# A critical role of striatal $A_{2A}$R-mGlu$_5$R interactions in modulating the psychomotor and drug-seeking effects of methamphetamine

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**Running title:** $A_{2A}$R-mGlu$_5$R interaction in psychostimulant addiction

**Keywords:** $A_{2A}$ Receptor, cocaine, conditioned-place preference, methamphetamine, mGlu$_5$ receptor, stereotypy

## ABSTRACT

Addiction to psychostimulants is a major public health problem with no available treatment. Adenosine $A_{2A}$ receptors ($A_{2A}R$) co-localize with metabotropic glutamate 5 receptors ($mGlu_5R$) in the striatum and functionally interact to modulate behaviors induced by addictive substances, such as alcohol. Using genetic and pharmacological ***antagonism*** of $A_{2A}R$ in mice, we investigated whether $A_{2A}R$-$mGlu_5R$ interaction can regulate the locomotor, stereotypic and drug-seeking effect of methamphetamine and cocaine, two drugs which exhibit distinct mechanism of action. Genetic deletion of $A_{2A}R$, as well as combined administration of sub-threshold doses of the selective $A_{2A}R$ antagonist (SCH 58261, 0.01 mg/kg, i.p.) with the $mGlu_5R$ antagonist, 3-((2-methyl-4-thiazolyl)ethynyl)pyridine (MTEP; 0.01 mg/kg, i.p.), prevented methamphetamine- but not cocaine-induced hyperactivity and stereotypic rearing behavior. This drug combination also prevented methamphetamine rewarding effects in a conditioned-place preference paradigm. Moreover, $mGlu_5R$ binding was reduced in the nucleus accumbens core of $A_{2A}R$ knockout (KO) mice supporting an interaction between these receptors in a brain region crucial in mediating addiction processes. Chronic methamphetamine, but not cocaine administration, resulted in a significant increase in striatal $mGlu_5R$ binding in wild-type mice, which was absent in the $A_{2A}R$ KO mice. These data are in support of a critical role of striatal $A_{2A}R$-$mGlu_5R$ functional interaction in mediating the ambulatory, stereotypic and reinforcing effects of methamphetamine but not cocaine-induced hyperlocomotion or stereotypy. The present study highlights a distinct and selective mechanistic role for this receptor interaction in regulating methamphetamine induced behaviors and suggests that combined antagonism of $A_{2A}R$ and $mGlu_5R$ may represent a novel therapy for methamphetamine addiction.

## INTRODUCTION

Cocaine and methamphetamine (MAP) are highly addictive and commonly abused psychostimulant substances and their use is a public health concern. There are currently no specific therapeutic agents with established efficacy for the treatment of either MAP or cocaine addiction. There is substantial evidence supporting a key role of the adenosine $A_{2A}$ receptors ($A_{2A}R$) in regulating the behavioral properties of drugs of abuse (Brown and Short, 2008). However, its role in modulating the behavioral and neurochemical effects of psychostimulant substances remains largely unclear, mainly due to the discrepancies between outcomes from studies using pharmacological manipulation of $A_{2A}R$ in rodents compared with studies using the $A_{2A}R$ knockout (KO) mouse model. For instance, pharmacological activation of $A_{2A}R$ attenuates cocaine self-administration (Knapp et al., 2001) and decreases both cocaine and MAP locomotor sensitization (Filip et al., 2006; Shimazoe et al., 2000). In contrast, global deletion of the $A_{2A}R$ gene has been shown to attenuate psychostimulant-induced hyperlocomotion and motor sensitization (Chen et al., 2000). ***Evidence supports both facilitatory as well as antagonistic roles of striatal postsynaptic and presynaptic $A_{2A}R$, respectively, in modulating psychostimulant-mediated responses (Shen et al., 2008).***

Expression of $A_{2A}R$ in the brain ***is highly enriched in the striatum*** (Rosin et al., 1998), where pre- and post-synaptic receptors are known to play differential role in the modulation of behavioral responses to psychostimulants (Golembiowska and Zylewska, 1997; Quarta et al., 2004; Rodrigues et al., 2005; Shen et al., 2008). $A_{2A}R$ post-synaptically co-localize with $D_2R$ in striatopallidal GABAergic neurons (Canals et al., 2003; Fink et al., 1992; Hillion et al., 2002) and functional antagonistic $A_{2A}R$-$D_2R$ interactions have been demonstrated to negatively modulate the locomotor and rewarding effects of psychostimulant drugs (Ferre et al., 1997; Filip

et al., 2006; Poleszak and Malec, 2002). $A_{2A}R$ are also located pre-synaptically on cortical glutamatergic afferents projecting to the striatum (Ciruela et al., 2006; Popoli et al., 1995; Schiffmann et al., 2007). In contrast to the antagonistic $A_{2A}R$-$D_2R$ interaction, activation of pre-synaptic $A_{2A}R$ positively modulates the behavioral responses of psychostimulants by facilitating accumbal DA and glutamate release (Golembiowska and Zylewska, 1997; Quarta et al., 2004; Rodrigues et al., 2005). Studies carried out in forebrain-specific and striatal-specific $A_{2A}R$ KO mice indicated a dominant role of the facilitatory effect of pre-synaptic extra-striatal $A_{2A}R$ over the antagonistic effects of post-synaptic striatal $A_{2A}R$ on modulating the behavioral effects of cocaine and phencyclidine (Shen et al., 2008).

There is increasing evidence suggesting that $A_{2A}R$ are also co-localized and functionally interact with the $mGlu_5R$ (Ferre et al., 2002). In particular, $mGlu_5R$ are expressed post-synaptically in striatopallidal GABAergic neurons, where they are co-expressed and functionally interact with $A_{2A}R$ to synergistically overcome $D_2R$-mediated effects, both at the behavioral and molecular level (Coccurello et al., 2004; Ferre et al., 2002; Kachroo et al., 2005; Popoli et al., 2001). Moreover, supra-additive effects of co-administration of sub-threshold doses of $mGlu_5R$ and $A_{2A}R$ antagonists have been reported in the facilitation of glutamate release to the striatum, suggesting presynaptic $A_{2A}R$-$Glu_5R$ interactions to also regulate glutamatergic striatal neurotransmission (Rodrigues et al., 2005). These receptor interactions have been shown to modulate motor deficits in an animal model of Parkinson's disease (Kachroo et al., 2005; Popoli et al., 2001) and to also regulate alcohol-seeking (Adams et al., 2008) and cocaine conditioning, behavior but not cocaine-induced hyperactivity (Brown et al., 2012). However, the role of $A_{2A}R$-$mGlu_5R$ interactions on addictive-related behavioral effects of MAP is currently unknown.

The aim of the present study was to characterize the role of $A_{2A}R$ and its interaction with $mGlu_5R$ on the behavioral and neurochemical effects associated with MAP and cocaine use. Therefore, we firstly investigated the effect of genetic and pharmacological ***antagonism*** of $A_{2A}R$ on the motor-activating effects of chronic MAP and cocaine. To assess the role of $A_{2A}R$-$mGlu_5R$ interactions in regulating the motor and rewarding effects of MAP, we tested the effect of the combination of subthreshold doses of $A_{2A}R$ and $mGlu_5R$ antagonists on MAP-induced hyperactivity, stereotypic rearing and conditioned-place preference behavior of mice. The effect of $A_{2A}R$ and $mGlu_5R$ antagonists on MAP-induced hyperactivity was compared to the respective behavioral effects of cocaine. We further biochemically explored the presence of $A_{2A}R$-$mGlu_5R$ interactions by assessing the effect of $A_{2A}R$ deletion on striatal $mGlu_5R$ binding in treatment-naïve mice and following chronic MAP or cocaine administration in wild type (WT) and $A_{2A}R$ KO mice. Finally, given the evidence supporting a possible functional $D_2R$-$A_{2A}R$-$mGlu_5R$ trimeric receptor interaction in the striatum ***(Cabello et al., 2009)***, we also investigated the effect of $A_{2A}R$ deletion on striatal $D_2R$ binding, in treatment-naïve mice and following chronic MAP and cocaine administration.

# MATERIALS AND METHODS

## Animals

The methodology for the generation of $A_{2A}R$ KO mice (CD-1 background) used in the current study has been previously described (Ledent et al., 1997). Male, 8–12 week-old, WT and $A_{2A}R$ KO, were housed individually in a controlled environment (12:12 hour light/dark cycle - lights on 06:00). Food and water were available *ad libitum*. All procedures received a favorable opinion from the University of Surrey Animal Welfare and Ethical Review Body and were approved by the UK Home Office under The Animals (Scientific Procedures) Act 1986. For genotyping, tail DNA was extracted using the DNeasy tissue kit, according to the manufacturer's instructions (QIAGEN, Germany). Genotyping was performed as previously described (Ledent et al., 1997).

## Confirmation of genotype

To confirm the genotype of the animals used in our studies, brain sections from all experimental WT and $A_{2A}R$ KO mice were processed for $A_{2A}R$ binding, using 10 nM [$^{3}$H]CGS21680 (PerkinElmer, USA) according to Bailey et al. (2002). $A_{2A}R$ were only detected in striatal regions of WT mice and absent in $A_{2A}R$ KO mice (Supplementary Figure S1).

## Chronic psychostimulant administration paradigms

A group of WT and $A_{2A}R$ KO mice (n = 7-11/group) were treated with a chronic steady-dose 'binge' cocaine administration paradigm as described by Metaxas et al. (2012), consisting of 3 injections per day (at 11:00, 12:00 and 13:00) of either cocaine (15 mg/kg/injection, s.c., Sigma-Aldrich, UK) or saline (10 ml/kg/day, s.c.), for 14 days. Another cohort of WT and $A_{2A}R$ KO mice (n= 6/group) were administered with chronic steady-dose of MAP (1 mg/kg/day, i.p.;

Sigma-Aldrich, Dorset, UK) or saline (10 ml/kg/day, i.p.) for 10 days, according to Zanos et al. (2014b).

**Ambulatory and stereotypic rearing activity**

Ambulatory activity was measured in locomotor chambers (40 cm length x 20 cm width x 20 cm height; Linton Instrumentation, Norfolk, UK). Each cage has two sets of 16 photocells, 2.5 cm apart, located 1 cm or 6 cm (referred to here as "vertical beams") above the cage floor. Ambulatory time was defined by the total active time, and vertical activity (i.e., rearing) was defined by vertical beam-breaks, recorded every 5 min. Each daily session began with placing the mice in the locomotor chambers for 1 hour prior to drug administration, for assessing basal activity. Subsequently, locomotor activity was measured for either 60 min following each of the 3 daily cocaine/saline injections (total time: 3 hours) or for 3 hours following MAP/saline injection. ***Since there were no differences between the three daily cocaine injections in either WT or $A_{2A}$R knockout mice (data not shown), total three-hour daily aggregated locomotor responses of cocaine-treated animals are reported.***

**Effects of pharmacological co-antagonism of $A_{2A}$R and $mGlu_5$R on cocaine- and methamphetamine-induced ambulatory and stereotypic rearing behavior**

To investigate the effects of pharmacological co-antagonism of $A_{2A}$R and $mGlu_5$R on cocaine- and MAP-induced ambulatory and rearing behavior, sub-threshold doses of $A_{2A}$R antagonist, SCH 58261 (Tocris Biosciences, UK) and $mGlu_5$R antagonist, 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP; Tocris Biosciences, UK) were determined. WT, 9 week-old mice were injected i.p. with either vehicle (20% DMSO-saline), MTEP (0.5, 0.25, 0.1 or 0.01 mg/kg) or SCH 58261 (1, 0.25, 0.1 or 0.01 mg/kg) following 1 hour habituation in locomotor chambers. They were then immediately placed back into the locomotor chambers for a further 30

minutes and ambulatory as well as rearing activity were measured. Subsequently mice received an injection of either MAP (1 mg/kg; i.p.) or saline (10 ml/kg; i.p.) and placed back into the chambers. Ambulatory time and rearing activity were recorded following MAP or saline administration for a period of 3 hours. The dose of 0.01 mg/kg, i.p. was identified as sub-threshold for both SCH 58261 and MTEP from the aforementioned dose-response experiments, as this dose did not alter MAP-induced ambulatory time or vertical activity of mice (Supplementary Figure S3; Also see Results section). Both SCH 58261 and MTEP were dissolved in DMSO and diluted to the required concentration using saline solution with a final concentration of 20% DMSO, in accordance with Kuzmin et al. (1999). ***To determine the effects of sub-threshold co-antagonism of $A_{2A}R$ and $mGlu_5R$ on psychostimulant-induced motor effects, another cohort of WT mice were pre-treated with either vehicle (20% DMSO-saline), SCH 58261 (0.01 mg/kg; i.p.), MTEP (0.01 mg/kg; i.p.), or a combination of both ligands (SCH 58261, 0.01 mg/kg and MTEP 0.01 mg/kg; i.p) 30 min prior to MAP (1 mg/kg, i.p./ 1 injection) or cocaine (15 mg/kg, i.p./3 injections; 1 hour apart) or saline (10ml/kg, i.p) administration, and ambulatory time and vertical activity were recorded for a total of 3 hours as described above.***

**Effects of pharmacological co-antagonism of $A_{2A}R$ and $mGlu_5R$ on methamphetamine-induced conditioned place preference**

Male, 9-week old, WT mice were used for the conditioned place preference (CPP) studies. We used a CPP apparatus (Opto-Max Activity Meter v2.16, Columbus Instruments, OH, USA), as previously described by Zanos et al. (2014a) and Bailey et al. (2010). Briefly, the MAP-induced CPP protocol consisted of a habituation phase, a pre-conditioning test, six conditioning sessions and a post-conditioning test (Figure 3A). On day 1 (i.e., habituation) and day 2 (i.e., pre-conditioning), WT mice were placed in the CPP apparatus and were allowed to explore both

compartments for 20 min. During the conditioning phase, mice were administered with MAP 1 mg/kg, i.p. and placed in their least preferred compartment on alternating days (i.e., days 3, 5 and 7) and saline (10 ml/kg, i.p.) in their preferred compartment on days 4, 6 and 8 (Figure 3A) for 45 minutes. During the post-conditioning test session (i.e., day 9), mice were pre-treated with an i.p. injection of vehicle (20% DMSO-saline), selective $A_{2A}$ receptor antagonist SCH 58261 (0.01 mg/kg), selective and non-competitive $mGlu_5R$ antagonist MTEP (0.01 mg/kg), or a combination of both antagonists (SCH 58261, 0.01 mg/kg and MTEP 0.01 mg/kg). Following a 30-min period in their home cages, mice were then placed in the CPP apparatus and were allowed to explore both compartments for 20 min. Time spent in each CPP compartment was measured during the last 15 min of both pre- and post-conditioning sessions.

To ascertain that the combined antagonism of $A_{2A}R$ and $mGlu_5R$ did not have any rewarding or aversive effects on its own following repeated administration, the effect of i.p. co-administration of SCH 58261 (0.01 mg/kg) and MTEP (0.01 mg/kg) was compared to vehicle (20% DMSO-saline) in a CPP paradigm, consisting of one habituation (20 min), one pre-conditioning (20 min), three 45-min conditioning (each consisting of daily vehicle injection in the morning and SCH 5826/ MTEP administration 4 hours later) and one post-conditioning phase (20 min). Time spent in each compartment was assessed during the last 15 min of the 20-min pre- and post-conditioning sessions.

**Effects of $A_{2A}R$ deletion on $mGlu_5R$, $D_2R$ and DAT binding in treatment-naïve animals or following chronic cocaine and MAP administration**

Since cocaine, but not MAP, acts primarily via blocking dopamine transporters (DAT) to exert its acute rewarding effects, DAT binding was also assessed in brains of naïve WT and $A_{2A}R$ KO mice to assess baseline differences. Quantitative autoradiography of $mGlu_5R$, $D_2R$ and DAT procedures were performed as detailed previously (Bailey et al., 2008; Bailey et al., 2007;

Georgiou et al., 2014), with minor modifications. Briefly, adjacent frozen coronal brain sections from naïve as well as chronically cocaine- or MAP-treated WT and $A_{2A}R$ KO mice and their respective saline controls were obtained. For the determination of total $mGlu_5R$, $D_2R$ and DAT binding, 10nM [$^3$H]2-methyl-6-(phenylethynyl)-pyridine ([$^3$H]MPEP; American Radiolabelled Chemicals, Missouri, USA), 4 nM [$^3$H]raclopride (PerkinElmer, USA) and 4nM [$^3$H]mazindol (PerkinElmer, USA) were used respectively. Non-specific binding was determined in the presence of 10 μM fenobam (Tocris Biosciences, UK) for $mGlu_5R$ binding, 10 μM sulpiride (Tocris Biosciences, UK) for $D_2R$ binding and 10 μM mazindol (Sigma-Aldrich, Poole, UK) for DAT binding. ***For the DAT binding, desipramine 0.3μM was also used in order to block the binding of [$^3$H]mazindol to norepinephrine uptake sites (Metaxas et al., 2010).*** Following a 60-min incubation period (on ice for [$^3$H]MPEP binding and room temperature for [$^3$H]raclopride and [$^3$H]mazindol), slides were rinsed in ice-cold Tris-HCl buffer, dried and apposed to Kodak MR films (Amersham International, UK) with [$^3$H]microscale standards, for a period of 3 weeks for $mGlu_5R$ binding, 6 weeks for $D_2R$ receptor binding and 5 weeks for DAT binding. Films were then developed using 50% Kodak D19 developer (Sigma-Aldrich, Poole, UK). Quantitative autoradiographic analysis was carried out by reference to the mouse brain atlas of Franklin and Paxinos, (2007) and binding was analyzed as previously described (Kitchen et al., 1997), using MCID image analyser (Image Research, Ontario, Canada).

## Statistics

All data are expressed as mean ± SEM. Comparison of chronic cocaine and MAP-induced ambulatory time and stereotypic behaviors was carried out using three-way repeated measures ANOVA for factors 'genotype', 'treatment' (i.e. saline or cocaine/MAP) and 'day'. For the dose-response experiments, one-way ANOVA was used to compare the effects of $A_{2A}R$ and $mGlu_5R$

antagonist pre-treatment with the vehicle control group. Analysis of the effects of pharmacological ***antagonism*** of $A_{2A}R$ or $mGlu_5R$ or the combination of these antagonists on cocaine- and MAP-induced locomotor and rearing responses was conducted using two-way ANOVA for factors ‘treatment’ (i.e., saline or MAP) and ‘ligand pre-treatment’ (vehicle, SCH 58261, MTEP, SCH 58261 + MTEP). Analysis of the effects of pharmacological ***antagonism*** of $A_{2A}R$ and/or $mGlu_5R$ on MAP-induced CPP was assessed by using two-way ANOVA for factors ‘treatment’ (i.e., vehicle, SCH 58261, MTEP, SCH 58261 + MTEP) and ‘CPP phase’ (i.e., pre-Cond, post-Cond). In treatment-naïve animals, differences in quantitative autoradiographic binding were assessed using two-way ANOVA for factors ‘genotype’ and ‘brain region’. The effects of $A_{2A}R$ genetic deletion on $D_2R$ and $mGlu_5R$ binding following chronic cocaine and MAP administration were assessed using two-way ANOVA for factors ‘genotype’ and ‘treatment’ (i.e. saline or cocaine/MAP) in each brain region. ANOVAs were followed by a *post-hoc* Tukey test when significance was reached (i.e., $p<0.05$). All relevant F-values and p values are provided in Table 1. All statistical analyses were performed using Statistica *V10* (StatSoft Inc., USA).

## RESULTS

**Basal ambulatory time and vertical locomotor activity of WT and $A_{2A}$R KO mice**

To investigate whether there are any baseline locomotor alterations induced by the genetic deletion of $A_{2A}$R, basal (i.e., treatment-naïve) locomotor activity of WT and $A_{2A}$R KO mice, defined as the total ambulatory time or vertical beam-breaks for 1 hour prior to any treatment injection on Day 1 of the chronic cocaine and MAP administration paradigms. There was no difference in either the ambulatory time or vertical activity between WT and $A_{2A}$R KO mice (Supplementary Figure S2).

**Genetic deletion of $A_{2A}$R modulates methamphetamine- but not cocaine-induced ambulatory and stereotypic rearing activity**

Chronic treatment with either cocaine or MAP increased ambulatory time of WT as well as $A_{2A}$R KO mice compared to the control saline group (Figure 1A, C; Table 1). While no significant genotype effect on ambulatory time was observed following cocaine treatment (Figure 1A), MAP-induced increase of ambulatory time was significantly attenuated in the $A_{2A}$R KO compared to WT mice (genotype effect: $F_{(1, 20)} = 7.78$, $p<0.05$; Figure 1C; Table 1)

Cocaine administration caused a significant increase in vertical (stereotypic) activity (Figure 1B; Table 1); no significant effect of genotype on cocaine-induced vertical activity was observed. In contrast, MAP-induced increase of vertical activity was abolished in the $A_{2A}$R KO (Figure 1D; Table 1). Vertical activity in MAP-treated $A_{2A}$R KO mice did not differ from that of saline-treated $A_{2A}$R KO mice.

**$A_{2A}$R-mGlu$_5$R receptor interaction modulates methamphetamine-induced hyperactivity and stereotypic rearing behavior**

In order to assess the role of $A_{2A}R$-$mGlu_5R$ interaction in the modulation of MAP-induced hyperactivity and stereotypic rearing behavior, WT mice were treated with a combination of sub-threshold doses of the $A_{2A}R$ antagonist, SCH 58261 and the $mGlu_5R$ antagonist, MTEP identified in a pilot study. A dose-response study was carried out with administration of SCH 58261 or MTEP 30 min prior to MAP treatment. MAP-induced ambulatory time was significantly decreased following pre-treatment with SCH 58261 at the dose of 1 mg/kg, (Supplementary Figure S3A; Table 1), and by pre-treatment with MTEP at the doses of 0.5 mg/kg and 0.25 mg/kg (Supplementary Figure S3B; Table 1). Similarly, MAP-induced vertical (rearing) behavior was significantly reduced following pre-treatment with either SCH 58261 at doses of 1 mg/kg, 0.25 mg/kg and 0.1 mg/kg (Supplementary Figure S3C; Table 1) or MTEP at doses of 0.5 mg/kg, 0.25 mg/kg and 0.1 mg/kg (Supplementary Figure S3D; Table 1). In contrast, pre-treatment with SCH 58261 or MTEP at a dose of 0.01 mg/kg did not alter MAP-induced hyperlocomotion (Supplementary Figure S3), and was thereby chosen as the sub-threshold dose. Neither SCH 58261, nor MTEP or their combined administration, at a dose of 0.01 mg/kg, has altered basal ambulatory time or stereotypic rearing activity (Supplementary Figure S4).

To investigate the role of the $A_{2A}R$-$mGlu_5R$ in the locomotor and stereotypic rearing behaviors induced by cocaine and MAP, sub-threshold doses of MTEP (0.01 mg/kg) and SCH 58261 (0.01 mg/kg) were injected separately or in combination 30 min prior to saline, MAP (1 mg/kg, i.p.) or cocaine (15 mg/kg, i.p.) injection. Administration of cocaine or MAP significantly increased both ambulatory time (Figure 2A, C; Table 1) and vertical activity (Figure 2B, D; Table 1). SCH 58261 (0.01 mg/kg) or MTEP (0.01 mg/kg) pre-treatment, when administered alone, did not alter cocaine- or MAP-induced ambulatory time or vertical activity (Figure 2). While co-administration of SCH 58261 (0.01 mg/kg, i.p.) and MTEP (0.01 mg/kg, i.p.) did not affect

ambulatory time or vertical activity induced by cocaine (Figure 2A, B; Table 1), it significantly reduced by 30% and 47% MAP-induced stimulation of these respective behaviors (Figure 2C, D; Table 1). Pre-treatment with either MTEP (0.01 mg/kg) or SCH 58261 (0.01 mg/kg) alone, or in combination, did not alter the ambulatory time or vertical locomotor response in saline-treated animals (Figure 2).

**$A_{2A}$R-mGlu$_5$R interaction mediates methamphetamine-induced conditioned place preference**

We further investigated the role of $A_{2A}$R-mGlu$_5$R receptor interaction in the reinforcing properties of MAP by using a CPP paradigm (Figure 3A). Sub-threshold doses of SCH 58261 and MTEP were administered separately or in combination 30 min prior to post-conditioning test session of a MAP-induced CPP protocol. WT mice pre-treated with vehicle, SCH (0.01 mg/kg, i.p.) and MTEP (0.01 mg/kg, i.p.) alone exhibited a MAP-induced CPP, as illustrated by an increase in the time spent in the drug-paired compartment during the post-conditioning phase compared to the pre-conditioning phase (Figure 3B). In contrast, pre-treatment with a combination of SCH 58261 (0.01 mg/kg, i.p.) and MTEP (0.01 mg/kg, i.p.) prevented the acquisition of MAP-induced CPP (Figure 3B; Table 1).

A possible mechanism by which the combined antagonism of $A_{2A}$R and mGlu$_5$R may modulate the reinforcing effects of MAP in the CPP paradigm is by inducing reward. Thus, we assessed the rewarding or aversive properties of the combination of MTEP and SCH 58261 ligands using the CPP paradigm. Co-administration of SCH 58261 (0.01 mg/kg, i.p.) and MTEP (0.01 mg/kg, i.p.) did not induce any conditioned place preference or aversion compared to the vehicle control group (Figure 3C; Table 1).

**Chronic cocaine administration does not alter $mGlu_5R$, or $D_2R$ binding in wild-type or $A_{2A}R$ KO mice**

Chronic cocaine treatment did not induce any changes in the $mGlu_5R$ (Figure 4 A, B) binding or dopamine $D_2R$ (Supplementary Figure S5A,B) in WT or $A_{2A}R$ KO mice in any of the analyzed brain regions (Supplementary table 1). No genotype or 'genotype' x 'treatment' interaction was observed (Table 1).

**Genetic deletion of $A_{2A}R$ prevented methamphetamine-induced striatal $mGlu_5R$ up-regulation**

Chronic MAP administration caused a significant increase in $mGlu_5R$ binding in the nucleus accumbens core (AcbC) and shell (AcbSh) of WT mice compared to saline controls (Figure 4C, D; Table 1). In the $A_{2A}R$ KO mice, this effect of chronic MAP treatment was not present (Figure 4B, D). Chronic MAP administration did not induce any significant alterations in any other brain regions analyzed (Supplementary Table 2).

**Chronic methamphetamine treatment did not alter $D_2R$ in wild-type or $A_{2A}R$ KO mice**

Chronic MAP treatment did not induce any changes in the dopamine $D_2R$ binding (Supplementary Figure S5C, D) in WT or $A_{2A}R$ KO mice, and no genotype effect was observed (Table 1).

**Decreased $mGlu_5R$ binding in the striatum of treatment-naïve $A_{2A}R$ KO mice**

We investigated the effects of global $A_{2A}R$ deletion on $D_2R$, DAT and $mGlu_5R$ in the brain (Figure 5). Quantitative autoradiographic binding of the striatal $D_2R$ and DAT showed no significant genotype effect or 'genotype' x 'brain region' interaction (Figure 5, Table 1). However, compared to WT mice, $mGlu_5R$ binding was significantly lower in the nucleus

accumbens core of $A_{2A}R$ KO mice, but not in the other brain regions analyzed (Figure 5C, F; Supplementary Table 3).

## DISCUSSION

This is the first study to demonstrate the critical role of $A_{2A}R$ via its striatal interaction with $mGlu_5R$ in mediating the ambulatory, stereotypic rearing behavior and rewarding properties of MAP. Interestingly, and in contrast with MAP, neither $A_{2A}R$ nor the $A_{2A}R$-$mGlu_5R$ interaction modulates the motor enhancing properties of cocaine in mice. These findings demonstrate a differential role of the $A_{2A}R$ and the $A_{2A}R$-$mGlu_5R$ interactions in modulating the effects of two mechanistically distinct psychostimulants. In addition, we showed that $A_{2A}R$ deletion does not only alter striatal $mGlu_5R$ binding under physiological conditions, but also prevents the MAP-induced upregulation of striatal $mGlu_5R$ binding, thus further supporting the evidence for a functional striatal $A_{2A}R$-$mGlu_5R$ interaction.

We show that both pharmacological ***antagonism*** and genetic deletion of $A_{2A}R$ attenuate MAP-induced stimulation of ambulatory activity. In addition, the present study is the first to demonstrate complete abolition of MAP-induced stereotypic rearing behavior in $A_{2A}R$ KO mice, supporting a main facilitatory role for $A_{2A}R$ in mediating the motor enhancing properties of MAP. Activation of the pre-synaptic $A_{2A}R$ localized on cortical glutamatergic afferents has been shown to positively modulate the behavioral responses of psychostimulants by facilitating glutamate release in the striatum ***(Golembiowska and Zylewska, 1997; Quarta et al., 2004; Shen et al., 2008).*** Since enhanced striatal glutamatergic release has been associated with the manifestation of stereotypic rearing behavior (Presti et al., 2004), it is likely that the positive modulatory effect of $A_{2A}R$ on glutamate release in the striatum might be at least partly responsible for the stereotypic-inducing properties of MAP. Interestingly MAP-induced

dependence/psychosis has been shown to be associated with a polymorphism of the $A_{2A}R$, *ADORA2A* gene (Kobayashi et al., 2010) supporting a role for $A_{2A}R$ in the psychotic effect of the drug. Given that selective increase of psychostimulant-induced repetitive rearing behavior in rodents has been previously associated with psychotic consequences of drugs of abuse (Reeves et al., 2003), the abolition of MAP-induced stereotypic rearing activity in $A_{2A}R$ knockout mice may suggest a key role for $A_{2A}Rs$ in facilitating MAP-induced psychotic effects. ***"While in the present study we show complete abolition of MAP-induced repetitive rearing behavior in mice lacking the $A_{2A}R$ gene, as well as following combined antagonism of $A_{2A}R$ and $mGlu_5R$, alterations in other stereotypic behaviors including circling, sniffing, grooming and head-weaving need to be further investigated"***

To further investigate whether the effects of $A_{2A}R$ ***antagonism*** involves its interaction with $mGlu_5R$, we investigated the effect of co-administration of sub-threshold doses of $A_{2A}R$ and $mGlu_5R$ antagonists on the ambulatory and stereotypic rearing behaviors induced by MAP. In line with the findings from the genetic deletion of $A_{2A}R$, we also showed that sub-threshold co-antagonism of $A_{2A}R$ and $mGlu_5R$ reduced ambulatory time and completely prevented MAP-induced rearing activity. Overall, these data clearly suggest a functional $A_{2A}R$-$mGlu_5R$ interaction in the positive modulation of the motor enhancing properties of MAP. These findings are in agreement with studies showing synergistic interactions between $A_{2A}R$ and $mGlu_5R$ in the drug-seeking effect of alcohol and cocaine (Adams et al., 2008; Brown et al., 2012), as well as, as well as in the manifestation of motor responses associated with Parkinson's disease (Ferre et al., 2002; Kachroo et al., 2005). Specifically, combination of sub-threshold doses of $A_{2A}R$ and $mGlu_5R$ antagonists improved motor deficits in bilaterally 6-hydroxy-dopamine-lesioned rats (Coccurello *et al*, 2004) as well as in DA-depleted mice (Kachroo *et al,* 2005), and prevented the conditioned cue-induced reinstatement of alcohol-seeking (Adams et al., 2008).

The synergistic interaction of $A_{2A}R$ with $mGlu_5R$ observed in the present study is consistent with evidence for the existence of heterodimeric $A_{2A}R$-$mGlu_5R$ complexes within the striatum (Ferre et al., 2002). The presence of a functional $A_{2A}R$-$mGlu_5R$ interaction is further supported by the significant reduction of $mGlu_5R$ binding in the ventral striatum of $A_{2A}R$ KO mice compared to WT. While it is possible that this down-regulation of $mGlu_5R$ binding reflects compensatory neuroadaptations due to the deletion of $A_{2A}R$, these observations may also have resulted from the inability of $mGlu_5R$ to interact with $A_{2A}R$ in the KO mice. These results are consistent with Brown et al. (2012), who demonstrated a decrease in $A_{2A}R$ binding in the striatum of mice treated with the $mGlu_5R$ antagonist MTEP. Moreover, the involvement of functional interactions between striatal $A_{2A}R$ and $mGlu_5R$ in the actions of MAP is supported by our findings showing complete abolition of chronic MAP-induced up-regulation of $mGlu_5R$ binding in the nucleus accumbens of $A_{2A}R$ KO mice. In line with cocaine (Ghasemzadeh et al., 1999) and morphine (Narita et al., 2005), chronic MAP administration induced an up-regulation of $mGlu_5R$. This is however, the first study to show that $A_{2A}R$ positively modulate this effect. The abolition of MAP-induced $mGlu_5R$ up-regulation along with the concomitant attenuation of MAP-associated hyperactivity and stereotypic rearing in $A_{2A}R$ KO mice, indicate a potential role of the $mGlu_5R$ up-regulation in the hyperactivity associated with MAP use.

Interestingly, the results from the present study demonstrate that $A_{2A}R$ is not involved in mediating the ambulatory or stereotypic rearing effects of cocaine, since genetic deletion of $A_{2A}R$ did not alter cocaine-induced enhancement on ambulatory time or vertical activity. The differential role of $A_{2A}R$ receptors in regulating MAP- and cocaine-associated ambulatory and stereotypic rearing effects may lie on their distinct mechanism of action. ***While MAP administration enhances striatal DA release primarily through facilitating vesicular DA release into the synaptic cleft by reversing DAT transporter action, cocaine increases***

***extracellular striatal DA levels by blocking DAT, thereby preventing DA reuptake.*** Given the dominant role of pre-synaptic striatal $A_{2A}R$ in positively modulating the behavioral responses of psychostimulants by facilitating DA and glutamate release (Golembiowska and Zylewska, 1997; ***Mark et al., 2004;*** Quarta et al., 2004) *versus* the antagonistic effect of post-synaptic $A_{2A}R$, the differential role of $A_{2A}R$ receptors in regulating MAP- and cocaine-induced hyperactivity is therefore perhaps not surprising. As a result, the lack of effect of $A_{2A}R$ or combined ***antagonism*** of $A_{2A}R$ and $mGlu_5R$ on motor activating properties of cocaine is likely to reflect a lack of pre-synaptic $A_{2A}R$ involvement in cocaine's mechanism of action. ***Moreover, it has been recently shown that MAP mechanism of action involves a DA-independent mechanism, by direct modulation of hippocampal glutamatergic synaptic transmission (Zhang et al., 2014), further supporting a differential regulation of these psychostimulants.*** Given that DAT is the prime target for cocaine, the lack of effect of $A_{2A}R$ on DAT binding might further explain the lack of $A_{2A}R$ involvement in the motor enhancing effects of cocaine. ***However, Short et al., (2006) found decreased DAT binding in the caudate putamen of $A_{2A}R$ KO mice. These discrepancies might reflect differences in the genetic background of the animals used. While Short et al., (2006) used $A_{2A}R$ KO mice on a CD-1 backcrossed with C57BL/6J for four generations, in the present study $A_{2A}R$ KO mice were bred exclusively on a CD-1 backound. In fact, phenotypic differences have been identified between $A_{2A}R$ KO mice bred on a CD-1 and C57BL/6J background (Castane et al., 2006; Chen et al., 2000; Shen et al., 2008).***

Unlike MAP, chronic cocaine administration did not alter $mGlu_5R$ binding. Additionally, $A_{2A}R$ deletion did not affect $mGlu_5R$ binding in chronically cocaine-treated mice suggesting a lack of $A_{2A}R$-$mGlu_5R$ in the locomotor-enhancing effects of cocaine. This is in agreement with the absence of any effect of combined administration of sub-threshold doses of $A_{2A}R$ and $mGlu_5R$ antagonists on cocaine-induced ambulatory and stereotypic rearing activity observed in our

study. Nonetheless, this does not necessarily preclude an involvement of this receptor interaction on other behavioral effects of cocaine. Indeed, although Brown *et al,* (2012) did not detect an $A_{2A}R$-$mGlu_5R$ interaction in regulating the acute enhanced locomotor responses of cocaine, they provided evidence for the involvement of such an interaction in mediating the reinforcing effects of cocaine.

In order to further assess the role of $A_{2A}R$-$mGlu_5R$ interaction in the reinforcing properties of MAP, we investigated the effects of co-administration of sub-threshold doses of $A_{2A}R$-$mGlu_5R$ antagonists in MAP-induced CPP. This is the first study, to our knowledge, to show complete abolition of the expression of MAP-induced place preference by sub-threshold co-antagonism of $A_{2A}R$ and $mGlu_5R$, supporting the key modulatory role of the functional $A_{2A}R$-$mGlu_5R$ interaction in the reinforcing properties of MAP. ***In line with our findings, Chesworth et al., (2015) have recently shown that*** $A_{2A}R$ ***KO mice do not develop CPP to MAP, further supporting a key role for the*** $A_{2A}R$ ***in mediating the reinforcing properties of MAP.*** Interestingly, genetic deletion of $mGlu_5R$ did not affect the development of MAP-induced CPP in mice (Chesworth et al., 2013), whereas activation of $A_{2A}R$ reduced the development of MAP-induced CPP (Kavanagh et al., 2015), suggesting a distinct function of $A_{2A}R$-$mGlu_5R$ interaction *vs* $A_{2A}R$ and $mGlu_5R$ on their own on the development of MAP-induced CPP. Since combined administration of $A_{2A}R$-$mGlu_5R$ antagonists was also able to prevent MAP-induced locomotor and stereotypic responses, it is hence plausible that these MAP-related behaviors are regulated by common neural circuits likely localized in the striatum, a brain area underlying Pavlovian conditioning responses (Robbins et al., 2008). Specifically, the mesolimbic DAergic system, projecting from the ventral tegmental area to the Acb, has been implicated in both the locomotor and the reinforcing properties of psychostimulant drugs of abuse (Koob, 1992). Similarly, psychostimulant-induced stereotypic behavior has been demonstrated to involve the DAergic

system in the striatum and more specifically the DA $D_2R$ (Amalric and Koob, 1993; Berke and Hyman, 2000). Interestingly, in our study, $A_{2A}R$ gene deletion did not affect striatal $D_2R$ binding either in treatment naïve mice, or in chronically MAP- or cocaine-treated mice suggesting a lack of involvement of $A_{2A}R$-$D_2R$ interactions in the actions of psychostimulants, at least at the receptor binding level. Although there is evidence suggesting that post-synaptic $A_{2A}R$-$D_2R$ interactions can negatively modulate the behavioral effects of psychostimulants (Ferre et al., 1997; Filip et al., 2006; Poleszak and Malec, 2002), it has been suggested that the actions of presynaptic $A_{2A}R$ receptors dominate over the postsynaptic $A_{2A}R$ receptors (Shen et al., 2008), which may explain why $A_{2A}R$ receptors do not modulate $D_2R$ binding following chronic psychostimulant treatment. ***However, multiple allosteric interactions have been described for $A_{2A}R$-$D_2R$ heteromer, which can differentially modulate G protein-dependent and independent signalling (Navarro et al., 2014).***

In summary, our findings support the existence of functional striatal interactions between $A_{2A}R$ and $mGlu_5R$ in modulating MAP- but not cocaine-induced locomotor and stereotypic rearing responses. We also demonstrated a key role of this interaction in positively modulating MAP-seeking behavior. These pre-clinical data highlight the potential of therapeutic agents which simultaneously target $A_{2A}R$ and $mGlu_5R$ for the treatment of MAP addiction. The fact that this combination of sub-threshold doses of $A_{2A}R$ and $mGlu_5R$ antagonists is neither sedating (Supplementary Figure S4), nor rewarding or aversive (Figure 3C) and at the same time it effectively prevents the motor enhancing and reinforcing effects of MAP, makes its potential progress towards clinical development for the treatment of MAP addiction especially appealing.

## Acknowledgments

The authors thank Nicole Pochanis and Aya Osman for their contribution in brain sectioning. Special thanks to Athanasios Metaxas for his involvement in the technical part of the DAT binding.

## Funding and Disclosures

This study was supported by a RCUK academic fellowship, a Royal Society Research grant (RG120556) and European Commission (Contact Number: LSHM-CT2004-005166). The sponsors had no involvement in the design of the study and in the collection, analyses and interpretation of the data, nor in the writing of the report and the decision to submit this article for publication. Sherie R. Wright, Panos Zanos, Polymnia Georgiou, Ji-Hoon Yoo, Susanna Hourani, Ian Kitchen, Raphaelle Winsky-Sommerer and Alexis Bailey report no conflict of interest and no biomedical financial interest from this research.

## Author Contribution

SRW, AB and IK were responsible for the design of the study. CL provided the $A_{2A}R$ knockout mice. SRW, PZ, PG and JHY contributed to the acquisition of animal data. SRW, PZ and JHY contributed to the binding assays. SRW and PZ performed data analysis with RWS contribution, as well as interpretation of findings. SRW and PZ drafted the manuscript. AB, IK, SMH and RWS provided critical revision of the manuscript. All authors critically reviewed content and approved final version for publication.

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**Table 1:** *Relevant effects of behavioral and biochemical data*

| | **Factor effect** | | **Interaction effect** | | ***N* (per group)** |
|---|---|---|---|---|---|
| ***Overall effects for Figure 1*** | | | | | |
| *Chronic Cocaine* | *Factor 'treatment'* | | *Factor 'treatment' x 'genotype'* | | |
| Ambulatory Time | $F_{[1,30]} = 60.91$; | $p<0.001$ | $F_{[1,30]} = 0.001$; | $p=0.99$ | 5-11 |
| Rearing activity | $F_{[1,30]} = 25.06$; | $p<0.01$ | $F_{[1,30]} = 0.001$; | $p=0.98$ | 5-11 |
| *Chronic Methamphetamine (MAP)* | *Factor 'treatment'* | | *Factor 'treatment' x 'genotype'* | | |
| Ambulatory Time | $F_{[1,20]} = 190.78$; | $p<0.001$ | $F_{[1,20]} = 13.01$; | $p=0.33$ | 6 |
| Rearing activity | $F_{[1,20]} = 25.58$; | $p<0.001$ | $F_{[1,20]} = 12.27$; | $p<0.01$ | 6 |
| ***Overall effects for Figure 2*** | | | | | |
| *Cocaine* | *Factor 'treatment'* | | *Factor 'treatment' x 'ligand'* | | |
| Ambulatory Time | $F_{[1,42]} = 1098.5$; | $p<0.001$ | $F_{[3,42]} = 0.10$; | $p=0.96$ | 6-7 |
| Vertical activity | $F_{[1,42]} = 106.79$; | $p<0.001$ | $F_{[3,42]} = 0.22$; | $p=0.88$ | 6-7 |
| *MAP* | *Factor 'treatment'* | | *Factor 'treatment' x 'ligand'* | | |
| Ambulatory Time | $F_{[1,40]} = 123.30$; | $p<0.001$ | $F_{[3,40]} = 3.48$; | $p=0.03$ | 6 |
| Vertical activity | $F_{[1,40]} = 87.99$; | $p<0.001$ | $F_{[3,40]} = 3.55$; | $p=0.02$ | 6 |
| ***Overall effects for Figure 3*** | *Factor 'ligand'* | | *Factor 'ligand' x 'CPP phase'* | | |
| *MAP-incuced CPP* | $F_{[1,50]} = 21.52$; | $p<0.001$ | $F_{[3,50]} = 6.032$; | $p<0.01$ | 6-7 |
| | *Factor 'ligand'* | | *Factor 'ligand' x 'CPP phase'* | | |
| *SCH 58261 (0.01) + MTEP (0.01) -induced CPP* | $F_{[1,20]} = 0.14$; | $p=0.71$ | $F_{[1,20]} = 0.01$; | $p=0.93$ | 6 |
| ***Overall effects for Figure 4*** | | | | | |
| *Chronic Cocaine - mGlu5 receptor autoradiography* | *Factor 'treatment'* | | *Factor 'treatment' x 'genotype'* | | |
| AcbC | $F_{[1,18]} = 0.002$; | $p=0.96$ | $F_{[1,18]} = 0.22$; | $p=0.65$ | 5-8 |
| AcbSh | $F_{[1,18]} = 0.19$; | $p=0.66$ | $F_{[1,18]} = 0.02$; | $p=0.89$ | 5-8 |
| Tu | $F_{[1,17]} = 0.28$; | $p=0.61$ | $F_{[1,17]} = 0.49$; | $p=0.49$ | 5-7 |
| CPu | $F_{[1,18]} = 0.31$; | $p=0.58$ | $F_{[1,18]} = 0.10$; | $p=0.76$ | 5-8 |
| *Chronic MAP - mGlu5 receptor autoradiography* | *Factor 'treatment'* | | *Factor 'treatment' x 'genotype'* | | |
| AcbC | $F_{[1,18]} = 1.59$; | $p=0.22$ | $F_{[1,18]} = 7.98$; | $p<0.05$ | 5-6 |
| AcbSh | $F_{[1,18]} = 2.25$; | $p=0.15$ | $F_{[1,18]} = 6.64$; | $p<0.05$ | 5-6 |
| Tu | $F_{[1,17]} = 2.01$; | $p=0.17$ | $F_{[1,17]} = 0.49$; | $p=0.06$ | 5-6 |
| CPu | $F_{[1,19]} = 0.84$; | $p=0.37$ | $F_{[1,19]} = 5.96$; | $p<0.05$ | 5-6 |
| ***Overall effects for Figure 5*** | | | | | |
| *Treatment-naïve WT and $A_{2A}R$ KO mice* | *Factor 'genotype'* | | *Factor 'genotype x brain region'* | | |
| *$D_2$ receptor autoradiography* | $F_{[1,40]} = 3.88$; | $p=0.56$ | $F_{[3,40]} = 0.13$; | $p=0.94$ | 6 |
| *mGlu5 receptor autoradiography* | $F_{[1,38]} = 5.19$; | $p<0.05$ | $F_{[3,38]} = 2.96$; | $p<0.05$ | 5-6 |
| ***Overall effects for Supplementary Figure 3*** | | | | | |
| *SCH 58261* | *Factor 'ligand'* | | | | |
| Ambulatory Time | $F_{[4,19]} = 2.28$; | $p=0.09$ | | | 3-8 |
| Vertical activity | $F_{[4,19]} = 9.82$; | $p<0.001$ | | | 3-8 |
| *MTEP* | *Factor 'ligand'* | | | | |
| Ambulatory Time | $F_{[4,19]} = 2.64$; | $p<0.05$ | | | 3-8 |
| Vertical activity | $F_{[4,19]} = 5.86$; | $p<0.01$ | | | 3-8 |
| ***Overall effects for Supplementary Figure 2*** | | | | | |
| *Effects of ligand - Treatment-naïve mice* | *Factor 'ligand'* | | | | |
| Ambulatory Time | $F_{[3,65]} = 0.81$; | $p=0.50$ | | | 16-21 |
| Vertical activity | $F_{[3,65]} = 0.21$; | $p=0.89$ | | | 16-21 |
| ***Overall effects for Supplementary Figure 5*** | | | | | |
| *Chronic Cocaine - $D_2$ receptor autoradiography* | *Factor 'treatment'* | | *Factor 'treatment' x 'genotype'* | | |
| AcbC | $F_{[1,16]} = 0.77$; | $p=0.39$ | $F_{[1,16]} = 0.004$; | $p=0.95$ | 4-5 |
| AcbSh | $F_{[1,17]} = 0.25$; | $p=0.62$ | $F_{[1,17]} = 0.02$; | $p=0.90$ | 4-5 |
| Tu | $F_{[1,19]} = 0.34$; | $p=0.57$ | $F_{[1,19]} = 0.04$; | $p=0.83$ | 4-6 |
| CPu | $F_{[1,19]} = 1.05$; | $p=0.32$ | $F_{[1,19]} = 0.64$; | $p=0.43$ | 4-6 |
| *Chronic MAP - $D_2$ receptor autoradiography* | *Factor 'treatment'* | | *Factor 'treatment' x 'genotype'* | | |
| AcbC | $F_{[1,18]} = 1.17$; | $p=0.29$ | $F_{[1,18]} = 0.98$; | $p=0.33$ | 5-6 |
| AcbSh | $F_{[1,18]} = 0.62$; | $p=0.44$ | $F_{[1,18]} = 0.01$; | $p=0.90$ | 5-6 |
| Tu | $F_{[1,17]} = 1.76$; | $p=0.20$ | $F_{[1,17]} = 0.03$; | $p=0.87$ | 5-6 |
| CPu | $F_{[1,19]} = 0.54$; | $p=0.47$ | $F_{[1,19]} = 0.01$; | $p=0.91$ | 5-6 |

## LEGENDS FOR TABLES AND FIGURES

**Table 1:** ***Relevant effects for biochemical and behavioral data.***

**Supplementary Table 1:** ***Quantitative autoradiography of mGlu$_5$R in WT and adenosine A$_{2A}$R KO mice following chronic cocaine administration.***

**Supplementary Table 2:** ***Quantitative autoradiography of mGlu$_5$R in WT and adenosine A$_{2A}$R KO mice following chronic methamphetamine administration.***

**Supplementary Table 3:** ***Quantitative autoradiography of mGlu$_5$R in treatment-naive WT and adenosine A$_{2A}$R KO mice.***

**Figure 1:** ***Adenosine A$_{2A}$R deletion attenuates hyperactivity and prevents stereotypic rearing behavior following chronic methamphetamine, but not cocaine administration.*** Ambulatory time in wild-type (WT) and adenosine A$_{2A}$R knockout (KO) mice was measured daily during chronic cocaine (3 x 15 mg/kg/day, 14 days, n = 7-11/group) or methamphetamine (MAP, 1 mg/kg/day, 10 days, n = 6/group) administration. Cocaine-induced **(A)** ambulatory time and **(B)** vertical activity, as well as MAP-induced **(C)** ambulatory time and **(D)** vertical activity are represented as the cumulative mean ± SEM, for a period of 3 hours daily. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs* WT Saline; $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ *vs* A$_{2A}$R KO MAP.

**Figure 2:** ***Co-antagonism of A$_{2A}$R and mGlu$_5$R reduces methamphetamine- but not cocaine-induced hyperactivity and stereotypic rearing behavior.*** Wild type mice were pre-treated with sub-threshold doses of A$_{2A}$R antagonist SCH 58261 (SCH, 0.01 mg/kg, i.p.), mGlu$_5$R antagonist MTEP (0.01 mg/kg, i.p.) or a combined administration of both (SCH 0.01 mg/kg + MTEP 0.01 mg/kg, i.p.), followed by an acute treatment with saline, methamphetamine (MAP, 1 mg/kg, i.p. n = 5-6/group) or cocaine (15mg/kg, i.p., n = 5-6/group). Cocaine-induced **(A)** ambulatory time

and **(B)** vertical activity, as well as MAP-induced **(C)** ambulatory time and **(D)** vertical activity are represented as the cumulative mean ± SEM, for a period of 3 hours. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

**Figure 3:** ***Co-antagonism of $A_{2A}R$ and $mGlu_5R$ prevents methamphetamine-induced conditioned place preference.*** **(A)** Experimental protocol of the different phases of the CPP paradigm. Wild type mice underwent the following protocol: Habituation phase; Pre-conditioning (Pre-Cond) phase: assessment of spontaneous place preference; Conditioning phase: 6 days, with saline injection (10 ml/kg, i.p., even days) in the preferred compartment and administration of methamphetamine (MAP; 1 mg/kg, i.p. odd days) in the least-preferred compartment; Post-conditioning (Post-Cond) session: assessment of conditioning with no injection. For the Post-Cond phase, mice were subdivided into four different experimental groups to receive vehicle (20% DMSO, i.p.; n=8), SCH 58261 (SCH, 0.01 mg/kg, i.p.; n=7), MTEP (0.01 mg/kg, i.p.; n=7) or a combination of SCH 0.01 mg/kg i.p. and MTEP 0.01 mg/kg, i.p.; n=7 Following a period of 30 min, mice were tested for post-conditioning during a 20-min session. **(B)** Time spent in the MAP-paired compartment for each phase of the CPP paradigm (analysis of the last 15 min of the 20-min session). Data are expressed as mean ± SEM$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. **(C)** Time spent in the MTEP/SCH- or saline-paired compartment (analysis of the last 15 minutes of the 20-min session) during the pre-conditioning (Pre-Cond) and post-conditioning (Post-Cond) phases of the CPP paradigm. Data are expressed as mean ± SEM, n = 6.

**Figure 4:** ***Absence of methamphetamine-induced striatal $mGlu_5R$ upregulation in adenosine $A_{2A}R$ knockout mice.*** Wild-type (WT) and $A_{2A}R$ knockout (KO) mice were treated with a chronic saline /cocaine (3 x 15 mg/kg/day, 14 days, n = 7-11/group) or saline/MAP (1 x 1

mg/kg/day, 10 days, n=6/group). Representative autoradiograms of [$^3$H]MPEP binding to $mGlu_5R$ receptors in coronal brain sections of mice underwent chronic **(A)** saline/cocaine or **(B)** saline/MAP administration. Binding levels are represented as a pseudo-colour interpretation of black and white film images in fmol/mg of tissue equivalent. Quantitative $mGlu_5R$ binding levels in the striatum of WT and $A_{2A}R$ KO mice following chronic **(C)** saline/cocaine or **(D)** saline/MAP administration. Data are expressed as the mean specific binding ± SEM. $^*p<0.05$. Abbreviations: *AcbC*, nucleus accumbens core; *AcbSh*, nucleus accumbens shell; *CPu*, caudate putamen; *Tu*, olfactory tubercle.

**Figure 5:** ***Decreased striatal $mGlu_5R$ binding in treatment-naïve adenosine $A_{2A}R$ knockout mice.*** Representative autoradiograms of **(A)** [$^3$H]MPEP binding to $mGlu_5R$ (n=5-6/genotype), **(B)** [$^3$H]raclopride binding to dopamine $D_2$ receptors ($D_2R$; n=6/genotype) and **(C)** [$^3$H]mazindol binding to dopamine transporters (DAT; n=4-6/genotype), in coronal brain sections of treatment-naïve CD-1 wild-type (WT) and $A_{2A}R$ knockout (KO) mice (n=5-6/group). Binding levels are represented using a pseudo-colour interpretation of black and white film images in fmol/mg of tissue equivalent. Quantitative **(D)** $mGlu_5R$, **(E)** $D_2R$ and **(F)** DAT binding levels in treatment-naïve WT and $A_{2A}R$ KO mice. Data are expressed as the mean specific binding ± SEM. $^*p<0.05$. Abbreviations: *AcbC*, nucleus accumbens core; *AcbSh*, nucleus accumbens shell; *CPu*, caudate putamen; *Tu*, olfactory tubercule.

**Supplementary Figure S1:** ***Confirmation of mouse genotype by autoradiographic binding of $A_{2A}R$.*** Representative computer-enhance pseudo-colour autoradiograms of brain sections incubated with [$^3$H]-CGS 21680 from mice used in the cocaine as well as MAP studies to label $A_{2A}R$.

**Supplementary Figure S2:** ***No differences in basal ambulatory and vertical activity between wild-type and $A_{2A}R$ knockout mice.*** Cumulative basal **(A)** ambulatory time and **(B)** vertical (rearing) locomotor activity of wild-type (WT) and adenosine $A_{2A}R$ knockout (KO) mice were recorded for 1 hour prior to any drug treatment injection on Day 1 of chronic saline, cocaine or MAP administration protocol. Data are represented as mean ± SEM (n = 24 – 29; mice from both cocaine and MAP experiments).

**Supplementary Figure S3:** ***Dose-response effect of SCH 58261 and MTEP on methamphetamine-induced motor activity.*** Male wild-type mice were pre-treated with either $A_{2A}R$ antagonist SCH 58261 (1, 0.25, 0.1 or 0.01 mg/kg, i.p., n = 3 – 4/ group) or $mGlu_5R$ antagonist MTEP (0.5, 0.25, 0.1 or 0.01 mg/kg, i.p., n = 3 - 4/group) followed by an acute methamphetamine (MAP, 1 mg/kg, i.p.) administration. The effects of SCH 58261 or MTEP pre-treatment on **(A, C)** ambulatory time and **(B, D)** vertical (rearing) locomotor activity were recorded for 3 hours following acute MAP administration. Data are represented as mean ± SEM. $^{*}p<0.05$, ** $p<0.01$

**Supplementary Figure S4:** ***Effect of SCH 58261 and MTEP on ambulatory and vertical activity of mice.*** Wild-type (WT) were treated with either vehicle (10ml/kg; 20% DMSO; n=16), SCH 58261 (SCH, 0.01 mg/kg, i.p.; n=16), MTEP (0.01 mg/kg, i.p.; n=16) or a combination of SCH 0.01 mg/kg i.p. and MTEP 0.01 mg/kg, i.p. (n=21) and tested for (A) ambulatory time and (B) stereotypic rearing activity. Data are represented as mean ± SEM

**Supplementary Figure S5:** ***Chronic methamphetamine or cocaine administration does not alter dopamine $D_2R$ binding in WT and $A_{2A}R$ knockout mice.*** Wild-type (WT) and $A_{2A}R$ knockout (KO) mice were treated with a chronic saline/cocaine (3 x 15 mg/kg/day, 14 days; n=4-6/group) or saline/MAP (1 x 1 mg/kg/day, 10 days; n=5-6/group). Representative

autoradiograms of [$^3$H]raclopride binding to dopamine $D_2R$ in coronal brain sections of mice underwent a chronic **(A)** saline/cocaine or **(C)** saline/MAP administration. Binding levels are represented using a pseudo-colour interpretation of black and white film images in fmol/mg of tissue equivalent. Quantitative $D_2R$ binding levels in the striatum of WT and $A_{2A}R$ KO mice treated with a chronic **(B)** saline/cocaine or **(D)** saline/MAP administration. Data are expressed as the mean specific binding ± SEM. Abbreviations: *AcbC*, nucleus accumbens core; *AcbSh*, nucleus accumbens shell; *CPu*, caudate putamen; *Tu*, olfactory tubercule.