# *Ex Vivo* Receptor Binding in Rats as a Rapid Screen for CNS Penetration and Occupancy of Abuse-related Molecular Targets

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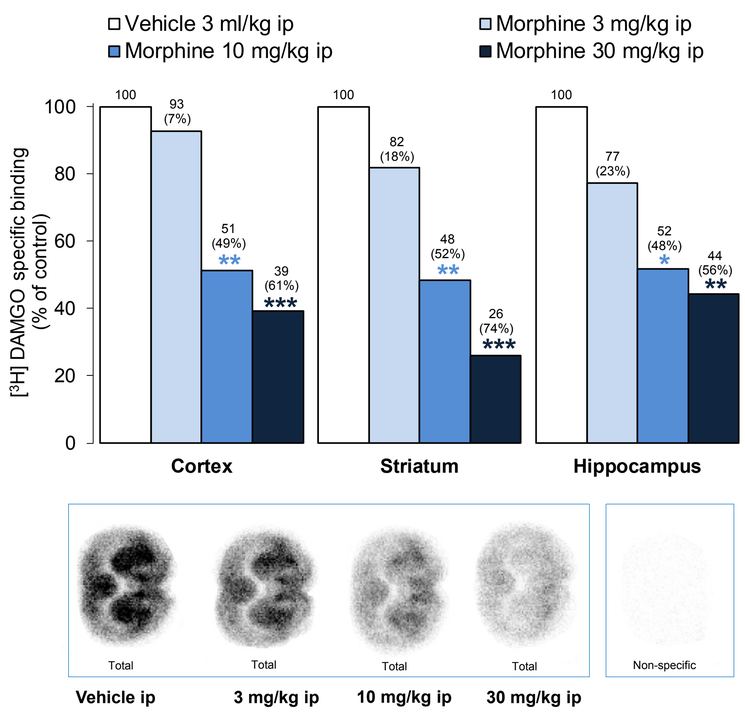
**INTRODUCTION** The molecular targets, e.g. transporters, receptors and ion channels, mediating the actions of substances of abuse are well established. Moreover, radioligand's to label them are available for many of them. We have determined the *ex vivo* occupancy of various drug targets by a range of CNS-active compounds to explore whether this technique can be used to screen for brain penetration and engagement with abuse-related targets. We have also conducted a literature search to determine which substances of abuse are brain penetrant and engage with target receptors *in vivo*.

**METHOD** "In house" ex vivo occupancy studies: Male, Sprague Dawley rats (300±50 g) were administered vehicle, morphine (3, 10 and 30mg/kg ip), buprenorphine (0.1, 0.3 and 1.0mg/kg ip), (-)-pentazocine (5, 10 and 20mg/kg ip), rimonabant (3 and 10mg/kg po), haloperidol (10mg/kg po), risperidone (10mg/kg po), nomifensine (10mg/kg po) and duloxetine (10mg/kg po) and terminated 30 ((-)-pentazocine) or 60 minutes later (all other compounds). For autoradiography, whole brains were removed, two coronal cuts were made, one at the level of the optic chiasm and one at the level of the cerebellum to produce an anterior brain block containing the cortex and striatum and a posterior brain block containing the amygdala and the hippocampus. Brain blocks were placed onto cork disks, covered with Tissue Tek™ and rapidly frozen in isopentane. Coronal sections (20 μm) containing the cortex, striatum, amygdala and the hippocampus were cut. Three adjacent sections were mounted onto each slide. Of these, two sections were used to measure total binding and one section was used to measure non-specific binding. Sections were incubated in 50mM Tris buffer plus additives containing either [$^3$H]DAMGO (2nM), [$^3$H]U-69,593 (2.5nM), [$^3$H]rimonabant (1nM) and [$^3$H]raclopride (1nM) for 10, 90, 15 or 10 minutes, respectively. Non-specific binding was determined by 50μM (-)Naloxone, 10μM U-69,593, 1μM rimonabant and 1μM(-)sulpiride for [$^3$H]DAMGO, [$^3$H]U-69,593, [$^3$H]rimonabant and [$^3$H]raclopride autoradiography, respectively. Binding was terminated by aspiration and sections washed in buffer (3x5 minutes for $^3$H]DAMGO and [$^3$H]U-69,593, 3x15 minutes for [$^3$H]rimonabant , 2x2 minutes for [$^3$H]raclopride ). β-emitting tritium radioactivity bound to the sections was rapidly quantified using a Biospace β-Imager (15 slides per run with a 16hr exposure time). For homogenate binding, whole brains were removed, frontal cortex and striata dissected and frozen at -20°C until required. On the assay day, homogenates were prepared and binding to frontal cortical 5-HT (SERT) and striatal dopamine uptake sites (DAT) were determined using [$^3$H]citalopram (1.3nM) and [$^3$H]WIN 35428 (24nM). Non-specific binding was defined by paroxetine (0.5μM) and GBR 12936 (1μM). Binding was terminated by filtration under vacuum using a Skatron cell harvester, through Skatron 11734 filters and radioactivity determined by liquid scintillation counting.
The literature search was conducted using the PubMed database using the following combined search terms: "drug name", "ex vivo" and "binding".

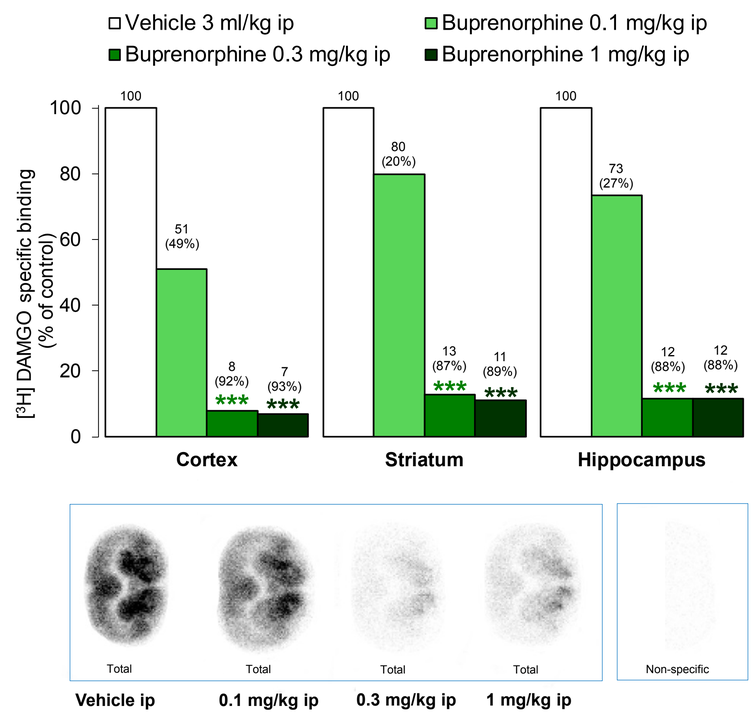
## RESULTS

- Morphine (10 and 30mg/kg ip) occupied μ-opioid receptors labelled by [$^3$H]DAMGO in the rat cortex, striatum and hippocampus (Figure 1).
- Buprenorphine (0.1, 0.3 and 1mg/kg ip) also occupied μ-opioid receptors labelled by [$^3$H]DAMGO in rat cortex, striatum and hippocampus (Figure 2).
- (-)-Pentazocine (5, 10 and 20 mg/kg ip) occupied κ-opioid receptors labelled by [$^3$H]U-69,593 in rat striatum (Figure 3).
- Validation of $CB_1$ *ex vivo* receptor binding was undertaken using the inverse agonist rimonabant (3 and 10 mg/kg po) which significantly inhibited [$^3$H]rimonabant specific binding in the rat cortex (47*** and 87 %***), amygdala (33** and 84 %***) and hippocampus (27** and 77 %***).
- Validation of $D_2$ *ex vivo* receptor binding was undertaken using risperidone and haloperidol (10 mg/kg po) which significantly occupied $D_2$ receptors labelled by [$^3$H]raclopride in the rat striatum (71*** and 94 %***).
- Validation of DAT *ex vivo* transportor binding using [$^3$H]WIN 35,428, which labels the cocaine binding site on the dopamine reuptake transporter, was validated using nomifensine (10 mg/kg po) which occupied 35% of the DAT sites.
- Validation of SERT *ex vivo* transportor binding using [$^3$H]citalopram to label 5-HT reuptake sites was undertaken with duloxetine (10 mg/kg po) and significantly occupied 70%*** of 5-HT reuptake sites.
- The literature search confirmed and extended our findings that *ex vivo* occupancy is a technique that has been utilised in many studies to determine target engagement with a wide range of drugs of abuse (Table 1).

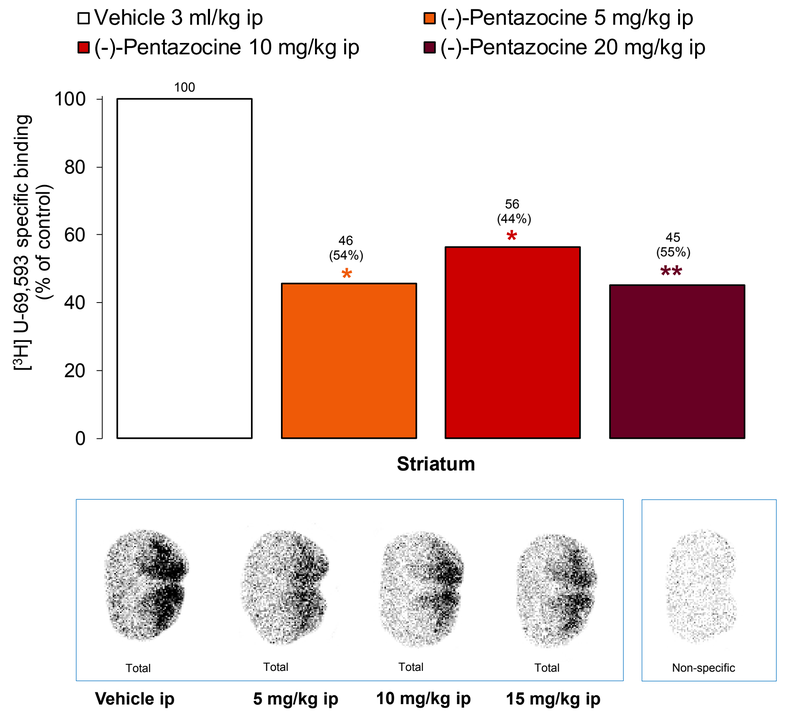
### Figure 1: Mu Opioid Receptor - Morphine




### Figure 2: Mu Opioid Receptor - Buprenorphine




### Figure 3: Kappa Opioid Receptor - (-)-Pentazocine




Results are mean specific binding as a % of control taken as 100% (n=3-5 morphine, 4-5 buprenorphine and (-)-pentazocine. For statistical analyses, data were square root transformed and analysed by one-way ANOVA followed by Williams' test. Significant differences versus vehicle: *p<0.05, **p<0.01, ***p<0.001. Images are autoradiograms of coronal brain sections in rat cortical and striatal sections and are representative samples of each treatment group.

## Table 1: Summary of target engagement of drugs of abuse using *ex vivo* occupancy studies in the literature

| Substance of abuse | Receptor or Target | Radioligand | References |
|---|---|---|---|
| **Opiates** | | | |
| **Mu agonists** | | | |
| Buprenorphine | MOR | [$^3$H]DAMGO | This poster |
| Heroin | MOR | [$^3$H]Naloxone | Sim-Selley et al 2000 |
| Morphine | MOR | [$^3$H]DAMGO | This poster<br>Takai et al 2018 |
| Oxycodone | MOR | [$^3$H]DAMGO | Takai et al 2018 |
| **Kappa agonists** | | | |
| Bremazocine | KOR | [$^3$H]Bremazocine | Yoo et al 2014[1] |
| Nalorphine | KOR | $^3$H]Bremazocine | Shaw et al 1989[1] |
| (-)-Pentazocine | KOR | [3H]U69,593 | This poster |
| Tifluadom | KOR | [$^3$H]Bremazocine | Shaw et al 1989[1] |
| U-50,488 | KOR | [$^3$H]Bremazocine | Shaw et al 1989[1] |
| **NMDA antagonists and dissociative anaesthetics** | | | |
| Ketamine | NMDA | [$^3$H]MK-801 | Lord et al 2013[1]<br>Murray et al 2000[1A, B] |
| Phencyclidine | NMDA<br>$D_2$ | [$^3$H]MK-801<br>[$^3$H]Raclopride | Murray et al 2000[1A, B] |
| **Cannabanoids** | | | |
| $\Delta^9$-THC | CB1 | [$^3$H]WIN 55212-2 | Petitet et al 1999[1] |
| 5F-AKB48 | CB1 | [$^3$H]CP55,940 | Canazza et al 2016[1B] |
| AKB48 | CB1 | [$^3$H]CP55,940 | Canazza et al 2016[1B] |
| JWH-018 (Spice cannabinoid) | CB1 | [$^3$H]CP55,940 | Vigolo et al 2015[1B]<br>Canazza et al 2016[1B] |
| JWH-073 | CB1 | [$^3$H]CP-55,940 | Ossato et al 2016[1B] |
| JWH-250 | CB1 | [$^3$H]CP-55,940 | Ossato et al 2016[1B] |
| WIN-55,212 | CB1 | [$^3$H]WIN 55,212-2 | Petitet et al 1999[1] |

| Substance of abuse | Receptor or Target | Radioligand | References |
|---|---|---|---|
| **Stimulants** | | | |
| Amphetamine | DAT | [$^3$H]WIN 35,428 | Scheffel et al 1996[1A] |
| D-Amphetamine | $D_2$ | [$^3$H]Spiperone | Vassout et al 1993[A] |
| Methamphetamine | DAT<br>$D_2$ | [$^{11}$C]WIN 35,428<br>[$^{11}$C]Raclopride | Villemagne et al 2008[2A, B]<br>Sato et al 2006[1A] |
| Cocaine | DAT<br>$D_2$ | [$^3$H]WIN 35,428<br>[$^3$H]Spiperone | Letchworth et al 2001[2A]<br>Mash et al 2002[3]<br>Peraile et al 2010[1]<br>Vassout et al 1993[A] |
| Modafinil | DAT | [$^{11}$C]WIN35,428 | Madras et al 2006[2A] |
| Nicotine | nAChR<br>$D_1$ | [$^3$H]Methyl-carbamylcholine<br>[$^3$H]SCH-23390 | Lapchack et al 1989<br>Goutier et al 2015 |
| Methylphenidate | DAT<br>$D_1$<br>$D_2$ | [$^3$H]WIN 35,428<br>[$^3$H]SCH 23390<br>[$^3$H]Spiperone | Robison et al 2017<br>Vassout et al 1993[A] |
| Mazindol | DAT | [$^{125}$I]RTI-55 | Staley et al 1994[3] |
| **5-HT2A agonists and hallucinogens** | | | |
| DOI | 5-$HT_{2A}$<br>5-$HT_{2C}$<br>$D_1$ | [$^3$H]Ketanserin,<br>[$^3$H]Mesulergine<br>[$^3$H]SCH23390 | Schindler et al 2012<br>Kettle et al 1999 |

| Substance of abuse | Receptor or Target | Radioligand | References |
|---|---|---|---|
| **Ethanol and GABAergics** | | | |
| Diazepam | $GABA_A$ chloride ion channel | [$^{35}$S]Butylbiclophosphorothionate<br>[$^3$H]Flunitrazepam<br>[$^3$H]GABA | Sanna et al 1991<br>Greenblatt and Sethy 1990[1]<br>Komiskey ,1987<br>Komiskey et al 1988 |
| Ethanol | $GABA_A$ chloride ion channel | [$^{35}$S]Butylbiclo-phosphorothionate<br>[$^3$H]GABA | Sanna et al 1991<br>Komiskey et al 1988 |
| Midazolam | $GABA_A$ chloride ion channel | [$^3$H]Flumazenil<br>[$^3$H]Flunitrazepam | Liefaard et al 2007<br>Misaka et al 2010 |
| Pentobarbital | $GABA_A$ chloride ion channel | [$^3$H]Ro 15-1788 (flumazenil) | Miller et al 1988[A] |
| Pregabalin | $GABA_A$ | [$^3$H]Gabapentin<br>[$^3$H]Pregabalin | Bian et al 2006[1] |
| **MDMA, other entactogens and designer drugs** | | | |
| MDMA | SERT<br>5-$HT_2$<br>DAT | [$^{123}$I]beta-CIT<br>[$^{125}$I]MIL<br>[$^3$H]WIN35,428 | Reneman et al 2002[A]<br>Scheffel et al 1992[A, B]<br>Peraile et al 2010[1] |
| Mephedrone | DAT<br>SERT<br>5-$HT_{2A}$<br>$D_2$ | [$^3$H]WIN35,428<br>[$^3$H]Paroxetine<br>[$^3$H]Ketanserin<br>[$^3$H]Raclopride | Martinez-Clemente et al 2011[B] |

A = *In vivo* receptor occupancy study but the technique can be transferred to ex vivo occupancy studies using this radioligand
B = *In vitro* displacement study but the technique can be transferred to ex vivo occupancy studies using this radioligand
1 = Experiments performed in mice.
2 = Experiments performed in monkeys
3 = Experiments performed in humans

**Abbreviations**
$\Delta^9$-THC = $\Delta^9$-tetrahydrocannabinol
DAMGO = [D-Ala$^2$, NMe-Phe$^4$, Gly-ol$^5$]-enkephalin
DOI = 2,5-dimethoxy-4-iodoamphetamine
KOR = kappa opioid receptor
MDMA = 3,4-methylenedioxymethamphetamine
[$^{125}$I]MIL = N1-Methyl-2-[$^{125}$I]lysergic acid diethylamide
MOR = mu opioid receptor
5MeO-DMT = 5-methoxy-N,N-dimethyltryptamine

## CONCLUSIONS

- Our *ex vivo* receptor binding results demonstrated occupancy of abuse-related targets in discrete brain regions after administration of a range of agonists, partial agonists and antagonist reference compounds.
- The literature search confirmed and extended our findings that this technique has been employed with a wide range of abuse-related targets in addition to the above for investigating target engagement with other abused drugs including ethanol, MDMA, other entactogens and "legal highs", NMDA antagonists and dissociative anaesthetics.
- These findings demonstrate the value of *ex vivo* receptor binding as a technique to assess brain penetration and as a screen to investigate target engagement and therefore possible abuse potential of CNS-active drugs.

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