

**The role of the metabotropic glutamate 5 and  
adenosine 2A receptors in  
methamphetamine addiction**

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## Abstract

Methamphetamine (METH) is a highly addictive psychostimulant for which there are no pharmacotherapies. Current theories of drug addiction suggest a dysregulation of dopamine and glutamate systems in the development and maintenance of addiction. Two receptors which modulate dopamine and glutamate transmission, and which have been implicated in animal models of drug-taking behaviour for other drugs of abuse (e.g. alcohol, cocaine, opiates) are the metabotropic glutamate 5 (mGlu5) and the adenosine 2A ( $A_{2A}$ ) receptors. This project used germline KO mice to identify the role of these receptors in METH-induced behaviour, and determine a neural locus where these receptors might act to mediate this behaviour.

Germline deletion of mGlu5 resulted in a deficit in extinction learning for METH in an operant self-administration paradigm, and an increased propensity to reinstate to drug-associated cues. mGlu5 KO mice also demonstrated enhanced locomotor activity when re-exposed to a drug-associated context compared to wildtype (WT) littermates, suggesting mGlu5 may modulate the contextual salience of drug-associated cues and contexts. In contrast,  $A_{2A}$  KO mice exhibited abolished conditioned place preference (CPP) and a reduction in the motivation to self-administer METH under high response requirements. There was also a reduction in sucrose self-administration under higher reinforcement schedules in  $A_{2A}$  KO mice, suggesting this receptor is involved in the rewarding and motivational properties of both METH and sucrose.

c-Fos immunohistochemistry was used to determine a locus where  $A_{2A}$  could mediate the rewarding properties of METH, as assessed by CPP. Initially, Fos-immunoreactivity (IR) was examined following the expression of METH CPP in  $A_{2A}$  WT and KO mice; however, there was a global reduction in Fos-IR throughout the forebrain in  $A_{2A}$  KO mice, preventing the identification of a potential locus. A second experiment was conducted in  $A_{2A}^{loxP/loxP}$  mice, examining Fos-IR following the expression vs. non-expression of CPP. This experiment identified the nucleus accumbens (NAcc) shell and the infralimbic cortex as regions activated following the expression of METH CPP. From this, it was hypothesised that  $A_{2A}$  activity in the NAcc shell might mediate

METH reward. This hypothesis was addressed using viral mediated knockdown of  $A_{2A}$ . Adeno-associated virus encoding Cre-recombinase (AAV-Cre) or mCherry (a control fluorophore) were microinjected into the rostral medial NAcc shell of  $A_{2A}^{loxP/loxP}$  mice. This resulted in a deletion of approximately 20% of  $A_{2A}$  in the rostral medial NAcc shell. There was no effect of AAV-Cre mediated deletion on the expression of METH reward or METH-induced locomotor behaviour. Furthermore, there was no correlation between the degree of knockdown and CPP, supporting the conclusion that a ~20% knockdown of  $A_{2A}$  in the rostral medial NAcc shell had no effect on METH reward.

In summary, the findings of this thesis implicate  $A_{2A}$  in reward and motivated behaviour for METH, but also in these behaviours for natural reinforcers such as sucrose. Although neural correlates suggested increased activity in the NAcc shell during the expression of METH reward-context associations, I was unable to confirm the involvement of  $A_{2A}$  in this behaviour using region specific receptor knockdown. In contrast, mGlu5 appears involved in cognitive processes associated with recognition of drug-associated stimuli and the extinction of drug-taking behaviour.

## Declaration

This is to certify that:

- the thesis comprises only my original work towards the PhD except where indicated in the Preface,
- due acknowledgment has been made in the text to all other material used,
- the thesis is fewer than 100,000 words in length, exclusive of tables, figures, references and appendices

Signed,

Rose Chesworth

Date:

## **Preface**

The author would like to acknowledge the following people for their contributions toward data collection and for their technical assistance. The mice used for self-administration in Chapters 3 and 4 were cannulated by Prof. Andrew Lawrence with the assistance of the following laboratory members at various times: Dr. Robyn Brown, Dr. Heather Madsen, Nicola Chen and Karlene Scheller. For the publication in Chapter 3, assistance with experimental design and manuscript revisions were provided by Prof. Andrew Lawrence, Dr. Robyn Brown, and Dr. Jee Hyun Kim. Also, Dr. Jee Hyun Kim assisted with the perfusions performed in Chapter 5. All other work was conducted by the author.

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## Publications in Peer-Reviewed Journals

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## Abstracts

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## List of Abbreviations

AAV – adeno-associated virus

A<sub>2A</sub> – adenosine 2A receptor

ALP – active lever press

AMPA -  $\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA - analysis of variance

CDPPB: 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide

CPP – conditioned place preference

Cre – Cre-recombinase

CREB - cAMP response element-binding protein

CS+ – conditioned stimulus

D<sub>1</sub> – dopamine D<sub>1</sub> receptor

D<sub>2</sub> – dopamine D<sub>2</sub> receptor

DAB - 3,3'-diaminobenzidine tetrahydrochloride

DARPP-32 - cAMP-regulated phosphoprotein 32 kDa

DAT – dopamine transporter

dmPFC - dorsomedial prefrontal cortex

ERK - extracellular signal-regulated kinases

FR – fixed ratio

GABA -  $\gamma$ -Aminobutyric acid

GLT-1 - glial glutamate transporter 1

GluA – AMPA receptor subunit

GluN - NMDA receptor subunit

IL - infralimbic cortex

ILP – inactive lever press

IR – immunoreactivity

IVSA – intravenous self-administration



KO – knockout

LTD – long term depression

LTP – long term potentiation

MDMA - 3,4-methylenedioxyamphetamine

METH – methamphetamine

mGlu – metabotropic glutamate receptor

MPEP - 2-methyl-6-(phenylethynyl)-pyridine

mPFC – medial prefrontal cortex

mRNA – messenger RNA

MSN – medium spiny neuron

MTEP - 3-((2-methyl-4-thiazolyl)ethynyl)pyridine

NAcc – Nucleus accumbens

NAM – negative allosteric modulator

NDS – normal donkey serum

NHS – normal horse serum

NMDA - N-methyl-D-aspartate

NR – NMDA receptor subunit

PAM – positive allosteric modulator

PBS - phosphate-buffered saline

PCR – polymerase chain reaction

PFA – paraformaldehyde

PFC – prefrontal cortex

PKA - cAMP-protein kinase A

PL – prelimbic cortex

PR – progressive ratio

RM - repeated measures

SD – standard deviation

SEM – standard error of the mean

SERT – serotonin transporter

TLP – total lever press

VMAT-2 - vesicular monoamine transporter-2

VP – ventral pallidum

VTA – ventral tegmental area

WT – wild type

# Chapter 1

## Introduction

### 1.1. Addiction: definition, prevalence, and importance

Drug addiction is a chronic, relapsing disorder characterised by compulsive drug use, despite known negative consequences [1]. This condition affects millions around the world, decreasing quality of life not only through issues directly associated with drug use (e.g. violence, poverty) but also through increased risk of chronic health and psychiatric disorders (e.g. depression, cardiovascular disease, cancer). The World Health Organisation (WHO) estimates drug abuse disorders affect up to 16% of the population worldwide, or 1.1 billion people [2]. The financial and humanitarian burden of these estimates is considerable; in 2010, disorders associated with alcohol and drug abuse accounted for more than 36 million disability adjusted life years [3]. Importantly, drug prevention programs can reduce drug-related harm and considerably reduce subsequent costs in related areas (e.g. health care, crime) [4].

The global cost of drug abuse and dependence is reflected in Australian statistics. In 2003, complications arising from alcohol, tobacco and illicit drug use contributed to 13% of the total burden of disease and injury in Australia [5]. Continuing burden is placed on society by the use of illicit substances; the estimated cost of illicit drug abuse was \$8.2 billion in 2004-05 [6]. The Australian Federal Government expenditure on the control of drug abuse mirrors this burden; the total estimated expenditure on illicit drug abuse alone in 2009/10 was \$1.7 billion, with more than a third (>\$500 million) allocated to programs to assist the control of drug-taking [7]. Furthermore, national strategies such as the National Drug Strategy, the National Drugs Campaign, and the National Amphetamine-Type Stimulant Strategy have been recently implemented to help reduce drug use and the harmful effects this has on the individual and society. It is clear that substantial resources are being put into the control, restriction and

treatment of drug abuse and addiction. For the sake of brevity this review will focus on the treatment of drug addiction. Current treatment programs focus strongly on counselling-based approaches, often proving most successful when paired with pharmacotherapeutic intervention [8]. However, for a number of drugs of abuse, pharmacotherapeutic options are lacking.

## **1.2. Methamphetamine abuse and treatment options**

One area of considerable interest in addiction research is the development of therapeutic treatments for psychostimulant addiction. Currently there are no Food and Drug Association (FDA) approved treatments for any illicit psychostimulants (e.g. amphetamine, ecstasy), despite abuse of this drug class remaining high in Australia compared to the rest of the world [9]. Almost 10% of the population have used psychostimulants in their lifetime, and 4-5% of the population report recent psychostimulant use [10]. Of these, methamphetamine (METH) is a highly addictive psychostimulant with significant health, financial and social consequences. Importantly, Australia has some of the highest *per capita* usage rates of METH in the world [11, 12], with over 6% of the population reporting having tried METH and 2-3% of the population reporting regular METH use [11, 13, 14]. This is particularly concerning as the standardised mortality risk (the risk of mortality adjusted for age, sex and ethnicity) is greater for METH users compared to cannabis, alcohol and cocaine [15]. Furthermore, a number of reports highlight the strain METH use places upon hospitals, mental health services and law enforcement agents. Specifically, there are increased acute episodes of aggression and psychosis [16-20], persistent mental health problems (e.g. anxiety, depression [21, 22]), and increased crime and violent offences [21, 23]. For the individual, chronic METH use has been associated with a number of physical and mental health complications, such as cardiovascular complaints (e.g. heart rate variability, increased blood pressure), increased risk of stroke [18, 19]; for reviews see [24, 25]); cognitive decline [26] and psychological conditions (e.g. increased anxiety, depression and paranoia [21, 27, 28]).

Despite the social and economic burden of METH abuse there are currently no clinically validated pharmacotherapies for METH addiction [11, 29], which has been identified as a barrier to rehabilitation [11, 29]. Outpatient studies suggest pharmacological treatment trials for METH addiction have little effect on reducing METH use (for reviews, see [30-33]). Bupropion, a dopamine and noradrenaline reuptake inhibitor, was first reported to reduce the acute METH-induced 'high' and cravings [34]. However, in a 12 week randomized, placebo-controlled trial, bupropion demonstrated no overall effect on METH use or cravings for the drug, although an *ad hoc* analysis of a subset of participants with low baseline METH use showed reduced METH use [35, 36]. A recent randomised pilot trial also indicates bupropion is not effective in reducing METH use and craving in adolescents [37]. A randomised controlled trial of imipramine, a tricyclic antidepressant, demonstrated 150mg imipramine per day for 180 days did not reduce METH withdrawal-associated depression scores, craving or abstinence in an outpatient setting [38]. A double blind, within subjects cross over design trial found isradipine (a calcium channel blocker, given at a dose of 15mg/day) only reduced METH liking and preference when administered 2hr prior to METH use, but not when administered on days leading up to METH administration, suggesting isradipine only acutely reduces measures of METH liking [39]. Attempted substitution therapy using methylphenidate (a dopamine and noradrenaline reuptake inhibitor) at a maximum daily dose of 54mg for 22 weeks did not demonstrate efficacy over placebo in reducing METH use and craving in a recent randomized, double-blind, placebo-controlled trial of outpatients in both Finland and New Zealand [40]. Aripiprazole, a partial dopamine D<sub>2</sub> (D<sub>2</sub>) family agonist, did not reduce METH consumption or abstinence in randomised, double blind, placebo controlled trials lasting up to 12 weeks [41, 42]. Recent double-blind, placebo-controlled pilot trials using 200-400mg modafinil for 3-7 days (which has complex mechanisms of action involving modulation of GABA, glutamate, dopamine and histamine systems [43]) for treatment of the cognitive deficits induced by repeated METH use have shown some efficacy for improvement of working memory and verbal recall, but not other cognitive faculties [44, 45]. Importantly, a recent randomised controlled trial

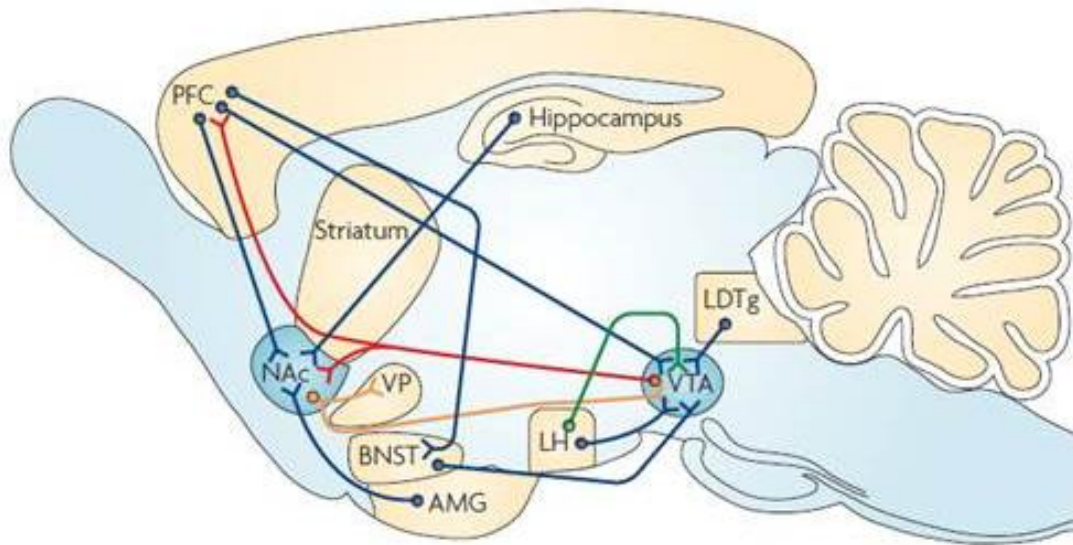
demonstrated no effect of daily 400mg modafinil treatment for 12 weeks on METH use or craving [46].

It is clear pharmacological treatment for METH abuse is lacking. This is a major cause for concern, as the combination of pharmacological treatment with psychological therapy (e.g. counselling) often leads to the greatest improvement of outcomes for substance abuse patients [8]. Moreover, a treatment strategy targeted at reversing the long term effects of METH use on the brain may be of more benefit than current therapeutic options, some of which appear to merely reverse acute drug effects (e.g. bupropion [34]). For this it is crucial to understand the acute and long term effects of METH consumption on the brain.

### **1.3. Methamphetamine – pharmacology; acute and long term effects**

The acute effects of METH include increased alertness, confidence and euphoria [47-49], as well as elevated blood pressure, heart rate [48, 50] and decreased appetite [51]. Pharmacologically, METH acts acutely as an indirect agonist, increasing dopamine, serotonin and noradrenaline release from presynaptic vesicular stores by reversing the function of vesicular monoamine transporter-2 (VMAT-2) and disrupting cytosolic pH ([52]; for reviews see [53-56]), and blocking their reuptake at the presynaptic membrane ([57, 58]; for reviews see [53-55]). This results in excess neurotransmitter concentration at the synaptic cleft. Acute METH administration also increases striatal extracellular glutamate concentration from presynaptic stores; however the mechanism driving this is unclear [59-63].

METH has a major influence upon the mesocorticolimbic pathway, which connects the ventral tegmental area (VTA) to medial prefrontal cortex (mPFC), the nucleus accumbens (NAcc), hippocampus and amygdala; this pathway is primarily involved in reward-related and motivated behaviours [53]. METH also modulates the nigrostriatal pathway, which connects the substantia nigra and dorsal striatum, and is important for motor control and learning [53] (Figure 1.1).



**Figure 1.1. Simplified mesolimbic dopamine circuit, through which drugs of abuse mediate reward.** Glutamatergic projections are depicted in blue; dopaminergic projections in red; GABAergic projections in orange; orexinergic projections in green. Abbreviations: PFC: prefrontal cortex; NAc: nucleus accumbens; VP: ventral pallidum; BNST: bed nucleus of the stria terminalis; LH: lateral hypothalamus; VTA: ventral tegmental area, LDTg: laterodorsal tegmental nucleus. Published in [64].

Repeated METH use results in the downregulation of neurotransmitter systems which mediate the acute effects of the drug. Rodent studies demonstrate that METH self-administration (between 1-6 weeks) reduces dopamine and serotonin transporter expression (DAT and SERT respectively) [65, 66], and decreases dopamine D<sub>2</sub> receptor expression in the VTA and substantia nigra pars compacta [67]. Neurotoxic dosing regimens of METH (e.g. 5 treatments of 15mg/kg METH at 6 hour intervals [68, 69]) reduce dopamine D<sub>1</sub> and D<sub>2</sub> expression in rats 18 hours after METH administration, while extracellular dopamine and its metabolites are reduced 1-4 weeks into withdrawal [68, 70]. These changes occur primarily in components of the mesocorticolimbic and nigrostriatal pathways, e.g. striatum, NAcc, prefrontal cortex (PFC), VTA and substantia nigra [67, 70-72].

In addition, glutamatergic dysfunction is apparent in rodent studies of chronic METH use; however, the directionality of glutamate dysfunction is contested. Increased prefrontal and

striatal glutamate receptor expression [e.g. N-methyl-D-aspartate (NMDA), and NMDA subunit NR2] as well as increased glutamate transporter 1 (GLT-1) expression has been demonstrated following repeated daily METH treatment in rats (dose range 1-30mg/kg s.c. or 4mg/kg i.p. for 1-3 weeks) [70, 73-75]. K<sup>+</sup>-evoked *in vitro* glutamate release is enhanced in the rat striatum following chronic METH administration [76], an effect also observed in anaesthetised rats [77]; however, *in vitro* glutamate release is reduced in the hippocampus [76]. Some studies suggest reduced glutamatergic function following repeated METH treatment. 2-3 weeks of METH treatment decreases striatal mRNA and protein expression of the AMPA receptor subunits GluA1 and GluA2, and NMDA NR1, NR2A and NR2B in Sprague Dawley rats and Swiss mice [76, 78, 79]. Two weeks of METH treatment also reduces acetylation of histones containing genes for GluA1 and GluA2 subunits in the striatum of Sprague-Dawley rats [78]. These discrepant results may be due to differences in METH dose, frequency of administration, brain region examined or the interval between treatment and expression studies. Interestingly, there have been reports of recovery of serotonin and noradrenaline transporter function in the striatum and frontal cortex [65], and NMDA expression in the striatum [80] following abstinence (7-30 days) in Sprague-Dawley rats.

Imaging studies in human METH abusers reflect the animal data above, in that they also demonstrate changes to the dopaminergic and glutamatergic systems following repeated METH use. Imaging studies using positron emission tomography demonstrate a loss of DAT and D<sub>2</sub> in the striatum and PFC in chronic METH users [81-85]; preliminary evidence suggests that low D<sub>2</sub> availability in the dorsal striatum may be predictive of relapse [86]. Human imaging studies show reductions in brain glutamate concentration in frontal grey matter in recently abstinent METH users, an effect correlated with METH craving [87]. While increased glutamate concentration has been observed in white matter, this effect has not been reported for frontal grey matter [88].



From the evidence presented it is clear that chronic METH use results in a number of behavioural and neural changes, particularly in the dopaminergic and glutamatergic systems; however, the specific neural changes which lead to the addicted state, and the means by which they do this is controversial. Indeed, not all the neural changes induced by repeated METH use necessarily lead to addiction, and there has been considerable research into the neurotoxic effects of repeated METH use; see [89, 90]. It is critical to understand how the brain changes as a result of repeated drug use, reflecting the transition from casual to compulsive use, in order to accurately target and ameliorate neural adaptations involved in addiction. While research into the neural changes which accompany the transition to METH addiction is somewhat lacking, it is possible to extrapolate from other drugs of abuse which neural changes may underlie the transition from casual to compulsive drug use.

#### **1.4. Neural changes corresponding with the development and persistence of addictive behaviour**

##### *1.4.1. Drugs of abuse acutely enhance dopaminergic signalling in the mesocorticolimbic system*

The acute reinforcing effects of drugs of abuse have been attributed to the large increase in dopamine transmission in the mesocorticolimbic system. Human imaging studies show temporal correspondence with self-reported 'high' and radio-labelled drug concentration in the basal ganglia [91]. Rodent studies suggest the acute effects of a variety of drugs of abuse are attributable to increased synaptic levels of dopamine in the NAcc [92-94]. It is noteworthy that acute administration of various drugs of abuse also increases extracellular concentrations of a number of other neurotransmitters (e.g. noradrenaline, serotonin [53, 95] for reviews see [96, 97]). Important for later discussion is the fact that psychostimulants also acutely increase striatal extracellular glutamate concentration from presynaptic stores [59-63, 71].

Increased extracellular dopamine is critical for learning about the reinforcing properties of drugs of abuse, and directing subsequent drug-taking behaviour [98]. Specifically, it has been

suggested dopamine transmission in the mesocorticolimbic system allows for the efficient acquisition of rewards by signalling ‘saliency’ (irrespective of whether the outcome is positive or negative [99]) and ‘prediction-error’ (the difference between the predicted reward and the current reward obtained [100, 101]). Because the magnitude of extracellular dopamine released from drug administration is considerably larger than that of biologically-relevant stimuli, the learning about the drug and cues predicting the drug are also correspondingly stronger [102]. Furthermore, unlike natural rewards, drugs of abuse continue to release dopamine in reward-relevant areas even after the association between drugs and their relevant cues has been established, leading to stronger associations between drug, reward and drug-relevant cues [102]. As a result, it has been suggested that drugs of abuse ‘hijack’ normal learning systems [103], inflating the importance of drug-taking and drug-related cues, and inducing progressively greater orientation of behaviour toward drug-seeking and drug-taking [104].

#### *1.4.2. Persistent changes to the dopaminergic system following chronic drug use*

Changes to dopaminergic signalling and receptor expression are observed in humans and rodents following repeated drug use (see Figure 1.2a). Reductions in baseline dopamine release in the NAcc [105, 106] and decreased D<sub>2</sub> expression in the dorsal and ventral striatum, as well as the VTA, are consistently observed after administration of ethanol, opiates and psychostimulants in rodent studies [107-110]. Similarly, decreased D<sub>2</sub> expression and reduced dopamine release in the striatum is also observed in abstinent human addicts [111, 112]. It is possible some of those changes to the dopaminergic system subserve some behavioural phenotypes exhibited by addicts. Decreased D<sub>2</sub> expression has been linked to increased impulsivity in rodents [113, 114] and human METH users [85]. Reduced D<sub>2</sub> expression is also correlated with reduced glucose metabolism in areas involved in decision making and impulsivity (e.g. cingulate gyrus and orbitofrontal cortex function [115]; see [116] for a review).

Indeed, a shift in focus from larger but delayed rewards to smaller and immediate reward often observed in addicts [117] is interpreted as increased impulsivity.

Also, hyper-responsiveness to drug-associated cues [118], and the propensity for drug-associated cues to trigger relapse [119] may also be somewhat explained by an altered dopaminergic system. Human and rodent studies suggest that with repeated drug use, the role of dopamine signalling changes from indicating reward, to predicting reward [120, 121]. Rats responding to cues associated with drug infusions show enhanced dopamine release in the dorsal striatum in the absence of the actual drug infusion [122]. This effect is blocked by microinjection of the relatively non-selective dopamine receptor antagonist  $\alpha$ -flupenthixol into the dorsal striatum [123]. In human cocaine users, cocaine associated stimuli evoke greater dopamine release in the dorsal striatum than neutral stimuli, and this effect is correlated with self-reported degree of drug craving [124]. It is clear that dopaminergic signalling is not only a critical component of acute reward, but it also plays a significant role in promoting drug-seeking in response to environmental cues and potentially increasing impulsivity in chronic drug use.

#### *1.4.3. Persistent changes to the glutamatergic system following chronic drug use*

Drugs of abuse acutely increase presynaptic glutamate release; however, with repeated drug-administration, striatal basal extracellular glutamate concentration is reduced [126]. Importantly, the reduction in extracellular glutamate concentration following repeated drug use appears to precipitate relapse [125]. The reduction in extracellular glutamate is driven by reduced expression of the glial glutamate transporter 1 (GLT-1), which is responsible for 90% of brain glutamate uptake [125-128]. Chronic cocaine administration decreases GLT-1 expression in the NAcc [127]; this reduces clearance of synaptic glutamate [128] and can lead to stimulation of extrasynaptic metabotropic glutamate receptors (mGlu), such as mGlu5. Notably, pharmacological stimulation of mGlu5 promotes reinstatement of cocaine-seeking in rats, which is an animal model of relapse [129]. Thus, when presynaptic glutamate is released (e.g. in the

presence of drug cues [130-132]), glutamate clearance via GLT-1 is reduced, which leads to a surge in extracellular glutamate that can promote relapse [126, 133, 134].

Importantly, normalising GLT-1 expression reduces reinstatement. Specifically, administration of N-acetylcysteine, which increases GLT-1 expression, reverses drug- and cue-induced reinstatement of drug-seeking behaviour (for cocaine: [133, 135-137] and heroin: [138]). Similar effects have also been demonstrated for cocaine drug-induced reinstatement with the GLT-1 regulator ceftriaxone [128]. Preliminary human studies in drug addicts also indicate a reduction in cocaine craving [136], desire to use cocaine [139] and a trend for a reduction in marijuana use [140] following N- acetylcysteine treatment; however, this treatment is yet to be shown effective for METH addiction [141].

#### *1.4.4. Altered AMPA and NMDA subunit receptor expression following chronic drug use*

Changes to glutamate receptor expression, particularly the subunits of the AMPA and NMDA receptors, occur following chronic drug treatment in the NAcc and mPFC (see Figure 1.2b). Importantly, increased AMPA and NMDA subunit expression, particularly in the NAcc, increases the propensity for reinstatement, while a reduction in AMPA and NMDA subunit expression reduces reinstatement propensity [142, 143]. Repeated cocaine or nicotine treatment increases expression of the AMPA subunit GluA1 in the NAcc after a period of withdrawal [130, 144] and cue-induced reinstatement is associated with increased accumbal GluA1 expression in rats ([145] for reviews, see [142, 143]). Viral overexpression GluA1 in the NAcc increases, whereas overexpression of a pore-dead mutant GluA1 decreases AMPA-induced cocaine-seeking in rats [146]. Also, blockade of GluN1 and GluN2 in the NAcc core reduces cue-induced reinstatement of nicotine-seeking [130], while GluN2B antagonist administration into the NAcc shell reduces morphine-primed reinstatement of place preference [147].

Conversely, chronic cocaine has the opposite effect on AMPA and NMDA subunit expression in the dorsomedial PFC (dmPFC) compared to the NAcc. Cocaine self-administration reduces

dmPFC AMPA (GluA1, GluA2) and NMDA (GluN1, GluN2B) subunits in rats [148]; similar effects are observed following repeated experimenter administered cocaine in rats [149]. The effect of reversing changes to AMPA or NMDA subunit expression in the mPFC on reinstatement has not been reported; however, cocaine-primed reinstatement is suppressed by AMPA injections into the infralimbic cortex of the rat mPFC [150]. It is possible drug-induced changes to AMPA and NMDA subunit expression in the NAcc and dmPFC play an important role in the vulnerability to relapse, as reversing these changes, notably in the NAcc, can reduce cue-induced and drug-primed reinstatement for nicotine [130].

#### *1.4.5. Altered synaptic plasticity following chronic drug use*

Plasticity at glutamatergic synapses, in the form of long term potentiation (LTP, synaptic strengthening) and long term depression (LTD, synaptic weakening) following acute and chronic drug administration is observed in critical components of addiction circuitry, including the VTA, NAcc, mPFC and amygdala [151]. Persistent LTP of AMPA-mediated currents in VTA dopaminergic cells is observed following a single drug exposure in mice [152, 153], while repeated cocaine exposure in rats induces LTP which persists months into abstinence, in both VTA dopaminergic neurons [154] and layer 5 pyramidal cells of the mPFC [155]. LTP on GABAergic MSNs in the NAcc is impaired in rats undergoing extinction or abstinence following cocaine self-administration [156, 157]. Interestingly, the direction of plasticity (i.e. enhancement or impairment) following cocaine exposure may depend on the withdrawal period. Five once-daily cocaine administrations in mice reduces LTP in the NAcc 24 hr after the cessation of drug treatment, but can enhance LTP two weeks after cessation of drug treatment [158]. Also, enhanced LTP in hippocampal CA1 neurons is observed in rats 3 days following cessation of cocaine self-administration, but LTP is reduced after 100 days of withdrawal [159]. Thus, it appears that while most drugs of abuse appear to enhance LTP, this effect can depend on the brain region examined and the withdrawal period employed.

In addition, impairments in the induction of LTD are also observed within addiction circuitry following chronic drug use. Impaired LTD in the NAcc core [157, 160, 161], mPFC layer 5 pyramidal neurons [162, 163] and VTA [164] is observed in rats and mice following cocaine self-administration or repeated experimenter administered cocaine or amphetamine.

Changes to LTP and LTD are important to the study of addiction because they have been associated with the development and expression of addiction-relevant behaviour. Indeed, a number of studies demonstrate altered synaptic plasticity following self-administration [154, 159, 160, 165], extinction training [157, 166-168], as well as following the expression of behavioural hallmarks of addiction [169-171]. Importantly, restoration of drug-induced changes to synaptic strength is protective against cue- and drug-primed reinstatement [172], while cue-induced reinstatement of cocaine-seeking transiently increases synaptic strength and MSN spine head density in the NAcc [173]. Considering the involvement of LTP and LTD in learning and memory (for reviews, see [174, 175]) it has been proposed that plasticity induced by drug use serves as a form of maladaptive learning [176, 177], and that persistent plasticity within addiction circuitry may underlie relapse propensity in abstinent addicts [178-180]. However, it is important to note that the mode of drug delivery (passive vs. self-administered), withdrawal conditions (abstinence with or without extinction) and brain region examined can all modify the direction and strength of drug-induced plasticity.

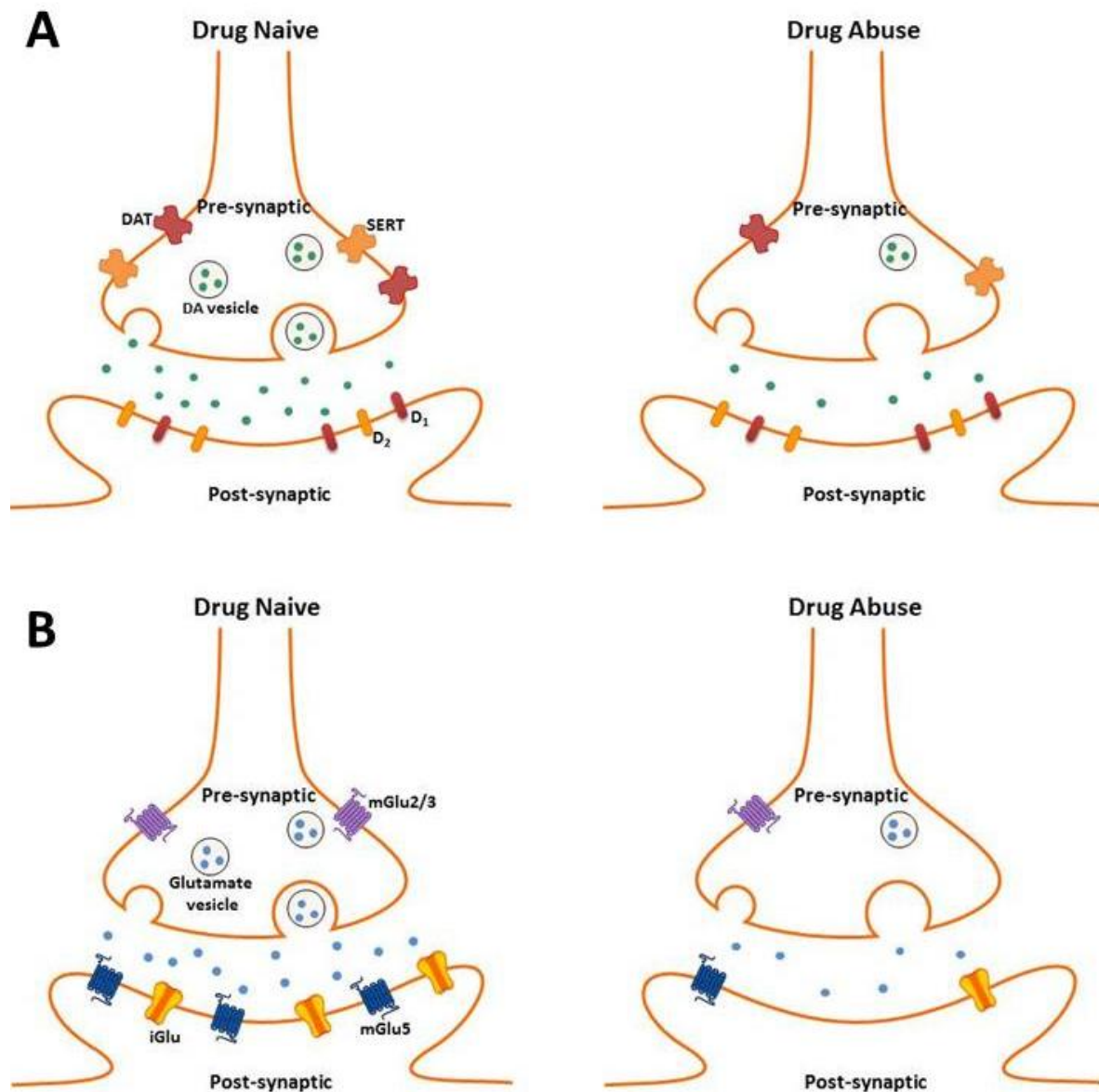
#### *1.4.6. Chronic drug use also alters metabotropic glutamate receptor expression*

Metabotropic glutamate receptors (mGlu) are divided into 3 classes: group I (mGlu1, mGlu5), group II (mGlu2, mGlu3) and group III (mGlu4, mGlu6, mGlu7 and mGlu8). mGlu receptors regulate synaptic plasticity and glutamate transmission [181-183], and dysregulation of these processes appears critical in the development and maintenance of addiction. There are changes to expression levels and synaptic plasticity induced by mGlu following chronic drug administration, in particular, mGlu2, mGlu3 and mGlu5 (see Figure 1.2b). Chronic cocaine

administration reduces mGlu2/3 protein content in the PFC and NAcc in Sprague-Dawley rats [184, 185]; however, no change to mGlu2/3 protein levels has also been reported following chronic cocaine self-administration in Wistar rats [186]. Chronic morphine administration reduces mGlu2/3 induced LTD in the NAcc in mice [187], while chronic cocaine reduces mGlu2/3 induced LTD in the mPFC in rats [163]. Reduced mGlu5 surface expression in the NAcc and striatum is observed following self-administration of cocaine in Sprague-Dawley and Wistar rats [186, 188]; this effect is also apparent after extinction training in Sprague-Dawley rats [156]. Also, mGlu5-induced LTD is reduced in the nucleus accumbens following chronic cocaine treatment [162], or in the dorsal striatum following cocaine self-administration in rats [188], which was not present following sucrose self-administration [188]. Importantly, recent human imaging studies highlight a reduction in striatal mGlu5 expression in chronic cocaine abusers [189], as well as a reduction in mGlu5 binding in the medial orbitofrontal cortex and caudate-putamen of smokers [190, 191], and a reduction in mGlu2/3 transcripts in the anterior cingulate cortex of alcoholics [192]. These human data strengthen the validity of the preclinical findings outlined above.

Importantly, reversing drug-induced changes to mGlu2/3 and decreasing mGlu5 signalling can ameliorate drug-seeking in preclinical models. Pharmacological restoration of drug-induced decreases in mGlu2/3 signalling can reduce cocaine self-administration under a progressive ratio schedule of reinforcement and reduce cue-induced cocaine-seeking in rats [193, 194]. These effects appear dependent on actions of mGlu2/3 in the NAcc and VTA [195-197]. A reduction in mGlu5 signalling decreases relapse-like behaviour for cocaine in rats [129, 188], and for morphine in mice [198]. Considering mGlu5 expression is reduced following cocaine self-administration, it is counterintuitive that a further reduction in mGlu5 signalling should reduce relapse propensity. However, considering relapse can be driven by a surge of glutamate in the NAcc [126, 199], it is possible that pharmacologically blocking glutamate transmission with mGlu5 antagonists can reduce reinstatement. The reduction in mGlu5 expression may be a

compensatory mechanism which occurs through repeated exposure to drugs of abuse which acutely increase glutamate release [126].



**Figure 1.2. Neural changes which occur during addiction. A)** Simplified schematic of changes to dopaminergic synapses in the nucleus accumbens following repeated drug use. Changes depicted here include a reduction in D<sub>1</sub> and D<sub>2</sub> expression, reduced dopamine transporter expression and a reduction in dopamine release. **B)** Simplified schematic of changes to glutamatergic synapses in the nucleus accumbens following repeated drug use. Changes depicted here include a reduction in mGlu2/3, mGlu1 and mGlu5 expression, and a reduction in glutamate release. Abbreviations: DAT: dopamine transporter, DA: dopamine; SERT: serotonin transporter, D<sub>1</sub> / D<sub>2</sub>: dopamine 1 / 2 receptor; mGlu2/3/5: metabotropic glutamate receptor; iGlu: ionotropic glutamate receptor. Figure created using Powerpoint slides courtesy of motifolio.com.



#### *1.4.7. Synthesis: how changes in dopaminergic and glutamatergic systems result in addiction*

From the review thus far, it is clear a number of changes occur in both the dopaminergic and glutamatergic systems with chronic drug use. How these changes contribute to the development of addiction, and propensity of addicts' for relapse has been the subject of much debate; however, some theories will be outlined and synthesised.

Dopamine release signals salience of reward and helps to establish drug-taking. Following repeated drug use, reductions in D<sub>2</sub> receptors and dopamine release may induce a state of dysphoria, and reduce the reward value of naturally occurring stimuli (e.g. food, social interaction) [200]. This may prompt drug-seeking to achieve either relief from negative states [201], or may result in drug-seeking over natural reward-seeking due to a loss of pleasure from natural reward.

Furthermore, altered plasticity (in the form of LTP and LTD) can strengthen the connection between drug effects and cues present during drug-taking activities, increasing the likelihood of drug-taking in response to these cues and contexts [64]. Indeed, persistent aberrant plasticity, notably in the form of impaired corticoaccumbal LTD, is suggested to underlie some behavioural inflexibility in responding to drug-associated cues [169] and habitual behaviour [202], which may underlie behavioural hallmarks of addiction [170]. Prefrontal glutamatergic projections to limbic structures control responses to drug-associated cues and contexts; however, in the presence of strengthened connections which strongly promote drug-seeking, and reductions in extracellular glutamate and glutamate receptor expression, prefrontal control of drug-seeking behaviour is compromised [126]. In addition, increased impulsivity in addicts (often associated with a reduction in PFC and anterior cingulate function) may also contribute to the loss of control addicts experience with drug-taking [120]. The combination of aberrant plasticity, which can strengthen connections between drug-associated cues and drug-taking, in combination with impaired prefrontal inhibition of behaviour and increased impulsivity, may

provide a framework to explain compulsive drug-seeking and relapse despite detrimental consequences to the user (see also [203]).

It is clear that while a number of different neural systems and substrates contribute to the expression of addiction, the dopaminergic and glutamatergic systems are prominent in the compulsive nature of drug-taking and susceptibility to relapse. This is highly relevant to METH addiction, for, as mentioned above, both dopaminergic and glutamatergic systems appear to be downregulated following chronic METH administration, in both rodent and human studies. In light of the importance of dopaminergic and glutamatergic system dysregulation, the following sections will focus on two potential therapeutic targets for METH addiction: the metabotropic glutamate 5 receptor, which regulates glutamatergic signalling [204], and the adenosine 2A receptor, which regulates both glutamatergic and dopaminergic signalling [205].

### **1.5. The metabotropic glutamate 5 receptor as a potential therapeutic target**

mGlu5 is well-positioned in the brain to mediate addiction relevant behavioural and neural processes. mGlu5 is expressed in brain regions important for both learning and addiction, such as the NAcc, dorsal striatum, hippocampus, cerebral cortex and lateral septum [206, 207]. mGlu5 is predominantly located postsynaptically on the perisynaptic annulus of dendritic spines [206, 208]; however, a small percentage of mGlu5 receptors are located presynaptically in the hippocampus and striatum [207, 209]. On the post synaptic cell, mGlu5 is structurally linked to NMDA receptors through scaffolding proteins such as Homer and Shank ([210, 211] for a review, see [212]). mGlu5 postsynaptic activation potentiates NMDA currents, likely via activation of protein kinase C [204]. In addition, mGlu5 is coupled to  $G_q/11$  proteins, which activate the phospholipase C pathway and increase intracellular calcium levels from inositol 1,4,5 triphosphate mediated stores [213-215].

Recently, there has been considerable interest in mGlu5 as a target for addiction therapeutics. Despite the clear involvement of ionotropic glutamate receptors (e.g. AMPA, NMDA) in

addiction, pharmacological modulation of these receptors has serious side effects (e.g. memory loss, disorientation, symptoms of psychosis [216, 217]); thus research targeting other glutamate receptors is warranted. Of the various mGlu receptors, mGlu5 (as opposed to mGlu2 or mGlu3) was investigated in this thesis due to the abundant expression of this receptor in brain regions associated with reward and learning, and the difficulty in producing highly selective ligands for mGlu2 and mGlu3 [212, 217]. Importantly, pharmacological and genetic studies implicate mGlu5 in the reinforcing effects of a number of drugs of abuse, as well as the extinction and reinstatement of drug-seeking behaviour.

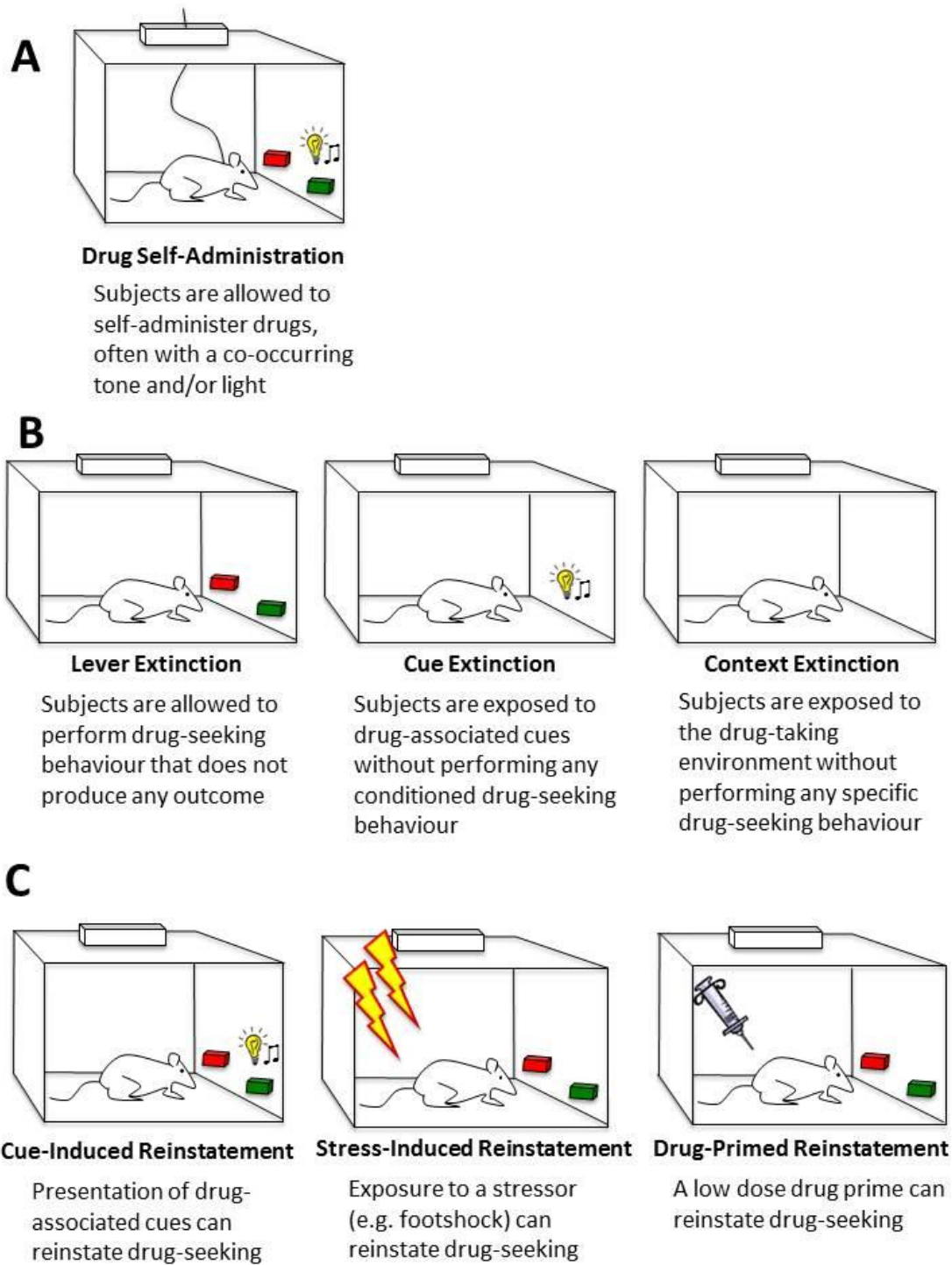
#### *1.5.1. Involvement of mGlu5 in conditioned place preference*

A reduction in mGlu5 signalling reduces the rewarding nature of a drug associated context, measured through the conditioned place preference paradigm (CPP). In this preclinical paradigm, repeated pairings of a drug (e.g. cocaine, alcohol, morphine) with a context produces a preference for this environment as measured by time spent in this context (compared to time spent in that context prior to conditioning, or time spent in the saline context); this preference is considered indicative of the conditioned rewarding nature of the drug [218, 219]. Systemic administration of the mGlu5 negative allosteric modulator (NAM) 2-Methyl-6-(phenylethynyl)pyridine (MPEP) prior to conditioning blocks the acquisition of place preference for cocaine, but not morphine, ethanol, nicotine, or amphetamine in mice [220]; however, i.c.v. MPEP blocks acquisition of morphine CPP in mice [221]. Administration of MPEP prior to test reduces the expression of CPP for morphine [222] and amphetamine in rats [223], as well as ethanol in mice [224], but not cocaine [222] or 3,4-methylenedioxymethamphetamine (MDMA) in rats [223]. In considering these discrepant results, it is important to note that the MPEP dose used to reduce the expression of morphine and amphetamine also reduced locomotor activity in these tests, which may have confounded the expression of CPP [222, 223]. Indeed, the reduction in acquisition of cocaine CPP and expression of ethanol CPP also only occurred at the higher end

of the dose range tested [220, 224]. Thus, it appears the reduction in the acquisition and expression of CPP by MPEP to drugs of abuse is dose dependent.

### *1.5.2. Involvement of mGlu5 in operant drug self-administration*

In order to detail the involvement of mGlu5 in drug self-administration, preclinical rodent paradigms used to assess this behaviour must first be outlined. Drug self-administration paradigms permit rodents to self-administer drugs of abuse within operant chambers. Animals are presented with an active lever, which when depressed the requisite number of times will deliver drug intravenously [225] (see Figure 1.3a). Drug reward is often presented in concurrence with a light or tone, which, through association with drug reinforcement, become conditioned stimuli (CS+) [225]. Other discriminative stimuli (e.g. scents, wall textures) may also be present to distinguish the drug-taking context. An inactive lever is also present, permitting discrimination for the active lever over the inactive lever [225]. Rodents learn to self-administer drugs of abuse according to different reinforcement schedules. Fixed reinforcement (FR) schedules require a fixed number of lever presses to obtain a drug reward, while progressive reinforcement (PR) schedules require an increasing number of lever presses to obtain a drug reward [226].



**Figure 1.3. Preclinical models of drug self-administration, extinction and reinstatement.** Self-administration is described in **A**). Green indicates the active, and red indicates the inactive lever. Different types of extinction are described in **B**). Different types of reinstatement are described in **C**). Modified from [227].

Pharmacological antagonism of mGlu5 reduces drug self-administration (for a summary of this section, see Table 1.1-Table 1.3). Treatment with the mGlu5 NAM 3-((2-methyl-4-thiazolyl)ethynyl)pyridine (MTEP) reduces self-administration of cocaine and METH in rats, with no effect on responding for a food reward [228, 229]. MTEP administration reduces operant self-administration of alcohol in two rat strains when administered both acutely and chronically [230]; similar findings have been reported in mice for alcohol [231] and also morphine [198].

There is also evidence to suggest mGlu5 antagonism is involved in the motivation to obtain drugs of abuse; MTEP decreases METH self-administration in Sprague-Dawley rats on fixed and progressive ratio schedules of reinforcement [232]. Furthermore, MPEP administration reduces breakpoint for nicotine and cocaine in Wistar rats [233], as well as ethanol in alcohol-preferring rats [234].

Studies using the germline knockout (KO) mice for mGlu5 somewhat reflect the pharmacological effects of mGlu5 antagonism on drug self-administration. Chiamulera and co-workers [235] demonstrated abolished self-administration of cocaine in mGlu5 KO mice; however, recently this has not been replicated [236]. Supporting the latter finding, cocaine self-administration is unaltered in mice with knockdown of mGlu5 in D<sub>1</sub> expressing neurons [237]. mGlu5 KO mice demonstrate lower self-administration of ethanol in line with pharmacological studies [238]; however, reduced ethanol self-administration in mGlu5 KO mice is likely due to increased sensitivity to the drug causing satiety, as CPP to a lower dose of ethanol was present in mGlu5 KO but not wild type (WT) mice [238]. It is possible developmental compensation may account for differences between pharmacological and germline genetic KO studies. Importantly, these KO studies indicate that while mGlu5 may be sufficient to acutely reduce drug self-administration, it is not necessary for this behaviour.

### *1.5.3. Role of mGlu5 in extinction learning*

Extinction learning is a process whereby the contingency between drug-seeking behaviour or drug predictive stimuli and drug reward is reduced, resulting in a decrease in drug-seeking behaviour [239]. This can occur in the form of a reduction in an operant drug-response (i.e. reduction in lever pressing for a drug), or a reduction in the association between a CS+ or context and drug availability [240, 241]. In preclinical animal models, extinction can involve presenting an operant lever that no longer provides drug infusions when pressed (henceforth referred to as lever extinction), or may constitute exposure to drug-associated cues or contexts in the absence of drug availability (henceforth referred to as cue or context extinction) (see Figure 1.3b) [227]. In a clinical setting, extinction procedures often involve cue exposure therapy, where cues associated with drug-taking (e.g. needles) are presented without drug reward [227, 242].

During extinction, a new contingency is learnt (e.g. lever pressing now no longer results in drug administration); however, the old contingency (e.g. that lever pressing results in drug administration) is not forgotten [239]. This is evident through tests of reinstatement in animals and relapse in humans, where exposure to cues signalling drug availability, stress or drug primes are employed to restore extinguished drug-seeking (see Figure 1.3c). If extinction is successful, the newly learnt contingency is expressed and drug-seeking is suppressed. If extinction is not successful, cues, stress or drug primes can restore drug-seeking [227, 239]. Recently, there has been a focus on enhancing extinction learning to prevent relapse [243, 244], as the facilitation of extinction learning may effectively inhibit the motivational salience and learned habits associated with drug cues [245].

mGlu5 modulates extinction learning in various types of behavioural tasks (e.g. [246, 247]), and this effect also appears to extend to extinction learning in addiction-relevant models. Systemically, the mGlu5 NAM MTEP inhibits extinction of a context associated with cocaine self-administration in Sprague-Dawley rats, in the absence of lever or cue extinction [244]. Also,

global genetic deletion of mGlu5 in mice inhibits extinction of cocaine CPP [236]. Conversely, mGlu5 positive allosteric modulators (PAMs), which increase receptor activity indirectly via activation of an allosteric site on the protein, facilitate the extinction of cocaine CPP and lever extinction following cocaine self-administration in Sprague–Dawley rats [248, 249]. It appears the facilitatory effect of mGlu5 signalling on extinction learning may be dependent on its actions in the NAcc. Ghasemzadeh *et al.* [250] demonstrated that lever extinction training and withdrawal without extinction following cocaine self-administration both reduce mGlu5 protein expression in NAcc shell, but not in the NAcc core or dorsal striatum in Sprague-Dawley rats; however, increased expression of the scaffolding protein PSD-95 in the NAcc core was observed in extinguished animals only. Similar experiments were performed by Knackstedt and colleagues [156]; however, the authors reported decreased surface expression of mGlu5 in the NAcc core (but not the shell) following lever extinction training after cocaine self-administration in Sprague-Dawley rats. In concordance with Ghasemzadeh *et al.* [250], Knackstedt and colleagues [156] reported increased expression of PSD-95, Homer1b/c, and also neuronal activity regulated pentraxin following lever extinction. It is possible overexpression of Homer1b/c is driving the facilitation of extinction learning, as Homer1b/c overexpression in the NAcc core inhibited subsequent cue-induced reinstatement of cocaine-seeking [156]. While a recent study [188] demonstrated inhibition of lever extinction following MTEP administration into the dorsal striatum, but not the NAcc shell, the study design (extinction was performed after abstinence and subsequent context reinstatement), makes comparisons between this study and those previously detailed difficult. Thus, it appears that lever extinction reduces mGlu5 expression and increases the expression of proteins which modulate the clustering of glutamate receptors in the PSD, within subregions of the NAcc.

mGlu5 signalling in other areas also known to regulate extinction learning (e.g. infralimbic cortex [150]) has only recently been investigated. A recent study demonstrated systemic administration of the mGlu5 PAM CDPPB facilitated the extinction of ethanol self-administration and subsequently reduced cue-induced ethanol-seeking in rats [251]. This effect was attributed



to enhanced mGlu5 activity in the infralimbic cortex, as the CDPPB-induced facilitation of extinction learning was blocked by infusion of the mGlu5 NAM MTEP into the IL [251]. This study suggests mGlu5 signalling in the infralimbic cortex mediates extinction learning for cocaine self-administration; further investigation of the mechanism/s behind this behavioural outcome (e.g. involvement of ERK 1 / 2 signalling pathway [252, 253]) and the applicability of this finding to other drugs of abuse is warranted.

#### *1.5.4. Role of mGlu5 in cue-and drug-primed reinstatement*

mGlu5 has also been implicated in the reinstatement of drug-seeking following extinction training. MPEP administration reduces drug-induced reinstatement of nicotine-seeking [254], as well as cue-induced reinstatement of cocaine-seeking [255] and ethanol-seeking [256]. Knockdown studies support these pharmacological findings; mice with knockdown of mGlu5 in D<sub>1</sub> expressing neurons demonstrate attenuated cue-induced reinstatement of cocaine self-administration [237]. Furthermore, the NAcc appears a potential site of action of mGlu5 antagonists on drug-seeking, as NAcc shell infusions of MPEP reduce drug-induced cocaine-seeking [257], and NAcc core MTEP infusions attenuate context-induced relapse for cocaine following abstinence and cue-primed reinstatement of cocaine-seeking following extinction training [188].

The literature outlined above suggests that using acute pharmacological methods, mGlu5 antagonists alter many aspects of drug-taking and drug-seeking behaviour. However, more recent studies using genetic KO mice for cocaine-related behaviours suggest this receptor has a critical function in extinction and reinstatement behaviour [236, 237]. Interestingly, almost all studies which pharmacologically modulate mGlu5 to examine extinction have been conducted using cocaine (except one recent study using alcohol [251]); only two studies have examined the role of mGlu5 in addictive behaviours for METH [232, 258], and none have used genetic means. Thus, one of the aims of the present thesis was to identify if there was a critical role of

mGlu5 in addiction relevant paradigms for METH, using mGlu5 KO mice. Considering recent interest in the role of mGlu5 in extinction learning, modulation of this receptor may provide a new therapeutic avenue for METH addiction, by assisting in relapse prevention through enhancement of extinction learning.

In considering mGlu5 as a potential therapeutic target, complications may arise due to the widespread expression of this receptor throughout limbic structures [206, 207], and the difficulties associated with using systemically administered pharmaceuticals to modulate mGlu5 activity in a region-specific manner. However, these potential complications may be overcome by targeting an mGlu5 heteromer, which is expressed in specific tissues. Indeed, mGlu5 appears to form receptor complexes with the adenosine 2A receptor in the striatum and hippocampus [259, 260]; these structures are strongly implicated in circuitry relevant to both extinction and addiction [239, 261, 262]. Furthermore, there is now considerable interest in the adenosine 2A receptor as a target for addiction therapeutics [263-265]. Also, there is functional evidence to suggest these receptors interact to modulate addiction behaviour for alcohol and cocaine [266, 267]. Thus, investigation of both the mGlu5 and adenosine 2A systems is warranted, to elucidate how these receptors modulate aspects of METH addiction, if they interact to modulate METH addiction, and determine if potential receptor interactions may provide new drug targets.

<b>Behavioural Measure</b>	<b>Pharmacological reduction of mGlu5 signalling</b>	<b>Pharmacological enhancement of mGlu5 signalling</b>	<b>Genetic knockout or knockdown</b>
↓Self-administration	[228, 229, 232, 254, 268-270]		[235]
↑or – Self-administration			– [236]
↓ Breakpoint	[186, 233]		
↑or – Breakpoint			↑ [236]
↓ Extinction	[244]		[236]
↑or – Extinction		– [258] ↑ [248, 249, 271]	
↓ Reinstatement /relapse	[232, 255, 257, 269, 270, 272-274]		[237]
↑or – Reinstatement / relapse		↑ [129] – [258]	
↓ CPP	[223, 267]		
↑or – CPP	– [220, 222, 223, 275]		– [236]
↓ Locomotor Sensitization	[275-278]		[279]
↑or – Locomotor Sensitization			

**Table 1.1. Role of pharmacological reduction, enhancement and genetic knockout or knockdown of mGlu5 signalling on addiction-relevant behaviour for psychostimulants.**

<b>Behavioural Measure</b>	<b>Pharmacological reduction of mGlu5 signalling</b>	<b>Pharmacological enhancement of mGlu5 signalling</b>	<b>Genetic knockout or knockdown</b>
↓Self-administration	Ethanol: [231, 280-283] Opiates: [198]		
↑or – Self-administration			
↓ Breakpoint	Ethanol: [234]		
↑or – Breakpoint			
↓ Extinction			
↑or – Extinction		Ethanol: [251]	
↓ Reinstatement /relapse	Ethanol: [256, 280, 284, 285]. Opiates: [198]		
↑or – Reinstatement / relapse			
↓ CPP	Ethanol: [224, 286]. Opiates: [221, 222, 275, 287]		
↑or – CPP	– Ethanol: [220]. – Opiates: [220, 288]		↑Ethanol: [238]
↓ Locomotor Sensitization	Ethanol: [289] Opiates: [290]		
↑or – Locomotor Sensitization	– Opiates: [275]		

**Table 1.2. Role of pharmacological reduction, enhancement and genetic knockout or knockdown of mGlu5 signalling on addiction-relevant behaviour for ethanol and opiates.**

Behavioural Measure	Pharmacological reduction of mGlu5 signalling	Pharmacological enhancement of mGlu5 signalling	Genetic knockout or knockdown
↓ Self-administration	[269, 270, 281, 291]		
↑ or – Self-administration	– [228, 229, 232, 254, 282, 283]		
↓ Breakpoint	[233, 234]		
↑ or – Breakpoint			
↓ Extinction			
↑ or – Extinction	↑ [271]		
↓ Reinstatement / relapse	[269, 270, 273, 292]		[292]
↑ or – Reinstatement / relapse	– [232, 255, 257, 272, 274, 284]		
↓ CPP			
↑ or – CPP	– [223]		
↓ Locomotor Sensitization			
↑ or – Locomotor Sensitization			

**Table 1.3. Role of pharmacological reduction, enhancement and genetic knockout or knockdown of mGlu5 signalling on addiction-relevant behaviour for food or sucrose.**

## 1.6. The adenosine 2A receptor as a potential therapeutic target

Interest in adenosine as a mediator in addiction has grown over the last decade due to the modulatory effects of adenosine on dopaminergic and glutamatergic systems, which, as outlined earlier, play important roles in the development and maintenance of addiction. Adenosine is ubiquitous in the CNS, and plays an important role in energy transfer, signal transduction and acts as both an excitatory and an inhibitory neuromodulator [293, 294]. There are four G-protein coupled adenosine receptors ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ ,  $A_3$ );  $A_1$  and  $A_{2A}$  are predominantly located in the central nervous system and appear involved in drug-taking, while  $A_3$  and  $A_{2B}$  receptors are predominantly located in the peripheral nervous system, and have been implicated in processes such as inflammation and the immune response [295]. This thesis will focus on  $A_{2A}$ ; reviews of the function of the  $A_1$  receptor are detailed elsewhere [296].

### 1.6.1. $A_{2A}$ receptor location and signalling

The  $A_{2A}$  receptor ( $A_{2A}$ ) is predominantly expressed within the basal ganglia (e.g. caudate putamen, NAcc [297]), but also the hippocampus [298], cerebral cortex [299] and olfactory tubercle [297, 300, 301]. It is located predominantly in the postsynaptic cell on dendrites and dendritic spines (although some presynaptic localisation has been observed), almost entirely at asymmetric (i.e. excitatory) synapses [302].  $A_{2A}$  is coupled to  $G_{s/olf}$  proteins, through which it stimulates adenylyl-cyclase and activates the cAMP-protein kinase A (PKA) signalling pathway [205, 303].  $A_{2A}$  activation also leads to the phosphorylation of a number of PKA substrates, such as dopamine- and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) and cAMP response element-binding protein (CREB) [304, 305], and can increase immediate early gene expression (e.g. *c-fos* [306]).

A<sub>2A</sub> appears to colocalise with other receptors (e.g. D<sub>1</sub>, D<sub>2</sub>) to modulate neurotransmitter release and transmission. Antagonistic receptor-receptor interactions exist between D<sub>2</sub> and A<sub>2A</sub> in the dorsal and ventral striatum ([307, 308]; see [309] for a review), and colocalisation of A<sub>2A</sub> and D<sub>2</sub> has been demonstrated in striatopallidal GABAergic medium spiny neurons (MSNs) [205]. It appears A<sub>2A</sub> forms heteromeric complexes at the membrane level with the D<sub>2</sub> [310]. Stimulation of A<sub>2A</sub> decreases D<sub>2</sub> affinity [311], thus decreasing signalling, neuronal excitability and neurotransmitter release [312, 313]. At the intracellular level, antagonistic A<sub>2A</sub>-D<sub>2</sub> interactions modulate gene expression and protein phosphorylation [313]. A<sub>2A</sub> also interacts with D<sub>1</sub> at a network level; D<sub>1</sub>-A<sub>2A</sub> double KO mice demonstrate reduced ethanol consumption compared to D<sub>1</sub>KO or A<sub>2A</sub>KO mice alone [314]. Thus it appears that A<sub>2A</sub> acts at the network, membrane and intracellular level to modulate dopaminergic signalling, which may have functional implications for addiction-relevant behaviours and neural processes.

In addition to interactions with D<sub>2</sub>, there appears to be a synergistic relationship between A<sub>2A</sub> and mGlu5, which can alter neurotransmitter release. Subthreshold doses of A<sub>2A</sub> and mGlu5 agonists have been shown to synergistically facilitate glutamate release, where this effect is not present following stimulation of each receptor individually [315]. GABAergic neurotransmission in the ventral striatum is strongly potentiated by agonism of mGlu5 and A<sub>2A</sub> together; facilitation by these receptors is much stronger when stimulated together than separately, and is blocked by D<sub>2</sub> antagonism [316]. The synergistic effects of mGlu5 and A<sub>2A</sub> on neurotransmitter release may occur through the formation of heteromeric complexes containing mGlu5 and A<sub>2A</sub>, as these receptors appear to colocalise in striatal membranes [259]. Considering the colocalisation of these two receptors and their synergistic effects on neurotransmitter release, it is critical to examine how the signalling of each receptor individually modulates different types of addiction-relevant behaviour. This will help identify how each receptor contributes to addiction, and any overlap in behavioural phenotypes may indicate potential receptor interactions.

### *1.6.2. Pharmacological modulation of A<sub>2A</sub> alters addiction-relevant behaviour*

There is considerable pharmacological and genetic animal model evidence suggesting a role for A<sub>2A</sub> in the modulation of addiction-relevant behaviour (see Table 1.4-Table 1.6). A<sub>2A</sub> antagonists decrease the self-administration or consumption of ethanol and opiates in rats [266, 317-320] and Δ<sup>9</sup>-tetrahydrocannabinol in monkeys [321]. A<sub>2A</sub> antagonists administered to Wistar rats dose-dependently reduce acquisition of place preference for amphetamine [322], and reduce expression of CPP to cocaine [323]. However, A<sub>2A</sub> agonists can show similar effects to A<sub>2A</sub> antagonists on drug self-administration. A<sub>2A</sub> agonist administration reduces cocaine self-administration and cue-induced reinstatement in Wistar and Sprague-Dawley rats [324, 325] and ethanol consumption in the home cage in C57BL/6J mice [326]. From pharmacological studies, it appears there is a role for A<sub>2A</sub> in addiction-like behaviour for many drugs of abuse; however, the direction of this involvement (i.e. whether A<sub>2A</sub> agonism or antagonism would be beneficial for reducing addiction-like behaviour) is unclear. A genetic approach may clarify if enhanced or reduced A<sub>2A</sub> signalling is beneficial for drug addiction.

### *1.6.3. Genetic animal models for A<sub>2A</sub> modulation*

Genetic deletion of the A<sub>2A</sub> has provided interesting insights into the functional specificity of this receptor in addiction-relevant behaviour. A<sub>2A</sub> KO mice show lower sensitivity to, and thus increased consumption of 20% w/v ethanol [327]. This effect is concentration dependent, for A<sub>2A</sub> KO mice demonstrate no change to ethanol consumption at a lower ethanol concentration (5% w/v) [314]. A<sub>2A</sub> KO mice demonstrate increased sensitivity to the anxiolytic and locomotor stimulating effects of ethanol [326], and dose-dependent ethanol CPP, depending on background strain [326]. With respect to opiates, A<sub>2A</sub> KO mice demonstrate decreased morphine self-administration, as well as reduced breakpoint on a progressive ratio schedule [328];



however, there is no effect of receptor deletion on cue-induced responding for morphine following withdrawal [328]. CPP to morphine is also absent in  $A_{2A}$  KO mice [328, 329]. The results outlined above suggest a reduction in the rewarding properties of opiates, but altered sensitivity to the psychomotor and consummatory properties of ethanol.

The response of  $A_{2A}$  KO mice to psychostimulants is complex, and behavioural responses to psychostimulants appear dependent on the location of  $A_{2A}$  deletion. In response to psychostimulants,  $A_{2A}$  KO mice bred on a CD-1 background demonstrate a lower rate of cocaine self-administration and reduced motivation to obtain cocaine [330], yet demonstrate intact CPP for cocaine [330], indicating  $A_{2A}$  may differentially modulate operant and pavlovian conditioning. Recent studies comparing striatal and forebrain deletion of  $A_{2A}$  suggest opposing roles for this receptor within and outside the striatum. This has been observed in response to sensitization, which is an enhanced locomotor response following repeated drug administration, and is considered to reflect neural adaptations which can precipitate future drug-taking [331]. Forebrain-specific  $A_{2A}$  KO mice show an attenuation of amphetamine- and cocaine-induced behavioural sensitization [332-334], yet striatal  $A_{2A}$  KO mice show enhanced cocaine-induced locomotor activity [333, 334]. Thus, the location of the deletion, as well as the type of conditioning employed, may recruit  $A_{2A}$  in different ways.

From the discussion above, it is clear the modulatory role of  $A_{2A}$  signalling in drug-taking and drug-seeking is complex. Pharmacologically,  $A_{2A}$  agonism and antagonism can reduce both drug-taking and drug-seeking behaviour. While genetic deletion studies confirm that lack of  $A_{2A}$  typically reduces drug-taking behaviour, this may be dependent on the behaviour of interest (e.g. self-administration vs. psychomotor sensitization), the paradigm used (e.g. operant or pavlovian conditioning) and also  $A_{2A}$  location (i.e. striatum only  $A_{2A}$  KO mice demonstrate the opposite phenotype to forebrain  $A_{2A}$  KO mice in response to cocaine). The varied behavioural responses of  $A_{2A}$  KO to different drugs of abuse makes it difficult to accurately predict the

response of these KO mice to different drug classes. Thus, in assessing the potential therapeutic value of  $A_{2A}$  modulation for METH addiction, it is imperative to determine how  $A_{2A}$  KO mice respond to METH, as this may be different to the response to other drugs within the psychostimulant class (e.g. cocaine). Furthermore, it is critical to assess the response of  $A_{2A}$  KO mice to a variety of drug-induced behaviours, as these KO mice demonstrate different phenotypes based on the paradigm used (e.g. locomotor sensitization to cocaine is intact in  $A_{2A}$  KO mice, but operant self-administration of cocaine is reduced [330]). Also, considering the role of striatal and forebrain  $A_{2A}$  in different psychostimulant-induced locomotor phenotypes, it is also of great interest to determine where  $A_{2A}$  may act within the brain to modulate METH-induced behaviours, as the location of this receptor may determine whether it facilitates or inhibits drug-taking and drug-seeking. Conditional deletion of  $A_{2A}$  in specific brain loci, achieved through viral mediated knockdown techniques, will help determine where  $A_{2A}$  acts to mediate METH-induced behaviours with a greater degree of anatomical specificity.

<b>Behavioural Measure</b>	<b>Pharmacological reduction of A<sub>2A</sub> signalling</b>	<b>Pharmacological enhancement of A<sub>2A</sub> signalling</b>	<b>Genetic knockout or knockdown</b>
↓ Self-administration		[324, 335]	[330, 336]
↑ or – Self-administration	– [335]		
↓ Breakpoint			[330]
↑ or – Breakpoint			
↓ Extinction		[337]	
↑ or – Extinction	– [337]		
↓ Reinstatement / relapse	[337]	[325, 338]	
↑ or – Reinstatement / relapse	↑ [338]	– [337]	
↓ CPP	[323]	[322, 323]	
↑ or – CPP			– [267, 330]
↓ Locomotor Sensitization	[332]	[339-341]	Forebrain-specific: [333, 334]
↑ or – Locomotor Sensitization	↑ [339]		– [330] Striatum-specific: ↑ [333, 334]

**Table 1.4. Role of pharmacological reduction, enhancement and genetic knockout or knockdown of A<sub>2A</sub> signalling on addiction-relevant behaviour for psychostimulants.**

<b>Behavioural Measure</b>	<b>Pharmacological reduction of A<sub>2A</sub> signalling</b>	<b>Pharmacological enhancement of A<sub>2A</sub> signalling</b>	<b>Genetic knockout or knockdown</b>
↓Self-administration	Ethanol: [266, 317, 318]	Ethanol: [342, 343] Opiates: [319]	Opiates: [328]
↑or – Self-administration	Ethanol: ↑ [342]		Ethanol: ↑ [326, 327] Ethanol: – [314]
↓ Breakpoint			Opiates: [328]
↑or – Breakpoint			
↓ Extinction			
↑or – Extinction			
↓ Reinstatement /relapse	Opiates: [320]		
↑or – Reinstatement / relapse			Opiates: – [328]
↓ CPP			Ethanol: [326] Opiates: [328, 329]
↑or – CPP			
↓ Locomotor Sensitization			
↑or – Locomotor Sensitization			Ethanol: – [326] Opiates: – [328]

**Table 1.5. Role of pharmacological reduction, enhancement and genetic knockout or knockdown of A<sub>2A</sub> signalling on addiction-relevant behaviour for ethanol and opiates.**

<b>Behavioural Measure</b>	<b>Pharmacological reduction of A<sub>2A</sub> signalling</b>	<b>Pharmacological enhancement of A<sub>2A</sub> signalling</b>	<b>Genetic knockout or knockdown</b>
↓ Self-administration	[344]	[326, 335, 342, 345, 346]	
↑ or – Self-administration	– [321, 347, 348]	– [335]	– [314, 327, 328]
↓ Breakpoint			
↑ or – Breakpoint			
↓ Extinction			
↑ or – Extinction			
↓ Reinstatement / relapse			
↑ or – Reinstatement / relapse		– [325, 338]	
↓ CPP			
↑ or – CPP			
↓ Locomotor Sensitization			
↑ or – Locomotor Sensitization			

**Table 1.6. Role of pharmacological reduction, enhancement and genetic knockout or knockdown of A<sub>2A</sub> signalling on addiction-relevant behaviour for food or sucrose.**

## 1.7. Hypotheses

It is hypothesised that germline deletion of mGlu5 or A<sub>2A</sub> will alter METH-induced addiction relevant behaviours (e.g. CPP, operant self-administration, locomotor sensitization), with no effect on addiction relevant behaviour for natural rewards. Due to the role of the mGlu5 in extinction learning, I hypothesise possible deficits in extinction learning and a subsequent reduction in reinstatement in KO mice in the operant self-administration paradigm. It is also possible that KO mice will demonstrate attenuated CPP to METH, as mGlu5 appears to modulate incentive learning. Genetic KO studies of the A<sub>2A</sub> in response to psychostimulants lead to the hypothesis that KO mice will show reduced METH operant self-administration and reduced breakpoint under a progressive ratio schedule, as well as an altered locomotor response to acute and chronic drug administration. The implication of A<sub>2A</sub> in the forebrain and striatum (notably the NAcc) in addiction-like behaviours [332, 333, 349, 350] suggests these areas may be responsible for altered behavioural responses to METH in A<sub>2A</sub> KO mice, and I hypothesise that conditional deletion of A<sub>2A</sub> in one of these regions may modulate METH-induced behaviour. I also hypothesise that the phenotypes of A<sub>2A</sub> and mGlu5 KO mice may be similar in some paradigms (e.g. CPP, self-administration or reinstatement). Similar phenotypes between these two KO mouse lines may permit the behavioural and molecular investigation of how these two receptors interact to modulate drug-taking and / or drug-seeking behaviour.

In short, these hypotheses can be summarised as such:

1. Germline deletion of mGlu5 will modulate aspects of drug-taking and drug-seeking behaviour for METH
2. Germline deletion of A<sub>2A</sub> will modulate aspects of drug-taking and drug-seeking behaviour for METH

3. The neural locus of effect of  $A_{2A}$  on METH-induced behaviour is confined to a region within the forebrain or striatum, and conditional knockdown of  $A_{2A}$  in a target region will alter METH-induced behaviour
4. Similarities will be present in the behavioural phenotypes of mGlu5 and  $A_{2A}$  KO mice, which will permit examination of interactions between these two receptors

### **1.8. Aims of this thesis**

The aims of this thesis were to determine whether there are critical roles of the mGlu5 and  $A_{2A}$  receptors in behaviours relevant for METH addiction. Importantly, I sought to establish if there are overlaps and/or dissociations in behaviours mediated by these receptors in response to METH, to discover effective therapeutics for METH abuse. To do this, mGlu5 and  $A_{2A}$  KO mice were tested in behaviours relevant to METH addiction (Chapters 3 and 4). As there was no overlap in the METH-induced behaviours in mGlu5 and  $A_{2A}$  KO mice, I sought to further investigate the neural locus of METH-induced behaviours in  $A_{2A}$  KO mice, considering the divergence in the effects of genetic deletion of  $A_{2A}$  in the striatum compared to the whole forebrain (see [333, 334]). I did this using a combination of molecular (Chapter 5) and genetic techniques (Chapter 6). From these experiments, I sought to establish where  $A_{2A}$  signalling was critical in mediating reward based behaviours for METH.

## Chapter 2

### General Methods

#### 2.1. Genotyping

##### 2.1.1. DNA extraction

DNA was extracted from mouse ear or tail samples using the following protocol (adapted from [351]). Samples were immersed in 600µL 50mM NaOH and incubated at 95°C for 10 min. When cool, samples were vortexed for 10 min, 150µL 1M Tris (pH 8.0) was added and samples were spun at 13,000 rpm in a Hitachi CT15RE centrifuge for 6 min. A DNA pellet was observed in all samples. Samples were subsequently kept at 4°C and 1µL supernatant was used for genotyping.

##### 2.1.2. Polymerase Chain Reaction (PCR)

Mouse genotyping was performed using PCR. A different mastermix and amplification program was employed for each mouse line (see Table 2.1 for mastermix and Table 2.2 for thermal cycler protocols). For all mouse lines, 1µL supernatant was added to the mastermix and the samples underwent polymerase chain reaction in a Biometra Thermocycler T3000 (Göttingen, Germany).

##### 2.1.3. Gel Visualisation

5-10µL of each sample was loaded into a 1-2% agarose/TBE gel containing 2% (w/v) SYBR Safe® (Life Technologies, Mulgrave, VIC, Australia) and run at 90-100V for 20-30 min in a gel electrophoresis tank (Bio-rad Mini Sub® Cell GT). 5µL of 1Kb Plus DNA ladder (Life



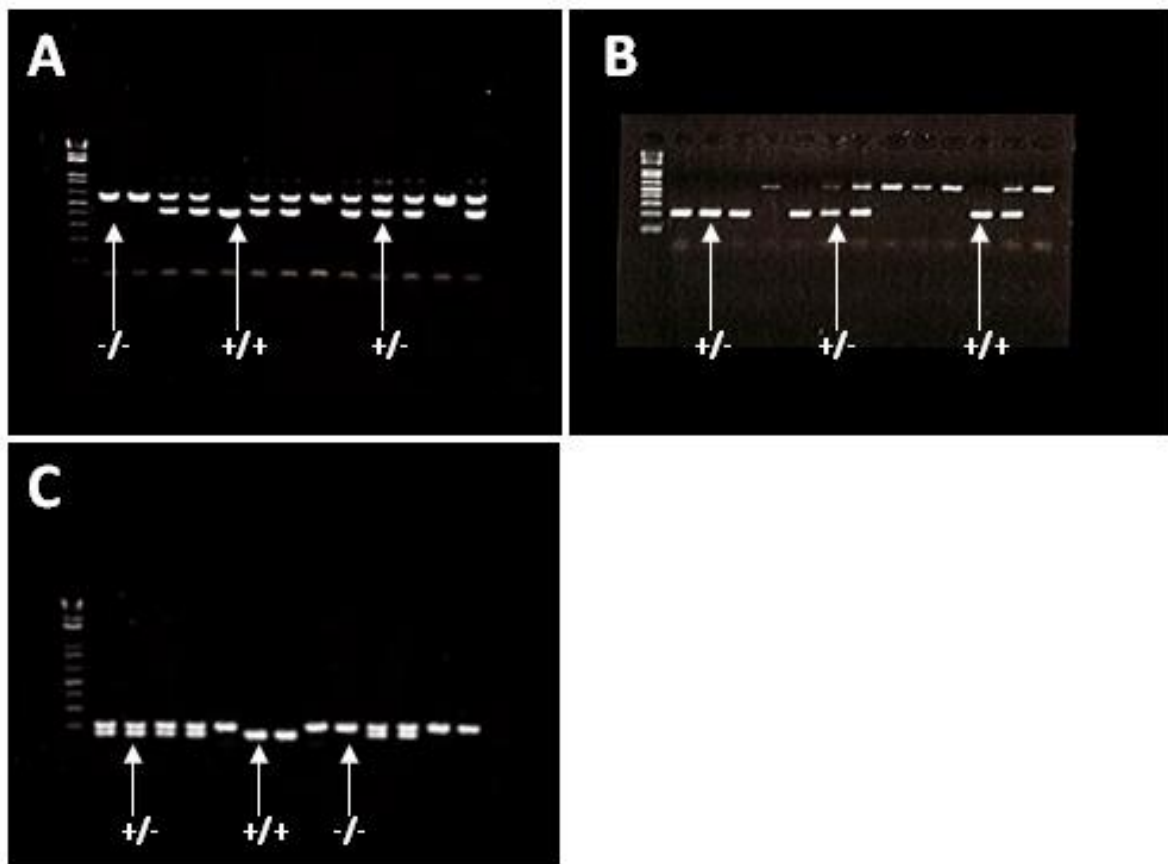
Technologies, Mulgrave, VIC, Australia) was run next to all samples. The gel was visualised using a Benchtop UV 2 Transilluminator (Upland, CA, USA) and Sony Video Graphic Printer (model: UP 897-MD; North Ryde, Australia). Examples of typical PCRs are provided in Figure 2.1.

Reagents	mGlu5 PCR	A <sub>2A</sub> PCR	A <sub>2A</sub> <sup>lox/lox</sup> PCR
Gotaq® Green Master Mix (Promega, Madison, WI, USA)	10µL	5µL	3.27µL
Deionised water	7.8µL	1.8µL	5.13µL
Primer 1	0.3µL reverse primer (sequence 5' – 3': CAC GAG ACT AGT GAG ACG TG)	0.4µL forward primer (sequence 5' – 3': AAG GAA GGG TGA GAA CAG AG)	0.2µL reverse primer (sequence 5'-3': ATT CTG CAT CTC CCG AAA CC)
Primer 2	0.6µL forward primer (sequence 5' – 3': CAC ATG CCA GGT GAC ATC AT)	0.4µL reverse primer (sequence 5' – 3': CAT GGT TTC GGG AGA TGC AG)	0.2µL forward primer (sequence 5'-3': GGG CAA GAT GGG AGT CAT T)
Primer 3	0.3µL reverse primer (sequence 5' – 3': CCA TGC TGG TTG CAG AGT AA)	0.4µL forward primer (sequence 5' – 3': CTC CAC CAT GAT GTA CAC CG)	0.2µL reverse primer (sequence 5'-3': CCT CAT CAT TCC TAC CCG CT)

**Table 2.1. Reagents used in polymerase chain reaction for genotyping mGlu5 KO, A<sub>2A</sub> KO and A<sub>2A</sub><sup>loxP/loxP</sup> mice.**

PCR step	mGlu5 PCR		A <sub>2A</sub> PCR		A <sub>2A</sub> <sup>lox/lox</sup> PCR	
	Temp (°C)	Duration	Temp (°C)	Duration	Temp (°C)	Duration
Initial denaturation	95	2 min	95	2 min	94	4 min
Denaturation	95	40 s	95	1 min	94	45 sec
Annealing	55	40 s	56	1 min	54	45 sec
Synthesis	72	1 min	74	1 min	72	1 min
Cycles	35		40		40	
Extension	72	5 min	74	5 min	72	10 min

**Table 2.2. Thermocycler protocols for polymerase chain reaction for genotyping mGlu5 KO, A<sub>2A</sub> KO and A<sub>2A</sub><sup>loxP/loxP</sup> mice.**



**Figure 2.1. Representative genotyping gels.** Representative genotyping gels for **A)** mGlu5 WT (+/+) and KO (-/-) mice, **B)**  $A_{2A}$  WT (+/+) and KO (-/-) mice, and **C)**  $A_{2A}^{lox-/lox-}$  and  $A_{2A}^{loxP/loxP}$  mice. Abbreviations: +/+ : wild type, +/- : heterozygous and -/- : null or *loxP/loxP*.

## 2.2. Animals

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia (Florey Animal Ethics Committee: ethics approval numbers: 11-015, 12-015, 14-002). All efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to *in vivo* techniques, if available.

Experiments were conducted using adult age-matched littermates. Mice were kept group housed in standard housing (*ad libitum* standard laboratory chow and water, tissues for nesting material) under a 12:12 h light-dark cycle, lights on at 7am, except during operant experiments. Mice were singly housed for operant experiments under a 12:12 h reverse light-dark cycle, lights on at 7am, and food restricted (no less than 85% of free feeding body weight). All mice were allowed at least 7 days to acclimatise to any change in housing conditions or light cycle before experimentation. Further information on the generation of each mouse strain and the mice used in each study are presented in the methods sections of chapters 3-6.

### **2.3. Behavioural Phenotyping**

In all experiments, genotypes were counterbalanced across test apparatus and sessions, and experiments were conducted blind to the genotype of the animals.

#### *2.3.1. Conditioned Place Preference (CPP)*

*Apparatus.* The CPP apparatus (Lafayette Instruments, USA) consisted of two main compartments with differences in visual (wall patterns) and tactile (floor texture) cues, separated by a neutral compartment. The time spent in each compartment, as well as general locomotor activity, was recorded via horizontal optic sensor beams and specific software for the apparatus (Motor Monitor™, Kinder Scientific, USA).

*Protocol 1 – Twice Daily Conditioning Sessions.* The CPP protocol was modified from that described previously [328, 352]. On day 1 (habituation), mice were placed in the central compartment and allowed free access to the entire apparatus. On days 2–5 (conditioning), mice

received injections of saline (10ml/kg) in the morning (approximately 2 h following onset of light phase) and METH (methamphetamine hydrochloride, 2mg/kg i.p., dissolved in saline, Sigma-Aldrich Australia) in the afternoon (approximately 8 h following onset of light phase). Following the injection, mice were immediately confined into one of the two conditioning compartments. A combination of unbiased and biased allocation was used. Specifically, mice with a neutral preference (45–55% for either side) were randomly allocated their drug-paired side (unbiased allocation). For the remainder of the mice, the drug was paired with the side which was least preferred (biased allocation). On test (day 6), mice were placed in the central (neutral) compartment and given free access to the CPP apparatus. All sessions were 30 min in duration and occurred at the same time each day. Place preference was calculated as a preference score (time spent in drug-paired zone - time spent in the saline-paired zone). Locomotor data were also collected throughout CPP testing to assess the development and expression of behavioural sensitization.

*Protocol 2 - Saline vs. METH Conditioning.* This protocol was designed to demonstrate the expression of a positive place preference following METH conditioning, and the expression of a neutral place preference following conditioning with saline. Habituation, conditioning and test sessions were identical to those described in protocol 2; however, half of the mice in this paradigm were conditioned with saline in *both* compartments, while the other half were conditioned with METH in the non-preferred compartment (as in protocol 1). Locomotor data were collected during conditioning and test sessions to assess the development of behavioural sensitization.

### 2.3.2. Operant Self-Administration

*Apparatus.* Self-administration of oral sucrose or intravenous METH was assessed using operant chambers (model ENV-307W, Med Associates, Vermont, USA) equipped with two levers, one paired with reinforcement (the active lever), the other resulted in no outcome when pressed (the inactive lever). A discrete light located above the active lever was turned on for 10s in conjunction with reinforcement (conditioned stimulus, CS+). A vanilla-scented piece of paper (discriminative cue) was placed below the active lever prior to each session. The chambers were housed in sound attenuated boxes and ventilated with fans.

*Sucrose training.* Self-administration procedures were conducted under a reverse dark-light cycle with singly housed mice, as published previously [198, 328, 353, 354]. All sessions were conducted during the first half of the dark cycle. Mice were taught to discriminate the active from the inactive lever with 8 days of sucrose training [198, 328, 353, 354], to ensure differences in METH self-administration were not due to an inability to learn an operant task. The volume of 10% sucrose (w/v) delivered was 5 $\mu$ l, over 1.7 s. Inclusion criteria were 75% discrimination for the active lever vs. the inactive lever with >100 active lever presses per day, for the last 3 days of training. Sucrose training sessions were 2 h.

*Surgery.* Mice were maintained under controlled anaesthesia using isoflurane (5% induction; 1.5-2.0% maintenance in air) plus meloxicam (3mg/kg i.p.; Boeringher Ingleheim, Inglehiem, Germany) and then implanted with indwelling venous cannulae as previously described [198, 328, 353, 354]. The catheter was secured to the skull using Loctite™ Marine Adhesive and supported with dental cement. Mice were treated with 0.2% neomycin antibiotic (Delta Veterinary Laboratories, Hornsby, Australia) diluted in heparinised saline following surgery and

for 2-4 days recovery post-surgery, prior to the commencement of behavioural experiments. Animals were flushed twice daily with 10 and 90 units of heparin in saline throughout the duration of experimentation.

*METH intravenous self-administration – general procedures.* For self-administration testing, mice were connected via the jugular catheter to an intravenous line (Tygon; Saint Gobain Performance Plastics, Campbellfield, VIC, Australia) that was connected to a 22 gauge swivel (Instech Solomon, Plymouth Meeting, PA, USA). The swivel was connected with BCOEX-T22 tubing (Instech Solomon, Plymouth Meeting, PA, USA) to a syringe filled with methamphetamine solution held in an infusion pump. Infusion volume was 19  $\mu$ l and duration of infusion 1.7 s. Sessions were terminated if a predetermined maximum number of drug infusions was attained (detailed in protocols below), and no drug was administered in the 10 s immediately after each drug infusion. During this period the CS+ light remained on, and any active lever presses were recorded as ‘time out’ responses. All self-administration sessions were two hours in length, while extinction and reinstatement sessions were 45 min and 1 hr respectively (maximum infusion contingency notwithstanding). During FR1 and PR self-administration, mice were tested periodically for patency using 0.02-0.03ml (mGlu5 WT and KO) or 0.05-0.08ml ( $A_{2A}$  WT and KO) of ketamine (Parnell Laboratories, Alexandria, Australia) dissolved in saline (15mg/ml). A greater volume of ketamine was used in  $A_{2A}$  WT and KO mice compared to mGlu5 WT and KO due to differences in mouse size, resulting from background strain differences (CD-1 vs. C57Bl/6). If signs of sedation or loss of motor coordination were not immediately apparent mice were excluded from the study. The protocols to assess METH self-administration differed between the two mouse lines used; see below.

*Protocol 1: METH intravenous self-administration in mGlu5 WT and KO mice.* Following recovery from surgery, mice were tested using an FR1 schedule of reinforcement (see Figure 2.2a for the timeline). Mice were tested using a 3µg/kg/infusion dose, which supports METH self-administration in mice as established in our laboratory [355]. Mice were given a maximum of 12 days to reach the following criteria and be considered as having ‘acquired’ the lever press response for METH: >6 infusions, with 75% discrimination for the active lever, maintained over three consecutive days. Mice that did not reach criteria were excluded from the study. Data collected from the three days during which mice met criteria were considered ‘FR1 Acquisition’. Mice were then tested for 5 days under an FR1 schedule to assess ‘Stable FR1’ responding. This was followed by two days of progressive ratio (PR) responding, interspersed with one day of FR1, to assess the motivation to self-administer METH (see [198, 328, 353, 354] for methods). The PR schedule required animals to make an increasing number of active lever responses to obtain a drug infusion, under the following schedule: 1, 3, 9, 13, 16, 18, 20, 22, 24, 25, 27, 28, 29, 31, 32, 34, 35, 37, 39, 41, 44, 47, 52, 64, 76, 88, 100, 112, 124, 136. Breakpoint was used to assess motivation to self-administer, and was defined as the point where an animal ceases to press the active lever for a drug infusion when the instrumental requirement is progressively increased [198, 328, 353, 354]. There was no designated duration of inactivity during the session which was considered to indicate breakpoint being achieved.

Extinction training followed PR testing, where responses on the active lever were no longer reinforced with a drug infusion. The CS+ light and vanilla discriminative cue were not present during extinction sessions. Daily extinction sessions ran for 45 min each. Mice needed to reach extinction criteria to be considered extinguished: 30% of averaged Stable FR1 active lever presses maintained over two consecutive days [356]. The day after extinction criteria was met, reinstatement testing (one hour) was conducted, where the CS+ light and vanilla discriminative stimulus were reintroduced to the operant chambers, but active lever responses remained

unreinforced. Mice were considered to have reinstated their operant responding if their active lever presses during the reinstatement test were double the number of active lever responses during the final 2 days of extinction, and at least ten active lever presses were made [357].

**A**

Day	8 days	4 days	up to 11 days	8 days	up to 10 days	1 day
Test	Sucrose FR1	Surgery + Recovery	FR1 acquisition	Stable FR1 + PR	Extinction	Reinstatement

**B**

Day	8 days	4 days	6 days	4 days	5 days	5 days
Test	Sucrose FR1	Surgery + Recovery	FR1 3 $\mu$ g/kg/inf	FR1 10 $\mu$ g/kg/inf	FR3 10 $\mu$ g/kg/inf + PR	FR3 30 $\mu$ g/kg/inf + PR

**Figure 2.2. Operant self-administration timeline for mGlu5 WT and KO mice, and A<sub>2A</sub> WT and KO mice.** Self-administration of sucrose and then METH in **A)** mGlu5 WT and KO mice, and **B)** A<sub>2A</sub> WT and KO mice.

*Protocol 2: Sucrose self-administration in mGlu5 WT and KO mice.* A separate cohort of mGlu5 WT and KO mice were tested for self-administration of 10% sucrose w/v. Experimental procedures were identical to IVSA procedures within each genotype (see Figure 2.3a for the timeline); however, mice did not undergo jugular catheter surgery, and were left undisturbed in the home cage for four days to correspond with surgery and recovery time in METH self-administration experiments. Also, the maximum number of sucrose deliveries was increased to 550.

*Protocol 3: METH intravenous self-administration in A<sub>2A</sub> WT and KO mice.* The response of A<sub>2A</sub> WT and KO mice to METH was assessed using different doses and under different schedules of reinforcement. Following recovery from surgery, mice were tested using an FR1 schedule of



reinforcement. Mice were given 6 days of FR1 training at 3µg/kg/infusion, then the dose was increased to 10µg/kg/infusion for 4 days (see Figure 2.2b for the timeline). A period of stable self-administration followed this, where mice were tested under FR3 at 10 and 30µg/kg/infusion for 4 days per dose. PR testing was assessed after 4 days of FR3 at 10 and 30µg/kg/infusion, where breakpoint was used to assess motivation to self-administer.

*Protocol 4: Sucrose self-administration in A<sub>2A</sub> WT and KO mice:* A<sub>2A</sub> WT and KO mice were tested in sucrose self-administration using a range of doses and schedules of reinforcement, to mimic the range of doses and schedules of reinforcement tested in METH IVSA (see Figure 2.3b for the timeline). Mice were taught to discriminate the active from the inactive lever as described above. Mice did not undergo jugular catheter surgery, and were left undisturbed in the home cage for two days to correspond with surgery and recovery time in METH self-administration experiments. Mice were then tested using 10% w/v sucrose under a FR1 and FR3 schedule (4 days each), followed by one day of PR testing. After this, the concentration of sucrose was reduced to 2.5% w/v and mice were subsequently tested under a FR1 and FR3 schedule (4 days each), followed by PR testing for one day. The maximum number of sucrose deliveries was 550.

**A**

<b>Day</b>	8 days	4 days	up to 11 days	8 days	up to 10 days	1 day
<b>Test</b>	Sucrose FR1	Surgery + Recovery	FR1 acquisition 10%	Stable FR1 + PR 10%	Extinction	Reinstatement

**B**

<b>Day</b>	8 days	2 days	4 days	4 days	1 day	4 days	4 days	1 day
<b>Test</b>	Sucrose FR1	Surgery + Recovery	FR1 10%	FR3 10%	PR	FR1 2.5%	FR3 2.5%	PR

**Figure 2.3. Sucrose self-administration timeline for mGlu5 WT and KO mice, and A<sub>2A</sub> WT and KO mice.** Self-administration of sucrose in **A)** mGlu5 WT and KO mice, and **B)** A<sub>2A</sub> WT and KO mice.

#### 2.4. Stereotaxic surgery

All mice were at least 3 months old prior to surgery, and were genotyped by PCR before viral injection (see section 2.1). Meloxicam (3mg/kg i.p.; Boeringher Ingleheim, Inglehiem, Germany) was administered immediately prior to surgery. Mice were anaesthetised with 5% isoflurane in air and maintained at 1-1.5% throughout surgery. Once anaesthetised mice were mounted in a stereotaxic frame (Stoelting, Dublin, Ireland), an incision was made in the skull, and bregma and lambda identified. Skull alignment was verified using the sagittal suture, and the skull was levelled across the anterior-posterior plane using bregma and lambda, as well as across the medial-lateral plane using two points made  $\pm$  2mm lateral of bregma. Coordinates were marked and then holes drilled into the skull. The coordinates for the NAcc shell used in the present experiments were determined from pilot experiments (data not shown), and were anteroposterior from bregma: +2.4 mm, mediolateral from the midline:  $\pm$  0.75 mm, dorsoventral from the skull surface: -4.85 mm. AAV-Cre or mCherry virus (see chapter 6 for more detail on these viruses) was injected bilaterally using a glass pipette connected to a 10 $\mu$ L Hamilton syringe, which was connected to an infusion pump mounted on the arm of the stereotaxic frame.

The injection volume was 500nL, at a rate of 500nL per min. The syringe was left in the brain for 2 min following virus infusion, and then raised 0.2mm and left for a further 4 min to prevent virus spreading up the injection tract. The pipette was then removed from the brain, the scalp incision was treated with Tricin antibiotic and sutured closed. Immediately after surgery, mice were allowed to recover for at least 1hr in an incubator (27°C), and then returned to the home cage. There was a three-week interval between surgery and place preference testing, to allow maximum viral transfection and transduction. Animals were then tested in METH place preference using protocol 1 (see section 2.3.1). At the end of all behavioural experiments, animals were perfused, brains collected and frozen, and double labelling fluorescence immunohistochemistry for Cre-recombinase (Cre) and A<sub>2A</sub> (see section 2.5.3).

## **2.5. Histology**

### *2.5.1. Tissue extraction and processing*

Brains for Fos-immunoreactivity (IR) studies in chapter 5 were collected 90 min after the start of the CPP test, as this time point reflects maximum Fos expression following behavioural testing [358, 359]. Brains from A<sub>2A</sub><sup>loxP/loxP</sup> mice used to assess spread and expression of Cre-recombinase and A<sub>2A</sub> in chapter 6 were collected 1 day after completion of experimental procedures. Mice were anaesthetised with 0.1mL sodium pentobarbitone (>60mg/kg ip) and then transcardially perfused with approximately 50mL of 0.1M phosphate-buffered saline (PBS) followed by 60-80ml of 4% paraformaldehyde (PFA; Sigma-Aldrich, St Louis, MO, USA) dissolved in PBS. Brains were post-fixed in 4% PFA for 1-2 hr after perfusion, and then stored in 20% sucrose dissolved in PBS overnight. Brains were frozen over liquid nitrogen the following day, and stored at -80°C until cut on a Leica cryostat CM1950 (Leica Biosystems, North Ryde,

Australia) into 40µm sections. Sections were cut in a 1 in 4 series into cryoprotectant [360] and stored at -20°C until processed.

### 2.5.2. *Fos immunohistochemistry and counting procedure*

Sections from the forebrain and midbrain were used for Fos immunohistochemistry, using a protocol adapted from Madsen *et al.* [361]. Sections were washed in 0.1M PBS (3 x 10 min), then quenched for 15 min (10% methanol, 10% hydrogen peroxide, 80% PBS). Following further washing (3 x 10 min), sections were incubated overnight at room temperature in a solution containing 0.1% goat polyclonal c-fos antibody (sc-52-G, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 0.5% normal horse serum (NHS), 0.5% Triton-X 100 (TX) and PBS. The next day, sections were washed (3 x 10 min) and then blocked for 30 min (0.5% NHS, 0.5% TX, PBS). Sections were then incubated for 1.5 hr at room temperature in a solution containing 0.2% biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA), 0.5% NHS, 0.5% TX, and PBS. Sections were then washed (3 x 10 min) and incubated for 1 hr in 0.4% Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) in PBS. Sections were again washed (3 x 10 min) prior to incubation with nickel enhanced 3,3'-diaminobenzidine tetrahydrochloride chromagen (DAB, Sigma-Aldrich, St Louis, MO, USA) solution containing 25% 0.1 M PBS and 0.004% w/v ammonium chloride/ammonium nickel (II) sulfate hexahydrate for 15 min. Immunoreactivity was developed by addition of 1% hydrogen peroxide. The reaction was terminated by addition of PBS. Sections were washed (3 x 10 min), slide mounted in 0.5% gelatine in dH<sub>2</sub>O, and dried. Slides were serially dehydrated with ethanol and cleared using X-3B before being coverslipped.

Slides were imaged using an Olympus BX51 microscope and Stereo Investigator software (MBF Bioscience, Williston, VT, USA). Each region of interest was counted in two sections per animal

by an experimenter blind to experimental conditions. Care was taken to ensure sections were matched at the same anatomic level for each mouse. The regions counted in each study are clarified in the specific methods in chapter 5.

### *2.5.3. Immunohistochemical procedures for double labelling of Cre-recombinase and A<sub>2A</sub>*

Double labelling of Cre and A<sub>2A</sub> was conducted using fluorescent immunohistochemistry. Sections were washed in 0.1M PBS (3 x 5 min), then blocked for 30 min using 10% normal donkey serum (NDS) with 0.5% TX in PBS. Sections were incubated at room temperature in primary antibodies overnight (A<sub>2A</sub>: 0.2% mouse monoclonal A<sub>2A</sub> antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cre-recombinase: 0.1% rabbit polyclonal Cre antibody, Merck Millipore, Darmstadt, Germany) in a PBS solution containing 0.5% TX and 1% NDS. The next day, sections were washed in PBS (3 x 5 min), then blocked for 30 min in 10% NDS and 0.5% TX in PBS. Sections were incubated at room temperature in fluorescent secondary antibodies (0.2% Alexa Fluor® 488 donkey anti-mouse IgG, 0.2% Alexa Fluor® 568 donkey anti-rabbit; Life Technologies, Mulgrave, VIC, Australia) and 1% NDS with 0.5% TX in PBS for 1.5-2 hr. Sections were washed again (3 x 5 min in PBS) and then mounted on slides in 0.5% gelatine in dH<sub>2</sub>O. Slides were covered and dried for 1-2 hr at room temperature prior to coverslipping using Dako Fluorescence Mounting Medium (Dako, North America Inc., CA, USA). Slides were imaged using an Olympus BX51 microscope and Stereo Investigator software (MBF Bioscience, Williston, VT, USA).

#### 2.5.4. *Site validation*

Sections from the forebrain containing the NAcc, as well as sections immediately rostral and caudal of this region, were used for verification of injection site of viruses. Cre-IR and mCherry fluorescence was observed under an Olympus BX51 microscope, and areas of viral expression were mapped onto a corresponding Mouse Brain Atlas page [362] to generate a representation of injection site location in the brain for each mouse. Injection sites were validated as 'hits' or 'misses' by an experimenter blind to the experimental groups.

#### 2.5.5. *Optical Density*

The expression of  $A_{2A}$  following viral treatment, as well as the area containing viral IR within the NAcc shell was quantitated using MCID Core Digital Imaging Software. Optical density has been employed previously to determine the degree of knockdown in animals treated with AAV-Cre [363-365]. Measurements of optical density (of  $A_{2A}$ ) and area size (of viral expression) were taken with the images used for site validation (above). Measurements were taken throughout the rostrocaudal axis and then averaged across hemispheres and across sections to produce a single score for each measure in each animal. All optical density measurements were calculated by [optical density of nominated region – optical density of background in the same section]. Lower optical density values indicate a reduction in the expression of  $A_{2A}$ .

## Chapter 3

# The Metabotropic Glutamate 5 Receptor Modulates Extinction and Reinstatement of Methamphetamine- Seeking in Mice

### 3.1. Introduction

As discussed in Chapter 1, mGlu5 appears to play a significant role in addiction relevant behavioural and neural processes. In particular, the involvement of mGlu5 in cognitive dimensions of addiction, such as the extinction of drug-seeking, has gained considerable attention in recent years (see [156, 236, 244, 248, 250, 251]). Indeed, facilitating extinction processes may present a novel therapeutic avenue for relapse prevention, as cues and contexts associated with drug-taking can precipitate relapse, and thus reducing the salience of these cues and contexts may reduce relapse propensity [227]. However, to date all studies which demonstrate that modulation of mGlu5 signalling affects extinction behaviour have only been conducted using cocaine or ethanol as reinforcers. While cocaine and METH are both classed as psychostimulants, there are different mechanisms of action and pharmacokinetic profiles between these two drugs; indeed, the mechanisms of action and pharmacokinetics are even more dissimilar between METH and alcohol [50, 53, 366-369]. Thus, it is important to determine whether mGlu5 is also implicated in extinction behaviour for METH. In the current set of experiments I employed a genetic approach to determine the role of mGlu5 in METH-driven behaviours. I did this because acute pharmacological studies demonstrate a role for mGlu5 many aspects of addiction, such as self-administration, extinction and reinstatement [228-230, 232-234, 244, 248, 249, 254, 256]; yet genetic studies suggest the critical role for mGlu5 lies in extinction and reinstatement [236, 237]. Also, considering the potential for altered

receptor expression following repeated mGlu5 agonist or antagonist administration, I used a genetic approach to model long term receptor up- or downregulation, as may occur following chronic agonist or antagonist treatment.

The following paper, published in *PLoS ONE* in July 2013 (volume 8, issue 7) outlines the behavioural response of mGlu5 KO mice to METH in the following addiction-relevant paradigms: conditioned place preference, locomotor sensitization, and operant self-administration. The response of mGlu5 KO mice to a natural reinforcer (sucrose) was also examined in the operant paradigm.



## 3.2. Publication

OPEN ACCESS Freely available online



# The Metabotropic Glutamate 5 Receptor Modulates Extinction and Reinstatement of Methamphetamine-Seeking in Mice

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### Abstract

Methamphetamine (METH) is a highly addictive psychostimulant with no therapeutics registered to assist addicts in discontinuing use. Glutamatergic dysfunction has been implicated in the development and maintenance of addiction. We sought to assess the involvement of the metabotropic glutamate 5 receptor (mGlu5) in behaviours relevant to METH addiction because this receptor has been implicated in the actions of other drugs of abuse, including alcohol, cocaine and opiates. mGlu5 knockout (KO) mice were tested in intravenous self-administration, conditioned place preference and locomotor sensitization. Self-administration of sucrose was used to assess the response of KO mice to a natural reward. Acquisition and maintenance of self-administration, as well as the motivation to self-administer METH was intact in mGlu5 KO mice. Importantly, mGlu5 KO mice required more extinction sessions to extinguish the operant response for METH, and exhibited an enhanced propensity to reinstate operant responding following exposure to drug-associated cues. This phenotype was not present when KO mice were tested in an equivalent paradigm assessing operant responding for sucrose. Development of conditioned place preference and locomotor sensitization were intact in KO mice; however, conditioned hyperactivity to the context previously paired with drug was elevated in KO mice. These data demonstrate a role for mGlu5 in the extinction and reinstatement of METH-seeking, and suggests a role for mGlu5 in regulating contextual salience.

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### Introduction

Methamphetamine (METH) is a highly addictive psychostimulant for which there are currently no approved pharmacotherapies to treat abusers [1,2]. Glutamatergic dysfunction has been implicated in the development and maintenance of addiction [3]. Indeed, overwhelming evidence from rodent models suggests chronic drug use results in the dysregulation of the glutamatergic system (e.g. [4–8]; for reviews see [3,9–11]). This is reflected in human imaging studies, which reveal reduced brain glutamate concentrations in frontal white and grey matter in recently abstinent METH users [12–15]. Furthermore, relapse of drug-seeking in animal models can be attenuated by reversing glutamatergic dysfunction [16–18]. There is some support for this in preliminary human studies in drug addicts; N-acetylcysteine administration (which restores glutamate homeostasis) reduces cocaine craving in addicts [19], however, N-acetylcysteine combined with naltrexone for METH dependence has no effect on METH use or craving. Hence, while there may be a role for

glutamate dysfunction in METH addiction (e.g. [12,13,20,21]), the nature of this dysfunction requires further investigation.

Of the various ionotropic and metabotropic glutamate receptors, the metabotropic glutamate 5 receptor (mGlu5) has provoked considerable interest as a potential therapeutic target for drug addiction [22–28], partly due to its distribution in the neural circuitry underlying reward consumption and seeking. Specifically, mGlu5 is predominantly located post-synaptically [29,30] in areas such as the hippocampus, cerebral cortex, nucleus accumbens (NAcc), lateral septum and the dorsal striatum [29,31]. Moreover, mGlu5 has been implicated in drug-taking behaviour; a reduction in mGlu5 signalling reliably decreases drug-taking and drug-seeking behaviour for alcohol [32–34], cocaine [35], METH [36,37], opiates [38] and nicotine [39,40]. A reduction in the acquisition of conditioned place preference (CPP) for cocaine [41,42] and morphine [43], as well as reduced expression of CPP for morphine [44], ethanol [45] and amphetamine [46] has also been reported following mGlu5 antagonist administration. Somatic signs of withdrawal to nicotine are attenuated [47], cocaine self-

administration abolished [41] and ethanol consumption reduced [48] in mGlu5 KO mice.

Although those studies suggest that a *reduction* in mGlu5 signaling may be a helpful approach to treat drug abuse, it is important to highlight that mGlu5 receptors play a critical role in long-term potentiation and depression [49–53], the putative cellular mechanisms for learning and memory [54,55]. Considering that addiction is characterised by dysfunction in learning processes [56,57], the implication of mGlu5 in learning processes suggests mGlu5 signalling is a potential target for addiction therapeutics. In support of this idea, recent reports suggest a role for mGlu5 in extinction and reinstatement of drug-seeking behaviour. Administration of the mGlu5 positive allosteric modulator (PAM), 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), facilitates the acquisition and consolidation of extinction of cocaine self-administration [58], as well as enhancing extinction of cocaine CPP [59]. Unlike cocaine, CDPPB has no effect on extinction of METH self-administration [60]. Gass and colleagues [36] report reduced cue-induced and drug-primed reinstatement of METH self-administration following MTEP administration; yet extinction was not examined in that study.

The involvement of mGlu5 in behaviours relevant for METH addiction, particularly extinction and reinstatement, is not clear from the current pharmacological literature. In addition, issues of tolerance and dose have been raised with pharmacological approaches [60,61]. Thus, in order to clarify the role of mGlu5 in these behaviours we utilised a model of genetic deletion. Specifically, the current study examined how mGlu5 KO mice responded to METH in a range of addiction-relevant behavioural paradigms. We also examined the response of mGlu5 KO mice to a natural reward (sucrose) in an operant paradigm, to delineate possible differences in extinction and reinstatement for METH and a natural reward. Using this genetic approach we sought to resolve if mGlu5 is necessary or sufficient for METH-driven behaviours.

## Methods

### Animals

mGlu5 KO mice [49] on a C57BL/6J background (Grm5tm1Rod; stock 003558) were obtained from Jackson Laboratories (Bar Harbour, ME, USA). All experimental subjects were fully backcrossed onto the C57BL/6 background (>10 generations). Mice were kept in standard housing (*ad libitum* standard laboratory chow and water, tissues for nesting material) under a 12:12 h light-dark cycle unless otherwise specified. Experiments were conducted using age-matched adult male mice littermates; cohort 1 [WT (n = 15), mGlu5 KO (n = 22)] was used for conditioned place preference, cohort 2 [WT (n = 17), mGlu5 KO (n = 13)] was used for intravenous self administration, cohort 3 [WT (n = 8), mGlu5 KO (n = 7)] was used for sucrose self-administration. In all experiments, genotypes were counterbalanced across test apparatus and sessions, and were conducted by an experimenter blind to the genotype of the animals.

### Ethics Statement

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia (Florey Animal Ethics Committee: ethics approval number: 11–015). All efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to *in vivo* techniques, if available.

## Behavioural Phenotyping

**Conditioned Place Preference (CPP).** The CPP apparatus (Lafayette Instruments, USA) consisted of two main compartments with differences in visual (wall patterns) and tactile (floor texture) cues, separated by a neutral compartment. The time spent in each compartment, as well as general locomotor activity, was recorded via horizontal optic sensor beams and specific software for the apparatus (Motor Monitor<sup>TM</sup>, Kinder Scientific, USA).

The CPP protocol was modified from that described previously [38,62]. Before each session mice were habituated to the experimental room for at least 30 min. On day 1 (habituation), mice were placed in the central compartment and allowed free access to the entire apparatus. On days 2–4 (conditioning), mice received injections of saline (10 ml/kg) or METH (2 mg/kg *i.p.*, dissolved in saline, Sigma-Aldrich Australia) and were immediately confined into one of the two conditioning compartments. A combination of unbiased and biased allocation was used. Specifically, mice with a neutral preference (45–55% for either side) were randomly allocated their drug-paired side (unbiased allocation). For the remainder of the mice, the drug was paired with the side which was least preferred (biased allocation); approximately 55% of mice demonstrated a side preference. On test, mice were given free access to the CPP apparatus.

All sessions were 30 min in duration and occurred at the same time each day. Place preference was calculated as a preference score (time spent in drug-paired zone - time spent in the saline-paired zone). Locomotor data was also collected throughout CPP testing to assess the development of behavioural sensitization [62].

**Intravenous Self-Administration (IVSA).** Operant self-administration of oral sucrose or intravenous METH (3 µg/kg/infusion) was assessed using operant chambers (model ENV-307W, Med Associates, Vermont, USA) equipped with two levers, one paired with reinforcement (the active lever), the other resulted in no outcome when pressed (the inactive lever). A stimulus light located above the active lever was turned on for 10 s in conjunction with reinforcement (conditioned stimulus, CS). A vanilla-scented piece of paper (discriminative cue) was placed below the active lever prior to each session. The chambers were housed in sound attenuated boxes and ventilated with fans.

Self-administration procedures were conducted under a reverse dark-light cycle with singly housed mice, as published previously [38,63–65]. Mice were given at least 7 days to acclimatise to the reverse light cycle and to single-housing. All sessions were conducted during the first half of the dark cycle. Mice were taught to discriminate the active from inactive lever with 8 days of sucrose training [38,63–65], to ensure differences in METH self-administration were not due to an inability to learn an operant task. The volume of sucrose delivered was 5 µl, over 1.7 s. Inclusion criteria were 75% discrimination for the active lever vs. the inactive lever with >100 active lever presses per day, for the last 3 days of training. Sucrose training sessions were 2 h.

After instrumental training, mice were anaesthetised using isoflurane (1.5–2.0% in air) plus meloxicam (3 mg/kg *i.p.*) and then implanted with indwelling venous cannulae as previously described [38,63–65]. Mice were treated with neomycin antibiotic diluted in saline following surgery and during the 4 days recovery post-surgery, prior to the commencement of behavioural experiments.

For self-administration testing, mice were connected via the jugular catheter to an intravenous line (Tygon; Saint Gobain Performance Plastics, Campbellfield, VIC, Australia) which in turn was connected to a 22 gauge swivel (Instech Solomon, Plymouth Meeting, PA, USA). The swivel was connected with BCOEX-T22 tubing to a syringe filled with methamphetamine

solution held in an infusion pump. Following recovery from surgery, mice were tested using an FR1 schedule of reinforcement. Infusion volume was 19  $\mu$ l and duration of infusion 1.7 s. Sessions were terminated if a predetermined maximum number of drug infusions (100) was attained, and no drug was administered in the 10 s immediately after each drug infusion. During this period the stimulus light remained active, and any active lever presses were recorded as 'time out' responses. All sessions were two hours in length (maximum infusion contingency notwithstanding). Mice were given a maximum of 12 days to reach the following criteria and be considered as having 'acquired' the lever press response for METH: >6 infusions, with 75% discrimination for the active lever, maintained over three consecutive days. Mice that did not reach criteria were excluded from the study. Data collected from the three days during which mice met criteria were considered 'FR1 Acquisition'. Mice were then tested for 5 days under a fixed ratio 1 (FR1) schedule to assess 'Stable FR1' responding. This was followed by two days of progressive ratio (PR) responding, interspersed with one day of FR1, to assess the motivation to self-administer METH (see [38,63–65] for methods). Breakpoint was used to assess motivation to self-administer, and was defined as the point where an animal ceases to press the active lever for a drug infusion when the instrumental requirement is progressively increased [38,63–65]. Extinction training followed PR testing, where responses on the active lever were no longer reinforced with a drug infusion. The stimulus light and discriminative cue were not present during extinction sessions. Extinction sessions ran for 45 min. Mice needed to reach extinction criteria to be considered extinguished: 30% of averaged Stable FR1 active lever presses maintained over two consecutive days [66]. The day after extinction criteria was met, reinstatement testing (one hour) was conducted, where the stimulus light and vanilla discriminative stimulus were reintroduced to the operant chambers, but active lever responses remained unreinforced. Mice were considered to have reinstated their operant responding if their active lever presses during the reinstatement test were double the number of active lever responses during extinction, and at least ten active lever presses were made [67]. Throughout the experiment mice were tested periodically for patency using 0.02–0.03 ml of 15 mg/kg ketamine (Parnell Laboratories, Alexandria, Australia); if signs of hypnosis were not apparent mice were excluded from the study.

A third cohort of mice was tested for self-administration of 10% sucrose w/v. Experimental procedures were identical to IVSA procedures; however, mice did not undergo jugular catheter surgery. Also, the maximum number of sucrose deliveries was increased to 550 during FR1 acquisition and Stable FR1 training.

### Statistical Analysis

Three- and two-way repeated measures (RM) analysis of variance (ANOVA) with factors 'days', 'lever type' and/or 'drug' and between factor 'genotype' were conducted. Where appropriate, this was followed by one-way ANOVA split by corresponding factor with a Bonferroni correction ( $p = .05/\text{number of independent variables}$ ). One-way ANOVA with the between factor 'genotype' was used to assess latency to acquisition/extinction. A log-rank (Mantel-Cox) test was used to assess duration of extinction, and Fisher's exact test was used to assess the propensity to reinstate. Data presented as means  $\pm$  standard error of the mean (SEM). Data analysis conducted using SPSS Statistics version 20 and GraphPad: Prism version 5.

## Results

### CPP

Preference for the METH-paired compartment during the CPP test was significantly increased from preference during habituation in both genotypes, evidenced by a main effect of 'day' [ $F(1,35) = 59.2, p < .001$ ] and no effect of 'genotype' [ $F(1,35) = .1, p = .8$ ]. A significant interaction [ $F(1,35) = 7.1, p = .01$ ] suggests a greater increase in preference score in WT compared to mGlu5 KO mice; however, one-way ANOVA split by 'genotype' demonstrates a significant increase in preference score in both genotypes [WT:  $F(1,14) = 28.5, p < .001$ ; mGlu5 KO:  $F(1,21) = 25.6, p < .001$ ; Fig. 1a].

### Locomotor Sensitization

Hyperactivity in mGlu5 KO mice was present upon exposure to a novel environment ['time'  $\times$  'genotype' interaction,  $F(1,175) = 6.1, p = .001$ ; data not shown], similar to the phenotype of this mouse reported previously [68]. The development of sensitization to METH (2 mg/kg i.p.) was present in both genotypes [main effect of 'days'  $F(3,105) = 26.4, p < .001$ , no effect of 'genotype'  $F(1,35) = .7, p = .4$ ; Fig. 1b]. Locomotor activity was heightened following METH administration compared to saline administration [main effect of 'drug'  $F(1,35) = 479.1, p < .001$ ]. A significant interaction between 'drug'  $\times$  'days' [ $F(3,105) = 106.6, p < .001$ ] suggests locomotor activity increased under METH compared to saline treatment during conditioning (Fig. 1b). Indeed, one-way ANOVA split by 'drug' and 'genotype' revealed a significant increase in locomotor activity on METH conditioning days (*vs.* METH conditioning day 1), and a decrease in locomotor activity on saline conditioning days (*vs.* saline conditioning day 1) in both genotypes (Fig. 1b).

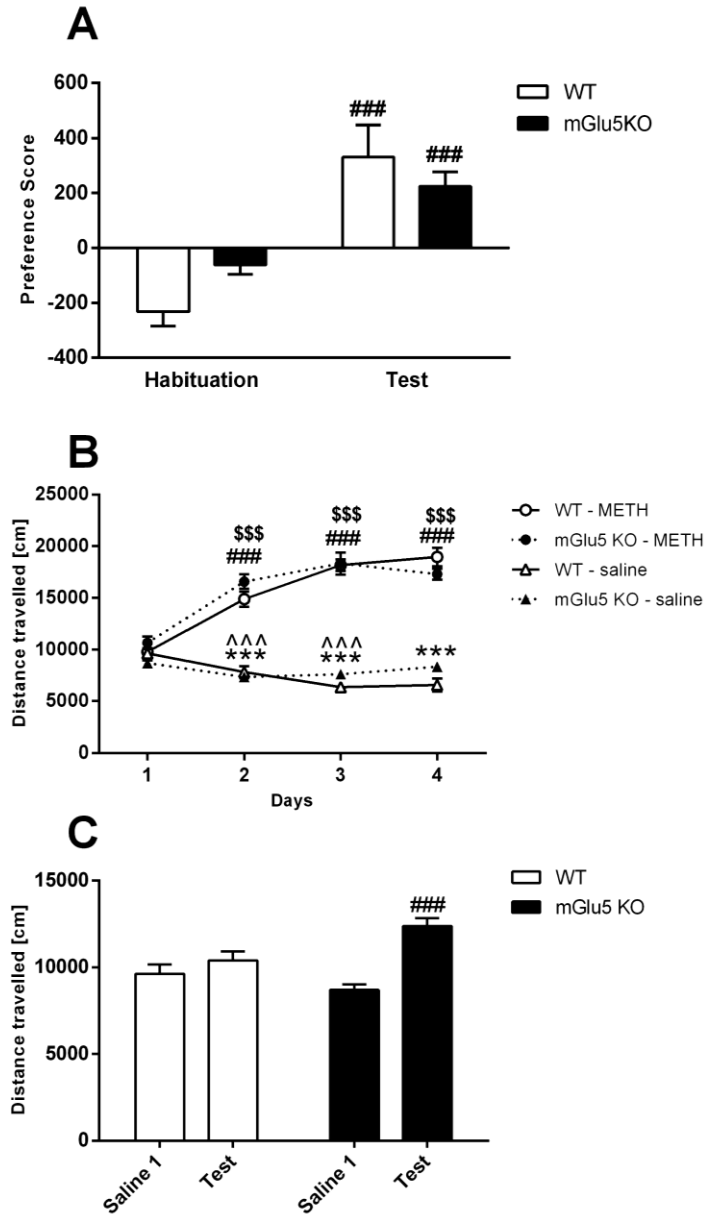
### Conditioned Hyperactivity

Conditioned hyperactivity was present on CPP test day [main effect of 'day'  $F(1,35) = 48.4, p < .001$ ; not of 'genotype'  $F(1,35) = .9, p = .3$ ; Fig. 1c]. There was a significant 'day'  $\times$  'genotype' interaction [ $F(1,35) = 20.8, p < .001$ ], suggesting conditioned hyperactivity on test day was more pronounced in mGlu5 KO mice (Fig. 1c). A significant 'day'  $\times$  'time'  $\times$  'genotype' interaction [ $F(5,175) = 2.4, p = .04$ ] suggests conditioned hyperactivity was present in KO mice throughout the entire test session, but was only present in WT mice in the first 5 minutes of the test (data not shown).

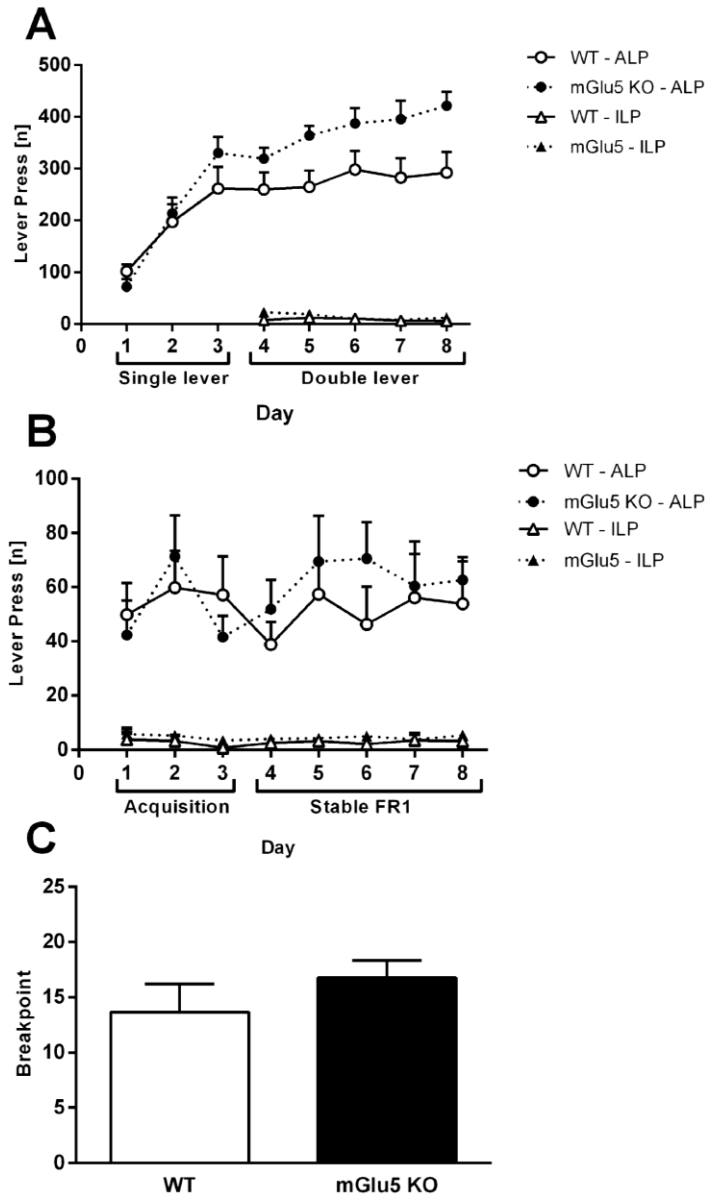
### Instrumental Learning

Acquisition of the single lever instrumental response was similar between the genotypes [main effect of 'days'  $F(2,58) = 50.6, p < .0001$ , n.s. main effect of 'genotype'  $F(1,28) = .2, p = .7$ ; Fig. 2a]. During double lever training, both genotypes showed clear discrimination for the active lever over the inactive lever [main effect of 'lever type'  $F(1,28) = 295.7, p < .001$ ; n.s. main effect of 'days'  $F(4,116) = 1.9, p = .1$ , Fig. 2a]. mGlu5 KO mice made more active lever presses than WT mice on days 4–8 [main effect of 'genotype'  $F(1,28) = 6.7, p = .02$ ; interaction between 'genotype' and 'lever type'  $F(1,28) = 5.3, p = .03$ ; Fig. 2a].

*IVSA.* 3 WT mice were excluded as they did not reach FR1 acquisition criteria; data from these mice was excluded from instrumental analyses. 3 mice were excluded due to loss of patency during the experiment; data for these mice was included in analyses until mice lost patency. 1 WT was excluded from the extinction analysis, as its score was >14 standard deviations above the mean. The data from this mouse was also excluded from the reinstatement analysis.



**Figure 1. METH CPP Preference Score, Locomotor Sensitization and Conditioned Hyperactivity. A)** Preference score in WT and mGlu5 KO mice at habituation and test. Preference score is defined as (time spent in the METH-paired compartment – time spent in the saline-paired compartment). **B)** Locomotor sensitization in WT and mGlu5 KO mice following acute saline or 2 mg/kg METH i.p. injection over 4 consecutive days. **C)** Conditioned hyperactivity in WT and mGlu5 KO mice during the CPP test. Data (mean ± SEM) analysed using two- or three-way RM ANOVA, followed by one-way ANOVA split by the factor ‘genotype’ with a Bonferroni correction. In Figs **A)** and **C)**, significant effects of ‘day’ (vs. habituation) are represented by ‘#’ (###*p*<.001); significant effects of ‘genotype’ (vs. WT on the same day) are represented by ‘\*’ (\*\**p*<.01). In Fig **B)**, significant effects of ‘day’ (vs. METH day 1) are indicated by ‘\$’ for WT mice (\$\$\$*p*<.001), ‘#’ for mGlu5 KO mice (###*p*<.001). Significant effects of ‘day’ (vs. saline day 1) are indicated by ‘\*’ for WT mice (\*\**p*<.001) and ‘†’ for mGlu5 KO mice (†*p*<.001). A significant ‘day’ × ‘genotype’ interaction was present in both Fig. A) and C), suggesting **A)** a greater change degree of change from habituation to test in WT compared to mGlu5 KO mice, and **C)** a greater change degree of change from habituation to test in mGlu5 KO compared to WT mice. Abbreviations: Saline 1 - day 1 of saline treatment. doi:10.1371/journal.pone.0068371.g001



**Figure 2. Instrumental learning, METH self-administration and progressive ratio testing.** **A**) Self-administration of 10% sucrose solution with a fixed ratio 1 schedule in WT and mGlu5 KO mice. Single lever training (days 1–3) was followed by double lever training (days 4–8). A significant main effect of ‘lever type’ suggests clear discrimination for the active lever in each genotype. **B**) Acquisition and stable self-administration of 3  $\mu\text{g}/\text{kg}/$  infusion METH in WT and mGlu5 KO mice. **C**) Final breakpoint reached within a two-hour test using a progressive ratio schedule of reinforcement. Data (mean  $\pm$  SEM) analysed using two- or three-way RM ANOVA, followed by one-way ANOVA split by the factor ‘genotype’ with a Bonferroni correction where appropriate. Significant main effects of ‘lever type’ during sucrose and METH acquisition and self-administration suggest clear discrimination for the active lever in each genotype. doi:10.1371/journal.pone.0068371.g002

During FR1 acquisition, there was no difference in the number of lever presses made or infusions received between the genotypes

[no main effect of ‘genotype’ for lever press:  $F(1,22) = 0.1$ ,  $p = .9$ ; infusions:  $F(1,22) = 1.1$ ,  $p = .3$ ; Table 1]. Both genotypes showed

clear discrimination for the active lever over the inactive lever [main effect of 'lever type'  $F(1,22) = 73.0, p < .001$ ; Fig 2b]. During Stable FR1 training, both genotypes made a similar number of infusions [WT:  $42.2 \pm 6.5$ ; mGlu5 KO:  $43.8 \pm 6.1$ ;  $p > .05$ ] and showed clear discrimination for the active lever over the inactive lever [main effect of 'lever type',  $F(1,20) = 63.4, p < .001$ ; Fig. 2b]. There was no difference between the genotypes in the motivation to self-administer METH, as assessed by a PR [n.s. main effect of 'genotype'  $F(1,18) = .1, p = .7$ ; n.s. effect of 'days'  $F(1,18) = .8, p = .4$ ; data presented as average breakpoint across two days of PR; Fig. 2c].

During extinction training, mGlu5 KO mice demonstrated a significantly longer latency to extinguish their responding for drug reinforcement [log-rank test,  $\chi^2 = 5.0, df = 1, p = .03$ ; Fig. 3a]. This was confirmed with a one-way ANOVA main effect of 'genotype' on the latency to extinguish [ $F(1,18) = 4.7, p = .04$ ; Fig. 3b]. Lever presses on the final two days of extinction training were averaged to produce an extinction lever press score. In order to compare Stable FR1 responding to extinction, responding was expressed as lever presses per minute to account for session duration. Both genotypes demonstrated a significant reduction in their extinction lever press score compared to averaged Stable FR1 [main effect of 'day'  $F(1,18) = 41.0, p < .001$ ; no main effect of 'genotype'  $F(1,18) = .3, p = .6$ ; Table 1]. During reinstatement, where cues signalling the availability of drug reinforcement were reintroduced, there was a low level of lever pressing in WT mice (Fig. 3c; n.s. effect of 'genotype'). We applied reinstatement criteria (at least 2x extinction score and  $>10$  active lever presses) to assess differences in the propensity to reinstate. The number of mGlu5 KO that met reinstatement criteria was greater than WT mice (2/8 WT mice and 12/13 mGlu5 KO mice reinstated; Fisher's exact test:  $p = .004$ ; Phi coefficient:  $-0.685$ ; Fig. 3d). Examining mice that did meet reinstatement criteria, both genotypes showed enhanced responding on the active lever during reinstatement compared to that of the final two days of extinction training [main effect of 'day'  $F(1,11) = 16.0, p = .002$ ]. There was a main effect of 'genotype' [ $F(1,11) = 8.7, p = .01$ ], and a 'genotype'  $\times$  'day' interaction [ $F(1,11) = 8.5, p = .01$ ], suggesting lower active lever pressing

during reinstatement in mGlu5 KO mice compared to WT upon reinstatement (Table 1).

### Sucrose Self-administration

Both genotypes acquired the operant response for 10% sucrose solution during the initial 8 days of training, with clear discrimination for the active lever (data not shown). During FR1 acquisition and Stable FR1, the number of sucrose deliveries self-administered was similar between the genotypes [FR1 acquisition: WT:  $241 \pm 42$ , mGlu5 KO:  $228 \pm 21$ ,  $F(1,13) = .1, p = .8$ ; Stable FR1: WT:  $271 \pm 36$ , mGlu5 KO:  $215 \pm 31$ ,  $F(1,13) = 1.3, p = .3$ ]. There was clear discrimination for the active lever in both genotypes during Stable FR1 [main effect of 'lever type'  $F(1,13) = 68.5, p < .0001$ ; no interaction; data not shown]. The motivation to self-administer sucrose demonstrated no difference in the breakpoint between the two genotypes [average breakpoint WT:  $34.8 \pm 3.4$ , mGlu5 KO:  $28.2 \pm 2.5$ , unpaired t-test,  $t = 1.5, df = 13, p = .2$ ].

Unlike METH IVSA, both genotypes met extinction criteria within two days of testing (percentage of Stable FR1 day 1 WT:  $15.9 \pm 4.3$ , mGlu5 KO:  $9.5 \pm 1.9$ , day 2: WT:  $16.1 \pm 4.2$ , mGlu5 KO:  $11.4 \pm 3.1$ ). The extinction lever press score was significantly reduced from that of Stable FR1 in both genotypes [main effect of 'day'  $F(1,13) = 67.5, p < .0001$  but not of 'genotype'  $F(1,13) = .7, p = .4$ , no interaction; Fig. 4a].

In the reinstatement test, there was a similar proportion of WT and mGlu5 KO mice which met reinstatement criteria (WT: 5/8, mGlu5 KO: 6/7; Fisher's exact test  $p = .6$ ; Phi coefficient:  $-0.134$ ). Within the reinstating mice, both genotypes demonstrated significantly greater active lever pressing during reinstatement compared to extinction [main effect of 'day'  $F(1,10) = 39.5, p < .001$ ; Fig. 4b]. This response was not different between the genotypes [no main effect of 'genotype'  $F(1,10) = .9, p = .4$ ; no interaction; Fig. 4b].

### Discussion

The current study provides evidence for a distinct role for mGlu5 in the extinction of operant responding for METH, but not sucrose. mGlu5 KO mice showed an enhanced propensity for cue-induced operant METH- but not sucrose-seeking. KO mice also demonstrated enhanced conditioned hyperactivity to a previously METH-paired context. Interestingly, mGlu5 does not appear critical for the self-administration or motivation to self-administer METH. Furthermore, loss of mGlu5 signalling does not impair the acquisition of CPP or development of locomotor sensitization to METH. The phenotype observed suggests impaired inhibition of METH-seeking operant behaviour in the absence of METH. Additionally, mGlu5 KO mice show augmented or enduring responding to METH-paired cues and contexts in the absence of the drug, putatively suggesting a role for mGlu5 in mediating the salience of environmental cues and contexts associated with drug availability.

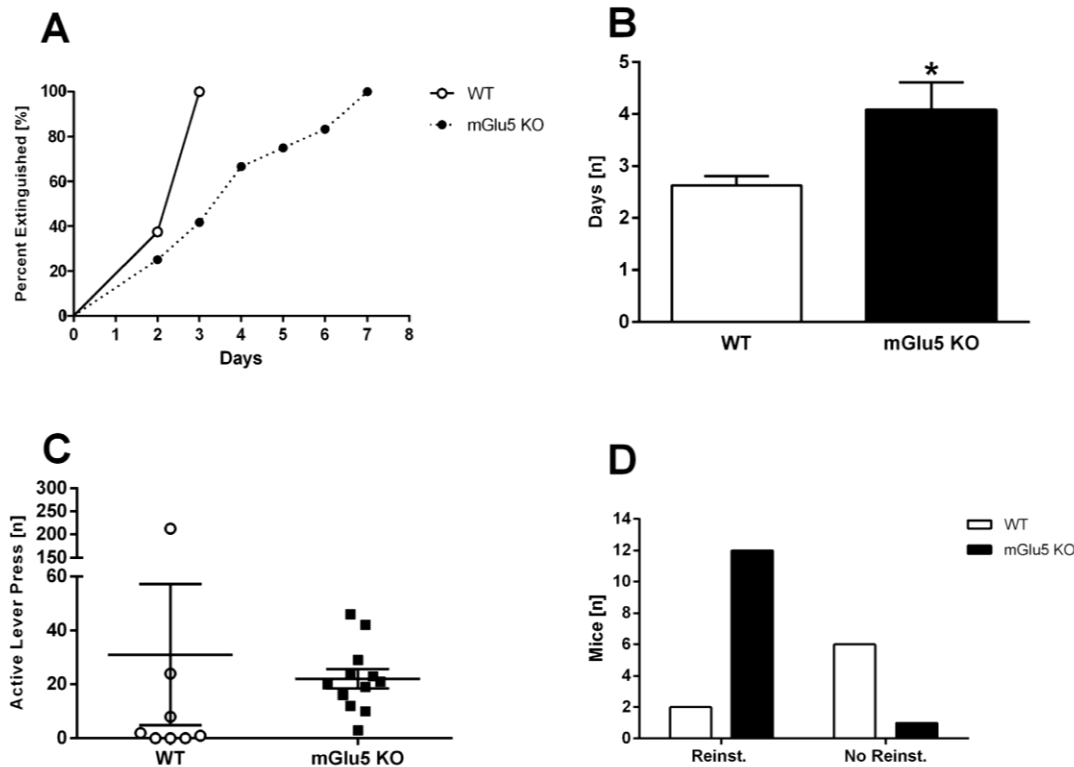
### mGlu5 is not Critical for Acquisition of Operant Responding, Place Preference or Locomotor Sensitization

mGlu5 KO mice did not demonstrate altered acquisition or motivation to self-administer METH, nor was the acquisition of METH CPP, development of locomotor sensitization or expression of sensitization different to WT mice. This suggests mGlu5 may be sufficient, but not necessary, for the development of these METH-induced behaviours, as acute pharmacological studies have demonstrated reduced METH self-administration [36], reduced cocaine locomotor sensitization [69], and reduced the

**Table 1.** Lever Responses and Infusions during METH IVSA FR1 Acquisition, Self-administration, Extinction and Reinstatement.

Measure	WT	mGlu5 KO
FR1 Acquisition Infusions	40.5+6.1	32.8+4.1
Stable FR1 Infusions	39.9+6.2	43.8+6.1
Stable FR1 ALP per minute	.48+.13	.52+.07
Extinction ALP per minute	.11+.04 \$	.16+.03 \$\$
Extinction Final ALP	4.6+1.5	6.9+1.5 ###
Extinction Final ILP	1.3+1.0	2.0+0.6
Reinstatement ALP	118.5+94.5	23.8+3.4 ###
Reinstatement ILP	4.0+4.0	3.2+1.6

Stable FR1 infusions averaged over 5 days of stable self-administration; extinction data averaged over the final two days of extinction. Data presented as means  $\pm$  SEM. Data analysed using two- or three-way RM ANOVA, followed by one-way ANOVA split by corresponding factor, where appropriate. Significant effects of 'lever type' are indicated by hash symbols (vs. inactive lever; ##  $p < .01$ , ###  $p < .001$ ); significant effects of 'genotype' are indicated by asterisks (\*  $p < .05$ ; significant effects of 'day' are indicated by \$ (†  $p < .05$ ). Abbreviations: ALP - active lever press; FR1 - fixed ratio 1; ILP - inactive lever press. doi:10.1371/journal.pone.0068371.t001



**Figure 3. Extinction and reinstatement of the operant response for METH.** **A)** Percentage of mice extinguished per day. mGlu5 KO mice took a significantly greater number of days to extinguish the operant response. Data (mean  $\pm$  SEM) analysed using a log-rank test; there was a significant effect of 'genotype' ( $p = .03$ ). **B)** Average number of days to extinguish the operant response. mGlu5 KO mice took significantly longer to extinguish their operant responding than WT. Data (mean  $\pm$  SEM) analysed using one-way ANOVA; a significant effect of 'genotype' indicated by '\*' ( $*p < .05$ ). **C)** Active lever pressing during reinstatement. Data (mean  $\pm$  SEM) analysed using one-way ANOVA. **D)** Proportion of WT and mGlu5 KO mice meeting reinstatement criteria. Data analysed using a Fisher's exact test. Abbreviations: Reinst - mice which met reinstatement criteria; No Reinst - mice which did not meet reinstatement criteria.  
doi:10.1371/journal.pone.0068371.g003

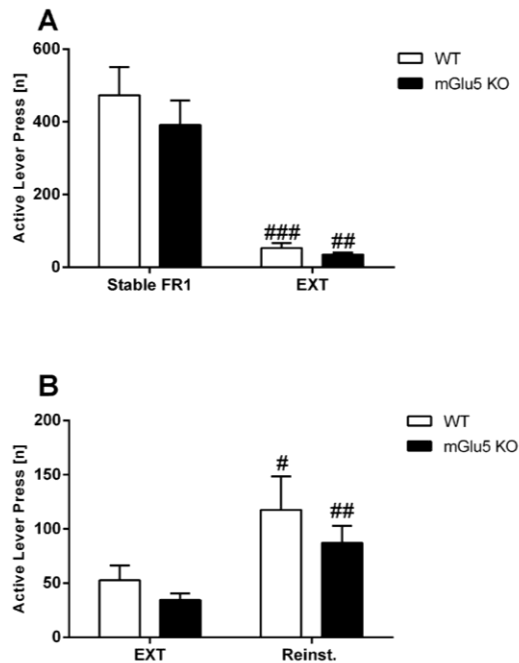
maintenance of METH CPP [70] following MTEP treatment. It appears mGlu5 is neither sufficient nor necessary for acquisition or maintenance of natural reward self-administration, as we found no effect of mGlu5 deletion on the acquisition and maintenance of sucrose self-administration. This is consistent with pharmacology-systemic MTEP does not reduce operant responding for a food reward [35]. Importantly, our study was designed to address the question of necessity. The data suggest that mGlu5 signalling is not necessary to acquire and/or support METH-driven behaviours. Nevertheless, we do not preclude the possibility that under other regimes/doses of METH an effect of genotype may emerge.

#### mGlu5 Moderates Operant Extinction of METH but not Sucrose

Despite a lack of phenotype for operant self-administration of METH, mGlu5 KO mice displayed a clear deficit in the extinction of METH-seeking. The increased latency to extinguish the operant response for METH in mGlu5 KO mice suggests deficits in METH operant extinction learning, which essentially requires the animal to actively inhibit responding to a lever that was previously rewarding. Notably, this effect was not observed for

sucrose, suggesting a differential role for mGlu5 in extinction learning for drug compared to natural rewards. This is important when considering mGlu5 as a potential therapeutic target, as it suggests mGlu5 may modulate drug-specific cognitive processes, without affecting cognitive processes involved in natural reward processing. Our findings are consistent with the literature; in rats, the mGlu5 PAM CDPBP facilitates extinction of cocaine CPP [59] and operant cocaine self-administration [58].

It is possible the operant extinction deficits observed are due to modulatory effects of mGlu5 on contextual salience. Indeed, mGlu5 may be necessary for learning to inhibit context-drug associations. That is, mGlu5 KO mice may have showed persistent operant responding during extinction despite the absence of drug, because previous context-METH associations were too salient in these mice compared to WT. Support for this is provided by the elevated conditioned hyperactivity observed in the CPP test session, where KO mice responded with greater locomotor activity than WT to a drug-paired environment in the absence of drug availability. Similarly, mGlu5 KO mice displayed exaggerated conditioned locomotor activity upon return to a cocaine-paired context in the absence of the drug [68]. Also, mGlu5 on dopamine



**Figure 4. Extinction and reinstatement of the operant response for sucrose.** **A)** Average active lever presses during Stable FR1 and the final 2 days of extinction. Both genotypes significantly decreased their active lever presses during both days of extinction. **B)** Average active lever presses during extinction and cue-induced reinstatement. Both genotypes increased their active lever pressing in the reinstatement test following extinction. Data (mean±SEM) analysed using two-way RM ANOVA, followed by one-way ANOVA split by the factor 'genotype' with a Bonferroni correction. Significant effects of 'day' (vs. Stable FR1) are represented by "#" ( $^{##}p<.01$ ;  $^{###}p<.001$ ). Abbreviations: EXT - final extinction score; FR1- fixed ratio 1; Reinst - reinstatement. doi:10.1371/journal.pone.0068371.g004

D<sub>1</sub> expressing neurons have been implicated in the acquisition of the incentive value of a conditioned stimulus, suggesting a role for mGlu5 in assigning valence to reward related stimuli [71]. Identifying which facets of extinction learning mGlu5 may be modulating is important for treatment of human addicts; the efficacy of cue exposure therapy is lacking (for a meta-analysis, see [72]), thus reducing the salience of drug-associated contexts may provide a more effective treatment approach to reducing relapse. Future experiments will undoubtedly address the role of mGlu5 in mediating the salience of cues and contexts associated with drug availability.

In contrast to METH, we found that mGlu5 KO and WT littermates demonstrated comparable extinction for a sucrose reinforcer. Our findings parallel those of Eiler et al. [73], where mGlu5 KO mice showed no differences in extinction of the operant response for sucrose pellets. The different phenotypes in mGlu5 KO mice in relation to extinction of operant responding for food and METH is presumably due to different neural adaptations which occur following self-administration and/or extinction of a drug versus a natural reinforcer. Self-administration of either cocaine or sucrose has the capacity to induce plasticity at glutamatergic synapses in midbrain dopamine neurons yet only in

the case of cocaine does this potentiation persist beyond 21 days of abstinence [76]. Moreover, increased phosphorylation of the 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptor subunit GluA1 (associated with the presence of high-conducting Ca<sup>2+</sup> permeable GluA2-lacking AMPA receptors and thus changes in synaptic plasticity) is observed in other mesocorticolimbic regions (dorsal and ventral striatum) during cocaine, but not sucrose withdrawal [74]. Furthermore, firing rates in the NAcc are elevated following abstinence and during extinction from cocaine self-administration [75], but not sucrose [76]. It is possible that similar adaptations may occur following METH self-administration and/or extinction of METH self-administration. Deletion of mGlu5 may affect normal neural adaptations which occur in response to METH and/or operant extinction of METH, but not sucrose.

#### Deletion of mGlu5 Enhances Propensity to Reinstatement Operant METH- but not Sucrose-seeking

Upon exposure to cues signalling drug-availability, mGlu5 KO mice demonstrated a higher propensity to reinstate drug-seeking. mGlu5 KO mice demonstrated lower reinstatement magnitude (i.e. decreased active lever pressing); however, the low number of reinstating WT mice and the variability in their reinstatement magnitude complicates interpretation. While acute antagonism of mGlu5 signalling reduces the magnitude of reinstatement for METH [77], cocaine [78], ethanol [79] and nicotine [80], differences in the propensity to reinstate are rarely examined. Furthermore, numerous discrepancies between genetic and pharmacological studies using mGlu5 antagonists and mGlu5 KO mice have been documented (e.g. [41,42]). A number of factors may make comparisons between acute pharmacological and genetic studies difficult; notably, tolerance to mGlu5 antagonists [61,81-83] and receptor desensitization [82,83] have been reported. Furthermore, it is possible that developmental compensation resulting from embryonic deletion of mGlu5 may affect the behaviours observed; mGlu5 KO mice show increased dendritic spine density [84], and increased spine diameter has been linked to cue-induced reinstatement [85].

The expression of extinction - and thus reinstatement propensity - likely depends upon contextual associations. Chaudhri and colleagues [86] demonstrated that if extinction occurs in multiple contexts (e.g. A, B, C), reinstatement is lower in a new context (D) than if extinction was only learnt in one context (A) for an equivalent period. Furthermore, Torregrossa et al. [87] demonstrated treatment with the NMDA agonist D-cycloserine enhances extinction in a different context, an effect mediated by the NAcc. This suggests glutamatergic tone in the accumbens, where mGlu5 is expressed, is important for the generalisation of extinction learning in one context to another. While our study did not examine extinction in multiple contexts, it is possible that mGlu5 KO mice failed to generalise what was learnt in the extinction context, where there was no discriminative cue and no CS, to the reinstatement context, where the discriminative cue and CS were present.

Furthermore, if there is a lack of generalisation in KO mice, this may be due to the discriminative cue, CS and operant chamber forming a compound stimulus representing drug availability (see [88]). During extinction, the repeated exposure to the context without METH served as context extinction sessions. However, the context in combination with the discriminative cue and CS (i.e., the reinstatement context) was never extinguished. The latter may present a compound cue signalling drug availability and hence the salience of the context. As discussed earlier, mGlu5 can regulate drug contextual salience [71]. If mGlu5 regulates or



inhibits responding to drug-associated cues or contexts, then loss of mGlu5 signalling may result in a disproportionate significance given to these cues or contexts. This may explain why reinstatement occurred more reliably in KO compared to WT mice.

Interestingly, WT mice demonstrated a lower propensity to reinstate to METH, but not sucrose, compared to mGlu5 KO mice. It is possible that in WT mice sucrose is a more preferable and potentially more salient reward compared to METH. Rats demonstrate a preference for saccharin self-administration over cocaine [89,90]. This effect may be dependent on the training period because Galuska and colleagues [91] demonstrated METH self-administration and reinstatement of METH-seeking are enhanced by extended exposure, but not a shorter exposure period; an effect not present for sucrose (see also [92]). In that study, the demand curve (i.e. how self-administration decreases with increases in response requirements) for sucrose was higher than that of METH before extended exposure to both reinforcers, suggesting that a shorter period of exposure may make sucrose a more desired reinforcer than METH [91]. Hence, it is possible the self-administration period in the current study was not long enough to reliably induce reinstatement to METH in all WT mice, while at the same time being sufficient for robust reinstatement of sucrose-seeking. This is an important point; the sucrose reinstatement data provide validation of the paradigm in WT mice. Accordingly, the relatively modest reinstatement of METH-seeking in WT mice presumably reflects a comparatively lesser “value” than sucrose under the regime tested.

In comparison to METH, mGlu5 deletion had no effect on the propensity or magnitude of cue-induced reinstatement of sucrose-seeking. This is in accordance with a number of pharmacological studies [36,78,80,93]; yet our findings did not replicate those of Eiler and colleagues [73], who demonstrated reduced reinstatement of food-seeking in mGlu5 KO mice. It is important to note that in the current study, if reinstatement criteria were not applied, mGlu5 KO mice demonstrated reduced cue-induced reinstatement for sucrose (data not shown). Reinstatement criteria were applied in the current study to provide a parallel for the METH self-administration findings and to accurately reflect the behavioural spectrum observed when analyzing individual mice compared to populations [94]. Also, procedural differences between the two studies may account for the divergence in phenotype. Importantly, the Eiler *et al.* study administered food rewards during the cue-induced reinstatement test, making the distinction between cue-induced and food-primed reinstatement unclear. The compound effect of food-primed and cue presentation may affect reinstatement in a different manner to each of these stimuli alone, and makes direct comparisons between the two studies difficult. Indeed, the paradigm used by Eiler and colleagues is essentially a study of reacquisition followed by rapid re-extinction more so than reinstatement.

#### Possible Circuitry Modulating Extinction and Reinstatement Behaviour in mGlu5 KO Mice

mGlu5 signalling in a number of regions may be required to mediate extinction and reinstatement of operant METH-seeking.

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Extinction circuitry overlaps considerably with reinstatement circuitry [95]. This circuit connects the medial prefrontal cortex (infralimbic and prelimbic cortices) and basolateral amygdala to the NAcc core and shell, which projects to areas associated with motor output, the substantia nigra and ventral pallidum [95–99]. mGlu5 is expressed in most of the regions implicated in this circuit [31,79,100,101]. Within this circuit, mGlu5 activity in the infralimbic (IL) and NAcc may be mediating the observed extinction and reinstatement phenotypes. Administration of the mGlu5 antagonist MPEP into the IL reduces recall of extinction learning [101]. The IL is recruited only after more than one day of extinction training [98]; the current study demonstrated a difference in extinction learning after day 1 (Fig. 3a), consistent with the notion that mGlu5 signalling in the IL modulates extinction. A reduction in mGlu5 activity in the IL may also be involved in the reinstatement phenotype observed; inactivation of IL promotes cue-induced reinstatement [98], while enhancement of AMPA activity in the IL suppresses cue-induced cocaine reinstatement [102].

Reduced mGlu5 signalling in the NAcc may also account for the extinction deficit and reinstatement propensity in mGlu5 KO mice. Suto and colleagues [103] demonstrated that extinction training enhances extracellular glutamate levels in the NAcc core and shell, compared to yoked saline and cocaine controls. Also, the extinction of cocaine self-administration results in reduced cell surface expression of mGlu5 in the NAcc core [104]. However, in contrast to our findings, pharmacological studies suggest reduced mGlu5 signalling in the NAcc reduces reinstatement, where we found the opposite effect. Intra-NAcc shell microinjections of MPEP reduce drug-primed reinstatement for cocaine [105], while mGlu5 agonist administration potentiates cue-induced reinstatement for cocaine [106]. Considering the critical role the NAcc core and shell play in reinstatement [10], the nature of the role of mGlu5 in the accumbens on reinstatement behaviour requires further investigation.

#### Conclusion

The present study highlights a role for mGlu5 in the extinction and reinstatement of operant METH-, but not sucrose-, seeking. This, in combination with the enhanced conditioned hyperactivity during the CPP test, implicates mGlu5 in the contextual salience of drug-related cues and environments. Future studies will delineate the anatomic loci where mGlu5 signalling contributes to the extinction and reinstatement of METH-seeking.

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#### Author Contributions

Conceived and designed the experiments: RC AL RB JK. Performed the experiments: RC RB. Analyzed the data: RC. Wrote the paper: RC. Revised the manuscript: RB JK AL.

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## Chapter 4

# Adenosine 2A receptors modulate reward behaviours for methamphetamine

### 4.1. Introduction

As discussed in Chapter 1,  $A_{2A}$  presents a potential target for modulating METH-induced behavioural and neural adaptations. Indeed, previous studies demonstrate that both pharmacological [323-325] and genetic [330, 332-334] manipulation of  $A_{2A}$  alters rewarding and reinforcing behaviour for other psychostimulants, such as cocaine and amphetamine. However, previous research using cocaine and amphetamine does not present a clear indication of how genetic deletion of  $A_{2A}$  will affect drug-induced behaviour for METH. Genetic deletion studies reveal a complex role for  $A_{2A}$  in a variety of behaviours relevant to addiction, including operant self-administration, place preference and locomotor sensitization. Indeed, while the reinforcing efficacy of cocaine is reduced in  $A_{2A}$  KO mice (measured through an operant self-administration paradigm), the conditioned rewarding nature of cocaine remains intact (measured by place preference) [330]. Also, the enhancement or reduction of locomotor sensitization following repeated cocaine treatment is dependent on localised deletion of  $A_{2A}$  [332-334]. Considering the complex phenotype of  $A_{2A}$  KO mice in response to cocaine, it is important to assess the response of these KO mice to METH. Furthermore, it is of interest to compare the response of  $A_{2A}$  KO mice to different psychostimulants, as similarities and differences in behavioural phenotypes may indicate similar mechanisms of action. Finally, the following experiments were designed to pave the way for future studies; following the identification of a phenotype in  $A_{2A}$  KO mice, I sought to then examine the neural locus of effect of  $A_{2A}$ , to determine where  $A_{2A}$  was acting to modulate reward and reinforcing behaviours.

## 4.2. Methods

### 4.2.1 *Animals and behavioural methods*

There were 4 experiments completed for this chapter, each using a separate cohort of mice. Experiments 1 and 2 assessed the response of  $A_{2A}$  KO mice and their WT littermates in METH CPP and sensitization (see General Methods, section 2.3.1, protocol 1). Two doses of METH were used; experiment 1 used 1mg/kg METH (WT n = 15,  $A_{2A}$  KO n = 14), while experiment 2 employed 2mg/kg METH (WT n = 14,  $A_{2A}$  KO n = 11). Experiment 3 examined intravenous self-administration of METH (WT n =16,  $A_{2A}$  KO n =17; see General Methods, section 2.3.2, protocol 2). The response of  $A_{2A}$  KO mice and their WT littermates to a natural reinforcer was assessed in Experiment 4 using self-administration of 10% sucrose (w/v) (see General Methods, 2.3.2, protocol 4, WT n =7 ,  $A_{2A}$  KO n = 5). At the conclusion of all behavioural experiments, mice were anaesthetised with 100mg/kg pentobarbital i.p. and culled via cervical dislocation.

### 4.2.2 *Statistics*

For behavioural studies, two-way repeated measures (RM) analysis of variance (ANOVA) with factors 'days', 'lever type' and/or 'drug' and between factors 'genotype' or 'group' was conducted. Where appropriate, this was followed by one-way ANOVA split by corresponding factor with a Bonferroni correction ( $p = .05 / \text{number of independent variables}$ ). One-way ANOVA with the between factor 'genotype' was used to assess differences in breakpoint. Correlations and simple linear regression were used to assess the relationship between responding at FR3 and PR at different doses. Data from the final four days of FR1 3 $\mu$ g/kg/infusion were analysed and compared to the four days at other doses to keep the number of days analysed at each dose consistent.

Data are presented as mean  $\pm$  standard error of the mean (SEM). Data analysis was conducted using SPSS Statistics version 20 and GraphPad: Prism version 5.

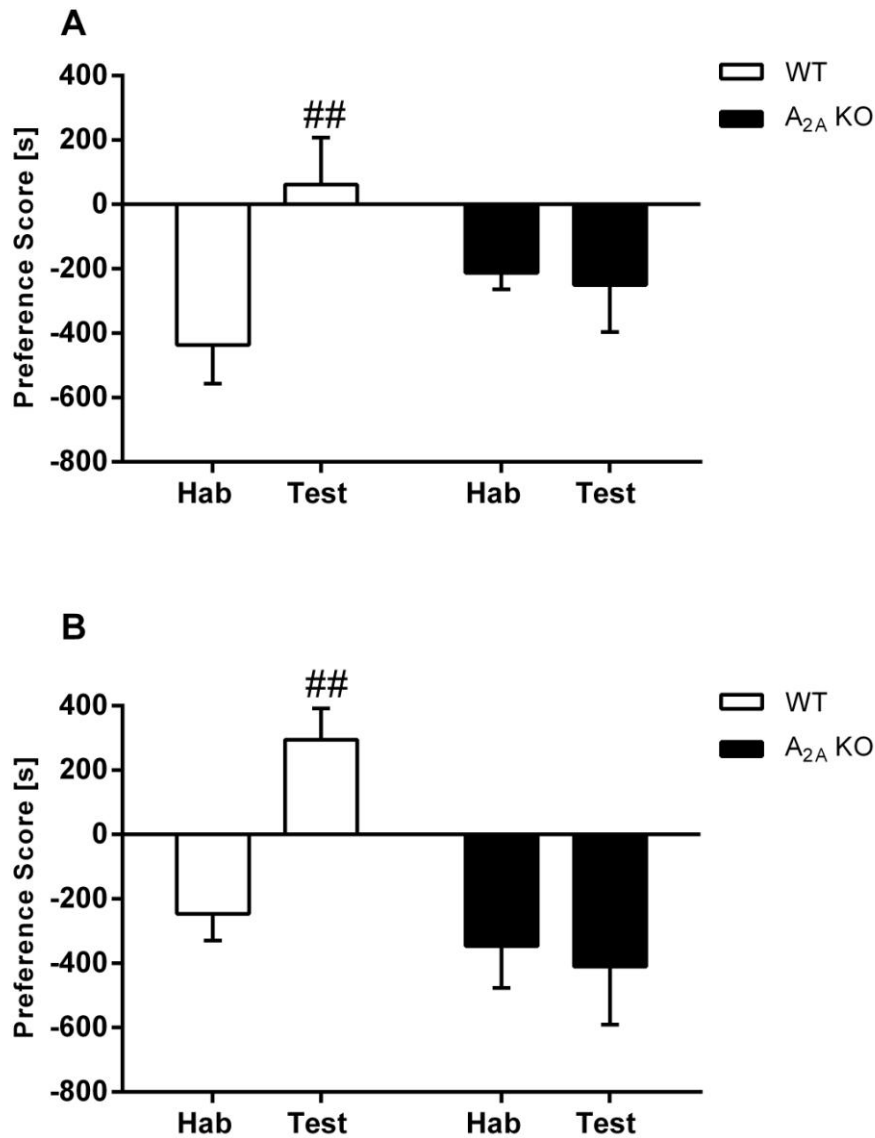
### 4.3. Results

#### 4.3.1. Experiment 1 and 2: *A<sub>2A</sub>* KO mice fail to exhibit a place preference to either 1mg/kg or 2mg/kg METH.

The expression of place preference was assessed using a preference score, where a positive preference score was indicative of a preference for the METH compartment. At the lower METH dose (1mg/kg), there was a change in the preference score between habituation and test in WT mice only [significant interaction between 'day' and 'genotype',  $F(1,27) = 6.4, p = .01$ ; trend for a main effect of 'day'  $F(1,27) = 3.8, p = .06$ ; n.s. main effect of 'genotype'  $F(1,27) = .2, p = .7$ ]. Figure 4.1a shows that the preference for the METH compartment changed from a negative preference at habituation, to a neutral preference at test in WT mice. The preference for *A<sub>2A</sub>* KO mice remained unchanged between habituation and test. Indeed, this interpretation of the significant day by genotype interaction is supported by one-way ANOVA split by 'genotype' with a Bonferroni correction, which revealed a main effect of 'time' in WT mice [ $F(1,14) = 10.4, p = .006$ ] but not in *A<sub>2A</sub>* KO mice [ $F(1,13) = .1, p = .7$ ].

At the higher dose (2mg/kg), WT mice demonstrated a clear preference for the METH compartment at test while *A<sub>2A</sub>* KO mice did not (Figure 4.1b) [significant interaction between 'time' and 'genotype'  $F(1,20) = 6.1, p = .02$ ]. There was also a main effect of 'genotype' [ $F(1,20) = 10.7, p = .004$ ]. Again, one-way ANOVA split by 'genotype' with a Bonferroni correction revealed a main effect of 'time' in WT mice [ $F(1,11) = 15.2, p = .003$ ] but not in *A<sub>2A</sub>* KO mice [ $F(1,9) = .1, p = .8$ ]. Data from two WTs and one *A<sub>2A</sub>* KO mouse were excluded at the 2mg/kg dose as they were more than 2.5 standard deviations (SD) from the mean. Collectively, these results demonstrate

that conditioning with either dose of METH did not result in the expression of a place preference in  $A_{2A}$  KO mice.



**Figure 4.1. Place preference following conditioning with 1 or 2mg/kg METH in  $A_{2A}$  WT and KO mice.** Preference score [s] in  $A_{2A}$  KO mice and WT littermates following conditioning with **A)** 1mg/kg METH and **B)** 2mg/kg METH. Preference score is defined as (time spent in METH-paired compartment during habituation – time spent in METH-paired compartment during test). Data presented as means  $\pm$  SEM, and analysed using two-way RM ANOVA, followed by one-way ANOVA split by the factor ‘genotype’ with a Bonferroni correction if appropriate. Significant effects of ‘day’ (vs. habituation) are represented by hash symbols ( $###p < .01$ ). Abbreviations: Hab: Habituation. Cohort 1 (1mg/kg METH) WT n = 15,  $A_{2A}$  KO n = 14; Cohort 2 (2mg/kg METH) WT n = 12,  $A_{2A}$  KO n = 10.

#### 4.3.2. Experiment 1 and 2: *A<sub>2A</sub>* KO mice sensitize to both 1mg/kg and 2mg/kg METH.

Locomotor activity was analysed as a difference score [distance travelled (cm) under METH treatment - distance travelled (cm) under saline treatment, on the same day]. Four daily 1mg/kg METH treatments produced sensitization in both genotypes [main effect of 'days'  $F(3,81) = 34.5$ ,  $p < .0001$ , n.s. main effect of 'genotype'  $F(1,27) = 1.6$ ,  $p = .2$ ]. Intriguingly, the sensitization profiles were different between genotypes [significant 'days' by 'genotype' interaction  $F(3,81) = 4.4$ ,  $p = .006$ ]; thus, locomotor activity continued to increase throughout conditioning in WT mice, but stabilised at a lower level following the second day of conditioning in *A<sub>2A</sub>* KO mice (Figure 4.2a). Unlike the lower dose, conditioning with a higher dose of METH (2mg/kg) resulted in similar sensitization profiles in both WT and *A<sub>2A</sub>* KO mice (Figure 4.2b) [main effect of 'days'  $F(3,60) = 11.8$ ,  $p < .001$ ; n.s. main effect of 'genotype'  $F(1,20) = .1$ ,  $p = .8$ , no interaction]. Together, these results suggest *A<sub>2A</sub>* KO mice sensitize to both doses of METH; however, the degree of sensitization was slightly lower in KO mice at the 1mg/kg dose.

#### 4.3.3. Experiment 1 and 2: Expression of conditioned hyperactivity is present in *A<sub>2A</sub>* KO mice.

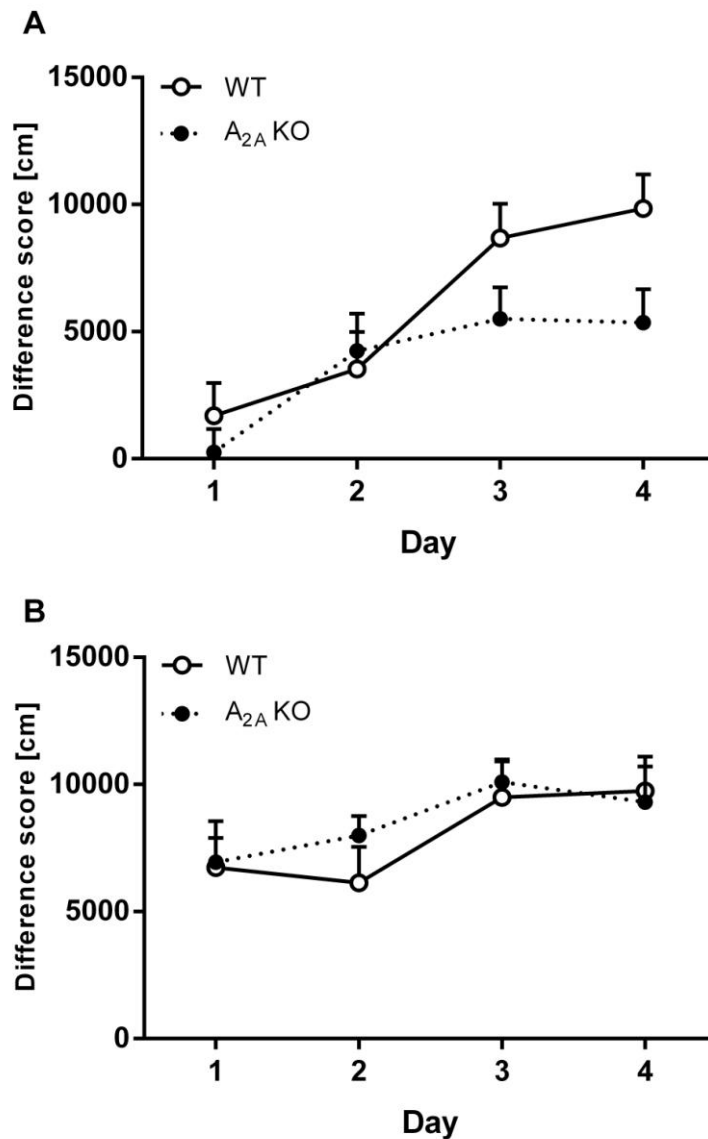
Conditioned hyperactivity is an increase in activity elicited by a context previously paired with a stimulus (e.g. drug), in the absence of that stimulus [370]. Locomotor activity at test was compared to locomotor activity on saline day 1, to assess if this was enhanced by repeated drug-context pairings. Notably, locomotor activity at test was not compared to that at habituation, due to high levels of novelty-induced locomotor activity at habituation (data not shown).

Upon re-exposure to the conditioning context there was an increase in locomotor activity at test compared to saline day 1 [main effect of 'time' for 1mg/kg METH:  $F(1,27) = 36.8$ ,  $p < .001$ ; 2mg/kg METH:  $F(1,20) = 20.7$ ,  $p < .001$ ] (Figure 4.3a, Figure 4.3b). Both genotypes demonstrated conditioned hyperactivity [one-way ANOVA split by 'genotype' with a Bonferroni

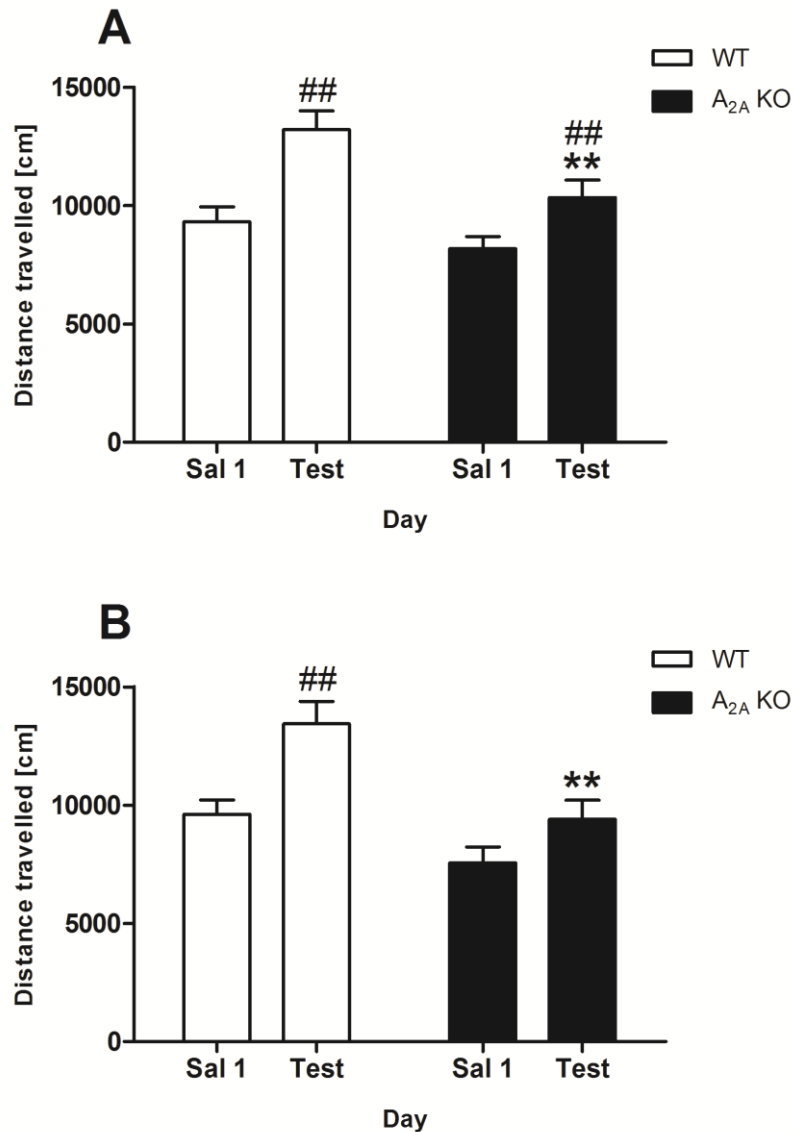


correction ( $p = .05 / 3 = .017$ ) effect of 'time' WT:  $F(1,14) = 26.4, p < .001$ ;  $A_{2A}$  KO:  $F(1,14) = 11.4, p = .005$ ]. A main effect of 'genotype' [ $F(1,27) = 6.0, p = .02$ ] in the 1mg/kg group suggests lower overall locomotor activity in  $A_{2A}$  KO mice. There was a weak trend for an interaction [ $F(1,27) = 3.0, p = .09$ ], suggesting genotype differences in locomotor activity tended to be more pronounced at test. This interpretation was confirmed using Bonferroni post-hoc tests (Figure 4.3a).

In the 2mg/kg group, there was main effect of 'genotype' [ $F(1,20) = 11.1, p = .003$ ] but no interaction, suggesting reduced overall locomotor activity in  $A_{2A}$  KO mice. One-way ANOVA ANOVA split by 'genotype' with a Bonferroni correction indicates conditioned hyperactivity was present only in WT mice following conditioning with 2mg/kg METH [WT:  $F(1,11) = 18.0, p = .001$ ;  $A_{2A}$  KO:  $F(1,9) = 4.9, p = .05$ ]. Bonferroni post-hoc tests also demonstrated a reduction in locomotor activity in  $A_{2A}$  KO mice at test, compared to WT littermates (Figure 4.3b).



**Figure 4.2. Locomotor sensitization during conditioning with 1 or 2mg/kg METH in A<sub>2A</sub> WT and KO mice.** Locomotor sensitization over 4 consecutive days to **A**) 1mg/kg METH and **B**) 2mg/kg METH. Sensitization is presented as a difference score [distance travelled (cm) under METH treatment - distance travelled (cm) under saline treatment, on the same day]. A significant interaction between 'days' and 'genotype' at the 1mg/kg dose indicates locomotor activity continued to increase over the 4 days of conditioning in WT mice, but stabilised after 2 days in A<sub>2A</sub> KO mice. Data presented as means  $\pm$  SEM, and analysed using two-way RM ANOVA, followed by one-way ANOVA split by the factor 'genotype' with a Bonferroni correction if appropriate. Cohort 1 (1mg/kg METH) WT n = 15, A<sub>2A</sub> KO n = 14; Cohort 2 (2mg/kg METH) WT n = 12, A<sub>2A</sub> KO n = 10.



**Figure 4.3. Conditioned Hyperactivity in WT and A<sub>2A</sub> KO mice following conditioning with 1 or 2mg/kg METH.** Distance travelled (cm) during the first saline conditioning session (Sal 1) and at test in A<sub>2A</sub> KO mice and WT littermates, following conditioning with **A)** 1mg/kg METH, of **B)** 2mg/kg METH. Conditioned hyperactivity is defined as an increase in locomotor activity following reexposure to a drug-paired stimulus, in the absence of the drug itself. Data presented as means  $\pm$  SEM, and analysed using two-way RM ANOVA, followed by one-way ANOVA split by the factor 'genotype' with a Bonferroni correction if appropriate. Bonferroni post-hoc tests were used to further probe genotype effects. Significant effects of 'genotype' (WT vs. A<sub>2A</sub> KO, on the same day) are denoted by asterisks ( $*p < .01$ ) and significant effects of 'day' (Sal 1 vs. test) are indicated by hash symbols ( $##p < .01$ ). Abbreviations: Sal 1: Saline treatment day 1. Cohort 1 (1mg/kg METH) WT n = 15, A<sub>2A</sub> KO n = 14; Cohort 2 (2mg/kg METH) WT n = 12, A<sub>2A</sub> KO n = 10.

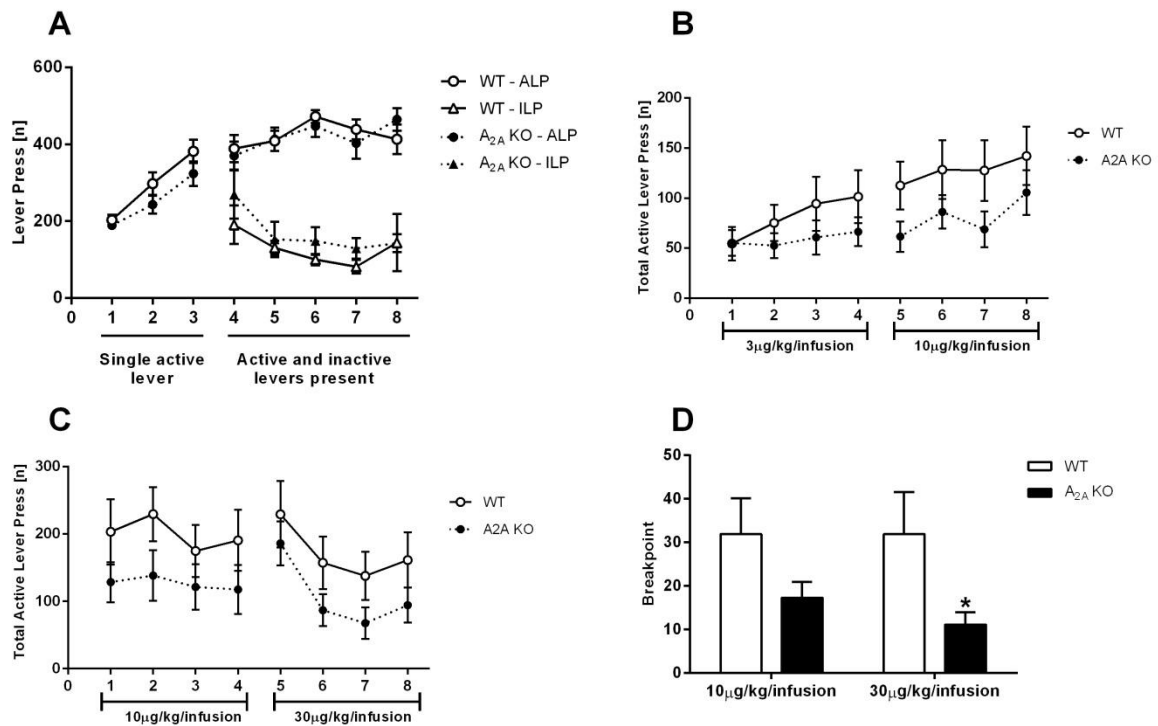
#### 4.3.4. Experiment 3: Sucrose self-administration is unaltered in $A_{2A}$ KO mice.

Prior to self-administration of METH, mice were trained in operant chambers to self-administer 10% sucrose (w/v) for 8 days. Animals were trained for 3 days with the active lever only, and then trained for 5 days with both active and inactive lever present. All mice increased their active lever pressing over the first three days of self-administration [main effect of 'days'  $F(2,62) = 46.2, p < .0001$ ]; both genotypes self-administered similar volumes of sucrose [no main effect of 'genotype'  $F(1,31) = 2.1, p = .2$ ] (Figure 4.4a). When the inactive lever was presented, both genotypes displayed clear discrimination for the active over the inactive lever [main effect of 'lever type'  $F(1,30) = 122.5, p < .0001$ ] (Figure 4.4a). Both genotypes administered similar levels of sucrose [n.s. main effect of 'genotype'  $F(1,30) = .3, p = .6$ ], replicating previous findings [328]. Self-administration of sucrose was relatively stable throughout the training period in both genotypes [n.s. main effect of 'days'  $F(4,120) = 1.4, p = .2$ , no interactions] (Figure 4.4a).

#### 4.3.5. Experiment 3: FR1 acquisition and stable self-administration are unaltered in $A_{2A}$ KO mice.

There was clear discrimination for the active lever over the inactive lever throughout acquisition and stable self-administration ( $p$ 's  $< .05$ ). Therefore, inactive lever responding is not presented on graphs for the purposes of clarity; however, Table 4.1 displays the average active and inactive lever presses at each dose and schedule of reinforcement.

During acquisition (Figure 4.4b),  $A_{2A}$  KO mice displayed similar active lever pressing to WT mice at the  $3\mu\text{g}/\text{kg}/\text{infusion}$  dose [n.s. main effect of 'genotype'  $F(1,30) = 8, p = .4$ ]. There was a trend for reduced active lever pressing in KO mice at the  $10\mu\text{g}/\text{kg}/\text{infusion}$  dose [main effect of 'genotype'  $F(1,31) = 3.7, p = .07$ ]. There was no significant effect of 'day' at either dose ( $p$ 's  $> .05$ ).



**Figure 4.4. Self-administration and motivation to self-administer sucrose or METH in A<sub>2A</sub> WT and KO mice.** **A)** Active and inactive lever pressing during the final 5 days of sucrose training in WT and A<sub>2A</sub> KO mice using a fixed ratio 1 schedule of reinforcement. A significant interaction between ‘days’ and ‘lever type’ suggests discrimination improved as training progressed. **B)** Active lever pressing in WT and A<sub>2A</sub> KO mice for 3 and 10 µg/kg/infusion METH using a fixed ratio 1 schedule of reinforcement. **C)** Active lever pressing in WT and A<sub>2A</sub> KO mice for 10 and 30 µg/kg/infusion METH using a fixed ratio 3 schedule of reinforcement. **D)** Motivation to self-administer 10 and 30 µg/kg/infusion METH, as assessed by a progressive ratio schedule of reinforcement. Breakpoint (the maximum number of lever presses achieved in order to receive a drug reward) is presented for each genotype. Data presented as means ± SEM, and analysed using three- or two-way RM ANOVA for **A)**, **B)** and **C)**, followed by one-way ANOVA split by the factor ‘genotype’ with a Bonferroni correction if appropriate. Data for **D)** was analysed by one-way ANOVA. Significant effects of ‘genotype’ indicated by asterisks (\**p* < .05). Abbreviations: ALP: active lever press, ILP: inactive lever press. WT n =16, A<sub>2A</sub> KO n =17.

Reinforcement schedule	Dose	Lever Type	WT	A <sub>2A</sub> KO
FR1	3µg/kg/inf	Active	97.6 + 17.5 ###	53.8 + 12.5
FR1	3µg/kg/inf	Inactive	35.6 + 8.5	26.8 + 9.6
FR3	10µg/kg/inf	Active	119.5 + 21.6 ##	73.7 + 15.8 ##
FR3	10µg/kg/inf	Inactive	54.9 + 9.4	29.8 + 6.5
FR3	10µg/kg/inf	Active	188.8 + 36.8 ##	133.1 + 31.1
FR3	10µg/kg/inf	Inactive	62.6 + 14.1	57.1 + 16.7
FR3	30µg/kg/inf	Active	177.1 + 40.2 ###	109.4 + 24.3 ##
FR3	30µg/kg/inf	Inactive	54.6 + 15.5	45.4 + 13.6

**Table 4.1. Discrimination for the active lever in IVSA.** Lever presses on the active and inactive lever during IVSA acquisition and stable self-administration. Data presented as means ± SEM, and analysed using three-way RM ANOVA followed by two-way ANOVA split by genotype and a Bonferroni correction. Significant effects of 'lever type' indicated by hash symbols (vs. inactive lever at the same dose and schedule of reinforcement, ## $p < .01$ , ### $p < .001$ ). WT  $n = 16$ , A<sub>2A</sub> KO  $n = 17$ . Abbreviations: inf: infusion.

When moved to a FR3 schedule of reinforcement (Figure 4.4c), at both doses tested, A<sub>2A</sub> KO mice also showed similar lever pressing for METH compared to WT littermates [n.s. main effects of 'genotype' at 10µg:  $F(1,31) = 1.7, p = .2$ ; 30µg:  $F(1,28) = 1.5, p = .2$ ]. The significant effect of 'days' at the 30µg/kg/infusion dose [ $F(3,84) = 10.0, p < .0001$ ], and significant linear and quadratic contrasts [linear:  $F(1,28) = 9.3, p = .005$ ; quadratic:  $F(1,28) = 14.0, p = .001$ ] suggests a reduction and then stabilization of self-administration at this higher dose (see Figure 4.4c). This interpretation is supported by a significant 'day' by 'lever type' interaction [ $F(3,84) = 6.9, p < .0001$ ], suggesting that in both genotypes, active lever responses reduced and stabilized over the four days of testing at this dose, while inactive lever responses remained unchanged.

#### 4.3.6. *Experiment 3: Progressive ratio responding is reduced in A<sub>2A</sub> KO mice in a dose related manner*

Motivation to self-administer was assessed through the breakpoint reached in a two hour session of progressive ratio testing. Motivation to self-administer METH appeared reduced in A<sub>2A</sub> KO mice at 10µg/kg/infusion METH, an effect that failed to reach significance [n.s. main effect of 'genotype'  $F(1,31) = 2.76, p = 0.1$ ]. When tested at 30µg/kg/infusion, however, A<sub>2A</sub> KO mice showed a marked and significantly reduced breakpoint compared to WT [ $F(1,29) = 4.52, p = .04$ ] (Figure 4.4d).

#### 4.3.7. *Experiment 3: Relationship between FR1, FR3 and PR responding.*

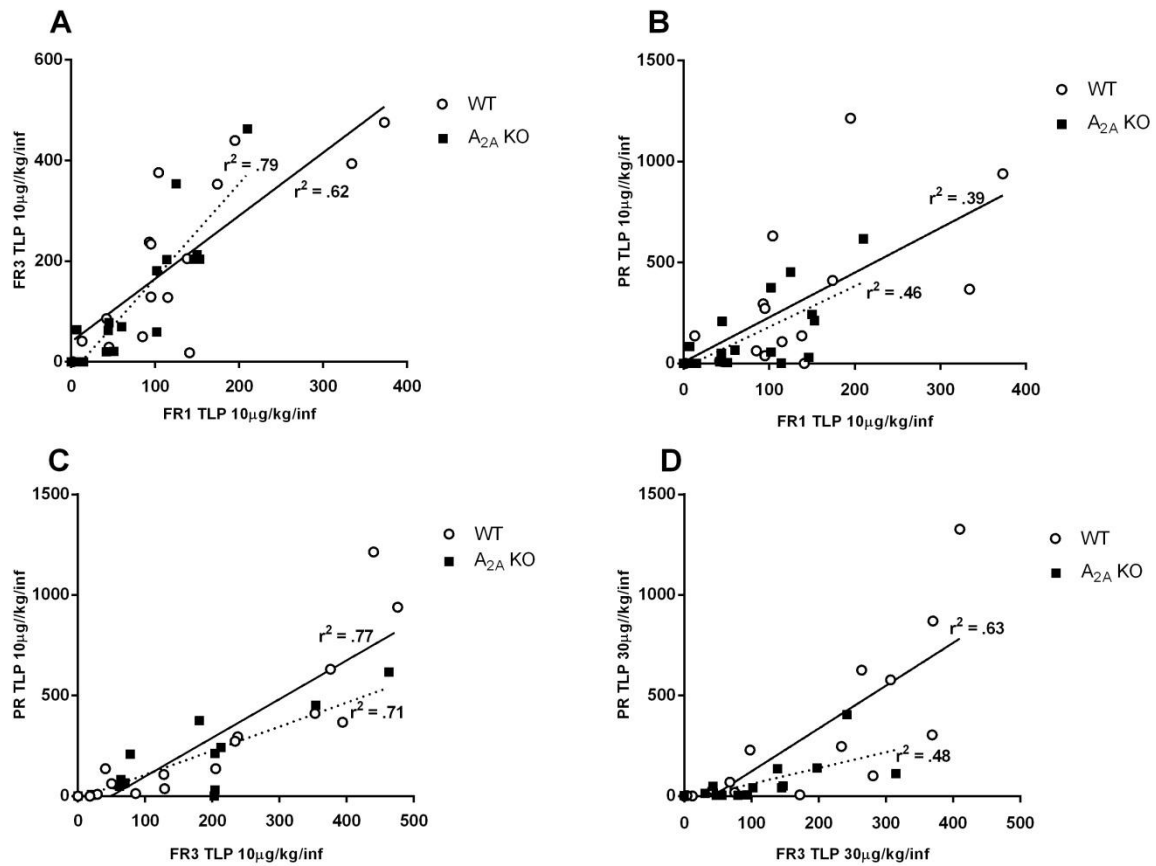
Linear regression was conducted to assess the relationship between responding for METH under different schedules of reinforcement in WT and A<sub>2A</sub> KO mice. This was performed to assess if self-administration behaviour and the motivation to self-administer were strongly related variables; a weak relationship may suggest the two variables were affected by different underlying constructs. Furthermore, I sought to assess if this relationship was affected by deletion of A<sub>2A</sub> receptors.

Table 4.2 and Table 4.3 display the correlations between FR and PR variables; relationships between variables were assessed at the same dose. I first examined how responding for 10µg/kg/infusion METH under an FR1 schedule of reinforcement related to other schedules of reinforcement. There was a strong and significant correlation between lever pressing under FR1 and FR3 ( $r = .79$  and  $.89$  for WT and A<sub>2A</sub> KO mice, respectively:  $.89$ ; both  $p$ 's <  $.001$ ). Linear regression demonstrates the slope of the lines was not different between the genotypes, suggesting responding under FR3 could be predicted from FR1 to a similar degree between the genotypes [ $F(1,29) = 2.4, p = .1$ ] (Figure 4.5a). At the same dose, FR1 lever pressing was also

significantly correlated with PR lever pressing, although to a lesser extent ( $r = .63$  and  $.68$  for WT and  $A_{2A}$  KO mice respectively, both  $p$ 's  $< .001$ ). Linear regression again demonstrates similar slopes between the genotypes [ $F(1,29) = 0.1, p = .9$ ] (Figure 4.5b). The intercepts of the slopes for both regressions were not different ( $p$ 's  $> .05$ ).

I then examined how responding for METH under an FR3 schedule of reinforcement related to other schedules of reinforcement. Significant correlations occurred between FR3 and PR lever pressing at both doses (see Figure 4.5c and Figure 4.5d); however, there were significant genotype differences at this higher schedule of reinforcement. Specifically, linear regression of FR3 onto PR responding at the  $10\mu\text{g}/\text{kg}/\text{infusion}$  dose demonstrated a significant difference in the slope of the best fit lines between the two genotypes [ $F(1,29) = 4.3, p = .04$ ] (Figure 4.5c). Similarly, at the higher  $30\mu\text{g}/\text{kg}/\text{infusion}$  dose, the slopes of the fit lines were different between the two genotypes [ $F(1,27) = 4.9, p = .04$ ] (Figure 4.5d). Despite these differences, the intercepts of the slopes for the both regression analyses were not different ( $p$ 's  $> .05$ ). These results suggest that while responding under FR3 and PR is related in both genotypes, the *strength* of this relationship is significantly reduced in  $A_{2A}$  KO mice.





**Figure 4.5. Correlations between active lever pressing during fixed ratio 1, fixed ratio 3 and progressive ratio testing in  $A_{2A}$  WT and KO mice.** Correlations between active lever pressing during **A)** fixed ratio 1 and fixed ratio 3 at 10 $\mu$ g/kg/infusion METH; **B)** fixed ratio 1 and progressive ratio at 10 $\mu$ g/kg/infusion METH; **C)** fixed ratio 3 and progressive ratio at 10 $\mu$ g/kg/infusion and **D)** fixed ratio 3 and progressive ratio at 30 $\mu$ g/kg/infusion. In both **C)** and **D)**, the slopes are significantly different between the genotypes (both  $p$ 's < .05). Abbreviations: TLP: total active lever press; FR: fixed ratio; PR: progressive ratio. WT n =16,  $A_{2A}$  KO n =17.

Variable	ALP 10µg FR1	ALP 10µg FR3	ALP 30µg FR3	PR 10µg ALP	PR 30µg ALP
ALP 10µg FR1	1	.79**	.68**	.63**	.5
ALP 10µg FR3	.79**	1	.91**	.88**	.72**
ALP 30µg FR3	.68**	.91**	1	.78**	.79**
PR 10µg ALP	.63**	.88**	.78**	1	.82**
PR 30µg ALP	.5	.72**	.79**	.82**	1

**Table 4.2. Relationship between fixed and progressive ratio responding during IVSA in WT mice.** Correlation analysis of total active lever pressing during fixed and progressive ratio responding in WT mice, using two doses of METH (10 and 30µg/kg/infusion). <sup>a</sup>Correlation is significant at the 0.05 level (2-tailed); <sup>b</sup>Correlation is significant at the 0.01 level (2-tailed). Abbreviations: ALP: average active lever pressing for this dose; FR1/3: fixed ratio 1/3; PR: progressive ratio. WT n = 16.

Variable	ALP 10µg FR1	ALP 10µg FR3	ALP 30µg FR3	PR 10µg ALP	PR 30µg ALP
ALP 10µg FR1	1	.89**	.63**	.68**	.68**
ALP 10µg FR3	.89**	1	.59*	.86**	.82**
ALP 30µg FR3	.63**	.59*	1	.63**	.69**
PR 10µg ALP	.68**	.86**	.63**	1	.89**
PR 30µg ALP	.68**	.82**	.69**	.89**	1

**Table 4.3. Relationship between fixed and progressive ratio responding during IVSA in A<sub>2A</sub> KO mice.** Correlation analysis of total active lever pressing during fixed and progressive ratio responding in A<sub>2A</sub> KO mice, using two doses of METH (10 and 30µg/kg/infusion). \*Correlation is significant at the 0.05 level (2-tailed); \*\*Correlation is significant at the 0.01 level (2-tailed). Abbreviations: ALP: average active lever pressing for this dose; FR1/3: fixed ratio 1/3; PR: progressive ratio. A<sub>2A</sub> KO n = 17.

#### 4.3.8. Experiment 4: Sucrose self-administration under FR3 and PR schedules of reinforcement

During all phases of sucrose self-administration, there was discrimination for the active lever over the inactive lever (main effects of 'lever type', all  $p$ 's < .05). Average active and inactive lever presses are displayed in Table 4.4; however, for clarity, inactive lever presses are not displayed in figures.

Both WT and  $A_{2A}$  KO mice acquired stable self-administration of sucrose following initial lever training using 10% sucrose, with each genotype showing discrimination for the active lever and making a similar number of lever presses over the final 5 days of training [main effect of 'lever type'  $F(1,10) = 15.9, p = .003$ , but not of 'genotype'  $F(1,10) = 1.9, p = .2$ , or of 'days'  $F(4,40) = .4, p = .8$ ; data not shown].

When moved to an FR3 schedule of reinforcement with 10% sucrose,  $A_{2A}$  KO mice made fewer lever presses compared to WT mice [main effect of 'genotype'  $F(1,10) = 6.8, p = .03$ ] (see Fig. 5a). Responding was stable across the 4 days of training [no main effect of 'days'  $F(3,30) = 1.0, p = .4$ ]. There was an interaction between 'lever type' and 'genotype' [ $F(1,10) = 6.1, p = .03$ ], which suggests discrimination for the active lever was worse in  $A_{2A}$  KO mice compared to WT littermates. When the sucrose concentration was reduced to 2.5%,  $A_{2A}$  KO mice made a similar number of lever presses as WT littermates [no main effect of 'genotype'  $F(1,10) = 1.7, p = .2$ ] (Figure 4.6a). There was also a main effect of 'days' [ $F(3,30) = 3.6, p = .02$ ], indicating a small but significant reduction in lever pressing as training progressed.

Motivation for sucrose was examined under a progressive ratio schedule of reinforcement. In a similar manner to FR3 responding,  $A_{2A}$  KO mice had a lower breakpoint for 10% sucrose, but not 2.5% sucrose [main effect of 'genotype' for 10% sucrose  $F(1,10) = 41.3, p < .001$ , but not 2.5% sucrose  $F(1,10) = 4.2, p = .07$ ] (Figure 4.6b).

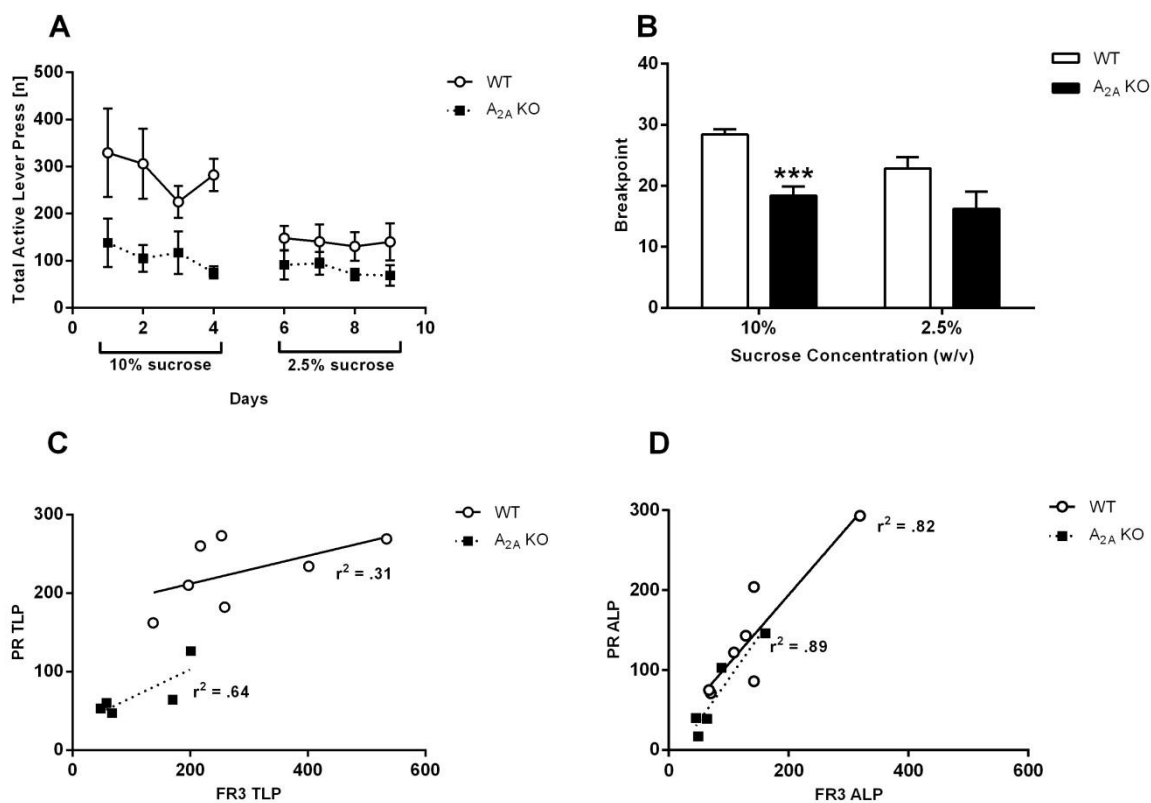
#### 4.3.8. Relationship between FR3 and PR responding for sucrose

Linear regression was conducted to assess the relationship between responding for sucrose under FR3 and PR schedules in WT and  $A_{2A}$  KO mice. At the higher sucrose concentration (10%), the correlation between FR3 and PR responding failed to reach significance in both genotypes (WT  $r = 0.55$ ,  $A_{2A}$  KO  $r = 0.80$ ,  $p = .2$  and  $.1$  respectively). Linear regression demonstrated similar slopes between the genotypes [ $F(1,8) = .5$ ,  $p = .5$ ], but different intercepts [ $F(1,8) = 22.5$ ,  $p = .001$ ], suggesting the strength of the relationship between FR3 and PR is similar between the genotypes, but that  $A_{2A}$  KO mice make fewer lever presses under both reinforcement schedules (Figure 4.6c).

At the lower sucrose concentration (2.5%), there were strong and significant correlations between FR3 and PR responding in both genotypes (WT  $r = 0.90$ ,  $p = .005$ ,  $A_{2A}$  KO  $r = 0.94$ ,  $p = .02$ ). Linear regression demonstrated similar slopes and intercepts between the genotypes [slope:  $F(1,8) = .3$ ,  $p = .6$ ]; intercept:  $F(1,8) = 1.1$ ,  $p = .3$ ]. This suggests the strength of the relationship between these two variables is similar between WT and  $A_{2A}$  KO mice, and that both genotypes made a similar number of lever presses at FR3 and PR (Figure 4.6d).

Reinforcement Schedule / Concentration	Lever Type	WT	A <sub>2A</sub> KO
FR1 10%	Active	209.5 ± 46.7 ^	136.6 ± 46.9 ^
FR1 10%	Inactive	58.6 ± 15.5	37.3 ± 13.7
FR3 10%	Active	285.7 ± 59.3 ##	109 ± 34.6 ^
FR3 10%	Inactive	48.9 ± 12.5	44.6 ± 18.3
FR3 2.5%	Active	139.6 ± 33.1 ^	81.5 ± 22.4
FR3 2.5%	Inactive	47.3 ± 11.4	44.5 ± 18.4
PR 10%	Active	227.1 ± 16.6 ##	70 ± 14.3 #
PR 10%	Inactive	64.3 ± 26.1	25.6 ± 10.7
PR 2.5%	Active	142.0 ± 30.7 #	69.0 ± 24.0
PR 2.5%	Inactive	54.6 ± 14.7	20.4 ± 7.6

**Table 4.4. Lever presses on the active and inactive lever during sucrose self-administration.** Data presented as means ± SEM, and analysed using three-way RM ANOVA followed by two-way ANOVA split by genotype and a Bonferroni correction. Significant effects of 'lever type' following a Bonferroni correction indicated by hash symbols (vs. inactive lever at the same concentration and schedule of reinforcement, #*p* < .01, ##*p* < .001); trends for an effect of 'lever type' indicated by '^' (^*p* < .05).



**Figure 4.6. Sucrose self-administration in WT and A<sub>2A</sub> KO mice.** **A)** Active lever presses for 10% sucrose and 2.5% sucrose in WT and A<sub>2A</sub> KO mice under and FR3 schedule of reinforcement. **B)** Motivation to self-administer 10% and 2.5% sucrose as assessed by a progressive ratio schedule of reinforcement. Breakpoint (the maximum number of lever presses achieved in order to receive a drug reward) is presented for each genotype. **C)** Correlations between FR3 and PR for 10% sucrose in each genotype. **D)** Correlations between FR3 and PR for 2.5% sucrose in each genotype. Data from **A)** and **B)** presented as means  $\pm$  SEM, and analysed using three-way RM ANOVA (**A)** or one-way ANOVA (**B)** Data from **C)** and **D)** analysed using linear regression. Abbreviations: TLP: total active lever press; FR: fixed ratio; PR: progressive ratio. WT n = 7, A<sub>2A</sub> KO n = 5.

#### 4.4. Discussion

The present study demonstrates a modulatory role for A<sub>2A</sub> in the rewarding and motivational properties of METH and sucrose. Mice lacking A<sub>2A</sub> did not exhibit a place preference to METH, and demonstrated a reduction in the motivation to self-administer METH under higher order schedules of reinforcement. Locomotor sensitization was present in KO mice, as was METH self-administration under lower order schedules of reinforcement. Sucrose self-administration under higher order schedules was reduced in A<sub>2A</sub> KO mice. Collectively, these data suggest A<sub>2A</sub> can modulate reward and motivated behaviour for both drug and natural reinforcers.

##### 4.4.1. *A<sub>2A</sub> deletion abolishes METH place preference*

CPP measures the rewarding or aversive properties of an unconditioned stimulus [371]. A<sub>2A</sub> KO mice failed to exhibit place preference to both 1 and 2mg/kg METH, suggesting a reduction in the conditioned rewarding nature of METH in these mice. This finding is largely consistent with previous findings; A<sub>2A</sub> KO mice fail to obtain a place preference for morphine [328, 329] and nicotine [372], but not cocaine [267], and demonstrate dose-dependent place preference for ethanol, depending on background strain [326]. Importantly, the presence of cocaine-induced CPP in A<sub>2A</sub> KO mice suggests the absence of place preference for METH is a drug-specific effect, and does not reflect Pavlovian contextual learning deficits. Indeed, A<sub>2A</sub> KO mice show enhanced working memory in the Morris water maze [373] and spatial memory in the Y-maze [374], which also suggests the absence of METH CPP in these mice is not likely due to generalised learning deficits.

There are other possible explanations for the lack of CPP observed in KO mice. The lack of place preference may be due to increased sensitivity to METH in KO mice. That is, the optimal dose for producing place preference may be much lower in A<sub>2A</sub> KO mice compared to WT, so that the

dose used in the current study was aversive. Indeed, increased sensitivity to the anxiolytic and locomotor stimulant effects of ethanol, with a concurrent reduction in CPP, has been demonstrated in  $A_{2A}$  KO mice bred on a CD-1 background [326]. While a full dose response curve would help resolve this issue, our data demonstrate an absence of place preference to the lower METH dose (1mg/kg) in  $A_{2A}$  KO mice, but a neutral preference in WT mice. This suggests a continued absence of expression of reward in KO mice, even at the lower limit of the WT dose response curve. Importantly, there were no indications of a leftward shift in the dose response curve in  $A_{2A}$  KO mice, which would suggest increased sensitivity to the drug. Thus, the absence of CPP under the conditions tested suggests that loss of  $A_{2A}$  signalling interferes with the expression of the rewarding properties of a context associated with drug experiences.

#### *4.4.2. METH-induced locomotor sensitization is present in $A_{2A}$ KO mice*

Unlike CPP, locomotor sensitization was present in  $A_{2A}$  KO mice at both doses tested. This was demonstrated by an increase in locomotor activity over the treatment period. Interestingly however, while present sensitization was reduced in  $A_{2A}$  KO mice at the 1mg/kg dose, but not at the higher 2mg/kg dose. The similar sensitization profile of KO mice at the 2mg/kg dose may indicate possible ceiling effects, where a maximum limit to locomotor activity may have obscured genotype differences. Indeed, maximum locomotor activity on day 4 in WT mice under 1mg/kg METH is comparable to maximum locomotor activity on day 4 in both genotypes at 2mg/kg METH. This suggests an upper limit to locomotor activity (prior to onset of stereotypies) may have been reached at the higher dose. Importantly, despite a reduced development of sensitization in  $A_{2A}$  KO mice at 1mg/kg METH, both genotypes *did* exhibit sensitization, suggesting  $A_{2A}$  signalling is not necessary for this behaviour.



The presence of locomotor sensitization but abolition of CPP in KO mice suggests these behaviours are driven by distinct neural mechanisms. Support for this notion was provided recently, when Seymour and Wagner [375] demonstrated no relationship between the magnitude of cocaine place preference and sensitization. Our data suggest  $A_{2A}$  receptors are necessary for the expression of CPP, but not sensitization. Similar findings were reported by Soria and colleagues [330], who demonstrated abolished CPP but intact sensitization to cocaine in  $A_{2A}$  KO mice (although this appears dependent on background strain, see [376]). The necessity of  $A_{2A}$  receptors in these behavioural tasks may depend on interactions between  $A_{2A}$  and  $D_2$  receptors on medium spiny neurons (MSNs).  $D_1$  and  $D_2$  receptor have opposing roles in the development of behavioural sensitization -  $D_1$  MSN activity promotes locomotion and the development of sensitization, while  $D_2$  MSN activity inhibits this process [377, 378]. Importantly, loss of NMDA mediated signalling on  $D_1$  expressing MSNs prevents the development of amphetamine sensitization, but a balanced reduction in NMDA signalling onto both  $D_1$  and  $D_2$  MSNs supports sensitization [379]. This suggests glutamatergic inputs onto  $D_1$ , but not  $D_2$  MSNs are critical for the development of psychostimulant sensitization [380]. As  $A_{2A}$  forms receptor complexes with  $D_2$ , but apparently not  $D_1$  MSNs [313], it is possible that  $A_{2A}$  deletion affects the 'indirect',  $D_2$ -mediated pathway, but not the 'direct',  $D_1$ -mediated pathway. Thus, sensitization could still occur through activation of the  $D_1$ -mediated pathway in  $A_{2A}$  KO mice.

#### *4.4.3. $A_{2A}$ KO mice demonstrate reduced self-administration of METH and sucrose under more demanding reinforcement schedules*

$A_{2A}$  KO mice demonstrated unaltered acquisition and self-administration of METH, suggesting the reinforcing efficacy of METH at low reinforcement schedules is largely unchanged in KO

mice. This is a surprising finding considering other studies demonstrate a reduction in self-administration of cocaine, morphine and MDMA in  $A_{2A}$  KO mice under similar reinforcement schedules (e.g. FR1, FR3) [330, 336]. It should be noted that the present study is not entirely inconsistent with these findings though; examination of Figure 4.4b and Figure 4.4c indicates a trend for a reduction in lever pressing in  $A_{2A}$  KO mice. Hence,  $A_{2A}$  signalling may be involved but not necessary for METH self-administration at low reinforcement schedules.

Nevertheless, ablation of  $A_{2A}$  signalling resulted in a dose-related reduction in the motivation to self-administer METH. That is, we observed a reduction in the breakpoint of  $A_{2A}$  KO mice for 30 $\mu$ g/kg/infusion METH, as well as a similar but non-significant effect at the lower 10 $\mu$ g/kg/infusion dose. This is an interesting finding, as it suggests a more modulatory role for  $A_{2A}$  in relation to METH than has been described for other drugs of abuse. Previous findings, including those from my laboratory, have found a global reduction in self-administration and motivation to self-administer cocaine and morphine following  $A_{2A}$  genetic deletion [328, 330]. However, the present data suggest  $A_{2A}$  modulates the motivation to obtain METH, an effect that only becomes apparent at higher doses and under more demanding response requirements.

Interestingly, a similar phenotype in  $A_{2A}$  KO mice was observed in response to sucrose self-administration. That is,  $A_{2A}$  KO mice demonstrated lower sucrose self-administration at higher schedules of reinforcement (FR3, PR), but not at lower reinforcement schedules (FR1, see also [328]). Furthermore, this effect was also dose-related, for the reduction in sucrose self-administration was only apparent at the higher concentration (10% sucrose) but not at the lower concentration (2.5% sucrose). The phenotype of  $A_{2A}$  KO mice in response to sucrose has clear parallels with the phenotype of these mice in response to METH – self-administration of sucrose or METH is reduced in KO mice, as the dose / concentration of reinforcer and response requirements increase. This suggests a more global role for  $A_{2A}$  in modulating motivated

behaviour for both natural and drug reward, specifically under higher reinforcement schedules, but not under low reinforcement schedules (e.g.  $A_{2A}$  antagonists have no effect on responding for a food reward under FR1 conditions [321]).

These findings are in accordance with previous research demonstrating that  $A_{2A}$  signalling can modulate motivated behaviour for a food reward under higher reinforcement schedules. A number of studies demonstrate that administration of the  $A_{2A}$  antagonist MSX-3 can ameliorate deficits in motivated behaviour induced by the vesicular monoamine transport inhibitor tetrabenazine (which causes dopamine depletion) [381-383]. Importantly, these effects are present only when response requirements are high and there is a baseline reduction in motivated behaviour, as MSX-3 has no effect on responding for food pellets on an FR5 schedule of reinforcement in rats [347, 348]. However, these studies suggest that  $A_{2A}$  antagonism can enhance motivated behaviour, rather than reducing it, as reported here. The reduction in motivated behaviour observed for both METH and sucrose under high response requirements may be due to neuroadaptations in  $A_{2A}$  KO mice. Baseline extracellular dopamine is reduced in  $A_{2A}$  KO mice [384], and dopamine depletion reduces responding for a food reward with high response requirements [385-388]. Thus it is possible that the low baseline striatal extracellular dopamine in  $A_{2A}$  KO mice may be causing the reduction in motivated responding for METH and sucrose. Returning extracellular dopamine in  $A_{2A}$  KO mice to WT levels and assessing subsequent motivated behaviour would help address this question.

#### *4.4.4. Consumption and the motivation to self-administer METH but not sucrose are dissociated in $A_{2A}$ KO mice*

To further investigate the involvement of  $A_{2A}$  in the motivation to obtain METH and sucrose, linear regression was employed. This analysis was used to account for spread within the data

and individual variability within populations [389]. When predicting motivated performance (i.e. PR responses) from self-administration behaviour (i.e. FR1, FR3), different results were found for different reinforcers.

For METH, the strength of the relationship between FR3 and PR was weaker in  $A_{2A}$  KO mice compared to WT littermates. This confirms that METH supports self-administration in  $A_{2A}$  KO mice at low reinforcement schedules, but this support dissipates when more effort is required to obtain the reward. Importantly, this relationship is observed even in  $A_{2A}$  KO mice with higher levels of FR3 responding, suggesting higher levels of METH self-administration do not necessarily entail equally high levels of motivation to obtain METH in KO mice. Adding to this, the intercepts of the lines were similar for both FR3/PR regressions, suggesting the differences in slope became apparent as task demands increased.

For sucrose, the strength of the relationship between FR3 and PR was not different between WT and  $A_{2A}$  KO mice. The intercept was lower in  $A_{2A}$  KO mice at the 10% sucrose concentration, recapitulating the reduction in FR3 and PR responding at this concentration. Unlike the regression analysis for METH, the sucrose analysis suggests that both WT and  $A_{2A}$  KO mice with higher levels of sucrose FR3 self-administration also demonstrated higher motivation during PR testing to obtain sucrose. This finding is interesting as it may suggest a somewhat stronger effect of  $A_{2A}$  deletion on motivated behaviour for drug reward over natural reward. Indeed, the reduction in breakpoint in  $A_{2A}$  KO mice appears more pronounced for the different doses of METH (10 $\mu$ g /kg: 45% reduction, 30 $\mu$ g/kg: 65% reduction) than for sucrose (2.5%: 30% reduction, 10%: 35% reduction). However, considering the slope differences between WT and  $A_{2A}$  KO mice for METH are modest, this interpretation should be treated with caution until further empirical support is provided.

An important control demonstration in the correlation analysis was the medium to strong positive correlations ( $r^2 > 0.4-0.8$ ) observed between FR and PR responding in both genotypes, across different reinforcers, doses / concentrations and schedules of reinforcement. This suggests the mice that responded more strongly during self-administration (FR1 or FR3) were more likely to demonstrate higher responding when presented with greater task requirements. While not unexpected, this is an interesting finding because recent research assessing relationships between variables in addiction relevant paradigms have failed to demonstrate previously expected relationships (e.g. no relationship between anxiety or novelty-seeking with sensitization [375]). Indeed, the strong correlation between FR and PR responding suggests the two measures may be affected by similar underlying constructs. It is important to note that despite these variables being correlated in both genotypes, the relationship between FR3 and PR was significantly weaker in  $A_{2A}$  KO mice than WT littermates.

#### *4.4.5. Conclusions*

The current study demonstrates that ablation of  $A_{2A}$  signalling reduces motivational behaviour required to obtain METH and sucrose, and prevents the expression of preference of a context previously associated with the drug. Importantly, these findings suggest  $A_{2A}$  modulates the motivation to obtain a reinforcer, but this occurs within a limited dose / concentration window. It appears  $A_{2A}$  signalling is not necessary for the locomotor sensitizing properties of METH, or acquisition and maintenance of self-administration behaviour. Taken together, these data suggest  $A_{2A}$  signalling is implicated in behaviours associated with the motivation to obtain both drug and natural reward.

## Chapter 5

### Neural loci implicated in METH place preference

#### 5.1. Introduction

The previous two chapters detailed the characterisation of mGlu5 and A<sub>2A</sub> KO mice in response to METH in various behavioural paradigms, to assess their potential as targets for addiction therapeutics. These experiments also addressed if deletion of these receptors resulted in similar behavioural phenotypes, which could suggest potential interactions between mGlu5 and A<sub>2A</sub> signalling in regulating drug-taking and drug-seeking behaviour. However, it seems mGlu5 and A<sub>2A</sub> modulate quite distinct behavioural domains. Indeed, there appears a critical involvement of A<sub>2A</sub> in signalling drug reward, and mGlu5 in cognitive processes associated with inhibition of drug-seeking. The absence of any overlap in the phenotypes observed did not present any clear direction for further investigations into interactions between these two receptors. While previous research indicates functional interactions between these receptors [266, 267], these occur in multiple behavioural paradigms (operant self-administration, CPP), using different drugs of abuse (cocaine, alcohol) and different species (mouse, rat). Thus, a number of variables would need to be addressed to investigate mGlu5-A<sub>2A</sub> interactions for METH in a hypothesis driven manner (e.g. use of operant or place preference paradigm, employment of pharmacological or genetic techniques, use of systemic / whole brain genetic deletion or localised pharmacological or genetic manipulation). Considering these variables, I sought to further investigate the neural locus of effect of a particularly prominent phenotype in A<sub>2A</sub> KO mice, that is, the absence of conditioned place preference. Furthermore, considering the expression of mGlu5-A<sub>2A</sub> heteromers on striatal MSNs, the present experiments may guide future research examining region-specificity in potential mGlu5-A<sub>2A</sub> interactions.

In Chapter 4, I reported that  $A_{2A}$  KO mice do not express a place preference for METH. This was a particularly robust effect, occurring at both doses tested (1 and 2mg/kg). Importantly, a number of studies suggest that these doses are rewarding in the place preference paradigm in mice [355, 390-396], suggesting  $A_{2A}$  signalling is necessary for the expression of context-reward associations. One question that arises from this finding is that of the neural locus / loci where  $A_{2A}$  mediates this type of reward-based learning. This question is of particular interest, as previous research demonstrates opposing effects of striatum-specific vs. whole forebrain  $A_{2A}$  deletion on cocaine-induced locomotion, suggesting that the neural loci where  $A_{2A}$  signals can determine either the enhancement or suppression of drug-induced behaviour [333]. It is possible that reward behaviour is also modulated in a similar location-dependent manner.

Currently, a few studies have examined how region-specific pharmacological modulation of  $A_{2A}$  alters reward for non-drug reinforcers and addiction-relevant behaviour. There appears a role for  $A_{2A}$  signalling in the NAcc in mediating reward. Intra-accumbal administration of the  $A_{2A}$  antagonist MSX-3 reverses deficits induced by the  $D_2$  antagonist eticlopride in an effort related choice task for high carbohydrate pellets in Sprague–Dawley rats [397]. Also, other studies in rats demonstrated that intra-accumbal treatment with the  $A_{2A}$  agonist CGS 21680 inhibits the expression of cocaine sensitization [340], and pharmacological enhancement or suppression of  $A_{2A}$  signalling in the NAcc core reduces and enhances drug-primed reinstatement of cocaine seeking respectively [338]. It is important to note that currently no study has definitively examined where in the brain  $A_{2A}$  may mediate drug reward specifically, as opposed to other behaviours relevant to addiction e.g. sensitization, reinstatement. Thus, the experiments in the current chapter sought to clarify where  $A_{2A}$  acts within the mesocorticolimbic system to mediate associations with METH reward and addiction-relevant behaviour.

## 5.2. Methods

### 5.2.1. Animals and behavioural methods

Experiment 1 used A<sub>2A</sub> KO mice and their WT littermates (n = 5 WT, n = 11 A<sub>2A</sub> KO), while experiment 2 used WT and HET A<sub>2A</sub><sup>loxP/loxP</sup> littermates (n = 5 WT, n = 9 HET A<sub>2A</sub><sup>loxP/loxP</sup>). Mice in experiment 2 were not injected with AAV-Cre, and were thus considered as WT mice. These mice will henceforth be referred to as A<sub>2A</sub><sup>lox-/lox-</sup>. All mice were group housed as described in the General Methods (section 2.2). Place preference was conducted according to protocol 1 for animals in experiment 1, and according to protocol 2 for animals in experiment 2 (see General Methods, section 2.3.1). At the end of the experiment, animals were perfused, brains collected and frozen, and tissue processed for Fos immunohistochemistry according to General Methods, section 2.5.2. Immunohistochemistry was conducted on all tissue for experiment 1 at the same time; this was also the case for experiment 2. Regions counted in experiment 1 and experiment 2 are listed in Table 5.1. Care was taken to ensure sections were matched at the same anatomical level for each mouse. The methodology for determination of Fos-immunoreactivity (IR) in each experiment is outlined in section 5.2.2.

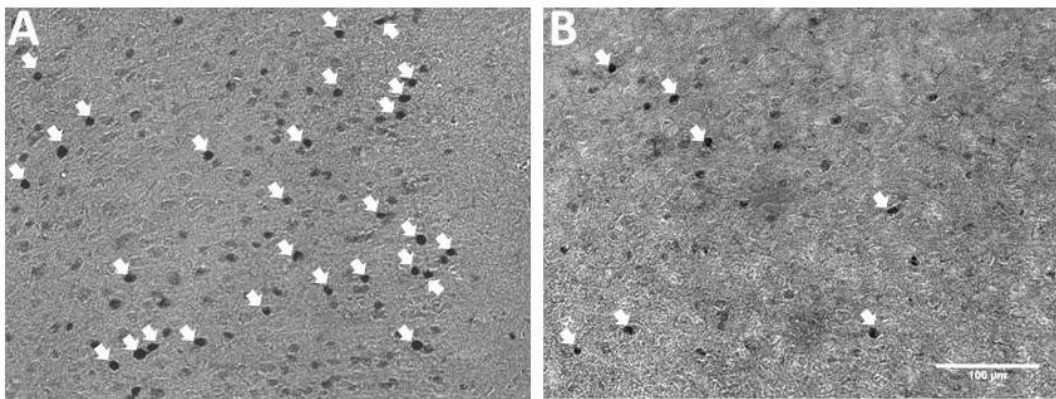


Experiment 1	AP 1	AP 2	Experiment 2	AP 1	AP 2
Lateral orbital cortex	+2.22	+1.98	Infralimbic cortex	+1.78	+1.54
Medial orbital cortex	+2.22	+1.98	Prelimbic cortex	+1.78	+1.54
Ventral orbital cortex	+2.22	+1.98	Nucleus accumbens core	+1.54	+1.1
Infralimbic cortex	+1.7	+1.42	Nucleus accumbens shell	+1.54	+1.1
Prelimbic cortex	+1.7	+1.42	Cingulate cortex	+1.1	+0.4
Cingulate cortex	+1.18	+0.5	Ventral pallidum	+0.62	+0.14
Piriform cortex	+0.74	+0.14	Dentate gyrus (hippocampus)	-1.46	-2.3
Clastrum	+1.42	+1.18	CA3 (hippocampus)	-1.46	-2.3
Lateral septum	+1.18	+0.74	Basolateral amygdala	-0.94	-1.34
Habenula	-1.34	-1.7	Somatosensory cortex 1, trunk region	-1.46	-1.82
Dentate gyrus	-1.82	-1.34	Somatosensory cortex 1, barrel field	-1.46	-1.82
Basolateral amygdala	-1.06	-1.94	Somatosensory cortex 2	-1.46	-1.82
Medial dorsal thalamus	-0.7	-1.06	Ventral tegmental area	-2.92	-3.52
Paraventricular thalamus	-0.7	-1.46			
Lateral hypothalamus	-0.7	-1.46			
Somatosensory cortex 1, trunk region	-1.7	-1.46			
Somatosensory cortex 1, barrel field	-1.7	-1.46			
Somatosensory cortex 2	-1.7	-1.46			
Lateral periaqueductal grey	-3.52	-4.24			
Dorsal periaqueductal grey	-3.52	-4.24			

**Table 5.1. List of regions and their anterior-posterior (AP) coordinates according to the Paxinos and Franklin Brain Atlas counted in histological experiments.** All regions were counted in two sections, matched across animals. Abbreviations: AP: anteroposterior from bregma; 1 and 2 refer to the first and second sections counted within each region.

### 5.2.2. Fos counting procedure

For both experiments, fos positive cells for each region of interest were counted in two sections, and the number of fos positive cells summed between the two sections to create a total score. Between experiments 1 and 2, the intensity of staining required for cells to be considered positive for Fos-IR differed. The results from experiment 1 (outlined below) indicated a genotype effect on Fos-IR, rather than region-specific activation following a behavioural test. In experiment 2, the required staining intensity for cells to be considered Fos positive was increased, to reduce potential false positives and increase specificity [398-400]. An example of cells that are considered Fos positive in experiment 1 and 2 is given in Figure 5.1.



**Figure 5.1. Examples of Fos positive selection criteria in experiment 1 and 2.** White arrows indicate cells that were considered positive for Fos-IR in experiment 1 (A) and experiment 2 (B). Scale bar = 100μm.

### 5.2.3. Statistics

Behavioural output from experiment 1 was analysed using two-way RM ANOVA for the variable 'preference score' with the repeated measure 'time' (habituation / test) and the between factor 'group' (WT / A<sub>2A</sub> KO mice preferring / A<sub>2A</sub> KO mice non-preferring). Delineation of A<sub>2A</sub> KO mice into preferring and non-preferring groups is outlined in section 5.3.1. Two-way ANOVA was followed by one-way ANOVA split by corresponding factor, with a Bonferroni correction ( $p = .05 / \text{number of independent variables}$ ). Fos raw counts from experiment 1 were analysed using one-way ANOVA for the variable 'raw Fos counts' with the factor 'group' (WT / A<sub>2A</sub> KO preferring / A<sub>2A</sub> KO non-preferring), followed by mutually orthogonal contrasts (WT vs. all A<sub>2A</sub> KO mice; A<sub>2A</sub> KO preferring vs. A<sub>2A</sub> KO non-preferring). Behavioural output from experiment 2 was analysed using two-way RM ANOVA for the variable 'preference score' with the repeated measure 'time' (habituation / test) and the between factor 'treatment group' (METH-conditioned / saline-conditioned). One-way ANOVA for the variable 'raw Fos counts' with the between factor 'treatment group' (METH-conditioned / saline-conditioned) were used to assess differences in Fos-IR in experiment 2. Data presented as mean  $\pm$  SEM.

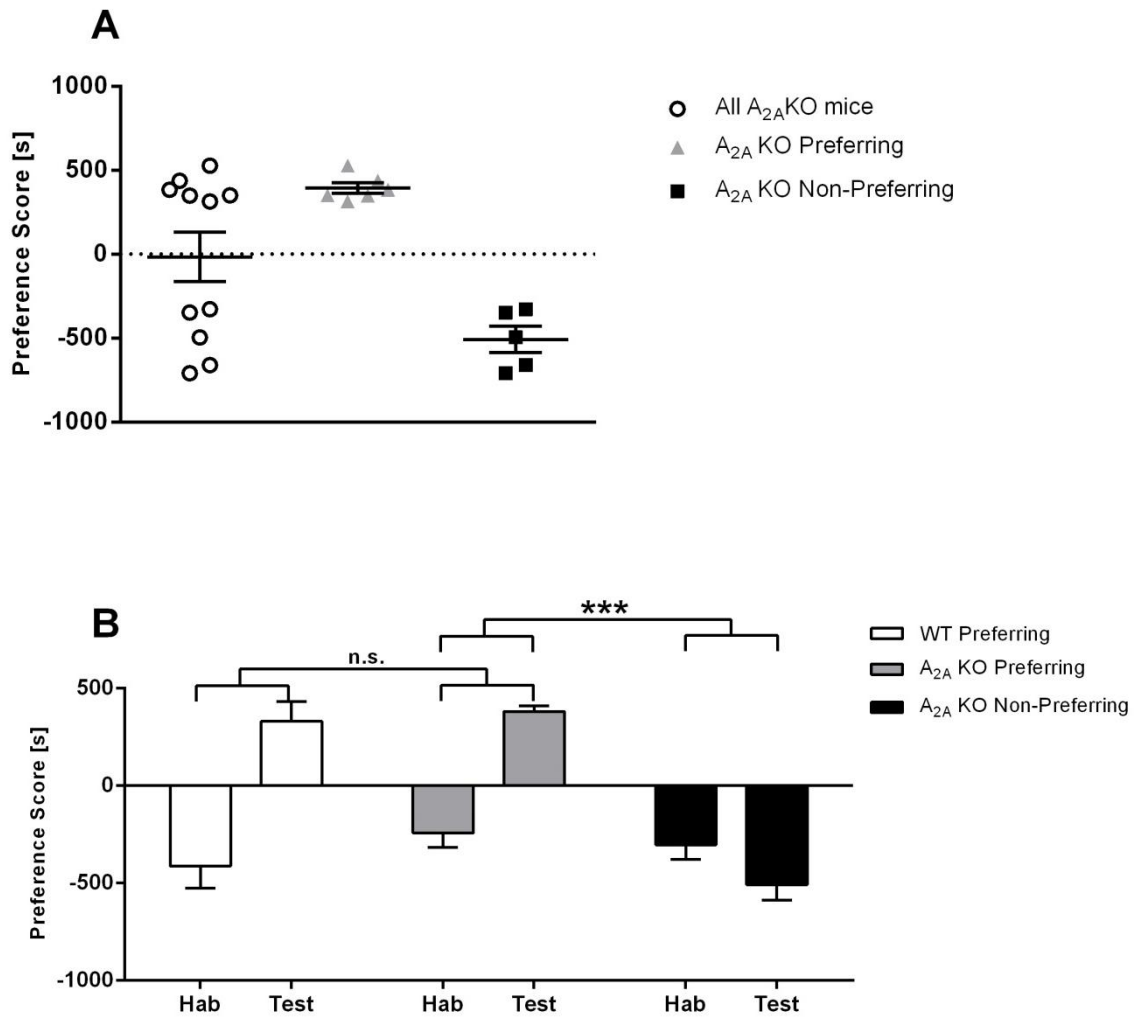
## 5.3. Results

### 5.3.1. Deletion of A<sub>2A</sub> did not uniformly reduce METH place preference

The expression of place preference was assessed using a preference score, described in section 4.3.1. One WT mouse was excluded as an outlier, as its preference score at test was more than 2.5 SDs below the mean. Intriguingly, there appeared a dichotomous split in the preference scores in A<sub>2A</sub> KO mice during the test session. Examination of Figure 5.2a demonstrates two subpopulations within the preference score of A<sub>2A</sub> KO mice, with approximately half of the A<sub>2A</sub> KO mice demonstrating a preference ( $n = 6$ ) and half not demonstrating a preference for the

METH-paired context ( $n = 5$ ). When all scores are coalesced for  $A_{2A}$  KO mice, the mean is not representative of any mice in this genotype (see Figure 5.2a). Thus, the results for KO mice were divided into two subgroups:  $A_{2A}$  KO mice that exhibited a METH place preference ( $A_{2A}$  KO preferring), and  $A_{2A}$  KO mice that did not exhibit a METH place preference ( $A_{2A}$  KO non-preferring).

Comparison of preference scores between WT,  $A_{2A}$  KO preferring and  $A_{2A}$  KO non-preferring mice demonstrates a change in preference score from habituation to test [main effect of 'time',  $F(1,13) = 50.1, p < .001$ ]. While WT and  $A_{2A}$  KO preferring mice demonstrated a change from a negative to a positive preference score with time (i.e. from habituation to test),  $A_{2A}$  KO non-preferring mice did not demonstrate a change in preference score over time (Figure 5.2b) [main effect of 'group'  $F(2,13) = 13.3, p < .001$ ; significant interaction  $F(2,13) = 31.0, p < .001$ ]. Orthogonal contrasts indicated the change in preference score from habituation to test was not significantly different between WT and  $A_{2A}$  KO preferring mice ( $p = .22$ ), but was significantly different between  $A_{2A}$  KO preferring and non-preferring mice ( $p < .001$ ). These data indicate METH conditioning produced a place preference in WT and  $A_{2A}$  KO preferring mice, but not in  $A_{2A}$  KO non-preferring mice (Figure 5.2b).



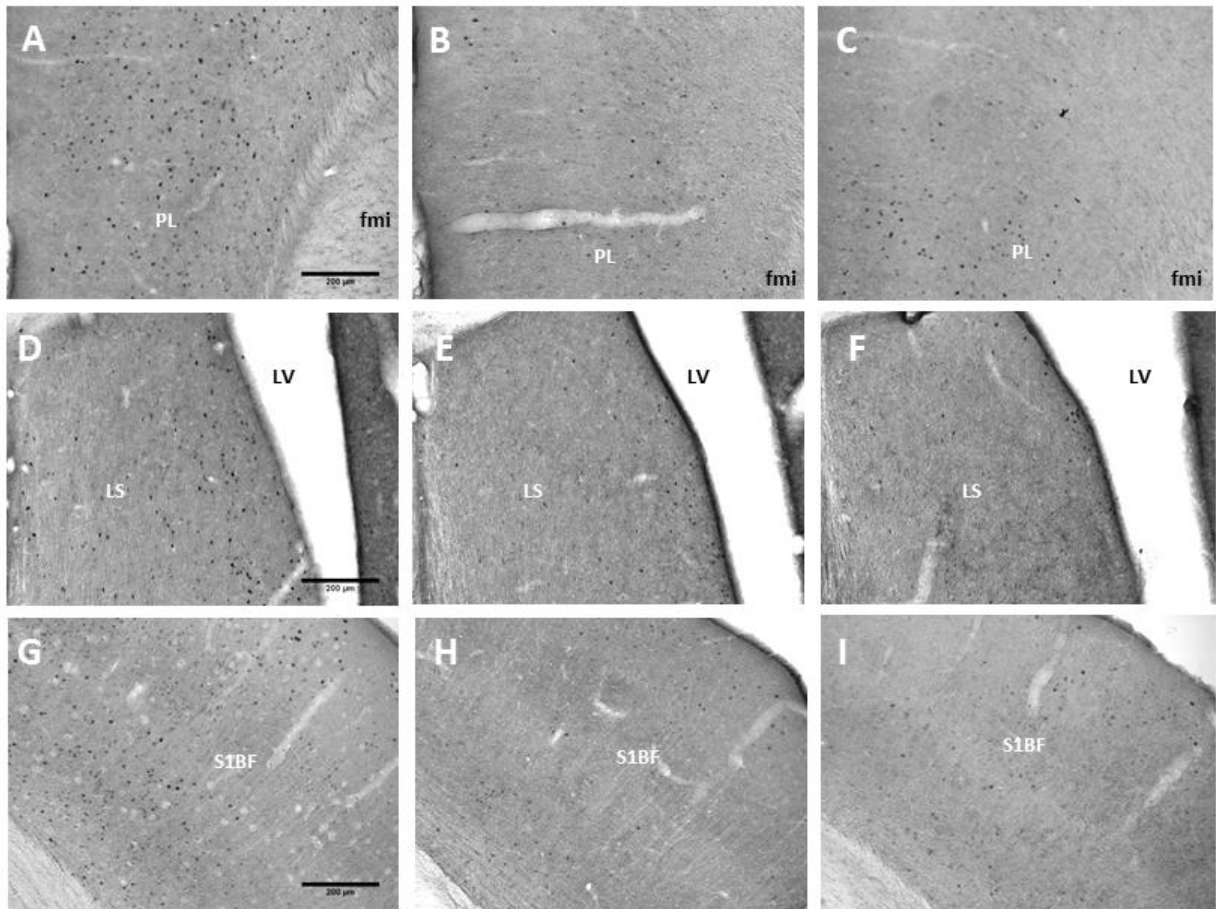
**Figure 5.2. Deletion of  $A_{2A}$  did not uniformly reduce METH place preference.** Preference score [s] in  $A_{2A}$  KO mice and WT littermates following conditioning with 2mg/kg METH. **A)** Preference scores [s] in  $A_{2A}$  KO mice have a bimodal distribution ('all  $A_{2A}$  KO mice'); hence this genotype was split into subgroups: ' $A_{2A}$  KO mice preferring' and ' $A_{2A}$  KO mice non-preferring'. **B)** Preference scores (habituation vs. test) in WT,  $A_{2A}$  KO preferring and  $A_{2A}$  KO non-preferring mice. Data presented as means  $\pm$  SEM, and analysed using two-way RM ANOVA, followed by orthogonal contrasts. Significant effects of 'group' (WT vs.  $A_{2A}$  KO preferring vs.  $A_{2A}$  KO non-preferring) indicated by asterisks (\*\*\*) ( $p < .001$ ). Abbreviations: Hab: habituation session. N = 5 WT, 6  $A_{2A}$  KO preferring and 5  $A_{2A}$  KO non-preferring mice.

### 5.3.2. *A<sub>2A</sub> KO mice show reduced Fos-IR, irrespective of expression of METH place preference*

Considering the expression of a METH place preference in some, but not all *A<sub>2A</sub>* KO mice, it was hypothesised the Fos-IR of *A<sub>2A</sub>* KO preferring mice would be similar to that of WT mice. However, in more than half of the regions counted (11/20 regions), there was greater Fos-IR in WT mice compared to *A<sub>2A</sub>* KO mice, irrespective of the expression of a place preference in KO mice (Table 5.2). Indeed, Fos-IR was similar between *A<sub>2A</sub>* KO preferring and *A<sub>2A</sub>* KO non-preferring mice. A significant effect of 'group' was present in the following regions: lateral orbital cortex [F(2,13) = 4.7, *p* = .03], ventral orbital cortex [F(2,13) = 5.5, *p* = .02], infralimbic cortex (IL) [F(2,13) = 5.5, *p* = .02], prelimbic cortex (PL) [F(2,13) = 7.4, *p* = .007], cingulate cortex [F(2,13) = 6.9, *p* = .01], claustrum [F(2,13) = 5.2, *p* = .02], lateral septum [F(2,13) = 4.7, *p* = .03], dentate gyrus [F(2,13) = 3.9, *p* < .05], somatosensory cortex 1 barrel field [F(2,13) = 9.8, *p* = .003], somatosensory cortex 1 trunk region [F(2,13) = 8.3, *p* = .005] and somatosensory cortex 2 [F(2,13) = 7.1, *p* = .008]. In all of these regions, orthogonal contrasts indicated a significant difference between WT and all *A<sub>2A</sub>* KO mice (irrespective of preference), but no difference between *A<sub>2A</sub>* KO preferring and non-preferring mice (Table 5.2; see also representative photomicrographs in Figure 5.3).

Region	WT	A <sub>2A</sub> KO Preferring	A <sub>2A</sub> KO Non-preferring
Lateral orbital cortex	41.8 ± 12.5 *	7 ± 1.8	12 ± 9.9
Medial orbital cortex	52.4 ± 13.7	23.7 ± 4.8	27.4 ± 12.1
Ventral orbital cortex	97.4 ± 23.2 **	27.2 ± 6.8	35.2 ± 17.6
Infralimbic cortex	47 ± 10.9 **	19.2 ± 3	22 ± 3.5
Prelimbic cortex	130.4 ± 30.9 **	41.3 ± 10.1	41.2 ± 9.6
Cingulate cortex	181.8 ± 35.5 **	76.8 ± 9.8	81 ± 17.7
Piriform cortex	137.2 ± 24.2	78.7 ± 10.9	86.2 ± 29.3
Clastrum	61.8 ± 18.2 **	25.3 ± 4	17.2 ± 2.4
Lateral septum	203 ± 40.6 **	96.3 ± 18.4	98.6 ± 22.2
Habenula	37.4 ± 4.6	25.2 ± 5.9	34.8 ± 5.8
Dentate gyrus (hippocampus)	51 ± 6.2 *	33.5 ± 4.7	38.4 ± 1.2
Basolateral amygdala	33 ± 8.8	14.3 ± 5.8	11.8 ± 4.6
Medial dorsal thalamus	42.6 ± 5.9	23.7 ± 6	28.4 ± 7.3
Paraventricular thalamus	67.8 ± 6.6	43.5 ± 7.3	58.8 ± 13.4
Lateral hypothalamus	89.6 ± 19.2	44.3 ± 11.1	45.2 ± 8.3
Somatosensory cortex 1, barrel field	96.2 ± 27.8 ***	12.2 ± 4.1	12.4 ± 2.2
Somatosensory cortex 1, trunk region	246.2 ± 60.7 ***	59.2 ± 15.8	63 ± 24
Somatosensory cortex 2	74 ± 20.1 **	15.7 ± 5.5	15.2 ± 9.4
Lateral periaqueductal grey	67.2 ± 19.1	48.7 ± 2.5	59.6 ± 10.3
Dorsal periaqueductal grey	26.4 ± 7.9	16.2 ± 2.9	15 ± 3.4

**Table 5.2. Total Fos-IR neurons in WT, A<sub>2A</sub> KO preferring and A<sub>2A</sub> KO non-preferring mice (experiment 1).** Data presented as means ± SEM, and analysed using one-way ANOVA followed by mutually orthogonal contrasts. Significant orthogonal contrasts showing effects of 'group' (WT vs. all A<sub>2A</sub> KO mice) indicated by asterisks (\**p* < .05, \*\**p* < .01, \*\*\**p* < .001). N = 5 WT, 6 A<sub>2A</sub> KO preferring and 5 A<sub>2A</sub> KO non-preferring mice.



**Figure 5.3. Representative photomicrographs of Fos immunoreactivity in WT,  $A_{2A}$  KO preferring and  $A_{2A}$  KO non-preferring mice.** Fos immunoreactivity in the prelimbic prefrontal cortex, lateral septum and the barrel field of the somatosensory cortex respectively, for WT mice in **A), D), G)**, for  $A_{2A}$  KO preferring mice in **B), E), H)** and for  $A_{2A}$  KO non-preferring mice in **C), F), I)**. Abbreviations: PL: prelimbic cortex, fmi: forceps minor of the corpus callosum, LS: lateral septum, LV: lateral ventricle, S1BF: somatosensory cortex 1, barrel field. N = 5 WT, 6  $A_{2A}$  KO preferring and 5  $A_{2A}$  KO non-preferring mice. Scale bar = 200 $\mu$ m.



### 5.3.3. Conditioning with METH, but not saline, produces a place preference

Experiment 2 was conducted to determine a region/s specifically activated following the expression of METH place preference, as experiment 1 failed to demonstrate region-specific Fos activation following expression of METH CPP. Animals in experiment 2 were conditioned either with METH in one compartment and saline in another (METH-conditioned), or saline in both compartments (saline-conditioned). One mouse (METH-conditioned) was excluded from the analysis due to a technical fault during habituation where data was not recorded.

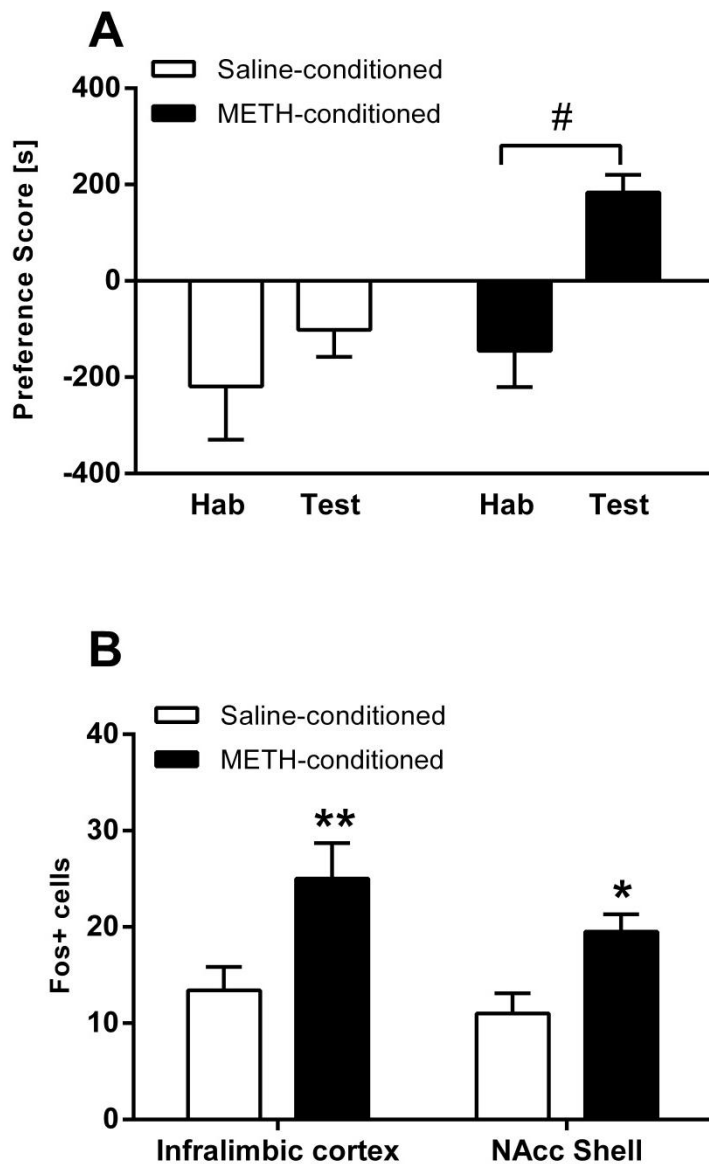
METH-conditioned mice demonstrated a preference for the METH-paired context [main effect of 'treatment'  $F(1,91) = 9.2, p = .01$ ]. Importantly, there was no difference between WT and  $A_{2A}^{lox- /lox-}$  mice [ $F(1,9) = .6, p = .4$ ], justifying the pooling of genotypes in this experiment. Scores tended to increase from habituation to test [trend for a main effect of 'time'  $F(1,9) = 4.6, p = .06$  no interactions]; this was mainly driven by METH-conditioned mice, where there was approximately a >2 fold increase in scores from habituation to test, whereas there was approximately a 0.5 fold increase in saline-conditioned mice. Examination of Figure 5.4a suggests METH-conditioned mice developed a positive preference following conditioning [one-way ANOVA with Bonferroni correction,  $F(1,5) = 12.8, p = .02$ ], whereas saline-conditioned mice show a non-significant increase in their preference score following conditioning [one-way ANOVA with Bonferroni correction,  $F(1,6) = .5, p = .4$ ].

5.3.4. Enhanced Fos-IR in the NAcc shell and IL following METH-conditioning, but not saline-conditioning

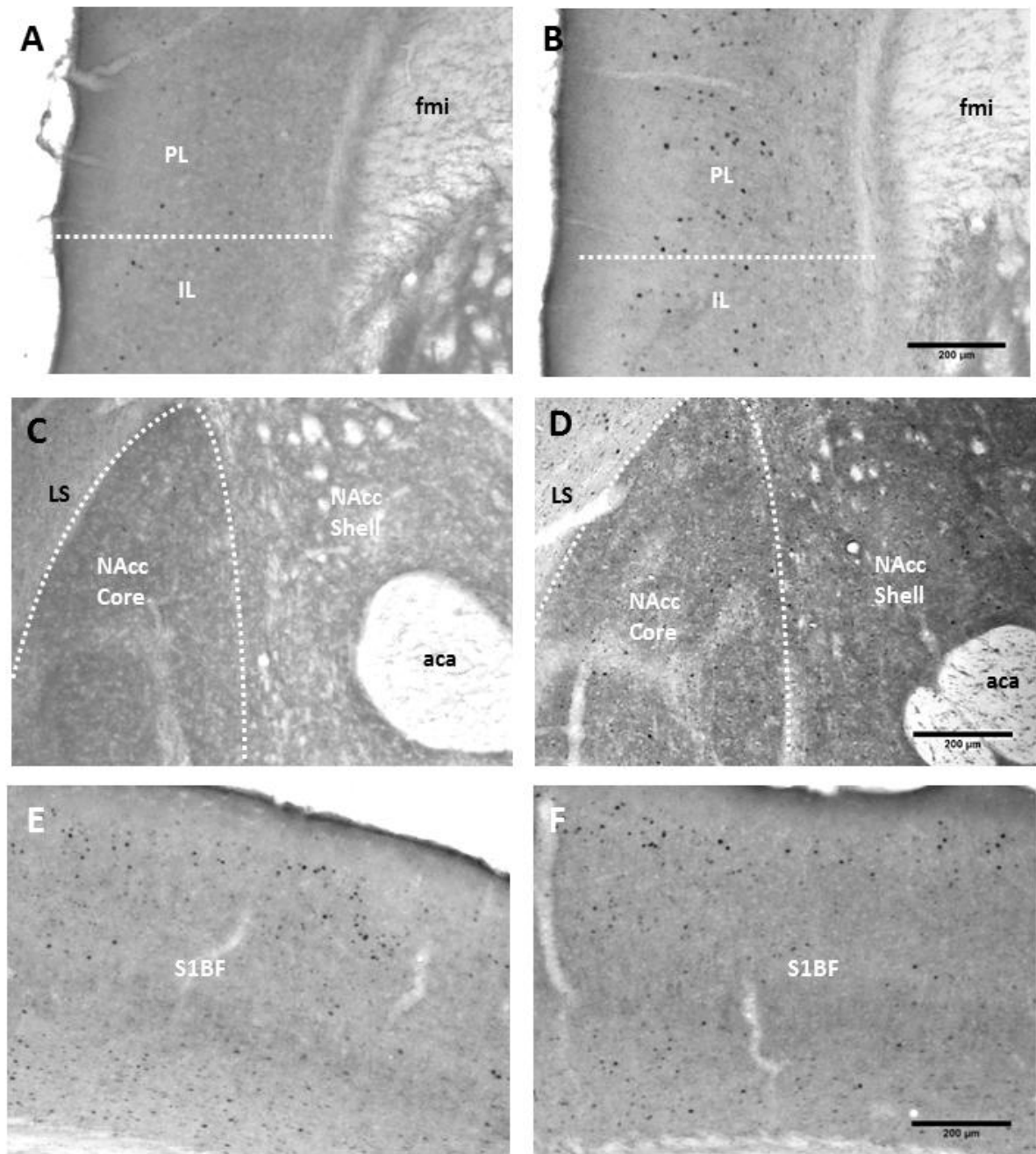
Mice conditioned with METH demonstrated greater Fos-IR in the NAcc shell [ $F(1,11) = 6.3$ ,  $p = .03$ ] and IL [ $F(1,11) = 10.4$ ,  $p = .008$ ] compared to mice conditioned with saline (Figure 5.4b, **Table 5.3**). Fos-IR was not different between the treatment groups in the other regions counted (see **Table 5.3**).

Region	Saline-conditioned	METH-conditioned
Infralimbic cortex	13.4 ± 2.4	26.3 ± 3.3 **
Prelimbic cortex	28.1 ± 6.5	41.7 ± 5.5
Nucleus accumbens core	2 ± 1	4.3 ± 1.4
Nucleus accumbens shell	11 ± 2.1	18.5 ± 2.1 *
Cingulate cortex	56 ± 14.8	57.5 ± 5.5
Ventral pallidum	11.1 ± 2.9	9 ± 2.5
Dentate gyrus (hippocampus)	27.6 ± 5.7	39.8 ± 6
CA3 (hippocampus)	16.4 ± 3.7	18.5 ± 2.1
Basolateral amygdala	22.7 ± 1	25.8 ± 3.5
Somatosensory cortex 1, trunk region	26 ± 8.1	19.2 ± 2.9
Somatosensory cortex 1, barrel field	71.3 ± 13.3	61.3 ± 7.7
Somatosensory cortex 2	39.6 ± 8.6	31.7 ± 5.4
Ventral tegmental area	12.3 ± 2.7	11.3 ± 1.7

**Table 5.3. Total Fos immunoreactive neurons in METH-conditioned and saline-conditioned mice (experiment 2).** Data presented as means ± SEM, and analysed using single sample t-tests for each region. Significant effects of ‘treatment’ (saline-conditioned vs. METH-conditioned) indicated by asterisks (\* $p < .05$ , \*\* $p < .01$ ). N = 7 saline-conditioned mice, 6 METH-conditioned mice.



**Figure 5.4. Conditioning with METH induces a place preference, and is associated with increased Fos immunoreactivity in the infralimbic cortex and nucleus accumbens shell. A)** Mice conditioned with METH, but not saline, exhibit a preference for the METH-paired context. **B)** Mice conditioned with METH exhibit enhanced Fos immunoreactivity in the infralimbic cortex and the NAcc shell. Data presented as mean  $\pm$  SEM, and analysed in **A)** using two-way RM ANOVA, followed by one-way ANOVA split by the factor 'genotype' with a Bonferroni correction if appropriate. Data in **B)** analysed using one-way ANOVA. Significant effects of 'day' (vs. habituation) are represented by hash symbols ( $\#p < .05$ ); significant effects of 'treatment' (vs. saline) are represented by asterisks ( $*p < .05$ ,  $**p < .01$ ). Abbreviations: Hab: habituation session; NAcc Shell: nucleus accumbens shell. N = 7 saline-conditioned mice, 6 METH-conditioned mice.



**Figure 5.5. Representative photomicrographs of Fos immunoreactivity in METH-conditioned and saline-conditioned mice.** Fos immunoreactivity in the medial prefrontal cortex, nucleus accumbens and the barrel field of the somatosensory cortex respectively, for saline-conditioned mice in **A), C), E)** and in METH-conditioned mice in **B), D)** and **F)**. Abbreviations: PL: prelimbic cortex, IL: infralimbic cortex, fmi: forceps minor of the corpus callosum, LS: lateral septum, NAcc shell: nucleus accumbens shell, NAcc core: nucleus accumbens core, aca: anterior commissure, anterior part, S1BF: somatosensory cortex 1, barrel field. N = 7 saline-conditioned mice, 6 METH-conditioned mice. Scale bar = 200 $\mu$ m.

## 5.4. Discussion

The current set of experiments sought to define a locus where  $A_{2A}$  may mediate the expression of a place preference to METH. In experiment 1, I was unable to observe differential IR of the immediate early gene c-Fos between WT and  $A_{2A}$  KO mice that corresponded to the expression of a METH place preference. However, in experiment 2, region-specific Fos-IR was observed following expression of a positive place preference for METH, compared to a neutral preference for saline. Furthermore, the region-specific activation of Fos in experiment 2 overlaps well with the known distribution of  $A_{2A}$ , providing a potential locus for experiments using conditional genetics.

### *5.4.1. Stress may interact with $A_{2A}$ deletion to modulate expression of place preference*

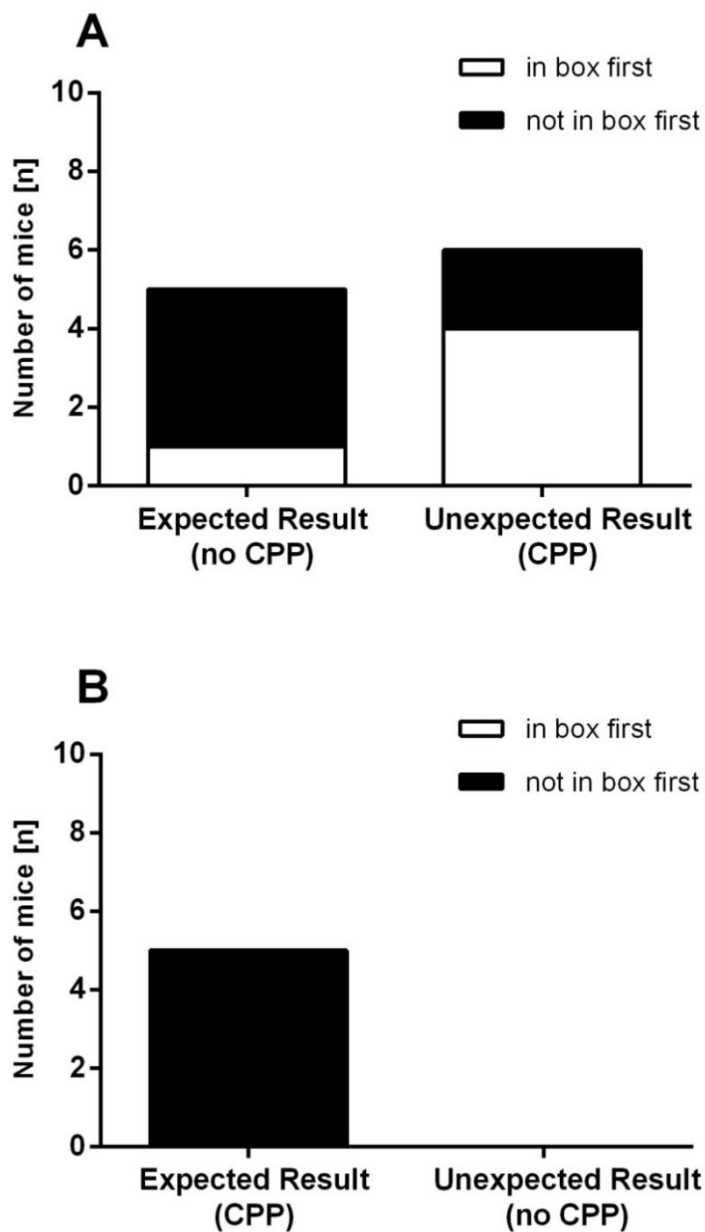
Experiment 1 did not replicate the place preference phenotype of  $A_{2A}$  KO mice in response to 2mg/kg METH. Rather,  $A_{2A}$  KO mice formed two subpopulations; mice that exhibited a place preference for METH (preferring) and mice that did not exhibit a place preference for METH (non-preferring) (see Figure 5.2a). This was an intriguing finding, as previously  $A_{2A}$  KO mice demonstrated an absence of METH place preference at both 1 and 2mg/kg METH. Interestingly, the dichotomy of  $A_{2A}$  KO mice in METH place preference indicates this receptor may modulate the expression of CPP, as some  $A_{2A}$  KO mice must have acquired METH CPP to express it in special circumstances. Indeed, this finding demonstrates our findings are not confounded by potential learning deficits in  $A_{2A}$  KO mice, as acquisition of a METH place preference is intact in  $A_{2A}$  KO preferring mice.

The design of experiment 1 in the present chapter suggests that the emergence of dichotomy in place preference is due to the potential stress effects on the expression of learnt behaviour in  $A_{2A}$  KO mice. Mice which were behaviourally tested and then sacrificed in experiment 1 may

have experienced a greater level of stress compared to mice which were previously only behaviourally tested (as in Chapter 4) because in experiment 1, mice were staggered when placed into the CPP apparatus. This was done to ensure all mice were perfused 90 min after the start of the test, as Fos expression is maximal following this delay [358, 359]. Thus, the experimenter repeatedly entered and exited the test room during the behavioural test, potentially disrupting animals already being tested in the room. Examination of Figure 5.6a and Figure 5.6b suggests expression of place preference in  $A_{2A}$  KO mice (an 'unexpected result') was more common in mice placed in the CPP box first, and thus exposed to more disruption during the test. Indeed, the majority of  $A_{2A}$  KO mice (4/6 mice) which were removed first from their home cages expressed a place preference (an 'unexpected result'), whereas most  $A_{2A}$  KO mice (4/5 mice) which were not removed from their home cages first, did not express place preference (an 'expected result'). Unfortunately, in the current experiment no WT mice were removed first from their home cages, making it unclear as to whether WT mice would also demonstrate altered expression of CPP following this mild stress. Importantly,  $A_{2A}$  KO mice in Chapter 4 which did not experience staggered CPP box entry did not exhibit a dichotomous preference score, as observed in Chapter 5, suggesting the results obtained may be due to the slight change in protocol. Regardless, it is possible that  $A_{2A}$  KO mice may be more susceptible to stress compared to WT littermates, as  $A_{2A}$  KO mice display an anxiogenic phenotype on the elevated plus maze [314], and exhibit greater basal plasma corticosterone levels compared to WT littermates [401].

Importantly, reducing  $A_{2A}$  activity under conditions of stress appears to be beneficial in cognitive tasks. Cognitive deficits in the Morris Water Maze induced by maternal separation are attenuated by systemic pharmacological  $A_{2A}$  antagonism in rats [402], while stress induced deficits in escape behaviour are ameliorated by systemic  $A_{2A}$  antagonist administration in rats [403]. Hence, if a reduction in  $A_{2A}$  signalling during stress can be beneficial in cognitive tasks, it

is possible that  $A_{2A}$  KO mice that experienced more stress were able to retrieve and express the place preference memory, whereas  $A_{2A}$  KO mice experiencing less stress could not.



**Figure 5.6. Analysis of preference scores in experiment 1 according to test schedule.** Expression of place preference in experiment 1 in **A**)  $A_{2A}$  KO and **B**) WT mice, according to whether they were placed in the CPP apparatus first ('in box first'), or second or third ('not in box first'), from their home cage group. The expected result in WT mice was the expression of a place preference, whereas in  $A_{2A}$  KO mice the expected result was the absence of place preference. Visual inspection of **A**) and **B**) suggests WT and  $A_{2A}$  KO mice demonstrate the expected result when not placed in the CPP box first, but  $A_{2A}$  KO mice demonstrate an unexpected result (i.e. exhibiting CPP) when placed in the box first.

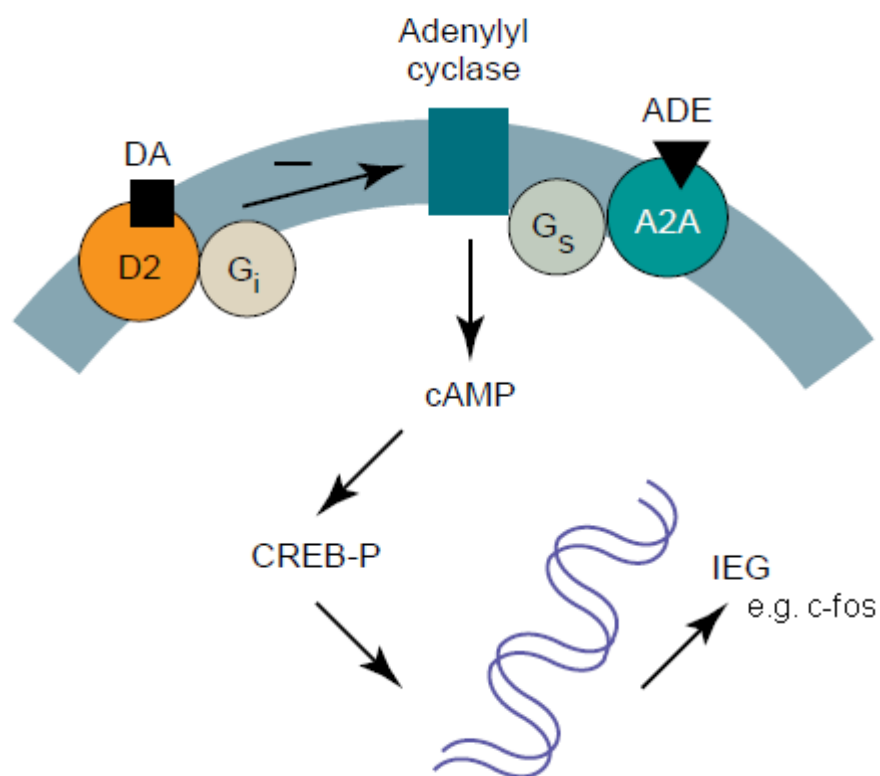
#### 5.4.2. *A<sub>2A</sub> KO mice show global reductions in Fos-IR, irrespective of place preference*

Considering the expression of place preference in some, but not all *A<sub>2A</sub> KO* mice, I expected a similar pattern of Fos-IR in WT and *A<sub>2A</sub> KO* preferring mice, which would be different to Fos-IR in *A<sub>2A</sub> KO* non-preferring mice. It was surprising then that Fos-IR was similar between *A<sub>2A</sub> KO* preferring and non-preferring mice. Fos has previously been employed as a marker of neuronal activity following expression [391, 404] and reinstatement of place preference [389]. However, there was an overarching effect of genotype on Fos-IR, making it difficult to identify regions specifically activated following the expression of place preference. Interestingly, the pattern of Fos-IR in different neural structures corresponds with general behavioural arousal [405, 406], and there was negligible Fos-IR in some regions previously associated with expression of place preference [e.g. dorsal and ventral striatum [391]]. This suggests Fos-IR in experiment 1 may have been dictated by the general response to the behavioural test (i.e. arousal, stress, sensory stimulation), rather than reflecting the expression of place preference. While we were able to address which regions were activated following CPP expression in experiment 2, it is interesting to speculate on the cause of the genotype effect on Fos-IR in *A<sub>2A</sub> KO* mice.

It is possible genetic deletion of *A<sub>2A</sub>* altered intracellular mechanisms which reduced Fos-IR across a number of forebrain regions. Post-synaptic *A<sub>2A</sub>* activates the cAMP-protein kinase A (PKA) signalling pathway and leads to the phosphorylation of transcription factors, such as cAMP response element-binding (CREB) [205, 309, 407]. This can activate expression of immediate early genes, such as c-Fos [309, 408] (see Figure 5.7). The effects of this intracellular signalling pathway have been demonstrated *in vivo*, as *A<sub>2A</sub>* antagonism reduces striatal Fos expression [409]. Importantly, genotype effects on Fos-IR were observed in regions where *A<sub>2A</sub>* is expressed (e.g. hippocampus, lateral septum, thalamus, hypothalamus, somatosensory cortex; [300]). Furthermore, there are also connections between *A<sub>2A</sub>* expressing regions (e.g. striatum,



hippocampus) to non- $A_{2A}$  expressing regions where we observed reduced Fos-IR in  $A_{2A}$  KO mice (e.g. mPFC) [410, 411]. Thus, it is possible that genotype effects of Fos-IR in the forebrain of  $A_{2A}$  KO mice could be a downstream effect of genetic deletion of this receptor. Examining the expression of IEGs which are not downstream of  $A_{2A}$  may clarify this hypothesis.



**Figure 5.7.**  $A_{2A}$  signalling cascade, which leads to activation of immediate early genes such as c-Fos. Modified from [309].

#### 5.4.3. Increased Fos-IR in NAcc shell and IL following expression of METH CPP

The genotype effects of Fos-IR in experiment 1 prevented the identification of a locus through which  $A_{2A}$  could be mediating place preference. Thus, a modified protocol was employed in

experiment 2, where  $A_{2A}^{lox-/lox-}$  mice were conditioned with METH or saline to determine Fos-IR following the expression of a positive, as opposed to a neutral place preference. Importantly, experiment 2 was conducted in mice which were essentially of the same genotype (i.e. WT and HET  $A_{2A}^{loxP/loxP}$  mice which were not treated with AAV-Cre) to prevent genotype effects on Fos-IR. Furthermore, I demonstrated statistically that there was no effect of genotype on CPP expression, validating the coalescing of these genotypes. These mice were used in experiment 2 as I intended to use these mice in future studies using conditional genetic manipulation.

The expression of a positive place preference for METH was associated with increased Fos-IR in the IL and NAcc shell. These findings are similar to those of Chiang and colleagues [391], who demonstrated increased Fos-IR in the mPFC and NAcc core following expression of METH place preference. I was able to expand on the findings of Chiang and colleagues by specifying the involvement of the IL, as opposed to the mPFC as a whole, in the expression of METH place preference. While the results of experiment 2 did not replicate enhanced Fos-IR in the NAcc core of Chiang *et al.* [391], there was a non-significant increase in Fos-IR in the NAcc core (see **Table 5.3**). The discrepancies between experiment 2 and that of Chiang *et al.* may be due to methodological differences. In experiment 2, Fos-IR was quantified using a total count from two anatomic sections, whereas Chiang *et al.* only counted in one section. Fos-IR in the posterior NAcc core in experiment 2 was somewhat greater in METH-conditioned mice compared to saline-conditioned mice (although this effect failed to reach significance), whereas in the anterior section Fos-IR was similar between treatment groups (data not shown). The posterior region counted in the present study has a similar anatomical locus to the NAcc core region counted in the study by Chiang and colleagues. Our method of summing counts from these two regions appears to have reduced the treatment group difference, which may explain the lack of treatment effect in the current study in the NAcc core. Other methodological differences (e.g.

counting method, region delineation, staining protocol differences) may also account for differences between the current study and that of Chiang *et al.*

#### *5.4.4. Involvement of NAcc shell and IL in expression of METH place preference*

The present findings demonstrate that activation of the NAcc shell and IL is associated with the expression of METH place preference. The neural circuitry mediating place preference involves complex reciprocal projections within the mPFC, forebrain and limbic system. Within this circuit, the mPFC appears involved in the recognition of rewarding stimuli, whereas the NAcc appears implicated in directing behavioural output.

Differential activation of the NAcc shell was observed at test in METH-conditioned mice compared to saline-conditioned mice. The NAcc as a whole has been strongly implicated in the acquisition of place preference [412-414]; however, the role of the NAcc in the expression of place preference has received less attention. Nonetheless, more recent studies demonstrate pharmacological antagonism of D<sub>1</sub> receptors on NAcc neurons prevents the expression of amphetamine CPP [415], and activation of inhibitory GABAergic neurons in the NAcc prevents the expression of cocaine CPP [416]. These data suggest activity within the NAcc is required for expression of CPP. Indeed, CPP expression may be modulated by NAcc as it is an interface between neural structures responsible for behavioural output and cognition [417, 418]. In particular, it appears activation of the NAcc shell, but not the core, may be required for expression of CPP, as pharmacological antagonism of NR2B-containing NMDA receptors in the NAcc shell by ifenprodil prevents expression of morphine CPP [419]. The shell and core regions of the NAcc appear to modulate different behavioural responses; the shell appears to regulate context driven drug seeking, while the core appears to regulate discrete cue driven drug seeking [417, 420]. Enhanced Fos-IR in the NAcc shell, but not core, in the present experiments is

consistent with CPP that relies heavily on contextual learning. It is possible that following the processing of reward-associated context, firing in the NAcc shell directs behavioural output and leads to the expression of place preference.

The differential Fos-IR in the IL between METH- and saline-conditioned mice may be due to the involvement of the mPFC in the recognition of reward associated stimuli. The mPFC appears involved in cognitive processes guiding behaviour towards reward associated stimuli, rather than in detecting reward *per se* [421-423]. There appears a role for the IL in the extinction of operant drug-seeking when reward is not present [150, 239, 424, 425], and in cognitive tasks assessing reward devaluation and habitual behaviour [426-429]. However, these functions do not appear compatible with the current experiment. IL activation in experiment 2 does not appear to correlate with extinction learning, as the time spent in the METH-paired zone in METH-conditioned mice was stable throughout the test (no effect of 'test duration',  $F < 0.2$   $p > 0.9$ ; data not shown). Also, it is unlikely IL activation was observed due to expression of a habit, as the short nature of the conditioning protocol was unlikely to produce habit-like behaviour [430, 431]. It is possible IL activation may be associated with inhibition of actions which do not result in reward. Reversible inactivation of the IL in rats disinhibits unreinforced actions, suggesting IL activity inhibits responses which do not result in reward [432]. Also, increased brain activity (measured by *vivo* oxygen amperometry) is observed in rats withholding a response to non-reinforced cues [433]. Thus it is possible IL activation may orient an animal toward a reward-associated stimulus (i.e. approach towards the METH-paired compartment) by inhibiting of behaviour not associated with reward (i.e. relative lack of approach towards the saline-paired compartment).

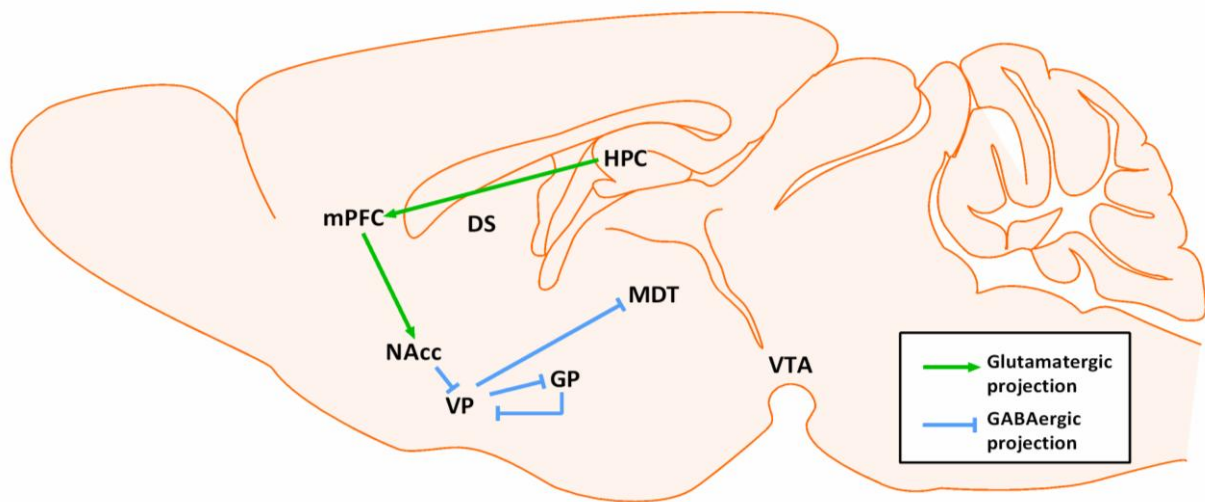
#### *5.4.5. NAcc shell and IL activation within a simplified circuit for expression of place preference*

The region- and reward-specific Fos-IR neuron counts observed in experiment 2 may be explained by the following neural circuitry (see Figure 5.8). Exposure to a drug-paired context causes retrieval of information in the HPC, and activates glutamatergic projections from the HPC to mPFC or NAcc [434, 435]. Following the retrieval of contextual information, recollection of the previous salient rewarding stimulus may have occurred in the IL, and this information was transmitted to the NAcc shell by glutamatergic projections [419, 436]. Excitatory input onto the NAcc shell may have caused activation of GABAergic MSNs [437]; dense GABAergic projections from the NAcc shell may then inhibit the VP, which is considered a major behavioural output structure [438-440]. However, considering recent studies indicate activation, not inhibition, of VP is required for expression of CPP [416, 441, 442], it is possible that expression of place preference relies on output from VP to other structures, such as the globus pallidus and medial dorsal thalamus [443]. There are inhibitory GABAergic projections from the VP to the globus pallidus and medial dorsal thalamus [444, 445]. Thus, NAcc shell mediated inhibition of VP GABAergic signalling results in a net excitatory output from the VP to other behavioural output structures, permitting expression of place preference. In addition, there are also reciprocal GABAergic projections from the VP to the NAcc which may form a feedback system to modulate behavioural output [444].

There are some aspects of this simplified model which require further exploration. The current experiment did not show differential activation of hippocampal regions or the BLA, both of which are implicated in encoding and retrieval of associations between reward and contexts or discrete cues [446-448]. It is possible retrieval of cue or contextual information – irrespective of whether it is associated with a positive or neutral stimulus – may engage a specific set of neurons; however, greater neural activation may occur when the valence of these

representations is interpreted in the other brain regions (e.g. mPFC). Indeed, other studies have found no difference in phosphorylated ERK 1/2 or Fos-IR in the amygdala or hippocampus following expression of morphine or METH CPP respectively [391, 419], suggesting that while these regions may form part of the circuit mediating expression of CPP, they may not be differentially activated following this behaviour. This explanation, however, requires further testing.

It is also intriguing that differential activation of the VTA was not observed following expression of place preference, considering that cholinergic VTA signalling appears to play a critical role in expression of cocaine place preference [449]. VTA activation is strongly implicated in the acute rewarding effects of drugs of abuse [450-453], and it appears the VTA is necessary for acquisition of place preference [449, 454]. It is possible that VTA Fos-IR was unchanged because METH can act at dopaminergic nerve terminals and cause dopamine release regardless of the activity of neurons. Further exploration of the role of the VTA in CPP expression is warranted.



**Figure 5.8. Proposed simplified circuit for the expression of METH place preference.** Abbreviations: HPC: hippocampus, mPFC: medial prefrontal cortex, DS: dorsal striatum, NAcc: nucleus accumbens, VP: ventral pallidum, GP: globus pallidus, MDT: mediodorsal thalamus, VTA: ventral tegmental area.

#### 5.4.6. Neural loci where $A_{2A}$ may act to mediate place preference

Experiment 2 was conducted to determine a possible locus whereby  $A_{2A}$  could mediate expression of place preference. Within the simplified circuit described above, it is possible  $A_{2A}$  mediates expression of METH place preference through actions in the NAcc shell.  $A_{2A}$  is densely expressed in the NAcc shell, but not the IL [300]. Furthermore, the modulation of CPP expression by the NAcc [415, 416], and the involvement of  $D_2$  striatopallidal neurons in reward inhibition – neurons which  $A_{2A}$  colocalises with [455], further strengthens the argument for  $A_{2A}$  mediating METH CPP expression through activity in this region. Experiments using viral mediated deletion of  $A_{2A}$  in chapter 6 were conducted to clarify if  $A_{2A}$  in the NAcc shell modulates expression of METH place preference.

#### *5.4.7. Conclusions*

The present Chapters' experiments followed up the absence of place preference observed in  $A_{2A}$  KO mice in Chapter 4, by seeking to determine the neural locus of effect of  $A_{2A}$  in this phenotype. In the first experiment, low baseline Fos-IR in  $A_{2A}$  KO mice - irrespective of their place preference - prevented identification of loci where  $A_{2A}$  was activated following expression of METH place preference. However, by using a different experimental design in experiment 2, I demonstrated activity in the IL and NAcc shell is associated with the expression of METH place preference. Importantly, as  $A_{2A}$  is expressed in the NAcc shell but apparently not the IL, it is possible METH place preference is modulated by  $A_{2A}$  receptors in the NAcc shell. This hypothesis was examined further in Chapter 6.



## Chapter 6

### Effect of A<sub>2A</sub> knockdown in the nucleus accumbens shell on METH CPP

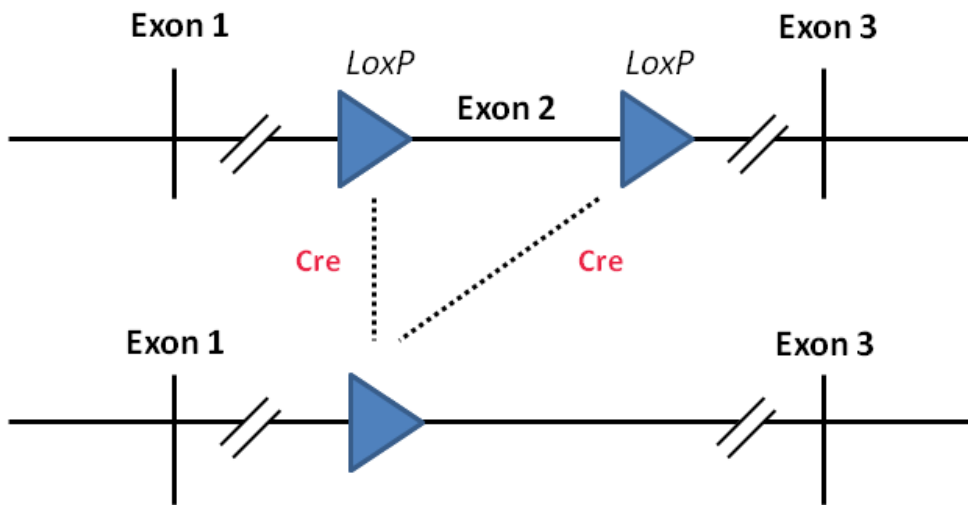
#### 6.1. Introduction

Previous Chapters demonstrated a critical role for A<sub>2A</sub> signalling in conditioned reward for METH (Chapter 4), and identified the NAcc shell as a putative neural locus (Chapter 5) for this behaviour. The current Chapter sought to determine the role of A<sub>2A</sub> signalling in the NAcc shell in conditioned reward for METH. This is an important final study, because the pattern of Fos-IR in Chapter 5 provides only correlational evidence for the NAcc shell mediating METH conditioned reward, and does not demonstrate cause / effect. Also, the region-specific pattern of activation following CPP expression does directly implicate A<sub>2A</sub> in this behaviour; rather, it demonstrates activation of a region where A<sub>2A</sub> is expressed. Thus, this final experiment is critical to demonstrate whether A<sub>2A</sub> signalling within the NAcc shell mediates conditioned reward for METH.

The experiments in this Chapter used conditional genetics to address whether A<sub>2A</sub> signalling in the NAcc shell mediates the expression of METH CPP. A genetic, as opposed to a pharmacological approach was taken for a number of reasons. Genetic approaches do not face issues of drug tolerance with repeated injections, which may lead to reduced drug efficacy over time, or lack of drug specificity, which can produce off-target effects. Furthermore, drug half-lives can limit the duration of the effect observed, which, if not observed within the correct time window, can lead to false negative data. In addition, issues of developmental compensation, which can occur using germline knockout techniques, can be overcome with conditional genetic

approach due to receptor deletion occurring in adulthood. Finally, this genetic approach ensures consistency between techniques used in Chapters 4 and 6.

Knockdown of  $A_{2A}$  in the NAcc shell was accomplished using Cre-Lox technology. This system uses a recombinant adeno-associated virus coding for cre-recombinase (AAV-Cre), which recognises the 34-bp sequence *loxP* and catalyzes recombination between pairs of *loxP* sites [456]. Thus, sequences flanked by *loxP* sites are effectively 'deleted' following viral transduction (Figure 6.1). Mice with *loxP* sites within the introns flanking exon 2 of the *ADORA2A* gene (coding for  $A_{2A}$ ) have been crossed with *Dlx5/6-Cre* or *L7ag13-Cre* transgenic mice in other laboratories to delete  $A_{2A}$  in the striatum or entire forebrain in mice [332, 333]. This method of conditional knockdown of  $A_{2A}$  has provided insight into region-specific roles of  $A_{2A}$  in mediating psychomotor activity. However, the use of viral microinjections can improve upon this technique by permitting far greater anatomic specificity. Also, the risk of developmental compensation is omitted by microinjecting AAV-Cre and knocking down  $A_{2A}$  in adulthood, after development is complete. Thus, this chapter details the microinjection of AAV-Cre into the NAcc shell, to determine the role of  $A_{2A}$  in this region on the expression of METH place preference.



**Figure 6.1. Schematic detailing cre-recombinase mediated knockdown of  $A_{2A}$  in  $A_{2A}^{loxP/loxP}$  mice.** Exon 2 of  $A_{2A}$  is flanked by *LoxP* sites, which the DNA recombinase cre recognizes. Cre catalyzes recombination between pairs of *LoxP* sites, effectively deleting any sequence in between.

## 6.2. Methods

### 6.2.1. Animals

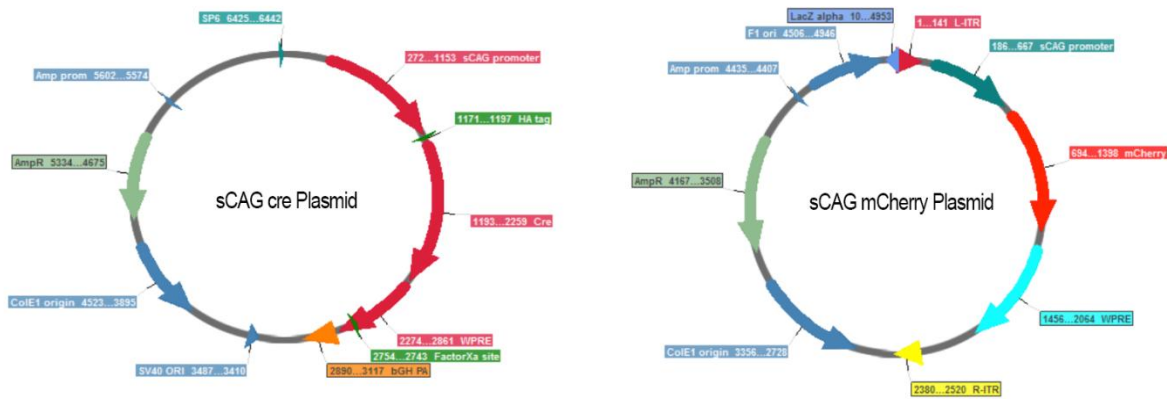
$A_{2A}^{loxP/loxP}$  and their WT littermates were kindly donated from Dr Joel Linden (University of Virginia, Charlottesville, VA, USA) via Dr Jiang-Fan Chen (Boston University School of Medicine, Boston, MA, USA). The production of these animals has been described previously [332] (see also <http://jaxmice.jax.org/strain/010687.html>). All mice were genotyped by PCR (see section 2.1) and experiments were conducted in age-matched male and female adult  $A_{2A}^{loxP/loxP}$  mice. Mice were group housed as described in section 2.2.

Twenty-six  $A_{2A}^{loxP/loxP}$  male and female littermates were used in the following experiments. Male and female mice were used in the current experiments due to low levels of breeding in this mouse line. All mice were group housed prior to stereotaxic surgery, as described in the General Methods (section 2.2). Following surgery, mice were singly housed for 7 days to facilitate

recovery. Mice were group housed again following the recovery period and remained this way until the end of experiments.

### *6.2.2. Adenoviral constructs*

Viral constructs used in this study were kindly provided by our collaborators, Professor Ross Bathgate and Dr. David Hawkes (Florey Institute of Neuroscience & Mental Health). The virus contained a plasmid with DNA coding for Cre recombinase (Cre) driven by a chicken  $\beta$ -actin promoter (Figure 6.2). The serotype was 2/1, which has been shown to preferentially transduce in neurons over astrocytes in the striatum [457]. The control virus used was a recombinant AAV 2/1 serotype virus containing a plasmid with DNA coding for mCherry driven by the same chicken  $\beta$ -actin promoter. mCherry is a red fluorescent protein (i.e. a fluorophore) with high photostability, is resistant to photobleaching and relatively low molecular weight, compared to other red fluorescent proteins [458]. The titre of the Cre virus was  $7.51 \times 10^{10}$  genomic copies per ml, while the titre of the mCherry virus was  $6.52 \times 10^{11}$  genomic copies per ml. The viral titre used for experiments was AAV-Cre:  $3.755 \times 10^7$ , mCherry:  $1.63 \times 10^8$  per infusion.

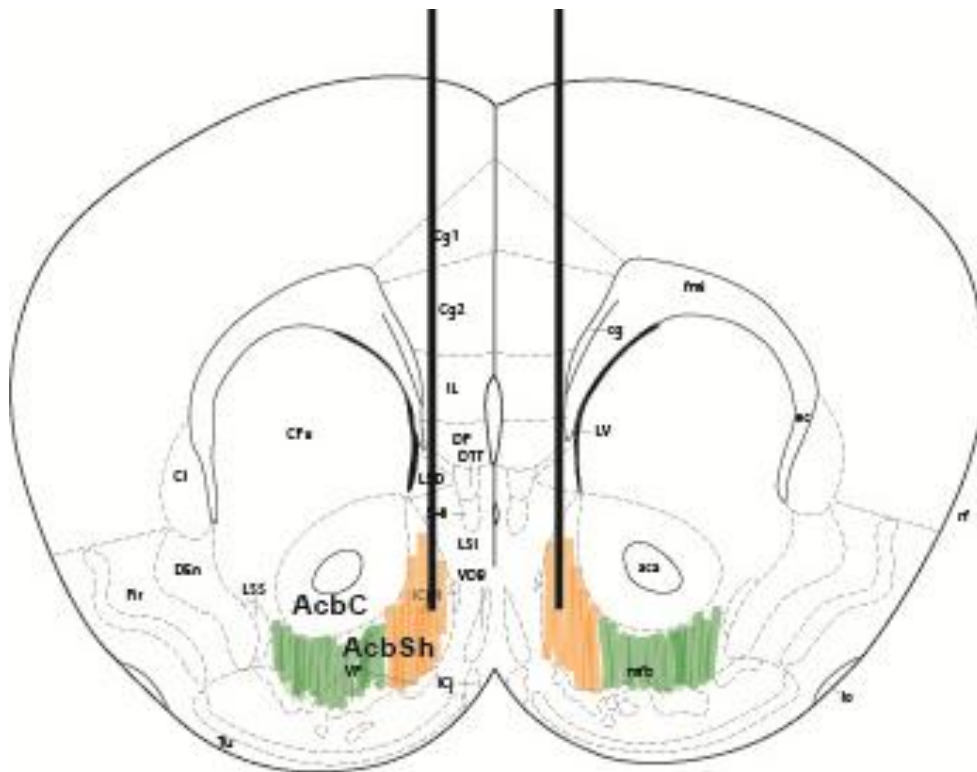


**Figure 6.2. Map of plasmids used for Cre recombinase virus and mCherry virus.**

### 6.2.3. Stereotaxic surgery and behavioural methods

AAV-Cre and mCherry were injected into the NAcc shell in  $A_{2A}^{loxP/loxP}$  mice according to section 2.4. The coordinates used were +2.4 anteroposterior from bregma,  $\pm$  0.75 mediolateral from bregma and -4.85 dorsoventral of the skull surface. These were chosen to target the rostral medial NAcc shell, to allow viral spread along the anteroposterior axis and minimise potential backtracking into the core. This strategy targeted the medial NAcc shell and minimised viral infusion into the lateral shell (see Figure 6.3).

Three weeks after stereotaxic surgery, animals were tested in METH place preference using protocol 1 (see section 2.3.1). Following the completion of behavioural experiments, animals were perfused, brains collected, frozen and sectioned before double labelling fluorescence immunohistochemistry for Cre and  $A_{2A}$ , according to General Methods, section 2.5.3.



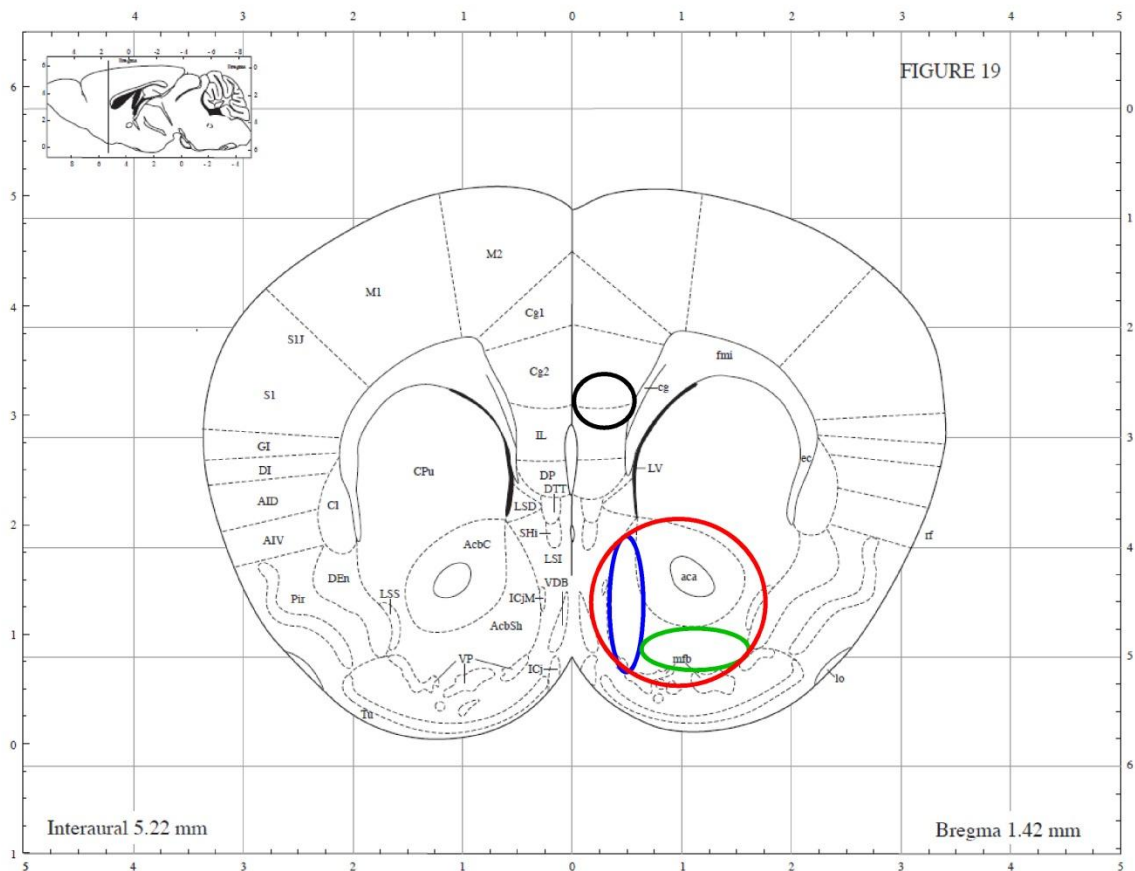
**Figure 6.3. Delineation of anticipated viral spread in the medial and lateral NAcc shell.** Expected spread of virus in the medial NAcc shell (orange), leaving the lateral NAcc shell relatively devoid of viral transfection. Parallel black lines indicate glass pipette injectors.

#### 6.2.4. Site validation

Sections from the forebrain containing the NAcc, as well as sections immediately rostral and caudal of this region, were used for verification of injection site. Site validation was conducted according to General Methods, section 2.5.4. Injection sites were validated by an experimenter blind to experimental groups, and classed as ‘hits’ (viral IR present in NAcc shell only), ‘mixed hits’ (viral IR present in NAcc shell with some minor spread into the NAcc core) and ‘unilateral hit’ (viral IR present in NAcc shell in one hemisphere only). Data from mice classified as ‘hits’ or ‘mixed hits’ were included in behavioural and optical density analyses.

### 6.2.5. *Optical density*

A<sub>2A</sub> IR following viral treatment, as well as the area encompassed by Cre-/mCherry-IR within the NAcc shell was quantified according to methods outlined in section 2.5.5. Optical density was only assessed in mice that were considered a 'hit' or 'mixed hit'. Measurements were taken throughout the rostrocaudal axis and then averaged across hemispheres and across sections to produce a single score for each measure in each animal. For each animal and each hemisphere, the optical density and area measurements were taken for the entire NAcc, as well as the medial and lateral NAcc shell. Area measurements were also taken for where AAV-Cre or mCherry were expressed ('hit areas'). In each section, background optical density was also measured; this was an area with very low or no A<sub>2A</sub> IR (e.g. mPFC or lateral septum). Figure 6.4 demonstrates how these regions were delineated. All optical density measurements were calculated by [optical density of nominated region – optical density of background in the same section]. Lower optical density values indicate a reduction in A<sub>2A</sub> IR.



**Figure 6.4. Delineation of regions within the nucleus accumbens shell and surrounding areas for optical density.** Optical density and area measurements were taken for the following regions: Entire NAcc (red), medial NAcc shell (blue), lateral NAcc shell (green). Optical density was also assessed in a region with no A<sub>2</sub>A staining (i.e. background, black).

### 6.2.6. Statistics

For behavioural studies, two-way repeated measures (RM) analysis of variance (ANOVA) with the repeated factor 'days' and between factor 'viral treatment' was conducted. Where appropriate, this was followed either by polynomial contrasts or by one-way ANOVA split by corresponding factor with a Bonferroni correction ( $p = .05 / \text{number of independent variables}$ ). Two-way ANOVA was also used to assess the size of viral IR in the medial NAcc shell, with repeated factor 'region' and between factor 'viral treatment'. One-way ANOVA with the between factor 'viral treatment' was used to assess total locomotor activity during habituation, as well as

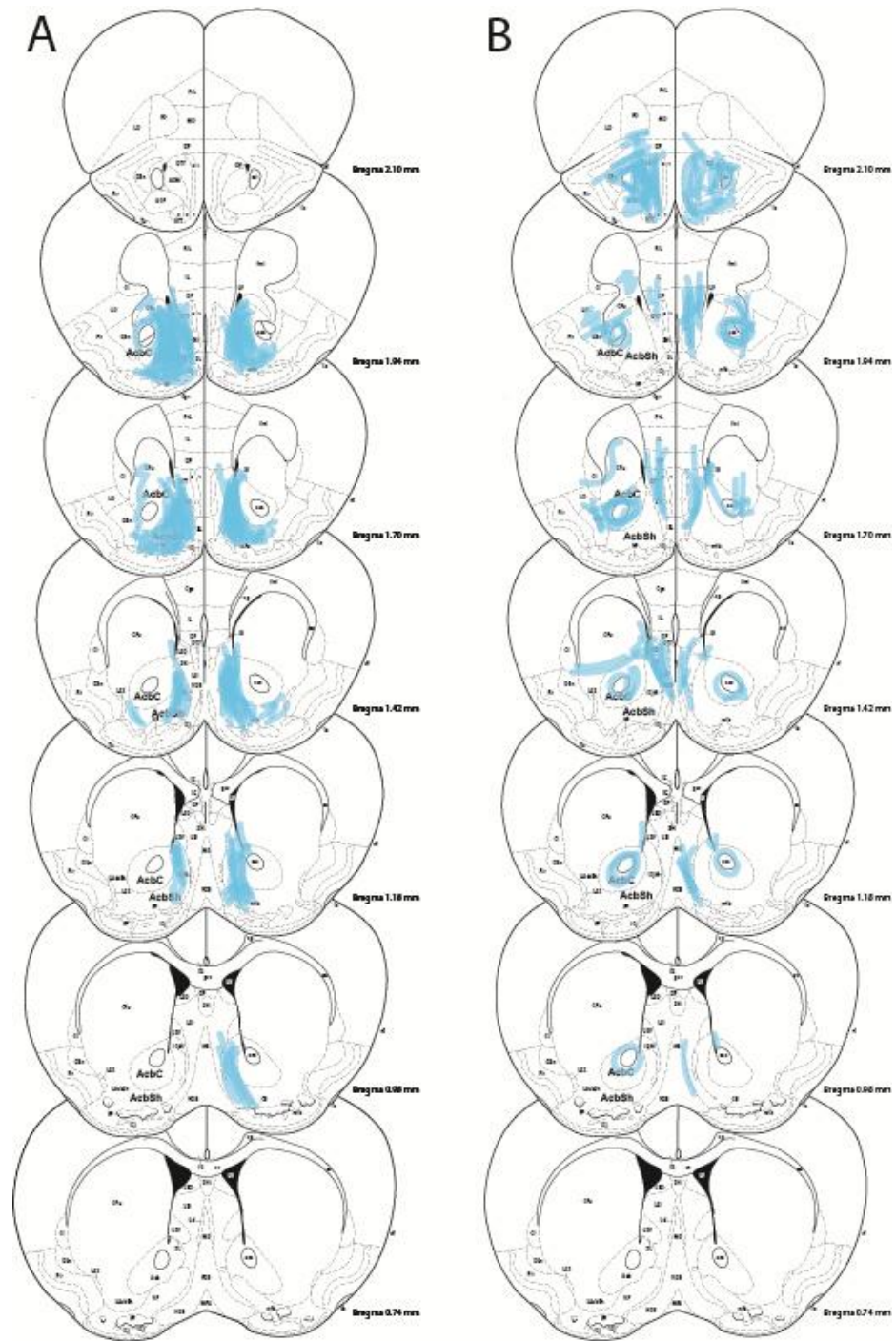


optical density and area measurements in AAV-Cre and mCherry treated mice. Correlations and simple linear regression were used to assess the relationship between the degree of viral mediated knockdown and the expression of METH CPP. Data are presented as mean  $\pm$  SEM. Data analysis was conducted using SPSS Statistics version 20 and GraphPad: Prism version 5.

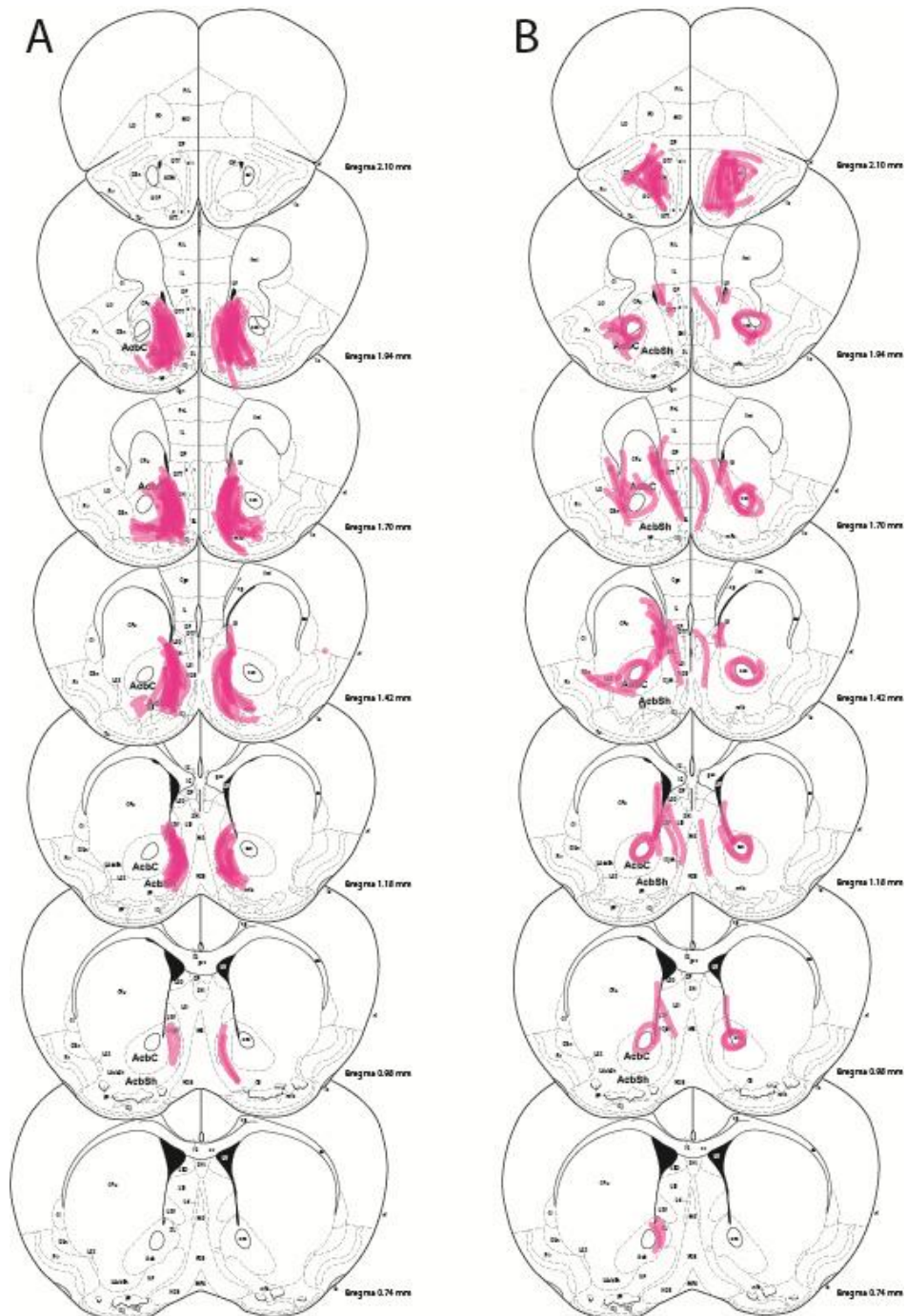
### **6.3. Results**

#### *6.3.1. Site validation*

Cre- / mCherry-IR throughout the rostrocaudal axis for AAV-Cre and mCherry treated mice is presented in Figure 6.5 and Figure 6.6. The distribution of hits, mixed hits and unilateral hits was evenly spread between the two treatment groups. In AAV-Cre treated mice, there were 4 hits, 9 mixed hits and 3 unilateral hits. In mCherry treated mice, there were 3 hits, 7 mixed hits and 2 unilateral hits. Due to the low number of hits (as opposed to mixed hits) and subsequent lack of statistical power, hits and mixed hits were collapsed into one group, giving a total of 10 mCherry and 13 AAV-Cre mice to be used in behavioural analyses. Importantly, animals with a mixed hit demonstrated Cre-/mCherry-IR predominantly in the NAcc shell, with limited spread into the NAcc core (see Figure 6.5 and Figure 6.6). Furthermore, visual examination of Cre-/mCherry-IR in Figure 6.5 and Figure 6.6 indicates the rostral medial portion of the NAcc shell was the predominant locus.



**Figure 6.5. Viral spread in  $A_{2A}^{loxP/loxP}$  mice injected with AAV-Cre.** Cre-IR in  $A_{2A}^{loxP/loxP}$  mice injected with AAV-Cre. Areas shaded indicate **A)** where Cre-IR was observed within the NAcc shell and **B)** where Cre-IR was observed outside of the NAcc shell. AAV-Cre n = 13.

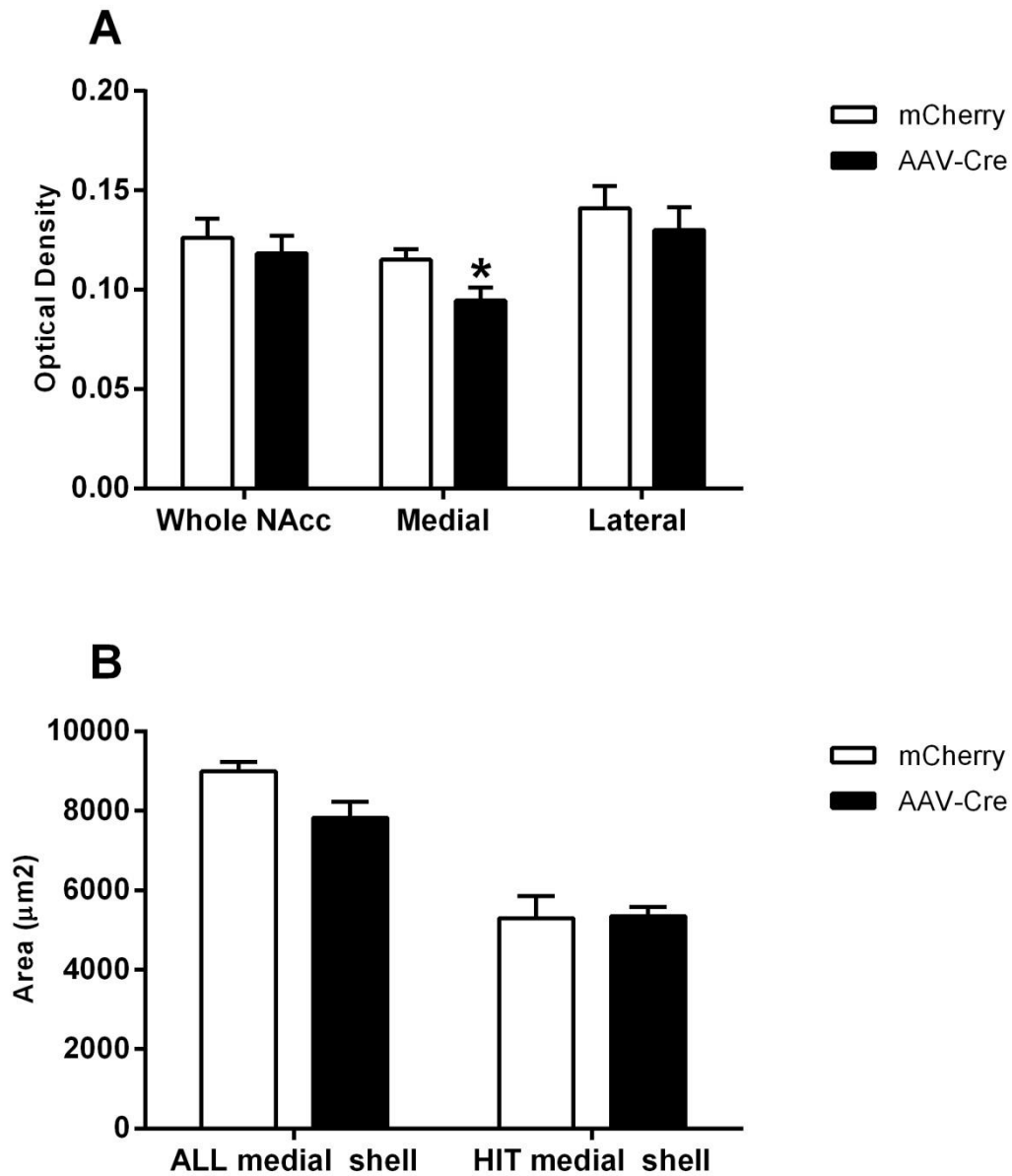


**Figure 6.6. Viral spread in  $A_{2A}^{loxP/loxP}$  mice injected with mCherry.** mCherry-IR in  $A_{2A}^{loxP/loxP}$  mice injected with mCherry. Areas shaded indicate **A)** where mCherry-IR was observed within the NAcc shell and **B)** where mCherry-IR was observed outside of the NAcc shell. mCherry n = 10.

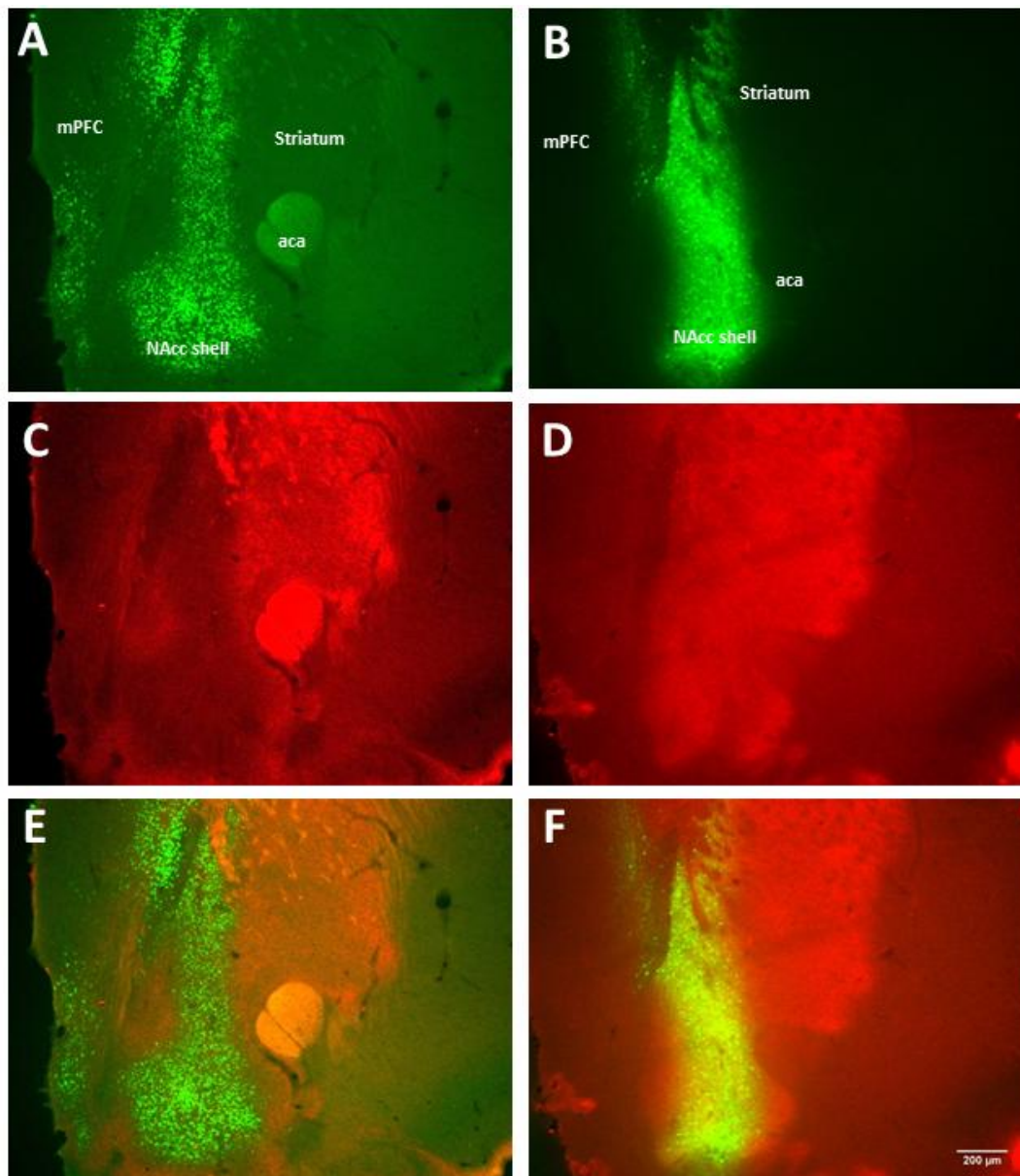
### 6.3.2. Optical density

Considering that site validation indicated the predominant locus of Cre-/mCherry-IR was the medial NAcc shell, optical density was compared between treatment groups in the medial and lateral NAcc shell, to determine if A<sub>2A</sub> IR was specifically reduced in the medial shell. Optical density was also compared between treatment groups in the NAcc as a whole to determine if the spread of AAV-Cre outside the NAcc shell (i.e. mixed hits) caused a significant reduction in A<sub>2A</sub> IR.

The optical density of A<sub>2A</sub> protein was reduced in the medial NAcc shell of AAV-Cre mice compared to AAV-mCherry treated mice [F(1,21) = 4.9, *p* = .04] but not in the lateral NAcc shell [F(1,21) = .2, *p* = .6] or the entire NAcc [F(1,21) = .5, *p* = .5] (Figure 6.7a). The average degree of knockdown in AAV-Cre mice (compared to AAV-mCherry mice) was 20 ± 6 %. Area measurements indicate the size of the whole NAcc and the lateral shell were not different between the treatment groups [NAcc mCherry: 16565 ± 750 μm<sup>2</sup>, NAcc Cre: 16178 ± 741 μm<sup>2</sup>, F(1,21) = .1, *p* = .7, lateral shell mCherry: 4590 ± 300 μm<sup>2</sup>, lateral shell Cre: 3959 ± 223 μm<sup>2</sup>, F(1,21) = 3.0, *p* = .1], but the area measurements for the medial shell were slightly smaller in AAV-Cre treated mice [mCherry: 8995 ± 232 μm<sup>2</sup>, Cre: 7825 ± 406 μm<sup>2</sup>, F(1,21) = 5.3, *p* = .03]. Importantly, the area within the medial shell where Cre-/ mCherry-IR was present was not different between treatment groups, and consisted of approximately 69 ± 7 % of the medial shell [no effect of 'viral treatment' F(1,21) = 1.6, *p* = .2, main effect of 'region' F(1,21) = 110.2, *p* < .001] (Figure 6.7b). Representative photomicrographs of Cre- and mCherry-IR, and subsequent knockdown of A<sub>2A</sub> protein is depicted in Figure 6.8.



**Figure 6.7. Optical density of  $A_{2A}$  protein and area measurements following injection of AAV-Cre or mCherry into the NAcc shell of  $A_{2A}^{loxP/loxP}$  mice. A)** Optical density of  $A_{2A}$  protein in the entire NAcc (core and shell), the medial shell (medial), and the lateral shell (lateral). Data analysed using one-way ANOVA. Significant effects of ‘viral treatment’ indicated by asterisks ( $*p < .05$ ). **B)** Area measurements outlining the size of the entire medial shell (ALL medial shell) compared to the area containing Cre- or mCherry-IR within the medial shell (HIT medial shell). ALL mCherry  $n = 12$ , ALL Cre  $n = 16$ ; HIT mCherry  $n = 10$ , HIT Cre  $n = 13$ .



**Figure 6.8. Representative images of AAV-Cre and mCherry immunoreactivity in the NAcc shell of  $A_{2A}^{loxP/loxP}$  mice.** Photomicrographs of the NAcc shell in  $A_{2A}^{loxP/loxP}$  mice treated with AAV-Cre depicting **A)** Cre-IR, **C)**  $A_{2A}$ -IR and **E)** merged. In mice treated with AAV-mCherry, photomicrographs depict **B)** mCherry-IR **D)**  $A_{2A}$ -IR and **F)** merged. Abbreviations: mPFC: medial prefrontal cortex; aca: anterior commissure, anterior; NAcc shell: nucleus accumbens shell. Scale bar = 200 $\mu$ m.

### 6.3.3. *A<sub>2A</sub> knockdown in the rostral medial NAcc shell has no effect on baseline locomotor activity or development of sensitization*

For all behavioural data, there were no main effects of 'sex' on any parameters. Thus, data from male and female mice were collapsed. Locomotor activity during habituation was similar between viral treatment groups [total distance travelled: mCherry: 12392 ± 663.4 cm, Cre: 12326 + 541.2 cm;  $F(1,21) = .1, p = .9$ ]. Sensitization was assessed using a difference score [distance travelled (cm) under METH treatment - distance travelled (cm) under saline treatment, on the same day]. Four daily 2mg/kg METH treatments produced a similar sensitization profile in both viral treatment groups [main effect of 'days'  $F(3,63) = 8.7, p < .001$ , no main effect of 'viral treatment'; Figure 6.9a]. METH-induced locomotor activity increased in a linear fashion as days progressed [significant linear 'days' contrast  $F(1,21) = 15.5, p = .001$ ]. These results suggest rostral medial NAcc shell A<sub>2A</sub> knockdown had no effect on baseline or METH-induced locomotor activity.

### 6.3.4. *A<sub>2A</sub> knockdown in the rostral medial NAcc shell has no effect on expression of place preference or conditioned hyperactivity*

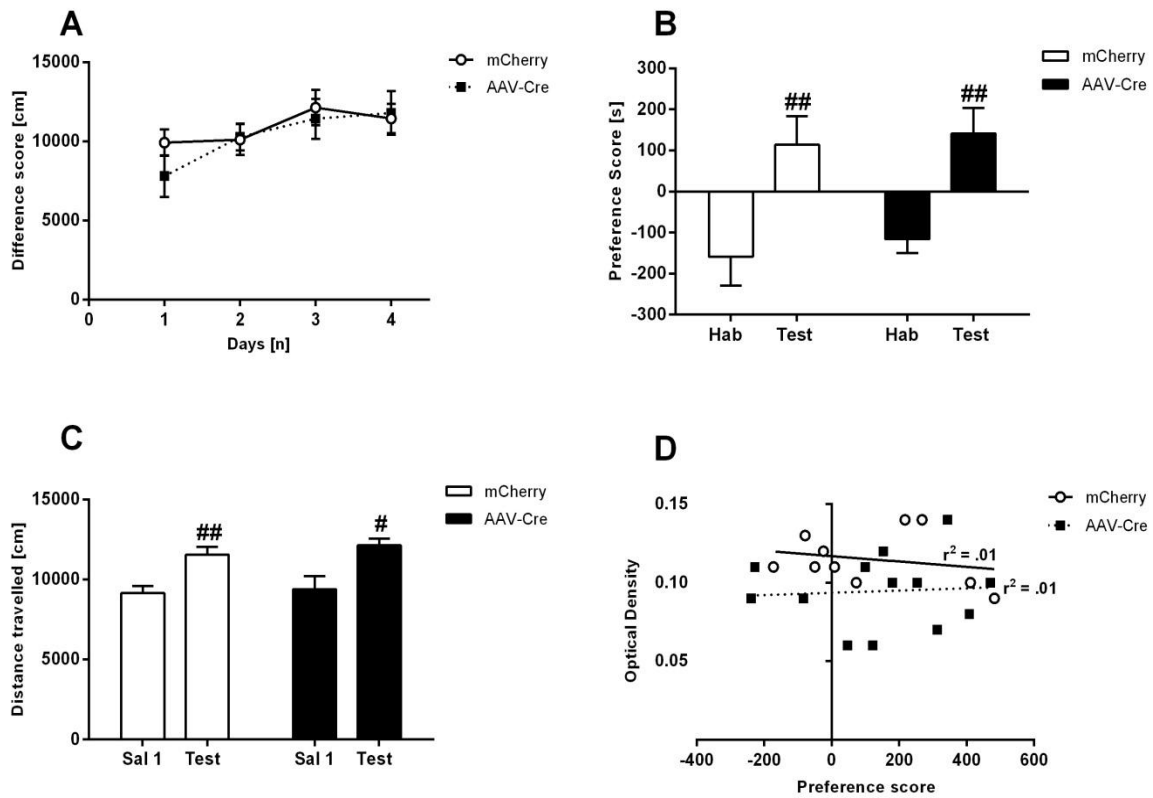
The expression of place preference was assessed using a preference score. Both AAV-mCherry and AAV-Cre treated mice expressed a positive place preference following four days of conditioning with 2mg/kg METH [main effect of 'days'  $F(1,21) = 21.5, p < .001$ , no main effect of 'viral treatment'  $F(1,21) = .3, p = .6$ ] (Figure 6.9b). The preference for the METH compartment was confirmed in each treatment group with one-way ANOVA split by 'viral treatment', which demonstrated an effect of 'days' in both mCherry and Cre expressing mice [mCherry:  $F(1,9) = 13.6, p = .005$ ; Cre:  $F(1,12) = 9.7, p = .009$ ].

Conditioned hyperactivity was also present in both viral treatment groups at test [main effect of 'time'  $F(1,21) = 16.9, p = .001$ , no main effect of 'viral treatment'  $F(1,21) = .6, p = .4$ ] (Figure 6.9c). The expression of conditioned hyperactivity was confirmed with one-way ANOVA split by 'viral treatment', indicating both treatment groups exhibited enhanced locomotor activity to the conditioned context at test compared to saline day 1 [main effect of 'time' in mCherry:  $F(1,9) = 13.6, p = .005$  and Cre:  $F(1,12) = 7.9, p = .016$ ].

#### *6.3.5. The degree of A<sub>2A</sub> knockdown in the rostral medial NAcc shell is not associated with METH CPP preference score*

Regression analysis was also conducted to examine if the degree of knockdown was associated with the expression of METH CPP. The correlations between METH CPP preference score and optical density of A<sub>2A</sub> protein in the medial NAcc shell were low in both treatment groups and were not significant (mCherry  $r = -.13, p = .7$ , Cre  $r = .1, p = .9$ ) (see Figure 6.9d). Simple linear regression demonstrated similar slopes between the two treatment groups [ $F(1,19) = .3, p = .6$ ], suggesting medial NAcc shell A<sub>2A</sub> optical density measurements predicted METH CPP preference score to a similar degree between the two treatment groups. The intercepts were significantly different between the two treatment groups, reflecting the reduction in A<sub>2A</sub> optical density in AAV-Cre treated mice [ $F(1,19) = 5.1, p = .04$ ].





**Figure 6.9. Sensitization, conditioned place preference and conditioned hyperactivity in  $A_{2A}^{loxP/loxP}$  mice treated with mCherry or AAV-Cre. A)** Locomotor sensitization over 4 consecutive days to 2mg/kg METH is not different between AAV-Cre and mCherry treated mice. Sensitization is presented as a difference score [distance travelled (cm) under METH treatment - distance travelled (cm) under saline treatment, on the same day]. **B)** Expression of METH place preference is unaltered in AAV-mCherry and AAV-Cre treated mice. Preference score is defined as (time spent in METH-paired compartment during habituation - time spent in METH-paired compartment during test). **C)** Enhanced locomotor activity at test in both viral treatment groups (compared to Sal 1) suggests expression of conditioned hyperactivity is unaltered. **D)** Correlations between  $A_{2A}$  optical density in the medial NAcc shell and preference score. The correlations between these two measures are not significant for either treatment group. Data presented as means  $\pm$  SEM. Data in **A), B), C)** analysed using two-way RM ANOVA followed by polynomial contrasts or one-way ANOVA split by 'viral treatment' with a Bonferroni correction. Significant effects of 'time' indicated by hash symbols ( $*p < .05$ ,  $##p < .01$ ). Data in **D)** analysed with linear regression. Abbreviations: Hab: habituation, Sal 1: saline treatment day 1. mCherry n = 10, AAV-Cre n = 13.

## 6.4. Discussion

The experiments in this chapter sought to determine the effect of  $A_{2A}$  knockdown in the NAcc shell on METH CPP. Viral-mediated knockdown of  $A_{2A}$  was conducted by injecting AAV-Cre into the NAcc shell of  $A_{2A}^{loxP/loxP}$  mice, and following a 3 week transduction period, mice were tested in METH CPP. Site validation demonstrated the predominant locus of viral IR was the rostral medial NAcc shell, with a small amount of Cre-/mCherry-IR present in the rostral NAcc core. The amount of knockdown was quantified using optical density, and was approximately 20%. AAV-Cre treatment had no effect on the development of sensitization, the expression of CPP or of conditioned hyperactivity. These results suggest a modest reduction of  $A_{2A}$  expression in the rostral medial NAcc shell has no effect on METH CPP and associated behaviours.

### 6.4.1. Knockdown of $A_{2A}$ was localised to the rostral medial NAcc shell

In this chapter, the predominant locus of viral IR was the rostral medial NAcc shell. The rostral portion of the medial shell was targeted to minimise spread into adjacent areas, as the rostral NAcc shell extends in both a dorsoventral and mediolateral manner. Caudal regions of the NAcc shell do not extend as widely across the mediolateral axis and may have increased the likelihood of spread into surrounding regions and compromised anatomic specificity.

Despite the targeting of this rostral locus, Cre-/mCherry-IR was observed in adjacent structures. These regions included the NAcc core, dorsal medial striatum, orbitofrontal cortices, and mPFC. While there was considerable viral IR in the orbitofrontal cortices, and some Cre-/mCherry-IR in the mPFC, the absence of  $A_{2A}$  expression in these regions [300, 459] suggests viral spread within these regions would likely have had no effect on behavioural outcomes.

The main structure outside the NAcc shell with A<sub>2A</sub> where Cre-/mCherry-IR was observed was the NAcc core. However, it is unlikely that a reduction of A<sub>2A</sub> in the NAcc core was a confounding factor within the present experiments. First, suggests that while Cre-IR was present in the NAcc core in some mice, the degree of Cre-IR in the core was much lower compared to that of the shell. Also, optical density measurements of A<sub>2A</sub> protein demonstrated that the only region with a significant reduction in A<sub>2A</sub>-IR was the medial shell. The optical density of A<sub>2A</sub> in the NAcc as a whole structure (i.e. core and shell) was not different between AAV-mCherry and AAV-Cre treated mice, suggesting that the reduction in A<sub>2A</sub> IR in the medial shell of AAV-Cre treated mice was not also evident in other structures. Thus, it appears that A<sub>2A</sub> IR was significantly reduced in AAV-Cre treated mice only in the rostral medial NAcc shell, and not in surrounding structures.

#### 6.4.2. Factors influencing the degree of knockdown in AAV-Cre treated mice

In A<sub>2A</sub><sup>loxP/loxP</sup> mice treated with AAV-Cre, the reduction in A<sub>2A</sub> IR was approximately 20% compared to AAV-mCherry controls. This is a modest reduction, as other studies demonstrate AAV-Cre mediated reduction in receptor expression can range from 50% to almost 100% knockdown using a similar volume of AAV-Cre virus (i.e. 250-1000µl [363-365, 460, 461]). Factors which could contribute to the degree of knockdown obtained include viral titre, incubation time and the volume of virus used. The viral titre used in the present experiments was lower than most reports (e.g. most viral titres are within the range of 1 x 10<sup>12</sup> or 1 x 10<sup>13</sup> per infusion [363-365, 460, 462]). However, Yu *et al.* [461] employed a viral titre of 1 x 10<sup>7</sup> genomic copies per infusion, which is similar to the titres in the present experiments (AAV-Cre: 3.755 x 10<sup>7</sup>, mCherry: 1.63 x 10<sup>8</sup> per infusion). Importantly, Yu and colleagues demonstrated significant receptor knockdown and subsequent behavioural modification using this titre [461]. This suggests that the viral titre used, while on the low end of what is used in the literature, is

comparable to that which has previously induced receptor knockdown and behavioural change in these mice.

Another factor which could influence the degree of knockdown is the incubation time of the virus (i.e. the time between viral infusion and test). There was a period of three weeks between infusion and test to permit transfection and transduction. It is unlikely that the incubation time was too short in the current experiments, as behavioural modification following a 2 week incubation period has been reported previously [363-365]. Indeed, most reports employ an incubation time of between 2-5 weeks, suggesting the present incubation time of 3 weeks fell within a validated range [363-365, 461-463].

It is possible that there was an insufficient volume of virus injected to cause recombination throughout the NAcc shell. The viral volume used in the current set of experiments (500nl bilaterally) is not dissimilar from that used in other experiments targeting the entire NAcc (e.g. 250nl bilaterally, [364, 365]). The volume used in the present experiments was chosen because validation experiments demonstrated smaller volumes increased the likelihood of missing or incompletely hitting the target structure (data not shown). Importantly, the volume of virus was chosen to specifically cause recombination in most of the medial NAcc shell. The volume used was sufficient to cause Cre- or mCherry-IR in approximately 70% of the NAcc medial shell, suggesting most of the medial shell experienced recombination, and that the volume injected was appropriate for the target structure. Furthermore, while a larger volume of virus could have been injected, this would likely result in spread of the virus into the NAcc core and reduce the anatomic specificity of the experiment.

In order to prevent viral spread into surrounding structures, the lateral NAcc shell was not targeted. Injections into this region were expected to cause viral spread into the NAcc core and striatum, through backtracking up from the injection site. Thus, the volume of virus injected was

designed to cause knockdown specifically within the medial NAcc shell, minimising viral spread into adjacent regions and permitting greater anatomic specificity, with the obvious caveat that this would result in a partial knockdown of A<sub>2A</sub> in the NAcc shell as a whole. This was considered the best compromise between degree of knockdown on the one hand vs. the confound that would have been caused by extensive spread of Cre into the NAcc core and dorsal striatum if a greater volume of virus had been injected.

#### *6.4.3. Knockdown of A<sub>2A</sub> in the rostral medial shell was insufficient to alter METH CPP*

The present experiments demonstrate that approximately 20% knockdown of A<sub>2A</sub> in the rostral medial NAcc shell is insufficient to alter METH-induced behaviours. Factors which could influence the knockdown have been discussed as being appropriate to ensure sufficient anatomic specificity. Thus, the absence of a behavioural phenotype resulting from Cre mediated knockdown of A<sub>2A</sub> in the rostral medial NAcc shell may be due to other influences, such as the degree of knockdown obtained, and / or the involvement of other structures in METH-induced behaviours.

The present experiments demonstrate a relatively low degree of A<sub>2A</sub> knockdown (20%) compared to that demonstrated in a number of reports using this technique (50-100%, [363-365, 461-463]). Importantly, these studies show that 50-100% reduction in receptor expression is sufficient to induce behavioural modification [363-365, 461-463]). While the 20% knockdown of A<sub>2A</sub> was statistically significant in this chapter, it is possible this finding may not be biologically significant. This interpretation is strengthened by the correlation analysis performed. If the greater degree of knockdown were correlated with lower CPP score, this would provide support for even a modest degree of knockdown inducing some behavioural outcome. However, as the correlation analysis demonstrated no relationship between the A<sub>2A</sub> IR

and CPP preference score, this suggests that the knockdown obtained is seemingly not associated with behavioural outcomes at least at this dose of METH (2mg/kg). Future experiments may examine lower doses of METH (e.g. 1mg/kg), as effects of A<sub>2A</sub> knockdown may be present at different doses.

Adding to this, while the reasons for targeting the rostral medial NAcc shell have been outlined above, it is possible that targeting the entire NAcc shell may have produced behavioural change. Notably, a recent study by Vincent and colleagues [460] showed that a complete reduction in glucocorticoid receptor expression throughout the entire rostrocaudal axis of the dorsal raphe nucleus could reduce anxiety- and depression-like behaviour, whereas there was no effect from a partial reduction which did not extend throughout the entirety of this structure. In the present chapter, A<sub>2A</sub> reduction extending further along the rostrocaudal and mediolateral axes of NAcc shell may have altered METH-induced behaviour. Taking these two factors together – the modest reduction in A<sub>2A</sub> IR and the limited target loci – it is not surprising that the present experiments did not replicate the phenotype of a global KO mouse, where there was complete ablation of A<sub>2A</sub> throughout the entire forebrain.

In addition, A<sub>2A</sub> activity in regions near or connected to the NAcc shell, such as the NAcc core or VP could also be mediating the expression of place preference. While there have only been a few studies, there are findings that A<sub>2A</sub> activity in NAcc core or VP can mediate drug-induced and motivated behaviour. For example, A<sub>2A</sub> agonist administration in the NAcc core reduces reinstatement, whereas NAcc core A<sub>2A</sub> antagonist administration exacerbates reinstatement [337]. Similarly, NAcc core A<sub>2A</sub> antagonist administration ameliorates D<sub>2</sub>-induced deficits in motivated behaviour for sucrose pellets [397]. Also, the VP appears to be a critical output structure for NAcc A<sub>2A</sub> mediated behaviours, as reversible disconnection of the VP from the NAcc prevents A<sub>2A</sub> agonist mediated reductions in motivated behaviour [464]. These studies

suggest  $A_{2A}$  activity in surrounding neural loci with connections to the NAcc shell could also mediate the expression of drug-induced and motivated behaviours, and potentially also METH-induced behaviour. Indeed, it is entirely possible that  $A_{2A}$  acts within multiple regions concurrently to mediate METH induced behaviour, and thus targeting only one region does not sufficiently disrupt this behaviour.

Finally, it is also likely that there are other receptor systems and neural structures involved in mediating METH place preference because this behaviour is mediated by complex neural circuitry and receptor systems (some of which were outlined in the discussion of chapter 5). Such receptor systems (e.g. dopaminergic, glutamatergic, opioid) mediate place preference through actions in the NAcc shell. Dopamine  $D_1$  and  $D_2$  receptor antagonism in the NAcc shell of rats prior to conditioning impairs the acquisition of nicotine CPP [465, 466], while 6-hydroxydopamine lesions in the NAcc shell of rats reduces acquisition and expression of amphetamine CPP [467]. A reduction in glutamatergic neurotransmission in the NAcc shell has also been implicated in the expression of CPP, as viral overexpression of GLT-1 in the NAcc shell of rats reduces methamphetamine CPP [468]. In addition, microinjection of the  $\mu$ -opioid receptor antagonist CTAP into the NAcc shell of rats reduces the expression of cocaine CPP [469]. Importantly, receptor systems which do not appear to interact with  $A_{2A}$  (e.g. opioid receptors) can also mediate the expression of psychostimulant CPP. It is possible that if there is a modest reduction in  $A_{2A}$  signalling in the NAcc shell, there are other receptor systems that could maintain the development and expression of METH CPP.

#### 6.4.4. *No effect of A<sub>2A</sub> knockdown on METH-induced locomotor behaviour*

AAV-Cre mediated knockdown of A<sub>2A</sub> had no effect on the development of sensitization or the expression of conditioned hyperactivity. This is an interesting finding, as A<sub>2A</sub> signalling in the striatum has previously been shown to modulate psychostimulant induced locomotor behaviour. Genetic deletion of striatal A<sub>2A</sub> increases cocaine-induced locomotor activity [333], while A<sub>2A</sub> agonist administration in the NAcc core decreases the expression of cocaine sensitization [340]. These studies suggest that reducing A<sub>2A</sub> signalling in the striatum enhances psychostimulant-induced locomotor activity. It is possible that the modest reduction in A<sub>2A</sub> expression in the NAcc shell may have in fact had the potential to increase METH-induced locomotor behaviour, but with the high dose of METH used (2mg/kg), ceiling effects on locomotor activity may have prevented the expression of this behaviour (see section 4.4.2 for a discussion of this). It is of interest to examine if the reduction of A<sub>2A</sub> in the rostral medial shell has any effect on locomotor activity when lower doses of METH (e.g. 1mg/kg) are used.

A<sub>2A</sub> signalling within the striatum is critical for psychostimulant-induced locomotor activity [333, 334]; however, it is of interest to determine if there is an essential striatal region mediating this behaviour. It is possible NAcc shell A<sub>2A</sub> signalling modulates METH-induced locomotor behaviour, as this region is necessary for the development of cocaine-induced locomotor sensitization [470]. Insufficient knockdown of A<sub>2A</sub> in the present experiment may have prevented there being any change to this behaviour. In addition, A<sub>2A</sub> may act within other regions of the striatum to mediate METH-induced locomotor behaviour. The NAcc core and the VP are identified as critical regions mediating psychostimulant sensitization - importantly, these regions exhibit dense A<sub>2A</sub> expression [300] - along with other regions such as the VTA and PFC [471]. It is quite possible that regions other than the NAcc shell within the limbic-motor circuit



facilitate the development of sensitization and expression of conditioned hyperactivity, leaving these behaviours intact in AAV-Cre treated mice.

#### *6.4.5. Future directions*

The data presented in this Chapter suggest a number of avenues for future research, which could address some issues raised in the current study. Future experiments could attempt to increase the degree of knockdown obtained. This may involve increasing the viral titre (which was relatively low compared to many AAV-Cre studies) and / or including a second injection site in the caudal NAcc shell (the virus volume injected at this site would need to be low due to potential spread into the NAcc core). Additionally, future experiments may examine the role of  $A_{2A}$  in multiple regions concurrently, such that knockdown of  $A_{2A}$  occurs in multiple regions in the same experiment (e.g. NAcc core and shell, or NAcc shell and VP). This approach may determine if  $A_{2A}$  activity in multiple regions, rather than one specific region, modulates METH CPP and / or psychomotor behaviour.

## Chapter 7

### General Discussion

The present thesis investigated how mGlu5 and A<sub>2A</sub> receptors modulate METH-induced behaviours, to examine whether these receptors were appropriate therapeutic targets for METH addiction. It was of particular interest to determine not only how, but also where in the brain, these receptors could modulate METH-induced behaviour. A combination of behavioural analysis, immunohistochemistry and viral-mediated receptor knockdown was used to address these aims.

#### 7.1. A summary of key findings

The involvement of mGlu5 in behaviours relevant to METH addiction was described in Chapter 3. Germline genetic deletion of mGlu5 resulted in a deficit in operant extinction learning, and an increased propensity to reinstate to drug-associated cues. These changes were independent of self-administration behaviour, which was unchanged in mGlu5 KO mice. Importantly, altered extinction and reinstatement behaviour in mGlu5 KO mice was specific to a METH reinforcer, as this phenotype was not present for a sucrose reinforcer. In addition to these findings, mGlu5 KO mice also show enhanced conditioned hyperactivity when re-exposed to a drug-associated context. These findings suggest mGlu5 may modulate the contextual salience of drug-associated cues and contexts, in line with previous studies examining cocaine-driven behaviours in this mouse line [236, 472].

Chapter 4 demonstrated an involvement of A<sub>2A</sub> in the rewarding and motivational properties of both METH and sucrose. A<sub>2A</sub> germline KO mice did not acquire a METH CPP and showed

reduced motivation to self-administer METH under high response requirements. The reduction in motivation to self-administer METH appears to extend to reinforcers in general, as sucrose self-administration under higher reinforcement schedules (FR3, PR) was also reduced in KO mice. Other aspects of drug-induced behaviour (e.g. locomotor sensitization, conditioned hyperactivity) were present but attenuated in  $A_{2A}$  KO compared to WT mice.

Experiments in Chapter 5 sought to determine the putative neural loci of a particularly prominent behavioural phenotype in  $A_{2A}$  KO mice, namely, the lack of place preference. Brains of  $A_{2A}$  WT and KO were collected following CPP testing, and processed for c-Fos immunohistochemistry. Compared to WTs,  $A_{2A}$  KO mice demonstrated reduced Fos-IR across a wide number of regions in the forebrain, in a pattern that did not reflect the preference behaviour of KO mice for a METH-paired context. As this experiment failed to indicate a potential neural region where  $A_{2A}$  could be mediating METH place preference, an additional experiment was conducted to address this question. CPP was conducted in  $A_{2A}^{lox-/lox-}$  mice using METH or saline treatment conditions, which led to a place preference in METH- conditioned mice, and no place preference in saline-conditioned mice. This behavioural difference was associated with increased Fos-IR in the IL and NAcc shell of METH conditioned mice compared to saline conditioned mice, with no observed differences in any other brain region examined. Considering the dense presence of  $A_{2A}$  in the NAcc shell, in contrast to its absence in IL [300], and the involvement of the NAcc shell in place preference [465-467, 469] it was hypothesised from these experiments that  $A_{2A}$  may mediate the expression of place preference through activity in the NAcc shell.

In Chapter 6, viral knockdown of  $A_{2A}$  in the rostral medial NAcc shell was accomplished through stereotaxic injections of AAV-Cre into  $A_{2A}^{loxP/loxP}$  mice. This technique resulted in a knockdown of approximately 20% of  $A_{2A}$  in the rostral medial NAcc shell. METH place preference learning and

testing was conducted 3 weeks after stereotaxic surgery. There were no differences between AAV-Cre treated mice and mCherry controls in the preference for a METH-paired context, the development of locomotor sensitization or the expression of conditioned hyperactivity. There was no correlation between the degree of knockdown and the preference score, suggesting AAV-Cre mediated knockdown of  $A_{2A}$  is not related to the expression of a METH place preference, at least under the conditions tested. The results from this chapter suggest that a 20% knockdown of  $A_{2A}$  in the rostral medial NAcc shell has no effect on METH place preference or METH-induced motor behaviour.

In summary, Chapters 4-6 implicate  $A_{2A}$  in reward and motivated behaviour for METH, but also in these behaviours for natural reinforcers such as sucrose. In addition, it is possible  $A_{2A}$  activity in the NAcc shell mediates reward behaviour for METH; however, experiments from this thesis were unable to confirm this. In contrast, Chapter 3 suggests mGlu5's involvement in cognitive processes associated with recognition of drug-associated stimuli and the extinction of drug-seeking behaviour.

## **7.2. Confirmation of some, but not all of the hypotheses of this thesis**

These data confirm some, but not all of the original hypotheses of this thesis. The overarching hypotheses of this thesis were

1. Germline deletion of mGlu5 will modulate aspects of drug-taking and drug-seeking behaviour for METH
2. Germline deletion of  $A_{2A}$  will modulate aspects of drug-taking and drug-seeking behaviour for METH

3. The neural locus of effect of  $A_{2A}$  on METH-induced behaviour is confined to a region within the forebrain or striatum, and conditional knockdown of  $A_{2A}$  in a target region will alter METH-induced behaviour
4. Similarities will be present in the behavioural phenotypes of mGlu5 and  $A_{2A}$  KO mice, which will permit examination of interactions between these two receptors

Some of these hypotheses were confirmed, as germline deletion of mGlu5 and  $A_{2A}$  did modulate some aspects of drug-seeking and drug-taking for METH. Indeed, the implication of mGlu5 in extinction behaviour is largely consistent with genetic and pharmacological studies suggesting mGlu5 mediates extinction of drug-seeking in different drugs of abuse [236, 244, 248, 249, 251, 271]. Similarly, the results of this thesis are consistent with previous genetic and pharmacological studies demonstrating diminished  $A_{2A}$  signalling reduces self-administration and the motivation to self-administer other drugs of abuse [266, 317, 318, 328, 330, 336]. Reinstatement or relapse after abstinence was not assessed in the  $A_{2A}$  receptor experiments in this thesis; however, it is possible this behaviour is unaltered in  $A_{2A}$  KO mice, as cue-induced morphine-seeking is unaffected in these mice [328]. Taken together, these results support there being similar neuroadaptations for different drugs of abuse within mesocorticolimbic circuitry underlying certain aspects of drug-taking, drug-seeking and addiction [97]. Importantly, points of divergence between the current findings for METH and other drugs of abuse may indicate how to appropriately target receptor systems according to drug class, which will be discussed later in this chapter.

There was insufficient evidence to confirm some of the above hypotheses. Notably, it was hypothesised that the neural locus of  $A_{2A}$  on METH-induced behaviour would be located within either the forebrain or striatum. Addressing this question was of particular relevance because a previous study has shown that  $A_{2A}$  deletion in the striatum enhances, while  $A_{2A}$  forebrain

deletion reduces psychostimulant induced locomotor activity [333]. Experiments in Chapter 5 indicated a putative location for the actions of A<sub>2A</sub> on METH-mediated behaviour; however, Chapter 6 was unable to confirm that this change in neural activity in Chapter 5 (as measured by Fos-IR) in NAcc shell was causal in the role of A<sub>2A</sub> in METH-mediated behaviours. As discussed previously, the limitations associated with the use of viral techniques in Chapter 6 do not rule out a role for A<sub>2A</sub> in the NAcc shell in METH-induced reward behaviour. Indeed, a number of studies suggest the NAcc shell is a critical region for drug reward as measured by CPP [473-476], suggesting this region is still a potential locus for mediating METH reward. It is possible that improvements to the efficacy of the viral technique used may indicate more definitively if NAcc shell A<sub>2A</sub> mediates METH reward.

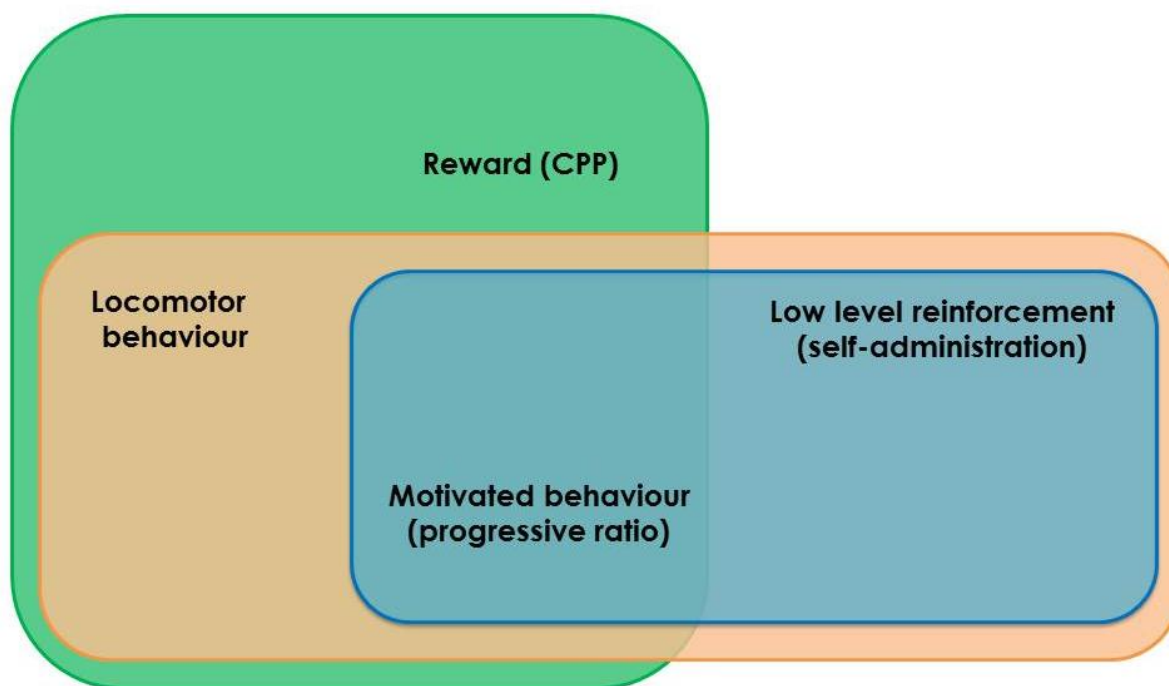
In contrast, some of the original hypotheses of this thesis were inconsistent with the present findings. A critical question of this thesis was whether mGlu5 and A<sub>2A</sub> KO mice exhibit similar behavioural phenotypes. This is because previous research demonstrated that these two receptors act synergistically to facilitate behavioural outcomes (e.g. as demonstrated in [266, 267]). I hypothesised that this synergy might be expressed through similar phenotypes in the two KO mice, providing a behavioural target through which to investigate potential interactions between mGlu5 and A<sub>2A</sub>. However, the results of Chapters 3 and 4 demonstrate that behavioural deficits in A<sub>2A</sub> KO mice were quite distinct from those in mGlu5 KO mice. Such differences in behavioural phenotypes were a surprising but important finding, because it suggests these two receptor systems are necessary for distinct outcomes in response to METH. It is important to note that this finding does not rule out potential interactions between these two receptors for METH-induced behaviour, and does not invalidate previous pharmacological research demonstrating mGlu5-A<sub>2A</sub> interactions at a membrane [259], synaptic [316, 477] and functional level [266, 267, 478]. For example, germline deletion used in the present thesis may involve compensatory mechanisms that could also occur with repeated up- or down-regulation of a

receptor system, which is not apparent with acute pharmacological studies. Importantly, germline deletion helps to illuminate which receptor systems, chronically acting in within different components of the mesocorticolimbic circuitry are necessary for addiction relevant behaviour. This distinction is critical in regards to chronic pharmacological therapy, for if a receptor is sufficient, but not necessary for a behaviour, the efficacy of the therapeutic for that receptor may be reduced because of other receptor systems which can compensate for the loss of signalling from this receptor. Importantly, the data from this thesis suggest the chronic unavailability of each receptor alters specific behavioural domains, which suggests that chronic pharmacotherapy targeting both receptor types may not be as synergistic or effective as acute pharmacotherapy.

Overall, the present findings illustrate the complex nature of how different drugs of abuse and related behaviours interact with receptors in the brain. The findings on mGlu5 have been extensively discussed in the publication that forms Chapter 3 of the present thesis. Therefore, I will focus on discussing the implications of the present results on  $A_{2A}$  in light of how METH compares to other psychostimulants in instrumental and Pavlovian associative learning memory, to understand the unique qualities of abusing and seeking METH.

### **7.3. The role of $A_{2A}$ in METH behaviour is unique compared to other psychostimulants**

The involvement of  $A_{2A}$  signalling - as assessed by genetic deletion - for the locomotor, rewarding and reinforcing effects of METH is different to other psychostimulants, and is presented compared to cocaine and MDMA in Figure 7.1.



**Figure 7.1. Schematic of behaviours altered in  $A_{2A}$  KO mice by the psychostimulants METH, cocaine and MDMA.** Green balloon indicates METH, orange balloon indicates cocaine, blue balloon indicates MDMA. Points of overlap indicate where  $A_{2A}$  deletion alters behaviour for all three drugs of abuse.

One key point of difference in the involvement of  $A_{2A}$  in these psychostimulants is the specific involvement of  $A_{2A}$  in conditioned rewarding properties of METH (as measured by CPP), but not for cocaine or MDMA. Examining the cellular mechanism driving the  $A_{2A}$ -mediated reduction in METH reward was outside the scope of this thesis; however, it is interesting to speculate on why this behaviour is not uniform across psychostimulants. It is possible that these differences are explained by NAcc dopamine release following drug administration. Accumbal dopamine release is critical for the development of CPP [479, 480]. Importantly, cocaine-induced NAcc dopamine release is present (although attenuated) in  $A_{2A}$  KO mice [481], and cocaine CPP is unaltered in these mice [267, 330]. In contrast, nicotine fails to increase NAcc dopamine release in  $A_{2A}$  KO mice, and these mice also do not exhibit nicotine CPP [372]. While NAcc dopamine release following METH administration has not been assessed in  $A_{2A}$  KO mice, it is possible



METH-induced dopamine release is attenuated, resulting in a lack of CPP for this drug. This point of divergence between different psychostimulants may demonstrate how to employ  $A_{2A}$  modulators to target specific behaviours in abusers of different drugs, which will be discussed in more detail below.

Motivation to self-administer as measured by progressive ratio is reduced in  $A_{2A}$  KO mice across all psychostimulants tested. As discussed in Chapter 4, neuroadaptations in  $A_{2A}$  KO mice may account for the reduction in motivated behaviour across various reinforcers. On the other hand, drug-induced locomotor activity is altered only for METH and cocaine, which may be explained by the different pharmacokinetic / dynamic properties of each drug. Both METH and cocaine bind with high affinity to DAT, preventing dopamine reuptake and enhancing dopamine release in the striatum [482]. In contrast, MDMA primarily binds to SERT and greatly increases extracellular serotonin in the striatum, and causes a comparatively lower degree of dopamine release [483-485]. Considering that striatal dopamine release induced by psychostimulants can cause locomotor stimulation [486, 487], and  $A_{2A}$  modulates dopamine transmission [296], it is possible that  $A_{2A}$  modulates psychostimulant-induced locomotion under conditions of relatively high extracellular dopamine.

#### **7.4. Interactions between $A_{2A}$ , stress and METH associated memory**

One of the most interesting and unexpected observations from the present thesis is the finding from Chapter 5 that suggests stress may impact on  $A_{2A}$  modulation of METH-associated memory. To our knowledge, this is the first observation of  $A_{2A}$  interacting with stress to mediate the retrieval of a drug-related memory, although such an interaction has previously been shown in the acquisition and retention of memory in cognitive tasks that do not involve drugs of abuse [402, 403]. It is also intriguing to observe that stress drastically changed the behaviour of an

apparently homogenous cohort of KO mice, which demonstrates the importance of individual differences even within inbred mouse strains [389]. Importantly, the findings from Chapter 5 tie in with a recent human imaging study, where  $A_{2A}$  antagonist administration enhanced brain activity in cocaine-dependent patients during a difficult – and possibly more stressful - working memory task, but not during easier memory tasks [488]. The regions activated in this study were associated with working memory and inhibitory control (e.g. lateral orbitofrontal cortex, left insula, left superior and middle temporal pole) [488], and suggests my preclinical demonstration of  $A_{2A}$  modulating cognition under stress may also apply to a human drug abuse setting. The apparent interaction between stress and  $A_{2A}$  signalling observed in Chapter 5 may also have implications for the therapeutic targeting of this receptor. Stress can precipitate reinstatement of METH-seeking [489, 490]. If reducing  $A_{2A}$  signalling during stress can help retrieve a drug-context memory, then enhancing  $A_{2A}$  signalling during stress may help prevent this retrieval, and reduce the likelihood of relapse-like behaviour.

### **7.5. Therapeutic utility of pharmacologically targeting mGlu5 and $A_{2A}$ for METH addiction**

I sought to determine whether mGlu5 and  $A_{2A}$  were appropriate pharmacological targets for METH addiction, and if they interacted to assist the development of more precise, effective addiction therapeutics. While I was unable to confirm mGlu5- $A_{2A}$  interactions, or a neural locus of effect of each receptor systems, it is nonetheless important to discuss whether the data presented suggest either mGlu5 or  $A_{2A}$  are suitable therapeutic targets for METH addiction.

The results from Chapter 3 suggest a role for mGlu5 signalling in the extinction of METH self-administration, with no effect on the extinction of sucrose self-administration. These findings are consistent with a number of other research reports that show a reduction in mGlu5

signalling interferes with extinction [236, 244, 248, 249, 271, 491]. Therefore, drugs that enhance mGlu5 signalling during behavioural therapy may assist in the extinction of drug-associated behaviours and contexts. However, modulation of mGlu5 signalling may need to be adjusted to suit the patient's circumstances, as reducing mGlu5 signalling can prevent relapse-like behaviour in preclinical models [232, 255-257, 269, 270, 272, 280, 284, 285]. Thus, it would be beneficial to increase mGlu5 signalling during extinction but decrease mGlu5 signalling when a patient was likely to encounter drug-associated cues and contexts that could initiate relapse.

In addition, considering the relatively widespread distribution of mGlu5 throughout the forebrain [206, 207] and the anatomic specificity of mGlu5 signalling in different addiction-relevant behaviours (e.g. [255, 492]), it would be of considerable interest to develop mGlu5 modulators which can target specific neural structures (using methods such as e.g. using RNA interference or targeting mGlu5 / A<sub>2A</sub> heteromers; for a discussion, see [493]). Targeting specific populations of mGlu5-expressing cells may reduce unwanted side effects. Also, as mGlu5 has been shown preclinically to alter performance in cognitive tasks ([494-497] for a review, see [245]), it is important to consider that therapeutics targeting mGlu5 may affect cognitive performance in humans. Thus, therapeutics targeting mGlu5 may be most efficacious when targeted to a specific stage of addiction (e.g. extinction / relapse) and brain region.

A<sub>2A</sub> mediates the rewarding properties of METH, and may also interact with stress to modulate memory for METH-associated contexts, providing a slightly unconventional therapeutic avenue for METH addiction. Current theories of drug addiction suggest the rewarding and hedonic aspects of drug-taking are generally confined to earlier stages of addiction, and do not drive critical aspects of addiction such as relapse, loss of behavioural control and aberrant cue reactivity [97, 203]. However, this generalisation may not be appropriate for METH, as Newton and colleagues [498] demonstrated METH users continue to use the drug or relapse after

abstinence primarily due to the reinforcing effects of the drug, rather than other factors such as habits, impulsivity, craving and pain avoidance. This suggests that at least a subset of METH users continue to take the drug for the reinforcing effects experienced. If a reduction in the rewarding nature of the drug can be achieved through  $A_{2A}$  antagonist administration, this may assist in reducing drug use. In addition, as discussed earlier, enhancing  $A_{2A}$  signalling may also help prevent the retrieval of drug-context associations, and thereby help prevent relapse. Thus, as with mGlu5, the use of  $A_{2A}$  modulators to mediate METH addiction may need to suit specific behaviour targets at different points during the addiction cycle.

## **7.6. Limitations and future directions**

There are a number of limitations of the present thesis that warrant further investigation. The involvement of  $A_{2A}$  in relapse and reinstatement was unexplored in the present experiments. The response of  $A_{2A}$  KO mice to various types of reinstatement for METH needs to be investigated, as pharmacological studies implicate  $A_{2A}$  signalling in cue-induced and drug-primed cocaine reinstatement [325, 337, 338]. As discussed earlier, the distinct behavioural profile of  $A_{2A}$  KO mice to different psychostimulants suggests the generalisation of findings between drug classes may not always be accurate, supporting the need to assess different types of reinstatement (e.g. cue-induced, drug- and stress-primed) in  $A_{2A}$  KO mice for METH. Also, considering the interaction between stress and  $A_{2A}$ , and the absence of pharmacological or genetic studies investigating the role of  $A_{2A}$  in stress-induced reinstatement, it is of great interest to determine the role of  $A_{2A}$  in this behaviour.

The behavioural characterisation of  $A_{2A}$  KO mice has also raised questions about the involvement of developmental neuroadaptations in METH-induced behaviour. It is important to confirm whether the phenotype observed is due to a reduction in  $A_{2A}$  signalling, or the

neuroadaptations induced by germline deletion of  $A_{2A}$ , to determine the involvement of  $A_{2A}$  signalling in motivated behaviour. Manipulating basal extracellular dopamine levels in  $A_{2A}$  KO mice to WT levels will help address this issue (e.g. via chronic treatment with L-DOPA via osmotic minipumps). Furthermore, improving on the degree of viral mediated knockdown of  $A_{2A}$ , and potentially deleting  $A_{2A}$  within multiple regions in the striatopallidal circuit may help address if, and where,  $A_{2A}$  acts to mediate METH reward.

The mechanism/s by which mGlu5 modulates the extinction of operant METH seeking is another avenue of potential research. This is a pressing question due to the relatively widespread distribution of mGlu5 throughout the brain, particularly in the mPFC, striatum, amygdala and hippocampus [206, 207]. It is possible mGlu5 mediates extinction through a circuit projecting from the IL to the NAcc shell. A previous study has shown the mGlu5 PAM CDBBP administered into the IL facilitates the extinction of alcohol seeking, with a concurrent increase in MSN spine density and changes to the spine class morphology [251]. This suggests that mGlu5-mediated changes in extinction learning correspond with structural and functional plasticity in IL. In addition, cocaine lever extinction reduces mGlu5 expression in the NAcc shell [250], and repeated cocaine exposure reduces mGlu5-mediated LTD in the NAcc shell [162]. Thus, it is possible that mGlu5 mediated plasticity in the IL-NAcc circuit may modulate extinction. While addressing this hypothesis was outside the scope of this thesis, the brain regions and mechanisms driving mGlu5 mediated extinction of drug-seeking is a critical area of future research.

Finally, it is possible to use other methods to explore potential interactions between mGlu5 and  $A_{2A}$ . Other genetic methods that could be used to explore potential interactions between these receptors include breeding double KO mice of mGlu5 and  $A_{2A}$  (for example, [314]) or combining pharmacology with a germline KO approach (for example, [267]). Also, if future genetic studies

suggest no interaction between mGlu5 and A<sub>2A</sub>, it would be important to determine why these differences exist between pharmacological and genetic studies, and how this could impact on the therapeutic potential of pharmacologically targeting mGlu5 and A<sub>2A</sub>.

## **7.7. Conclusions**

The present thesis demonstrates distinct roles for the mGlu5 and A<sub>2A</sub> receptors in modulating addiction-relevant behaviours for METH. Germline receptor deletion demonstrates these receptors are necessary for specific behavioural domains, namely, that mGlu5 modulates operant extinction learning for METH, while A<sub>2A</sub> modulates the rewarding and motivational properties of the drug. It is possible A<sub>2A</sub> modulates METH reward through activity in the NAcc shell, but the present experiments were unable to confirm this locus. These experiments suggest that targeting both mGlu5 and A<sub>2A</sub> may have therapeutic utility in specific behaviours or phases of addiction. Determining where and how these receptors act to mediate METH-induced behaviour is important in enhancing their therapeutic utility.

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