucrose. This would help in determining the degree of selectivity of MOM Sal B in attenuation of cocaine-seeking behaviour. The results from such tests have been discussed in the following chapter (Chapter 3).

Herkinorin has a higher affinity for MOPr than KOPr (Harding et al., 2005; Tidgewell et al., 2006; Prisinzano and Rothman, 2008), and was tested for its capacity to reinstate cocaine induced behaviour. Herkinorin pre-treatment had no significant effect on cocaine prime induced drug-seeking (Fig 2.10; Tab 2.3). However, it was interesting to observe that there was a slight trend towards a decrease in lever press responding in rats pre-treated with high doses (0.3, 1.0 mg/kg) of herkinorin.

MOPr agonists such as methadone and its derivatives have been used clinically for opioid maintenance therapy (Paulus and Halliday, 1967; Section 1.1, Chapter 1). However, one of the problems associated with methadone is that it is itself addictive and in some cases might also lead to fatal overdosing (Reingardiene et al., 2009). It is well known that KOPr activation opposes MOPr activation related behaviours (Pan, 1998; Section 1.7; Chapter 1). Therefore, developing Sal A analogues which activates MOPr as well as KOPr may have the potential to be developed into pharmacotherapies to manage opioid withdrawal effects. A recent study by Groer and colleagues, (2007) also showed that herkinorin, unlike other MOPr agonists, did not promote beta-arrestin recruitment to MOPr or cause receptor internalization. Further detailed behavioural and neurochemical studies with herkinorin are required to explore its usefulness in drug withdrawal. Future studies with herkinorin as maintenance therapy has been discussed in Chapter 4. Since herkinorin is the only Sal A analogue with MOPr affinity, a sense of interest has been generated in developing herkinorin analogues possessing analgesic effects with fewer adverse effects compared to selective MOPr agonists (Harding et al., 2005; Tidgewell et al., 2006; 2008; Prisinzano and Rothman, 2008).

KOPr ligands having affinities for other opioid receptors have previously been suggested to attenuate cocaine induced behaviours with safer adverse effect profile than selective KOPr agonists (Archer et al., 1996; Bowen et al., 2003; Stevenson et al., 2004; Kreek et al., 2005; Bart et al., 2005). Additionally, bremazocine and cyclazocine (KOPr agonists with MOPr antagonist actions) have been shown to modulate cocaine induced behaviours in laboratory animals (Archer et al., 1996; Vanderschuren et al., 2000; Glick et al., 1998; Section 1.7; Chapter 1). The present study showed that nalmefene, which is a MOPr antagonist having partial agonist activity at KOPr attenuated cocaine prime induced drug-seeking behaviour (0.3, 1.0 mg/kg; Fig 2.11a). This study is the first to present pre-clinical evidence suggesting that nalmefene attenuates cocaine induced

behaviour (Tab 2.3). Ligands activating KOPr and antagonising MOPr functions have been proposed as anti-addiction agents with possibly less adverse effects as compared to selective KOPr agonists (Mello and Negus, 2000; Bowen et al., 2003; Wang et al., 2009). Since nalmefene has been shown to attenuate cocaine induced drug-seeking in rats, it would be interesting to note the extent to which a single exposure to nalmefene produces KOPr related adverse effects. Further work in this regard was undertaken and is described in the following chapter (Chapter 3).

In order to determine if KOPr activation by Sal A, DS1 and nalmefene was in fact due to selective KOPr activation cocaine-seeking behaviour in the presence of KOPr antagonist nor-BNI was also determined. Animals pretreated with Nor-BNI 30 min before injection of KOPr agonists (Sal A, DS1 and nalmefene) completely reversed the attenuation in drug-seeking behaviour, thus confirming a KOPr mediated effect (Fig. 2.2b; 2.6b; 2.11b; Tab 2.3). Nalmefene is also an antagonist at MOPr (Bart et al., 2005; Ingman et al., 2005), therefore, the role of MOPr antagonism in the nalmefene induced anti-cocaine effects needs to be further characterised. KOPr activation has been shown to reduce extracellular DA levels in the NAc, an effect reversed with nor-BNI pre-treatment (Spanagel et al., 1990). Thus, a reduction in extracellular DA levels in the NAc by KOPr activating compounds might reflect its ability to attenuate cocaine-seeking behaviour. To validate this hypothesis future studies are required and have been discussed in Chapter 4.

It was interesting to note an enhancement in active lever-pressing in animals pre-treated with nor-BNI (181.8 ± 39.38, Fig 2.2b; 251.2 ± 37.21, Fig 2.11b) compared to control (0 mg/kg) animals (107.1 ± 7.3, Fig 2.2a; 166.2 ± 42.39, Fig 2.11a). Similarly, Beardsley and colleagues (2005) also reported a non-significant potentiation of cocaine prime induced reinstatement in rats pre-treated with JDTic, a selective KOPr antagonist. Thus, enhancement in the cocaine's reinforcing effects by KOPr antagonists might be

via the blockade of endogenous KOPr tone. This also shows that opposite effects are produced by KOPr antagonists' vs KOPr agonists on cocaine seeking (Fig 2.2, 2.11).

As explained in Section 2.1, the cocaine induced reinstatement paradigm has been extensively used to determine the effectiveness of pharmacological agents in producing anti-addiction effects. The within-session reinstatement paradigm however has its limitations. This model does not take into account the neuroadaptations associated with long term withdrawal from the drug self-administration. Such neuroadaptations are considered critical for the emergence of negative reinforcement and for expression of craving and relapse (Self, 2004; Self et al., 2004; Wolf, 2010; Koob & Volkow, 2010; Feltenstein & See, 2008). Therefore, the effect of modulating KOPr system on long term withdrawal from cocaine self-administration needs further assessment and can be performed using a between session reinstatement test. Such studies have the potential to determine the effect of KOPr activation on neuroadaptations associated with withdrawal and relate them to relapse.

The within-reinstatement model exhibits low predictive power by itself and results obtained using this paradigm fail to discriminate between false positives and compounds producing anti-addiction behaviour. This can be illustrated by considering the possible effects of dopamine agonists and antagonists on this model. The neuroleptic haloperidol or the D1 antagonist SCH23390, at doses that do not induce catalepsy or motor inhibition by themselves, may block cocaine-induced reinstatement of drug seeking. However, we might not think of them as potential or safe therapeutics for cocaine abuse. These probable results would, at least, imply the role of DA receptors in cocaine-induced reinstatement (Self, 2004). Additionally, it would be interesting to consider the effect of DA agonist on measuring cocaine-seeking behaviour in this model. One example is apomorphine, a classical mixed D1/D2 receptor agonist which, at low doses, produces sedative, yawning and motor inhibition

effects (Ernst, 1967). If apomorphine attenuates cocaine-seeking at this dose, it might reflect a false positive effect. Furthermore, low doses of apomorphine via D2 auto-receptors have been shown to inhibit the synthesis and release of DA (Anderson and Gazzara, 1993), an effect similar to that elicited by KOPr activation (Shippenberg et al., 2007). Apomorphine suppresses cocaine-induced reinstatement of drug-seeking (De Vries et al., 1999), however, it is ineffective at treating cocaine addiction. Also the D3 receptor agonist' quinpirole and 7- OH-DPAT have been shown to produce similar inhibitory profiles in attenuating cocaine self-administration (Caine and Koob, 1993), but are unlikely to be used as an anti-addiction pharmacotherapies. Taken together, these data suggest that the within-session reinstatement model could be applied as a preliminary screening tool to identify the effectiveness of neoclerodane diterpenes as anti-addiction compounds. Further screening is therefore required to coin the effectiveness of KOPr compounds in modulating addictive behaviours and such experiments are described in detail in chapter 4.

KOPr compounds that attenuate cocaine-seeking using the within-session reinstatement model will need further evaluation to discriminate them from false positives. To do so, we have applied control experiments which were aimed to measure motor suppression during reinstatement test and effect of KOPr compounds on natural reward related behaviour in rats. Data from these experiments have been described in the following chapter (Chapter 3).

2.6. Summary and conclusions

The results from this chapter indicate that Sal A and its analogues MOM Sal B, DS1 and partial KOPr agonist/ MOPr antagonist, nalmefene attenuated cocaine prime-induced drug seeking behaviour in rats in a similar way to traditional KOPr agonists. Sal A and DS1 also attenuated expression of cocaine behavioural sensitization, with

DS1 also attenuating cocaine induced hyperactivity in rats. Thus given the anti-cocaine profile for these novel KOPr analogues, it will be interesting to note if they produce KOPr agonist related adverse effects such as anhedonia, depression, aversion and motor suppression. The next chapter deals with the adverse effect profiles of these novel KOPr agonists.

Chapter.3. Side effect profile of novel KOPr agonists

3.1. Introduction

3.1.1 Adverse effects of traditional KOPr agonists

Although, KOPr agonists attenuate cocaine induced behaviours, they are also associated with producing adverse effects such as conditioned place and taste aversion, depression, hallucination, sedation, emesis and decreased responding for food reinforcements in laboratory animals (Todtenkopff et al., 2004; Butelman et al., 2010; Mello and Negus, 2000; Section 1.5, Chapter 1). Also, clinically, KOPr activation has been shown to induce depersonalization, confusion, visual distortions and hallucinations (Pfeiffer et al., 1986; Walsh et al., 2001a; Johnson et al., 2011; Section 1.5, Chapter 1). These adverse effects associated with KOPr agonists have limited their development as anti-addiction therapeutics (Walsh et al., 2001a). Due to the undesirable effects associated with KOPr agonists, we performed behavioural adverse effect screening on the compounds which successfully attenuated cocaine-seeking behaviour (Chapter 2).

3.1.2. Pre-clinical models to screen side effect profile of KOPr agonists

The following section gives a brief account on the behavioural models used to screen the adverse effect profile for novel KOPr agonists. The spontaneous open field activity test has been used to determine the motor suppressant effects of traditional KOPr agonists (U50488H, U69593, spiradoline) pre-clinically (Sction 1.5, Chapter 1; Wadenberg, 2003; Lahti et al., 1982; 1985). The principle for sucrose reinforcement test and CTA has been described in Section 1.3, of Chapter 1. A recent study by Davis and colleagues (2009) has shown that a single pairing of U50488H with novel tasting saccharin solution produces taste aversion. Also, a previous study by Mucha and Herz, (1985) showed that traditional KOPr agonists (U50488H, bremazocine, ethylketazocine, trifluadom) produced taste and place aversion in a dose dependent manner. Therefore, in this present study, CTA was applied to measure the aversive behaviour produced by a single KOPr agonist exposure in rats.

The FST (Porsolt et al., 1979) has been used extensively as an animal model of depressive like behaviours (Porsolt et al., 1979; Carlezon et al., 2006) and to screen pharmacological anti-depressants in laboratory animals (Detke et al., 1995). In the FST, the animals are habituated to force swimming in a cylindrical chamber for 15 min. The next day, animals having been pharmacologically treated during the intervening period, are again placed in the cylindrical chamber and the frequency of these behaviours (climbing, swimming and immobility) is noted for 5 min. If the treated animal spends more time immobile and relatively less time swimming or climbing, it then indicates possible depressive effects induced by the pharmacological agent (Porsolt et al., 1979; Carlezon et al., 2006). On the other hand, more time spent either climbing or swimming (active behaviours) and less time spent immobile, indicates potential anti-depressant like effect of the administered pharmacological agent (Detke et al., 1995).

3.1.3. KOPr mediated SERT modulation

SERT belongs to the sodium-chloride symporter protein family and plays a prominent role in 5HT neurotransmission by clearing 5HT from the synaptic cleft (Blakely et al., 1994; Saier, 1999; Zahniser and Doolen, 2001; Rudnick, 2006). Alteration of5HT neurotransmission has been implicated in numerous psychiatric disorders such as depression (Owens and Nemeroff, 1994), anxiety (Suranyi-Cadotte et al., 1990), bipolar disorder (Bellivier et al., 1997; Velayudhan et al., 1999) and addiction to psychostimulants (Heinz et al., 2001; Bauman and Rothman, 1998). It is well documented that KOPr's are co-localized with DAT in the NAc (Svignos et al., 2001) and KOPr agonists modulate DA neurotransmission by increasing DAT function in the NAc (Thompson et al., 2000). However, little is known about their effects on modulating the function of other monoamine transporters. Therefore, it would be interesting to understand whether KOPr activation by both established and novel KOPr agonists modulates 5HT systems.

3.1.4. Live cell ASP^+ uptake accumulation model

4-(4-(dimethylamino)-styryl)-N-methylpyridinium (ASP^+), an analogue of the neurotoxin 1-methyl-4-phenylpyridinium (Javitch et al., 1985) is a monoamine transporter substrate which fluoresces in a lipid environment (Schwartz et al., 2003; 2005; Blakely and DeFelice, 2007). ASP^+ has been used to measure real-time function of DAT (Bolan et al., 2007; Zapata et al., 2007), NET (Schwartz et al., 2003) and SERT (Oz et al., 2010) in single cells. The transport of ASP^+ can be classified into two stages. Initially, ASP^+ binds rapidly to the monoamine transporter which is followed by its linear uptake into the cell. This can be visualized as accumulation of ASP^+ fluorescence within the cell (Schwartz et al., 2005; Fig. 3.1). Recent reports have shown that ASP^+ binds to SERT at micromolar concentrations and accumulates in cells expressing SERT (Schwartz et al., 2003; Oz et al., 2010). Additionally, the uptake of ASP^+ is saturable, temperature dependent, and is dependent on sodium and chloride ions (Schwartz et al., 2003; Oz et al., 2010). Radio-ligand uptake assays are usually employed to measure SERT function (Owens et al., 2001). The advantage of using the ASP^+ uptake technique is that modulation of SERT function can be observed in real-time, whereas this cannot be done by using the radio-ligand uptake assays (Oz et al., 2010). This technique can also determine alterations in transporter functional kinetics using single cells over a period of time, whereas radio-ligand assays require addition of ligands followed by washout periods (Bylund and Toews, 1993) which limits the collection of rapid kinetic data. Within cell- and between cell- ASP^+ uptake protocols can be used to determine monoamine transporter function immediately or after incubating cells with drug treatment (Oz et al., 2010; Bolan et al., 2007; Zapata et al., 2007; Schwartz et al., 2003). The rationale behind this study was to determine if U50488H, Sal A and DS1 had the ability to directly modulate SERT function *in vitro* in cells transiently expressing both SERT and KOPr. Development of an *in vitro* cell model will help to determine the

mechanism by which KOPr agonists modulate addictive behaviours. Overall, this may aid in the development of more effective and better tolerated anti-addiction pharmacotherapies.

Results from the previous chapter (Chapter 2) indicate that Sal A, DS1, MOM Sal B and nalmefene attenuate cocaine prime induced drug-seeking, an effect similar to what has been reported using the traditional KOPr agonists. Therefore, this chapter deals with studying the adverse behavioural effect profile of these compounds with respect to motor function, CTA and FST in rats. The 5HT system is implicated in the aetiology and treatment of depression (Pare, 1971; Pare et al., 1974). It is therefore possible that KOPr agonists induced depressive like symptoms might improve via modulating the serotonin systems. In order to test this, the effect of antagonising SERT function prior to novel KOPr agonist exposure on depressive like behaviours were measured using FST in rats. Additionally, we tested the effect of pre-treatment with established as well as novel KOPr agonists on modulating SERT function using live cell ASP^+ accumulation assay *in vitro*.

3.1.5. Aims

KOPr compounds (Sal A, DS1, MOM Sal B, nalmefene) with demonstrated efficacy for attenuating cocaine seeking behaviours as identified in Chapter 2, were tested for undesirable behavioural side-effects. The aims of the experiments described in this chapter were:-

1. To determine if selected KOPr compounds (Sal A, DS1, MOM Sal B, nalmefene) alter cocaine induced hyperactivity in cocaine self-administering rats (motor suppression during reinstatement tests).

2. To determine if single exposure to KOPr agonists (Sal A, DS1, MOM Sal B and nalmefene) alters the rats natural reward-seeking behaviour. This will be done by

measuring weather KOPr agonists disrupt operant responding to obtain 10% sucrose solution.

3. To determine if acute exposure to Sal A, DS1, MOM Sal B and nalmefene causes motor suppression (sedation) during recording of spontaneous open field activity.

4. To determine if acute exposure to Sal A, DS1 and nalmefene produces aversion using CTA in rats.

5. To determine if acute exposure to Sal A, DS1 and nalmefene produces depressive symptoms using the FST in rats.

6. To identify if SERT blockade improves KOPr agonist induced depressive like behaviour. In order to determine this, the effect of sub-chronic treatment with FLX on Sal A and DS1 induced depressive like behaviours will be determined by using the FST paradigm in rats.

7. To study the effect of classical KOPr (U50488H) and novel neoclerodane diterpenes (Sal A, DS1) KOPr agonists on modulating SERT function using live cell ASP^+ uptake assay *in vitro*.

3.2 Methods

3.2.1. Subjects

Animals previously self-administering cocaine and used in cocaine reinstatement tests were also used to measure cocaine induced hyperactivity. These were male Sprague Dawley rats, weighing 350-400 g. Drug naive male Sprague Dawley rats weighing 200-300 g were used for the spontaneous open field activity, CTA, FST and sucrose reinforcement tests. In addition rats (Male Sprague Dawley; 350-400 g) which were self-administering and tested for reinstatement but no longer had patent iv

cannulae were used for preliminary experiments with the KOPr compounds for the FST (Tab. 3.1).

Animals were housed individually (cocaine induced hyperactivity in self-administering rats, preliminary experiments with FST, sucrose reinforcements, CTA) or two per cage (spontaneous locomotion, FST in drug naive rats) in hanging polycarbonate cages. All rats were housed at least 5 days prior to the test in the animal facility in a temperature (19-21°C) and humidity controlled (55% relative humidity) room. Animals were handled for at least 5 days before the experiments to avoid handling stress. Lights in the room were maintained at 12:12 h, with lights on from 0700 h. All experimental procedures were approved by the Animal Ethics Committee of Victoria University of Wellington.

For locomotion tests, rats were placed in the activity chambers and were acclimatized for 30 min before they received drug treatment. For sucrose reinforcement tests, animals were maintained at approximately 85% of their initial feeding weights with free access to water throughout the experiment. For conditioned taste aversion, rats had free access to food but were water deprived for 23 hr (water habituation session) or 23 hr 20 min (saccharin sessions) per day for the entire duration of study. During habituation sessions, rats received water, whereas, on the pairing and test days, animals received a 0.1% novel tasting saccharin solution. Doses used to test the undesirable effects for each KOPr agonist (Sal A, DS1, MOM Sal B, nalmefene) were the minimal effective dose which attenuated cocaine reinstatement (0.3 mg/kg; Chapter 2).

Tab 3.1. Amount of cocaine consumed by rats used for preliminary FST experiments.

Sr. No.	Days exposed to cocaine (days)	Days abstinent from cocaine before FST (days)	Amount of cocaine consumed (mg/kg)
1	82	26	564
2	76	22	392
3	81	18	393
4	77	26	380
5	77	30	510
6	76	32	547
7	74	27	601
8	78	32	487
9	78	34	568
10	61	33	649
11	59	33	359
Avg	**75**	**29**	**496**

3.2.2. Cell culture maintenance for live cell ASP^+ uptake experiments.

HEK-293 (American type culture collection, USA) cells were maintained in culture in Dulbecco's Modified Eagle Medium (Gibco, Invitrogen) containing 10% foetal bovine serum (Gibco, Invitrogen) and 1 % penstrap (5,000 units of penicillin G sodium salt and 5,000 µg streptomycin sulphate/ml in 0.85% saline; Invitrogen). The cells were grown in a T75 tissue culture flask (BD Bioscience) and were maintained in a humidified atmosphere in an incubator at 37°C with 5% CO_2.

3.2.3. Drugs

Sal A, DS1 and MOM Sal B (from Dr. Thomas E. Prisinzano, University of Kansas) were suspended in 75% DMSO. Cocaine hydrochloride (Merck, Palmerston North, New Zealand), nalmefene (Sigma Aldrich, MO) and fluoxetine (Tocris, UK) were dissolved in 0.9% saline and ip and sc injections were administered in the volume of 1 mL/kg. For live cell confocal microscopy experiments, 4-(4-(dimethylamino)-styryl)-N-methylpyridinium (ASP^+; Sigma Aldrich, St Louis MO) was dissolved in Krebs Ringer Herpes buffer (KRH; pH 7.4). U50488H (Sigma Aldrich, St. Louis, MO), Sal A and DS1 were initially suspended in 100% DMSO followed by further dilution into KRH (pH 7.4).

3.2.4. Apparatus

3.2.4.1. Locomotion tests

As described in Section 2.2.4.1, Chapter 2.

3.2.4.2. Sucrose reinforcement

Training and testing procedures were conducted in eight modular test chambers (Med Associates, ENV-008) equipped with two retractable levers (Med Associates ENV-112CM) situated at the front of the chamber to either side. 10% sucrose solution was delivered by a liquid dipper (Med-Associates ENV-202M). The dipper was situated

in the centre, at the front of the chamber 2 cm from the chamber floor. The two retractable levers were positioned to the left and right 8 cm from the liquid dipper and 3 cm from the sides of the chamber. These chambers were located in an unlit, sound attenuated room. Sucrose delivery and data acquisition were controlled by Med Associates software (St Albans, VA, USA). Experiments were conducted between 0900 and 1600 hr.

3.2.4.3. Forced swim test

The FST apparatus consisted of a cylinder which was 44 cm high and 20 cm in diameter. The cylinder was filled with water maintained at 25 ± 1 °C. The water was filled up to 35 cm and the animal was placed in the water and behaviour was recorded by a camera connected to a nearby computer. The forced swimming behaviours (climbing, swimming or immobile) were recorded for a period of 5 min and later analysed by a blinded to treatment observer.

3.2.5. Procedure

3.2.5.1. Cocaine induced hyperactivity (in self-administering rats)

This test was conducted to measure weather KOPr agonists caused any disruption in motor function during the reinstatement test. Methods described by Morani et al., (2009) were followed. The conditions for this test were maintained similar to the reinstatement test. On the test day, rats previously self-administering cocaine received an injection of either Sal A (0.3 mg/kg, ip) 5 min, MOM Sal B (0.3 mg/kg, ip) 5 min, nalmefene (0.3 mg/kg, sc) 15 min and DS1 (0.3 mg/kg, ip) 45 min prior to an injection of cocaine (20 mg/kg, ip). Immediately following the cocaine injection, the rats were placed in the activity chambers and total activity, a compilation of horizontal and vertical activity, and ambulation at 5 min intervals was measured for a period of 1 hr. All injections were administered in the open field chamber. The experimenter remained outside the test room while the activity test was in progress.

3.2.5.2. Spontaneous open field activity (drug naive rats)

This test was performed to detect any sedative or motor suppression effects produced by an acute exposure to the KOPr agonists. For this study, we modified the methods of Frankowska et al, (2009) and Xu et al, (2010). On the test day, drug naive rats were injected with either vehicle (75% DMSO or 0.9% saline) or KOPr compounds 5 min (Sal A, 0.3 mg/kg, ip; MOM Sal B, 0.3 mg/kg, ip), 15 min (nalmefene, 0.3 mg/kg, sc) or 45 min (DS1, 0.3 mg/kg, ip) prior to measuring their spontaneous open field activity. Total activity, a compilation of horizontal and vertical activity, and ambulation at 5 min intervals was measured for a period of 1 hr. All injections were administered in the activity chamber with the experimenter remaining outside the test room while the activity test was in progress.

3.2.5.3. Sucrose reinforcement training and test

This test was performed to determine the effect of acute exposure to KOPr agonists on operant lever press responding to a palatable reward (10% sucrose solution). Methods described by Morani et al., (2009) were followed. Animals were maintained at approximately 85% of their initial feeding weights throughout the experiment. Initially rats were trained to obtain sucrose reinforcements using an auto-shaping procedure for 45 min daily for ten consecutive days. Once they were stably responding, the animals were trained to self-administer sucrose orally on a FR1 schedule of reinforcement. Following acquisition, the response requirement was increased to a FR-5 schedule. Daily 1 hr sessions were conducted until there was less than 20% variation in responding for three consecutive days. Once responding on the FR-5 schedule was stable, the effects of prior administration of Sal A (0.3, 1.0 mg/kg, ip), DS1 (0.3, 1.0 mg/kg, ip), MOM Sal B (0.3 mg/kg, ip), nalmefene (0.3 mg/kg, sc) and vehicle (75% DMSO; 0.9% saline) on sucrose-reinforced responding were noted. The injections were administered 5 min (Sal A, MOM Sal B), 15 min (nalmefene) and 45 min (DS1) prior to

the experiment and responding was measured for 60 min following drug/vehicle treatment.

3.2.5.4. Conditioned taste aversion

This test was performed to determine the aversive effects produced by a single injection of each of the KOPr agonists. To do this we measured the amount of novel tasting saccharin solution (which had been paired with KOPr agonist) consumed on test day. For this experiment, we modified methods described by Fenu et al., (2005) and Schenk et al., (1987).

Habituation: On day 1 of the experiment the rats were placed on a water deprivation schedule for 23 h. The following day, animals were given access to water for 1 h. The amount of water consumed was measured on a daily basis, to the nearest mL. This process was repeated untill the variation in water consumption was $\leq$ 2 mL for three consecutive days.

Pairing: The day after rats attained stable water consumption, they were subjected to pairing session. During these sessions, rats were presented with a novel 0.1% saccharin solution instead of water, and the amount of saccharin consumed (mL) was noted for 40 min. The animals were divided evenly into two groups (Vehicle or KOPr agonist treated), matched on saccharin intake and were immediately injected with either vehicle (75% DMSO or 0.9% saline) or KOPr compound (Sal A, DS1 or nalmefene) and were returned to their home cage. The following day (24 h post pairing sessions), rats were provided with drinking water for 60 min and were tested for the saccharin aversion the next day (24 h later).

Test day*:* 48 hr after the pairing session, rats were again presented with the saccharin solution and the amount of saccharin consumed was measured for 40 min (to the nearest mL). In order to determine any taste aversion produced by the novel KOPr compound, the amount of saccharin consumed on the pairing day and test day by the KOPr agonist

treated groups was compared to the corresponding vehicle treated group. All experiments were performed between 1100 – 1300 hr.

3.2.5.5. FST in drug naïve rats and preliminary experiments.

To initially screen the depressive effect of novel KOPr agonists, FST was carried out using animals which were previously subjected to self-administration training and reinstatement tests (Tab 3.1). These rats were withdrawn from the self-administration experiments due to the failure of their jugular cannulae. The average total amount of cocaine consumed by these rats was 496 mg/kg (range: 359-649 mg/kg). The average number of days these rats were exposed to cocaine was 75 days (range: 59-82 days). The average number of days they were abstained from cocaine was 29 (range: 18-34 days). Depressive effects produced by KOPr agonists in preliminary tests were later determined in drug naive animals. For this experiment, we modified methods of Carlezon et al., (2006).

Habituation day: Animals were habituated in the test room for 60 min prior to the commencement of experiments. Rats were singly placed in the FST chamber and habituated to forced swimming for 15 min. Rats initially tried to escape from the FST chamber but later they habituated to the swimming conditions which was observed as maintaining immobility. After the 15 min session, animals were removed from the chamber, dried using cloth towels and were placed under a heating lamp for at least 30 min. Water in the cylinder was changed every 1-2 rats (to remove animal litter). After the habituation sessions were completed, the rats were returned to their home cages.

Test day: Rats were injected with KOPr compounds 5 min (Sal A), 15 min (nalmefene) and 45 min (DS1) prior to the experiments. This second FST was carried out for a period of 5 min. The climbing, swimming and immobility behaviours were recorded using a camera connected to a nearby computer via Ethernet. To avoid any bias in

analysis, treatments were masked from the experimenter during subsequent video analysis. The incidence of each FST behaviour was analysed in bins of 5 sec interval.

3.2.5.6. Effect of serotonin transporter blockade by FLX on KOPr agonist induced depression in FST:

The habituation and test sessions were carried out as previously described (section 3.2.4.5). In between the habituation and test sessions, rats were treated with saline (1 mL/kg, sc) or FLX (5 mg/kg, sc; Detke et al., 1995; Estrada-Camarena et al., 2003), 30 min, 19 hr and 23 hr post habituation. The next day (24 hr after habituation), animals received the KOPr agonists (Sal A, DS1) and were subjected to FST for 5 min. The data obtained from these experiments were analysed as described in section 3.2.5.5.

3.2.5.7. Transfections and live-cell imaging to determine SERT function modulation by KOPr agonists (between cell designs).

Transfections:- For ASP^+ uptake experiments, HEK-293 cells were trypinised (TrypLETM express stable Trypsin-like enzyme with Phenol red, Invitrogen, New Zealand), washed and cultured in fresh antibiotic-free media (Dulbecco's modified Eagle medium (Gibco, Invitrogen) containing 10% fetal bovine serum) and were seeded in 35 mm sterile glass bottomed Delta T culture dishes (MatTek MA, USA) at 1.25 X 10^5 cells/mL, using 1 mL/dish. Transient transfections were performed with plasmids containing green fluorescence protein tagged human SERT (GFP-hSERT; a gift from Dr. Samanda Ramamoorthy, Medical University of South Carolina, USA; Ramamoorthy et al., 1993) and myc-tagged rat KOPr (myc-rKOPr; a gift from Dr. Lakshmi Devi, Mount Sinai School of Medicine, NY, USA; Li et al., 1993; Meng et al., 1993) using Lipofectamine 2000 (Invitrogen) and OptiMEM media (Invitrogen). The transfections were carried out according to the manufacturer's instructions (Appendix 3). The transfection solution contained 3 µL Lipofectamine and 1 µg plasmid DNA (GFP-

hSERT: 0.4 µg ; myc-rKOPr: 0.6 µg) and the volume made up to 100 µl using OptiMEM. Each 35 mm culture dish was treated with 100 µL of the transfection solution. The cells were transfected 24 hr post-seeding. Drug treatment and imaging experiments were performed 36-48 hr post transfection when cells were found confluent between 70-90%.

Live-cell ASP$^+$ uptake experiments:- On the day of the experiment, cells were treated with either drug (10 µM U50488H, 10 µM Sal A, 10 µM DS1) or vehicle (0.1% DMSO diluted in Krebs Ringer Herpes buffer; KRH, pH: 7.4, Appendix 4) and placed into the incubator for 30 min. Just before image capture, the media from the dish was replaced with KRH and the dish was mounted on a temperature (37°C) and humidity controlled stage (Tokai Hit; INU-ZILCS-Fl; Shizuoka-ken; Japan) and attached to an Olympus FV1000 laser scanning confocal microscope (Olympus, Wellington, New Zealand). Initially cells were visualized using a 40X water immersion objective (Olympus) and a monolayer of cells selected. The KRH was then removed without disturbing the dish and an initial image showing GFP-hSERT fluorescence was captured (Fig. 3.1A). Immediately after this, 1 ml of 10 µM ASP$^+$ solution in KRH (Sigma, MO; 1 mL/dish) was added, and live-cell ASP$^+$ uptake recorded for 5 min with images taken every 5 sec (Fig. 3.1B). The GFP-hSERT and ASP$^+$ were excited to fluoresce by using the solid state lasers (Olympus, Wellington, New Zealand). GFP-hSERT was excited at 473 nm, (485-545 filter) and ASP$^+$ was excited at 559 nm (570-670 filter).

Data analysis for live-cell ASP$^+$ uptake experiments:- Individual cells were selected in a region of interest using both differential interference contrast and GFP-hSERT images to localise the cell boundaries (Fig. 3.1C)**.** The GFP-hSERT and ASP$^+$ fluorescence were quantified for each cell using Fluoview FV1000 software (Olympus**;** Fig. 3.1D).

Non-transfected cells had shown some non-specific ASP^+ accumulation. This non-specific accumulation was subtracted from the accumulation measured in the transfected cells. In order for ASP^+ accumulation to be directly correlated to the amount of GFP-hSERT expressed on the cell surface, we expressed ASP^+ uptake as a percentage of SERT expression using the following formula:-

$$\textbf{Normalization of fluorescence intensity of } \mathbf{ASP^+} \textbf{ uptake (AFU)} = \frac{ASP^+ \text{ fluorescence intensity}_{\text{background corrected}}}{\text{GFP-SERT fluorescence intensity}} \times 100$$

All experiments were performed in duplicate from at least three separate transfections.

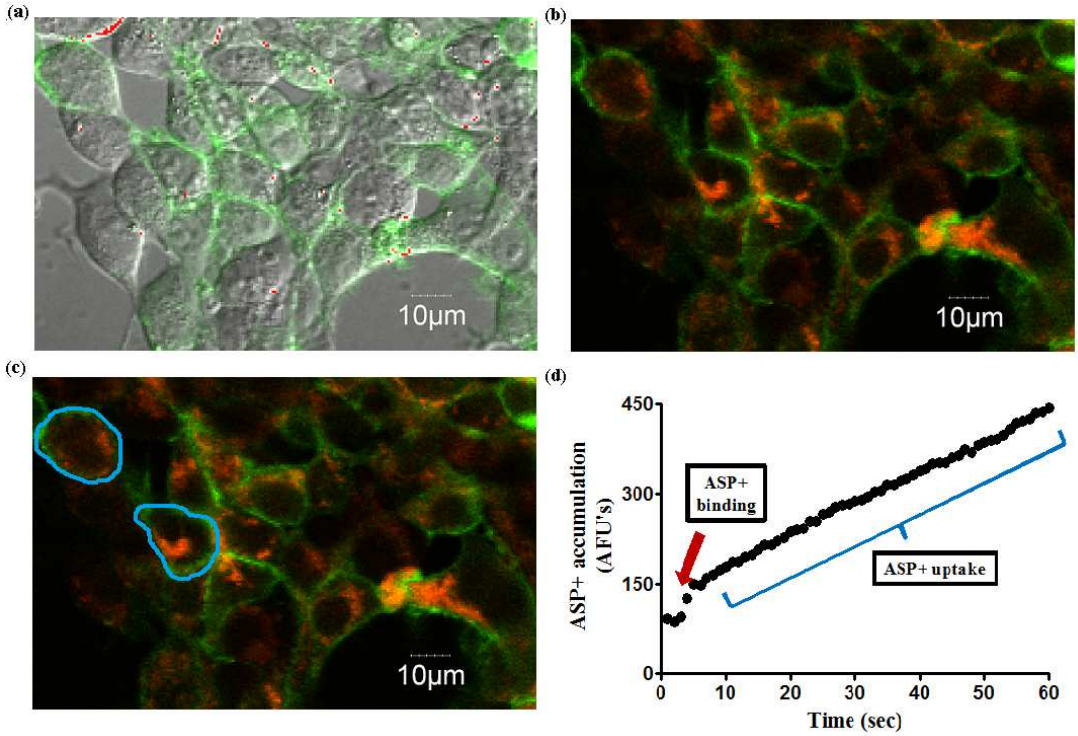


Fig. 3.1 ASP+ accumulation and fluorescence analysis.

A*. After incubating the cells with KOPr agonists (10 µM U50488H, Sal A, DS1) or vehicle (DMSO), each culture dish was placed on the inverted confocal microscope stage which focussed on the monolayer of cells visualised using differential interference contrast and GFP-hSERT fluorescence (green colour) to identify and locate cells. The GFP-hSERT fluorescence was captured just before adding* ASP^+*.* ***B.*** ASP^+ *was then added to the dish and uptake was measured every 5 sec for 5 min.* ***C.*** *After 5 min uptake, cells were circled and the amount of* ASP^+ *uptake accumulated (red colour) over 5 min was determined using Fluoview software (Version 2.0c).* ***D.*** *The binding to and uptake pattern of* ASP^+ *in a representative GFP-hSERT and myc-rKOPr transfected cell. Scale bar represents 10 µm.*

3.2.6. Statistical analysis

Data analysis was performed using Prism Graphpad software (Version 5.0; San Diego, CA). Statistical analysis for cocaine induced hyperactivity; spontaneous open field activity; sucrose reinforcements (for MOM Sal B, nalmefene) were performed using separate Student t-tests. The sucrose reinforcement tests for Sal A, DS1 and CTA (for Sal A, DS1 and nalmefene) were analysed using one way ANOVA followed by Tukey post-hoc tests. The effect of 5HT system modulation via SERT blockade using FLX on Sal A and DS1 induced immobility were analysed using a separate two-way ANOVA for each FST behaviour'. For comparing FST results in cocaine exposed and drug naïve rats, each forced swimming behaviour' was analysed using the Mann-Whitney test. Data for correlation experiments measuring ASP^+ uptake and GFP-hSERT expression levels were analysed using linear regression. ASP^+ accumulation experiments in non-transfected vs. transfected cells were analysed from the 5 min uptake values using student t-test. Changes in ASP^+ uptake in KOPr treated cells were analysed using one-way ANOVA followed by Dunnets multiple comparison tests (Oz et al., 2010). For analysing GFP-hSERT expression levels in cells selected for ASP^+ accumulation experiments, one-way ANOVA followed by Dunnets multiple comparison tests were performed.

3.3. Results

3.3.1. Effect of KOPr compounds on cocaine induced hyperactivity (cocaine self-administering rats).

The effect of Sal A (0, 0.3 mg/kg, ip), DS1 (0, 0.3 mg/kg, ip), MOM Sal B(0, 0.3 mg/kg, ip) and nalmefene (0, 0.3 mg/kg, sc) pre-treatment on cocaine induced hyperactivity in rats previously self-administering cocaine and subjected to reinstatement tests is shown in Fig 3.2. No significant reduction in cocaine produced hyperactivity was observed in either Sal A ($p = 0.46$; Fig 3.2a) and MOM Sal B pre-treated animals ($p = 0.43$; Fig 3.2c). A non-significant trend to increase total activity was noted in animals treated with nalmefene ($p = 0.216$; Fig 3.2d). However, rats that were pre-treated with DS1 showed a non-significant trend towards a decrease in cocaine produced hyperactivity ($p = 0.09$; Fig 3.2b).

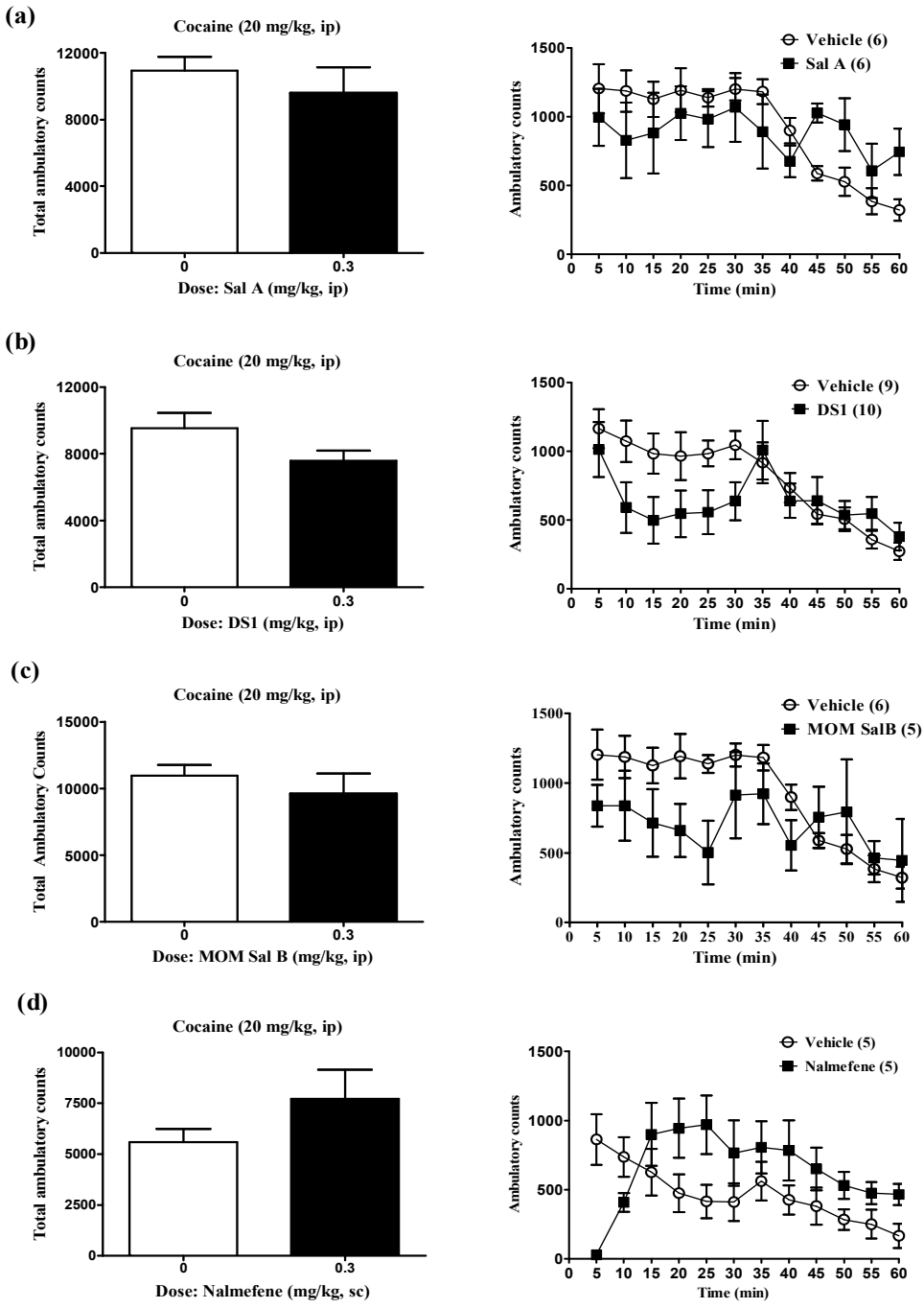


Fig 3.2. Effect of KOPr compounds on cocaine induced hyperactivity (cocaine self-administering rats).

Rats previously self-administering cocaine were initially treated with either (a) Sal A (0, 0.3 mg/kg; n= 6), (b) DS1 (0, 0.3 mg/kg; n= 9, 10), (c) MOM Sal B (0, 0.3 mg/kg; n=5, 6), (d) nalmefene (0, 0.3 mg/kg; n= 5). This was followed by a priming injection of cocaine (20 mg/kg) and total locomotion was measured for 60 min. Symbols in the left panel represent total ambulatory counts (+SEM) and in right panel indicates mean (± SEM) of ambulation measured every 5 min interval. Student t-test.

3.3.2. Effect of KOPr compounds on natural reward reinforcements

The effect of Sal A (0, 0.3, 1.0 mg/kg, ip); DS1 (0, 0.3, 1.0 mg/kg, ip); MOM Sal B (0, 0.3 mg/kg, ip) and nalmefene (0, 0.3 mg/kg, sc) on sucrose reinforced responding is shown in Fig. 3.3. Statistical analysis indicated no significant alteration in sucrose reinforcement observed in rats which were pre-treated with either Sal A [$F(2,27) = 0.058$, $p=0.944$; Fig. 3.3a]; DS1 [$F(2,18) = 1.11$, $p=0.35$; Fig. 3.3b] and nalmefene ($p>0.05$; Fig. 3.3d). However, a significant reduction in sucrose reinforcement was observed in rats pre-treated with MOM Sal B ($p=0.0073$; Fig. 3.3c).

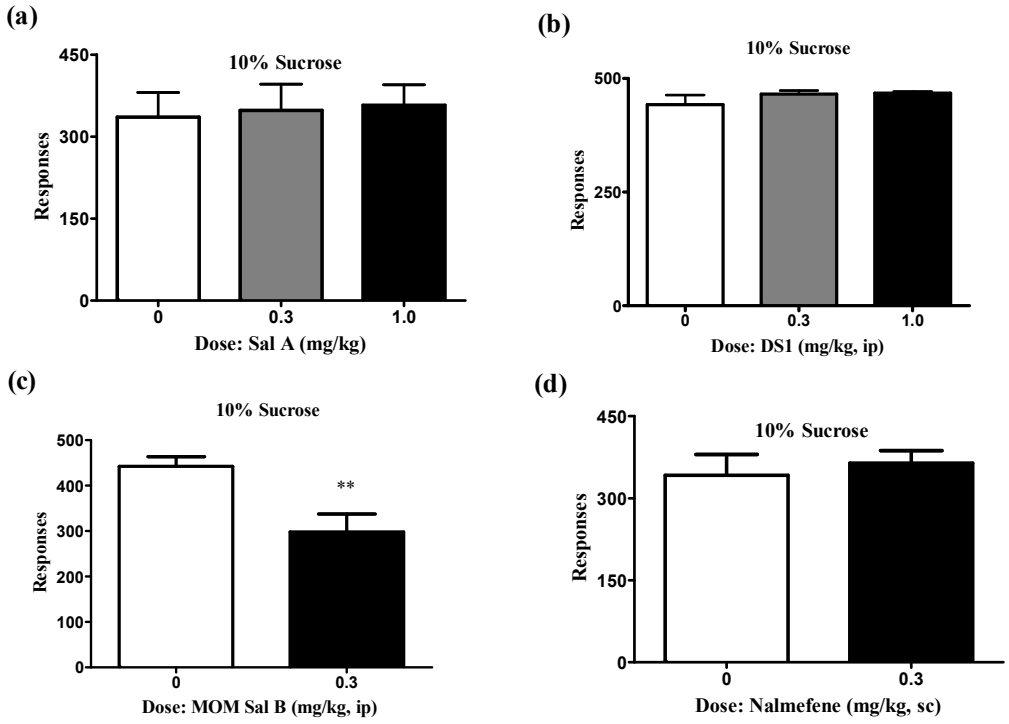


Fig. 3.3. Effect of KOPr compounds on natural reward reinforcements.

*Animals which were stably responding for sucrose reinforcements at a FR-5 schedule of reinforcement were injected with KOPr compounds prior to measuring sucrose reinforced lever press responding for 60 min. Bars indicate average number of "active" lever press responses (+SEM) for 10% sucrose solution following (a) Sal A (0, 0.3, 1.0 mg/kg; n=10), (b) DS1 (0, 0.3, 1.0 mg/kg, n=7), (c) MOM Sal B (0, 0.3 mg/kg, n=7), (d) nalmefene (0, 0.3 mg/kg, n=6) treatment. One way ANOVA followed by Tukey post-hoc tests was used for (a) and (b) and Student t-test for (c) and (d). **p<0.01 compared to 0 mg/kg.*

3.3.3. Effect of KOPr compounds on motor function.

Effect of an acute exposure to Sal A (0, 0.3 mg/kg, ip); DS1 (0, 0.3 mg/kg, ip); MOM Sal B (0, 0.3 mg/kg, ip) and nalmefene (0, 0.3 mg/kg, sc) on spontaneous open field locomotion activity in rats is shown in Fig. 3.4. Statistical analysis indicated no significant change in total locomotion activity observed for Sal A ($p=0.78$; Fig. 3.4a); DS1 ($p=0.54$; Fig. 3.4b) and MOM Sal B ($p=0.43$; Fig. 3.4c) pre-treated animals. However, rats pre-treated with nalmefene ($p=0.023$; Fig. 3.4d) significantly suppressed spontaneous open field activity.

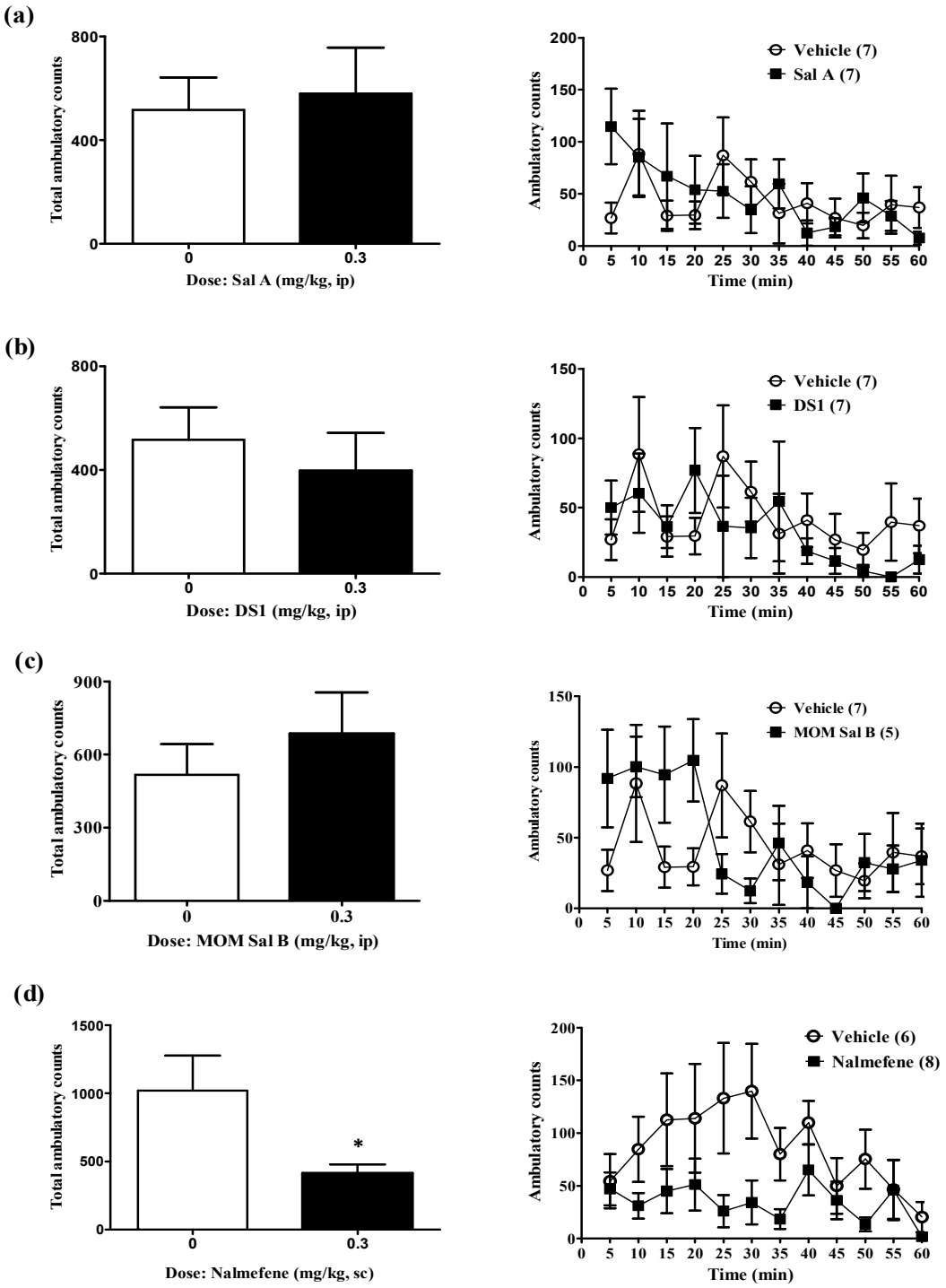


Fig. 3.4. Effect of KOPr compounds on motor function.

*Drug naïve rats were treated with (a) Sal A (0, 0.3 mg/kg; n= 7), (b) DS1 (0, 0.3 mg/kg; n= 7) and (c) MOM Sal B (0, 0.3 mg/kg; n= 5,7), (d) nalmefene (0, 0.3 mg/kg, n= 5,7) and spontaneous open field activity was measured for 60 min. Symbols in the left panel represent total ambulatory counts (+SEM) and in right panel indicates mean (± SEM) of ambulation measured every 5 min interval. *p<0.05, data compared with 0 mg/kg, Student t-test.*

3.3.4. Effect of KOPr compounds on conditioned taste aversion.

The amount of saccharin consumed by rats paired with novel tasting saccharin solution and Sal A (0, 0.3 mg/kg, ip); DS1 (0, 0.3 mg/kg, ip) and nalmefene (0, 0.3 mg/kg, sc), on pairing day and test day is shown in Fig 3.5. Statistical analysis showed no significant difference in saccharin drinking patterns observed for Sal A ($F(3,22) = 2.7$; $p > 0.05$; Fig 3.5a) and nalmefene ($F(3, 20) = 0.3$; $p = 0.8$; Fig 3.5c) pre-treated rats. However, DS1 pre-treated rats, showed a significant increase in saccharin drinking pattern on test day when compared with pairing day ($F(3, 22) = 7.3$; $p < 0.01$; Fig 3.5b).

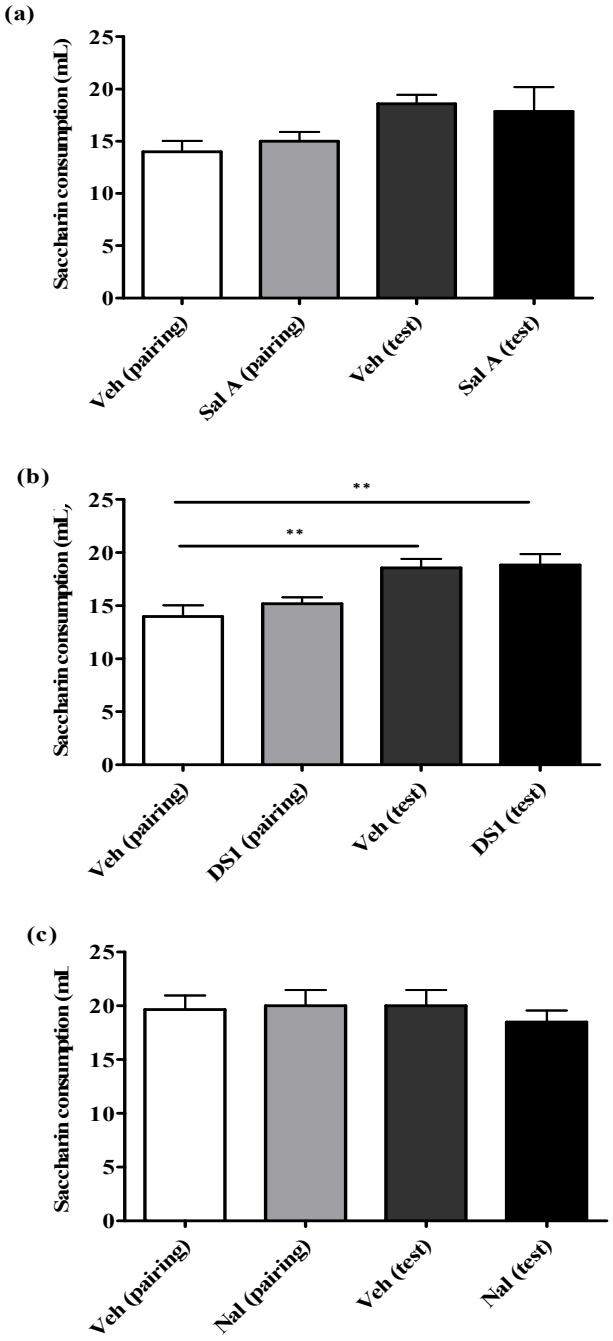


Fig. 3.5. Effect of KOPr compounds on conditioned taste aversion.

*Animals experienced a pairing of a novel tasting saccharin solution and KOPr compounds [(a) Sal A (0, 0.3 mg/kg; n= 6-7), (b) DS1 (0, 0.3 mg/kg; n= 6-7) and (c) nalmefene (0, 0.3 mg/kg; n= 6)]. They were again presented with the saccharin solution, 48 hr post pairing session, and the amount of saccharin consumed on pairing day and test day was measured for 40 min. Bars indicate average (+SEM) amount of 0.1% saccharin solution consumed by rats (mL) treated with vehicle or KOPr compounds on pairing and test day. **p<0.01, one way ANOVA followed by Tukey post-hoc test.*

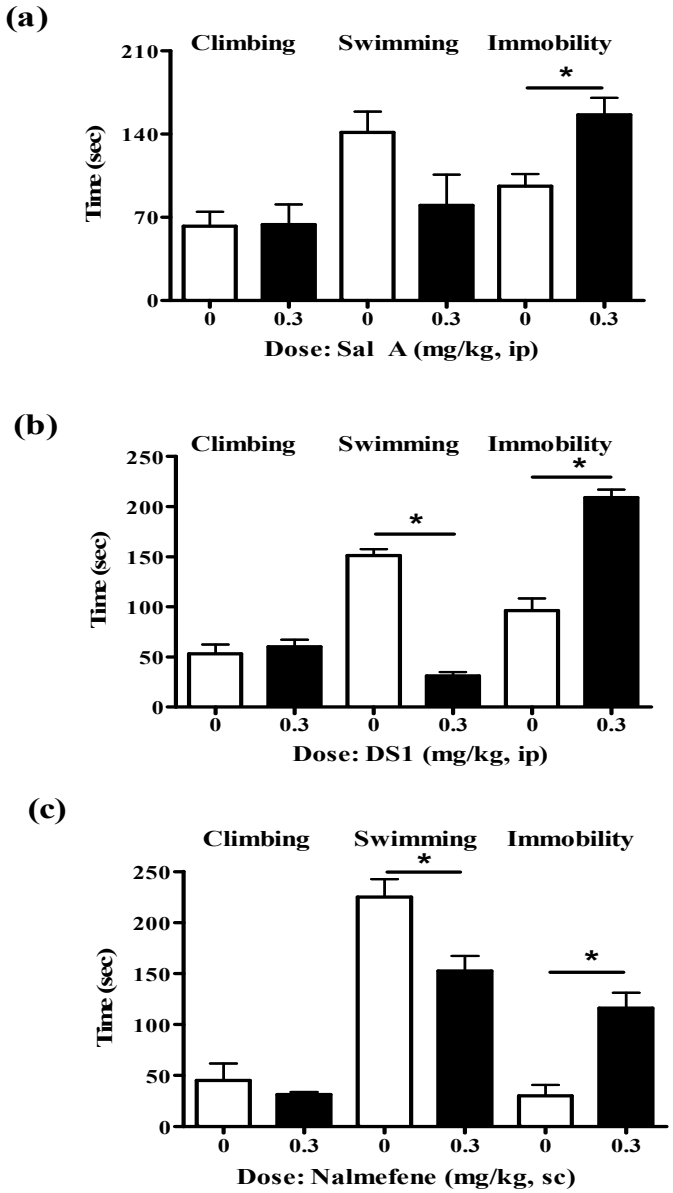
3.3.5. Effect of KOPr compounds on FST in rats with previous history of cocaine self-administration.

The effect of acute exposure to Sal A (0, 0.3 mg/kg, ip); DS1 (0, 0.3 mg/kg, ip) or nalmefene (0, 0.3 mg/kg, sc) in rats with a prior history of cocaine self-administration on forced swimming behaviours are shown in Fig. 3.6.

For Sal A pre-treated animals, statistical analysis indicated no significant difference in time spent climbing ($p=0.9$) or swimming ($p=0.15$). However, a significant increase in immobility ($p=0.04$) time was observed for Sal A pre-treated rats vs. vehicle treated rats (Fig. 3.6a).

For DS1 pre-treated rats, no significant difference in time spent climbing ($p=0.83$) was noted. However, a significant decrease in time spent swimming ($p=0.01$) and an increase in immobility ($p=0.01$) time were noted for DS1 pre-treated rats vs. vehicle treated rats (Fig. 3.6b).

For nalmefene pre-treated rats, no significant difference in climbing time ($p=1.0$) was noted. However, a significant reduction in swimming time ($p=0.03$) and a significant increase in time spent immobile ($p=0.02$) were observed for nalmefene pre-treated rats vs. vehicle treated rats (Fig. 3.6c).



<u>Fig. 3.6. Effect of KOPr compounds on FST in rats with previous history of cocaine self-administration.</u>

*Animals previously self-administering cocaine and subjected to reinstatement testing were habituated to the forced swimming sessions on day 1. The following day, animals received (a). Sal A (0, 0.3 mg/kg; n=4), (b). DS1 (0, 0.3 mg/kg; n=5) or (c) nalmefene (0, 0.3 mg/kg; n=4-5) and were subjected to FST. The forced swimming behaviours were recorded for a period of 5 min and analysed in bins of 5 sec. Data are expressed as mean time in sec (+SEM) for climbing, swimming or immobility during FST. *p<0.05. Data for 0.3 mg/kg compared with 0 mg/kg for corresponding climbing, swimming and immobile behaviours using Mann Whitney tests.*

3.3.6. Effect of KOPr compounds on FST in drug naive rats.

The effect of single injections of Sal A (0, 0.3 mg/kg, ip); DS1 (0, 0.3 mg/kg, ip) and nalmefene (0, 0.3 mg/kg, sc) on forced swimming behaviours in drug naive rats is shown in Fig. 3.7.

Significant reductions in climbing ($p=0.044$) and swimming ($p=0.005$) times were observed in rats pre-treated with Sal A. A significant increase in time spent immobile ($p=0.005$) was also noted for Sal A vs. vehicle pre-treated animals (Fig. 3.7a).

For DS1 pre-treated animals, a non-significant trend to decrease time spent as climbing was noted ($p=0.09$). However, a significant decrease in swimming time ($p=0.006$) and a significant increase in time spent immobile ($p=0.006$) was observed for DS1 vs. vehicle pre-treated animals (Fig. 3.7b).

For nalmefene pre-treated rats, statistical analysis indicated no significant difference in time spent as climbing ($p=0.48$). However, a significant decrease in swimming time ($p=0.025$) and a significant increase in immobility time ($p=0.034$) were observed for nalmefene vs. vehicle pre-treated animals (Fig. 3.7c).

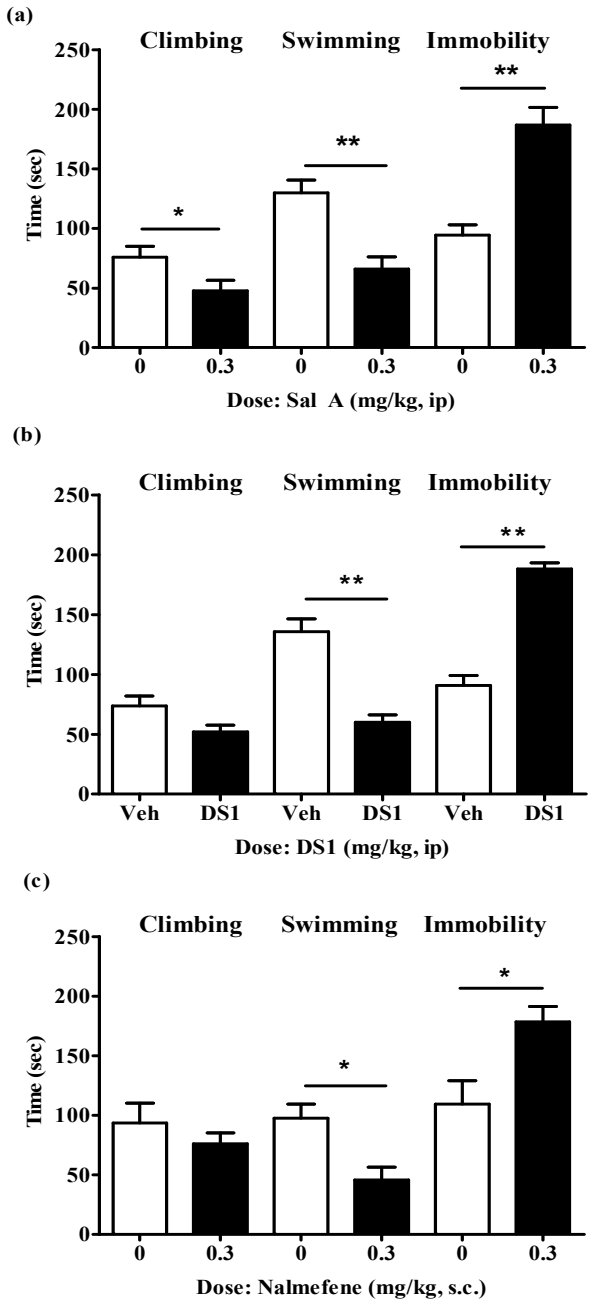


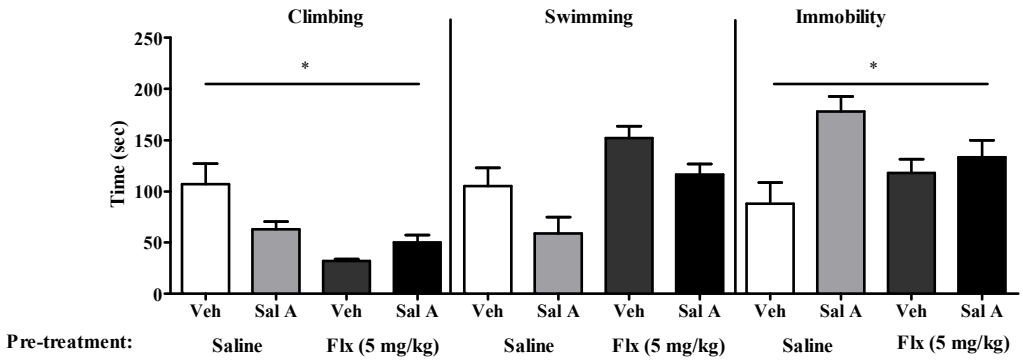
Fig. 3.7. Effect of KOPr compounds on FST in drug naive rats.

*Drug naive animals were habituated to the forced swimming sessions on day 1. The following day, animals received (a). Sal A (0, 0.3 mg/kg; n=6), (b). DS1 (0, 0.3 mg/kg; n=5-7) and (c). nalmefene (0, 0.3 mg/kg; n=6) and were subjected to FST. The forced swimming behaviours were recorded for a period of 5 min and analysed in a bin of 5 sec intervals. Data expressed as mean time in sec (+SEM) spent by animals for climbing, swimming and immobile behaviours during FST. *p<0.05, **p<0.01. Data for 0.3 mg/kg compared with 0 mg/kg for corresponding climbing, swimming and immobile behaviours. Mann Whitney test.*

3.3.7. Effect of SERT blockade by FLX on KOPr agonists induced immobility in FST.

The purpose of this test was to determine the role of SERT blockade via sub-chronic FLX pre-treatment on different forced swimming behaviours produced by Sal A and DS1 exposure in rats. Statistical analysis shows that FLX pre-treatment significantly reduced the amount of time spent immobile in animals pre-treated with Sal A [$F(1,17) = 4.95$, $P < 0.05$; Fig 3.8a] or DS1 [$F(1, 17) = 7.25$, $P = 0.015$; Fig 3.8b]. A significant increase in the amount of time spent climbing was also noted in Sal A [$F(1,17) = 8.01$, $P = 0.012$; Fig 3.8a] and DS1 [$F(1,17) = 5.44$, $P < 0.05$; Fig 3.8b] pre-treated animals, following FLX pre-treatment. No effect of FLX pre-treatment on swimming time was observed in Sal A [$F(1, 17) = 0.15$, $P = 0.7$; Fig 3.8a] or DS1 [$F(1, 17) = 1.75$, $P = 0.2$; Fig 3.8b] treated rats.

(a)



(b)

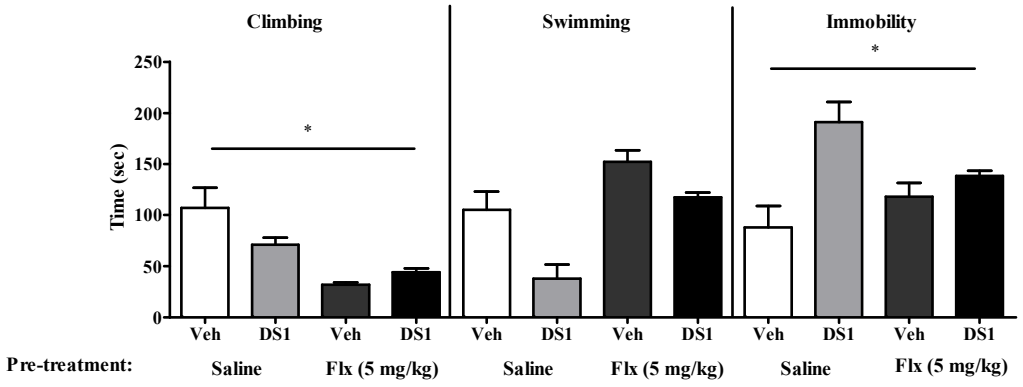


Fig. 3.8. Effect of SERT blockade by FLX pre-treatment on KOPr agonist induced immobility in FST.

*Drug naive rats were habituated to the forced swimming test on day 1. Rats received injections of FLX (0, 0.3 mg/kg, sc), 30 min, 19 hr and 23 hr post habituation sessions. The next day (24 hr following habituation sessions), animals received an injection of a) Sal A (0, 0.3 mg/kg) or b) DS1 (0, 0.3 mg/kg) and were subjected to FST. The forced swimming behaviours were recorded for a period of 5 min and analysed in a bin of 5 sec intervals. Data expressed as mean time in sec (+SEM) for climbing, swimming or immobile during FST. *$p<0.05$, two-way ANOVA for each FST behaviours. n= 5-6 for each group.*

3.3.8. Control experiments for ASP^+ uptake

Control experiments were performed to show a correlation between the level of ASP^+ accumulation and GFP-hSERT expression levels. An image of GFP-hSERT fluorescence was captured, followed by the addition of ASP^+ at time 0. The ASP^+ accumulation was measured every 5 sec for 5 min. Statistical analysis showed a linear correlation between GFP-hSERT expression and ASP^+ accumulation in transfected cells ($r^2 = 0.82$; Goodness of fit, $p<0.0001$; Spearman coefficient, $n = 80$; Fig. 3.9a).

The amount of ASP^+ accumulation in cells not expressing GFP-hSERT (non-transfected cells) was compared with those cells expressing GFP-hSERT (transfected cells). Our results showed a significant increase in ASP^+ accumulation in transfected cells vs. non-transfected cells ($p<0.0001$; Fig. 3.9b), thus indicating increased ASP^+ uptake in cells expressing GFP-hSERT.

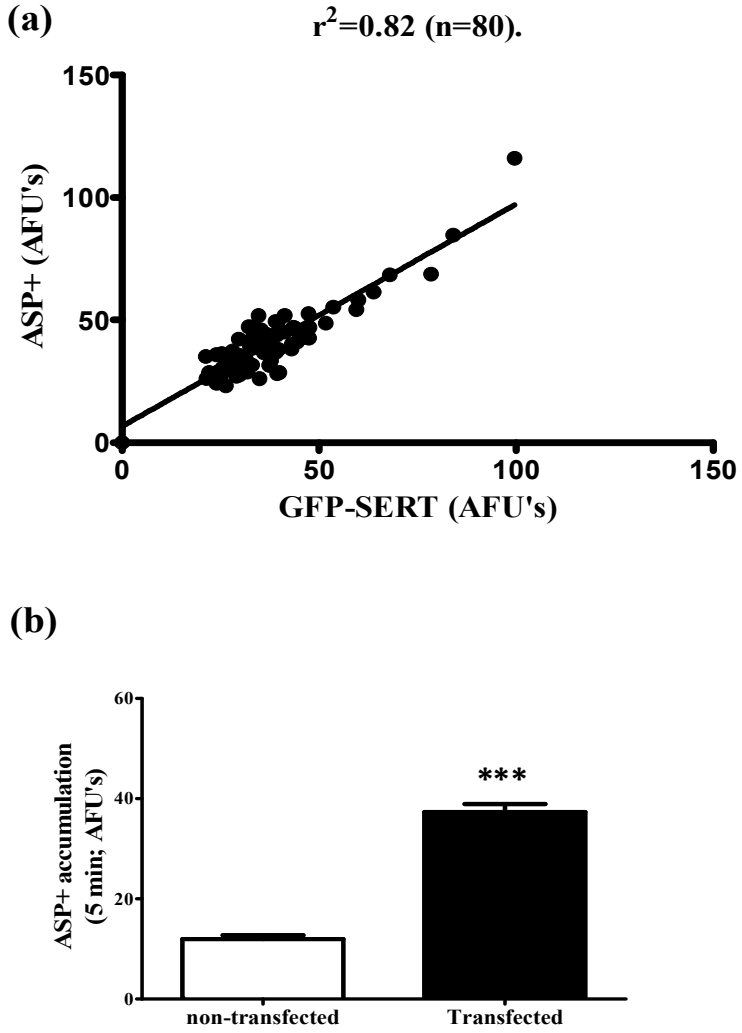


Fig. 3.9. Control experiments for ASP$^+$ uptake.

*a) ASP$^+$ uptake and GFP-hSERT expression levels. GFP-hSERT levels for each cell are shown at time 0 (x axis). The accumulation of ASP$^+$ after 5 min is shown for each cell on the y axis. ASP$^+$ and GFP-hSERT fluorescence are measured as arbitrary fluorescence units (AFU). Each dot indicates GFP-hSERT expression levels and corresponding ASP$^+$ accumulated at 5 min by that particular cell. Data were analysed using linear regression. n=80. b) ASP$^+$ accumulation in transfected vs. non-transfected cells. Bars indicate the amount of ASP$^+$ accumulated in transfected and non-transfected cells and measured as AFU. ***p<0.0001, data compared with a non-transfected group of cells using student t-test. Number of non-transfected cells = 24, transfected cells = 80.*

3.3.9. Effect of KOPr agonists pre-treatment on ASP^+ uptake

The effect of KOPr agonist pre-treatment on GFP-hSERT function in GFP-hSERT and myc-rKOPr co-transfected HEK-293 cells using the ASP^+ uptake method is shown in Fig. 3.10a. Cultures were pre-treated with 10 µM U50488H, Sal A or DS1 and following a 30 min incubation, SERT function was measured. Pre-treatment with the traditional KOPr agonist U50488H significantly decreased ASP^+ uptake ($p<0.001$) [$F(3,261) = 106.1$, $p<0.0001$]. However, there was no significant difference in ASP^+ accumulation in cells pre-treated with either Sal A or its structural analogue DS1 ($p>0.05$), thus indicating a potential difference in modulation of SERT function by novel and traditional KOPr agonists.

In order to verify that changes seen in ASP^+ uptake were specifically due to KOPr agonist treatment and not due to selection of cells with lower levels of GFP-hSERT expression levels, GFP-hSERT fluorescence intensity for cells pre-treated with KOPr agonists and selected for ASP^+ accumulation analysis were compared with vehicle treated cells. Statistical analysis revealed no significant difference in GFP-hSERT expression levels in cells analysed for ASP^+ uptake [$F(3,261) = 2$, $p = 0.11$; Fig. 3.10b].

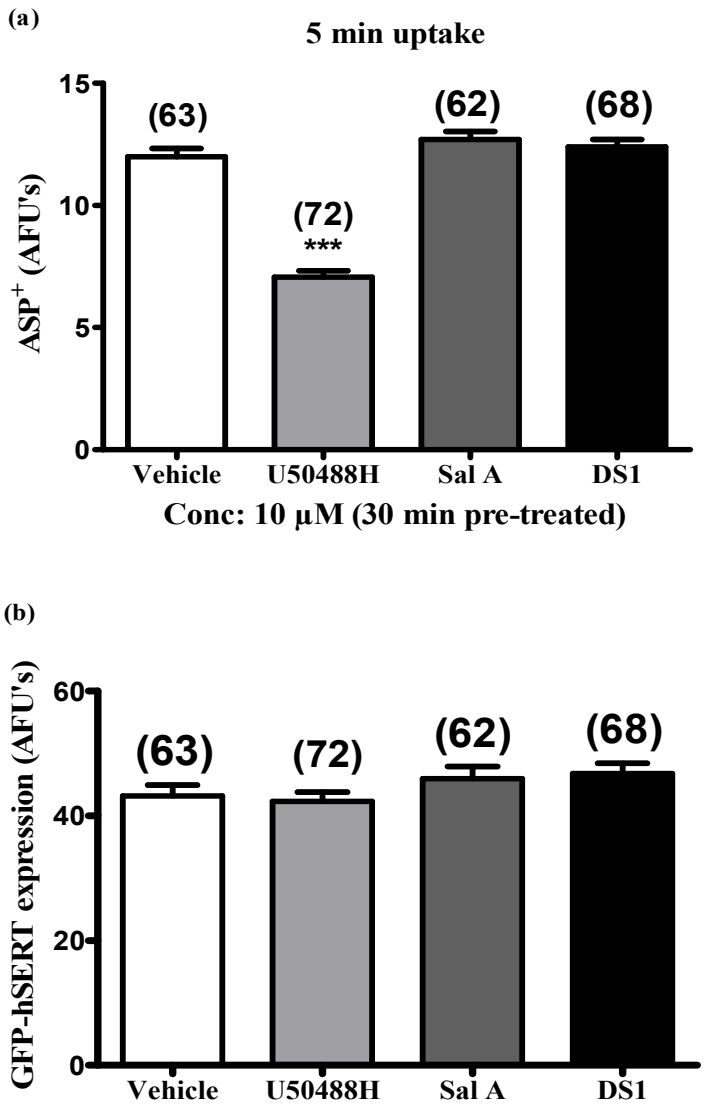


Fig. 3.10. Effect of KOPr agonists pre-treatment on ASP+ uptake

*a) HEK-293 cells co-transfected with GFP-hSERT and myc-rKOPr were pre-treated with KOPr agonists (U50488H, Sal A and DS1; 10 µM each) for 30 min and ASP^+ accumulation was recorded for 5 min. The quantification of ASP^+ fluorescence was measured as arbitrary fluorescence units (AFU). Figures indicates Mean (+SEM) AFU's. ***P<0.001, one-way ANOVA followed by Dunnet multiple comparison test. b) Symbols indicate the amount of GFP-hSERT expression measured in AFU's for cells selected for ASP^+ uptake analysis. One-way ANOVA followed by Dunnet multiple comparison tests. Numbers in parentheses indicate sample size.*

3.4. Summary of results.

Tab. 3.2. Control experiments to determine selectivity of KOPr agonists in attenuating reinstatement

Compounds tested	Cocaine induced hyperactivity in self-administering rats (motor suppression during reinstatement test)	Sucrose reinforcement test (effect on operant responding for natural reward)
Sal A	No effect	No effect
DS1	Non-significant trend to decrease	No effect
MOM Sal B	No effect	Significant attenuation
Nalmefene	Non-significant trend to increase	No effect

Tab. 3.3. Adverse effect profile of novel KOPr agonists

Compounds tested	Motor function (spontaneous open field activity)	Aversion (CTA)	Depressive behaviours (FST) (Cocaine exposed and drug naive rats)
Sal A	No effect	No effect	Inceased immobility
DS1	No effect	No effect	Inceased immobility
MOM Sal B	No effect	-	-
Nalmefene	Significant suppression	No effect	Inceased immobility

Tab. 3.4. Effect of FLX pre-treatment on KOPr agonist induced enhanced immobility in FST

Compound tested	Effect on depressive behaviour
Sal A	Improved
DS1	Improved

Tab. 3.5. Effect on SERT modulation by traditional and novel KOPr agonists by using live cell ASP^+ uptake.

Compound tested	Effect on ASP^+ uptake
U50488H	Decreased
Sal A	No effect
DS1	No effect

3.5. Discussion

In Chapter 2 we showed that a single injection of either Sal A, DS1, MOM Sal B or nalmefene attenuated cocaine prime induced reinstatement. In the present chapter we evaluated whether this attenuation in cocaine seeking behaviour was selective to reinstatement. We tested this by measuring cocaine induced hyperactivity (in cocaine self-administering rats) and disruption of responding for palatable reinforcements (sucrose reinforcement). We also performed studies to determine if side effects such as sedation, aversion and depressive like behaviour resulted following acute administration of novel KOPr agonists. Initially FST experiments were performed using animals with history of cocaine self-administration (Tab. 3.1). These pilot studies were necessary to develop this paradigm in our laboratory and to obtain permission from the Animal Ethics Committee to test the effect of KOPr compounds (Sal A, DS1, nalmefene) in drug naive rats. In order to determine a possible mechanism for the depressive like effects of KOPr agonist treatment, the effect of blocking SERT on KOPr agonists induced depressive behaviours was tested using FLX in rats. Additionally, the effect of SERT modulation by KOPr agonists was tested *in vitro* by using live cell ASP^+ uptake assay.

The results from the present study indicate that acute exposure to Sal A (0.3 mg/kg), DS1 (0.3 mg/kg; Fig 3.2a), MOM Sal B (0.3 mg/kg; Fig 3.2c) or nalmefene (0.3 mg/kg; Fig. 3.2d) did not produce any statistically significant motor suppression during the reinstatement test. However, on closer inspection, DS1 (0.3 mg/kg) exposure showed a non-significant trend towards a decrease in motor function (Fig 3.2b). Results from Chapter 2 indicate that DS1 pre-treatment significantly attenuated acute cocaine-induced hyperactivity in rats (Tab 2.4). The observed difference in DS1's ability to modulate cocaine-induced locomotion might be attributed to the multiple cocaine exposures as animals used in this study previously self-administered cocaine (Section

3.2.5.1). Drug naive animals were used for experiments outlined in Chapter 2 (Section 2.2.4.2 A). Acute nalmefene injections, on the other hand, showed a non-significant trend towards an increase in motor function (Fig 3.2d). Acute exposure to Sal A, DS1 or nalmefene, at doses that successfully attenuated cocaine seeking, did not suppress sucrose reinforcement (Fig. 3.3). In contrast, MOM Sal B significantly suppressed sucrose reinforcements (Fig. 3.3c). These results indicate that Sal A (Morani et al., 2009) and an analogue with similar potency at KOPr (DS1), attenuated cocaine prime induced drug-seeking behaviour without reducing sucrose reinforcements. This however cannot be said for the more potent and longer acting Sal A analogue, MOM Sal B (Tab 3.2).

Previous studies by June and colleagues (1998), showed that nalmefene pre-treatment attenuated ethanol-maintained operant lever press responding without affecting saccharin reinforcement. Their study also showed that the efficacy of nalmefene was dependent on the route of administration (sc > oral route) and pre-treatment time (15 min prior to the commencement of tests). Injections of nalmefene bilaterally into the NAc (1.0, 10 μg) and VTA (10 μg) also attenuated ethanol induced responding without affecting saccharin reinforcement (June et al., 2003). However, infusing a higher dose (40 μg) of nalmefene bilaterally into both the VTA and NAc suppressed ethanol and saccharin reinforcement. These findings indicate a dose related effect produced by nalmefene in selectively attenuating alcohol self-administration (June et al., 2003). Since the dose of nalmefene tested in this current study (0.3 mg/kg) attenuated cocaine induced reinstatement without suppressing sucrose reinforcements, this effect produced by nalmefene on cocaine seeking behaviour (Chapter 2) was deemed to be selective for cocaine (Tab 3.2).

The effects of a single injection of Sal A (0.3 mg/kg), DS1 (0.3 mg/kg), MOM Sal B (0.3 mg/kg) and nalmefene (0.3 mg/kg) on spontaneous open field activity in rats were determined. Our results indicate that acute injections of Sal A, MOM Sal B and

DS1 did not suppress spontaneous open field activity in rats (Fig. 3.4). These results show that attenuation of both cocaine induced behavioural sensitization by Sal A and DS1 (Chapter 2) and cocaine induced hyperactivity by DS1 in drug naive rats (Chapter 2) was a not due to motor suppression caused by Sal A and DS1 pre-treatment. Furthermore, previous reports have shown that acute exposure to Sal A did not suppress open field activity in rats (Hooker et al., 2009a; Carlezon et al., 2006). Similarly, MOM Sal B exposure did not produce motor suppression in rats (Wang et al., 2008).These published reports support our findings in the current study.

A single injection of nalmefene, however significantly suppressed motor function in the drug naive rats (Fig. 3.4d). Since, nalmefene pre-treatment did not decrease cocaine induced hyperactivity during reinstatement tests, an observable difference in motor function induced by nalmefene exposure might be attributed to the prior cocaine exposure in self-administering rats (Tab. 3.3). Additionally, as all compounds tested having selective affinity for KOPr (Sal A, DS1, MOM Sal B) did not produce locomotor suppression; a possible role of MOPr antagonism might be implicated in nalmefene produced motor suppression in drug naive rats. Further studies are required to explain this. On close observation, animals treated with 75% DMSO (Fig 3.4a, 3.4b, 3.4c) showed less locomotor activity compared to rats treated with 0.9% saline (Fig 3.4d). As nalmefene treated animals produced approximately similar ambulatory counts compared to the other KOPr compounds tested (Sal A, DS1, MOM Sal B, Fig 3.4); the suppression in motor function produced by nalmefene in the spontaneous locomotion test may reflect a floor effect.

In order to evaluate the aversive behaviours produced by acute exposure to novel KOPr agonists, a conditioned taste aversion model was used. Our results indicated that Sal A (0.3 mg/kg) and nalmefene (0.3 mg/kg) when paired with the novel tasting saccharin solution, did not induce aversive effects in rats (Fig. 3.5a, 3.5c; Tab. 3.3).

However, Sal A treated rats showed a non-significant trend to increase saccharin drinking on the test day compared to pairing day (Fig 3.5a). Rats paired with DS1 showed a significant increase in saccharin consumption on test day when compared to pairing day (Fig 3.5b; Tab 3.3). Previous reports suggest that Sal A at low doses produce a preference for conditioned behaviours in rats and zebrafish (Braida et al., 2007; 2008). The preference for a novel tasting saccharin solution produced by low dose of Sal A (0.3 mg/kg) and DS1 (0.3 mg/kg) may be a dose related effect. One aim of this study was to determine if the dose of KOPr agonist that successfuly attenuated drug-seeking had aversive properties. We measured this by using a taste aversion model and showed that acute exposure to all three KOPr compounds did not produce CTA (Tab 3.3). However, detailed studies are required to compare and contrast the aversive properties of different dose of Sal A, DS1 and nalmefene with standard aversive agent such as lithium chloride (Taraschenko et al., 2010). Such studies have the potential to further characterize the aversive properties of these KOPr compounds.

Recent studies have shown that systemic administration of Sal A (2.0 mg/kg) increases ICSS threshold in rats and decreases phasic DA release in the NAc (Ebnar et al., 2010). This same study also showed that Sal A (2 mg/kg) also significantly decreased sucrose reinforcements using both FR-5 schedule and progressive ratios schedule of reinforcement (Ebnar et al., 2010). In contrast, a lower dose of Sal A (0.25 mg/kg) did not modulate ICSS thresholds, alter sucrose reinforcement or decrease phasic DA release in NAc (Ebnar et al., 2010). Moreover, results from our current study indicate that Sal A at comparatively lower doses (0.3 and 1.0 mg/kg) did not suppress sucrose reinforcement using a FR-5 schedule of reinforcement (Morani et al., 2009). The ip route of administration for Sal A and the vehicle control (75% DMSO) in this study were comparable to those used by Ebnar et al, (2010), indicating that Sal A induced aversive effects might be dose related. Similarly, Sal A at higher dose produced

place aversion in C57BL/6J mice (1.0, 3.2 mg/kg; Zhang et al., 2005), zebrafish (80 μg/kg; Braida et al., 2007) and Wistar rats (160 μg/kg; Braida et al., 2008). Therefore, Sal A (0.3 mg/kg) produces anti-cocaine effects without producing aversion, motor suppression or anhedonia in rats (Morani et al., 2009).

Our initial studies investigating the depressive like effects produced by KOPr compounds were performed on rats with a previous history of cocaine self-administration. These results showed that Sal A (0.3 mg/kg), DS1 (0.3 mg/kg) and nalmefene (0.3 mg/kg) significantly increased immobility time in the FST (Fig. 3.6). These preliminary findings led to the subsequent FST studies in drug naive rats, which showed that all the KOPr compounds tested also produced pro-depressive behaviours (Fig. 3.7; Tab. 3.3).

Depression induced by Sal A and DS1 might not be due to suppression in motor function, as single exposure to either compound (0.3 mg/kg) had no effect on spontaneous open field activity (Tab 3.3). However, treatment with nalmefene (0.3 mg/kg) induced sedation, as shown by the results from spontaneous locomotion test (Fig 3.4d). Therefore, the depressive effects produced by nalmefene in drug naive rats might not be specific. However, nalmefene has been shown to stimulate the hypothalamic pituitary adrenal axis, which might explain the depressive effects observed in this current study (Geer et al., 2005). Since depression is one of the limiting factors in developing selective KOPr agonists as anti-addiction pharmacotherapies, future studies with nalmefene may help to address wether this behavioural profile is more likely induced by mixed KOPr ligands compared to selective KOPr agonists.

Previous reports have shown that Sal A produces both anti-depressant (Braida et al., 2008; 2009) and pro-depressive effects (Carlezon et al., 2006) in rats using the FST paradigm. The present study determined whether an acute injection of Sal A (0.3 mg/kg)

at a dose which attenuated cocaine seeking (Chapter 2) produced depressive effects or not. The results from this study suggest that acute exposure to Sal A (0.3 mg/kg) produces pro-depressive behaviour without suppressing spontaneous locomotion (Fig. 3.7a; Fig. 3.3a). These results are in agreement with the findings of Carlezon et al., (2006) which showed that pre-treating rats with Sal A, dose dependently (0.25 – 2.0 mg/kg) produced pro-depressive effects without suppressing their open field locomotion activity. The difference in the experimental design of this study was that Carlezon and colleagues, (2006) had pre-treated rats with Sal A, thrice, at 30 min, 19 hr and 23 hr post habituation, whereas, in our study, animals received a single injection of Sal A, 5 min prior to the FST. In contrast to these findings, Braida et al., (2009) showed that acute Sal A up to 1 mg/kg produced anti-depressant effects in rats using FST paradigm. The observable dissimilarities may be attributed to the difference in; route of administration for Sal A (i.p. current study, Carlezon et al., 2006 vs. s.c. Braida et al., 2009); time of Sal A pre-treatment (5 min, current study vs. 20 min Braida et al., 2009), and vehicle used to suspend Sal A (75% DMSO, current study, Carlezon et al., 2006 vs. 1:1:8, Ethanol: Tween 80: Water, Braida et al., 2009).

From the present findings, it was interesting to note that in drug naive rats, Sal A significantly decreased climbing time and swimming time, whereas DS1 significantly reduced time spent swimming, although a trend to decrease climbing behaviour was also noted (Fig 3.7a, 3.7b). In contrast nalmefene only decreased swimming time without any effect on climbing behaviour. Carlezon et al. (2006) also showed that Sal A decreased swimming time during the FST. A decrease in climbing time is believed to relate to NET modulation, whereas SERT is implicated in a reduction in swimming time (Detke et al., 1995). Taken together, these findings implicate a possible involvement of SERT and NET in depressive-like behaviours induced by Sal A and DS1 in the FST and only SERT in nalmefene induced depression. Further studies are required to clarify this.

In order to determine if Sal A and DS1 modulate depressive behaviour via SERT modulation, we measured depressive behaviours following SERT antagonist (FLX) pre-treatment followed by Sal A, DS1 or vehicle. Previous studies have shown that pre-treating rats with FLX (5, 10, 20 mg/kg, sc), 30 min, 19 hr and 23 hr post FST habituation induces anti-depressant effects using the FST model (Detke et al., 1995). In this present study, a low dose of FLX (5 mg/kg) that has been shown previously to have anti-depressant effects in the FST was used (Detke et al., 1995). The results from our study show that pre-treating rats with the minimum effective dose of FLX significantly improved Sal A (Fig 3.8a) and DS1 (Fig 3.8b) induced depressive behaviours. This was noted by a reduction in immobility times and enhancement in time spent climbing with both Sal A and DS1. Additionally, a non-significant increase in swimming time was observed for saline/Sal A vs. FLX/Sal A and saline/DS1 vs FLX/DS1 treated animals (Fig 3.8). Thus sub-chronic FLX treatment reduced depressive behaviours produced by KOPr agonists (Tab. 3.4).

Exposure to FLX (1- 20 mg/kg) dose dependently increases 5HT levels in raphae nucleus, ventral hippocampus and frontal cortex when measured using *in vivo* microdialysis (Malagie et al., 1995). As mentioned earlier, KOPr activation has been shown to decrease 5HT levels in brain regions implicated in depressive behaviours (Tao and Auerbach, 2005; Yilmaz et al., 2006). Therefore, modulating 5HT levels by FLX pre-exposure might be responsible for the observable improvement in KOPr agonist induced depression in FST. An increase in phosphorylated CREB levels in the NAc shell has been implicated in depressive like behaviours in the FST (Plaikas et al., 2001). Moreover, KOPr activation increases CREB phosphorylation in NAc, which is antagonised by FLX, desimipramine (NET inhibitor) and KOPr antagonists (Carlezon et al., 1998; Mague et al., 2003; Chartoff et al., 2009). Therefore, decreased CREB activation by FLX pre-treatment may be responsible for diminishing KOPr agonists'

induced depressive-like behaviours. Detailed studies on this will help us to better understand the mechanisms underlying depressive effects produced by novel KOPr agonists.

The model of FST described by Porsolt et al., (1979) has been used to pharmacologically evaluate anti-depressants (Castagne et al., 2010). In 1995, Detke and colleagues modified the FST apparatus and analysis of the forced swimming behaviours to enhance the efficiency of the paradigm to evaluate anti-depressants (see Detke et al., 1995). As FST has been previously been applied to measure depressive effects produced by KOPr agonists (Pliakas et al., 2001; Mague et al., 2003; Carlezon et al., 2006), we also used this model to measure KOPr agonist induced behavioural despair in this current study. However, the use of FST as a model to evaluate behavioural despair has its limitations (Borsini and Meli, 1988; Castagne et al., 2010). The major limitation with FST is that the immobility behaviour might be due to the animal getting adjusted to the swimming conditions rather than actual behavioural despair, which might lead to false result interpretation (Borsini and Meli, 1988; Castagne et al., 2010). Therefore future studies with Sal A, DS1 and nalmefene are needed to evaluate their effects in inducing anhedonia and behavioural despair. Such experiments have been mentioned in detail in Chapter 4.

Due to the behavioural results with FLX to improve Sal A and DS1 induced depressive behaviours in FST, we conducted preliminary experiments to determine if KOPr directly modulates SERT protein. To do this we used an *in vitro* cell model. Cells were treated to transiently express GFP-hSERT and myc-rKOPr, followed by treatment with traditional (U50488H) and novel (Sal A and DS1) KOPr agonist's, and SERT function was measured in real-time.

Control experiments were performed to validate that ASP^+ uptake is dependent on the level of GFP-hSERT expression in single cells (Fig. 3.9a). The amount of ASP^+

accumulated by non-transfected cells was significantly lower than the uptake shown by transfected cells (Fig. 3.9b). These findings are in accordance with the recently published findings by Oz et al., (2010). The results from the current study also indicate that a 30 min pre-incubation with the novel KOPr agonists Sal A (10 μM) and DS1 (10 μM) did not have any significant effect on modulating SERT function using the ASP^+ accumulation assay in single cells (Fig. 3.10a). However, pre-incubating cells with the classic KOPr agonist, U50488H (10 μM for 30 min) significantly attenuated ASP^+ uptake (Fig. 3.10a). This effect produced by the KOPr agonist was a SERT-selective effect as ASP^+ accumulation was dependent on SERT expression levels (Fig. 3.9a) and no significant difference in GFP-hSERT expression levels were observed in cells selected from each treatment group (Fig. 3.10b). This, to the best of our knowledge, is the first study to determine the effect of KOPr agonist in modulating SERT function *in vitro*. However, as acute KOPr agonists show anti-addiction properties (Chapter 2) with depression as an adverse effect (Chapter 3), understanding the effect of acute exposure to KOPr agonists on modulating SERT function is necessary to confirm and quantify the findings from the present study. Also, such study has the potential to flesh out the role of SERT modulation in KOPr agonists produced depression.

In this study, we captured the image of the whole cell SERT which included the amount of SERT expressed on the cell surface and within the cell. Also, the initial SERT measurements were recorded after KOPr compounds/vehicle incubations. The ASP^+ uptake has been shown to be mediated by the SERT expression levels on the cell surface (Oz et al., 2010; Schwartz et al., 2003). Thus, in this present experiment we cannot measure the effect on ASP^+ accumulation due to transporter internalization which might have occurred due to KOPr agonist pre-treatment. In order to quantify the amount of ASP^+ accumulation due to SERT internalization, further imaging experiments are required. Such studies can help in differentiating the effect of chronic

vs. acute KOPr activation on modulating cell surface SERT expression and transporter internalization.

Previous studies have shown that Sal A has a greater affinity for KOPr than U50488H and U69593 and equal affinity compared to DYN (Chavkin et al., 2004). Furthermore, Sal A pre-treatment has been shown to produce 40 times less KOPr internalization than U50488H (Wang et al., 2005), whereas, another study indicated Sal A and U50488H produced internalization to a similar extent (Wang et al., 2008). Since, in this experiment, the KOPr agonists were pre-incubated for 30 min, KOPr may have been internalised after treatment with U50488H which may not have occurred with Sal A or DS1. Therefore, further studies are required to compare and contrast the effect of traditional and novel Sal A like KOPr agonists on KOPr internalization. Since acute KOPr activation has been shown to produce anti-addiction behaviours in pre-clinical models, it would be interesting to note the effects of acute activation of KOPr on SERT function and kinetics. This model (ASP^+ uptake) can be used in future to study the effect of SERT modulation following acute exposure to novel and classical KOPr agonists. Such experiments may aid in the development of KOPr analogues that vary in their effects on SERT function.

3.6. Summary and Conclusion

The results from sucrose-reinforced responding showed that Sal A, DS1 and nalmefene attenuated cocaine seeking behaviour in a selective manner (Chapter 2), whereas MOM Sal B attenuated cocaine seeking in a non-selective manner. Sal A and DS1 did not affect motor function or produce aversion, while nalmefene suppressed motor function without producing aversion in cocaine naive rats. All three KOPr agonists induced depressive behaviours. Furthermore, we provide preliminary evidence to suggest that the Sal A and DS1 induced depressive behaviours may involve negative modulation of 5HT systems which was attenuated by SERT antagonist. Further *in vitro*

studies exploring SERT modulation by KOPr agonists indicated a potential difference in modulation of SERT function by classical KOPr agonist U50488H and novel neoclerodane diterpenes Sal A and DS1.

Chapter 4. General Discussion

4.1. Endogenous KOPr systems and cocaine addiction

As mentioned earlier (Chapter 1, Section 1.5), central KOPr are up-regulated by repeated cocaine exposures (Collins et al., 2002). During this initial hedonic stage of cocaine use, there is an increased sensitization of the midbrain dopamine system which has been shown to be attenuated by KOPr activation (Glick et al., 1996; Archer et al., 1996). Also, studies by Chefer and colleagues (2005) have shown that KOPr gene deletion in mice increases DA levels and decreases DA re-uptake in the NAc. The same study also showed that KOPr knock-out mice remained in persistent cocaine sensitized state following a single cocaine injection (Chefer et al., 2005). Thus indicating that the KOPr systems might be implicated in controlling the dopaminergic effects produced by cocaine and activating KOPr could prevent the development and progression of cocaine addiction during the initial stages of the addiction cycle (Shippenberg et al., 2001; 2007; Mysels and Sullivan, 2009; Bruijnzeel, 2009; Fig 4.1).

Withdrawl from drug use has been associated with increased CREB phosphorylation in the NAc and the production of depressive like symptoms in laboratory animals (Nestler and Carlezon, 2001). KOPr activation has also been shown to enhance CREB phosphorylation in the NAc (Pliakas et al., 2001). A decrease in DA levels in the NAc during abstinence from the drug use has also been reported (Weiss et al., 1992). KOPr agonists have been shown to produce stress and depressive like symptoms in laboratory animals (Carlezon et al., 1998; 2006; Mague et al., 2003). On the other hand, KOPr antagonism enhances extracellular DA levels and decreases phosphorylated CREB levels in the NAc (Mague et al., 2003; Plaikas et al., 2001; Spanagel et al., 1990). KOPr antagonists also produce anti-depressant like effects in pre-clinical models (Spanagel et al., 1992; Mague et al., 2003; Nestler and Carlezon, 2006). Therefore, antagonizing KOPr signalling during the withdrawal stage of addiction cycle might be useful as replacement therapy to aid in preventing relapse to

drug use (Shippenberg et al., 2007; Prisinzano, 2008). As there are no current pharmacotherapies available for cocaine addiction, manipulating the endogeneous KOPr systems might be a novel mechanism towards finding the treatment for cocaine addiction (Shippenberg et al., 2007; Mysels and Sullivan, 2009; Bruijnzeel, 2009; Fig 4.1). The present study addresses the first part of the problem, that is, developing safer KOPr agonists which could assist in breaking the addiction cycle with fewer KOPr agonists related adverse effects.

Reinstatement of extinguished cocaine self-administration by the presentation of stressors (foot-shock, forced swim stress; Erb et al., 1996) has been used pre-clinically to screen the anti-addictive potential of KOPr antagonists (Beardsley et al., 2005; 2010). Additionally, reinstatement of cocaine via stressors requires prior KOPr activation as KOPr knockout mice were insensitive to stress-produced reinstatement (Mclaughlin et al., 2003; Redila and Chavkin, 2008). KOPr antagonist administration does not modulate cocaine prime induced drug-seeking in laboratory rats (Beardsley et al., 2005). Furthermore, data from Chapter 2 shows that nor-BNI pre-treatment completely reversed Sal A (Fig 2.2b), DS1 (Fig 2.6b) and nalmefene (Fig 2.11b) produced attenuation of cocaine prime induced reinstatement. KOPr activation has also been shown to potentiate stress induced reinstatement in mice (Redila and Chavkin, 2008). Taken together, these findings suggest that the reinstatement of extinguished cocaine-seeking initiated by a priming injection of cocaine is attenuated by KOPr activation. In contrast, blocking KOPr signalling attenuates reinstatement of extinguished cocaine seeking initiated by presentation of environmental stressors. This effect might reflect a possible difference in the central neuronal circuit involved in drug prime vs. stress induced reinstatement (Kreibich and Blendy, 2004) and activating or deactivating KOPr systems may modulate these behaviours in an opposing manner (Schenk et al., 1999; Redila and Chavkin, 2008; Beardsley et al., 2005). Given the fact that both these

paradigms have been applied extensively to study cocaine reinstatement pre-clinically, they can also be used to evaluate the utility of KOPr agonists and antagonists as anti-cocaine agents.

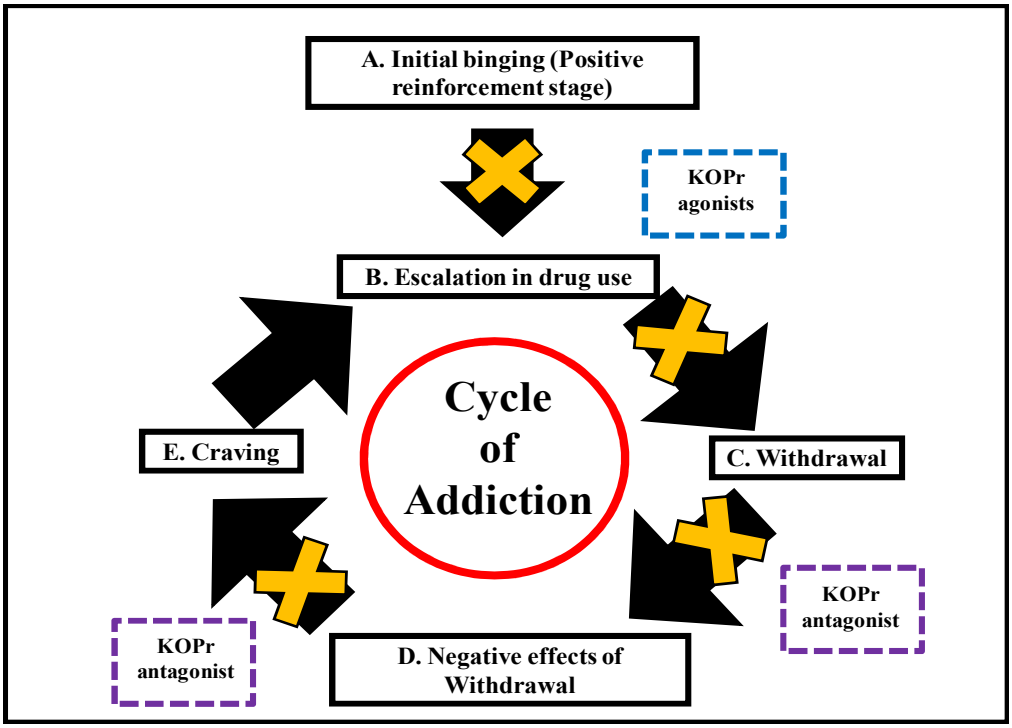


Fig.4.1. KOPr system modulation in addiction cycle

KOPr activation antagonises cocaine produced hedonic state and therefore could prove useful in managing the progression of the addiction cycle. KOPr antagonists, on the other hand produce anti-depressive effects and therefore antagonising KOPr systems could be useful in preventing relapse.

4.2. Limitations of classical KOPr agonists: rationale for Salvinorin A screening.

As described in Chapter 1 (section 1.5), the major limiting factor in developing synthetic KOPr agonists is the adverse effects associated with them (Prisinzano et al., 2005; Shippenberg et al., 2007; Wee and Koob, 2010). KOPr activation also produces dysphoria and psychotomimetic effects in humans (Walsh et al., 2001b; Pfeiffer et al., 1986). *Salvia divinorum* leaves have been used by the natives from Oaxaca, Mexico for the purpose of divination (Siebert et al., 1992; Ott, 1995; Valdes et al., 1994; Vorthermes and Roth, 2006). Also, its use as a recreational hallucinogen has recently gained widespread popularity among adolescents in the western countries (Vorthermes and Roth, 2006; Griffin et al., 2008; Kelly, 2011). With the discovery that Sal A produces its pharmacological effect via selectively binding and activating KOPr has opened new avenues in KOPr research (Roth et al., 2002; Prisinzano, 2005; Prisinzano et al., 2005; Pravett-Smith and Prisinzano, 2010). With this as a primary objective, several research groups have worked towards exploring the behavioural pharmacology of Sal A at KOPr (Carlezon et al., 2006; McCurdy et al., 2005; Fantagrossi et al., 2005; Zhang et al., 2005; Braida et al., 2007; 2008; 2009; Baker et al., 2009). However, few studies have aimed at comparing the anti-addiction pharmacology of Sal A to traditional KOPr agonists.

Sal A has been shown to attenuate cocaine prime induced reinstatement and suppress cocaine induced hyperactivity in laboratory animals (Chartoff et al., 2008; Morani et al., 2009). We have shown in this study that the Sal A induced attenuation of cocaine reinstatement has no effect on either natural reward reinforcements or suppression of motor function (Morani et al., 2009). On the other hand, the classical KOPr agonists U50488H and spiradoline attenuated cocaine produced behaviours in a non-selective manner (Glick et al., 1995; Wadenberg, 2003). A study by Chavkin and

colleagues (2004) showed that Sal A had a similar efficacy for KOPr to DYN whereas; it was more potent than both U50488H and U69593. Also the same study showed that Sal A had more affinity for KOPr compared to U50488H and U69593 (Chavkin et al., 2004). As Sal A has a different structure to traditional KOPr agonists (Fig 1.5), we hypothesized that Sal A might produce a better tolerated anti-addiction profile compared to classical KOPr agonists. If so, then compounds based on the structure of Sal A possessing better pharmacokinetics could be developed as potential anti-addiction agents. With this as a central hypothesis of this thesis, initial preliminary screenings were designed for Sal A, its structural analogues and nalmefene (MOPr antagonist/ partial KOPr agonist) aimed at exploring their behavioural anti-addiction pharmacology. The behavioural screening was extended further to measure the adverse effect profile of these KOPr compounds.

4.3. Pharmacological profiling of novel KOPr agonists.

The behavioural experiments used to screen Sal A, its analogues and nalmefene is shown in Fig 4.2. The initial aim of this thesis was to determine if Sal A, its structural analogues (DS1, MOM Sal B, EOM Sal B, herkinorin) and nalmefene produced anti-addiction behaviours in a similar way to traditional KOPr agonists. This was done using the cocaine prime-induced within reinstatement model (Fig 4.2). This model was initially reported by Worley et al, (1994) and has been used previously to screen traditional KOPr agonists for anti-addiction effects (Schenk et al., 1999; 2000; Morani et al., 2009). Our results show that Sal A (0.3, 1.0 mg/kg), DS1 (0.3, 1.0 mg/kg), MOM Sal B (0.3 mg/kg) and nalmefene (0.3, 1.0 mg/kg) all significantly attenuate cocaine prime induced drug seeking (Tab 4.1).

Once these compounds were shown to attenuate cocaine-seeking, the next step was to determine the selectivity in attenuation of cocaine-seeking behaviour. To do this

we determined if sedative effects occurred. Motor function was determined by measuring cocaine produced hyperactivity in animals self-administering cocaine and the suppression of natural reward seeking behaviour was measured using the sucrose reinforcement test (Morani et al., 2009; Fig 4.2). Our results show that none of the compounds tested (Sal A, DS1, MOM Sal B, nalmefene) produced motor suppression during the reinstatement tests (Tab 4.1). Also, Sal A, DS1 and nalmefene pre-treatment did not suppress sucrose reinforcement. However MOM Sal B did decrease sucrose reinforcement (Tab 4.1). These results showed us that Sal A, DS1 and nalmefene induced attenuation of cocaine-seeking behaviour in a selective manner.

The compounds which selectively attenuated cocaine seeking behaviour were further tested for their pharmacological activation of KOPr in attenuating cocaine seeking Our results showed that Sal A, DS1 and nalmefene induced attenuation of cocaine-seeking were mediated via KOPr activation (Tab 4.1) and this effect was prevented when KOPr activation was blocked by selective KOPr antagonist nor-BNI (Fig 4.2).

These KOPr compounds were also tested for their behavioural adverse effects. Since KOPr agonists have been shown to cause aversion, motor suppression and depressive effects, we applied CTA, spontaneous open field activity and FST respectively to test these behaviours (Fig 4.2). Sal A and DS1 pre-treatment did not suppress motor function or produce taste aversions (Tab 4.1). Although, nalmefene did not produce taste aversions, it significantly suppressed motor function (Tab 4.1). However, all three compounds produced depressive like effects in FST (Tab 4.1). These data showed that Sal A and DS1 showed better adverse effect profile than nalmefene.

The effect of blocking SERT on Sal A and DS1 induced depressive behaviour in FST was also assessed (Fig 4.2). SERT antagonism via sub-chronic FLX treatment significantly improved Sal A and DS1 induced depressive effects in the FST (Tab 4.1).

To further quantify the effect of Sal A, DS1 and U50488H on modulating SERT function, we used *in vitro* ASP^+ uptake paradigm in cells (Fig 4.2). Sal A and DS1 had no effect on SERT function, whereas U50488H pre-treatment decreased SERT function. This indicates a potential difference in the modulation of SERT function by classical and Sal A like KOPr agonists (Fig 4.2; Tab 4.1). However further studied are needed to clarify this possibility.

The effects of Sal A and DS1 were also tested for cocaine produced locomotion (acute, behavioural sensitization and stereotypy) to further characterize the behavioural effects of these compounds (Fig 4.2). Acute DS1 pre-treatment suppressed the expression of cocaine behavioural sensitization and locomotion activity following acute cocaine exposure (Tab 4.1). Although, Sal A attenuated cocaine sensitization, it potentiated motor function induced by acute cocaine (Tab 4.1). Overall, from this pharmacological profiling, we fleshed out three lead compounds Sal A, DS1 and nalmefene which can be further characterized for their anti-addiction effects.

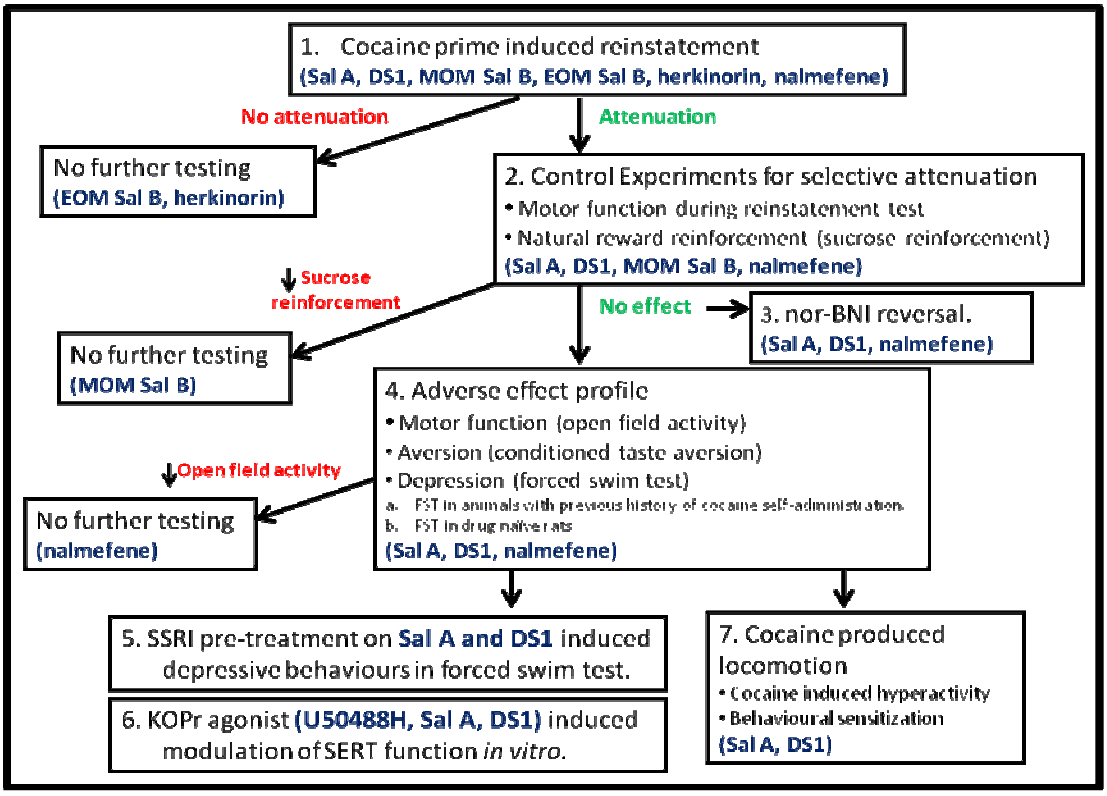


Fig 4.2. Pharmacological profiling of novel KOPr agonists.

Novel KOPr agonists were initially screened for their anti-cocaine profile followed by their selectivity in attenuation of cocaine-seeking. The compounds were further tested for their behavioural adverse effects. KOPr agonists Sal A and DS1 were also tested for their role in modulating serotonin transporter function and cocaine produced locomotion activity.

Tab 4.1. Pharmacological screening of KOPr agonists

	Sal A	DS1	MOM Sal B	EOM Sal B	Herkinorin	Nalmefene
ANTI-ADDICTION PROFILE						
Cocaine induced reinstatement	↓	↓	↓	**NE**	**NE**	↓
Motor suppression during reinstatement test.	**NE**	**NE**	**NE**	-	-	**NE**
Sucrose reinforcements	**NE**	**NE**	↓	-	-	**NE**
Cocaine induced hyperactivity	↑	↓				
Behavioural Sensitization	↓	↓				
ADVERSE EFFECT PROFILE						
Motor function	**NE**	**NE**	**NE**	-	-	↑
Taste aversion	**NE**	**CTP**		-	-	**NE**
Immobility time in FST	↑	↑	-	-	-	↑
Effect of FLX pre-treatment on immobility	↓	↓				
Effect on ASP+ accumulation	**NE**	**NE**				

↓ - attenuates/suppresses; ↑ - potentiates/ produces; **NE** - no effect

Effect of KOPr agonists were tested for their anti-addiction profile and side effect profile. KOPr agonists Sal A and DS1, having similar affinity for KOPr produced similar behavioural anti-addiction profile and adverse effect profile. More potent and long lasting Sal A analogue, MOM Sal B produced anti-addiction behaviour but also produced anhedonia. Mixed KOPr compound herkinorin had no effect on cocaine induced reinstatement. MOPr antagonist/ partial KOPr agonist, nalmefene produced anti-addiction behaviours without producing anhedonia and aversion. However, nalmefene produced motor suppression and depressive effects when tested in drug naive rats.

4.4. Significance of current results

In Tab 4.2, we compare the binding affinity and efficacy at KOPr (and MOPr for nalmefene and herkinorin) with the KOPr compounds ability to attenuate cocaine seeking behaviours. Both Sal A and DS1 which have a similar affinity for- and potency at- KOPr successfully attenuated cocaine seeking behaviour in a selective manner (Tab 4.2). The dose at which both these compounds attenuated cocaine reinstatement was also similar (0.3, 1.0 mg/kg). However, longer acting and more potent Sal A analogues such as EOM Sal B and MOM Sal B were ineffective in modulating cocaine seeking behaviour in a desirable manner. At 0.3 mg/kg dose, EOM Sal B showed a trend to increase responding which was an opposite effect to Sal A. Although, MOM Sal B attenuated cocaine-seeking at 0.3 mg/kg dose, this effect was non selective as it also suppressed natural reward. Therefore, the ability of neoclerodane diterpene KOPr agonists to selectively modulate cocaine induced behaviour may be dependent on the KOPr potency of these compounds.

It has been hypothesized that KOPr ligands with mixed affinity for other opioid receptors have the potential to be developed as anti-addiction pharmacotherapies (Stevenson et al., 2004; Archer et al., 1996; Bart et al., 2005; Kreek et al., 2005). With this in mind we performed an initial preliminary screening of nalmefene (MOPr antagonist/partial KOPr agonist) and herkinorin (MOPr/KOPr agonist) using cocaine prime induced drug seeking model. Acute nalmefene attenuated cocaine induced reinstatement in rats without suppressing sucrose reinforcements or producing taste aversion. Nalmefene has shown to bind with higher affinity to KOPr than MOPr and is an antagonist at MOPr (Bart et al., 2005; Tab 4.2). The endogeneous MOPr and KOPr activation produces opposing tonic effects (Pan, 1998). Additionally, as mentioned in Chapter 1 (Section 1.6), KOPr agonists and MOPr antagonists attenuated MOPr agonist-induced rewarding behaviours and -increase in DA levels in the NAc (Spanagel

et al., 1992). Therefore, nalmefene may have an application in modulating the progression and development of cocaine addiction.

Considerable evidence supports the idea for the development of compounds that reverse the decrease in central monoamine activity as they are have potential abilities in preventing relapse following withdrawal from chronic drug use. Ideally by activating DA and 5HT receptors (Gorelick et al., 2004; Grabowski et al., 2004; Karila et al., 2008; Rothman et al., 2008). However, serious adverse effects associated with agonists at monoamine transporters have also opposed this theory (Mello and Negus, 2000). Developing agents which indirectly modulate monoaminergic systems may decrease the adverse effects associated with direct monoaminergic activators (Mello and Negus, 2000; Lin et al., 2010). We show in this study that acute herkinorin treatment has no effect on cocaine-seeking (Tab 4.1). Although a higher dose of herkinorin (0.3, 1.0 mg/kg) showed a trend to suppress responses in a non-significant manner (Tab 4.2). As herkinorin binds with greater affinity to MOPr than KOPr and is an agonist at both the opioid receptors (Tidgewell et al., 2006; Tab 4.2), it has potential to be developed as a maintenance therapy that may prevent relapse. Thus, both nalmefene and herkinorin could potentially have an application as anti-addictives but at different stages of the addiction cycle. Further studies, are needed to test this hypothesis.

KOPr activation is associated with aversive and depressive effects (Carlezon et al., 1998; 2006; Todtenkopff et al., 2004; Mague et al., 2003; Ebnar et al., 2010) and the current results support this (Chapter 3; Tab 4.2). All three compounds tested showed depressive behaviours in drug naïve as well as cocaine exposed animals (Tab 3.3). The cocaine exposed animals were however abstinent from cocaine for a longer period of time (Tab 3.1). Abstinence from cocaine use has been shown to produce neuroadaptations which results in depressive behaviours (Carlezon et al., 1998; Nestler and Carlezon, 2006). Therefore, it might be possible that the depressive effects

produced in cocaine self-administering animals might be an additive effect of cocaine abstinence and KOPr activation. As KOPr agonists are proposed to break the hedonic stage of addiction cycle (Shippenberg et al., 2007; Mysels and Sullivan, 2009). A detailed study is required to evaluate the depressive effects produced by acute KOPr exposure in self-administering rats which have not been subjected to any abstinence or withdrawal. In the present thesis, FLX pre-treatment attenuated the time spent immobile in animals exposed to Sal A and DS1. This finding has the potential to reduce the depressive effects produced by KOPr agonists when used to break the addiction cycle. Moreover, recent findings suggest that FLX (3, 10 mg/kg) attenuates cue-induced and cocaine prime induced reinstatement in rats self-administering cocaine (Burmeister et al., 2003). Therefore, it would be interesting to determine the effect of co-administration of KOPr agonists with FLX on cocaine prime induced reinstatement and self-administration. It would also be interesting to see if this proposed combination of treatments modulates central monoamine neurotransmission. This study may identify potential solution to the depressive side-effects associated with the use of KOPr agonists as anti-addiction pharmacotherapies.

Initial ASP^+ uptake experiments identify a potential difference in the modulation of SERT by classical- and neoclerodane diterpene-KOPr agonists (Tab 4.1). Therefore, detailed studies are required to explain the role of the 5HT system modulation by classical and novel KOPr agonists and associate them to the observed depressive behaviours. Such studies could utilise *in vitro* models to allow manipulation of signalling systems and proteins.

In conclusion, this study has identified Sal A, DS1 and nalmefene as lead compounds which can be further characterized for their anti-addiction profile. Furthermore, Sal A and DS1 attenuated cocaine behavioural sensitization. One of the aims of this thesis was to identify if any longer-acting structural analogues of Sal A

could retain anti-addiction effects with fewer adverse effects. Our findings indicate that DS1 may be such an analogue. The favourable results with Sal A, DS1 and nalmefene from this thesis encourages a detailed behavioural profiling for their anti-addiction- and adverse-effects. Moreover, this study has utilised several experimental models to pharmacologically screen KOPr compounds for their anti-addiction behaviour. Using these preliminary experiments (Fig 4.2), we aim to screen more novel analogues for their anti-addiction pharmacology. This line of research has the potential to identify novel neoclerodane diterpenes as possible leads for the development of pharmacotherapies to treat psychostimulant addiction.

Tab 4.2. Binding affinities and efficacy for KOPr and its effect on cocaine seeking behaviour.

Compound	Binding affinity at KOPr *Ki* (nM)	Binding affinity at MOPr Ki (nM)	Potency at KOPr EC_{50} (nM)	Potency at MOPr (nM)	Cocaine seeking behaviour dose (mgkg)
Salvinorin A	2.4 ± 0.4[b] 1.9 ± 0.2[a]	>10000	1.8 ± 0.5[c] 40 ± 10[d]		0.3, 1.0 (selective attenuation)
DS-1	2.3 ± 0.1 [a]	6820 ± 660[a]	30 ± 5[d]		0.3, 1.0 (selective attenuation)
Nalmefene	0.083 ± 0.0008 [b]	0.24±0.006[b]	EC_{50} = 4.2±1.6[c] IC_{50} = 18±1.0[c]	IC_{50} = 13±2.3[c]	0.3, 1.0 (selective attenuation)
EOM Sal B	0.32 ± 0.02[b]	ND	0.14 ± 0.01[c]		0.03 (no effect); 0.1, 0.03 (trend to increase responding)
MOM Sal B	0.6 ± 0.07[b]	ND	0.40 ± 0.04[c]		0.03, 0.1 (trend to increase responding) 0.3 (non-selective attenuation)
Herkinorin	90 ± 2 [a]	12.1 ± 1[a]	1320±150[d]		0.3, 1.0 (non-significant trend to decrease responding)

[a]Data expressed as ± SD; [b]Data expressed as ± SEM; ND= not defined.[c]Enhancement of [^{35}S]GTP γS binding to Chinesse Hamster Ovary - human KOPr; EC50 ± SEM (nM). [d][^{35}S]GTP γS functional assay carried out in stably transfected Chinesse Hamster Ovary cells containing DNA for human KOPr ;EC50 ± SD nM. Data for Sal A, DS1 and herkinorin, modified from Harding et al., 2005; data for MOM Sal B , EOM Sal B, modified from Munro et al., 2008; data for nalmefene modified from Bart et al., 2005.

4.5. Future directions

Results obtained from this thesis have opened new avenues to further quantify the behavioural anti-addiction and side effect profiles for the lead compounds Sal A, DS1 and nalmefene. Additionally, there is a need to determine the mechanisms by which these compounds produced their anti-addiction and depressive behaviours. The following section gives a brief description of future possible studies with these compounds.

Effect of KOPr agonists in models of cocaine self-administration and reinstatement.

As discussed in Chapter 2 (Section 2.5, discussion), there is a need to test the lead KOPr compounds using models which show a well defined set of alterations in neuronal adaptations seen in pre-clinical studies (Self et al., 2004). To do this additional studies will be required to test the effects of Sal A, DS1 and nalmefene on cocaine prime induced reinstatement following extended extinction times or utilising a between-sessions reinstatement paradigm (Stretch et al., 1971; Ferragud et al., 2009). Keeping in mind the rationale for using KOPr agonists as cessation agents for cocaine self-administration, it would be imperative to test the effects of these lead compounds (Sal A, DS1 and nalmefene) on cocaine self-administration in animals' stably self-administering cocaine (Glick et al., 1996). Also, it would be interesting to observe the effects of these lead compounds on altering the monoamine transporter functions in animals self-administering cocaine. This can be achieved by measuring DAT and SERT function by using rotating disc electrode voltammetry (RDEV) in NAc and CPU brain regions. These tests would idealy be performed on animals following cocaine self-administration and reinstatement tests.

Endogenous KOPr antagonism on cocaine prime induced reinstatement.

As mentioned in Chapter 2 (Section 2.5, discussion), KOPr antagonism by nor-BNI reversed KOPr agonists induced attenuation of drug seeking behaviour. However, the effect of KOPr antagonism by itself on cocaine-seeking was not performed. Ideally this can be measured by determining the effects of KOPr antagonism with traditional (nor-BNI, JDTic) and novel antagonists on cocaine-prime induced -within-session reinstatement and -reinstatement after prolonged period of abstinence in rats (Worley et al., 1994; Beardsley et al., 2005). Such experiments could prove useful, as KOPr antagonists are proposed as maintainence agents to prevent relapse (Section 4.1). Also, determining the DAT and SERT functions in NAc and CPU regions by using RDEV in animals subjected to the reinstatement tests could help explain the mechanism of the observed behaviours.

Effect of KOPr antagonists on stressors induced cocaine seeking.

As explained in Section 4.1, reinstatement of extinguished cocaine self-administration by presentation of environmental stressors has been used for pharmacological evaluation of KOPr antagonists. Therefore, this paradigm could be useful in pharmacological screening of novel KOPr antagonists as agents to prevent relapse induced by stress. The reinstatement of cocaine-seeking could be initiated by the presentation of foot-shock stressors or forced swim stress in rats (Beardsley et al., 2005; McLaughlan et al., 2005a; 2005b). Also, determining the function of DAT and SERT in the NAc and CPU could help explain the mechanism of KOPr antagonists in this model. Such experiments could be performed using RDEV following reinstatement testing.

Effect of KOPr agonists on FST in cocaine self-administering animals.

As mentioned earlier (Section 4.4), KOPr agonists have shown to produce depressive like symptoms (increased immobility time) in animals self-administering cocaine (Fig 3.6; Tab 3.3; Chapter 3). However, these rats were abstained from cocaine (Tab 3.1). Therefore, in order to quantify the depressive effects produced by KOPr activation, effects of Sal A, DS1 and nalmefene need to be measured using FST in cocaine self-administering animals which have not been subjected to any abstinence. In addition to the measurement of the behaviours, determining the DAT, SERT function in brain regions subjected to reward (dorsal and ventral striatum) could explain the mechanism of depressive effects following KOPr activation (Section 1.5.5.3; 1.5.5.4). These experiments could be performed using the RDEV technique.

Effect of KOPr agonists on cocaine motor sensitization after prolonged abstinence.

The results from this present study showed the efficacy of both Sal A and DS1 to attenuate expression of locomotion sensitization when animals were challenged with the cocaine injection after a short period of abstinence (4 days). Previous reports suggest that expression of sensitization is a model which represents alteration in underlying neuronal circuits mediating psychomotor function and craving potential of the psychostimulants (Stekette, 2005; Robinson and Berridge, 2000; 2003; 2008). Also, challenging sensitized animals with a low dose of cocaine even after a long period of abstinence has shown to express locomotor sensitization (Heidbreder et al., 1995). Therefore, it would be interesting to test the effect of Sal A and DS1 on expression of sensitization after a prolonged period of abstinence (15, 30, 45 days). Additionally, the DAT and SERT function in NAc and CPU could be determined in these sensitized animals using RDEV. This experiment has the potential to provide evidence to link the

alteration induced by KOPr agonists in the monoamine transporter function at midbrain reward circuit with cocaine motor sensitization.

Effect of KOPr agonists on cocaine induced stereotypy.

KOPr agonists, Sal A and DS1 have shown to attenuate motor sensitization produced by high dose of cocaine (20 mg/kg; Tab 4.2). However, both Sal A and DS1 pre-treatment showed a non-significant enhancement in cocaine-induced stereotypy (Fig 2.4c, 2.8c). As suppression in forward locomotion by KOPr agonists might reflect an enhanced stereotypy, a detailed study on cocaine produced stereotypic behaviours is warranted. With similar experimental procedure to induce motor sensitization (mentioned in Section 2.2; Chapter 2), effect of KOPr compounds on cocaine produced stereotypic behaviours could be measured. The stereotypic behavioural counts could be determined using methods described by Kalivas et al., (1988) and Szumlinski et al., (2000).

Effect of SSRI+ KOPr agonists in cocaine prime induced drug-seeking

Both KOPr agonist and SERT antagonist, FLX treatment has shown to attenuate cocaine prime induced reinstatement in laboratory animals (Burmeister et al., 2003). Moreover, sub-chronic FLX pre-treatment attenuated KOPr agonists produced depressive behaviours in FST (Tab 4.1). Therefore, it would be interesting to test the effect of co-administration of FLX with Sal A/DS1 on cocaine-seeking behaviour. To test this, an initial screening could be performed using the within-session reinstatement model (Worley et al., 1994). Preliminary results from such experiment could open a new avenue of research using SERT antagonists in combination with KOPr agonists in tackling cocaine addiction without producing KOPr activation induced depressive effects.

Pre-clinical models to quantify behavioural despair induced by KOPr agonists

With the limitations of the FST paradigm (discussed in Chapter 3; Section 3.5), it would be interesting to characterize KOPr activation produced behavioural despair using paradigms which have been previously applied to study anhedonia pre-clinically. Such tests could be performed using social interaction effect (Sams-Dodd, 1997), sucrose drinking (Muscat and Wilner, 1992) and ICSS test in rats (Todtenkopff et al., 2004; Ebnar et al., 2010). The measurement of ICSS thresholds have been previously used to evaluate anhedonia like effects of Sal A (Ebnar et al., 2010) and hence could be useful in evaluating DS1 and nalmefene produced behavioural despair.

Herkinorin as maintenance therapy

Herkinorin is the only synthetic Sal A analogue with more affinity for MOPr and weak affinity for KOPr. Also, herkinorin is an agonist at MOPr and KOPr. The results from this thesis showed that herkinorin did not potentiate cocaine seeking in within session reinstatement test (Tab 4.1). This led to the hypothesis that neoclerodane diterpenes acting as agonists at MOPr and KOPr could be effective in relapse prevention. To prove this hypothesis, experiments could be performed by looking at the effect of herkinorin pre-treatment on cocaine prime induced reinstatement after a prolonged period of withdrawal (Ferragud et al., 2009). Also, by measuring the function of DAT and SERT in NAc and CPU could help explain the mechanism of action of herkinorin. Such experiments could be performed using RDEV technique. The preliminary results from such experiments with herkinorin have the potential to develop a new class of neoclerodanes diterpenes aimed at preventing relapse to cocaine use.

4.6. Summary and conclusions

This study shows that the novel KOPr activating neoclerodane diterpenes Sal A, DS1 and MOM Sal B attenuate cocaine induced behaviours in a similar way to traditional KOPr agonists. This effect was also observed for partial KOPr agonist/MOPr antagonist, nalmefene. However, longer acting and more potent Sal A analogue, MOM Sal B produced anhedonia which was not seen with either Sal A, DS1 or nalmefene. Also, Sal A and DS1 showed better adverse effect profile than nalmefene in drug naive rats. However, all three compounds tested (Sal A; DS1 and nalmefene) produced depression and preliminary evidence has been presented to suggest that Sal A and DS1 induced depression may involve serotonin neurotransmission. Finally, preliminary *in vitro* cell studies show KOPr activation by traditional agonist U50488H directly modulates serotonin transporter function. However, Sal A analogues may regulate SERT in a different manner compared to traditional KOPr agonists. Further studies are needed to determine the extent of these differences.

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Appendix

Appendix for Chapter 2

1. ***Test for patency of catheters (Pentobarbital test)***

- Initially try and withdraw some blood from the catheter using a syringe which has penicillin in heparinised saline.
- If you observe blood in the syringe, the line is OK. Flush back blood with 0.1 mL of heparinised penicillin solution.
- If you don't observe blood, then infuse 0.1 mL of pentobarbital (Provet, Auckland; 5 mg/mL). If the animal becomes unconscious, then the line is still OK. If the animal does not get unconscious, then the rat has either a chest leak or head leak and needs unblocking of its line.
- The cather patency test was performed when rats were ***not being trained*** for self-administration sessions.

Appendix for Chapter 4

1. ***Recipe for LB Broth (for 500 mL)***

- Tryptone (OXOID, UK) 5 g
- Sodium Chloride (LabServ) 5 g
- Yeast Extract (Sigma) 2.5 g
- Add distilled water and autoclave it.

2. ***Recipe for Agar plates (200 mL; each plate takes approx 20 mL)***

- Agar (Fischer Scientific)2 g
- Tryptone 2 g
- Sodium Chloride 2 g
- Yeast Extract 1 g

- Add distilled water and autoclave it. After autoclaving, cool the flask (until the flask is slightly warm) then add appropriate antibiotic, ie Ampicillin (Sigma, MO) for KOPr plasmid preparation (100 µg/mL) and Kanamycin (Tocris, UK) for SERT plasmid (50 µg/mL).
- Slightly shake the flask for gently mixing and then pour in the agar plates. Allow the agar to set for few hours and then store it in refrigerator. These plates can be stored up to 2 months.

3. Method for GFP-hSERT and myc-rKOPr plasmid Preparation:

- **Day 1:-** Take a scraping of the frozen transformed bacteria transfected with cDNA for GFP-hSERT (generous gift from Dr. Samanda Ramamoorthy) and myc-rKOPR (generous gift from Dr. Lakshmi Devi) from the vial stored at -80 °C. Plate the bacteria on a fresh agar plate. Incubate the plate for 24 hr at 37 °C and allow the bacterial colony to grow on agar plate in humid condition (place some water in a conical flask in the incubator).
- **Day 2:-** Add 2 mL of sterile LB broth in a sterile 15 mL tube which contains 100 µg/mL of ampicillin (for myc-rKOPr) and 50 µg/mL of kanamycin (for GFP-hSERT). Select a middle sized colony of bacteria from the agar plate and transfer it using a sterile pipette into the 15 mL tube and incubate for 24 hr at 37 °C with constant shaking at 280 rotation per minute in humid condition.
- Day 3:- Add 200 µL of the bacterial culture to 200 mL of LB broth which contains appropriate antibiotic in proper concentrations and incubate for 24 hr at 37 °C with constant shaking at 280 rotation per minute in humid condition.
- Day 4:- Centrifuge samples for 15-25 min at 4 °C. (Dupont Centrifuge, RC5C; GSA 1 rotor; rotor code: 10; Sorvall Instruments). Dispose off the supernatant and keep the pellet on ice. Then follow the instructions mentioned in the manufacturer's catalogue (Quagen)

4. ***Media for HEK-293 cells. (for 100 mL)***

- Dulbecco Modified Eagle's Medium (Gibco, Invitrogen) 89 mL.
- Fetal Calf Serum 10 mL (Invitrogen)
- Penstrap (Invitrogen)1 mL.

5. ***Media for HEK-293 cells. (without penstrap; for 100 mL)***

- Dulbecco Modified Eagle's Medium 90 mL.
- Fetal Calf Serum 10 mL.

6. ***Transient transfection of HEK-293 cells with myc-rKOPr and GFP-SERT.***

- **Mix A:-** 3 µL Lipofectamine (Invitrogen) + 47 µL OptiMEM (Invitrogen) (Keep it for 5 min).
- **Mix B:-** (0.4 µg/mL GFP-hSERT + 0.6 µg/mL myc-rKOPr) + make volume upto 50 µL using OptiMEM.
- Mix A + Mix B (Keep this for 20 min)
- Add 100 µL of Mix A + Mix B to each culture dish (1 mL/dish) and return dish back to incubator.

7. ***Krebs Ringer Herpes buffer (KRH).***

- Sodium Chloride 130mM
- Potassium Chloride (LabServ) 1.3mM
- Calcium Chloride (Fischer Scientific) 2.2mM
- Magnesium sulphate (LabServ) 1.2mM
- Potassium Dihydrogen Phosphate (LabServ) 1.2mM
- HEPES (Sigma) 10mM
- D-Glucose (Sigma) 10mM

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Effect of kappa-opioid receptor agonists U69593, U50488H, spiradoline and salvinorin A on cocaine-induced drug-seeking in rats

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ABSTRACT

Our previous work indicated that pretreatment with the selective kappa-opioid receptor (KOPr) agonist, U69593, attenuated the ability of priming injections of cocaine to reinstate extinguished cocaine-seeking behavior. The present study expanded these initial tests to include other traditional KOPr agonists, U50488H, spiradoline (SPR), and salvinorin A (Sal A), an active constituent of the plant *Salvia divinorum*. Following acquisition and stabilization of cocaine self-administration, cocaine-produced drug-seeking was measured. This test was conducted in a single day and comprised an initial phase of self-administration, followed by a phase of extinguished responding. The final phase examined reinstatement of extinguished cocaine self-administration followed by a priming injection of cocaine (20.0 mg/kg, intraperitoneal (I.P.)) in combination with the various KOPr agonists. Cocaine-induced drug-seeking was attenuated by pretreatment with U69593 (0.3 mg/kg, subcutaneous (S.C.)), U50488H (30.0 mg/kg, I.P.), SPR (1.0, 3.0 mg/kg, I.P.) and Sal A (0.3, 1.0 mg/kg, I.P.). Sal A (0.3, 1.0 mg/kg, I.P.) had no effect on operant responding to obtain sucrose reinforcement or on cocaine-induced hyperactivity. These findings show that Sal A, like other traditional KOPr agonists attenuates cocaine-induced drug-seeking behavior.

1. Introduction

Prior studies have demonstrated that KOPr agonists attenuate some of the neuro-chemical and behavioral effects of drugs of abuse (Heidbreder et al., 1993, 1995; Shippenberg et al., 1996; Thompson et al., 2000). These effects might be due to the interactions between KOPr and dopaminergic systems since pretreatment with kappa-opioid agonists decreased dopamine (DA) concentrations in terminal regions (Devine et al., 1993; Shippenberg et al., 1994; Maisonneuve et al., 1994; Heidbreder and Shippenberg, 1994; Heidbreder et al., 1996) and attenuated cocaine- (Shippenberg et al., 1994), amphetamine- (Gray et al., 1999) and heroin- (Xi et al., 1998) induced DA release in the ventral striatum. Also, many behavioral effects of psycho-stimulants like cocaine (Heidbreder et al., 1993, 1995), amphetamine (Gray et al., 1999) and nicotine (Hahn et al., 2000) were decreased by prior administration of KOPr agonists. Additionally, pretreatment with KOPr activating compounds attenuated cocaine (Glick et al., 1995; Mello and Negus, 1996, 1998; Negus et al., 1997; Schenk et al., 1999; Schenk and Partridge, 2001), morphine (Glick et al., 1995) and heroin (Xi et al., 1998) self-administration in laboratory animals. These findings suggest the possible role of KOPr agonists as pharmacotherapeutics in the treatment of addiction (Mello and Negus, 1998; Prisinzano et al., 2005; Shippenberg et al., 2007; Willmore-Fordham et al., 2007; Prisinzano and Rothman, 2008; Tomasiewicz et al., 2008).

During a binge of cocaine self-administration in abusers there is tolerance to many of the subjective effects (Fischman et al., 1985). Self-administration and drug craving, however, continue relatively unabated. Since exposure to cocaine produces craving for more cocaine (Jaffe et al., 1989), it is possible that continued exposure to cocaine during a binge maintains a cycle of drug-seeking and drug-taking, even after tolerance develops. If so, identification of factors that reduce the ability of cocaine to produce drug-seeking would be an important step in the development of effective treatments for cocaine abuse.

Animal models of drug-seeking have contributed greatly to investigations of these factors. A number of laboratories have used procedures developed by de Wit and Stewart (1981) to demonstrate the ability of cocaine to reinstate extinguished cocaine self-administration behavior. This effect appears to be mediated, at least in part, by dopaminergic mechanisms since cocaine-produced drug-seeking was attenuated by pretreatment with dopaminergic agonists and antagonists (Self et al., 1996; Khroyan et al., 2000, 2003; Alleweireldt et al., 2002).

Pretreatment with the kappa-opioid receptor agonist, U69593 attenuated cocaine-produced reinstatement of extinguished cocaine-

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taking behavior (Schenk et al., 1999, 2000). This effect was somewhat specific since reinstatement produced by experimenter-administered injections of the dopamine uptake inhibitors, GBR 12909 or WIN 35428, was not attenuated by pretreatment with U69593 (Schenk et al., 2000). The attenuation of drug-seeking involved effects at central KOPrs, as prior intracerebroventricular administration of nor-BNI, a kappa-opioid antagonist, reversed the effects of U69593 (Schenk et al., 1999).

Sal A, a neoclerodane diterpene, is an active constituent of the hallucinogenic sage, *Salvia divinorum*, and has been used in traditional, spiritual and ethnopharmacological practices by the Mazatec Indians of Oaxaca, Mexico (Valdes et al., 1983; Valdes, 1994; Siebert, 1994). Sal A binds selectively to the KOPr (Roth et al., 2002; Yan and Roth, 2004; Prisinzano, 2005) with greater efficacy than U69593 and U50488H (Chavkin et al., 2004). It has a rapid onset of action and a short elimination half life (56.6 ± 24.8 min) (Schmidt et al., 2005; Butelman et al., 2009). Accordingly, there has been recent interest in understanding the neuropharmacology of Sal A and comparing it with traditional KOPr agonists.

Sal A dose dependently produced antinociception in tail flick, hot plate and acetic acid induced writhing in mice that was antagonized by nor-BNI (McCurdy et al., 2006; John et al., 2006). Sal A also produced discriminative stimulus effect in rats (Willmore-Fordham et al., 2007; Baker et al., 2009) and rhesus monkeys (Butelman et al., 2004), decreased mobility in the forced swim test (Carlezon et al., 2006) and produced motor inco-ordination in mice (Fantegrossi et al., 2005; Zhang et al., 2005). The aim of our current study was to determine whether Sal A has similar effects to other KOPr agonists, U69593, U50488H and SPR on cocaine-produced drug-seeking in rats.

2. Methods

2.1. Subjects

Male Sprague–Dawley rats weighing 325–350 g were used. They were housed individually in hanging polycarbonate cages. The humidity (55%) and temperature (19–21 °C) were controlled and food and water were freely available except during testing. Animals tested for sucrose self-administration had free access to water at all times within the home cage and were maintained at approximately 85% of their initial feeding weight during the experiments by restricting access to food. The animal colony was maintained in the animal facility at School of Psychology, Victoria University of Wellington. All the experiments were conducted in accordance with the guidelines of the Animal Ethics Committee of Victoria University of Wellington. Lights were maintained on a 12:12 h cycle with lights on at 0700.

2.2. Surgery

Under deep anesthesia produced by ketamine/xylacine (90/9 mg/kg, I.P.), a silastic catheter was placed in the right jugular vein. The external jugular vein was isolated, the catheter was inserted and the distal end (22 gauge stainless steel tubing) was passed subcutaneously to an exposed portion of the skull where it was fixed to embedded jeweler's screws with dental acrylic.

Each day, the catheters were infused with 0.1 ml of a sterile saline solution containing heparin (30.0 U/ml), penicillin G Potassium (250,000 U/ml) and streptokinase (8000 U/ml) to prevent infection and the formation of clots and fibroids. The rats were allowed five days post surgery for recovery.

2.3. Apparatus

2.3.1. Cocaine self-administration

Self-administration testing was carried out in a humidity (55%) and temperature (19–21 °C) controlled environment in standard operant chambers (Med Associates, ENV-001) equipped with 2 levers. Depression of one lever (the active lever) resulted in a 0.l ml intravenous infusion of cocaine HCl dissolved in sterile physiological saline containing heparin (3.0 U/ml). Infusions were of 12 s duration. Coincident with drug delivery was the illumination of a stimulus light located directly above the active lever. This stimulus light remained illuminated throughout each 12 s infusion. Depression of the other lever (the inactive lever) was without programmed consequence.

Rats were maintained in the animal colony except during testing. Immediately prior to each daily test, the catheter lines were infused with 0.1 ml of the heparin–penicillin–streptokinase solution. The stainless steel catheter was connected to a length of microbore tubing and connected to the syringe. At the end of each test, the lines were again infused with 0.1 ml of the heparin–penicillin–streptokinase solution, the stainless steel tubing was plugged and the animal was returned to its home cage. Drug delivery and data acquisition were controlled by Med Associates software. Cocaine deliveries were made via mechanical pumps (Razel, Model A with 1.0 rpm motor equipped with 20 ml syringe).

2.3.2. Sucrose self-administration

Training and testing procedures were conducted in eight standard operant chambers (Med Associates, ENV001) in an unlit, sound attenuating room. Operant chambers were equipped with two retractable levers and a sucrose bottle delivering 0.1 ml of 10% sucrose solution onto a tray on the chamber wall according to the imposed schedule of reinforcement. Sucrose delivery and data acquisition were controlled by Med Associates software. Experiments were conducted between 0900 and 1600 hours.

2.3.3. Locomotor activity tests

Eight open field chambers (Med Associates) equipped with two banks of sixteen photocells on each wall were used to measure horizontal locomotion. The open field boxes were interfaced with a microcomputer located in an adjacent laboratory. Testing was conducted in the dark between 1000 and 1600 hours. White noise was continually present to mask extraneous disturbances.

2.4. Procedure

2.4.1. Cocaine self-administration training

Acquisition of cocaine self-administration was monitored during daily 2 h sessions. Each session began with an experimenter-delivered infusion of cocaine (0.5 mg/kg/infusion). Thereafter, depression of the active lever produced automated cocaine infusions according to an FR-1 schedule of reinforcement. The criterion for acquisition of cocaine self-administration consisted of at least 20 reinforced responses (10 mg/kg) during the 2 h session and a ratio of active:inactive lever responses of at least 2:1. Self-administration was considered acquired when these criteria were met for three consecutive days. Following acquisition, the response requirements were increased to FR-5. Daily 2 h sessions were conducted until there was less than 20% variation in responding on three consecutive days. During training, the cocaine infusion was always paired with the illumination of a house light located directly above the active lever.

2.4.2. Cocaine reinstatement test

Once responding on the FR-5 schedule was stable, the effect of prior administration of the kappa-opioid agonists, U69593, U50488H, SPR and Sal A on drug-seeking produced by cocaine was measured. As with our previous studies (Schenk et al., 1999, 2000), this test was conducted in a single day and consisted of three phases. The first phase was comprised of a 1 h period of cocaine self-administration (0.5 mg/kg/infusion, FR-5 schedule of reinforcement) in which the light stimulus was paired with cocaine infusions. After the 1 h self-administration period, the cocaine solution was replaced with heperanized saline and responding was reinforced with this vehicle infusion (FR-5 schedule of

reinforcement) and illumination of the light stimulus. Responding during this phase was measured for 3 h. At the start of the third phase during which responding was again reinforced according to an FR-5 schedule of reinforcement with an infusion of vehicle solution and illumination of the light stimulus, separate groups of rats ($n = 5$–7 per group) received an injection of U69593 (0.03, 0.1 or 0.3 mg/kg, S.C.), U50488H (3.0, 10.0 or 30.0 mg/kg, I.P.), SPR (0.3, 1.0 or 3.0 mg/kg, I.P.), Sal A (0.1, 0.3 and 1 mg/kg, I.P.) or vehicle. These injections were administered either 5 (Sal A), 15 (U69593) or 30 (U50488H, SPR) min prior to an injection of cocaine HCl (20 mg/kg, I.P.). Responding was measured for 60 min following the cocaine injection.

2.4.3. Sucrose reinforcement training and test

Animals were trained to self-administer sucrose using an auto-shaping procedure. Training sessions were 45 min duration on each of 10 training days. Once stable responding was produced, the animals were maintained on an FR1 schedule of reinforcement during which depression of the active lever (left lever) delivered 0.1 ml of a 10% sucrose solution. Following acquisition, the response requirements were increased to FR-5. Daily 1 h sessions were conducted until there was less than 20% variation in responding for three consecutive days. Once responding on the FR-5 schedule was stable, the effect of Sal A on sucrose-reinforced responding was measured. Rats received an injection of either Sal A (0.3 or 1.0 mg/kg, I.P.) or vehicle 5 min prior to sucrose self-administration testing and the number of responses was measured for 60 min.

2.4.4. Procedure for cocaine-produced locomotor activity

On the test day, separate groups of rats ($n = 6$ per group) received an injection of Sal A (0.0 or 0.3 mg/kg, I.P.) 5 min prior to an injection of cocaine (20 mg/kg, I.P.). Immediately following the second injection, the rats were placed in the activity chambers and total activity, a compilation of horizontal and vertical activity, was measured for a period of 60 min.

2.5. Data analysis

The number of responses produced during the 1 h period following the cocaine injection (20 mg/kg, I.P.) at the start of phase 3 was measured. Sucrose-reinforced responding was measured for a 1 h period following Sal A or vehicle exposure. Statistical analysis for cocaine-induced reinstatement and sucrose reinforcement consisted of one-way ANOVAs followed by Tukey post-hoc comparisons where appropriate. Student *t*-test was applied for locomotion test.

2.6. Drugs

Cocaine HCl (Merck Pharmaceuticals, Palmerston North, New Zealand), SPR and U50488H (Sigma-Aldrich, St. Louis, MO) were dissolved in physiological saline. U69593 (National Institute of Drug Abuse) was dissolved in an aqueous solution of 25% propylene glycol. Sal A (isolated by Dr. Thomas E. Prisinzano, University of Kansas, Lawrence, KS) was suspended in 75% DMSO. Sucrose was dissolved in tap water. Intravenous infusions were in a volume of 100 µl and S.C. or I.P. injections were in a volume of 1 ml/kg. All drug weights refer to the salt.

3. Results

Fig. 1 shows the responding during phases 1 and 2 for a representative group of rats from the present study. During the 1 h period of cocaine self-administration, responding was high. When saline was substituted for cocaine during phase 2, responding decreased to less than 20 responses during hour 3.

Fig. 2 shows the number of saline-reinforced responses produced during the 1 h period following the injection of cocaine at the start of phase 3 for rats that received systemic administration of the KOPr agonists, U69593, U50488H, SPR and SalA. Each of the KOPr agonists produced a dose-dependent reduction of cocaine-produced reinstatement (U50488H: $F(3,17) = 4.277$, $p < 0.05$; U69593: $F(3,20) = 3.103$, $p < 0.05$); SPR: ($F(3,21) = 7.899$, $p < 0.01$; Sal A: ($F(3,23) = 79.33$, $p < 0.0001$). Doses of 30.0 mg/kg U50488H, 0.3 mg/kg U69593, 1.0 or 3.0 mg/kg SPR and 0.3 or 1.0 mg/kg Sal A significantly decreased drug-seeking ($p < 0.05$).

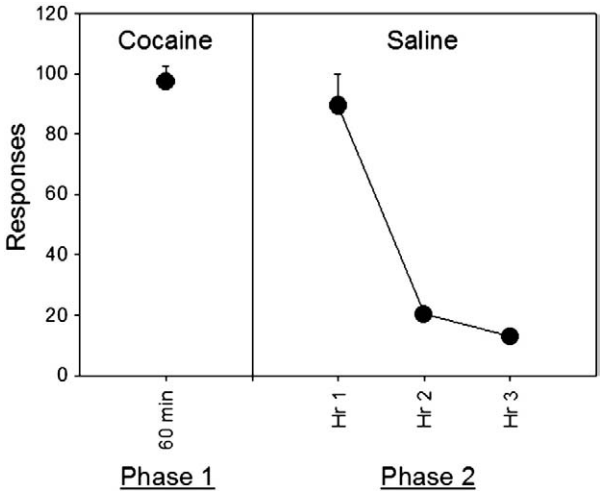

Fig. 1. Number of lever press responses (+SEM) during Phases 1 (cocaine self-administration) and 2 (extinction) ($n = 6$). During phase 1, responding is high (97.0 + 6.1) and by the end of phase 2, after the cocaine solution had been replaced with saline, fewer than 20 responses per hour were produced (16.3 + 1.3).

Fig. 3 shows the number of sucrose-reinforced responses produced during the 1 h period following the injection of Sal A (0.3 and 1.0 mg/kg, I.P.). Sal A did not significantly decrease sucrose self-administration ($F(2,27) = 0.06$, NS).

Fig. 4 shows the effect of Sal A (0.0 or 0.3 mg/kg, I.P.) on cocaine-produced locomotor activity. Sal A failed to decrease cocaine-produced hyperactivity.

4. Discussion

As has previously been shown, cocaine reinstated extinguished cocaine-taking behavior (Worley et al., 1994; Schenk and Partridge, 1999; Schenk et al., 1999). This effect was attenuated by pretreatment with KOPr agonists. High doses of SPR might have produced a non-selective effect on motor behavior as suggested by the decreased responding observed in a drug discrimination study (Holtzman et al., 1991). The attenuation of drug-seeking was also produced by a dose of SPR (1.0 mg/kg) that did not decrease responding in a drug discrimination task (Holtzman, 2000). It is therefore unlikely that the decrease in cocaine-seeking represents a generalized inability to perform the lever press operant at the dose used in this study. Similarly, decreased drug-seeking was produced by doses of U69593 and U50488H that failed to produce a generalized decrease in motor activity (Schenk et al., 1999; unpublished findings).

In a previous study, U50488H administered to mice 60 min prior to cocaine potentiated cocaine-induced conditioned place preference (CPP) but when administered 15 min prior to cocaine it suppressed the cocaine-CPP (McLaughlin et al., 2006). In this study we measured cocaine-induced drug-seeking during a 60 min period following U50488H administration and also observed a small but non-significant increase in responding.

In drug discrimination tests, Sal A and its synthetic derivatives substituted completely for U69593 (Baker et al., 2009), suggesting an effect mediated by the KOPr. Because of the interaction between KOPr and dopaminergic mechanisms, a focus on the effects of Sal A on dopamine-mediated behaviors has been of interest. Cocaine-seeking has been

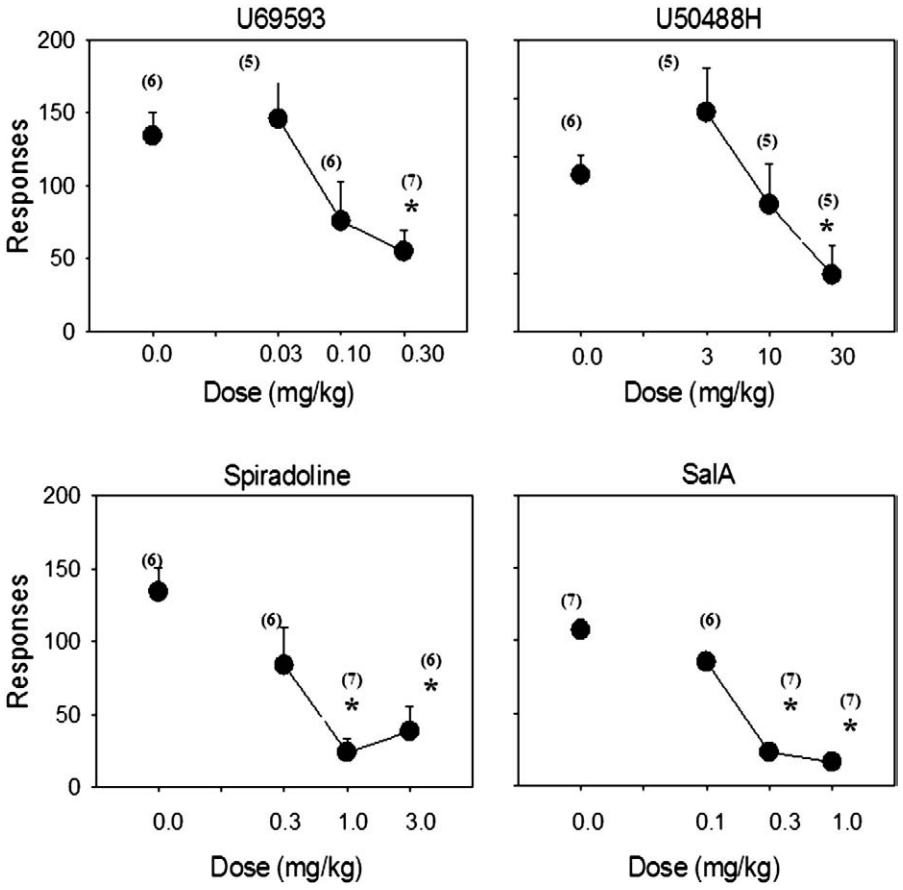

Fig. 2. Responses (+SEM) produced during phase 3 following injection of KOPr agonists and cocaine. Numbers in parentheses represent sample sizes for each group. *<0.05.

attributed to dopaminergic mechanisms and there are data to support the idea that Sal A modulates the effects of cocaine through interactions with D1 receptor mediated signaling in the dorsal striatum (Chartoff et al., 2008; Gehrke et al., 2008). Low doses of Sal A (40 μg/kg) increased DA levels in nucleus accumbens (NAc) (Braida et al., 2008) whereas higher doses decreased NAc DA levels (Zhang et al., 2005). Thus, the ability of moderate to high doses of Sal A to decrease cocaine-produced drug-seeking might be due to its effects on the dopaminergic system.

Alternatively, the decrease in drug-seeking might reflect a non specific effect. This possibility was tested by examining the effects of Sal A on responding maintained by a sucrose reinforcer. Conditions for this test were comparable to conditions of the reinstatement tests. In this experiment, responding maintained by sucrose was high and comparable to responding maintained by cocaine. Doses of Sal A (0.3, 1.0 mg/kg I.P.) that decreased cocaine-seeking failed to alter sucrose-reinforced responding.

Additionally, KOPr activation has shown to produce motor incoordination (Fantegrossi et al., 2005). A more recent report, however, failed to find any effect of Sal A on locomotor activity (Baker et al., 2009). The effect of Sal A on cocaine-produced hyperactivity was measured in the present study under conditions that were comparable to the conditions of the reinstatement tests. Sal A did not attenuate cocaine-produced hyperactivity. Therefore, the decrease in drug-seeking produced by Sal A cannot be attributed to either a generalized inability to perform the lever press operant or a disruption of motor activity.

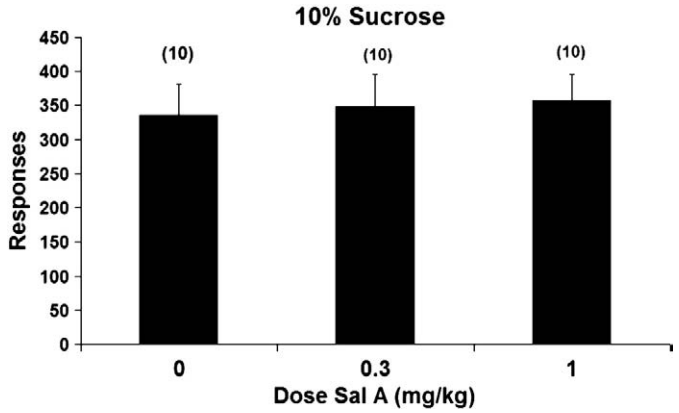

Fig. 3. Number of sucrose-reinforced responses (+SEM) produced by rats for 60 min after treatment with Sal A (0.3 and 1.0 mg/kg, I.P.). Numbers in parentheses represent sample sizes for each group.

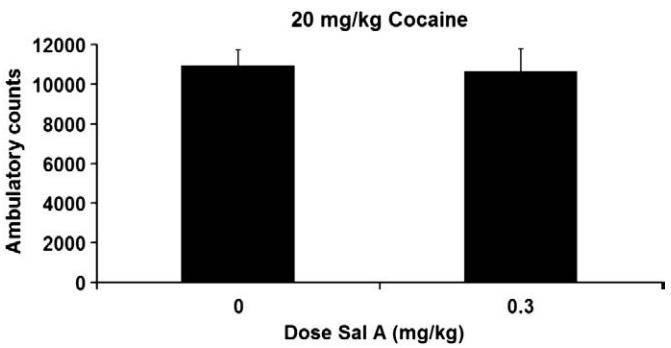

Fig. 4. Effect of Sal A (0 and 0.3 mg/kg, I.P.) on cocaine-induced locomotion. Symbols represent total ambulatory counts (+SEM) during the 60 min period following the cocaine injection ($n=6$).

Reinstatement of conditioned place preference (CPP) following extinction has also been used as a model of drug-seeking (Kreibich and Blendy, 2004) and a modulation of drug-seeking behaviors by traditional KOPr agonists has been demonstrated using this model. The effects appear, however, to be opposite to those produced when drug-seeking in a self-administration paradigm is measured. For example, foot-shock or forced swim stress was effective in reinstating both extinguished place preference (McLaughlin et al., 2003, 2006; Redila and Chavkin, 2008) and self-administration (Beardsley et al., 2005). The effect in the CPP paradigm was, however, blocked by pretreatment with the KOPr antagonist, nor-BNI (Redila and Chavkin, 2008; McLaughlin et al., 2003). The effect of KOPr antagonists might depend on the drug-seeking stimulus since the KOPr antagonist, JDTic, inhibited stress induced drug-seeking but had no effect on cocaine-produced reinstatement (Beardsley et al., 2005).

Further, KOPr knock out mice were insensitive to stress-produced reinstatement, as measured in the CPP paradigm, supporting the idea that reinstatement of CPP required KOPr activation. A further difference in the role of KOPr in reinstatement as measured in the CPP and self-administration paradigms is that U50488H potentiated the cocaine-produced place preference whereas it decreased drug-seeking following extinction of self-administration (present study). Additional studies will have to be conducted in order to tease apart the different aspects of the two paradigms.

In conclusion, Sal A, like traditional KOPr agonists attenuated cocaine-induced cocaine-seeking. Further studies on Sal A and its structural derivatives will help us understand the mechanism by which KOPr agonists attenuate drug-seeking behaviors and to determine whether Sal A produces the same magnitude of adverse effects that have limited the development of other KOPr agonists as anti-addiction pharmacotherapies.

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