

Themed Section: Opioids: New Pathways to Functional Selectivity

RESEARCH PAPER

Pharmacology and anti-addiction effects of the novel κ opioid receptor agonist Mesyl Sal B, a potent and long-acting analogue of salvinorin A

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BACKGROUND AND PURPOSE

Acute activation of κ opioid (KOP) receptors results in anticocaine-like effects, but adverse effects, such as dysphoria, aversion, sedation and depression, limit their clinical development. Salvinorin A, isolated from the plant *Salvia divinorum*, and its semi-synthetic analogues have been shown to have potent KOP receptor agonist activity and may induce a unique response with similar anticocaine addiction effects as the classic KOP receptor agonists, but with a different side effect profile.

EXPERIMENTAL APPROACH

We evaluated the duration of effects of Mesyl Sal B *in vivo* utilizing antinociception assays and screened for cocaine-prime induced cocaine-seeking behaviour in self-administering rats to predict anti-addiction effects. Cellular transporter uptake assays and *in vitro* voltammetry were used to assess modulation of dopamine transporter (DAT) function and to investigate transporter trafficking and kinase signalling pathways modulated by KOP receptor agonists.

KEY RESULTS

Mesyl Sal B had a longer duration of action than SalA, had anti-addiction properties and increased DAT function *in vitro* in a KOP receptor-dependent and *Pertussis* toxin-sensitive manner. These effects on DAT function required ERK1/2 activation. We identified differences between Mesyl Sal B and SalA, with Mesyl Sal B increasing the V_{max} of dopamine uptake without altering cell-surface expression of DAT.

CONCLUSIONS AND IMPLICATIONS

SalA analogues, such as Mesyl Sal B, have potential for development as anticocaine agents. Further tests are warranted to elucidate the mechanisms by which the novel salvinorin-based neoclerodane diterpene KOP receptor ligands produce both anti-addiction and adverse side effects.

LINKED ARTICLES

This article is part of a themed section on Opioids: New Pathways to Functional Selectivity. To view the other articles in this section visit <http://dx.doi.org/10.1111/bph.2015.172.issue-2>

Abbreviations

ASP+, 4-4(dimethylamino)-styryl)-N-methylpyridinium; CPP, conditioned place preference; DAT, dopamine transporter; dStr, dorsal striatum; ICSS, intracranial self-stimulation; Mesyl Sal B, (2S,4aR,6aR,7R,9S,10aS,10bR)-9-(methanesulfonyloxy)-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2H-naphtho[2,1-c]pyran-7-carboxylic acid methyl ester; MPE, maximum possible effect; NAcb, nucleus accumbens; norBNI, norbinaltorphimine; RDEV, rotating disk electrode voltammetry; SalA, salvinorin A; TIRFM, total internal reflective fluorescence microscopy; U50,488H, (*trans*-(±)-3,4-dichloro-N-methyl-N(2-[1-pyrrolidinyl]-cyclohexyl) benzenacetamide methanesulfonate; U69,593, (5 α ,7 α ,8 β)-(+)-N-methyl-N(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide

Introduction

Activation of κ opioid (KOP) receptors by traditional agonists such as U50,488H, U69,593 and the novel neoclerodane diterpene salvinorin A (SalA) have been shown previously to have anti-addiction effects in preclinical models of addiction (Negus *et al.*, 1997; Mello and Negus, 1998; Gray *et al.*, 1999; Schenk and Partridge, 1999; 2000; Schenk *et al.*, 1999; 2000; Morani *et al.*, 2009; Wee *et al.*, 2009; receptor nomenclature follows Alexander *et al.*, 2013). Unfortunately, side effects including sedation, aversion, dysphoria and depression limit their therapeutic use. SalA has been shown to have reduced side effects compared with the traditional KOP receptor agonists; however, pro-depressive effects were still observed (Morani *et al.*, 2012). The short duration of effects of SalA also make it undesirable as a potential therapeutic agent (Prisinzano, 2005; Butelman *et al.*, 2009; Teksin *et al.*, 2009; Ranganathan *et al.*, 2012). Utilizing the unique structure of SalA, several investigators have synthesized novel derivatives with varying opioid receptor selectivities, potencies and efficacies (Harding *et al.*, 2005; Prisinzano and Rothman, 2008; Lovell *et al.*, 2011). One SalA analogue, β tetrahydropyran Sal B, was shown to have anti-addiction effects in preclinical studies similar to those reported for SalA, although side effects were not reported (Prevatt-Smith *et al.*, 2011). The development of longer acting KOP receptor agonists possessing desirable anti-addiction effects and reduced side effects remains to be investigated along with the mechanism of action of such effects (Kivell *et al.*, 2014).

Acute administration of cocaine leads to increases in dopamine levels in the dorsal striatum (dStr) and nucleus accumbens (NAcb), leading to rewarding effects. It has been proposed that KOP receptor agonists oppose the effects of drugs, such as cocaine, by modulating the dopamine system (Spangler *et al.*, 1997; Shippenberg *et al.*, 2007; Wee and Koob, 2010). Acute administration of KOP receptor agonists such as: U50,488H, U69,593 and SalA have all been shown to cause a decrease in dopamine concentrations in the NAcb and dStr (Spanagel *et al.*, 1990; Shippenberg *et al.*, 2007). This decrease has been linked to reduced release of dopamine from the presynaptic neuron. KOP receptors are also localized in the same neurons as the dopamine transporter (DAT) in the NAcb (Svingos *et al.*, 2001), suggesting that KOP receptors may also play a role in regulating DAT function. DAT clears dopamine from the synapse and its activity is rapidly regulated by kinases.

Identification of functional agonists that are able to differentially activate signalling pathways may yield analogues with desirable anti-addiction effects without the unwanted side effects limiting their therapeutic use. There are currently

no reported potent SalA analogues with partial KOP receptor agonist or antagonist activities. Activation of p38-MAPK, β -arrestin recruitment and late activation of ERK1/2 have all been identified as candidate pathways for the undesirable effects of KOP receptor agonists (Bruchas *et al.*, 2007; 2011; Land *et al.*, 2009; Muschamp *et al.*, 2011). ERK1/2 activation is responsible for the discriminative stimulus effects induced by U50,488H (Yoshizawa *et al.*, 2011). Repeated stress has also been shown to activate ERK1/2 in the mouse striatum in a KOP receptor -dependent manner (Bruchas *et al.*, 2008). Mice, following the acute administration of the KOP receptor agonist U50,488H display potentiated ethanol-conditioned place preference (CPP) and forced swim stress potentiated ethanol-CPP. These effects were shown to be KOP receptor-dependent (Sperling *et al.*, 2010). Stress-induced potentiation of cocaine CPP was prevented by the KOP receptor antagonist norbinaltorphimine (norBNI) and was absent in mice lacking the prodynorphin gene (McLaughlin *et al.*, 2003), suggesting an important role for the KOP receptor system in modulating stress-induced behaviours.

Many experimental models are used to predict addiction behaviours in humans, including various measures of natural reward, CPP and intracranial self-stimulation (ICSS). No single model is able to successfully model such complex behaviours. However, drug self-administration models are considered to be the gold standard (Kmiotek *et al.*, 2012). Within self-administration models, different paradigms such as long and short access models, progressive ratio models and extinction models are commonly used to model various aspects of addiction and drug-reward properties (Dworkin and Stairs, 2002). All models have various strengths and weaknesses and model different aspects of addiction in humans. In this study, we used a drug-prime model of relapse in rats trained to self-administer cocaine following a short period of extinction. This model has been extensively used to measure the anticocaine-like effects of traditional KOP receptor agonists (Schenk and Partridge, 1999; 2000; Schenk *et al.*, 1999; 2000; Morani *et al.*, 2009); SalA (Morani *et al.*, 2009) and novel SalA analogues (Prevatt-Smith *et al.*, 2011; Morani *et al.*, 2013) allowing for direct comparisons between these compounds. Future work comparing acute and chronic administration will identify if differences between novel SalA analogues and traditional compounds are present. It is generally accepted that acute administration of KOP receptor agonists attenuate the rewarding effects of drugs of abuse, possibly by inducing punishing/aversive-like effects; whereas, chronic exposure to KOP receptor agonists may potentiate drug-seeking and/or drug taking, via stress mediated mechanisms and modulation of withdrawal (Wee and Koob, 2010; Graziane *et al.*, 2013).

In this study, the novel SalA analogue, Mesyl Sal B, was investigated. Mesyl Sal B is a selective KOP receptor agonist with a similar affinity for the receptor (K_i 2.3 nM for Mesyl Sal B, compared with 1.9 nM for SalA) and potency (EC_{50} of 30 ± 5 nM compared with 40 ± 10 nM for SalA) (Harding *et al.*, 2005). In this study, we showed that Mesyl Sal B was a longer acting KOP receptor agonist than SalA, and that it attenuated cocaine-prime induced drug seeking or taking following acute administration. We also showed that Mesyl Sal B increased DAT function and activated early, but not late, phase ERK1/2 in the dStr, NAcB and prefrontal cortex. Unlike SalA and U69,593, the increases in DAT function seen with Mesyl Sal B were not due to increases in cell-surface DAT expression.

Methods

Animals

All animal care and experimental procedures conform to the UK laws governing animal experimentation and were approved by the Animal Ethics Committee (VUW), New Zealand. The authors have consulted the ARRIVE guidelines (Kilkenny *et al.*, 2010) and reporting of experiments (McGrath *et al.*, 2010). A total of 128 male Sprague Dawley rats (250–400 g) were used for self-administration experiments and for brain tissue, and 27 male mice (B6-SJL, 23–27 g) were used for tail-withdrawal assays.

All rats were bred and housed in the Victoria University of Wellington, New Zealand (VUW) animal facility. Juvenile mice were purchased from the Malaghan Institute of Medical Research (Wellington, New Zealand), prior to housing within the VUW animal facility. All animals were maintained on a 12-h light/dark cycle and given free access to food and water except during experimental sessions. All procedures were conducted during the light cycle.

Hot water tail-withdrawal assay

To determine the duration of action of Mesyl Sal B, the antinociception hot water tail-withdrawal assay was carried out in mice ($n = 9$) following the method of Simonin *et al.* (1998). Briefly, mice were restrained in a Plexiglas tube (internal diameter of 24 mm) and allowed to acclimatize for 15 min. One-third of each mouse tail was marked and immersed in water ($50 \pm 0.5^\circ\text{C}$) with a maximum cut-off latency set at 10 s (Simonin *et al.*, 1998; Valjent *et al.*, 2002). Repeated measurements were carried out at 1, 5, 10, 15, 30, 45, 60, 90, 120, 150 min, and the time for the animal to show a tail-withdrawal response was recorded following i.p. administration of vehicle (80% propylene glycol, 20% DMSO diluted with the same volume PBS) or KOP receptor agonist dissolved in vehicle by an observer, unaware of the treatments given to the experimental groups. The maximum possible effect (MPE) of analgesia was then calculated using the following formula: $\% \text{ MPE} = 100 \times (\text{test latency} - \text{control latency}) / (10 - \text{control latency})$.

Cocaine self-administration

A total of 16 rats were used for self-administration experiments. Under deep anaesthesia [ketamine/xylazine (90/9 mg·kg⁻¹, i.p.)], a silastic catheter was inserted into the right external jugular vein. The distal end (22 gauge stainless steel

tubing) was passed subcutaneously to an exposed portion of the skull where it was fixed to embedded jeweller's screws with dental acrylic. Post-surgery catheters were infused daily with sterile saline solution (containing 30 U·mL⁻¹ heparin, 250 000 U·mL⁻¹ penicillin G potassium and 8000 U·mL⁻¹ streptokinase), and rats were rested for 5 days. Self-administration training and reinstatement tests were conducted in standard operant chambers equipped with two retractable levers (Med Associates, ENV-001, St. Albans, VT, USA). Self-administration procedures were as described previously (Schenk and Partridge, 1999; 2000; Morani *et al.*, 2009). Briefly, depression of the active lever led to a programmed intravenous (i.v.) infusion (0.1 mL) of cocaine-HCl dissolved in physiological saline containing heparin (3.0 U·mL⁻¹). Infusions lasted 12 s and were accompanied by illumination of a light above the active lever. Rats were trained to self-administer cocaine daily for 2 h sessions, and trained on a fixed response-1 (FR-1) schedule of reinforcement followed by FR-5. The reinstatement test was conducted in a single day and consisted of three phases. During the first phase, the animals were allowed to self-administer cocaine (0.5 mg·kg⁻¹·infusion⁻¹, FR-5) for 1 h, followed by a 3 h extinction phase when cocaine was replaced with heparinized saline (FR-5). The extinction period (3 h) was chosen because this was the time taken for responses to decline to less than 10% of baseline responding. Only animals that reached this criterion were used in experiments. At the beginning of the third phase (reinstatement), rats ($n = 5$ –6 per group) received Mesyl Sal B or vehicle. These injections were administered 45 min prior to the cocaine-prime (20 mg·kg⁻¹, i.p.), and responding under extinction was measured for 1 h. The 45 min pre-incubation for Mesyl Sal B was based on *in vivo* pharmacological studies (Figure 2). Some rats were also treated with the KOP receptor antagonist norBNI (2 mg·kg⁻¹, i.p.) 30 min prior to Mesyl Sal B (0.3 mg·kg⁻¹, i.p.) prior to reinstatement testing to evaluate KOP receptor-mediated effects.

Spontaneous locomotion activity

A total of 14 drug-naïve male rats were used to determine locomotor activity by previously described methods (Morani *et al.*, 2013). Briefly, following a 30 min habituation phase in the open field chamber (Med Associates ENV-520), drug-naïve rats were injected with either vehicle (75% DMSO, i.p.) or Mesyl Sal B (0.3 mg·kg⁻¹, i.p.), and ambulation counts were measured at 5 min intervals for 60 min.

Cell culture

HEK-293, green monkey kidney (COS7) and mouse neuroblastoma cells (N2A) (ATCC, Manassas, VA, USA) were transfected 48 h prior to experiments with 0.6 µg yellow fluorescent protein-tagged human DAT (YFP-hDAT) (J. Javitch, New York, NY, USA) and 0.4 µg myc-rKOP receptor (L. Devi, New York, NY, USA) using Lipofectamine 2000. HEK-293 and COS7 cells were maintained in DMEM containing 10% FBS, 1% penstrep antibiotic and 1% L-glutamine, and N2A cells were maintained in RPMI-1640 medium containing 10% FBS and 1% penstrep antibiotic.

Imaging experiments

ASP+ uptake. DAT function was measured using 4-(4-(dimethylamino)-styryl)-N-methylpyridinium (ASP+).

Methods were carried out as previously described (Schwartz *et al.*, 2003; Bolan *et al.*, 2007; Zapata *et al.*, 2007). In brief, transfected cells were placed into a temperature controlled Olympus Fluoview FV1000 confocal microscope (Sydney, NSW, Australia) in KREBs buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄·6H₂O, 1.2 mM KH₂PO₄, 10 mM HEPES, 10 mM D-glucose, pH 7.4), medium was aspirated and cells imaged (120 s, image every 5 s, YFP filter: 485 nm excitation; 545 nm emission, ASP+ filter: 570 nm excitation; 670 nm emission). ASP+ (10 μM) was added after capture of the first image followed by agonist (1–10 μM) or DMSO vehicle at 250 s. For cellular experiments KOP receptor specificity (norBNI, 1 μM, 30 min), *Pertussis* toxin (PTX 100 ng·mL⁻¹ 16–24 h) and ERK1/2 signalling pathways (U0126, 20 μM 30 min) were investigated, cells were incubated with the appropriate drug before ASP+ addition. For binding experiments, cells were incubated with agonist or DMSO vehicle for 250 s prior to ASP+ addition with images taken at maximum speed for 100 s.

Analysis for ASP+ uptake and binding studies. YFP-hDAT expressing cells were manually outlined on the image, and the accumulation of ASP+ over time determined. The rates of uptake for a period of 60 s before and 60 s following drug were calculated at the time of maximal effect. The change in slope was calculated as a percentage change [(slope after drug/slope before drug) × 100]. For binding studies, data from 3 to 7 s after ASP+ addition were analysed.

Total internal reflective fluorescence microscopy (TIRFM). Methods followed previously published protocols (Furman *et al.*, 2009; Kivell *et al.*, 2010). Cells were placed onto the stage of an Olympus FV1000 microscope in a cell chamber for 20 min in aerated culture medium (100X objective, 1.45 NA; Olympus, PLAPO, Sydney, NSW, Australia). A region of cells was selected using the TIRFM camera (QImaging Digital Camera 6.2.0.6; Olympus) and Image Pro Plus 7.0 acquisition software (Media Cybernetics, Rockville, MD, USA). A penetration depth of 100 nm and a refractive index of 1.52 were set. Images (473 nm laser, YFP filter: 485 nm excitation; 545 nm emission) were captured every minute for 40 min from time 0 with an exposure time of 3 s. After 10 min, agonists (10 μM) were added. Pixel intensities of individual cells at each time point were analysed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

ERK phosphorylation

Transfected HEK-293 cells were treated with 10 μM KOP receptor agonist or vehicle (0.28% DMSO in KREBS buffer) following serum starvation (30 min), lysed in RIPA buffer (10 mM Tris-HCL, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1% sodium deoxycholate; pH 7.5) and run on SDS-PAGE gels, followed by overnight transfer to PVDF membranes (Millipore Corporation, Billerica, MA, USA). Polyacrylamide gels were imaged on a Fuji FLA-5100 laser scanner before transferring to PVDF to confirm YFP-hDAT expression. Once transferred, membranes were probed with mouse monoclonal P-ERK 1/2 followed by anti-mouse Cy5, and then stripped and re-probed with rabbit monoclonal ERK 1/2, followed by anti-rabbit Cy3. ERK 1/2 signalling was inhibited using the MEK inhibitor U0126

(20 μM). To analyse data, band intensities were measured using ImageJ, and P-ERK1/2 was normalized to total ERK1/2 expression. To determine ERK phosphorylation *ex vivo*, vehicle (75% DMSO) or KOP receptor agonist (2.0 mg·kg⁻¹, i.p.) was administered at selected times (0, 10–15, 20, 60 and 120 min) and rats killed following CO₂ asphyxiation. A total of 40 rats were utilized to determine P-ERK1/2 levels. The brain was then rapidly removed and the dStr, NAcB and prefrontal cortex dissected. All brain regions were placed into microcentrifuge tubes. Tissue was lysed in 300 μL RIPA buffer containing protease and phosphatase inhibitors, on ice. Lysates were centrifuged at 16 000 g for 15 min, and the DNA pellet removed and processed as above.

Cell surface biotinylation

Protocols were followed as previously published (Kivell *et al.*, 2010). Transfected cells were treated with agonist or vehicle 30 min prior to biotin incubation (1 mg·mL⁻¹, EZ-link-NHS-Sulfo-SS-biotin). Cells were lysed in 400 μL of RIPA buffer for 1 h in the dark at 4°C with shaking. Lysates were centrifuged at 16 000 g for 30 min at 4°C and 50 μL removed (total fraction). Supernatant was incubated with 100 μL streptavidin beads for 1 h at room temperature, centrifuged (4°C for 1 min at 500 g) and 50 μL of supernatant removed (internal fraction). Bound proteins were eluted from washed beads with 50 μL of SDS-PAGE loading buffer (cell-surface fraction). Fractions were run on an SDS-PAGE gel, the gel was scanned to visualize YFP-hDAT, transferred overnight onto PVDF and blotted for GAPDH for verification of fraction identities.

Rotating disk electrode voltammetry (RDEV)

Methods were carried out as previously described (Povlock and Schenk, 1997; Danielson *et al.*, 2014). A total of 56 rats were used for these RDEV studies. DStr, NAcB and prefrontal cortex tissue was dissected from freshly killed drug-naïve rat brains, weighed, finely chopped in ice-cold PBS and transferred to microcentrifuge tubes. Tissue was washed eight times in oxygenated, 37°C Krebs buffer, allowing to tissue to settle between each wash. Tissue was resuspended in 300 μL of buffer and transferred to the RDEV chamber where the glassy carbon rotating electrode (Pine Instruments, AFMDO3GC, Durham, NC, USA) was lowered and rotated at 2000 r.p.m. A potential of +450 mV was applied and the resulting current recorded (eDAQ, Denistone, NSW, Australia). Tissue was left to reach baseline prior to KOP receptor agonist (500 nM) or DMSO vehicle addition. For the *low to infinite trans* model (Meiergerd and Schenk, 1994; Povlock and Schenk, 1997), increasing concentrations of dopamine were added, and the current allowed to reach baseline between each addition. For single additions, once the current had reached baseline, a single addition of dopamine was added (2 μM). For medial prefrontal cortex experiments, once baseline had been reached, the noradrenaline transporter inhibitor desipramine-HCl (1 μM) was added 5 min prior to dopamine addition. For the norBNI experiments, tissue was pre-incubated at 37°C with 1 μM norBNI in oxygenated, 37°C Krebs buffer for 30 min. To determine whether ERK activation was required for KOP receptor mediated increases in DAT function, dStr tissue was pre-incubated with U0126 (10 μM) or vehicle (0.02% DMSO in Krebs buffer) prior to KOP receptor agonist addition and determination of DAT function. Uptake

data were collected for 10 s beginning 1 s after addition of dopamine, and a linear regression calculated. Uptake was expressed as $\text{pmol}\cdot\text{s}^{-1}\cdot\text{g}^{-1}$ of tissue. For the *low to infinite trans*-model, uptake values were entered into GraphPad Prism, and a nonlinear regression (one-site binding hyperbole) was fitted to each repeat and the V_{max} and K_{m} values obtained.

Data analysis

Data are expressed as mean \pm SEM. Phase 3 of the reinstatement tests were analysed using one-way ANOVA, followed by Tukey's *post hoc* test. All imaging experiments were carried out over three transfections with at least two dishes per transfection analysed; n equals the number of animals studied. GraphPad Prism (San Diego, CA, USA) was used to determine statistical significance, with Student *t*-tests carried out for comparing a single treatment to control, one-way ANOVA followed by Bonferroni *post hoc* tests to compare multiple treatments, and two-way ANOVA used to compare multiple effects. Values of $P < 0.05$ were considered significant.

Materials

The KOP receptor agonist SalA was isolated and purified from *Salvia divinorum* leaves (>98% pure by HPLC) (Butelman *et al.*, 2007), and Mesyl Sal B was synthesized from SalA as described previously (Harding *et al.*, 2005). Desipramine HCl, dopamine HCl, U0126, U69,593, U50, 488H and norBNI were purchased from Sigma (Auckland, New Zealand), ASP+, and PTX from Tocris (Bristol, UK). Other chemicals were purchased as indicated: streptavidin beads and EZ-link NHS-Sulfo-SS-biotin (Pierce, Rockford, IL, USA), mouse monoclonal anti-GAPDH (Abcam, Cambridge, MA, USA), mouse monoclonal anti-P-ERK1/2 (1:500; Santa Cruz Biotechnology, Paso Robles, CA, USA), rabbit monoclonal anti-ERK1/2 (Cell Signalling Technologies, Danvers, MA, USA), anti-rabbit Cy3 and anti-mouse Cy5 (1:1000; GE Healthcare, Fairfield, CT, USA), YFP-hDAT (J. Javitch) and myc-rKOP receptor (L. Devi). Addition of these fluorescent tags does not alter the trafficking, localization or function of the proteins (Jordan and Devi, 1999; Zapata *et al.*, 2007).

Results

Mesyl Sal B is a neoclerodane diterpene prepared from SalA. Mesyl Sal B has a mesylate group at the C2 position (Figure 1) and has previously been reported to be a selective and potent full KOP receptor agonist with similar binding affinity to SalA at the KOP receptor (Table 1) and similar potency in GTP γ S assays [SalA: 40 ± 10 nM EC_{50} ; Mesyl Sal B: 30 ± 5 nM (means \pm SD)] (Harding *et al.*, 2005).

In vivo pharmacology

No previous *in vivo* work for Mesyl Sal B has been reported. Therefore, we determined its duration of action *in vivo*. Mesyl Sal B significantly increases the latency of tail-withdrawal at 30, 45 and 60 min compared with vehicle controls ($P < 0.05$); whereas, SalA had significant effects at 5 ($P < 0.05$), 10 ($P < 0.0001$) and 15 min ($P < 0.001$; $n = 9$ for all groups). Significant differences between SalA and Mesyl Sal B were observed at 10 min ($P < 0.0001$), with SalA showing significantly higher latencies (Figure 2).

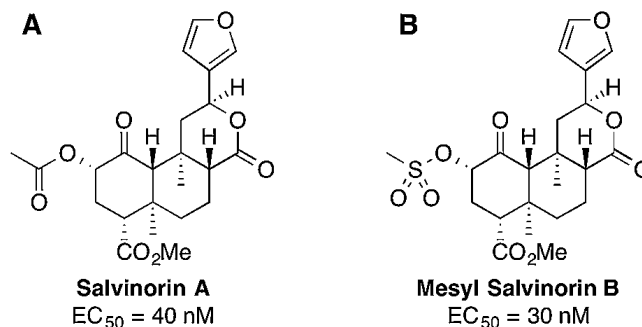


Figure 1

Chemical structures and *in vitro* functional potencies (EC_{50}) for SalA (A) and Mesyl Sal B (B). Reported previously in Harding *et al.* (2005). [35 S]GTP γ S assays in CHO cells stably expressing human KOP receptors. EC_{50} SalA 40 ± 10 nM ($EC_{50} \pm$ SD), E_{max} $120 \pm 2\%$ compared with U50, 488H [% stimulation of binding compared with U50, 488H (500 nM)]. Mesyl Sal B: EC_{50} 30 ± 5 ; E_{max} 112%.

Table 1

Binding affinities of salvinorin A and Mesyl Sal B at the KOP receptor using [125 I]IOXY as radioligand

Compound	$K_i \pm$ SD, nM		
	μ	δ	κ
SalA	>1000	$5\,790 \pm 980$	1.9 ± 0.2
Mesyl Sal B	6820 ± 660	>10 000	2.3 ± 0.1

Reported previously in Harding *et al.* (2005).

Traditional KOP receptor agonists and SalA have demonstrated anti-addiction effects. The effects of the longer acting SalA derivative, Mesyl Sal B, on addiction behaviours have not been tested. The effects of acute Mesyl Sal B administration on cocaine-prime induced reinstatement of drug seeking (Figure 3A) shows Mesyl Sal B ($0.3, 1.0$ mg \cdot kg $^{-1}$) dose-dependently attenuated this effect [$F(3,23) = 36.82, P < 0.0001$]. This effect was KOP receptor-dependent, as attenuation of reinstatement was prevented in rats pretreated with the KOP receptor antagonist norBNI ($p = 0.0002$) (Figure 3B). No effects on spontaneous locomotor activity were observed with Mesyl Sal B (0.3 mg \cdot kg $^{-1}$, *i.p.*) (Figure 3C).

Using ASP+ uptake methods, changes in DAT function in cells expressing YFP-hDAT and myc-rKOP receptor were measured. Mesyl Sal B dose-dependently (1 – 10 μ M) increased the rate of ASP+ uptake (Figure 4A) ($P < 0.001$). The increase in DAT function observed was similar to U50,488H with a 21% ($\pm 5\%$) increase (Figure 4B) ($P < 0.001$) and was approximately half that of SalA ($45 \pm 7\%$) ($P < 0.001$) (Figure 4C). The KOP receptor antagonist norBNI (1 μ M, 30 min) completely inhibited the increase caused by 10 μ M Mesyl Sal B (Figure 4A) ($P < 0.01$), U50,488H (Figure 4B) ($P < 0.001$) and SalA (Figure 4C) ($P < 0.001$).

The KOP receptor is a G_{oi} protein-coupled receptor. Pretreatment of cells with PTX (100 ng \cdot mL $^{-1}$ for 16–24 h) blocked the effects of 10 μ M Mesyl Sal B (Figure 4A) ($P < 0.01$),

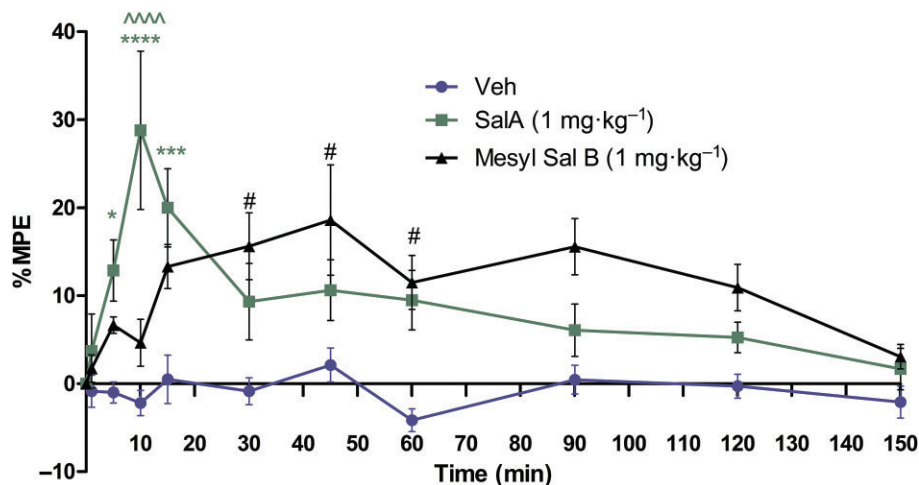


Figure 2

Duration of action of Mesyl Sal B *in vivo* in the hot water tail immersion assay in mice. Tail-withdrawal latency is expressed as percent maximal possible effect (% MPE \pm SEM) versus time. SalA (1 mg·kg⁻¹) has rapid analgesic effects compared with vehicle at 5, 10 and 15 min. Mesyl Sal B (1 mg·kg⁻¹) has a slower onset and longer duration of action with increased tail-withdrawal latencies at 30, 45 and 60 min. SalA and Mesyl Sal B showed significant differences at 10 min (SalA compared to veh * $P < 0.05$, *** $P < 0.001$ **** $P < 0.0001$, Mesyl Sal B compared to veh # $P < 0.05$, SalA compared with Mesyl Sal B ~~~~ $P < 0.0001$). Repeated measures ANOVA revealed a significant effect of time [$F(3603) = 4.952$; $P < 0.0001$], drug [$F(6625) = 19.88$; $P < 0.0001$] and a drug and time interaction [$F(3580) = 3.69$; $P < 0.001$]. Two-way ANOVA followed by Bonferroni post test ($n = 9$).

U50,488H (Figure 4B) ($P < 0.001$) and SalA (Figure 4C) ($P < 0.001$). The ability of Mesyl Sal B to increase ASP+ uptake was not restricted to HEK-293 cells, as this effect at 10 μ M was also seen in N2A cells (Figure 4D) ($P < 0.01$) and COS7 cells (Figure 4E) ($P < 0.01$). In addition to increasing ASP+ uptake, Mesyl Sal B also significantly increased ASP+ binding to DAT in HEK-293 cells (Figure 4F). Neither norBNI, PTX, nor vehicle (DMSO) had effects on ASP+ uptake on their own (data not shown).

Previous studies have shown KOP receptor agonists activate ERK1/2 (Belcheva *et al.*, 2005), and ERK1/2 activation is known to increase DAT function and cell-surface expression (Moron *et al.*, 2003). Therefore, ERK1/2 is a possible link between KOP receptor and DAT. We determined the effects of Mesyl Sal B on phosphorylation of ERK1/2 with a time-course experiment in HEK-293 cells transiently expressing YFP-hDAT and myc-rKOP receptor. Mesyl Sal B rapidly phosphorylated ERK1/2 with significant increases seen at 3 and 5 min (Figure 5A,B) ($P < 0.05$). ERK1/2 is activated by direct phosphorylation of MEK. The selective MEK inhibitor U0126 (20 μ M for 30 min) attenuated ERK1/2 activation (Figure 5C,D) ($P < 0.001$). To determine if the increase in ASP+ uptake following KOP receptor activation by Mesyl Sal B (10 μ M) was dependent on ERK1/2 activation, ASP+ uptake experiments were performed on cells pre-incubated with the MEK inhibitor U0126 (20 μ M, 30 min). U0126 inhibited the increase in ASP+ uptake by Mesyl Sal B (10 μ M) (Figure 5E) ($P < 0.001$). Although high doses of U0126 (50 μ M) altered DAT surface expression and may modulate ASP+ uptake, control experiments showed no effects on ASP+ uptake compared with controls (U0126, 20 μ M, 30 min) (data not shown).

To determine if the effects on ERK1/2 activation in cell-culture models translated to animal models, we also investigated the ability of Mesyl Sal B (2 mg·kg⁻¹, i.p.) to

Table 2

Kinetics of dopamine uptake in rat nucleus accumbens tissue

Treatment	V_{max} (pmol·s ⁻¹ ·g ⁻¹)	K_m (μ M)
Vehicle	740 (73)	0.71 (0.24)
U50,488H	1837 (406)*	1.95 (0.93)
Mesyl Sal B	1567 (228)**	1.89 (0.61)

Values in the Table are means (SEM). * $P < 0.05$, ** $P < 0.01$, significantly different from vehicle.

phosphorylate ERK1/2 in brain tissue taken from the rat dStr, NAcb and prefrontal cortex. Mesyl Sal B significantly increased ERK1/2 phosphorylation at 10–15 min in the dStr, NAcb and prefrontal cortex (Figure 6) ($P < 0.05$).

To determine whether Mesyl Sal B modulates DAT function in the rat brain, we performed RDEV on tissue from the rat NAcb that was pre-incubated with the KOP receptor agonist (500 nM, 4 min) prior to RDEV experiments. Mesyl Sal B caused a significant increase in V_{max} (1509 \pm 115, pmol·s⁻¹·g⁻¹) ($P < 0.05$) with no change in K_m (1.5 \pm 0.5 μ M) compared with vehicle control (V_{max} : 1026 \pm 181) (K_m : 1.5 \pm 0.6 μ M) similar to the effects seen with the positive control U50,488H compared with vehicle [V_{max} , 1979 \pm 335 pmol·s⁻¹·g⁻¹ ($P < 0.05$)] with no change in K_m (2.3 \pm 0.8 μ M) (Figure 7A; Table 2). To investigate the effects of Mesyl Sal B on different brain regions, a single-addition model (dopamine, 2 μ M) was used. Mesyl Sal B (500 nM) ($P < 0.05$), like control U50,488H ($P < 0.01$), showed a significant increase in dopamine clearance in the NAcb, dStr and prefrontal cortex ($P < 0.01$) that was prevented by norBNI (1 μ M, 30 min)

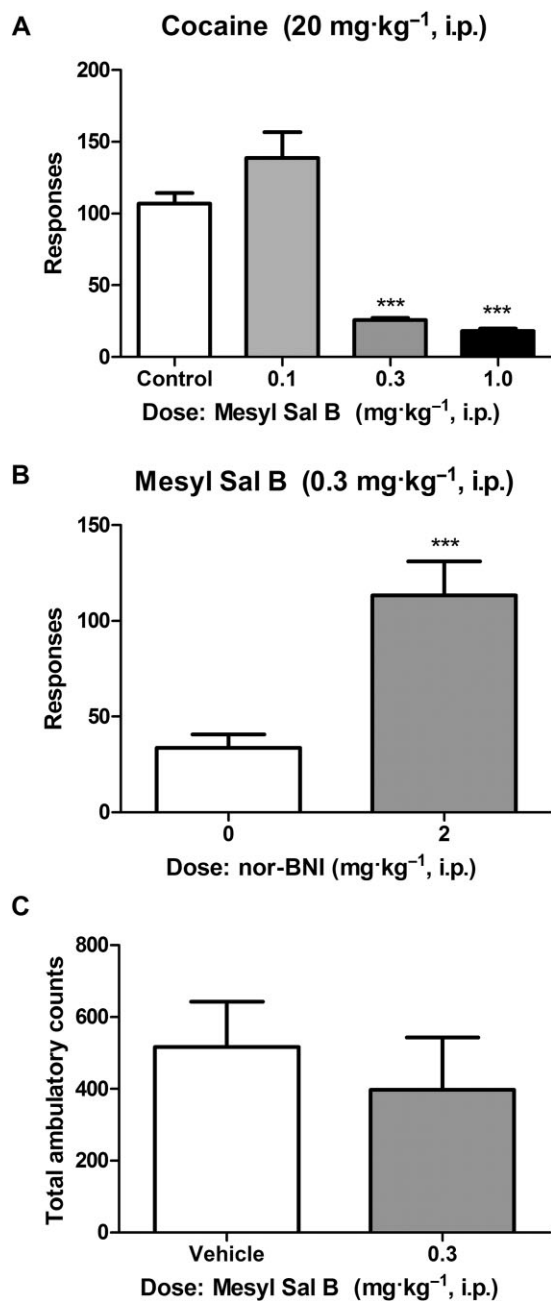


Figure 3

Mesyl Sal B attenuates cocaine-primed cocaine-seeking behaviour in rats. (A) Active lever press responses following Mesyl Sal B 45 min prior to a priming injection of cocaine (20 mg·kg⁻¹) *** $P < 0.001$, data compared with vehicle treated controls (0 mg·kg⁻¹): one-way ANOVA followed by Tukey's *post hoc* test: $n = 5-6$ for each group. (B) nor-BNI (30 min pretreatment, 2 mg·kg⁻¹, s.c.) followed by Mesyl Sal B (0.3 mg·kg⁻¹, ip) during phase 3 of reinstatement tests (mean \pm SEM). (C) Spontaneous locomotor activity in vehicle and Mesyl Sal B treated rats over 60 min (mean \pm SEM) (Student's *t*-test) ($n = 7$ for each group).

(Figure 7C) ($P < 0.05$). SalA showed a faster onset of action of 1 min compared with 4 min for U50,488H and Mesyl Sal B (data not shown), and significantly increased dopamine uptake in the dStr, NAcB and prefrontal cortex, respectively

($P < 0.001$, $P < 0.001$ and $P < 0.05$) at this time point. The increase in dopamine uptake was dependent on ERK1/2 activation as pre-incubation with MEK inhibitor U0126 prevented the Mesyl Sal B increase in dopamine uptake in dStr tissue (Figure 7D). Incubation with nor-BNI alone had no effect on dopamine uptake (Figure 7C).

An increase in V_{max} is often associated with an increase in cell-surface expression of transporters. To investigate whether the increase in V_{max} of DAT with Mesyl Sal B (Figure 7) was due to changes in cell-surface expression of DAT, we utilized an *in vitro* cell-culture model and performed cell-surface biotinylation and TIRFM experiments to determine if there were any changes in cell-surface DAT. In biotinylation experiments, a 30 min incubation with the KOP receptor agonists U69,593 (Figure 8B) and SalA (Figure 8C) (10 μ M) caused a significant increase in cell-surface DAT levels $37 \pm 15\%$ ($P < 0.05$), and $25 \pm 12\%$ ($P < 0.01$) respectively. Mesyl Sal B had no effect on cell-surface DAT levels ($P = 0.2063$) (Figure 8A). Results from TIRFM studies show that at 30 min following U69,593 (Figure 8E) and SalA [(Figure 8F) exposure, HEK-293 cells increased cell-surface expression of DAT ($P < 0.05$), an effect that is not seen with Mesyl Sal B (Figure 8D). No significant changes in cell-surface DAT were seen with short incubations of any of the KOP receptor agonists tested with either biotinylation or TIRFM protocols (Mesyl Sal B, SalA, U69,593; data not shown).

Discussion

SalA is a full agonist at KOP receptors (Roth *et al.*, 2002) and has similar efficacy to the traditional KOP receptor agonists U50,488H and U69,593 and the endogenous ligand dynorphin in GTP γ S assays (Chavkin *et al.*, 2004; Prevatt-Smith *et al.*, 2011). Acutely administered SalA has anti-addiction effects in rats (Morani *et al.*, 2009), similar to the effects seen with traditional KOP receptor agonists (Schenk and Partridge, 1999; Schenk *et al.*, 1999; Shippenberg *et al.*, 2007). SalA attenuated cocaine-induced drug-seeking behaviour without suppressing sucrose reinforcements (Morani *et al.*, 2009). Also, SalA suppressed cocaine-induced hyperactivity (Chartoff *et al.*, 2008) and cocaine behavioural sensitization without inducing sedation and aversion in rats at the dose required to attenuate drug seeking (Morani *et al.*, 2012). In the present study, Mesyl Sal B, like SalA, showed no sedative effects in spontaneous locomotion experiments. Although SalA has a better toxicity profile (Mowry *et al.*, 2003; Johnson *et al.*, 2011) and fewer undesirable side effects (Morani *et al.*, 2012) than traditional KOP receptor agonists, its poor pharmacokinetic properties make it undesirable for therapeutic development (Beguín *et al.*, 2006; Prisinzano and Rothman, 2008). With this in mind, the development of new SalA analogues with improved pharmacokinetics and reduced side effects has the potential to yield useful KOP receptor ligands for the treatment of addiction (Kivell *et al.*, 2014), pain (McCurdy *et al.*, 2006), neuroprotection (Su *et al.*, 2012) and for development of non-addictive analgesics (Groer *et al.*, 2007).

The C-2 position of SalA is an important position for its binding to KOP receptors and metabolic stability (Chavkin *et al.*, 2004; Prisinzano, 2005; Kane *et al.*, 2008). Mesyl Sal B

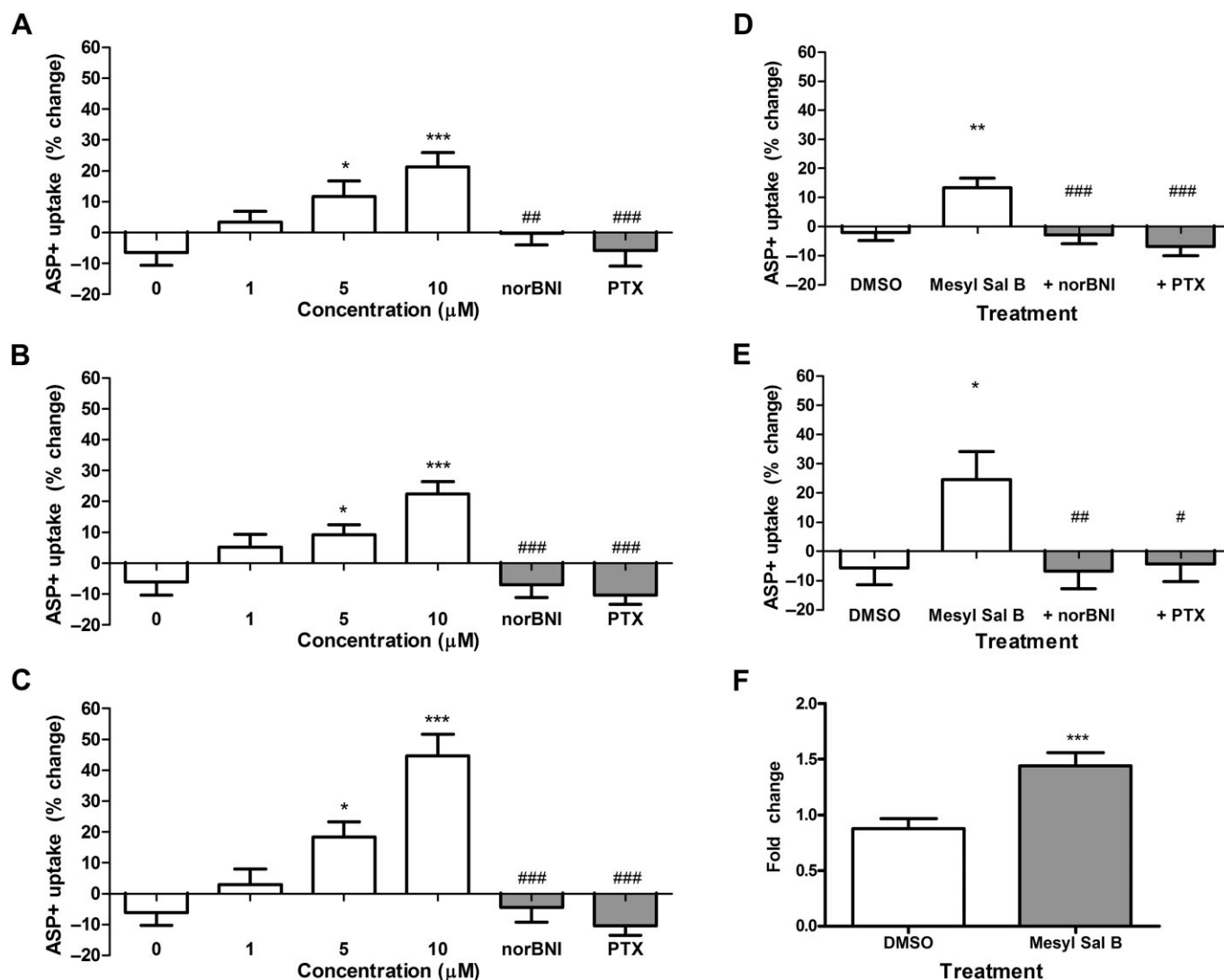


Figure 4

Mesyl Sal B modulates DAT function *in vitro* via a KOP receptor-dependent and PTX-sensitive mechanism in cells transiently expressing YFP-hDAT and Myc-rKOP receptor (A) Mesyl Sal B, (B) U50,488H and (C) SalA cause concentration-dependent increases in ASP+ uptake. These effects (at 10 μM Mesyl Sal B) were norBNI reversible (30 min pretreatment, 1 μM compared to 10 μM drug, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$). Mesyl Sal B (10 μM) also caused an increase in ASP+ uptake in (D) N2A and (E) COS7 cells. (F) ASP+ binding following Mesyl Sal B (10 μM). The addition of DMSO (at time 0) shows that the vehicle control did not lead to a significant decrease in uptake from vehicle for any of the agonists (one sample Student's *t*-test). Experiments were carried out over three transfections with at least two dishes used per transfection ($n = 46\text{--}64$ cells). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the vehicle control without KOP receptor agonist (mean \pm SEM).

has a mesylate group ($-\text{SO}_2\text{-CH}_3$) substitution at the C-2 position and retains selective KOP receptor binding affinity and potency (Harding *et al.*, 2005). No previous *in vivo* work has been reported on Mesyl Sal B; therefore, we determined its duration of action in mice relative to the known antinociceptive properties of KOP receptor agonists (Simonin *et al.*, 1998; John *et al.*, 2006; McCurdy *et al.*, 2006). Results show that Mesyl Sal B has a slower, more prolonged onset of action *in vivo* compared with SalA. The effects we show here for SalA are consistent with the literature with antinociceptive effects in the tail-flick assay also reported at 10 min with 1 $\text{mg}\cdot\text{kg}^{-1}$ (i.p.) SalA but not at 20 or 30 min (McCurdy *et al.*, 2006). Tail-flick assays performed in mice intrathecally administered

SalA also showed significant antinociceptive effects between 5 and 15 min (John *et al.*, 2006). The longer duration of effects seen with Mesyl Sal B in this study are likely to reflect an improvement in its metabolic stability and bioavailability.

Novel neoclerodanes based on the structure of SalA have been suggested as potential targets for the development of anti-addiction pharmacotherapies (Beguin *et al.*, 2006; Prisinzano and Rothman, 2008; Prevatt-Smith and Prisinzano, 2010). Here, we show that Mesyl Sal B retained the ability to dose-dependently attenuate cocaine-prime induced drug-seeking or drug-taking behaviour in a KOP receptor-dependent manner when administered acutely, just like the parent compound SalA (Morani *et al.*, 2009). While

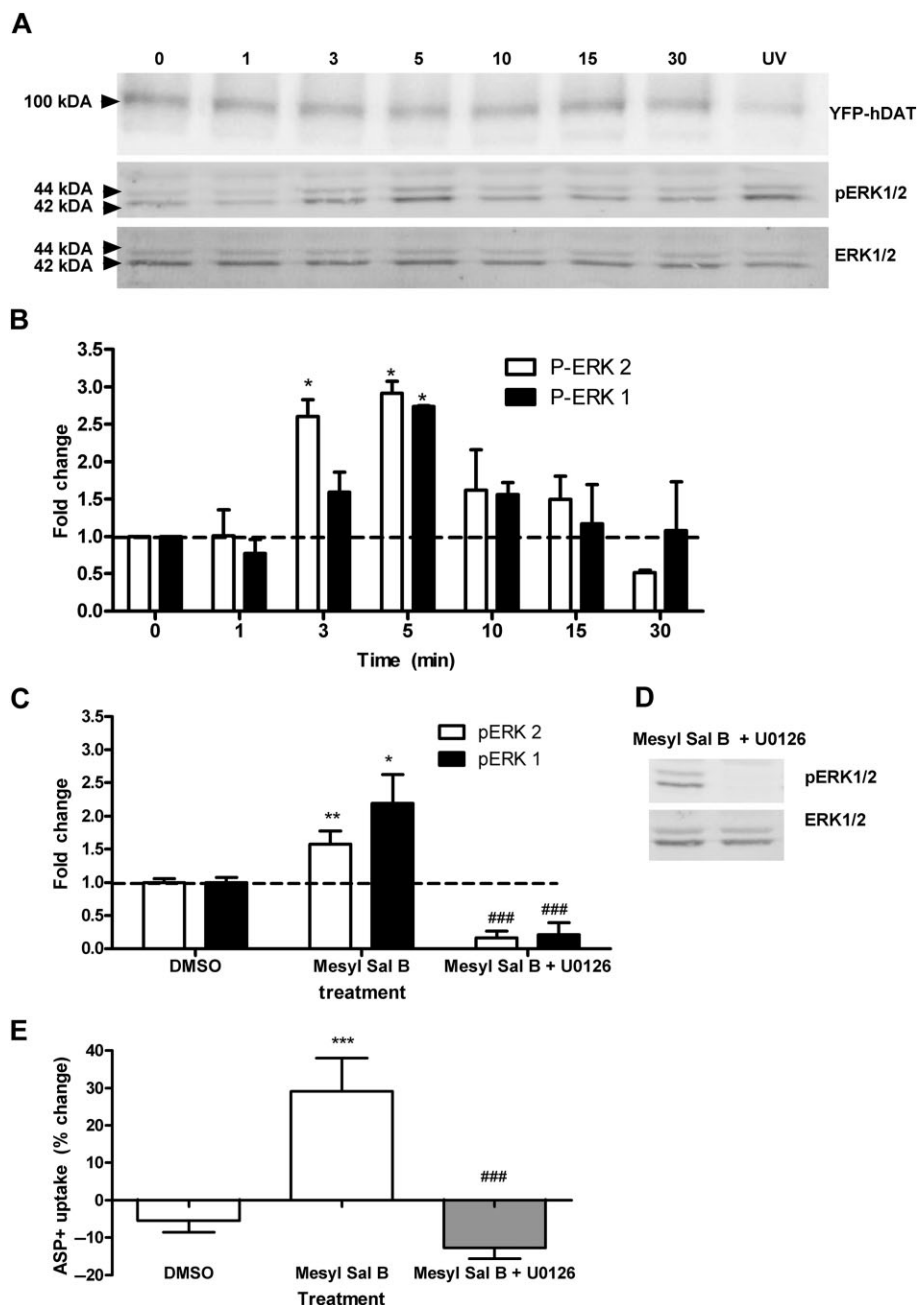


Figure 5

Mesyl Sal B modulates DAT function through an ERK1/2-dependent pathway. (A) Fluorescent scan of a representative gel showing YFP-hDAT expression (top), P-ERK1/2 expression (middle) and total ERK1/2 expression (bottom) after Mesyl Sal B treatment (10 μ M). (B) P-ERK1/2 expression was normalized to total ERK1/2 and expressed graphically as a fold change compared with time 0 (one-way ANOVA followed by Bonferroni post test, $*P < 0.01$, $n = 4$). (C & E) Inhibition of ERK1/2 activation by U0126 pretreatment (20 μ M, 30 min) one-way ANOVA followed by Bonferroni post test, $*P < 0.05$, $**P < 0.01$ compared with appropriate control, $###P < 0.001$ compared with appropriate Mesyl Sal B treated control, $n = 6$. (D) A representative Western blot of the effect of U0126 on the Mesyl Sal B-induced phosphorylation of ERK1/2. (E) The increase in uptake caused by Mesyl Sal B was inhibited by U0126; one-way ANOVA followed by Bonferroni post test, $***P < 0.001$ compared with DMSO control, $###P < 0.001$ compared with Mesyl Sal B treated control respectively ($n = 32$ –50). The addition of DMSO as a vehicle control did not lead to a significant decrease in uptake from 0 (one sample Student's *t*-test).

this model is limited to cocaine-produced reinstatement following extinction and does not attempt to evaluate cue or stress-induced behaviour, this widely used model does allow us to make direct comparisons with traditional and novel

KOP receptor agonists utilizing the same model (Schenk and Partridge, 1999; 2000; Schenk *et al.*, 1999; 2000; Morani *et al.*, 2009; 2013; Prevatt-Smith *et al.*, 2011). Our findings are consistent with ICSS measurements of reward following acute

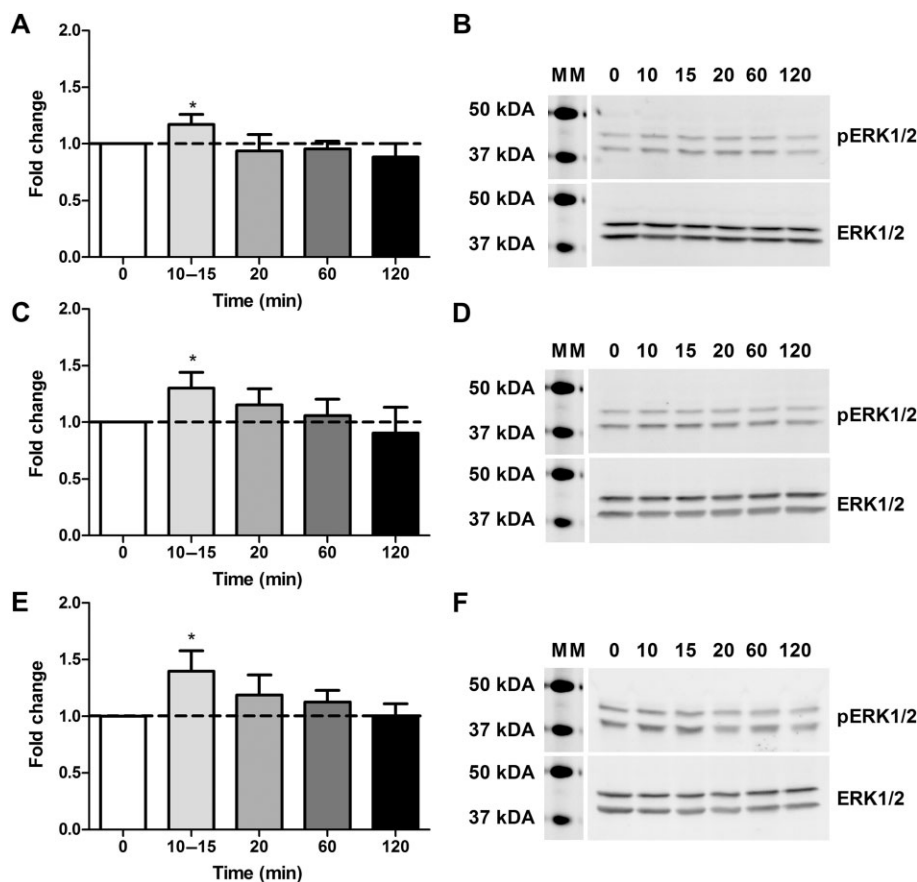


Figure 6

Mesyl Sal B modulates ERK phosphorylation *in vivo*. (A) P-ERK1/2 expression in the dStr, (C) NAcB and (E) prefrontal cortex was normalized to total ERK1/2 and expressed as fold change (mean \pm SEM) compared with time 0. ERK1/2 phosphorylation following Mesyl Sal B administration (Student's *t*-test, * $P < 0.05$, $n = 6-17$). Representative Western blot showing P-ERK1/2 expression (top) and ERK1/2 expression (bottom) after Mesyl Sal B administration in (B) dStr, (D) NAcB and (F) prefrontal cortex. MM = molecular weight marker.

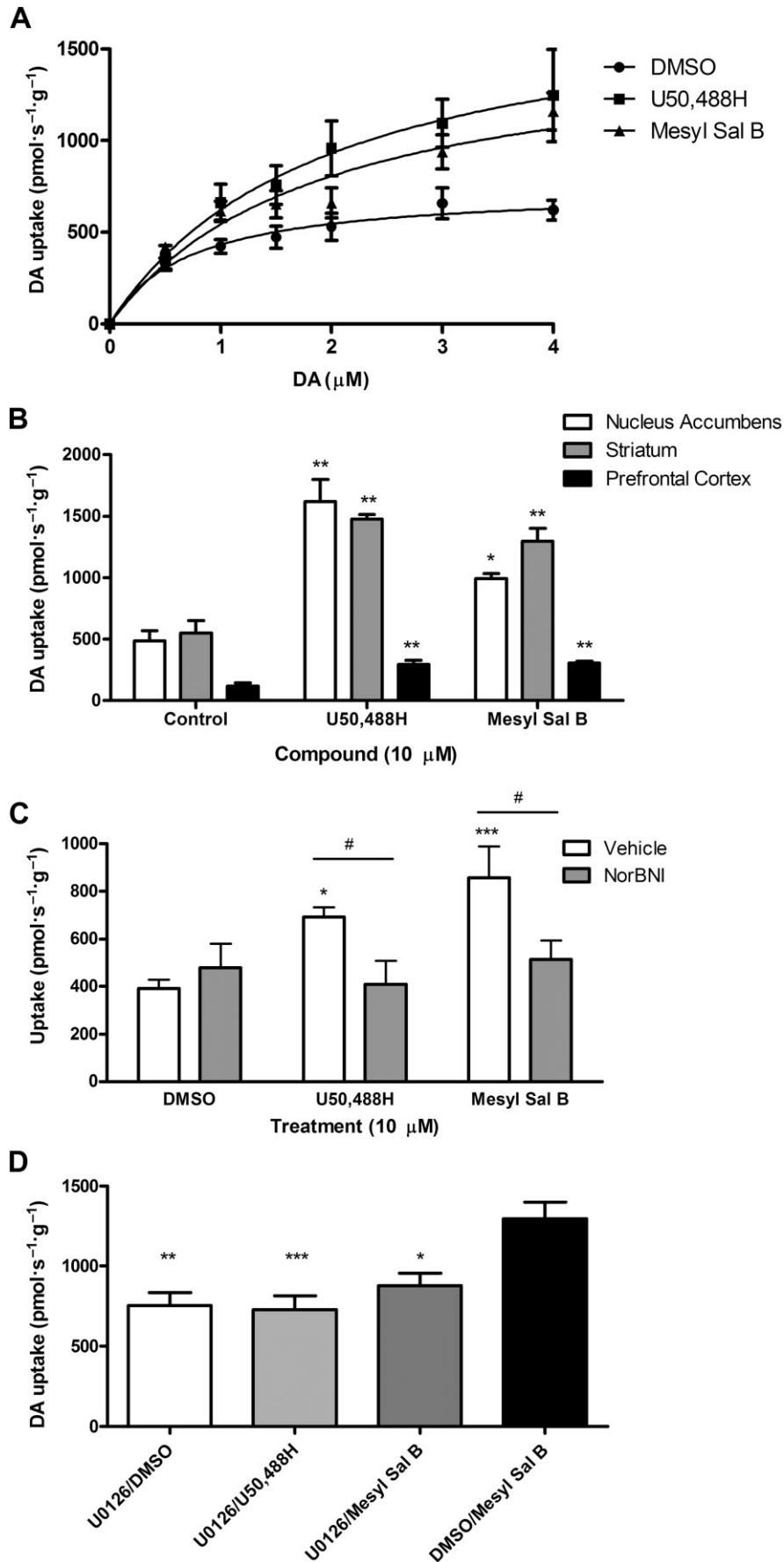
Figure 7

Mesyl Sal B modulates DAT function *ex vivo* via a KOP receptor dependent mechanism. (A) NAcB tissue was pre-incubated with vehicle (DMSO matched), or KOP receptor agonist U50,488H or Mesyl Sal B before sequential addition of dopamine. Data points shown are the mean \pm SEM ($n = 6-8$ tissue homogenates). Mesyl Sal B significantly increased V_{max} (1567 ± 228 , $\text{pmol}\cdot\text{s}^{-1}\cdot\text{g}^{-1}$) (** $P < 0.01$) with no change in K_m (1.9 ± 0.6 μM) compared with vehicle control. U50,488H also showed an increase in V_{max} [1837 ± 406 $\text{pmol}\cdot\text{s}^{-1}\cdot\text{g}^{-1}$] (* $P < 0.05$) with no change in K_m (1.9 ± 0.9 μM). (B) Mesyl Sal B (500 nM) and U50,488H showed a significant increase in dopamine clearance in the NAcB ($P < 0.05$ and $P < 0.01$, respectively), dorsal striatum ($P < 0.01$ and $P < 0.01$, respectively) and prefrontal cortex ($P < 0.01$ and $P < 0.01$, respectively) following a single addition of dopamine (2 μM). (C) This increase in dopamine clearance was inhibited by prior incubation of dStr tissue with norBNI (1 μM , 30 min) (# $P < 0.05$). Incubation with norBNI alone had no effect on dopamine uptake. Pre-incubation of dStr tissue with U0126 (10 μM) prevented Mesyl Sal B from increasing DAT function (Mesyl Sal B + U0126 compared with DMSO + U0126 ($P = 0.9368$); Mesyl Sal B/vehicle compared with Mesyl Sal B + U0126 (** $P < 0.001$); one-way ANOVA followed by Bonferroni post test, ($n = 6-7$). DA = dopamine.

SalA (Potter *et al.*, 2011). In a cocaine challenge following repeated SalA, a decrease in the reward effects of cocaine were also reported. However, the delayed effects of repeated SalA at 24 h were also shown to increase the rewarding impact of ICSS (Potter *et al.*, 2011). Acute activation of the KOP receptor system is widely accepted to have anticocaine effects (Schenk and Partridge, 1999; Schenk *et al.*, 1999; Morani *et al.*, 2009; Potter *et al.*, 2011); in contrast to delayed (Potter *et al.*, 2011) or chronic exposure which shows KOP receptor agonists may potentiate the rewarding effects of drugs by activating stress,

anxiety systems or negative emotional states (McLaughlin *et al.*, 2003; Koob and Le Moal, 2008; Wee *et al.*, 2009; Koob *et al.*, 2014). It is worth noting that studies on SalA have shown both pro- (Carlezon *et al.*, 2006; Morani *et al.*, 2009) and antidepressive effects (Braidia *et al.*, 2008; 2009; Harden *et al.*, 2012), whereas, traditional KOP receptor agonists are consistently reported to be pro-depressive and aversive (Van't Veer and Carlezon, 2013).

SalA has been shown previously to decrease dopamine levels in ventral (Carlezon *et al.*, 2006) and dStr (Chartoff



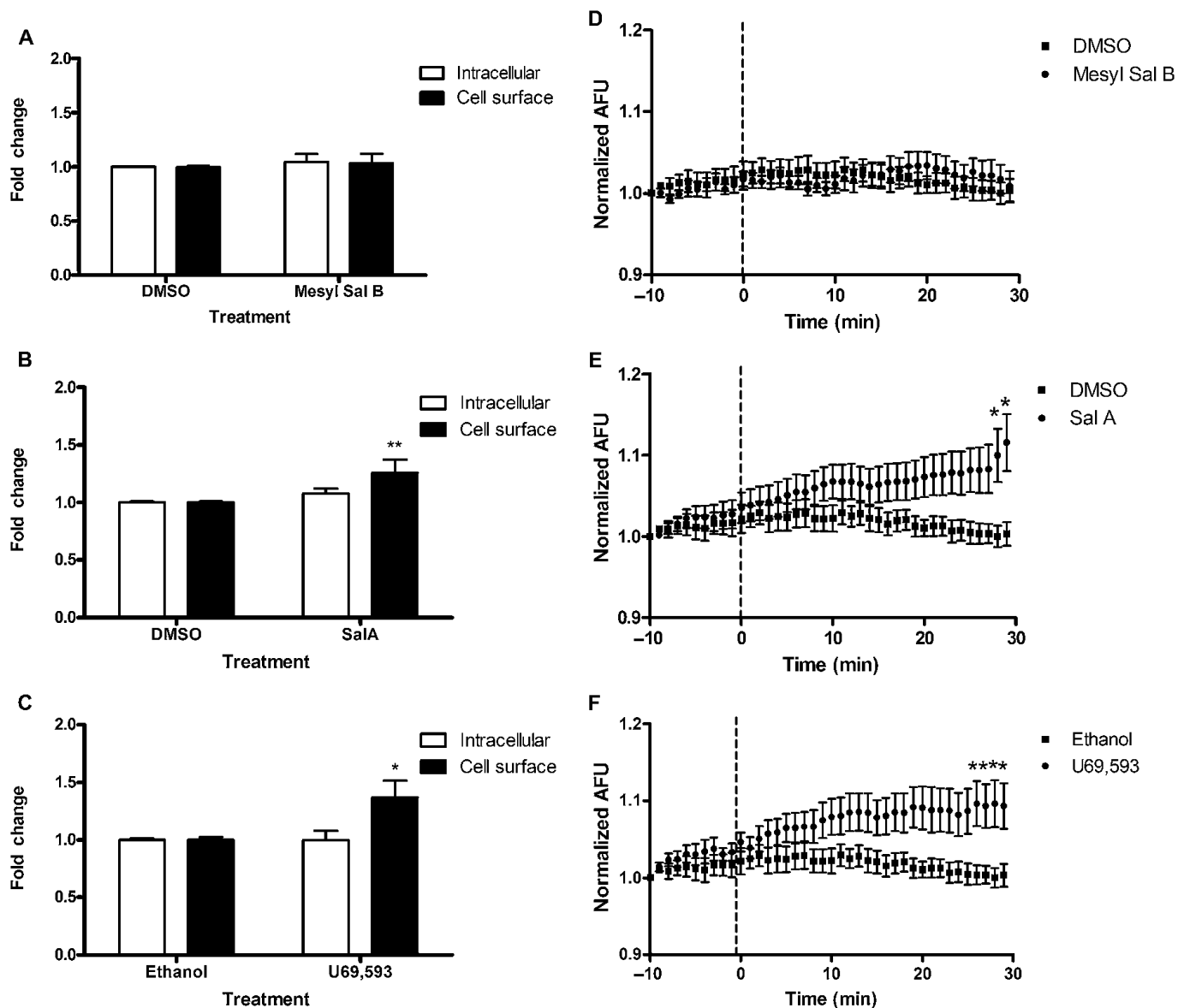


Figure 8

The increase in DAT function following Mesyl Sal B is not via an increase in DAT cell-surface expression in HEK-293 cells. (A–C) HEK-293 cells were incubated with KOP receptor agonists (10 μ M, 30 min) before biotinylation studies. (D–F) TIRFM studies were carried out on HEK-293 cells expressing YFP-hDAT and myc-rKOP receptor with drug addition occurring at 10 min. (A) Mesyl Sal B biotinylation (one-way ANOVA followed by Bonferroni post test; $n = 12$ –13), (D) Mesyl Sal B TIRFM experiments (two-way ANOVA followed by Bonferroni post test; $n = 9$ –10). (B) U69,593 biotinylation (one-way ANOVA followed by Bonferroni post test, $*P < 0.05$; $n = 6$ –8), (E) TIRFM studies with U69,593 (two-way ANOVA followed by Bonferroni post test, $*P < 0.05$, $**P < 0.01$; $n = 12$). (C) SalA biotinylation experiments (one-way ANOVA followed by Bonferroni post test, $**P < 0.01$; $n = 12$ –16) and (F) TIRFM studies with Sal A (two-way ANOVA followed by Bonferroni post test, $*P < 0.05$; $n = 7$ –9). T = total, I = internal, CS = cell surface.

et al., 2008; Gehrke *et al.*, 2008) and modulate cocaine-induced locomotor behaviours by interacting with dopamine D_1 receptor signalling in the dStr (Chartoff *et al.*, 2008). In the present study, SalA (Morani *et al.*, 2009) and Mesyl Sal B attenuated cocaine seeking via KOP receptor activation. Cocaine seeking has been attributed to dopaminergic mechanisms (Schmidt *et al.*, 2005), and KOP receptor activation has previously been shown to modulate dopamine neurotransmission (Thompson *et al.*, 2000). Therefore, the ability of

SalA and Mesyl Sal B to modulate dopamine neurotransmission is a possible mechanism underlying their anticocaine effects. It is well known that endogenous KOP receptor activation and administration of KOP receptor agonists attenuate dopamine release (Chefer *et al.*, 1999; 2000; Gray *et al.*, 1999; Thompson *et al.*, 2000; Carlezon *et al.*, 2006). Ultrastructural studies by Svingos *et al.* (2001) showed that KOP receptors and dopamine transporters are colocalized in the NAcB, identifying an additional mechanism whereby activation of KOP

receptors may regulate cocaine-induced behaviours. In the current study, we show that Mesyl Sal B, by activation of KOP receptors, increased DAT function in cells and brain tissue, similar to SalA and the traditional KOP receptor agonist U50,488H.

Many studies have shown differences in dopamine regulation between the NAc and dStr (Roitman *et al.*, 1999; Wu *et al.*, 2001; Richards and Zahniser, 2009), and there is evidence that the regulation of DAT by KOP receptors is different between these brain regions (Chefer *et al.*, 1999; Thompson *et al.*, 2000). In rats, *ex vivo* RDEV experiments showed increased dopamine uptake in the ventral but not dStr following acute exposure to U69,593 (Thompson *et al.*, 2000). Here, we are the first to show that Mesyl Sal B rapidly increases DAT function in HEK-293, N2A and COS7 cells and in rat brain tissue taken from the dStr, prefrontal cortex and NAc. Because these effects are seen *in vitro*, and in multiple neuronal and non-neuronal cell lines, it is unlikely that intact brain circuitry is required for these effects within individual cells. The differences between the study by Thompson *et al.* (2000) using U69,593 and the current study with Mesyl Sal B and U50,488H are that we observed increases in DAT function in all tissue coexpressing DAT and KOP receptors. These differences are likely to reflect our use of tissue treated *in vitro* rather than *ex vivo*. Quantitative microdialysis experiments in mice have shown no change in dopamine uptake *in vivo* in the dStr following SalA (Gehrke *et al.*, 2008) nor in the NAc in rats using fast-scan cyclic voltammetry (Ebner *et al.*, 2010). Further investigations are needed to determine if a direct interaction between KOP receptors and DAT exists *in vivo*.

It is established that SalA activates ERK1/2 rapidly at 15 min and repeated SalA activates ERK1/2 and CREB in the NAc (Potter *et al.*, 2011). Here, we show that acute Mesyl Sal B administration rapidly and transiently activates ERK1/2 *in vitro* at 3 and 5 min and at 10–15 min in the NAc, dStr and prefrontal cortex of the rat *ex vivo*. No change in late phase ERK1/2 activation was seen with Mesyl Sal B (Figure 6). Early phase ERK1/2 activation by KOP receptors is G-protein mediated and β -arrestin independent; whereas, late activation (2 h) is β -arrestin dependent (McLennan *et al.*, 2008). In astrocytes, U69,593 has been shown to cause both early- and late-phase ERK1/2 activation in contrast to the SalA analogue Mom Sal B, which only shows early phase ERK1/2 activation (McLennan *et al.*, 2008). The lack of late-phase ERK1/2 activation at 60 and 120 min with Mesyl Sal B (Figure 6) and SalA analogue Mom Sal B is in contrast to U69,593 which shows both early- and late-phase activation (McLennan *et al.*, 2008). Findings presented in this study support the proposal that SalA analogues possess different signalling properties to traditional KOP receptor agonists. Further studies into the recruitment of β -arrestin and p-38 MAPK activation are warranted to evaluate the ligand bias of these novel SalA analogues.

Differential ERK1/2 activation between brain regions may explain reported differences in dopamine regulation seen in previous studies. We show in the current study utilizing ASP+ uptake methods and RDEV that Mesyl Sal B activation of DAT is dependent on rapid ERK1/2 phosphorylation. Pre-incubation with the MEK inhibitor U0126 prevented the Mesyl Sal B induced increase in DAT function *in vitro* (Figure 5E) and in the dStr *ex vivo* (Figure 7). The effects seen

with Mesyl Sal B are consistent with the effects of SalA and U69,593.

A recent study by Zhou *et al.* (2013) identified two low MW KOP receptor agonist scaffolds that displayed potent G-protein activation and weak β -arrestin-2 recruitment (Zhou *et al.*, 2013). Recruitment of β -arrestin is associated with the dysphoric effects of KOP receptor agonists and requires late-phase activation of ERK1/2 (McLennan *et al.*, 2008; Al-Hasani and Bruchas, 2011). It has also been shown that p-38 MAPK activation in the dorsal raphe is required for KOP receptor-mediated dysphoria (Land *et al.*, 2009). Another KOP receptor ligand, 6'-guanidinonaltrindole, was shown to activate G-protein coupling to KOP receptors without β -arrestin-2 recruitment in transfected cells and activated Akt but not ERK1/2 in striatal neurons (Schmid *et al.*, 2013). There is growing support for research into the identification of biased ligands for the development of compounds with the desired therapeutic effects and fewer side effects (Melief *et al.*, 2010; Chavkin, 2011; Cox *et al.*, 2015; Kivell *et al.*, 2014; Thompson *et al.*, 2015).

Both Mesyl Sal B and U50,488H increase DAT function *in vitro* in tissue from the rat NAc (Figure 7). This effect is due to an increased V_{max} with no change in K_m . An increase in V_{max} is suggestive of an increase in cell-surface DAT or functional DAT on the cell surface. TIRFM and cell-surface biotinylation techniques have been used previously to investigate changes in cell-surface levels of transporters for dopamine (DAT) or for 5-HT (SERT) (Bolan *et al.*, 2007; Zapata *et al.*, 2007; Furman *et al.*, 2009; Kivell *et al.*, 2010; Perry *et al.*, 2010). Utilization of these two complementary techniques has identified differences between Mesyl Sal B, SalA and U69,593. We show that Mesyl Sal B had no effect on cell-surface DAT using biotinylation or TIRFM. SalA and U69,593 both increase cell-surface DAT. This suggests that Mesyl Sal B has a different mechanism of action in its modulation of DAT. It should also be noted that the effects on cell-surface expression of DAT with SalA and U69,593 only occur at around 30 min and were not observed at the earlier rapid time points when increases in DAT function are observed, suggesting other mechanisms are also likely to play a role. Despite this, it is clear that differences between Mesyl Sal B, SalA and classic agonists exist. Differences between SalA and traditional KOP receptor agonists have been reported in the literature previously. For example, SalA caused 40% less KOP receptor internalization compared with U50,488H in CHO cells (Wang *et al.*, 2005).

The interaction between KOP receptor activation and DAT is a new potential target for the development of therapeutic drugs to treat addiction, particularly if differences between classic agonists and novel agonists can be identified. The longer duration of action of Mesyl Sal B and the demonstration of anticocaine effects when administered acutely has identified Mesyl Sal B as a SalA analogue with improved pharmacokinetic properties that warrants further investigation into potential therapeutic utility.

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Author contribution

B. M. K. designed the study, contributed to analysis of data and wrote the paper. B. S. performed the experiments, data analysis and wrote the paper. A. W. M. E., L. W., A. S. M. and N. K. performed the research and analysed the data. T. E. P. and D. S. provided salvinorin A and Mesyl Sal B for the study, and T. E. P. provided input into the design of the study and critical review of the manuscript. J. H. M. provided critical input into the writing of the manuscript and analysis of data.

Conflict of interest

The authors have no competing interests to disclose.

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