Exploring the aversive and anxiogenic effects of novel kappa opioid receptor agonists in rats

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Abstract

Drug addiction is characterised by uncontrolled, compulsive drug use despite negative consequences. As this disease has a high social and economic cost, greater attention is required in finding an effective treatment for individuals suffering addiction. Kappa opioid receptor (KOPr) agonists demonstrate anti-addiction effects in the rodent cocaine drug-prime model of reinstatement. Salvinorin A (Sal A), a novel non-nitrogenous KOPr agonist, has demonstrated reduced side-effects compared to traditional agonists. However, its short half-life and duration of action limit clinical development. The design of novel Sal A analogues with improved pharmacokinetics, anti-addiction effects, and reduced side-effects is an important step towards the pharmaceutical development of KOPr agonists. β-Tetrahydropyran Sal B (β-THP Sal B), Mesyl Sal B, ethoxymethyl salvinorin B ether (EOM Sal B), and Ethynyl Sal A (Ethy Sal A) have demonstrated anti-addiction effects by reducing cocaine-seeking behaviour in rats, but their aversive and anxiogenic properties have yet to be examined. Here the conditioned place aversion (CPA) paradigm is used to evaluate aversion and the elevated plus maze (EPM), light/dark test, and open field are utilised to measure anxiety in male Sprague-Dawley rats.

EOM Sal B (0.1 mg/kg, i.p) and Ethy Sal A (0.3 mg/kg, i.p) did not produce aversive effects, whereas the traditional KOPr agonist U50,488 (10 mg/kg, i.p), Sal A (0.3 mg/kg, i.p), and the novel analogue β-THP Sal B (1 mg/kg, i.p) produced significant aversion using the CPA protocol.

In the EPM all the novel analogues, β-THP Sal B, EOM Sal B, Mesyl Sal B, and Ethy Sal, A did not show a reduction in time spent on the open arm. In addition, EOM Sal B showed a significant increase in time spent on the open arm compared with Sal A (0.3 mg/kg, i.p). Sal A

(0.3 and 1 mg/kg, i.p) showed significant anxiogenic effects, but the traditional agonist U50,488 did not. In the light/dark test Sal A (1 mg/kg, i.p) showed significant dose dependent anxiogenic effects with significant effects observed at 1 but not 0.3 mg/kg dose. This is in contrast to results observed in the EPM. The novel analogues EOM Sal B and β-THP Sal B demonstrated a non-significant trend toward anxiogenic behaviour in the light/dark test, but U50,488, Mesyl Sal B, and Ethy Sal A did not show significant reductions in time spent in the light box.

KOPr stimulation activates its associated G-proteins, allowing them to interact with several intracellular effectors. Activation of cAMP response element binding protein (CREB) can occur downstream of the KOPr signalling cascade. The phosphorylation of CREB is associated with dysphoria and stress-induced reinstatement of drug-seeking behaviour. An initial attempt to validate CREB assays was made.

The lack of behavioural anxiogenic and aversive side-effects with EOM Sal B, Mesyl Sal B and Ethy Sal A treatment demonstrates that the development of KOPr agonists with desirable effects and reduced side-effects is possible. These novel Sal A agonists provide promising candidates for pharmacotherapy development.

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Abbreviations

Amg: amygdala

BSA: bovine serum albumin

β-THP Sal B: β-tetrahydropyran Sal B

cAMP: cyclic adenosine monophosphate

CHO cells: Chinese hamster ovary cells

CPA: conditioned place aversion

CPP: conditioned place preference

CREB: cAMP response element binding protein

CRF: corticotrophin-releasing factor

CTA: conditioned taste aversion

DA: dopamine

DAT: dopamine transporter

DMEM: Dubecco's modified Eagle's medium

dStr: dorsal striatum

EOM Sal B: ethoxymethyl salvinorin B ether

EPM: elevated plus maze

ERK 1/2: extracellular signal-regulated kinases 1 and 2

Ethynyl Sal A: Ethy Sal A

FCS: Foetal calf serum

FST: forced swim test

GRK3: G-protein coupled receptor kinase 3

Hy: hypothalamus

KOPr: kappa opioid receptor

i.p.: intraperitoneal

i.m.: intramuscular

MAPK: mitogen-activated protein kinase

Mesyl Sal B: (2S,4aR,6aR,7R,9S,10aS,10bR)-9-(Methanesulfonyloxy)-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2H-napthol[2,1-c]pyran-7-carboxylic acid methyl ester

NAc: nucleus accumbens

norBNI: norbinaltorphimine

PBS: phosphate buffered saline

PCP: phencyclidine

PFC: prefrontal cortex

PI3 kinase: phosphoinositide 3-kinase

PKA: protein kinase A

PVDF: polyvinylidene difluoride

RB-64: 22-thiocyanatosalvinorin A

RIPA buffer: radioimmunoprecipitation assay buffer

Sal A: Salvinorin A

s.c.: subcutaneous

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SN: substantia nigra

TBS: Tris-buffered saline

T-TBS: Tween-20 Tris buffered saline

U50,488: 2-(3,4-dichlorophenyl)-N-methyl-N-[(2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide

U69,593: (5R,7S,8S)-(+)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8yl]

benzeneacetamide

1.   Introduction

Drug addiction is defined as a chronic relapsing brain disorder which is characterised by compulsive and uncontrolled drug use despite the negative consequences (Koob, 2008). In New Zealand around 3.5 percent of the total population is affected by this disorder and the budget for treatment is $120 million a year (National Committee for Addiction Treatment, New Zealand, 2011). Criminal activity and drug abuse are often linked; statistics from the National Committee for Addiction Treatment (2011) showed that 76% of the prison population abused alcohol, while 55% used cannabis, and 40% abused other drugs. With such high social and economic costs more attention is needed to develop new therapies for treating those suffering from addiction. However, despite decades of addiction research there has not yet been a treatment approved by the Food and Drug Administration (FDA) for psychostimulant abuse. The kappa opioid peptide receptor (KOPr) has shown promise as a new therapeutic target. Traditional KOPr agonists show anti-cocaine effects, but are also associated with negative behavioural side effects such as aversion, anxiety, depression, and sedation. This thesis will examine the aversive and anxiogenic effects of several structural analogues of the novel KOPr agonist Salvinorin A (Sal A), an agonist with reduced side effects but a poor pharmacokinetic profile. Research is currently aimed at developing efficacious Sal A analogues with improved pharmacokinetic properties that may be useful as an anti-addiction pharmacotherapy.

1.1   Behavioural Models

The clinical application of KOPr agonists as anti-addiction pharmacotherapies is currently limited by their side effects. The most common are aversion (Braida et al., 2008; Chefer et al., 2013; Mucha & Herz, 1985; Sufka et al., 2014; Suzuki et al., 1992; Tejeda et al., 2013; Zhang

et al, 2005), anxiety (Valdez & Harshberger, 2012), depression (Carlezon et al., 2006; Mague et al., 2003; Morani et al., 2012), and sedation (Mague et al., 2003; Paris et al., 2011; Zhang et al., 2005). These can be evaluated using preclinical behavioural models. For aversion, the conditioned place aversion (CPA) and conditioned taste aversion (CTA) tests; for anxiety, the elevated plus maze (EPM), open field, or light/dark tests; for depression or despair the forced swim test (FST); for evaluation of sedative effects the rotarod test for motor coordination, and open field activity test for locomotor activity can be used.

The theoretical idea behind behavioural models is that they should reproduce certain features of complex phenomena seen in humans. This is useful as it enables variables to be isolated and remain constant, a state impossible to achieve in humans. They are not meant to replicate all features of a disorder or side effect but rather to generate a general behavioural state that could be related to a treatment or psychiatric disorder (Lister, 1990). Therefore, in order to study novel compounds with the aim of pharmaceutical development we must use multiple different tests to determine the side effects profile of potential therapeutics.

The conditioned place preference (CPP) and CPA paradigms are the most popular models to study the rewarding and aversive effects of drug and non-drug compounds in animals due to their low cost and rapid data collection (Tzschentke, 2007). They are useful for screening the rewarding and aversive effects of a drug-induced state within the same system (Bals-Kubik, et al., 1993) and require little training and no surgery compared to self-administration models (Prus, James, & Rosecrans, 2009). These procedure involves creating an association between the properties of a drug (the unconditioned stimulus) and that of neutral environmental stimuli such as the colour, pattern and texture of a discrete chamber. The resultant behaviour of the animal toward these stimuli after conditioning indicates the preference or aversive

effects of the drug. Another way of testing these properties of a drug is in the conditioned taste aversion (CTA) procedure by paring its effects with a rewarding taste, such as saccharin solution (Anderson, et al., 2013). In contrast to CPA, the pairing of the drugs effect is with a novel taste rather than environmental stimuli.

The most commonly used tests of anxiety are the EPM, light/dark test, and open field. These tests utilise the conflict between normal exploratory behaviour and the fear of open and/or elevated places (open field and EPM) and brightly lit spaces (light/dark). To test for depressive side effects the FST can be utilised. It involves forced swimming behaviour where a position of immobility is believed to represent despair (Porsolt, Bertin, & Jalfre, 1977).

1.2   Addiction: Reward and Stress

An individual usually begins to recreationally take a drug for its positive effects. As drug use increases, they begin to take the drug compulsively and control over intake is lost. At this point the individual is part of an addiction cycle that involves three major steps. The first stage involves the intoxication or binge episode. This leads into the second stage which is characterised by abstinence and withdrawal resulting in the individual experiencing negative side effects. In the final stage, the individual will seek the drug to alleviate the negative emotional state characterised by anxiety and dysphoria. Drug use will lead back to an intoxication event completing the addiction cycle (Koob & Le Moal, 1997). This behaviour is thought to be reinforced though positive and negative reinforcement; the hedonic, rewarding effects of the stimulus and removal of the stressful, negative emotional state respectively. These effects complement each other, progressively becoming more intense and ultimately resulting in the pathology of addiction (Koob, 2013).

1.2.1 Reward

The natural reward pathway consists of dopaminergic neurons that originate in the ventral tegmental area (VTA) and substantia nigra (SN) and project to key regions of the brain. Projections from the VTA to the prefrontal cortex (PFC) and the nucleus accumbens (NAc) are known as the mesocortical and mesolimbic pathways respectively. Collectively these systems are known as the mesocorticolimbic pathway and are associated with motivation, reward, and the acute reinforcing effects of psychostimulants (Wise, 2009). Projections from the SN to the dorsal striatum (dStr) are known as the nigrostriatal dopaminergic pathway which is associated with motor function. Electrical stimulation of this pathway has also been shown to have rewarding properties (Wise, 2009) (Figure 1).

Dopamine release is increased in the NAc and VTA in response to naturally rewarding stimuli such as food and water (Yoshida et al., 1992). Cocaine increases extracellular dopamine levels in the NAc (Di Chiara & Imperato, 1988) by binding to and inhibiting the dopamine transporter (DAT), a transmembrane protein that removes dopamine from the synapse (Ritz, et al., 1987). Therefore, during cocaine use, dopamine is not cleared as rapidly and the post-synaptic neuron is excessively stimulated, resulting in the perceived 'high' experienced by drug users (Volkow et al., 1997).

1.2.2 Stress

Two brain stress systems have key roles in driving the negative reinforcement of drug taking behaviour, namely the dynorphin and corticotrophin-releasing factor (CRF) systems (George F Koob, 2013) (Figure 1).

Dynorphin is an opioid peptide widely distributed in the central nervous system with the highest amounts found in the hypothalamus, SN, pallidum, and dStr (Gramsch et al., 1982).

Dynorphin is the endogenous ligand for the KOPr (Chavkin et al., 1982) and has been shown to produce analgesic effects similar to other opioids (Baumeister et al., 1987) but divergent rewarding effects (Funada et al., 1993). Cocaine self-administration results in an increase in prodynorphin mRNA in the dorsal striatum (Daunais et al., 1995) and dynorphin peptides reduce dopamine release in the brain (Maisonneuve et al., 1994), suggesting that the dynorphin system acts as a natural suppressor of reward. In contrast, mice subjected to forced swimming stress show an increased place preference for cocaine compared with unstressed and prodynorphin gene knockout animals (McLaughlin et al., 2003). McLaughlin et al. (2003) demonstrates that stress induced activation of the dynorphin system potentiates the rewarding effects of cocaine.

CRF containing neurons can be found in the paraventricular nucleus of the hypothalamus, the central nucleus of the amygdala and the bed nucleus of the stria terminalis (collectively known as the extended amygdala) (Swanson et al., 1983). In response to stress, CRF is released from the paraventricular nucleus to stimulate the release of adrenocorticotropic hormone in the pituitary, resulting in production and release of cortisol by the adrenal gland. It has been proposed that the extended amygdala plays a key role in mediating the negative affect seen with drug withdrawal syndrome (Koob & Kreek, 2007). CRF has been shown to play a role in stress-induced but not drug-primed reinstatement (Erb et al., 2001). Additionally, activation of the CRF_1 receptor leads to anxiogenic behaviour whereas activation of the CRF_2 receptor leads to aversive behaviour (Bruchas et al., 2009). These results are dependent on the dynorphin/KOPr system as behavioural effects were reversible with administration of the KOPr antagonist norbinaltorphimine (norBNI) and were not observed in prodynorphin knock-out mice (Bruchas et al., 2009; Land et al., 2008).
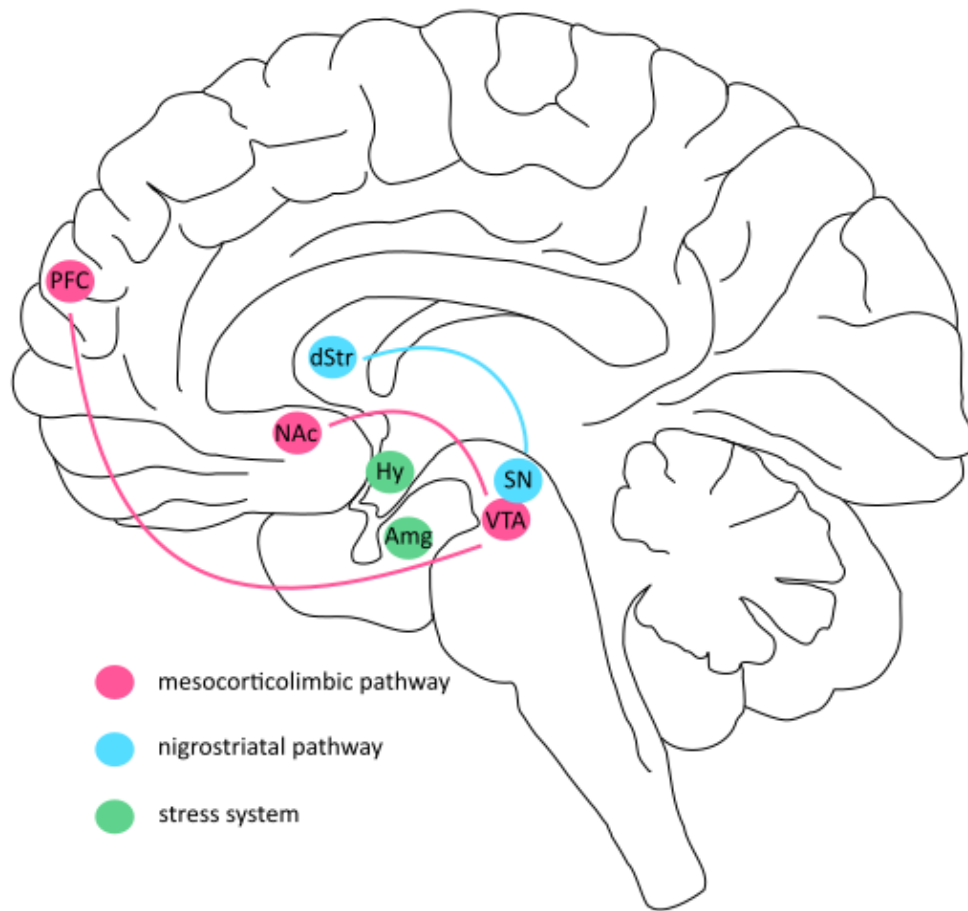
Figure 1   Areas of the brain involved in reward and stress

The regions of the brain involved in reward (mesocorticolimbic and nigrostriatal pathways) and stress. Projections from the VTA to the PFC comprise the mesocortical pathway, and projections from the VTA to the NAc the mesolimbic pathway. Collectively these pathways are known as the mesocorticolimbic pathway and are associated with motivation and hedonic effects. Projections from the SN to the dStr are known as the nigrostriatal system and are involved in movement and reward. CRF neurons are found in the hypothalamus and amygdala and are associated with the stress response. Amg = amygdala, dStr = dorsal striatum, Hy = hypothalamus, NAc = nucleus accumbens, PFC = prefrontal cortex, SN = substantia nigra, VTA = ventral tegmental area

1.2.3 CREB

Cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) is expressed ubiquitously in the brain and functions as a transcription factor. Activation of CREB can occur via the adenylyl cyclase, Ca^{2+} or mitogen activated protein kinase (MAPK) signalling pathways (Dash et al., 1991; Sheng et al., 1991; Xing et al., 1996). Phosphorylation of CREB at serine-133 results in transcription of target genes (Gonzalez & Montminy, 1989) including those for CRF and dynorphin (Cole et al., 1995; Itoi et al., 1996).

Administration of dopamine to primary cultures of rat striatal neurons resulted in an increase in cAMP via activation of the dopamine D1 receptor, leading to the phosphorylation of CREB and an increase in dynorphin mRNA (Cole et al., 1995). The same effect was seen with acute administration of amphetamine. It is proposed that this stimulant-induced increase in dynorphin levels contributes to the dysphoria seen with drug withdrawal (Cole et al., 1995). Activation of the CRF_1 receptor and subsequent phosphorylation of CREB, particularly in the NAc and lateral septum, has been shown to be necessary for stress-induced reinstatement of cocaine place preference (Kreibich & Blendy, 2004; Kreibich et al., 2009). CRF has been shown to act through CRF_1 receptors coupled to $G\alpha_s$. However, rather than the typical $G\alpha_s$ - adenylyl cyclase – protein kinase A (PKA) signalling pathway, the phosphorylation of CREB is through $G\beta\gamma$ activation of the MEK (MAPK kinase) pathway (Stern et al., 2011) (Figure 2).

Elevated CREB activity in the NAc resulted in increased immobility in the FST (Pliakas et al., 2001) in addition to reduced CREB levels displaying anti-depressant like effects (Newton et al., 2002). However, a reduction in CREB activity in the NAc caused by social isolation resulting in an increase in anxiety-like behaviours were reversed following restoration of CREB activity (Barrot et al., 2005). Carlezon Jr. et al. (2005) hypothesise that normally rewarding and

aversive stimuli result in a short term increase in CREB activity and that larger or more

sustained increases, such as with excessive stress or drug use, leads to dampening of

emotional reactivity. Further, sustained decreases in CREB activity, seen with social isolation,

result in an extreme response to emotional stimuli similar to a state of anxiety (Carlezon Jr.

et al., 2005).
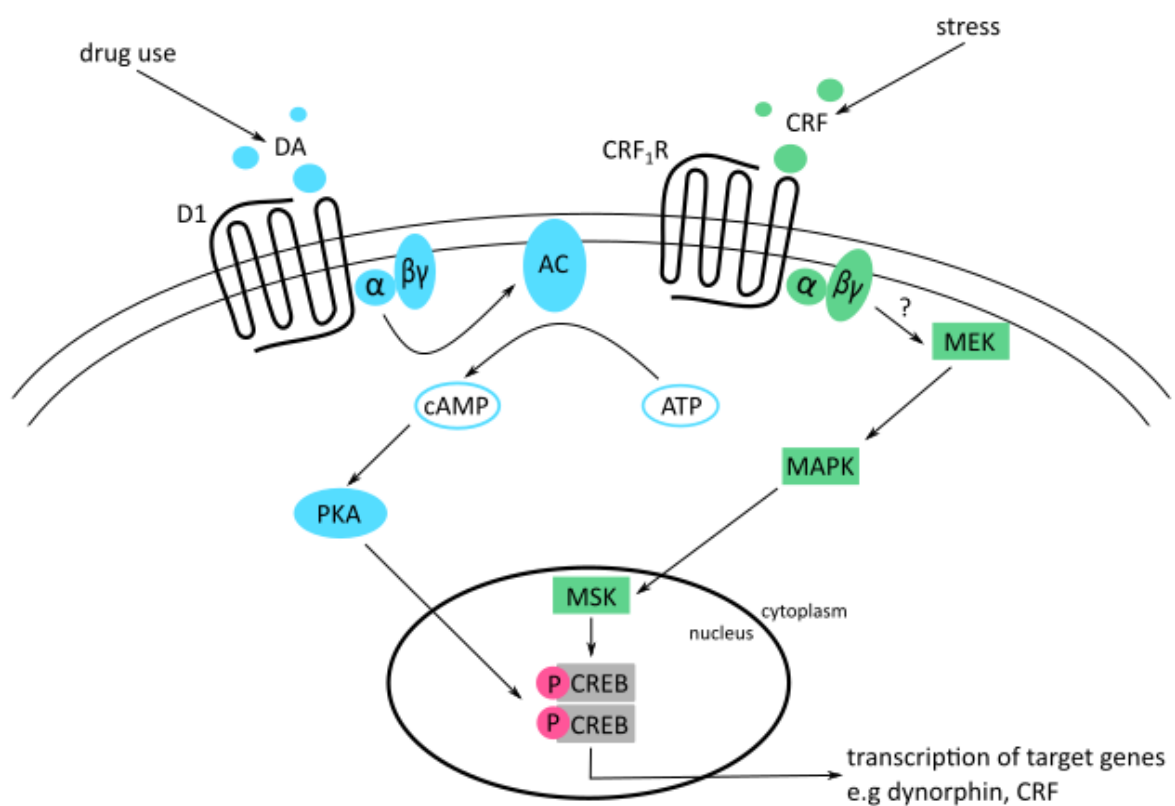
Figure 2   Signalling pathways leading to CREB phosphorylation

Psychostimulant drug use and stress lead to the phosphorylation of the transcription factor
CREB resulting in the production of dynorphin and CRF. AC = adenylyl cyclase, cAMP = cyclic
adenosine monophosphate, CREB = cAMP response element binding protein, CRF =
corticotrophin-releasing factor, CRF_1R = CFR_1 receptor, DA = dopamine, D1 = dopamine
receptor 1, MAPK = mitogen-activated protein kinase, MEK = MAPK kinase, MSK = mitogen-
and stress-activated protein kinase, PKA = protein kinase A.

1.3   Kappa opioid receptor

The KOPr is a G-protein coupled receptor (Avidor-Reiss et al., 1995) widely distributed in the central and peripheral nervous system. Autoradiography studies have shown dense distribution of KOPr in the dStr, NAc, and SN and moderate levels in the hippocampus, thalamus, hypothalamus, amygdala, medial geniculate nucleus and periaqueductal grey (Mansour et al., 1987; Wang et al., 2011).

KOPr stimulation activates its associated G-proteins, allowing them to interact with several intracellular effectors. The $G\alpha_i$ subunit decreases cAMP production through inhibition of adenylyl cyclase (Taussig et al., 1993) whereas the $G\beta\gamma$ subunits modulate calcium and potassium channel activity leading to the inhibition of Ca^{2+} influx and enhancing K^+ efflux (Rusin et al., 1997). Chronic KOPr activation results in phosphorylation of the receptor by G-protein coupled receptor kinase 3 (GRK3), leading to β-arrestin recruitment and internalisation of the receptor (Liu-Chen, 2004) (Figure 3). For a full review of KOPr signalling cascades see Bruchas & Chavkin, 2010.

Following KOPr activation, extracellular signal-regulated kinases 1 and 2 (ERK 1/2) are phosphorylated. This is believed to occur in two phases, with the early phase occurring at 5-15 minutes and a late phase around 2 hours after agonist treatment. The early phase involves phosphorylation via phosphoinositide 3-kinase (PI3 kinase), protein kinase C ζ, and Ca^{2+} (Belcheva et al., 2005) and the late phase via β-arrestin recruitment (McLennan et al., 2008). The early phase ERK 1/2 pathway has been associated with the anti-cocaine effects of KOPr agonists (Simonson et al., 2014) through increasing the expression of DAT and enhancing its clearance of dopamine (Morón et al., 2003).

β-arrestin recruitment activates late-phase ERK 1/2 and p38 MAPK signalling. This pathway is associated with the negative behavioural side effects seen with KOPr agonist treatment. For instance, repeated swim stress increased p38 MAPK phosphorylation in mice, and when inhibited prevented KOPr agonist induced CPA and immobility in the FST. These results were not seen in KOPr knockout animals or those pre-treated with KOPr antagonist norBNI (Bruchas et al., 2007). Further, p38 MAPK activation in dopaminergic neurons located in the VTA is required for KOPr initiated place aversion in mice (Ehrich et al., 2015).

Activation of CREB can also occur downstream of the KOPr signalling cascade. Repeated swim stress has been shown to result in phosphorylation of CREB in a KOPr dependent manner (Bruchas et al., 2007; Bruchas et al., 2008). This occurs through the ERK 1/2 signalling pathway independently of GRK3 (Bruchas et al., 2008) and p38 MAPK (Bruchas et al., 2007). Other behavioural side effects associated with the activation of CREB have already been discussed.

It is now widely accepted that G-protein coupled receptors have multiple conformational states which are dependent upon the properties of the ligand (Perez & Karnik, 2005). Functional selectivity, or ligand-directed signalling, is the concept that different conformational states are biased toward activating specific downstream pathways (Urban et al., 2007). This has a clear impact on the field of drug discovery as this raises the possibility of designing drugs that preferentially activate only a subset of desired functions while avoiding cellular signalling pathways associated with undesirable side effects. In the case of the KOPr receptor, agonists that activate the beneficial pathway (such as early phase ERK 1/2) and avoid the adverse pathways (such as late phase ERK 1/2, p38 MAPK, and CREB) would constitute the most promising therapeutic compounds.
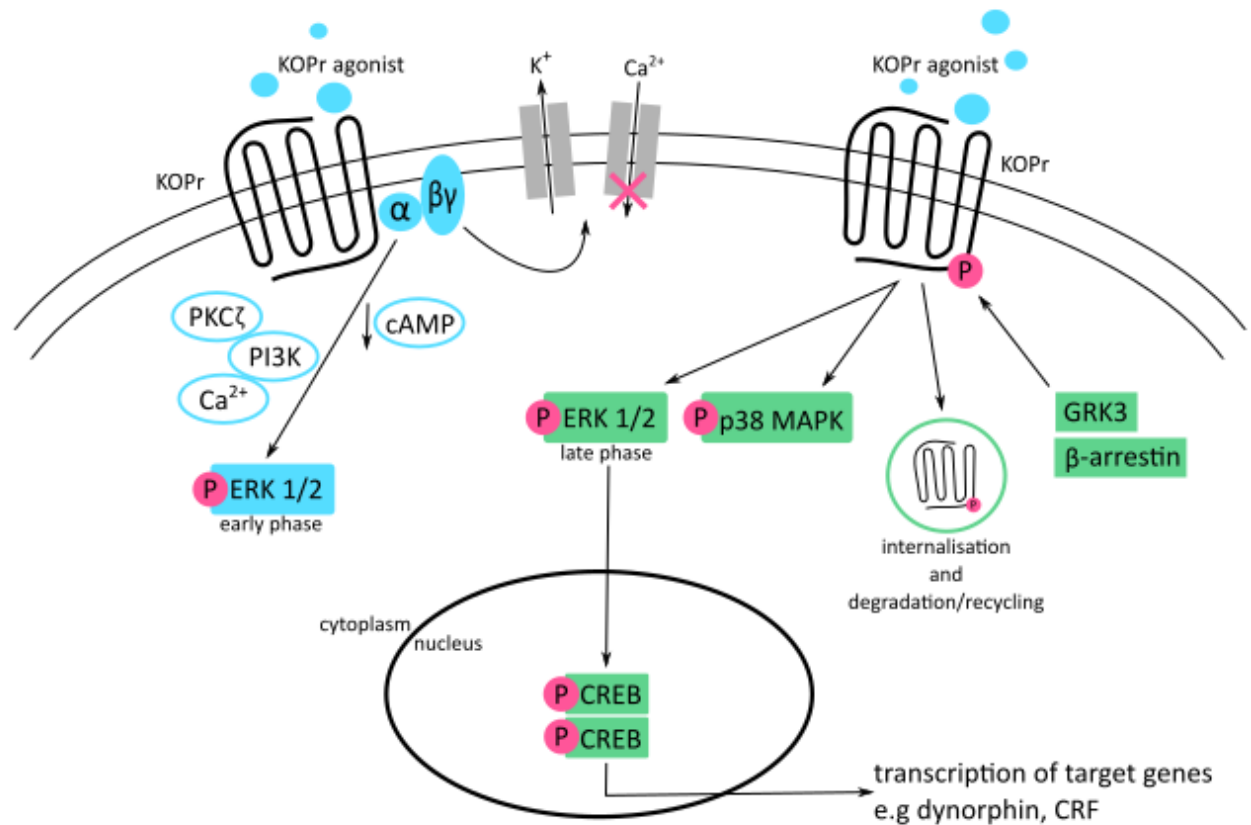
Figure 3   *Kappa opioid receptor signalling pathways*
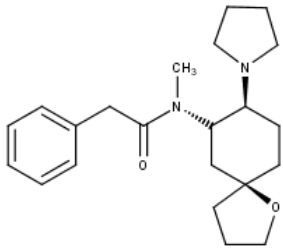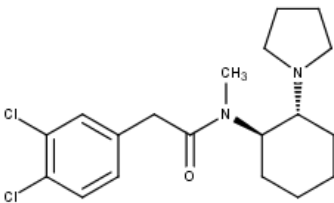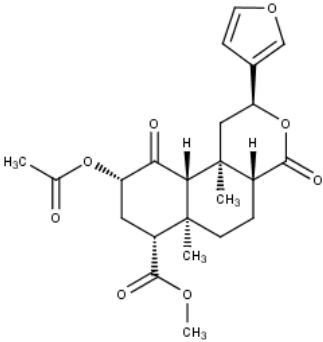
Binding of an agonist (e.g dynorphin) to the KOPr results in the activation of multiple G-protein coupled cell signalling cascades and β-arrestin signalling pathways. Functional selectivity or ligand biased signalling gives rise to the possibility of designing drugs that preferentially activate only a subset of desired functions while avoiding cellular signalling pathways associated with undesirable side effects. cAMP = cyclic adenosine monophosphate, CREB = cAMP response element binding protein, CRF = corticotrophin-releasing factor, ERK 1/2 = extracellular signal-regulated kinases 1 and 2, GRK3 = G-protein coupled receptor kinase 3, KOPr = Kappa opioid receptor, p38 MAPK = p38 mitogen-activated protein kinase, PI3K = phosphoinositide 3-kinase, PKC ζ = protein kinase C ζ.

The most extensively studied KOPr agonists are the acrylacetamides (5R,7S,8S)-(+)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide (U69,593) and 2-(3,4-dichlorophenyl)-N-methyl-N-[(2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide (U50,488) (Lahti et al., 1985; Von Voigtlander et al., 1983). These have been shown to attenuate cocaine-prime induced reinstatement in the rat self-administration model at 0.3 mg/kg (subcutaneous, s.c) and 30 mg/kg (intraperitoneal, i.p) respectively (Schenk et al., 1999; Morani et al., 2009). Further, addition of U69,593 to fentanyl resulted in a decrease in self-administration in rhesus monkeys (Negus et al., 2008). Additionally, animals that receive pre-exposure to cocaine in combination with U69,593 (0.04-0.16 mg/kg, s.c) or U50,488 (5 mg/kg, s.c) failed to develop a place preference with cocaine, an effect which was shown to be reversed with norBNI administration. Further, this treatment with U69,593, in combination with cocaine, was shown to be effective at preventing the usual elevation of dopamine levels that normally occur with repeated cocaine administration (Shippenberg et al., 1996). These results demonstrate a role for the KOPr receptor in the development of behavioural sensitisation to cocaine, suggesting a potential for KOPr agonists as therapeutics for the treatment of cocaine craving and relapse of drug-seeking behaviour.

KOPr agonists did progress into a clinical trial in the 1980s but were abandoned due to depressive and hallucinatory side effects in humans (Pfeiffer, et al., 1986). These traditional agonists have additional side effects including aversion (Mucha & Herz, 1985; Shippenberg & Herz, 1987), anxiety (Motta, et al., 1995), depression (Mague et al., 2003), and sedation (Mague et al., 2003; Mello & Negus, 2000), which has made them unsuitable for further development (Table 3). Thus, recent developments in the field of functional selectivity has shed new light of the development of novel KOPr agonists and provides the rational for the

development of biased agonists which activate systems relating to desirable effects while avoiding those cellular pathways associated with unwanted side-effects.

	U69,593	U50,488	Sal A
Structure			
$K_i \pm SEM^a$ (nM)	2.5 ± 0.3 [1]	0.42 ± 0.22 [1]	0.28 ± 0.22 [1]
EC_{50}^b (nM)	94 [2]	30 [2]	23 [2]
$E_{max} \pm SEM^c$ (%)	108 ± 6.6 [2]	101 ± 4.2 [2]	103 ± 2.9 [2]

[1] Béguin et al., (2008), [2] DiMattio, Ehlert, & Liu-Chen, (2015)

[a] binding affinity of traditional agonists and Sal A at the KOPr using [^{3}H]U69,593 as the radioligand in human embryonic kidney (HEK)-293 cells stably expressing KOPr

[b] EC_{50} = effective concentration required to observe 50% of the maximal response in Neuro-2a cells stably expressing human KOPr with the [^{35}S]GTP-γ-S functional assay

[c] E_{max} = the percentage at which compound stimulates [^{35}S]GTP-γ-S binding compared to U50,488 (10 µM) at the KOPr

Table 1   Structural and pharmacological comparison of traditional KOPr agonists and Salvinorin A

1.4   Salvinorin A and novel analogues

Sal A is a naturally occurring product isolated from the plant *Salvia divinorum*, a mint plant native to Mexico that is used traditionally as a medicine for various ailments and its hallucinogenic properties in spiritual practices (Valdés, 1994). It is a novel, non-nitrogenous neoclerodane diterpene with no structural resemblance to other hallucinogens or traditional KOPr agonists (Roth et al., 2002). Sal A has been found to be a potent and full agonist for the KOPr (Wang et al., 2005) (Table 1) and produces hallucinogenic effects in humans, reportedly more unique and intense than other hallucinogens (Valdés, 1994).

Sal A has also been shown to have anti-cocaine effects in several preclinical behavioural models of drug abuse. Sal A (0.3 mg/kg, i.p) decreased cocaine-induced behavioural sensitisation (Morani et al., 2009; Morani et al., 2012) and cocaine-prime induced drug-seeking behaviour in rats (Morani et al., 2009). Sal A also decreased self-administration of cocaine or remifentanil when co-administered with these drugs in rhesus monkeys (Freeman et al., 2014).

In mice, Sal A showed a dose-dependent decrease in dopamine levels in the caudate putamen in (0.32 – 3.2 mg/kg, i.p) with aversion and reduced locomotor activity demonstrated at 1.0 and 3.2 mg/kg, i.p (Zhang, et al., 2005). Sal A also produces place aversion in rats at a dose of 0.3 and 1 mg/kg, i.p (Sufka et al., 2014) but no taste aversion at 0.3 mg/kg, i.p (Morani et al., 2012) nor sedative effects at 0.125-2 mg/kg, i.p (Carlezon et al., 2006; Morani et al., 2012). In zebrafish it was found that lower doses of Sal A (0.2 and 0.5 µg/kg intramuscular, i.m) produced preference while higher doses (1 and 80 µg/kg i.m) produced aversion (Braida et al., 2007). This is consistent with findings in rats; Sal A conditioned preference is formed at 0.1 – 40 µg/kg and aversion is formed at 160 µg/kg (Braida et al., 2008). This suggests that

preference is observed in low doses and aversion in higher doses. This trend is also seen with depression in the FST as 10-1000 µg/kg, s.c demonstrated anti-depressive effects (Braida et al., 2009) and 0.25-2 mg/kg, i.p resulted in pro-depressive behaviour in rats (Carlezon et al., 2006; Morani et al., 2012). Anxiolytic effects of Sal A (0.1-160 µg/kg, s.c) in rats have also been demonstrated on the EPM (Braida et al., 2009) (Table 3).

Sal A has an improved side effects profile compared with traditional KOPr agonists but a short duration of action (Valdés, 1994). This makes it unsuitable for development as an anti-addiction agent. The emergence of longer acting analogues with improved side effect profiles is an important step forward in the development of anti-addiction pharmacotherapies. Munro et al. (2008) attempted to increase the affinity and potency of Sal A by adding standard protecting groups to the C-2 acetyl group of Sal A. A promising compound, ethoxymethyl salvinorin B ether (EOM Sal B), was synthesised and shown to have greater binding affinity to KOPr *in vitro* than Sal A (Table 2). This alkoxymethyl ether is more likely to remain stable *in vivo,* which would lengthen its duration of action compared with Sal A (Munro et al., 2008). EOM Sal B has recently been shown to attenuate cocaine-prime induced cocaine seeking (0.1 and 0.3 mg/kg i.p) (Kivell lab, unpublished data) and to not exhibit undesirable effects in the FST at 0.1 mg/kg, i.p in rats (Kivell lab, unpublished data) (Table 3).
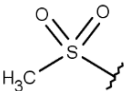
β-tetrahydropyran Sal B (β-THP Sal B) was synthesised as a constrained analogue of Sal A to help elucidate the structural basis for the changes in affinity and potency at the KOPr seen in previous studies (Prevatt-Smith et al., 2011). This compound was found to have a similar KOPr receptor binding affinity and potency compared with Sal A and is likely to have a greater duration of action *in vivo* due to the lack of a hydrolysable ester at C-2 (Table 2). At a dose of 1 mg/kg, i.p β-THP Sal B was found to attenuate cocaine-induced drug-seeking in rats and

produced a similar response as Sal A (0.3 mg/kg, i.p) (Prevatt-Smith et al., 2011) but with no pro-depressive effects in the FST (Kivell lab, unpublished data) (Table 3).

(2S,4aR,6aR,7R,9S,10aS,10bR)-9-(Methanesulfonyloxy)-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2H-napthol[2,1-c]pyran-7-carboxylic acid methyl ester (Mesyl Sal B) was synthesised to investigate the addition of a mesylate group at the C2 position of Sal A and its consequent effect on KOPr affinity and activity (Harding et al., 2005). It has similar binding affinity and potency compared to Sal A (Harding et al., 2005) (Table 2). Mesyl Sal B (1 mg/kg, i.p) has a slower onset and longer duration of action compared with Sal A (1 mg/kg, i.p) (Simonson et al., 2014). In rats, Mesyl Sal B also attenuated cocaine-prime induced reinstatement (0.3 and 1 mg/kg, i.p) without a decrease in locomotor activity (0.3 mg/kg, i.p) (Simonson et al., 2014) or a conditioned place aversion in rats (0.3 mg/kg, i.p) (Kivell lab, unpublished data). However, it has demonstrated pro-depressive effects in the FST at 0.3 mg/kg, i.p (Kivell lab, unpublished data) (Table 3).

Ethynyl Sal A (Ethy Sal A) was produced by Riley et al. (2014) to examine how functionalisation of the furan ring of Sal A effected binding and activity at the KOPr. Ethy Sal A was shown to have a similar potency to that of Sal A (Table 2) and was subsequently shown to significantly attenuate drug-seeking behaviour in the drug-primed cocaine self-administration model at a dose of 0.1 and 0.3 mg/kg, i.p with no observed sedative (Riley et al., 2014) or pro-depressive effects at 0.3 mg/kg, i.p (Kivell lab, unpublished data) (Table 3).
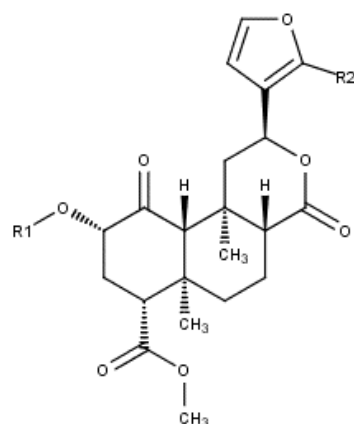
	Sal A	β-THP Sal B	EOM Sal B	Mesyl Sal B	Ethy Sal A
R1					-H
R2	-H	-H	-H	-H	
$K_i \pm SD^a$ **(nM)**	7.4 ± 0.7 [1,2] 1.9 ± 0.2* [3]	6.21 ± 0.40 [2]	3.13 ± 0.40 [2]	2.3 ± 0.1* [3]	-
$EC_{50} \pm SD^b$ **(nM)**	40 ± 10 [1,2,3] 0.030 ± 0.004# [4]	60 ± 6 [2]	0.65 ± 0.17 [2]	30 ± 5 [3]	0.019 ± 0.004# [4]
$E_{max} \pm SD^c$ **(%)**	120 ± 2 [1,2,3]	109 ± 3 [2]	127 ± 5 [2]	112 ± 4 [3]	-

[1] Lozama et al., (2011)   [2] Prevatt-Smith et al., (2011)   [3] Harding et al., (2005)   [4] Riley et al., (2014)

[a] binding affinity of Sal A and analogues at the KOPr using [³H]U69,593 or *[¹²⁵I]IOXY as radioligand, [b] EC_{50} = effective concentration required to observe 50% of the maximal response in CHO cells stably expressing human KOPr with the [³⁵S]GTP-γ-S functional assay or #forskolin-induced cAMP accumulation assay ($EC_{50} \pm SEM$), [c] E_{max} = the percentage at which compound stimulates [³⁵S]GTP-γ-S binding compared to U50,488 (500 nM) at the KOPr

Table 2   Pharmacological comparison of Sal A and its analogues at the KOPr

Adverse Effect	Procedure	U69,593	U50,488	Sal A	β-THP Sal B	EOM Sal B	Mesyl Sal B	Ethy Sal A
Drug-seeking	Drug prime reinstatement	↓ 0.32, s.c [1,2]	↓ 30, i.p [2]	↓ 0.3, 1.0, i.p [2]	↓ 1.0, i.p [17]	↓ 0.1, i.p [18]	↓ 0.3, i.p [19]	↓ 0.1, 0.3. i.p [20]
Aversion	CPA	↑ 0.32, s.c (rats and mice)[3,4]	↑ 1,2, s.c [7] 10, i.p[8]	↑ 0.3, 1.0, i.p[11] 160 µg/kg i.p[12] ↑ 1.0, 3.2, i.p (mice)[13] ↓ 0.1-40µg/kg i.p[12]	-	-	n.e 0.3, i.p [18]	-
	CTA	-	↑ 0.5-2, s.c [7]	n.e 0.3, i.p [14]	-	-	-	-
Depression	FST	↑ 0.3-10, i.p [5]	-	↑ 0.25-2.0, i.p [14,15] ↓ 10-1000 µg/kg, s.c [16]	n.e 1.0, i.p [18]	n.e 0.1, i.p [18]	↑ 0.3, i.p [18]	n.e 0.3, i.p [18]
Anxiety	EPM	↓ 100 µg/kg, i.p [6]	↓ 10,100 µg/kg, i.p [6] ↑ 10, i.p [9]	↓ 0.1-160 µg/kg, s.c [16]	-	-	-	-
	light/dark	-	-	-	-	-	-	-
Sedation	Locomotor activity	↑ 10, i.p [5]	↑ 0.3-10, i.p (mice)[10]	↑ 1.0, 3.2, i.p (mice)[13] n.e 0.125-2.0, i.p [14,15] n.e 0.001-1 µg/kg, s.c (mice)[16]	n.e 0.3, 1.0, i.p [18]	n.e 0.1, i.p [18]	n.e 0.3, i.p [19]	n.e 0.3, i.p [20]

Doses given in mg/kg and effect seen in a rat model unless otherwise stated, ↑ = presence of the effect, ↓ = attenuation of the effect, n.e = no effect

[1] Schenk et al., (1999)
[2] Morani et al., (2009)
[3] Tejeda et al., (2013)
[4] Chefer et al., (2013)
[5] Mague et al., (2003)
[6] Privette & Terrian, (1995)
[7] Mucha & Herz, (1985)
[8] Suzuki et al., (1992)
[9] Valdez & Harshberger, (2012)
[10] Paris et al., (2011)
[11] Sufka et al., (2014)
[12] Braida et al., (2008)
[13] Zhang et al., (2005)
[14] Morani et al., (2012)
[15] Carlezon et al., (2006)
[16] Braida et al., (2009)
[17] Prevatt-Smith et al., (2011)
[18] Kivell lab, unpublished data
[19] Simonson et al., (2014)
[20] Riley et al., (2014)

Table 3   Behavioural effects of acute KOPr agonist treatment

2.   Aims and Hypotheses

Given the social and economic impact of drug addiction on the public it is important that we address possible solutions to this problem. As KOPr agonists have been shown to be effective at decreasing drug-seeking behaviour in animal models they show potential as a therapeutic target. Sal A is one such compound that has this property, but unfortunately has too short a duration of action to be viable. Novel analogues of Sal A have been synthesised and demonstrate improved pharmacokinetics as well as decreased behavioural side effects such as depression and sedation. However, the aversive and anxiogenic effects have yet to be examined for the majority of these compounds.

This study aimed to assess these effects of novel KOPr agonists β-THP Sal B, EOM Sal B, and Ethy Sal A in rats using the CPA, EPM, and light/dark procedures at the doses at which they have demonstrated significantly reduced drug-seeking behaviour in the cocaine drug-primed model of reinstatement. Mesyl Sal B was also tested for possible anxiogenic effects, as previous experiments already demonstrated that it does not cause CPA (Kivell lab, unpublished data). It was hypothesised that the novel analogues of Sal A would have improved effects over the parent compound.

Activation of the KOPr can result in adverse behavioural effects. The phosphorylation of the transcription factor CREB has been associated with these side effects. This study aimed to assess the effects of the novel Sal A analogues on the levels of pCREB found in the NAc, dStr, and PFC in the rat brain. It was hypothesised that compounds that do not show aversive or anxiogenic effects would not demonstrate significant increases in pCREB.

3.   Methods

3.1   Animals

Male Sprague-Dawley rats (*Rattus norvegicus*) weighing between 280-430 g were used for all experiments and housed in groups of 2-4 per cage. Animals were housed in the animal facility of the School of Biological Sciences, Victoria University of Wellington in a temperature (20°C) and humidity (50%) controlled environment on a 12:12 light:dark cycle with the light cycle starting at 0700 h. All experiments were conducted during the day in the light cycle between 0830-1700 h and in the presence of white noise to mask background noises. Water and food (Diet 86, Sharpes Stock Feed) was available *ad libitum* except during testing sessions. Animals were handled for several days prior to experimentation to reduce experimenter stress. In accordance with the 3 Rs principle for ethical use of animals in research (Russell & Burch, 1959), rats were used in multiple behavioural experiments (Reduction) with a minimum rest period of seven days between tests (Refinement). All experiments were approved by the Victoria University of Wellington Animal Ethics Committee.

3.2   Drug administration

Rats were injected with U50,488 (10 mg/kg, i.p) (Sigma-Aldrich, St. Louis, MO, USA), Sal A (0.3 and 1 mg/kg, i.p), β-THP Sal B (1 mg/kg, i.p), Mesyl Sal B (0.3 mg/kg, i.p), EOM Sal B (0.1 mg/kg, i.p), and Ethy Sal A (0.3 mg/kg, i.p) (courtesy of Prof. T. Prisinzano, University of Kansas, Lawrence, KS, USA). All drugs were dissolved in a 2:1:7 mixture of DMSO, Tween 80 and miliQ water. The drugs were administered in a volume of 1 ml/kg. Control animals were injected with vehicle, i.p.

3.3 Behavioural Assays

3.3.1 Conditioned place aversion procedure

CPA procedures were performed as previously described by Tejeda et al. (2013). We used a three-chambered place preference apparatus which consisted of two large chambers (30 x 30 x 34 cm) which were connected by a smaller corridor (8 x 10 x 34 cm) with sliding doors to confine the animal to either chamber as required (PanLab, Harvard Apparatus, USA). One of the large chambers had a textured black floor with white walls with a black dot pattern while the other had a smooth white floor with black walls and a white stripe pattern. The corridor had a smooth grey floor with grey walls. Animals were recorded using a camera mounted directly above the apparatus and tracked using the SMART 3.0 software (PanLab, Harvard Apparatus, USA). The main fluorescent ceiling lights were switched off during experiments and lighting was provided by two free-standing LED lamps, one positioned toward the ceiling to provide ambient lighting for filming and the other directed at the corridor to discourage lingering. The average light intensity in each conditioning chamber was 20 lux whereas the corridor was 60 lux.

The day before experimentation rats were allowed to freely roam the apparatus for 15 min to habituate (day 0). On the first experimental day rats were again given free access to both chambers for 15 min. Time spent in each chamber was measured and the preferred chamber noted. Animals were excluded if they showed over 80% preference for a particular chamber or over 40% preference for the corridor. Rats were subjected to conditioning using a biased procedure on days two-seven whereby the compound of interest was administered in the preferred chamber. On days two, four and six, rats were injected with KOPr agonist or vehicle and placed in their preferred chamber and confined for 45 min. On days three, five and seven,

rats were injected with vehicle and confined to the opposite chamber for 45 min. Conditioning days were counterbalanced to reduce experimental differences. On the testing day animals were again placed in the corridor and given free access to both chambers for 15 min and the time spent in each chamber recorded.
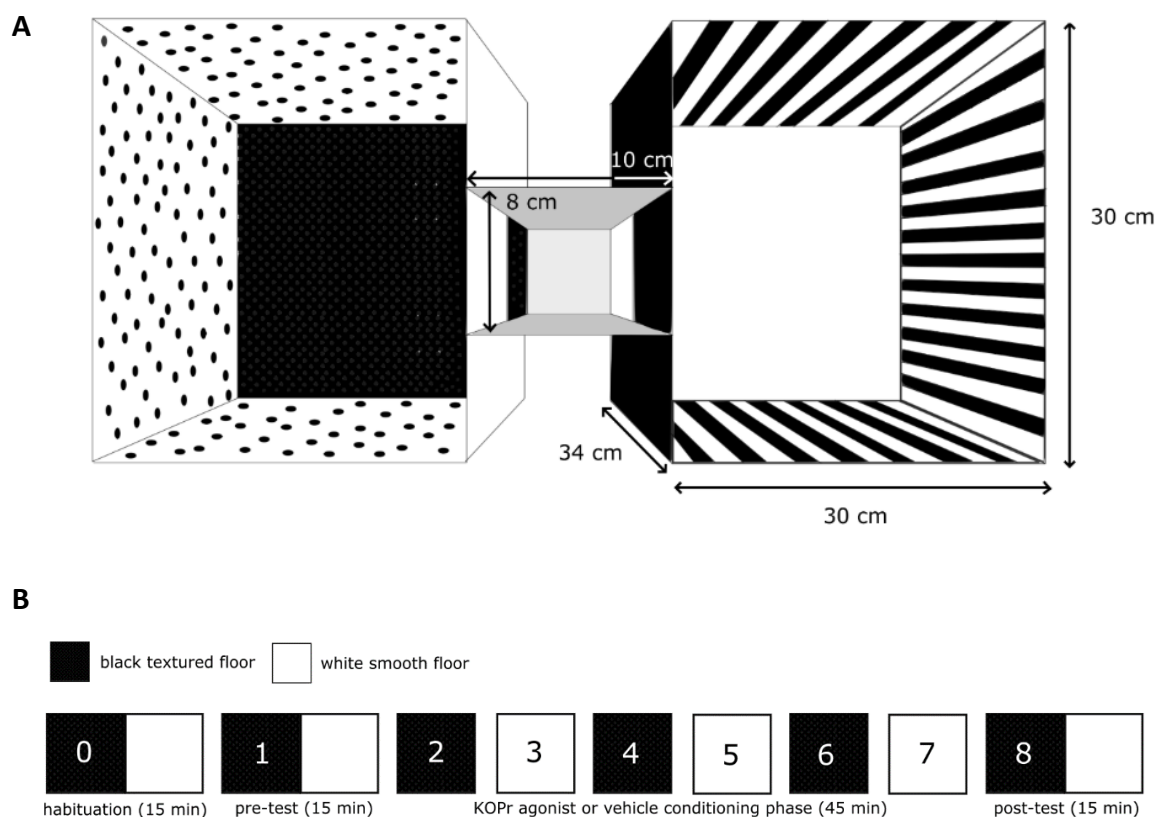
Figure 4   Conditioned place aversion apparatus and procedure

A: Diagram shows the three-chambered CPA apparatus.

B: Rats were initially habituated to the entire apparatus (Day 0) before testing for their baseline preference in the pre-test (Day 1). Rats were then divided into treatment groups (vehicle or KOPr agonist). During the conditioning phase (Days 2-7) animals were confined to one compartment and received either systemic vehicle or KOPr agonist treatment in their most preferred box (Days 2, 4, and 6) or vehicle in their least preferred box (Days 3, 5, and 7). The effect of the treatment was then assessed in the post-test by allowing access to the entire apparatus as in the pre-test (Day 8).

3.3.2 Elevated plus maze procedure

EPM was performed as previously described by Walf and Frye (2007). The maze was made of black plastic and consisted of four arms (50 cm x 10 cm each) elevated to 55 cm (Victoria University of Wellington, Wellington, NZ). The two open arms had 3 cm high clear plastic ledges and the two closed arms had 40 cm high black plastic walls. Lighting on the entire maze was provided by fluorescent ceiling lamps. Following vehicle or KOPr agonist injection rats were placed in the centre of the apparatus facing an open arm and filmed for 5 min (Sony HDR-SR5E digital camera recorder). Time spent on each arm and entries to the arms were measured by experimenters blind to the treatment of the animal. An arm entry was defined as having all four paws on the arm (Walf & Frye, 2007). Rats treated with anxiogenic compounds have a decreased open arm time and those treated with anxiolytic compounds increased open arm time when compared with vehicle treated rats (Pellow, et al., 1985). Animals that fell off the maze during testing were placed back on the maze for the duration of the experiment but were excluded from analysis.

3.3.3 Light/dark test procedure

Testing was performed in the same apparatus as CPA with the exception of an insert added to the black box to decrease the total area to 17 x 30 x 34 cm. The fluorescent room lights were turned off during experimentation and lighting levels were controlled by LED lamps to provide 100 lux in the light box, 70 lux in the corridor and 10 lux in the dark box. Following vehicle or KOPr agonist injection rats were placed in the black box and recorded for 15 min using SMART 3.0 software. Anxiogenic compounds are known to decrease the time spent in the light box (Merlo Pich & Samanin, 1989).
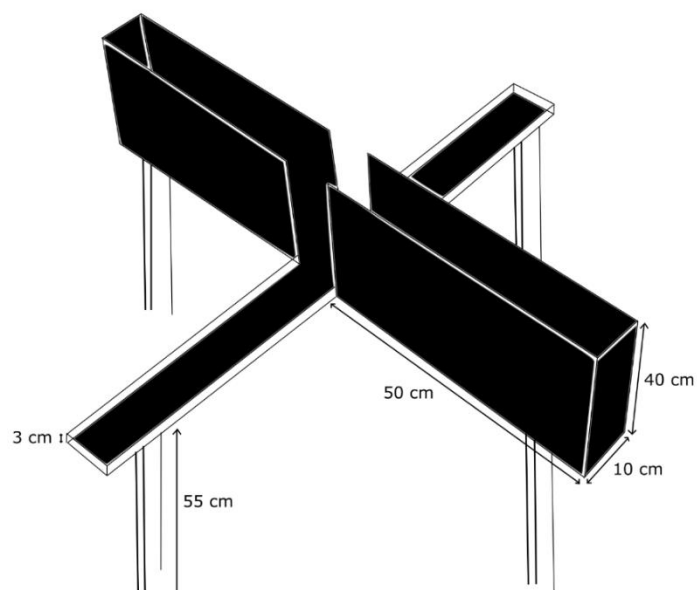
Figure 5   Elevated plus maze apparatus

Anxiogenic effects were examined in the EPM test. Animals were injected with KOPr agonist or vehicle and placed on the centre of the maze. Time spent on the open arm and open arm/total arm entries were used as anxiety-like behavioural measures, while closed arm entries were used to test for potential sedative effects.
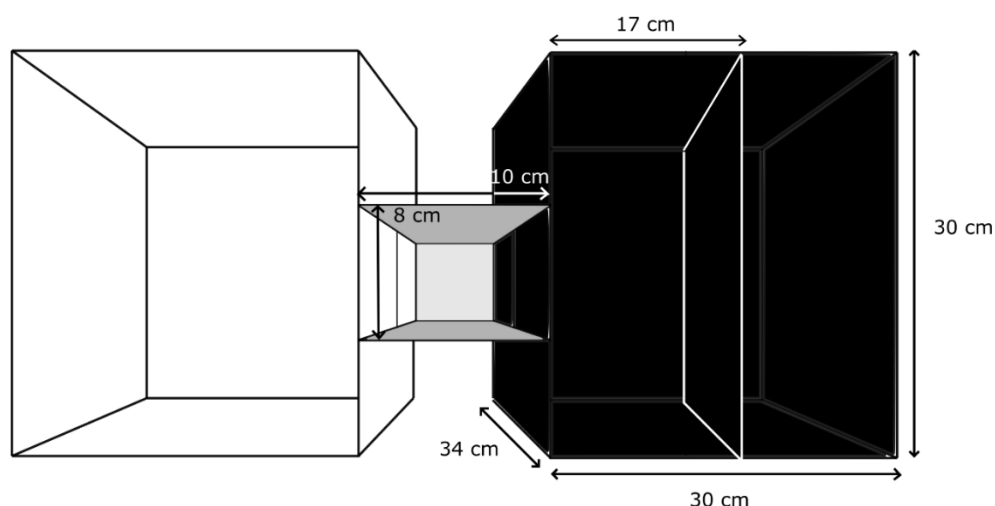
Figure 6   Light/dark test apparatus

Anxiogenic effects of test compounds were examined using the light/dark test. Animals were placed in the small, dark box following an injection with either vehicle or KOPr agonist and the time spent in the larger, brightly lit light box and total distance travelled in both compartments was recorded.

3.3.4   Open field test procedure

The open field arena (45 x 45 cm) was separated into two virtual zones using SMART 3.0 software with a 20 x 20 cm central square. Dark grey mats were placed in the floor of the arenas to enable accurate video tracking of the animal. The light level in the arena was 30 lux. Following either vehicle or KOPr agonist injection, rats were placed in the centre of the arena and recorded for 10 min using SMART 3.0 software and the time spent in each zone and total distance travelled recorded. Anxiogenic drugs are known to decrease time spent in the centre of the open field (Prut & Belzung, 2003).

3.4   Optimisation of CREB antibodies in Western Blotting

3.4.1   *In vitro* samples

The immortalised HEK-293 cell line was used to demonstrate the effectiveness of the pCREB and CREB antibodies prior to testing tissue samples. Cells were cultured in Dubecco's modified Eagle's medium (DMEM); (Invitrogen, Auckland, NZ) containing 10% v/v Foetal calf serum (FCS); (ICP Biologicals, Auckland, NZ) and 1% v/v penstrep antibiotic (penicillin G sodium 5000 units/ml and streptomycin sulphate 5000 units/ml dissolved in 0.85% saline). Cultures were grown in a humid Heracell incubator at 37°C with 1% CO_2. In preparation for experimentation cells were passaged and plated on 35 mm petri dishes at a density of 3.0 x 10^5 cells/ml in DMEM (10% FCS, 1% penstrep), or serum starved in low-serum DMEM (1% FCS, 1% penstrep) to remove basal phosphorylation, and left to incubate overnight. Cells were treated with forskolin (30 µM) (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes, washed twice with phosphate buffered saline (PBS); (0.14 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4; Appendix 1), and then lysed with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% SDS, 1% Sodium

deoxycholate; Appendix 1) and 1X Halt™ protease and phosphatase inhibitor (Thermo Scientific, Rockford, IL) for 60 min at 4°C.

3.4.2   *Ex vivo* samples

Rats were either untreated, injected with vehicle (0.9% saline, i.p) or cocaine (30 mg/kg, i.p). Rats were sacrificed via CO_2 asphyxiation and the brain rapidly removed and placed on ice. The NAc, dStr and PFC were identified using a brain matrix (Alto, AgnTho's AB, Sweden) and stereotaxic coordinates (Paxinos & Watson, 2005). Both hemispheres were pooled for analysis and mechanically homogenised or sonicated on ice (4 x 30 sec sonication, 30 sec rest) (Sonoplus mini20, Bandelin, Berlin, Germany) in RIPA buffer containing protease and phosphatase inhibitors.
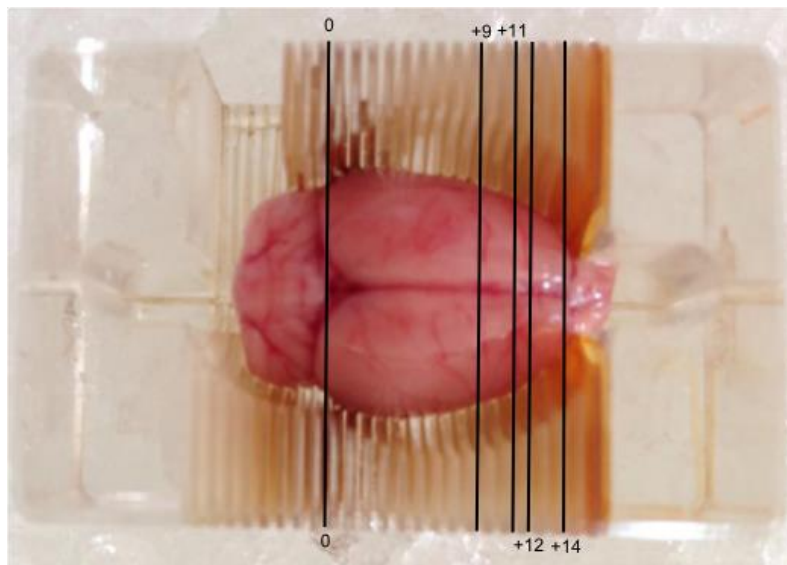

Figure 7   *Interaural coordinates for rat brain dissection*

Rat brains were removed and positioned in the brain matrix to measure interaural units. The brain regions of interest were dissected from 2 mm sections; dorsal striatum and nucleus accumbens from +9 to +11 and the prefrontal cortex from +12 to +14.

3.4.3   Protein quantification

Protein isolated from cultured cells and tissue samples was quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (BSA); (Sigma-Aldrich, St. Louis, MO, USA) standards were prepared in RIPA buffer (1000, 750, 500, 250, 125, 25, 0 µg/ml). Both standards and diluted samples were loaded in triplicate on a 96-well plate and 200 µl of the protein assay dye (Bio-Rad, Hercules, CA, USA) added, incubated for 5 min at room temperature and absorbance at 595 nm determined (VERSA$_{MAX}$ microplate reader, Molecular Devices Group, USA). Triplicate readings were averaged and graphed using Microsoft Excel 2013 to obtain a standard curve, which was used to determine the protein concentration of each sample. Protein samples (20 µg for *in vitro* samples and 100 µg for *ex vivo* samples) were reduced in reducing buffer (62.5 mM Tris-HCl, 2% SDS, 20% Glycerol, 1% Bromophenol Blue; Appendix 1) containing 9% β-mercaptoethanol before being loaded onto 15-well 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

3.4.4   Gel electrophoresis and Western blotting

SDS-PAGE gels (10% separating gel, 4% stacking gel) were cast and kept moist at 4°C prior to experimentation. Reduced samples were loaded in a total volume of 20 µl and electrophoresed at 120V for 1.5 hours in running buffer (3.5 mM SDS, 25 mM Tris HCl, 190 mM Glycine; Appendix 1) using the Mini-PROTEAN Tetra Cell system (Bio-Rad, Hercules, CA, USA). Polyvinylidene difluoride (PVDF) transfer membrane (pore size: 0.45 µm) (Immobilon-FL, Millipore, Billerica, MA, USA) was pre-soaked in methanol for 5 min followed by Western blot transfer buffer (190 mM Glycine, 25 mM Tris HCl, 20% methanol; Appendix 1) for a further 5 min. The transfer cassette was assembled in transfer buffer and contained, in layered order, a sponge, filter paper, protein gel, transfer membrane, filter paper, and

sponge. The cassette was then placed in transfer buffer in the transfer tank with an ice pack and then electrophoresed at 20V for 16 hours (Mini Trans-Blot Cell, Bio-Rad, Hercules, CA, USA).

Membranes were removed from the transfer cassette and washed three times with Tris-buffered saline (TBS); (50 mM Tris HCl, 150 mM NaCl; Appendix 1) and blocked with either TBS containing 5% w/v BSA or 5% w/v non-fat dry milk for 60 min at room temperature. The membrane was then incubated with primary antibody specific for pCREB (1:1000) (Phospho-CREB (Ser133) (1B6) mouse mAb #9196 and Phospho-CREB (Ser133) (87G3) rabbit mAb #9198, Cell Signalling Technology, Danvers, MA, USA) or CREB (1:1000) (CREB (48H2) rabbit mAb #9197, Cell Signalling Technology, Danvers, MA, USA) in Tween 20-TBS (T-TBS); (TBS containing 0.1% Tween 20) containing 5% w/v BSA or T-TBS containing 5% w/v non-fat milk powder overnight at 4°C.   Membranes were washed three times with T-TBS then immunolabeled with goat anti-rabbit conjugated Cy5 (PA45011, Amersham, GE Healthcare Life Sciences, Auckland, NZ) or goat anti-mouse conjugated Cy5 (PA45009, Amersham, GE Healthcare Life Sciences, Auckland, NZ) secondary antibodies at room temperature for 60 min. Membranes were subsequently washed three times in T-TBS and imaged using a FUJIFILM FLA-5000 laser scanner (Fujifilm, Tokyo, Japan) at an excitation wavelength of 635 nm at 450 V using a band pass filter (R665, Fujifilm, Tokyo, Japan) (Table 4).

Membranes were then blocked with TBS containing 5% w/v BSA for 60 min, incubated with an α-tubulin rabbit antibody as a loading control (ab18251, Abcam, Melbourne, Australia) in T-TBS containing 5% w/v BSA for 60 min at room temperature, washed three times with T-TBS and then probed with goat anti-rabbit conjugated Cy5 secondary antibody at room

temperature for 60 min. Membrane was again scanned after another three washes with T-TBS (Table 4).

Western blots were analysed using ImageJ software (NIH, Bethesda, Maryland, USA). Band densities were corrected against the background and for protein loading differences by comparing pCREB to α-tubulin.

3.5   Data analysis

Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA). All behavioural data was tested for normality using the D'Agostino and Pearson omnibus normality test, as some values were tied. Data sets with $p \leq 0.05$ in the normality test were subsequently analysed with nonparametric tests as a Gaussian distribution was not assumed. A paired Student's t-test was used to compare the pre- and post-test conditioning times in CPA. One-way ANOVA with Bonferroni's multiple comparisons tests were performed on data obtained from the EPM and the Kruskal-Wallis test with Dunn's multiple comparisons test in the light/dark test. Post-hoc testing was only performed if the result from the ANOVA/Kruskal-Wallis test was significant. P values reported for the post hoc tests are multiplicity adjusted p values due to the correction calculated for multiple comparisons. The vehicle and Sal A (0.3 and 1 mg/kg) data sets are repeated for comparison with the novel compounds in all behavioural tests. Western blots were analysed with Student t-tests to compare treatment groups to untreated controls. All values presented are the mean ± SEM and statistical significance was determined when $p \leq 0.05$.

Protein	Size (kDA)	Blocking solution		1° antibody			2° antibody			
		Solution	Time/Condition	Solution	Dilution	Time/Condition	Specificity	Solution	Dilution	Time/Condition
pCREB (mouse)	43	TBS cont. 5% w/v non-fat dry milk	60 min/RT	T-TBS cont. 5% w/v non-fat dry milk	1:1000	Overnight/4°C	Mouse Cy5	T-TBS	1:5000	60 min/RT
pCREB (rabbit)	43	TBS cont. 5% w/v BSA	60 min/RT	T-TBS cont. 5% w/v BSA	1:1000	Overnight/4°C	Rabbit Cy5	T-TBS	1:5000	60 min/RT
CREB (rabbit)	43	TBS cont. 5% w/v BSA	60 min/RT	T-TBS cont. 5% w/v BSA	1:1000	Overnight/4°C	Rabbit Cy5	T-TBS	1:5000	60 min/RT
α-tubulin (rabbit)	50	TBS cont. 5% w/v BSA	60 min/RT	T-TBS cont. 5% w/v BSA	1:5000	60 min/RT	Rabbit Cy5	T-TBS	1:5000	60 min/RT

Cont: containing; BSA: bovine serum albumin; TBS: Tris-buffered saline; RT = room temperature; T-TBS: 0.1% Tween 20 Tris-buffered saline

Table 4   Antibodies used and their conditions

4. Results

4.1 U50,488 and Sal A

The CPA assay was used to screen U50,488 and Sal A for aversive effects. Both compounds have been previously reported to show a significant decrease in time spent in an environment paired with the effects of these drugs at 10 mg/kg, i.p and 0.3 mg/kg, i.p respectively (Sufka et al., 2014; Suzuki et al., 1992). These results were replicated to act as a positive control for the protocol and for comparison with the novel Sal A analogues.

U50,488 (10 mg/kg, i.p) significantly decreased the time that the animals spent in their initially preferred chamber after conditioning ($t_{(10)}$ = 3.835, p = 0.0033; 47 ± 3% to 33 ± 3%). A non-significant decrease was observed in vehicle treated animals ($t_{(10)}$ = 1.864, p = 0.0919; 44 ± 4% to 37 ± 2%). Sal A (0.3 mg/kg, i.p) had a significant decrease in time spent in the drug paired chamber ($t_{(8)}$ = 3.742, p = 0.0057; 57 ± 5% to 37 ± 6%) (Figure 8A). It should be noted that this treatment also showed a significant increase in time spent in the neutral corridor ($t_{(8)}$ = 3.403, p = 0.0093; 25 ± 4% to 39 ± 5%). U50,488 conditioning also shows a trend towards an increase in time spent in the corridor but did not reach statistical significance ($t_{(10)}$ = 2.183, p = 0.054; 23 ± 3% to 30 ± 3%). Vehicle treated animals showed no change in time spent in the corridor ($t_{(10)}$ = 0.7895, p = 0.4482; 26 ± 3% to 29 ± 2%) (Figure 12A).

To examine the anxiogenic effects of U50,488 and Sal A the EPM and light/dark test were utilised. U50,488 has previously been shown to decrease open arm exploration at a dose of 10 mg/kg, i.p in Wistar rats (Gillett et al., 2013; Valdez & Harshberger, 2012). To our knowledge the effect of U50,488 and Sal A on behaviour in the light/dark test has not yet been reported.

In the present study, both the EPM and light/dark test demonstrated a significant effect of treatment on time spent on the open arm ($F_{(3,71)}$ = 3.687, p = 0.0158) or time spent in the light box ($H_{(3)}$ = 12.84, p = 0.0050).

In the EPM test U50,488 did not show a significant decrease in time spent on the open arm compared with vehicle (26 ± 4% to 37 ± 4%, p = 0.2324). However, both doses of Sal A demonstrated significant anxiogenic effects (0.3 mg/kg: 20 ± 4% p = 0.0156; 1 mg/kg: 22 ± 4% p = 0.0435) (Figure 9A). Consistent with the results seen in the EPM test, U50,488 did not show a significant decrease in time spent in the light box compared with vehicle (12 ± 2 to 17 ± 2 %, p = 0.4500) and the higher dose of Sal A (1 mg/kg) did (5 ± 1 to 17 ± 2 %, p = 0.0014). In contrast, the lower dose of Sal A (0.3 mg/kg) did not exhibit anxiogenic effects (14 ± 2 to 17 ± 2 %, p = ≥ 0.9999) in disagreement to the results seen in the EPM test (Figure 9B).

These results show that U50,488 and Sal A (0.3 mg/kg) show aversive effects. U50,488 did not demonstrate the expected anxiogenic effect in the EPM nor light/dark tests and Sal A at 1 mg/kg exhibits anxiogenic effects in both of the anxiety tests. Interestingly, the lower dose of Sal A (0.3 mg/kg) only produced significant anxiogenic effects in the EPM.

A

B
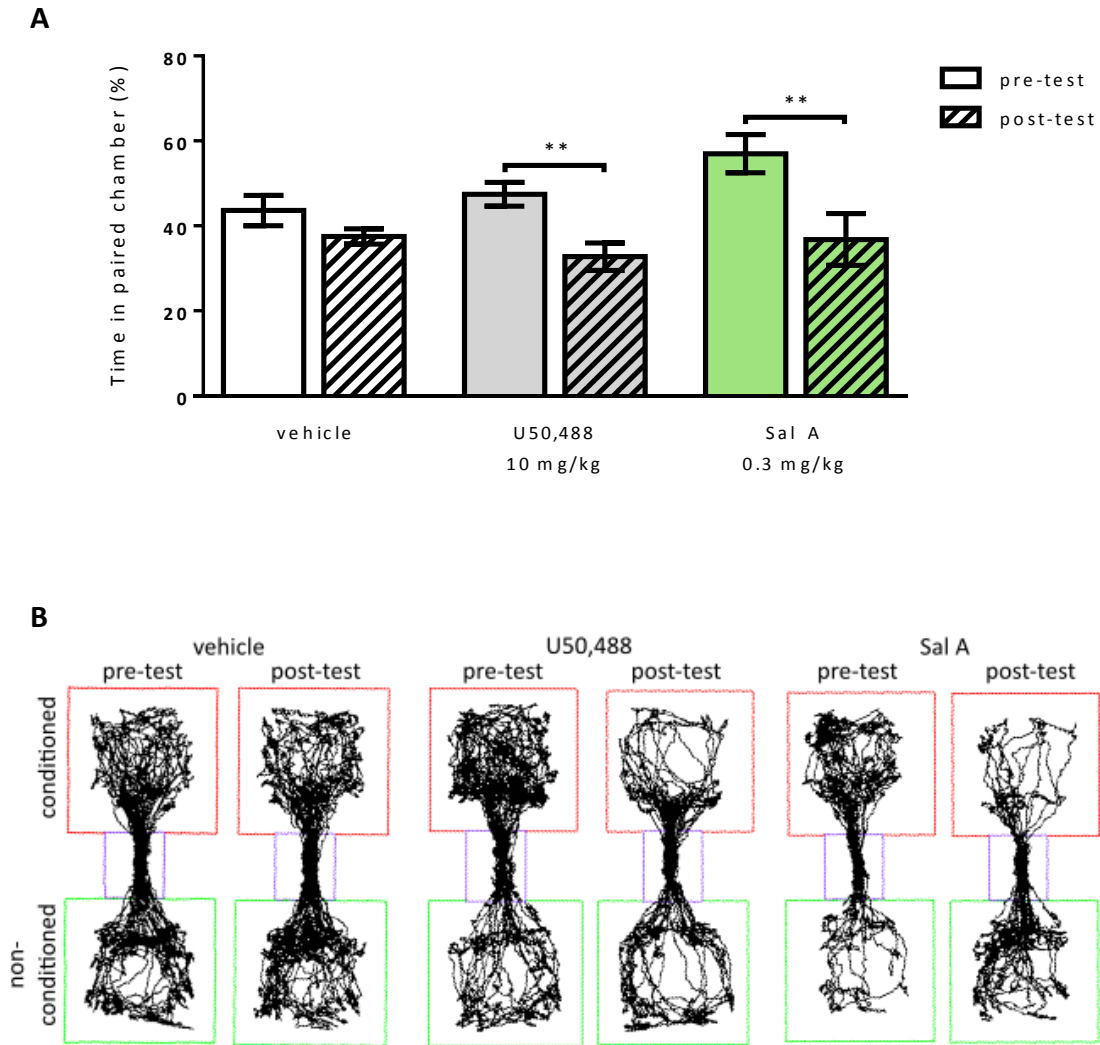
Figure 8   Both U50,488 and Sal A show aversive effects

A: Time spent in the paired compartments in pre- and post-tests. Rats treated with U50,488 and Sal A showed a significant decrease in time spent in the drug paired chamber. Paired Student t-test (n = 9-11).

B: Representative trajectory traces show explorative behaviour in the pre- and post-conditioning tests.

**p≤0.01
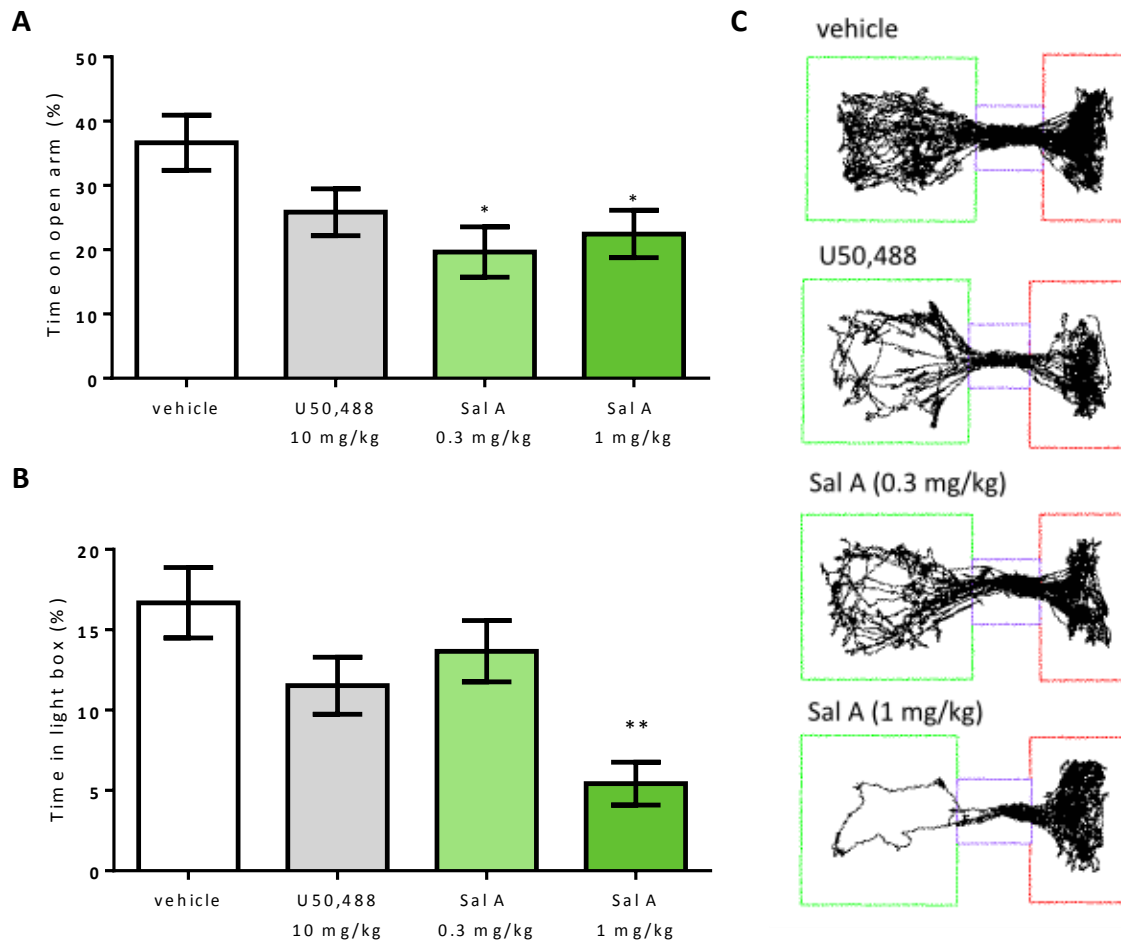
Figure 9   Treatment with Sal A, but not U50,488, results in anxiogenic effects

A: Rats administered Sal A but not U50,488 significantly altered time spent on the open arm when compared with vehicle treatment. One-way ANOVA with Bonferroni's multiple comparisons (n = 14-29).

B: Sal A significantly decreased time spent in the light box at 1 mg/kg but not 0.3 mg/kg or U50,488. Kruskal-Wallis with Dunn's multiple comparisons (n = 12-25).

C: Representative traces show explorative behaviour in the light/dark test.

*p≤0.05, **p≤0.01.

4.2   β-THP Sal B

Since it has been shown that Sal A produces aversion and anxiety in rats the novel compounds were tested to see if they showed reduced side effects.

In the CPA test, rats treated with β-THP Sal B demonstrated a significant reduction in the time they spent in the drug paired chamber ($t_{(6)}$ = 7.655, p = 0.0003; 49 ± 3% vs. 32 ± 5%) (Figure 10A) which was accompanied by a significant increase in the time they spent in the corridor ($t_{(6)}$ = 3.933, p = 0.0077; 23 ± 4% vs. 37 ± 3%) (Figure 12A) consistent with Sal A (0.3 mg/kg).

Both the EPM and light/dark test demonstrated a significant effect of treatment on time spent on the open arm ($F_{(3,71)}$ = 3.923, p = 0.0119) or in the light box ($H_{(3)}$ = 13.57, p = 0.0036).

β-THP Sal B does not show any difference in time spent on the open arm in the EPM test compared with vehicle (33 ± 3% vs. 37 ± 4%, p = ≥ 0.9999). Additionally, there were no significant differences between Sal A (0.3 mg/kg) and β-THP Sal B (20 ± 4% vs. 33 ± 3%, p = 0.2293). Sal A (0.3 mg/kg) demonstrated a significant anxiogenic effect compared with vehicle (20 ± 4% vs. 37 ± 4%, p = 0.0184), however due to the Bonferroni correction for multiple comparisons, Sal A (1 mg/kg) now only shows a non-significant trend towards an anxiogenic effect (22 ± 4%, p = 0.0526) (Figure 11A). In the light/dark test, β-THP Sal B showed a non-significant trend toward an anxiogenic effect in contrast to the results seen in the EPM test (8 ± 2 to 17 ± 2 %, p = 0.1596). No differences were seen between treatment with β-THP Sal B and Sal A (0.3 mg/kg) (8 ± 2 to 14 ± 2 %, p = 0.4855). Sal A (1 mg/kg) consistently showed anxiogenic effects despite the correction for multiple comparisons (5 ± 1 to 17 ± 2 %, p = 0.0034) (Figure 11B).

In summary, β-THP Sal B, like Sal A (0.3 mg/kg), did cause aversive effects. However, unlike Sal A (0.3) it did not show anxiogenic effects in the EPM test. It also did not demonstrate

anxiogenic effects in the light/dark test unlike a higher dose of Sal A (1 mg/kg). However it is important to note that β-THP Sal B did demonstrate a non-significant decrease in time spent in the light box.

A

B
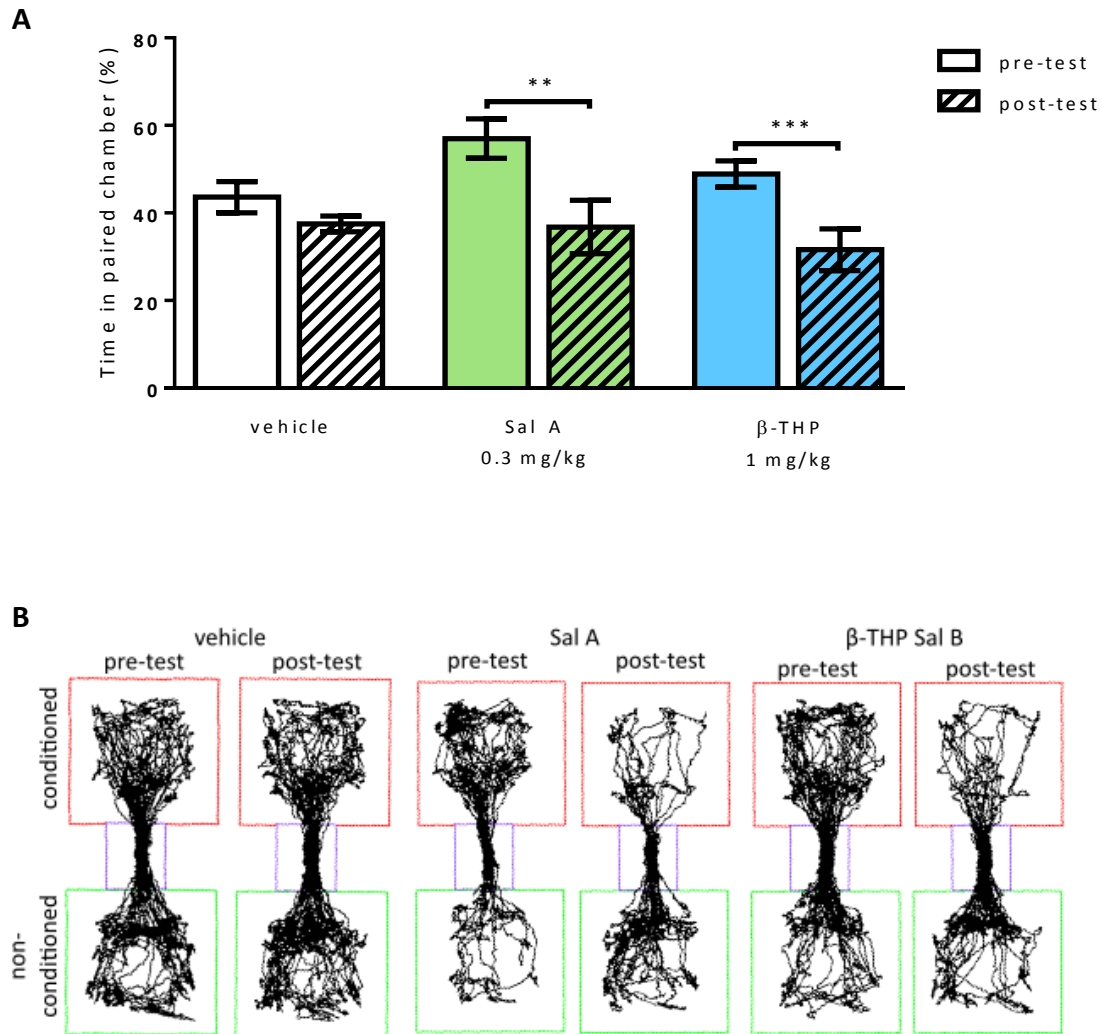
Figure 10   β-THP Sal B shows significant aversive effects

A: Time spent in the paired compartment before and after conditioning. Treatment with β-THP Sal B significantly decreased time spent in the drug paired chamber. Paired Student's t-test (n = 7-11).

B: Representative trajectory traces show explorative behaviour in the pre- and post-conditioning tests.

**p≤0.01, ***p≤0.001
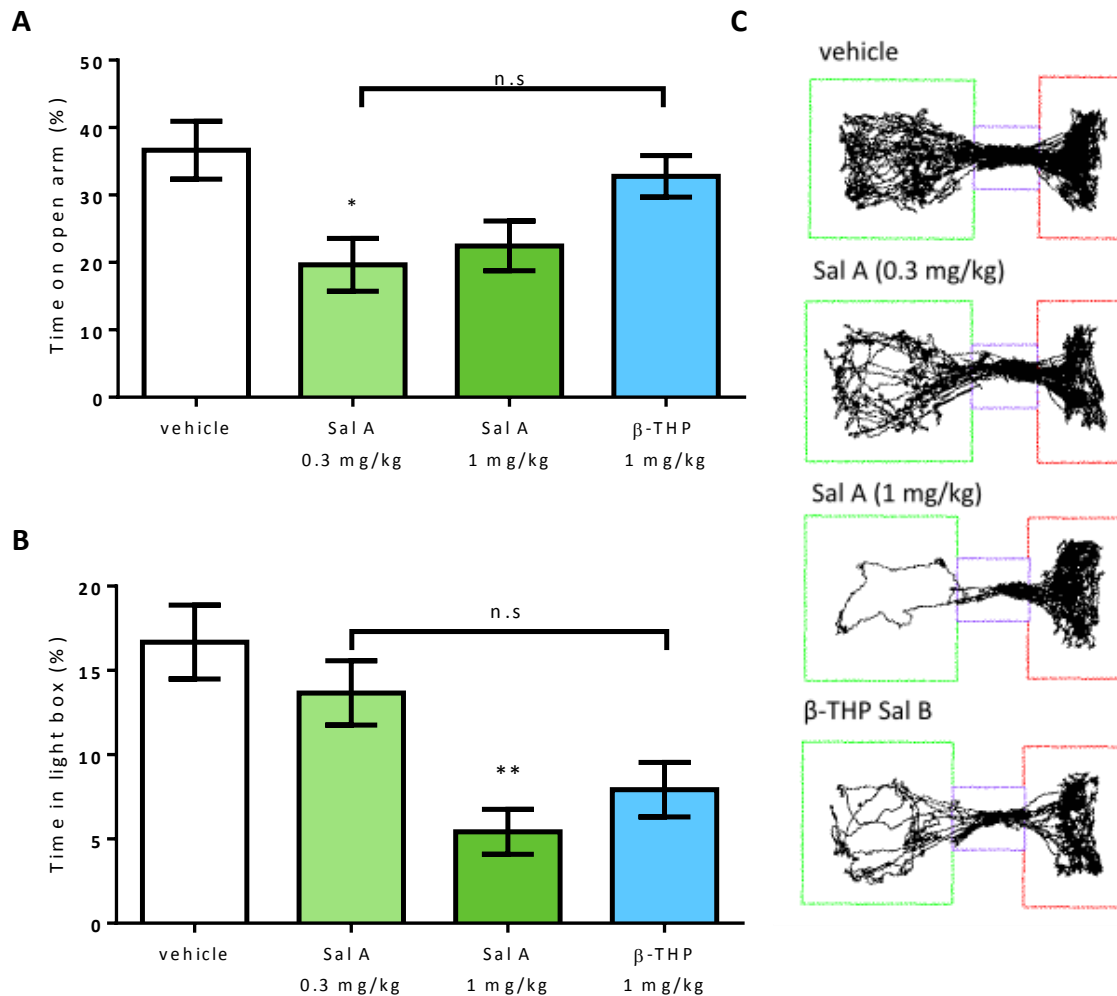
Figure 11   β-THP Sal B does not show anxiogenic effects

A: Rats administered β-THP Sal B did not reduce time spent on the open arm compared with vehicle and is not significantly different to Sal A treatment. One-way ANOVA with Bonferroni's multiple comparisons (n = 14-29).

B: Treatment with β-THP Sal B did not significantly reduce time spent in the light-box compared with vehicle or Sal A (0.3 mg/kg). Kruskal-Wallis with Dunn's multiple comparisons (n = 10-25).

C: Representative traces show explorative behaviour in the light/dark test.

*p≤0.05, **p≤0.01.
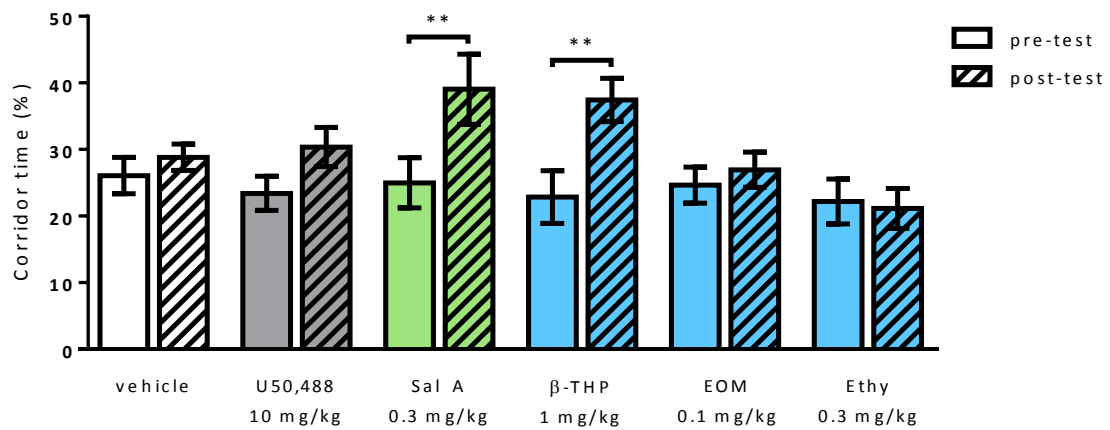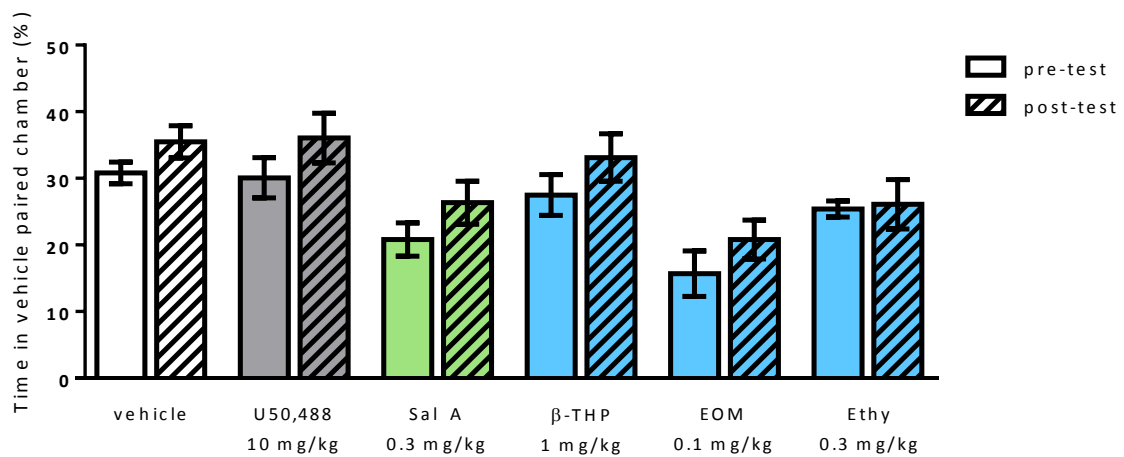
Figure 12   Aversive effects are accompanied by an increase in corridor time, not vehicle paired chamber time

A: KOPr agonist treatments that show aversive effects also show an increase in corridor time. Paired Student's t test (n = 7-11).

B: Aversive KOPr agonist treatment does not significantly increase time spent in the unpaired chamber. Paired Student's t test (n = 7-11).

**p≤0.01.

4.3   EOM Sal B

Conditioning the effects of EOM Sal B to the environment of the rats initially preferred chamber in the CPA test did not result in a significant decrease in time spent in this chamber ($t_{(7)}$ = 1.496, p = 0.1783, 60 ± 5% vs. 52 ± 3%) (Figure 13A). It should also be noted that there was not a significant change in time spent in the corridor ($t_{(7)}$ = 0.7091, p = 0.5012; 25 ± 3 vs. 27 ± 3%) (Figure 12A).

Treatment had a significant effect on time spent in the open arm in the EPM ($F_{(3,69)}$ = 5.185, p = 0.0027) and time spent in the light compartment of the light/dark test ($H_{(3)}$ = 13.36, p = 0.0036).

Rats administered EOM Sal B (43 ± 5%) did not spend less time on the open arm compared with vehicle (37 ± 4%; p = ≥ 0.9999), but did significantly increase open arm time when compared with Sal A (0.3 mg/kg) (20 ± 4%; p = 0.0111) (Figure 14A). Consistent with the effects seen with β-THP Sal B, EOM Sal B showed a non-significant decrease in light box time (9 ± 2% vs. 17 ± 2% for vehicle; p = 0.2361) in the light/dark test. In contrast to the results seen in the EPM test there was no significant difference between Sal A (0.3 mg/kg) treatment and EOM Sal B (14 ± 2% vs. 9 ± 2%; p = 0.7032) (Figure 14B).

To summarise, EOM Sal B did not show aversive or anxiogenic effects. It was shown to have a significant increase in time spent on the open arm compared with Sal A (0.3 mg/kg) in the EPM test, but no statistical difference in the light/dark test.

A

B
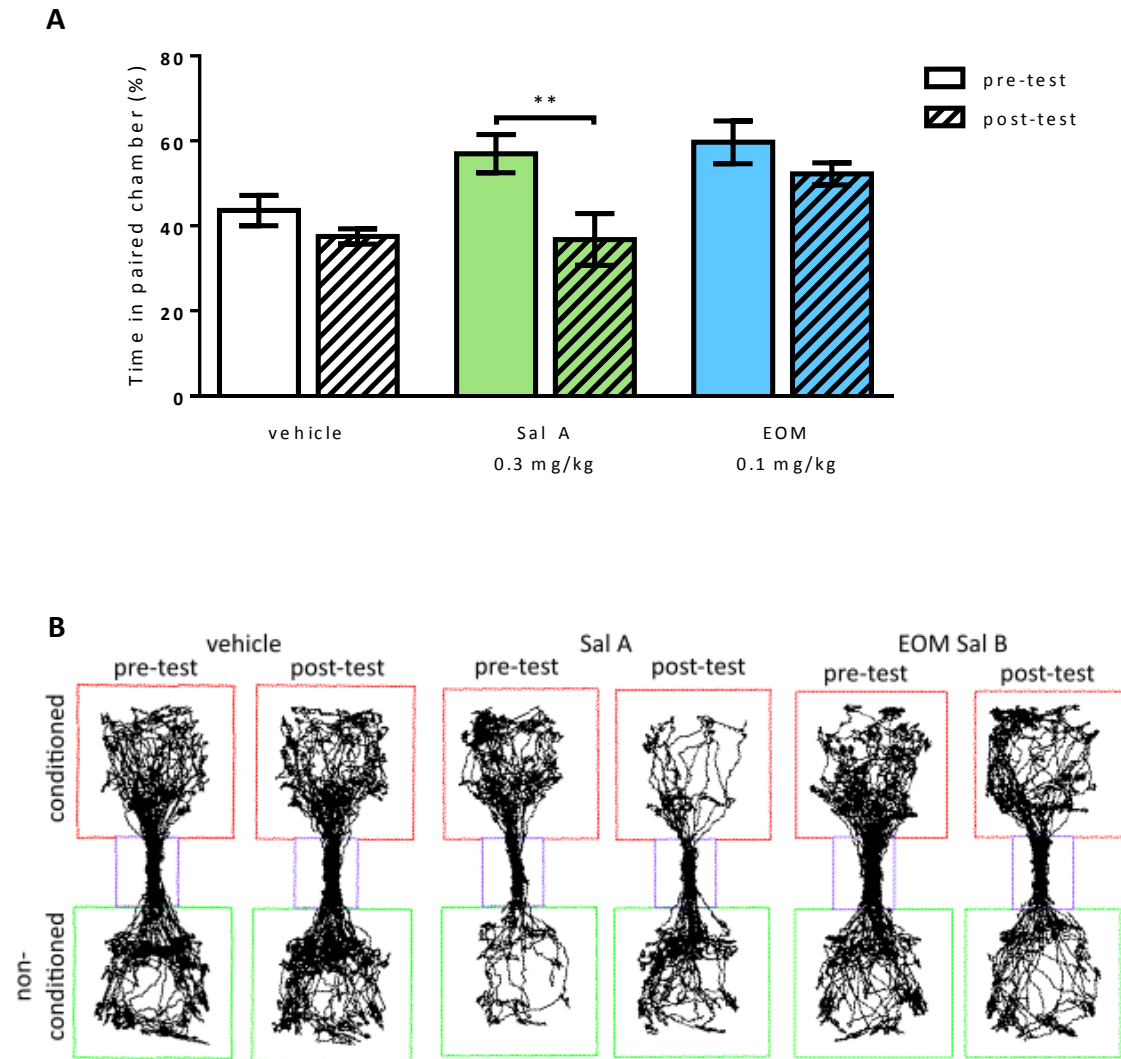
Figure 13   EOM Sal B does not show aversive effects

A: Comparison of the time spent in the initially preferred chamber pre- and post-conditioning. EOM Sal B did not result in a significant decrease in time spent in the initially preferred chamber. Paired Student's t-test (n = 8-11).
**B:** Representative behavioural traces in the pre- and post-conditioning tests.
$**p \leq 0.01$.
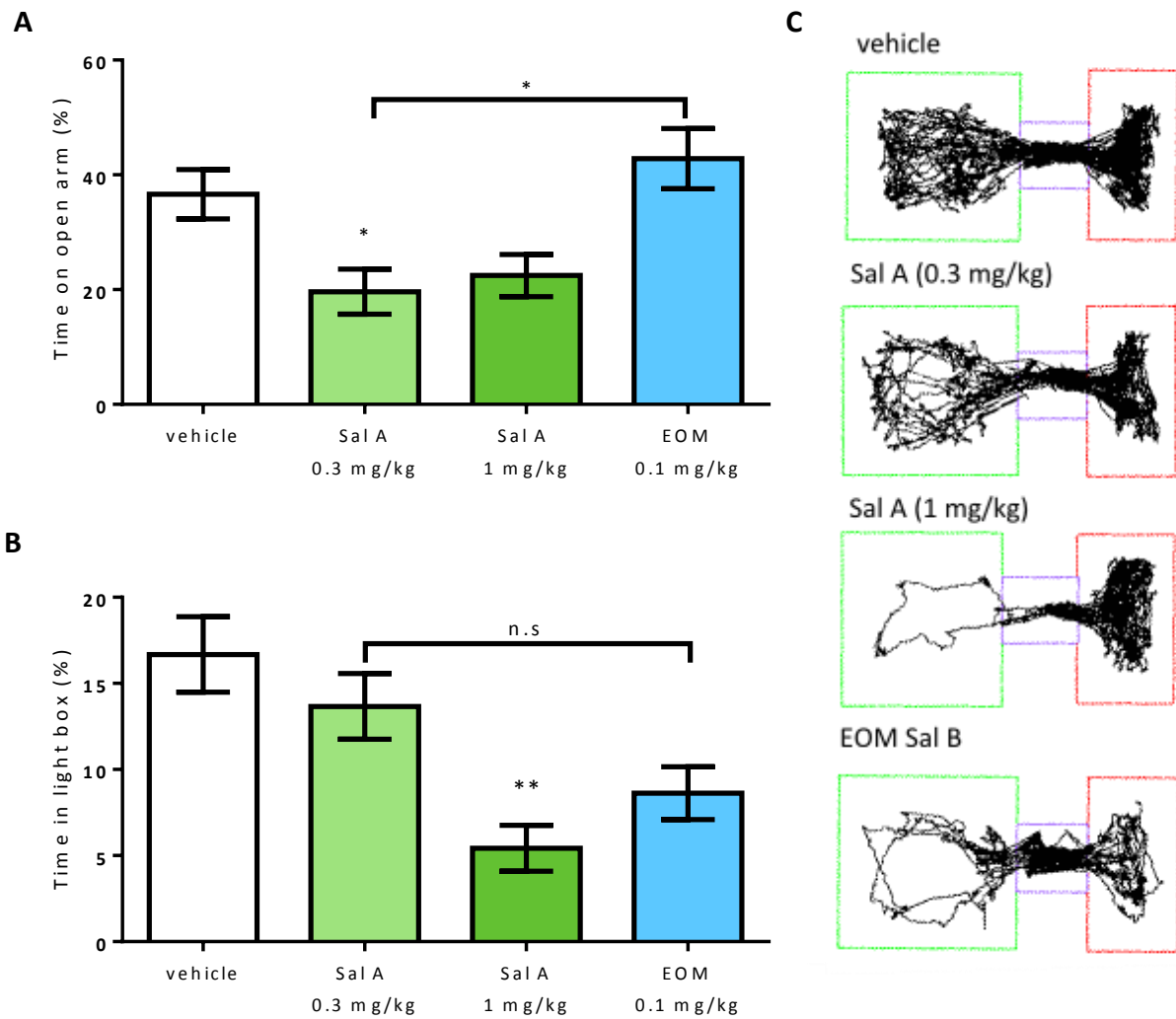
Figure 14   EOM Sal B does not show anxiogenic effects

A: Treatment with EOM Sal B did not decrease time spent on the open arm when compared with vehicle but significantly increased the time spent on the open arm compared with Sal A. One-way ANOVA with Bonferroni's multiple comparisons (n = 12-29).

B: EOM Sal B treatment does not significantly reduce time spent in the light box. There were no significant effects between Sal A and EOM Sal B. Kruskal-Wallis with Dunn's multiple comparisons (n = 10-25).

C: Representative trajectory traces of behaviour in the light/dark test.

*$p \leq 0.05$, **$p \leq 0.01$.

4.4   Mesyl Sal B

Mesyl Sal B was tested only in the EPM and light/dark tests as it had already been shown to have no significant aversive effects at 0.3 mg/kg, i.p (Kivell lab, unpublished data).

Post-hoc testing was performed on data collected from the EPM and light/dark tests as there was shown to be a significant relationship between drug treatment and time spent in the aversive parts of the arena (EPM: $F_{(3, 75)}$ = 3.708, p = 0.0152; light/dark: $H_{(3)}$ = 11.96, p = 0.0075).

In the EPM test Mesyl Sal B did not show a significant effect when compared with either vehicle (30 ± 3% vs. 37 ± 4%; p = ≥ 0.9999), or Sal A (0.3 mg/kg) (30 ± 3% vs. 20 ± 4 %; p = 0.4012) (Figure 15A). Corroborative results are observed in the light/dark test where Mesyl Sal B (17 ± 3) had no difference in light box time compared with vehicle (17 ± 2) nor Sal A (0.3 mg/kg) (14 ± 2; p = ≥ 0.9999) (Figure 15B).

Mesyl Sal B was shown to not have anxiogenic effects compared with vehicle treatment, and was not shown to be statistically different to Sal A (0.3 mg/kg) in these anxiety tests.
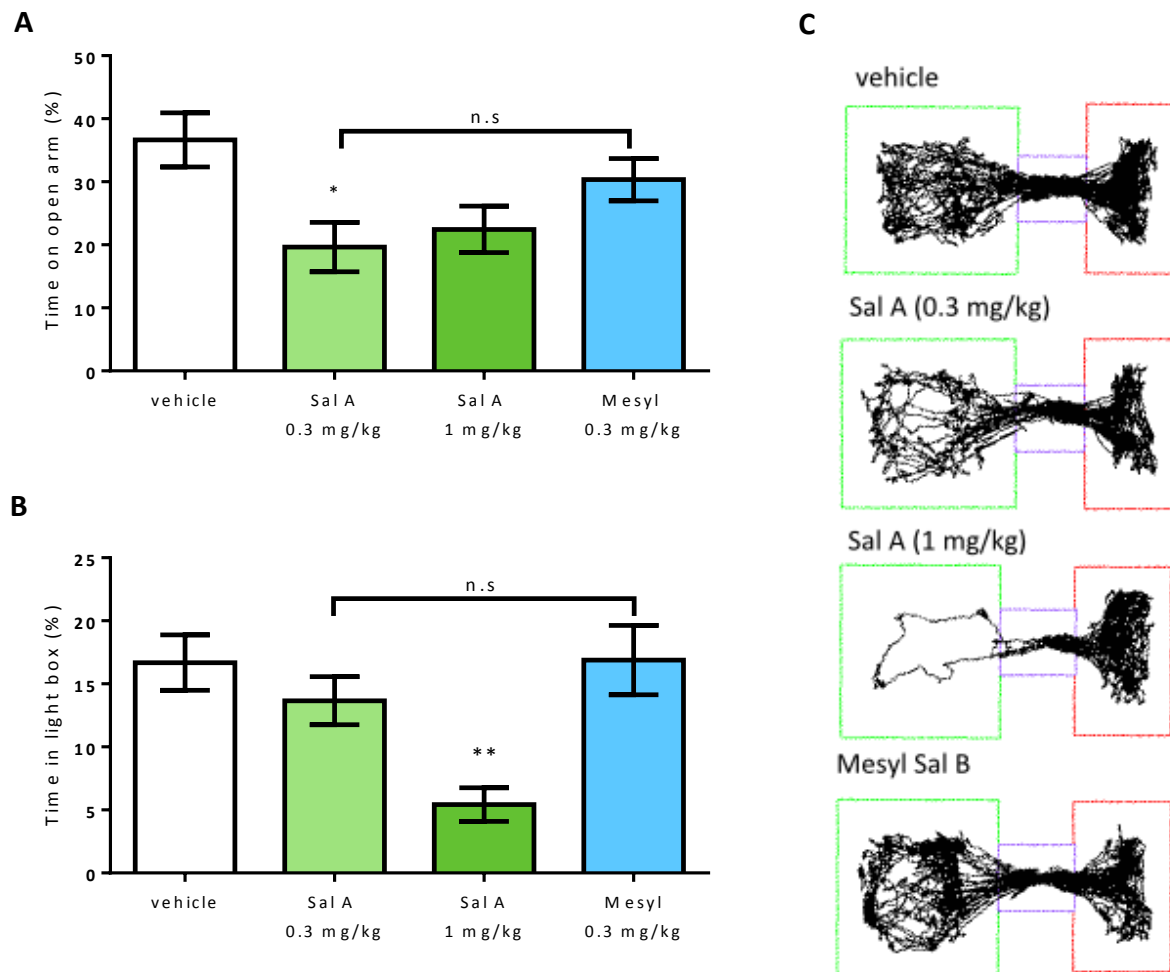
Figure 15   Mesyl Sal B does not show anxiogenic side effects

A: Administration of Mesyl Sal B did not reduce time spent in the open arm compared with vehicle and is not significantly different to Sal A (0.3 mg/kg) treatment. One-way ANOVA with Bonferroni's multiple comparisons (n = 15-29).

B: Treatment with Mesyl Sal B did not result in significant differences with time spent in the light box compared with vehicle or Sal A (0.3 mg/kg). Kruskal-Wallis with Dunn's multiple comparisons (n = 12-25).

C: Representative trajectory traces of explorative behaviour in the light/dark test.

$p \geq 0.05$, *$p \leq 0.05$, **$p \leq 0.01$.

4.5   Ethy Sal A

In the CPA paradigm Ethy Sal A did not cause a decrease in time spent in the drug-paired compartment ($t_{(7)}$ = 0.05355, p = 0.9588; 52 ± 3% to 53 ± 5%) (Figure 16A). This was accompanied with no change in corridor time ($t_{(7)}$ = 0.2331, p = 0.8223; 22 ± 3% to 21 ± 3%) (Figure 12A).

In both the EPM and light/dark tests there was a significant effect of treatment on time spent on the open arm ($F_{(3,69)}$ = 3.842, p = 0.0132) or time spent in the light box ($H_{(3)}$ = 11.61, p = 0.0088).

Ethy Sal A did not show a significant difference when compared with vehicle (39 ± 7% vs. 37 ± 4%; p = ≥ 0.9999) in the EPM test, however, there is a non-significant trend towards an increase in open arm time when compared with Sal A (0.3 mg/kg) (39 ± 7% vs. 20 ± 4%; p = 0.0704) (Figure 17A). In addition, no significant differences were observed with comparisons between vehicle (17 ± 2%), Sal A (0.3 mg/kg) (14 ± 2%) and Ethy Sal A (12 ± 2%; p = ≥ 0.9999) in the light/dark test (Figure 17B).

In conclusion, unlike Sal A, Ethy Sal A did not show aversive nor anxiogenic effects. However, Ethy Sal A only demonstrated a trend towards being significantly different to Sal A (0.3 mg/kg) in the EPM with no differences observed in the light/dark test.

A
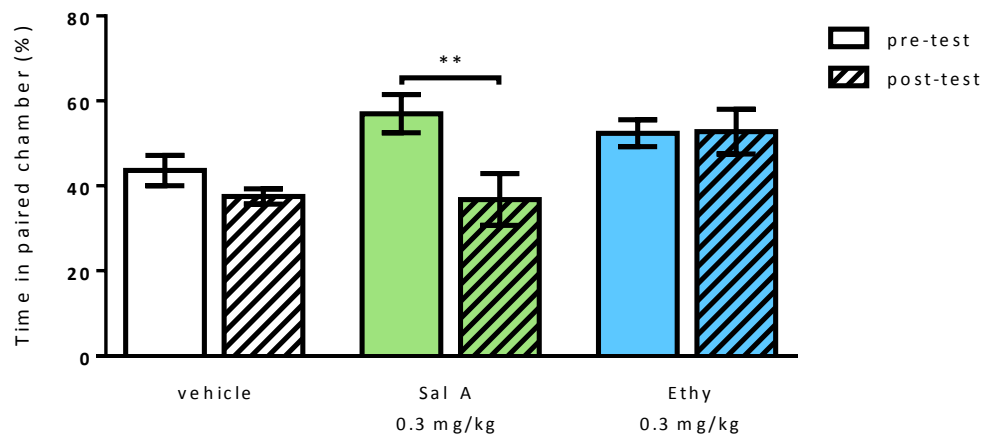
B
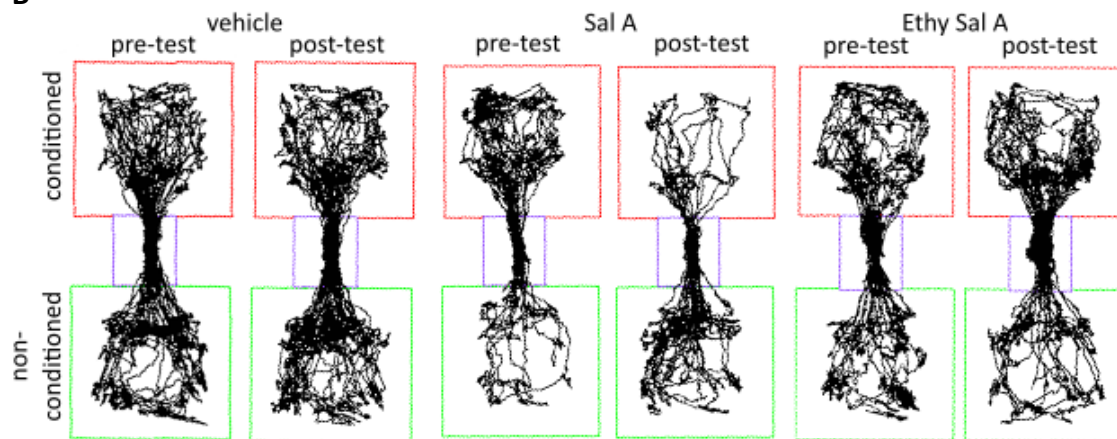
Figure 16   Ethy Sal A does not show aversive effects

A: Ethy Sal A did not significantly reduce the time spent in the preferred chamber after conditioning. Paired Student's t-test (n = 8-11).
B: Representative trajectory traces of behaviour in the pre- and post-conditioning tests.
**p≤0.01.
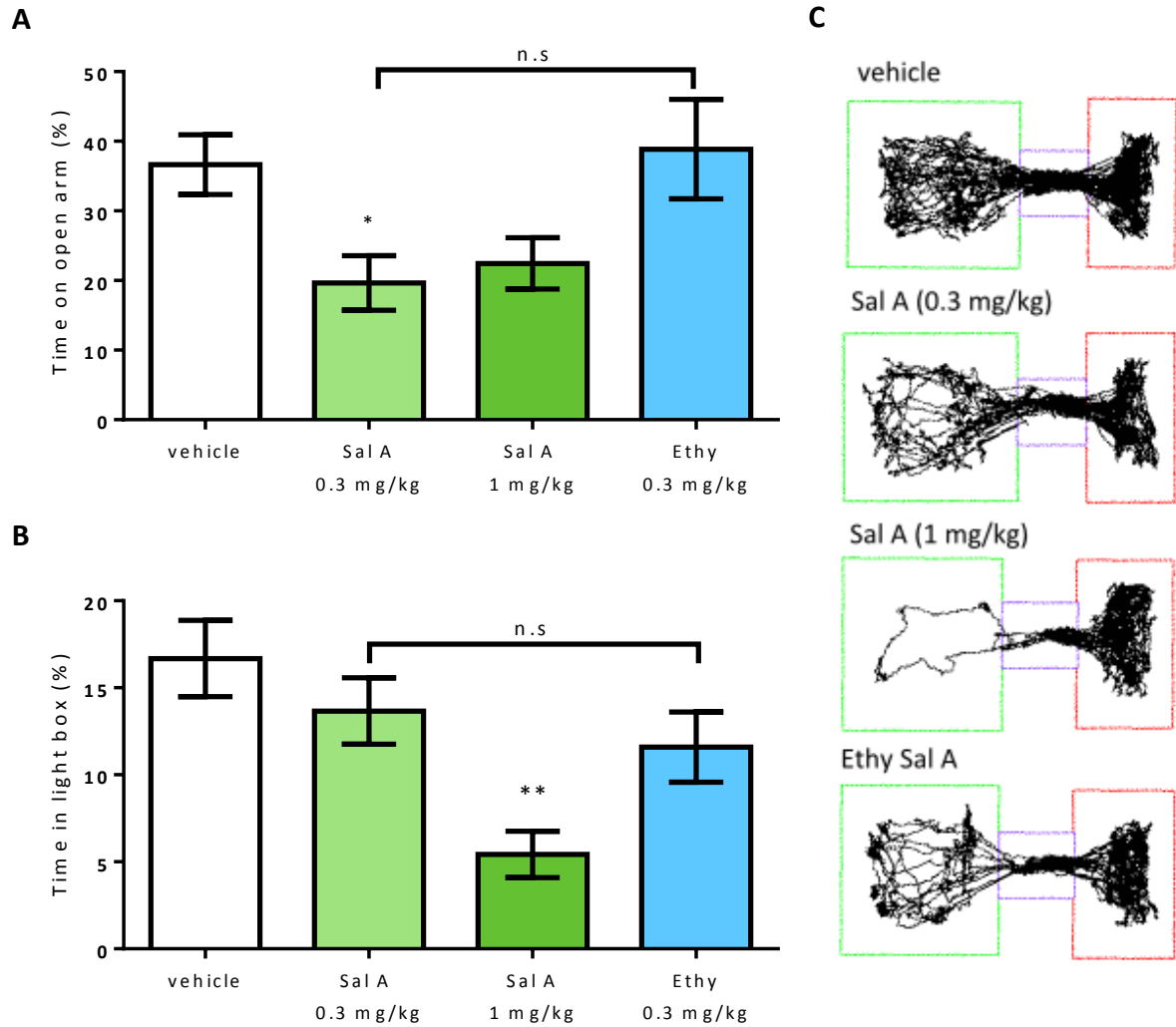
Figure 17   Ethy Sal A does not show anxiogenic effects

A: Administration of Ethy Sal A did not result in significant differences in open arm time when compared with either vehicle or Sal A (0.3 mg/kg). One-way ANOVA with Bonferroni's multiple comparisons (n = 12-29).

B: Ethy Sal A did not cause a significant decrease in light box time compared with vehicle or Sal A. Kruskal-Wallis with Dunn's multiple comparisons (n = 10-25).

C: Representative traces of behaviour in the light/dark test.

*p≤0.05, **p≤0.01.

4.6   Anxiogenic vs. sedative effects

As both the light/dark and EPM procedures utilise an approach/avoidance conflict they rely on normal exploratory behaviour, therefore sedative effects could be a confounding factor (Bouwknecht & Paylor, 2008). To examine the sedative effects of these compounds we counted the number of closed arm entries in the plus maze and the total distance travelled was measured in the light/dark test.

There was no relationship observed between treatment and closed arm entries in the EPM ($F_{(7, 124)}$ = 1.544, p = 0.1585) (Figure 18A). This indicates that none of the treatments resulted in a decrease in active behaviour and the significant anxiogenic effects are most likely not due to sedative effects.

Treatment did have a significant effect on total distance travelled in the light/dark test ($H_{(7)}$ = 18, p = 0.012) (Figure 18B). Administration of U50,488 (226 ± 19 cm), Sal A (0.3 mg/kg) (231 ± 21 cm), EOM Sal B (231 ± 17 cm) and Ethy Sal A (280 ± 36 cm) did not result in changes to distance travelled compared with vehicle (237 ± 14 cm; p = ≥ 0.9999). However, a non-significant decrease was seen with Sal A (1 mg/kg) treatment (187 ± 19 cm; p = 0.1928).

A

B
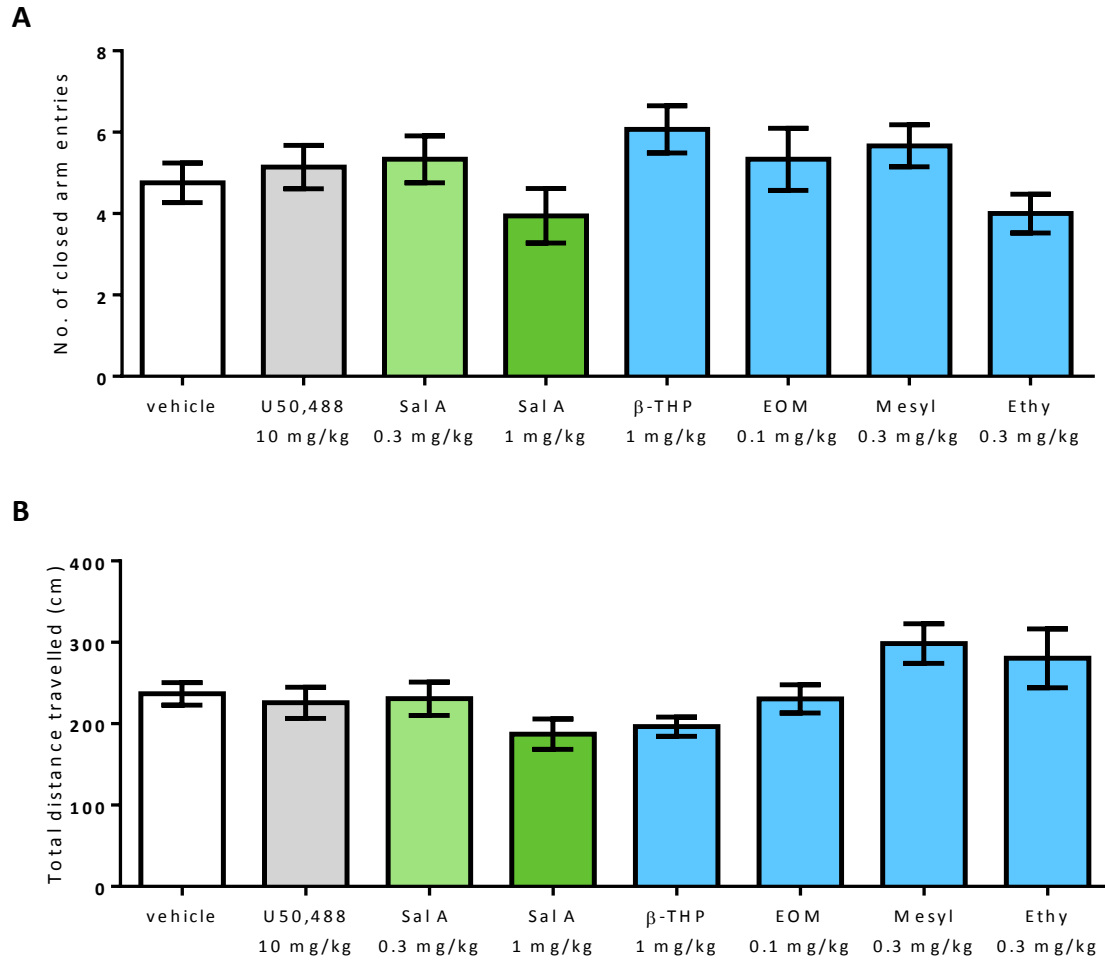
Figure 18   Anxiogenic effects were not due to sedation

KOPr agonist treatment does not significantly alter time spent on the open arm in the EPM test (**A;** One-way ANOVA, n = 12-29) or total distance travelled in the light/dark test (**B;** Kruskal-Wallis test, n = 10-25.).

4.7 Open field

Due to the light/dark and EPM tests showing differing effects with Sal A (0.3 and 1 mg/kg), EOM Sal B and β-THP Sal B, the open field test was also utilised to further evaluate anxiety-like behaviours. Initial testing was performed with vehicle and Sal A (0.3 and 1 mg/kg) (Figure 19A). Animals treated with vehicle spent only 5.9 ± 1.8% in the centre of the apparatus, which is too low to be able to accurately detect any increase in anxiety-like behaviour following a drug treatment. Despite such low values for time spent the centre of the arena, this didn't appear to be due to a decrease in general exploratory behaviour (Figure 19B).
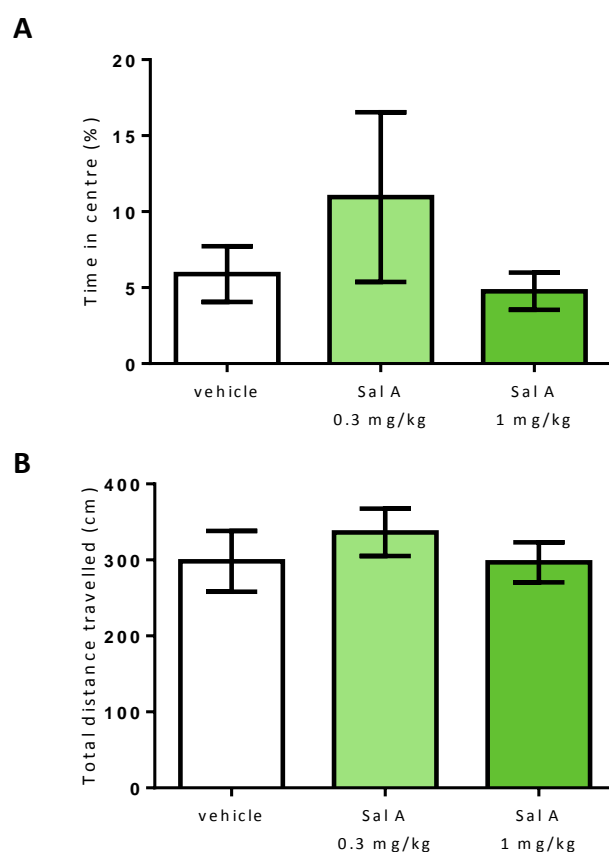
Figure 19   *Time spent in the centre not sufficient for anxiogenic effects to be detected*

Animals treated with vehicle in the open field test did not sufficiently explore the centre of the arena for a difference to be detectable with an anxiogenic drug treatment (**A,** n = 4-5). This was not due to sedative effects (**B**, n = 4-5).

4.8   Validation of pCREB and CREB antibodies

Activation of the KOPr can result in the phosphorylation of CREB (Bruchas et al., 2007, 2008) and subsequent expression of dynorphin and CRF (Cole et al., 1995; Itoi et al., 1996), activating the endogenous stress systems resulting in dysphoria, aversion, and anxiety (Bruchas et al., 2009; Cole et al., 1995; Funada et al., 1993; Pliakas et al., 2001). It was hypothesised that compounds that did not demonstrate aversive and anxiogenic effects would not show a significant difference in pCREB levels in the NAc, dStr and PFC.

Cell culture manipulation of pCREB was used to optimise a Western blotting procedure before beginning experimentation in tissue samples. Forskolin is a potent stimulator of cAMP pathways resulting in activation of PKA, which in turn phosphorylates CREB. HEK-293 cells were treated with forskolin (30 µM) for 20 mins to determine the efficacy of the Phospho-CREB (Ser133) (1B6) antibody; no signal was detected for pCREB, whereas a signal was detected for total CREB and the α-tubulin loading control (Figure 20).

A replacement Phospho-CREB (Ser133) (87G3) antibody was furnished by the supplier as the Phospho-CREB (1B6) antibody was deemed defective. Treatment of serum-starved HEK-293 cells with the same concentration of forskolin and Western blotting protocol resulted in a significant increase in pCREB when compared with untreated controls ($t(2) = 4.947$, $p = 0.0385$) (Figure 21).
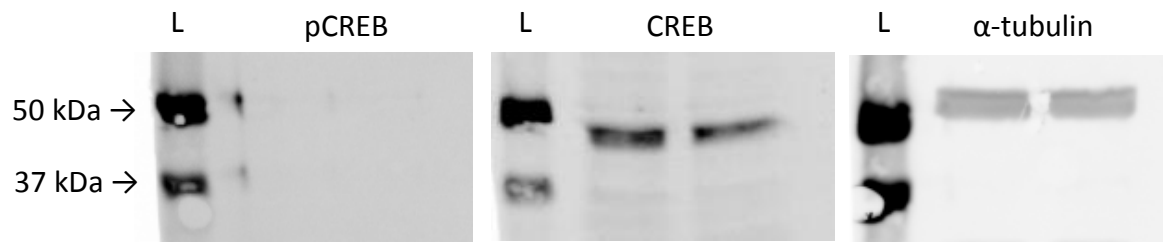
Figure 20   No signal was detected using Phospho-CREB (Ser133) (1B6)

Forskolin treated HEK-293 cells probed for pCREB (1B6 antibody), CREB (43 kDa) and α-tubulin (50 kDa) loading control. No signal was detected for pCREB. Total protein loaded per well = 20 µg, L: molecular weight marker.
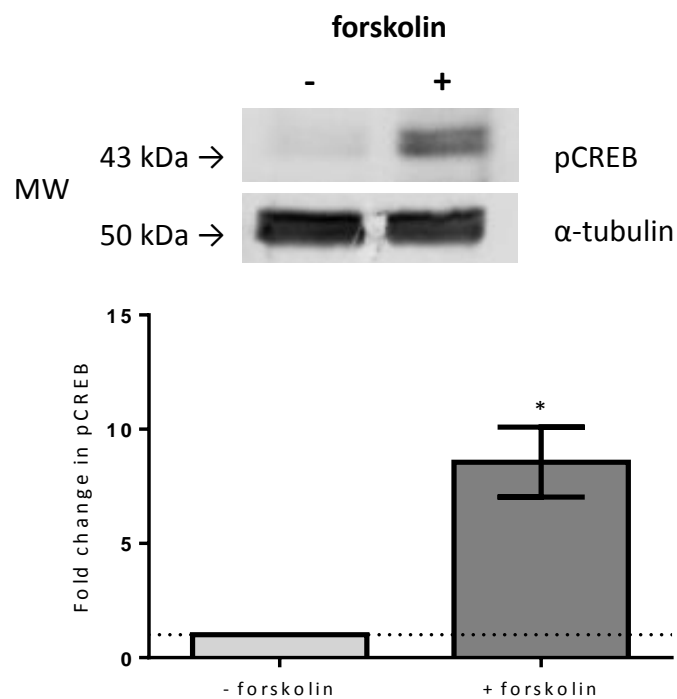
Figure 21   Forskolin phosphorylates CREB in vitro

Activation of CREB using forskolin treatment. To confirm the ability of the pCREB antibody to produce a signal, serum-starved HEK-293 cells were treated with forskolin (30 µM) for 20 mins. This significantly increased the amount of pCREB protein compared with untreated controls. Student's t-test, n = 2.

As a signal was detected for pCREB and CREB *in vitro* we attempted to confirm the same in tissue samples before commencing experimentation with KOPr agonists. Initially this was attempted in drug naïve animals as pCREB and CREB are present at basal levels in the brain (Turgeon et al., 1997), but a sufficient signal was not detectable for either CREB or pCREB. It has been shown that an acute injection of cocaine (30 mg/k, i.p) is sufficient to significantly increase phosphorylation of CREB from 5, 15, and 30 mins after cocaine administration in the NAc in male Sprague-Dawley rats (Nazarian et al., 2009). However, we were still unable to obtain a signal with CREB or pCREB (Figure 22). Hypothesising that perhaps insufficient homogenisation of the sample was the cause, samples were subjected to sonication. Unfortunately, this also did not result in a signal for the proteins of interest (Figure 24; Appendix 3). Further experimentation was not carried out due to time constraints.
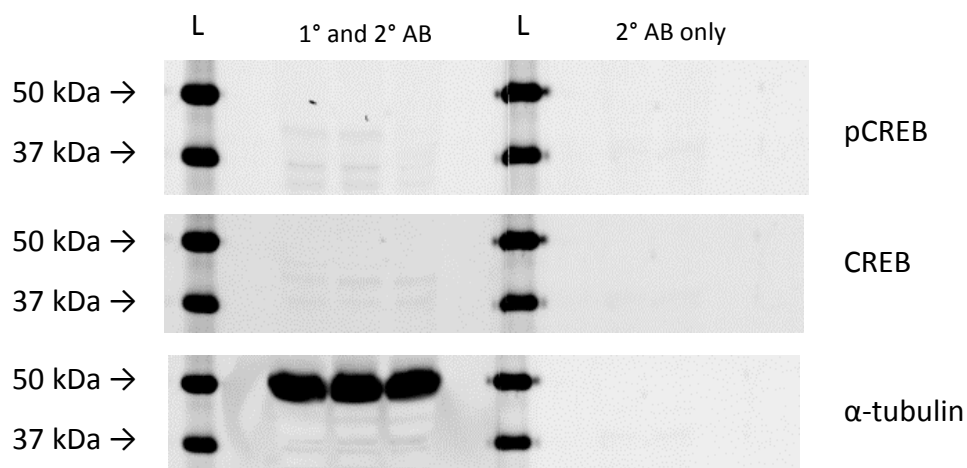
Figure 22   *No signal detected with ex vivo sample*

Membranes immunloabeled with Phospho-CREB (87G3) antibody, CREB antibody, and α-tubulin (50 kDa) loading control. All membranes were tested for non-specific binding of the secondary antibody. Total protein loaded per well = 100 µg.

5. Discussion

Traditional KOPr agonists such as U69,593 (0.3 mg/kg, i.p) and U50,488 (30 mg/kg, i.p) have been shown to be effective at decreasing cocaine-primed reinstatement of drug-seeking in preclinical animal models (Morani et al., 2009; Schenk et al., 1999) and so hold promise for the development of anti-addiction pharmacotherapies. Unfortunately, U69,593 and U50,488 have demonstrated many side-effects including aversion (Chefer et al., 2013; Mucha & Herz, 1985; Suzuki et al., 1992; Tejeda et al., 2013), depression (Mague et al., 2003), sedation (Mague et al., 2003; Paris et al., 2011), and anxiety (Valdez & Harshberger, 2012). Sal A is a novel KOPr agonist with proven anti-cocaine effects at 0.3 mg/kg, i.p (Morani et al., 2009, 2012), with anti-depressant and anxiolytic effects at 10-1000 µg/kg, s.c and 0.1-160 µg/kg, s.c respectively (Braida et al., 2009), with no sedation observed at 0.125-2 mg/kg, i.p (Carlezon et al., 2006; Morani et al., 2012). However, its undesirable pharmacokinetic profile limits its therapeutic utility (Valdés, 1994). Recently, work has been undertaken to produce analogues of Sal A which have an increased duration of action and a more favourable side-effects profile. Therefore this study aimed to screen structural analogues of Sal A for their aversive and anxiogenic effects at the minimum effective dose that significantly decreased cocaine-primed reinstatement of drug-seeking behaviour (Kivell lab, unpublished data; Morani et al., 2009; Prevatt-Smith et al., 2011; Riley et al., 2014; Simonson et al., 2014). The traditional agonist U50,488 was tested at 10 mg/kg, i.p, a dose that previously decreased cocaine self-administration in female Sprague-Dawley rats (Glick et al., 1995).

Here we present evidence that several structural analogues of Sal A do not produce these undesirable side-effects, providing promising candidates for anti-addiction pharmacotherapy development.

5.1 Aversion

Using a biased, three-chamber CPA paradigm we show that U50,488 (10 mg/kg, i.p) and Sal A (0.3 mg/kg, i.p) demonstrated significant aversion to the drug paired stimuli. In support of this, U50,488 (1 and 2 mg/kg, s.c and 10 mg/kg, i.p) demonstrated significant aversive effects in male Sprague-Dawley rats (Mucha & Herz, 1985; Suzuki et al., 1992), whereas Sal A showed significant aversive effects in male Wistar rats at 160 µg/kg, i.p (Braida et al., 2008) and at 0.3 and 1 mg/kg, i.p in male Sprague-Dawley rats (Sufka et al., 2014) using the CPA test. The same effect has been observed in murine models; U50,488 (2 mg/kg, s.c) caused aversive effects in male mu opioid receptor knockout and wild type mice (Skoubis et al., 2001) and Sal A (1 and 3.2 mg/kg, i.p) resulted in significant CPA in C57BL/6J mice (Zhang et al., 2005).

We hypothesised that novel analogues of Sal A would not show aversion. In support of this hypothesis the Sal A analogue Mesyl Sal B showed no aversive effects in both CPA and CTA tests at 0.3 mg/kg (Kivell lab, unpublished data). Here we show EOM Sal B and Ethy Sal A also had no aversive effects, however β-THP Sal B showed significant aversion at a similar level to Sal A (difference between pre- and post-test time for β-THP Sal B -17 ± 2% vs. Sal A -20 ± 5%).

U50,488, Sal A and β-THP Sal B all showed a significant decrease in time spent in the drug paired chamber. Interestingly, these compounds also showed an increase in time spent in the neutral corridor, an effect that was not seen with the vehicle paired chamber (Figure 12). This may be due to the natural tendency for rats to prefer novel, confined environments. This is supported by a study conducted by Morales et al. (2007) where the effects of U50,488 (1 mg/kg, i.p) were conditioned in Sprague-Dawley rats using both a two- and three-chambered compartment CPA apparatus design. It was shown that U50,488 place aversion is more prominent with the use of a two-chambered compartment apparatus, as there was a large

preference of the rats to the connecting chamber over the saline and U50,488 paired chambers in the three-chambered compartment design after conditioning (Morales et al., 2007). This appears to be unique to aversive drugs, since with the rewarding drug morphine (5 mg/kg, i.p), CPP was similar between the two apparatuses (Morales et al., 2007).

Morani et al. (2012) has previously evaluated the effects of Sal A (0.3 mg/kg, i.p) on CTA, another preclinical test commonly employed to screen for aversive effects. They found that when Sal A was administered after a novel saccharin tasting session, the subsequent time rats were presented with the solution there was no decrease in the amount consumed (Morani et al., 2012). This is in contrast to Sufka et al. (2014) and the present study where Sal A (0.3 mg/kg, i.p) resulted in a decrease in time spent in the environment where the drug was administered. While these aversion tests measure the effects of a drug on aversive behaviour by conditioning its effects to either a novel taste or place, differences between these tests have been noted previously. Gore-Langton et al. (2015) showed that the emetic lithium chloride caused a CTA but not CPA, and drugs of abuse such as cocaine (Isaac et al., 1989) and morphine (Simpson & Riley, 2005) produced both a CPP and CTA when administered at the same dose and route of administration in rats. Furthermore, the CTA procedure includes a water deprivation step. This causes stress to the animal, possibly resulting in a confounding factor (Anderson et al., 2013). Therefore, when testing compounds that are associated with the stress response, such as KOPr agonists, CPA is the most appropriate test to use.

Unlike the parent compound, the Sal A analogues EOM Sal B and Ethy Sal A show improvements as they do not produce aversive effects in the CPA paradigm. This finding is significant as the majority of KOPr agonists show aversive effects at the dose that decreases drug-seeking behaviour in the cocaine drug-prime reinstatement model (Table 3).

5.2   Anxiety

5.2.1   Anxiogenic effects

In this study anxiogenic effects were tested following KOPr agonist administration using the EPM and light/dark tests. The traditional agonist U50,488 (10 mg/kg, i.p) did not produce significant anxiety-like behaviours in either test, however there was a 30% decrease in time spent on the open arm and a 32% decrease in time spent in the light box compared with vehicle, suggesting a trend toward anxiogenic effects. Sal A was administered at 0.3 and 1 mg/kg, i.p; the low dose only showed anxiogenic effects in the EPM model, and the high dose exhibited a 68% decrease in time spent in the light box and a 47% decrease in time spent on the open arm. Previously Braida et al. (2009) showed that Sal A (0.1-160 µg/kg, s.c) produced anxiolytic effects in the EPM test using Sprague-Dawley rats. Combined with the results presented in this study, Sal A demonstrates anxiolytic effects at doses lower than that which has been shown to attenuate reinstatement behaviour (Braida et al., 2009), and consistent anxiogenic effects at a dose higher than 0.3 mg/kg. β-THP Sal B (1 mg/kg, i.p), EOM-Sal B (0.1 mg/kg, i.p) and Ethy Sal A (0.3 mg/kg, i.p) demonstrated a non-significant decrease in time spent in the aversive area of the light/dark test (32-53% decrease compared with vehicle), however no change in open arm time in the EPM test. Mesyl Sal B (0.3 mg/kg, i.p) was the only novel analogue to consistently show no anxiogenic behaviour in either model.

It has previously been shown that treatment with U50,488 (10 mg/kg, i.p) demonstrates anxiety-like behaviour in the EPM test in Wistar rats (Gillett et al., 2013; Valdez & Harshberger, 2012). To the best of our knowledge the effects of U50,488 on behaviour in the light/dark has not been reported. The difference in these results is most likely due to strain differences as it is known that there are variances in rat strains between the EPM and circular

light/dark tests. A study by van der Staay et al. (2009) compared Brown Norway, Lewis, Fischer 344, and Wistar Kyoto rats and showed that Fischer rats spent more time on the open arm in the EPM compared to the other three rat strains. In addition, Wistar Kyoto rats and Lewis rats spent more time in the dark compartment in the circular light/dark test than the Fischer and Brown Norway rats (van der Staay et al., 2009). It has also been suggested that Wistar rats are more sensitive to the aversive properties of stress than other rats strains, and are therefore more likely to demonstrate anxiety-like behaviour (Carr & Lucki, 2010). The results presented demonstrate that preclinical behavioural testing should be performed in multiple strains to obtain a comprehensive side-effects profile.

5.2.2   Paradigm considerations

In this study Sal A (0.3 mg/kg) showed opposite effects between the EPM and light/dark tests, and all the novel compounds, excluding Mesyl Sal B (0.3 mg/kg), showed a decrease in time spent in the light box but no change in open arm behaviour. However, it is known that results can differ between anxiety tests with factors such as age, sex, hereditary effects, and experimental model. For example, a single dose of phencyclidine (PCP) produces anxiolytic effects in the light/dark and EPM tests in Sprague-Dawley adult females, and yet anxiogenic effects in adult male rats. In addition, PCP treatment in adolescent animals had anxiogenic effects in the EPM but anxiolytic effects in the light/dark test (Turgeon et al., 2011). It has also been shown that maternal licking and grooming behaviour can affect the result of anxiety tests; juvenile animals that experience high levels of grooming spent more time on the open arm of the plus maze compared with those that experienced low levels. However, there was no difference between the two groups in the open field test of anxiety (Masís-Calvo et al., 2013). With the present study age, strain, and sex were consistent between the light/dark and EPM models. As we had used the same individual rats for these procedures, the

difference is unlikely due to hereditary effects. The discrepancies observed here are therefore most likely due to the EPM and light/dark tests examining differing aspects of anxiety with partially overlapping constructs (Ramos, 2008). This difference may be explored further with the use of the elevated T-maze. This test measures, using short, successive trials, the level of inhibitory avoidance (which is related to general anxiety disorder) and one-way escape (which is related to panic disorder) (McNaughton & Zangrossi Jr., 2008). The EPM and light/dark tests are mixed tests in that the animals can display a range of behaviour as they are free to explore the entire apparatus. This highlights the need for multiple tests to examine the full range of behavioural effects with complex emotions such as anxiety.

Therefore, to further examine the effects of KOPr agonists that showed differing results in the EPM and light/dark tests, the open field test of anxiety was utilised. However, it is difficult if not impossible to detect an increase in anxiety when an animal is already displaying high levels of anxiety behaviour (Bouwknecht & Paylor, 2008). In the open field test we examined the time spent in the centre vs. the periphery of the arena. Unfortunately, we experienced a floor effect whereby the vehicle control rats spent little time in the centre of the arena, therefore a decrease in time spent in the centre with anxiogenic compounds could not be observed. Both anxiogenic and anxiolytic effects can be examined by measuring time spent in the safest part of the open field (the corners) whereas only anxiolytic effects can be seen when measuring the most aversive part of the open field (the centre) (van der Staay et al., 2009). Therefore, when testing KOPr agonists which have previously demonstrated anxiogenic effects, it would be more accurate to examine time spent in the corners of the open field as opposed to the centre. This could not be performed in the current study due to the limitations of the recording software.

5.2.3   Sedative effects

As the anxiety tests utilised in this study all require normal exploratory behaviour, locomotion is an important factor that may confound the measures of anxiety. The anxiogenic effects noted for each compound tested were unlikely due to sedation as no treatment was significantly different to vehicle treated rats for either closed arm entries in the EPM nor total distance travelled in the light/dark test. This is supported by results seen in locomotor activity tests in rats where Sal A and all its novel structural analogues have not shown a reduction in activity at the doses tested here (Carlezon et al., 2006; Morani et al., 2012; Riley et al., 2014; Simonson et al., 2014, Kivell Lab, unpublished data) (Table 3). It should be noted that previously U50,488 (10 mg/kg, i.p) has been shown to produce sedation in mice (Paris et al., 2011), but as U50,488 did not exhibit anxiogenic effects in this study it is unlikely to have been a confounding factor in our models.

It is important to examine the results of the EPM and light/dark tests in the greater context of all behaviours recorded, such as entries into the safe and aversive zones and distance travelled throughout the arena, so as to get a complete picture of anxiogenic effects (Bouwknecht & Paylor, 2008). For instance, it has been shown that the percentage of open/total arm entries is a variable associated with anxiety (Cruz, Frei, & Graeff, 1994), and the percentage of distance travelled in the light box/total distance is associated with anxiety-like behaviour in the light/dark test (Arrant et al., 2013). In this study there were no significant differences in the open arm/total arm entries recorded for any treatment compared with vehicle (Figure 23A; Appendix 2), and only the higher dose of Sal A (1 mg/kg, i.p) showed a significant difference in the light box/total distance travelled (Figure 23B; Appendix 2). This is consistent with the conclusion that the higher dose of Sal A was the only compound tested to exhibit anxiogenic effects in the light/dark test.

5.2.4 Limitations

A major limitation with the use of these behavioural assays to screen for anxiogenic side effects is that the tests themselves elicit anxiety-like behaviours. This study compared treatment groups with vehicle control groups, therefore compounds that did not show anxiogenic effects did not increase basal anxiety levels. This may miss subtle effects caused by drug treatment that would be better seen in unstressed groups. Thus, in addition to behavioural studies, well-accepted physiological markers of stress intensity, such as adrenocorticotropic hormone, corticosterone, prolactin, and glucose, should be measured as their plasma levels are proportional to the intensity of the emotional response (Armario, 2006). Performing multiple behavioural tests combined with physiological measures would provide a comprehensive screen for anxiety-like behaviour.

The limitations discussed here are relevant to preclinical anxiety tests collectively and should not diminish the results presented in this study. The paradigms used are widely accepted in the literature and are valid methods for screening adverse effects. To conclude, Sal A shows anxiogenic effects at 0.3 mg/kg on the EPM and at 1 mg/kg in the light/dark test whereas its novel analogues do not. Here EOM Sal B (0.1 mg/kg) is shown to be significantly improved over Sal A (0.3 mg/kg) in the EPM, demonstrating that novel analogues of Sal A can show improved effects over their parent compound and show promise as potential therapeutics for treating drug addiction.

5.3 pCREB and CREB antibody validation

The phosphorylation of CREB is associated with dysphoria (Newton et al., 2002) and stress-induced reinstatement of drug-seeking behaviour (Kreibich & Blendy, 2004; Kreibich et al., 2009) through the regulation of dynorphin and CRF (Cole et al., 1995; Itoi et al., 1996). A

potential reason why the analogues of Sal A show reduced side effects, while still retaining their anti-addictive effects, is if these ligands show a signalling bias (Kivell et al., 2014). This concept is called functional selectivity or biased agonism, whereby G-protein coupled receptors have multiple conformational states resulting in the activation of different downstream signalling pathways dependent on the properties of the ligand (Perez & Karnik, 2005).

By understanding which pathways produce advantageous effects and those which produce unwanted effects, KOPr agonists could be screened prior to animal testing, enabling the identification of promising compounds in a faster, cheaper, and more efficient way. White et al. (2014) recently identified a range of KOPr agonists with signalling bias through parallel *in silico* and *in vitro* screening, including the G-protein-biased Sal A derivative 22-thiocyanatosalvinorin A (RB-64), previously synthesised by Yan et al. (2009). Subsequent *in vivo* studies with RB-64 (3 mg/kg, s.c) in male C57BL/6J wild type and β-arrestin knockout mice demonstrated KOPr mediated analgesic effects and CPA, suggesting G-protein signalling mediates these effects (White et al., 2015). This treatment also lacked anhedonic effects, measured using intracranial self-stimulation (ICSS), and motor incoordination and sedation, measured using the rotarod and novelty-induced locomotion assays respectively, suggesting that undesirable side-effects are mediated through β-arrestin signalling (White et al., 2015). It is interesting to note that this study suggests that aversion is mediated through G-protein rather than β-arrestin signalling as previously hypothesised (Bruchas & Chavkin, 2010) as activation of p38 MAPK resulted in KOPr dependent aversion (Bruchas et al., 2007; Ehrich et al., 2015). White et al. (2015) suggest that perhaps p38 MAPK was activated via a different signalling pathway or that aversion can be induced in a p38-independent manner. Still, these studies demonstrate the possibility of producing functionally selective agonists with a bias

towards desirable effects and reduced side-effects, and highlights the importance of combining signalling and behavioural data to identify the pathways associated with favourable therapeutic outcomes.

To examine the potential signalling bias of the tested compounds, we hypothesised that novel analogues of Sal A that did not show aversive or anxiogenic effects would show reduced phosphorylation of CREB which functions to activate endogenous stress and punishment pathways.

Optimisation of the Western blotting procedure was initially performed in HEK-293 cells treated with forskolin before progressing to experimentation with animals treated with novel KOPr agonists. Forskolin was used as a positive control as it stimulates cAMP pathways resulting in downstream phosphorylation of CREB. Phospho-CREB (Ser133) (1B6) was found to be defective, so further experiments used the Phospho-CREB (Ser133) (87G3) antibody. After validation in cells Western blots using tissue samples were conducted. Neither pCREB nor CREB was detectable in any tissue sample although α-tubulin gave a strong signal, confirming adequate protein loading. This was potentially due to a manufacturers issue with the antibody rather than experimenter error, however all antibodies was used and stored as per manufacturer's instructions, and both antibodies gave a signal in cell culture samples. Although it could be due to differences in human vs. rat CREB protein, this is unlikely as there are only three amino acid differences between human and rat CREB proteins (Meyer & Habener, 1993; The UniProt Consortium, 2014) (Figure 25; Appendix 3). After recent consultation with the manufacturer and supplier of the antibodies we were advised that they have not been able to detect pCREB when using the Phospho-CREB (87G3) in either mouse or rat brain lysates. Further experimentation should be conducted with an alternative antibody

when using tissue samples, or using HEK-293 cells transiently transfected with KOPr and β-arrestin.

5.4   Future directions

To further examine the effects of these novel analogues, dose-dependent studies should be performed, as Sal A has shown opposing effects at low vs. high doses. For instance, Sal A produced CPP at 0.1-40 μg/kg, s.c (Braida et al., 2008) but CPA at 160 μg/kg, 0.3 and 1 mg/kg i.p in rats (Braida et al., 2008; Sufka et al., 2014), and anxiolytic effects at 0.1-160 μg/kg, s.c in the EPM test in rats (Braida et al., 2009) with 0.3 and 1 mg/kg, i.p demonstrating anxiogenic effects in the present study. Activation of the KOPr receptor has also been shown to produce differing effects in males and females. Robles et al. (2014) demonstrated that female but not male California mice treated with 2.5 mg/kg U50,488, i.p produced significant CPA, and males but not females produced significant CPA at 10 mg/kg U50,488, i.p. It has also been shown that female dynorphin knockout C57BL/6N displayed reduced anxiety-like behaviour on the EPM compared with males (Kastenberger et al., 2012). Therefore, further studies on the aversive and anxiogenic effects of novel Sal A analogues would benefit from utilising female test subjects.

The Western blot is the most common method to quantify proteins in a biological sample. However, due to the difficulty in using this procedure to identify pCREB in our rat brain tissue samples, other methods may prove to be more effective. These include enzyme-linked immunosorbent assays (ELISAs), immunohistochemistry (IHC), or mass spectrometry.

5.5   Conclusions

Mesyl Sal B, EOM Sal B, and Ethy Sal A do not produce aversive effects in the CPA test or anxiogenic effects in the light/dark and EPM test. This study provides evidence that structural modification of the novel agonist Sal A can produce compounds with fewer side-effects than their parent compound, demonstrating their increased potential as effective anti-addiction pharmacotherapies compared with the traditional agonists and the parent compounds Sal A.

Compound	CPA	EPM	Light/dark
U50,488 (10 mg/kg)	Aversive	n.e	n.e
Sal A (1 mg/kg)	-	Anxiogenic	Anxiogenic
Sal A (0.3 mg/kg)	Aversive	Anxiogenic	n.e
β-THP Sal B (1 mg/kg)	Aversive	n.e	n.s anxiogenesis
EOM Sal B (0.1 mg/kg)	n.e	n.e	n.s anxiogenesis
Mesyl Sal B (0.3 mg/kg)	-	n.e	n.e
Ethy Sal A (0.3 mg/kg)	n.e	n.e	n.e

n.e = no effect, n.s non-significant

Table 5   Summary of the findings in the present study

6.   Supplementary Information

6.1   Appendix 1: Solutions

10X Phosphate Buffered Saline (PBS) pH 7.4

	Concentration	g/L
NaCl	1.4 M	80
KCl	26.8 mM	2.0
Na_2HPO_4	81.0 mM	26.8
KH_2PO_4	14.7 mM	2.4

RIPA buffer pH 7.5

	Concentration	g/L
Tris-HCl	10 mM	1.2
NaCl	150 mM	8.766
EDTA	1 mM	0.37
Triton-X-100	1%	10 ml
SDS	0.1%	1.0
Sodium deoxycholate	1%	10

5X Reducing Buffer

	Concentration
Tris HCl pH 6.8	62.5 mM
SDS	2%
Glycerol	20%
Bromophenol blue	1%

SDS-PAGE gels (makes 2)

10% separating gel	Volume
dH$_2$O	8 ml
1.5 M Tris pH 8.8	5 ml
10% SDS	200 µl
Acrylamide	6.66 ml
10% APS	100 µl
TEMED	10 µl

100% isopropanol used to cover the gel while it was setting; poured off before the stacking gel was added

4% stacking gel	Volume
dH$_2$O	6.1 ml
0.5 M Tris pH 6.8	2.5 ml
10% SDS	100 µl
Acrylamide	1.33 ml
10% APS	50 µl
TEMED	10 µl

10X Running buffer

	Concentration	g/L
SDS	35 mM	10
Tris HCl	250 mM	30.3
Glycine	1.9 M	144.1

Western transfer buffer

	Concentration	g/L
Glycine	190 mM	14.4
Tris HCl	25 mM	3.03
Methanol	20%	200 ml

10X TBS pH 7.5

	Concentration	g/L
Tris HCl	500 mM	60.5
NaCl	1.5 M	87.6

T-TBS

1X TBS containing 0.1% Tween 20
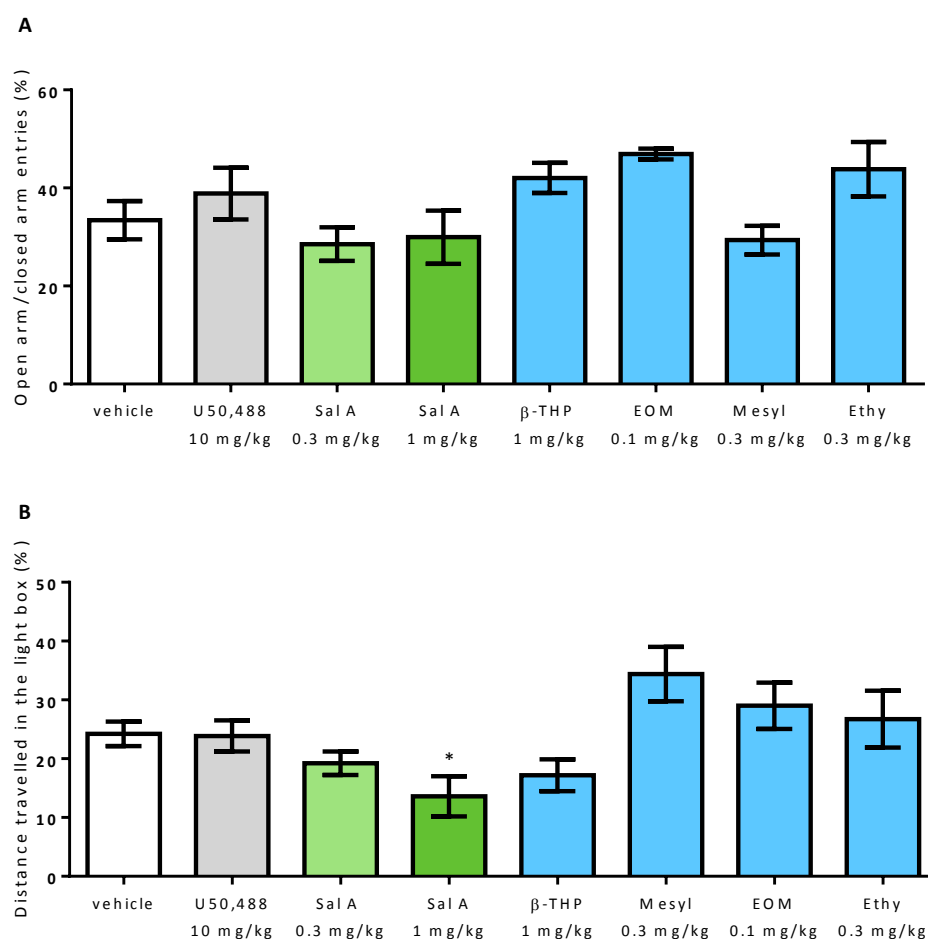
Figure 23   Additional anxiety measures in the EPM and light/dark tests

A: Percentage of open arm/total arm entries on the EPM. This has been shown to be a further measure of anxiety and give further indication of anxiogenic effects in addition to the time spent on the open arm. One-way ANOVA with Bonferroni post hoc test, n = 12-29.

B: Percentage of distance travelled in the light box/total distance travelled. This has also been shown to be a measure of anxiety-like behaviour. Sal A (1 mg/kg), the only KOPr agonist to show anxiogenic effects in the light/dark test, also shows a decrease in distance travelled in the light box which is consistent with anxiety-like behaviour. Kruskal-Wallis test with Dunn's multiple comparison, n = 10-25.

*p = ≤0.05 – data points presented as mean ± SEM.

6.3　Appendix 3: Additional Western blot optimisation data

A　　　　　　　　　　**B**　　　　　　　　　　**C**
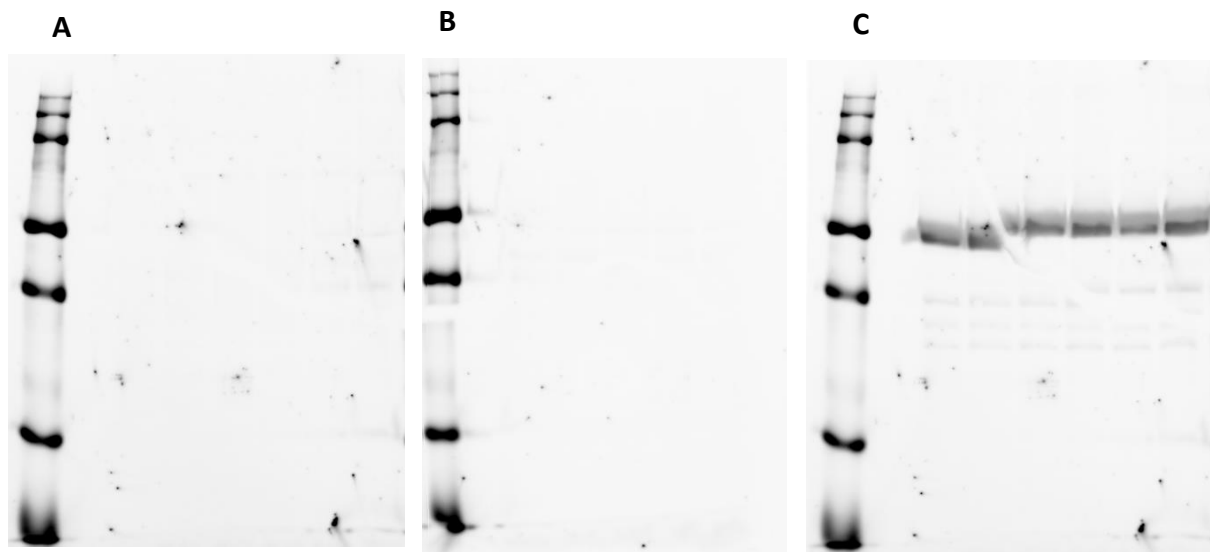
Figure 24　　Sonication of ex vivo samples did not increase protein signal

A: Membrane probed with Phospho-CREB (Ser133) (87G3) did give a signal.
B: No signal was detected with incubation with CREB protein antibody.
C: α-tubulin loading control.
Total protein loaded per well = 60 μg.

```
                   10         20         30         40         50
hCREB   MTMDSGADNQ QSGDAAVTEA ESQQMTVQAQ PQIATLAQVS MPAAHATSSA
rCREB   MTMESGAENQ QSGDAAVTEA ENQQMTVQAQ PQIATLAQVS MPAAHATSSA

                   60         70         80         90        100
        PTVTLVQLPN GQTVQVHGVI QAAQPSVIQS PQVQTVQSSC KDLKRLFSGT
        PTVTLVQLPN GQTVQVHGVI QAAQPSVIQS PQVQTVQSSC KDLKRLFSGT

                  110        120        130        140        150
        QISTIAESED SQESVDSVTD SQKRREILSR RPSYRKILND LSSDAPGVPR
        QISTIAESED SQESVDSVTD SQKRREILSR RPSYRKILND LSSDAPGVPR

                  160        170        180        190        200
        IEEEKSEEET SAPAITTVTV PTPIYQTSSG QYIAITQGGA IQLANNGTDG
        IEEEKSEEET SAPAITTVTV PTPIYQTSSG QYIAITQGGA IQLANNGTDG

                  210        220        230        240        250
        VQGLQTLTMT NAAATQPGTT ILQYAQTTDG QQILVPSNQV VVQAASGDVQ
        VQGLQTLTMT NAAATQPGTT ILQYAQTTDG QQILVPSNQV VVQAASGDVQ

                  260        270        280        300        310
        TYQIRTAPTS TIAPGVVMAS SPALPTQPAE EAARKREVRL MKNREAAREC
        TYQIRTAPTS TIAPGVVMAS SPALPTQPAE EAARKREVRL MKNREAAREC

                  320        330        340        350
        RRKKKEYVKC LENRVAVLEN QNKTLIEELK ALKDLYCHKS D
        RRKKKEYVKC LENRVAVLEN QNKTLIEELK ALKDLYCHKS D
```

Figure 25   Human and rat CREB proteins share 99.1% homology

The amino acid sequence for human (hCREB) and rat (rCREB) CREB. Only three amino acids differ between these two protein sequences. The pCREB antibodies were specific to Ser133 phosphorylation.

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