Neuropsychopharmacology (2009) 34, 844–856

www.neuropsychopharmacology.org

A Differential Role for the Adenosine A_{2A} Receptor in Opiate Reinforcement *vs* Opiate-Seeking Behavior

Robyn Mary Brown[1,2], Jennifer Lynn Short[2], Michael Scott Cowen[1,3], Catherine Ledent[4] and Andrew John Lawrence*[,1,3]

[1]Brain Injury and Repair Group, Howard Florey Institute, University of Melbourne, Parkville, VIC, Australia; [2]Monash Institute of Pharmaceutical Sciences, Parkville, VIC, Australia; [3]Centre for Neuroscience, University of Melbourne, Parkville, VIC, Australia; [4]Faculte de Medecine, Institut de Recherche Interdisciplinaire, Universite de Bruxelles, Brussels, Belgium

The adenosine A_{2A} receptor is specifically enriched in the medium spiny neurons that make up the 'indirect' output pathway from the ventral striatum, a structure known to have a crucial, integrative role in processes such as reward, motivation, and drug-seeking behavior. In the present study we investigated the impact of adenosine A_{2A} receptor deletion on behavioral responses to morphine in a number of reward-related paradigms. The acute, rewarding effects of morphine were evaluated using the conditioned place preference paradigm. Operant self-administration of morphine on both fixed and progressive ratio schedules as well as cue-induced drug-seeking was assessed. In addition, the acute locomotor response to morphine as well as sensitization to morphine was evaluated. Decreased morphine self-administration and breakpoint in A_{2A} knockout mice was observed. These data support a decrease in motivation to consume the drug, perhaps reflecting diminished rewarding effects of morphine in A_{2A} knockout mice. In support of this finding, a place preference to morphine was not observed in A_{2A} knockout mice but was present in wild-type mice. In contrast, robust cue-induced morphine-seeking behavior was exhibited by both A_{2A} knockout and wild-type mice after a period of withdrawal. The acute locomotor response to morphine in the A_{2A} knockout was similar to wild-type mice, yet A_{2A} knockout mice did not display tolerance to chronic morphine under the present paradigm. Both genotypes display locomotor sensitization to morphine, implying a lack of a role for the A_{2A} receptor in the drug-induced plasticity necessary for the development or expression of sensitization. Collectively, these data suggest a differential role for adenosine A_{2A} receptors in opiate reinforcement compared to opiate-seeking.
Neuropsychopharmacology (2009) **34,** 844–856; doi:10.1038/npp.2008.72; published online 4 June 2008

Keywords: adenosine A_{2A} receptor; knockout mice; morphine; intravenous self-administration; drug-seeking; reward

INTRODUCTION

Adenosine is a ubiquitous purine nucleoside that has a fundamental role in governing the physiological function of cells, including the processes underlying neurotransmission (for review, see Dunwiddie, 1985; Snyder, 1985; Brundege and Dunwiddie, 1997). Four adenosine receptors belonging to the family of G-protein-coupled receptors have been identified thus far: the A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm *et al*, 2001b, 1994). Of the known adenosine receptor subtypes, A_1 and A_{2A} receptors are primarily responsible for the central effects of adenosine (Fredholm *et al*, 2001a).

*Correspondence: Professor AJ Lawrence, Brain Injury and Repair Group, Howard Florey Institute, University of Melbourne, Parkville, Victoria 3010, Australia, Tel: +613 8344 0414, Fax: +613 9348 1707, E-mail: andrew.lawrence@florey.edu.au
Received 14 November 2007; revised 4 April 2008; accepted 14 April 2008

In contrast to the widespread distribution of A_1, A_{2B}, and A_3 receptors within the central nervous system, A_{2A} receptors are specifically enriched in the dendritic spines of striatopallidal neurons (Fink *et al*, 1992; Svenningsson *et al*, 1997; Hettinger *et al*, 2001; Rosin *et al*, 2003) where they are colocalized with dopamine D_2 receptors (Fink *et al*, 1992; Schiffmann *et al*, 1991). Indeed, the formation of heterodimers between these two receptors has been demonstrated (Canals *et al*, 2003). Adenosine A_{2A} receptor activation has been shown to modulate negatively the postsynaptic effects of dopamine at the biochemical, cellular, functional, and behavioral levels (Ferre *et al*, 1993, 1991a). Behaviorally, adenosine A_{2A} agonists produce dopamine D_2-like antagonist effects and *vice versa* (Ferre *et al*, 1997). For example, the adenosine A_{2A} receptor agonist CGS 21680 induces catalepsy and this is counteracted by administration of the D_2 antagonist BHT-920 (Ferre *et al*, 1991b). In addition to direct interactions with the dopamine D_2 receptor, A_{2A} receptors have been shown to interact indirectly with dopamine D_1 receptors to regulate D_1-mediated behavioral and cellular effects

(Pollack and Fink, 1996; Pinna *et al*, 1996; Svenningsson *et al*, 1998; Short *et al*, 2006b).

A critical role for the mesocorticolimbic dopamine system has long been established in the rewarding properties of drugs of abuse (Di Chiara and Imperato, 1988), and a growing body of evidence exists implicating the A_{2A} receptor in reward-related behavior. Adenosine, via A_{2A} receptors, modulates behaviors associated with acute and chronic exposure to cannabinoids (Soria *et al*, 2004), psychostimulants (Chen *et al*, 2000; Soria *et al*, 2006), nicotine (Castane *et al*, 2006), and alcohol (Arolfo *et al*, 2004; Short *et al*, 2006b). The bulk of evidence to date concerning adenosine involvement in opiate reward centers on opiate withdrawal. Thus, adenosine agonists reduce and antagonists increase symptoms associated with precipitated opiate withdrawal (Salem and Hope, 1997; Kaplan and Sears, 1996). Increased levels of adenosine metabolites in the nucleus accumbens (NAc) have been reported to occur during opiate withdrawal in rats (Salem and Hope, 1997) and adenosine kinase inhibitors significantly reduce morphine withdrawal symptoms in mice, an effect that can be blocked by the nonselective adenosine receptor antagonist caffeine (Kaplan and Coyle, 1998). In agreement with these findings, two studies (Bailey *et al*, 2004; Berrendero *et al*, 2003) have found an enhancement in the expression of some morphine withdrawal signs in opiate-dependent A_{2A} receptor knockout mice. These data are in apparent discord with the well-documented antagonistic relationship between adenosine A_{2A} receptors and dopamine D_2 receptors described previously and indicate a more complex role for adenosine A_{2A} receptors in the action of opiates, potentially including nondopaminergic mechanisms.

Aside from studies relating to opiate withdrawal, a small number of pharmacological studies have provided insight into the potential involvement of the A_{2A} receptor in other aspects of morphine reward and dependence. It has been suggested that adenosine receptor antagonists attenuate the development of morphine sensitization in mice (Weisberg and Kaplan, 1999), and nonselective adenosine antagonists inhibit morphine self-administration in rats (Sahraei *et al*, 1999). More recently, it was reported that administration of a selective A_{2A} receptor antagonist eliminated reinstatement of heroin-seeking in rats (Yao *et al*, 2006). The comparatively low selectivity profiles of some adenosine antagonists (Yang *et al*, 2007) make it difficult *in vivo* to clarify the participation of the different adenosine receptor subtypes in opioid dependence. As yet, there has been no study examining the impact of A_{2A} receptor deletion on aspects of morphine reward other than withdrawal. The generation of A_{2A} receptor knockout mice with complete and specific inactivation of the A_{2A} receptor (Ledent *et al*, 1997) provides a useful tool to extend these pharmacological data. The aim of the present study was to examine the effect of A_{2A} receptor deletion on morphine reward as well as morphine-seeking behavior. The extent to which A_{2A} receptor knockout and wild-type mice would self-administer morphine was assessed, as well as their motivation to obtain a morphine reward on a progressive ratio (PR) schedule. Cue-conditioned morphine-seeking behavior following a period of withdrawal was also measured. Differences in the 'hedonic' value attributed to morphine between genotypes were assessed using the conditioned place preference paradigm, and the impact of A_{2A} receptor deletion on drug-induced plasticity was assessed using the locomotor sensitization paradigm.

MATERIALS AND METHODS

Animals

All experiments were performed in adherence to the Prevention of Cruelty to Animals Act, 1986, under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. The adenosine A_{2A} receptor knockout mice used in the present study were bred on a CD-1 background, originally obtained from C Ledent (IRIBHN, Belgium). Wild-type mice used in the study were obtained from litters generated by heterozygous breeders. Mice were backcrossed for eight generations and were genotyped by routine PCR (Snell *et al*, 2000). All experiments were performed with adult male mice housed at the Integrative Neuroscience Facility, Howard Florey Institute. Mice used for intravenous self-administration were singly housed in a 12 h reverse light-dark cycle (dark 0700–1900 hours). Nesting material was available. Mice used in conditioned place preference and locomotor sensitization experiments were group housed (typically four per cage) with nesting material available and free access to food (standard mouse chow) and water.

Drugs

Morphine hydrochloride and cocaine hydrochloride were obtained from Glaxo Australia Pty Ltd (Boronia, Australia) and dissolved in sterile 0.9% saline. Ketamine was obtained from Parnell Laboratories (Alexandria, Australia) and midazolam from Roche Products (Dee Why, Australia), and both drugs were diluted with 0.9% saline. Neomycin sulfate was supplied by Delta Veterinary Laboratories (Hornsby, Australia) and was diluted in heparinized (10 U) 0.9% saline. Meloxicam was obtained from Boeringher Ingleheim (Inglehiem, Germany).

Operant Self-Administration

Apparatus. Operant self-administration of oral sucrose or intravenous morphine (0.1 mg/kg/infusion) was assessed using operant chambers (model ENV-307W, Med Associates, Vermont, USA) that were equipped with two levers, one paired with reward delivery (the active lever) and one which resulted in no outcome when pressed (the inactive lever). A stimulus light (conditioned stimulus, CS) was located above the active lever and flashed on for a period of 10 s when the active lever was pressed and drug/sucrose delivered. In addition to this visual cue, a tray was placed under the grid below the active lever with a piece of paper (1×1 cm) upon which two drops of vanilla essence was placed before each session. The vanilla-infused paper provided an olfactory cue (unconditioned stimulus, US) for the location of the active lever. The chambers were housed in sound attenuated boxes and ventilated with fans.

Self-Administration Protocol: Natural Reinforcement

Mice were engaged in an instrumental learning task for up to 8 days. The reward obtained was a delivery of sucrose to a liquid receptacle in the chamber (5 μl), and reward delivery was contingent upon an active lever press. A fixed ratio of 1 (FR1) active lever press per sucrose delivery was employed. All sessions were 2 h in length and held in the dark phase of the photoperiod at approximately the same time each day. Mice were assessed for self-administration of sucrose at concentrations of 2.5, 5, or 10%.

Surgery. Mice were anaesthetized using isofluorane (1.5–1.8%). and then implanted with indwelling venous cannulae (modified from Griffin and Middaugh, 2003). The cannulae consisted of a 3.5 mm length silastic tubing (inner diameter 0.012 inches × outer diameter 0.025 inches, Dow Corning Corporation, Midland, MI, USA) attached to a 22-gauge needle which was bent into U shape, then bent again at right angles to the luer. The catheter was inserted 1 cm into the jugular vein and anchored with suture. The remaining tubing ran subcutaneously behind the ear to exit on top of the head. The catheter port was attached to the skull using Loctite 454 instant adhesive (Loctite Australia Pty Ltd, Caringbah, Australia) followed by dental cement (Vertex-Dental, Zeist, Netherlands). While still under anesthesia the catheter was flushed with heparinized antibiotic (0.03 ml neomycin sulfate 4 mg/ml in 10 U heparin) and meloxicam (0.1 ml of 5 mg/ml, i.p.) was administered for pain relief. After surgery mice were allowed to recover for 24 h before training began. The day after surgery, the catheters were flushed with heparinized antibiotic and subsequent to that, heparinized saline twice daily to maintain catheter patency (typically before and after self-administration session). The patency of the catheters was evaluated periodically using 0.02–0.03 ml of ketamine (15 mg/ml) and midazolam (0.75 mg/ml) solution. If prominent signs of hypnosis were not apparent within 3 s of infusion the mouse was removed from the experiment.

Self-Administration Protocol: Morphine Reinforcement

Mice were connected via the jugular catheter to an intravenous line (Tygon, inner diameter 0.02 inches, outer diameter 0.06 inches) connected to a 22-gauge swivel (Instech Solomon, Plymouth Meeting, PA, USA). The swivel was connected to a syringe held in the infusion pump (model PHM-100SVA; Med Associates) with Bcoex-T22 tubing (inner diameter 0.24 inches × outer diameter 0.64 inches; Instech Soloman). As with responding for the natural reinforcer, CS and US were present and an FR1 schedule was employed. The injection volume was 19 μl and duration of injection was 2.3 s. A maximum of 50 drug infusions was set to prevent overdose, as well as a 10 s time-out period after each drug infusion. During the time-out period active lever presses were recorded but no drug infusion occurred. All sessions were 2 h in length unless 50 infusions were obtained (at which point the session was terminated) and held in the dark phase of the photoperiod, at approximately the same time each day. Initially mice were trained on cocaine (1 mg/kg per infusion, 1 day) before being moved onto morphine (0.1 mg/kg per infusion). Criteria were set at >6 infusions per session, a lever discrimination of at least 65% and less than 20% variation between infusions over 3 days. Mice who failed to meet criteria were excluded from the study. Subsequently, an additional cohort of mice ($n = 8$–14) were cannulated and their responding for morphine at a higher dose (0.2 mg/kg per infusion) was assessed.

'Breakpoint' was assessed using a PR schedule. Breakpoint represents the point at which an animal ceases to press the active lever for a drug infusion when the instrumental requirement is progressively increased. This reflects the motivation of an animal to self-administer a given drug and may therefore provide more accurate data pertaining to the reinforcing efficacy of that drug (Arnold and Roberts, 1997). The PR protocol utilized was modified from Thomsen *et al*, 2005, with 0.1 mg/kg morphine delivered per infusion. The breakpoint was defined as the last completed ratio, after which a period of 60 min ensued where no reinforcer was earned. If this did not occur, the session was terminated after 2 h and the breakpoint was defined as the final ratio completed within the 2 h session. Although a 'true breakpoint' as defined by Arnold and Roberts (1997) may not be reached in a 2 h session, the data yielded still provide useful information regarding the motivation to obtain a drug reward. In addition, this session length has been reliably used previously, both in rats (Cornish *et al*, 2008; Clemens *et al*, 2006) and mice (Soria *et al*, 2006; Trigo *et al*, 2006). After 3 weeks abstinence in the home cage, drug-seeking was precipitated by placing mice into the operant chambers with the US present. Drug-seeking was assessed under extinction conditions (FR1 response resulted in CS but no infusion of morphine) for 1 h. There was no maximum set as there was no risk of overdose.

Morphine-Induced Conditioned Place Preference

The conditioned place preference apparatus (Lafayette Instruments, IN, USA) consisted of two main compartments with differences in visual (wall patterns) and tactile (floor texture) cues, separated by a neutral compartment. The light intensity settings were set at 30 (80 lux) within the conditioning compartments and 90 (380 lux) in the central compartment, with these values referring to the settings on the equipment provided. 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 all three compartments for 15 min. 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, CA, USA). Locomotor activity was measured as the number of beam breaks, referred to as 'activity counts'. On days 2–9 (the conditioning phase) mice received alternating injections of morphine (10 mg/kg, i.p.) or vehicle and were immediately confined into one of the two conditioning compartments for 40 min (randomly allocated). On day 10 (test day), mice were once again allowed free access to all three compartments for 15 min. Place preference was determined as a positive difference between time spent in the drug-paired

compartment on test day compared to the saline-paired compartment.

Behavioral Sensitization

Sensitization to the locomotor responses induced by chronic morphine treatment was performed as previously described (McPherson and Lawrence, 2006). Briefly, locomotor activity responses induced by morphine (10 mg/kg, i.p.) or vehicle were evaluated using photo-optic locomotor cells (Truscan Photobeam; Coulbourn Instruments, Allentown, PA, USA) in a low luminosity (20 lux), controlled environment. Movement was measured over *x* and *y* axes by optic sensor beams. Before each session, mice were habituated to the experimental room for at least 30 min. Mice were habituated to the locomotor cells for 40 min per day over 3 consecutive days. On days 4–8, mice were placed in the locomotor cells immediately after morphine (10 mg/kg, i.p.) or vehicle injection, and their locomotor activity recorded for 40 min. Data were retrieved using Truscan software.

After completing chronic morphine or vehicle administration (days 4–8) mice remained without treatment in their home cage for 7 days (days 9–15). On day 16, mice received a challenge dose of morphine (5 mg/kg, i.p.) or vehicle and their locomotor activity was immediately measured for 40 min.

Statistical Analyses

For morphine self-administration (0.1 mg/kg per infusion) a one-way ANOVA was performed on the total lever presses across the genotypes (active and inactive levers), with Student—Newman–Keuls *post hoc* analyses where appropriate. For time course analyses (morphine and sucrose self-administration, PR cumulative responses), and analyses of the effect of genotype and dose on morphine administration a two-way ANOVA was performed, followed by either Student—Newman–Keuls or Holm–Sikak *post hoc* tests where appropriate. The data for lever discrimination were not normally distributed, and therefore a Mann–Whitney rank sum test was employed to compare between the genotypes. Comparisons of the PR data between the genotypes and the latency to reach sucrose criteria were analyzed using an unpaired *t*-test. Drug-seeking data and conditioned place preference data were analyzed by one-way ANOVA, with Student—Newman–Keuls post-tests to compare the different treatment groups and genotypes. Time course locomotor data were analyzed by three-way ANOVA with time, treatment, and genotype as factors, followed by Student—Newman–Keuls *post hoc* analyses where appropriate. Differences were deemed statistically significant if $p<0.05$.

RESULTS

Self-Administration of Sucrose is Similar in A_{2A} Knockout and Wild-Type Mice

As shown in Figures 1a, c, and e, no difference was observed between wild-type and A_{2A} receptor knockout mice with respect to self-administration of sucrose, a natural reinforcer, over a range of concentrations. Two-way repeated-measures ANOVA revealed no effect of genotype ($p>0.05$). There was no difference between the genotypes ($p>0.05$) with respect to latency to criteria (defined as 70 sucrose rewards in a single 2 h session). Analysis of discrimination data by Mann–Whitney rank sum tests revealed no difference between genotypes at 2.5 and 10% ($p>0.05$) but a difference at 5% ($t=947$, $p<0.01$) with A_{2A} receptor knockout mice displaying slightly worse discrimination for the active lever (83%) compared to wild type (94%) at this dose (data not shown).

Decreased Self-Administration of Morphine on FR1 and PR Schedules in A_{2A} Knockout Mice Compared to Wild Type

Both wild-type and A_{2A} receptor knockout mice reliably self-administered morphine at a dose of 0.1 mg/kg per infusion (Figure 2a). Analysis of aggregate data by two-way ANOVA revealed a main effect of lever identity ($F_{(1,417)}=160.937$, $p<0.001$). Student—Newman–Keuls *post hoc* comparisons revealed a significant preference for the active lever over the inactive lever in both wild-type ($q=18.199$, $p<0.001$) and A_{2A} knockout mice ($q=7.2213$, $p<0.001$), demonstrating that both genotypes exhibited significant discrimination in favor of the morphine-paired lever (see Figure 2a). Despite both genotypes displaying high discrimination for the morphine-paired lever, the level of discrimination was impaired in A_{2A} knockout mice compared to wild type. Analysis of the lever discrimination data via a Mann–Whitney rank sum test revealed a significantly lower discrimination for the active lever in A_{2A} knockout mice compared to wild type ($t=134$, $p<0.05$, data not shown).

As shown in Figures 2a and b, self-administration of morphine at the dose tested was significantly attenuated in A_{2A} knockout mice compared to wild-type mice. Analysis by two-way ANOVA revealed a main effect of genotype ($F_{(1,417)}=10.175$, $p<0.01$) and a significant interaction between lever (active *vs* inactive) and genotype ($F_{(1,29.674)}$, $p<0.001$; Figure 2a). Student—Newman–Keuls *post hoc* comparisons revealed significant differences between genotypes within active lever presses ($q=8.648$, $p<0.001$). Analysis of the number of morphine infusions over the 7-day period revealed a significant effect of genotype (two-way ANOVA; $F_{(1,184)}=28.948$, $p<0.001$). Student—Newman–Keuls *post hoc* analysis revealed that the genotype difference in infusions received and therefore the amount of morphine self-administered was evident on every day of self-administration ($p<0.05$).

Analysis of an additional cohort of mice at a higher dose of morphine (0.2 mg/kg per infusion) revealed similar results. As can be seen in Figure 2c, A_{2A} knockout mice also self-administered less morphine at this higher dose. Analysis by two-way ANOVA revealed a significant effect of genotype ($F_{(1,270)}=20.728$, $p<0.001$) and dose ($F_{(1,270)}=55.547$, $p<0.001$) but no interaction between these ($p>0.05$). Figure 2d displays representative event records for each genotype from an FR1 session (0.1 mg/kg per infusion), illustrating the pattern of drug self-administration within a session.

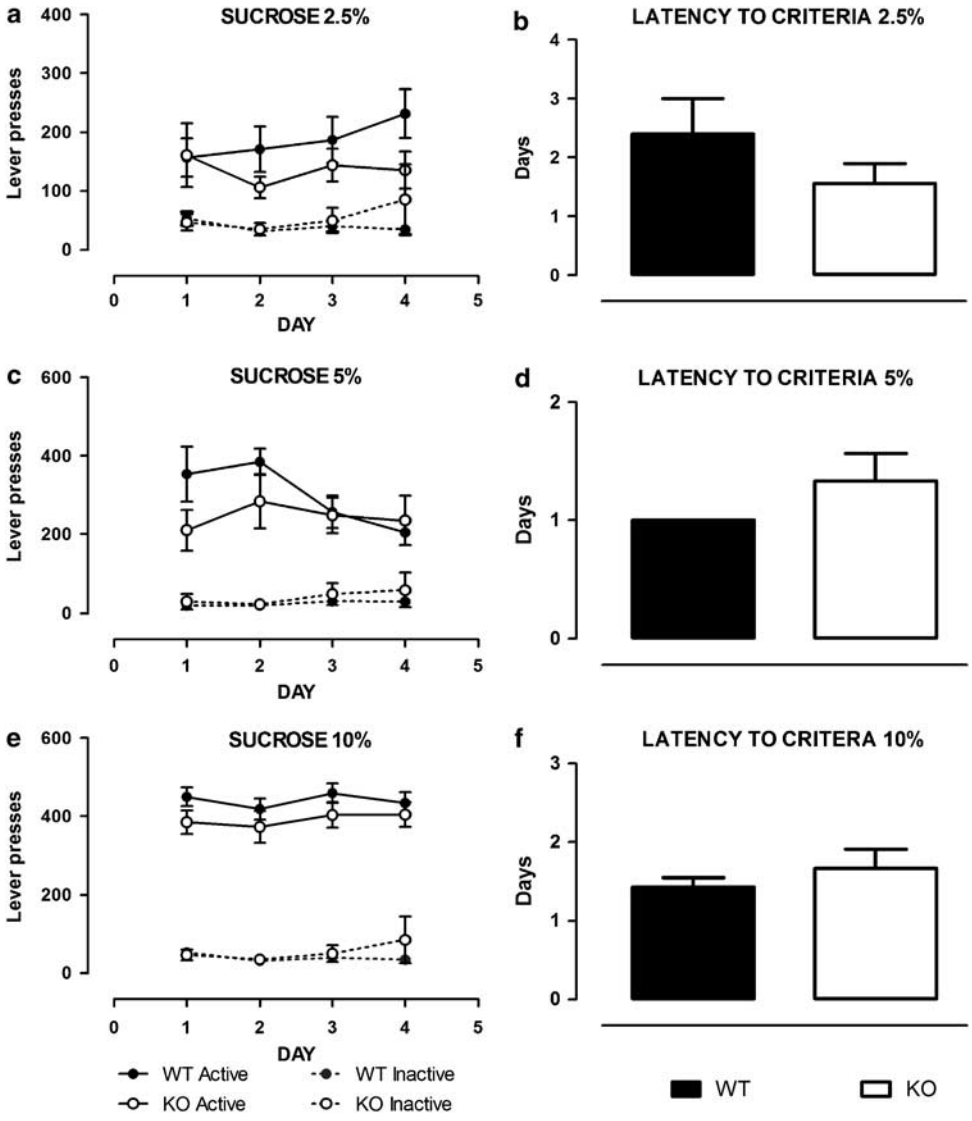

Figure 1 Sucrose self-administration (2.5, 5, and 10%, w/v) in A_{2A} knockout (KO) and wild-type (WT) mice (fixed ratio (FR) 1). Data are expressed as mean (±SEM), $n = 9$–31 per genotype. Latency to reach criteria defined as 70 sucrose rewards in one session. (a) Lever presses at 2.5%; (b) latency to reach criteria (days) at 2.5%; (c) lever presses at 5%; (d) latency to reach criteria (days) at 5%; (e) lever presses at 10%; and (f) latency to reach criteria (days) at 5%.

In addition to decreased baseline morphine self-administration, A_{2A} receptor knockout mice displayed a lower breakpoint compared to wild type when assessed using a PR schedule. As shown in Figures 3a, b, and c, unpaired *t*-tests revealed a significant difference between genotypes with respect to the final ratio obtained (breakpoint) ($t = 4.263$, $p < 0.001$), as well as the total number of lever presses for the session ($t = 3.694$, $p < 0.001$) and the number of drug infusions obtained ($t = 3.967$, $p < 0.001$). When assessing the proportion of mice that reached a 'true breakpoint' (when no reinforcer is earned for a 60 min period) it was discovered that 9 out of 17 knockout mice (53%) did indeed reach a 'true breakpoint' in the 2 h session, whereas only 2 from 16 wild-type mice (12.5%) did. This is apparent when examining the cumulative response record (Figure 3d) as one can observe a clear plateau in the responding of A_{2A} knockout mouse but not for the wild-type mice. Analysis of cumulative response record data (Figure 3d) by two-way ANOVA revealed a significant effect of genotype ($F_{(1,341)} = 10.980$, $p < 0.01$) and time ($F_{(11,341)} = 38.698$, $p < 0.001$), and a significant interaction between the two factors ($F_{(11,341)} = 7.872$). Holm–Sidak *post hoc* comparisons revealed that there was no difference between wild-type and knockout mice in the first four time bins ($p > 0.05$) but cumulative responses were significantly reduced in A_{2A} knockout mice compared to wild type in every 10 min time bin from 50 to 120 min ($t = 2.438$, 2.948, 3.197, 3.666, 3.883, 4.017, 4.649 and 4.991, respectively, $p < 0.05$). A plateau in responding was evident from 80 min onward for knockout mice. The cumulative levels of lever pressing at the 80 min time point were significantly higher than the 10 min time point for both A_{2A} knockout ($t = 3.954$, $p < 0.001$) and wild-type mice ($t = 9.904$, $p < 0.001$). When comparing the cumulative levels of responding between the last time bin (110–120 min) and the 80 min time point, a similarly significant increase was found in wild-type mice

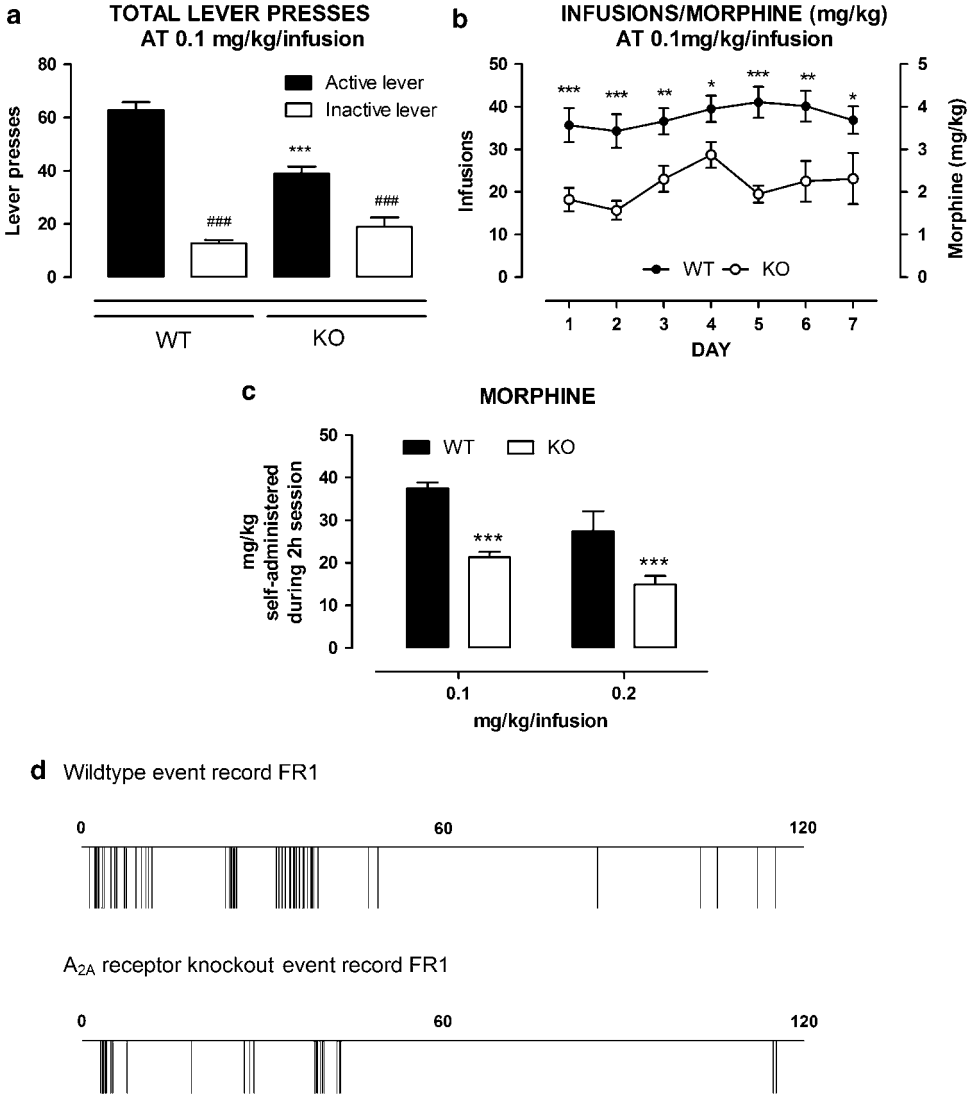

Figure 2 Morphine self-administration (0.1 mg/kg per infusion; Figure 2a, b, and d; both 0.1 and 0.2 mg/kg per infusion; Figure 2c) in A_{2A} knockout (KO) and wild-type (WT) mice (fixed ratio (FR) 1). Data are expressed as mean (± SEM), $n = 18$–20 per genotype for 0.1 mg/kg per infusion, $n = 8$–14 for 0.2 mg/kg per infusion. (a) Average of the total number of lever presses (on the active and inactive lever) over the 7 days of self-administration; $^{###}p < 0.001$ compared to active lever, $^{***}p < 0.001$ compared to WT (two-way ANOVA). (b) Number of drug infusions and dose of morphine obtained over 7 days in mg/kg; $^{***}p < 0.0001$, $^{**}p < 0.001$, $^{*}p < 0.05$ significant effect of genotype (two-way ANOVA with Student—Newman–Keuls post-tests). (c) Amount of morphine self-administered in 2 h sessions by A_{2A} KO and WT mice at different doses of 0.1 and 0.2 mg/kg per infusion; $^{***}p < 0.001$ significant effect of genotype (two-way ANOVA). (d) Event records for morphine self-administration during a single FR1 session for WT and A_{2A} KO mice. Each vertical line represents an infusion of morphine (0.1 mg/kg, i.v.).

($t = 3.376$, $p < 0.01$). This was not the case for A_{2A} knockout mice; there was no significant difference between any time bin between 80 and 120 min ($t = 0.936$, $p > 0.05$), suggesting a plateau in active lever responding had occurred.

Both A_{2A} Knockout and Wild-Type Mice Exhibit Robust Cue-Conditioned Morphine-Seeking Behavior after a Period of Withdrawal

Despite the significant differences in FR1 and PR sessions as described above, both genotypes exhibited robust cue-conditioned drug-seeking behavior after a period of 3 weeks abstinence (Figure 4). Lever presses on the active lever were significantly higher on test day (1 h session) compared to baseline responding (2 h session), despite the absence of drug infusions during the session. This significant cue-conditioned drug-seeking response for both genotypes was revealed by one-way ANOVA ($F_{(3,59)} = 13.12$), and Student—Newman–Keuls *post hoc* analyses for both wild-type ($q = 4.692$, $p < 0.001$) and A_{2A} knockout mice ($q = 7.578$, $p < 0.001$), confirming enhanced responding on the active lever on test day compared to the FR1 schedule. There was, however, no difference between genotypes in active lever presses on the test day ($q = 0.9493$, $p > 0.05$).

Absence of Conditioned Place Preference to Morphine in A_{2A} Knockout Mice

As Figure 5a demonstrates, a conditioned place preference to morphine (10 mg/kg, i.p.) was observed in wild-type mice

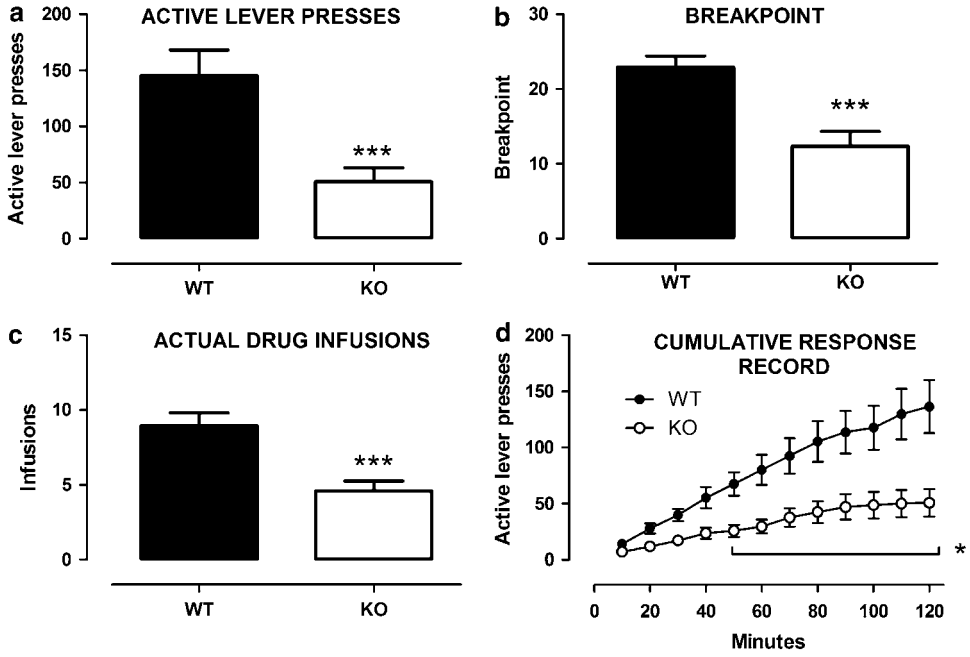

Figure 3 Morphine self-administration by A_{2A} knockout (KO) and wild-type (WT) mice on a progressive ratio (PR) schedule. All data are expressed as mean (±SEM), $n = 17–18$ per genotype. (a) Total active lever presses from the PR session; ***$p < 0.001$ (unpaired *t*-test). (b) Breakpoint (defined as the last completed ratio, after which a period of 60 min ensued where no reinforcer was earned. If this did not occur the session was terminated after 2 h and the breakpoint was defined as the final ratio completed within the 2 h session); ***$p < 0.001$ (unpaired *t*-test). (c) Actual drug infusions obtained during the PR session; ***$p < 0.001$ (unpaired *t*-test). (d) Cumulative response record for the PR session divided into 10 min time bins; *$p < 0.05$ compared to WT for that time bin (two-way ANOVA with Holm–Sidak post-tests).

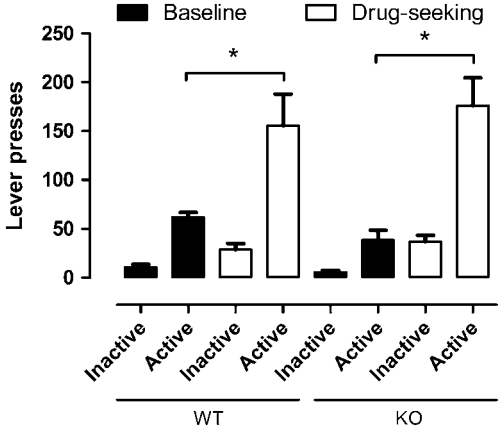

Figure 4 Cue-induced drug-seeking after a period of 3 weeks withdrawal in A_{2A} knockout (KO) and wild-type mice ($n = 12–18$ per genotype). The data are expressed as mean (±SEM). Total active lever presses during the drug-seeking session (cues present but no drug infusions, 1 h, represented by white bars) as compared to lever presses during final day of fixed ratio 1 (day 7, 2 h session, represented by black bars); *$p < 0.05$ (one-way ANOVA).

but not in A_{2A} knockout mice. One-way ANOVA revealed a significant effect of treatment ($F_{(3,44)} = 4.131$, $p < 0.01$) and a *post hoc* Student—Newman–Keuls multiple comparison test revealed a significant difference between time spent in the morphine-paired compartment *vs* time spent in the saline-paired compartment on test day in wild-type mice ($q = 4.953$, $p < 0.01$), but not knockout mice ($p > 0.05$).

A_{2A} Knockout Mice Fail to Display Tolerance to the Locomotor-Activating Properties of Morphine

During the preconditioning session locomotor activity was not significantly different between the two genotypes (data not shown). Time course data over the 4 days of morphine conditioning were analyzed by a two-way ANOVA, followed by Student—Newman–Keuls *post hoc* analyses where appropriate. As can be seen in Figure 5b, an effect of genotype was determined ($F_{(1,82)} = 21.54$, $p < 0.001$), and although there was no difference between genotypes on the first day of morphine treatment ($q = 0.572$, $p > 0.05$), the level of morphine-induced locomotor activity in wild-type mice was significantly less than that observed in A_{2A} receptor knockout mice on days 2 ($q = 4.264$, $p < 0.01$), 3 ($q = 3.949$, $p < 0.01$), and 4 ($q = 5.011$, $p < 0.001$).

The locomotor response to morphine in wild-type mice actually decreased to a point where it was not significantly different from the locomotor response to saline. This was revealed by a two-way ANOVA within genotypes. In wild-type mice, the response to morphine was significantly higher than the response to saline on days 1–3 ($q = 6.060$, 5.108, and 5.026, respectively, $p < 0.001$) but not day 4 ($q = 0.997$, $p > 0.05$). In contrast, for A_{2A} receptor knockout mice, the locomotor response to morphine was significantly higher than that to saline on days 2, 3, and 4 ($q = 3.549$, 4.964, and 3.477 respectively, $p < 0.001$). Furthermore, the sustained hyperactive response to morphine in A_{2A} knockout mice during place conditioning was confirmed with the locomotor cell data obtained during the chronic treatment regime for the sensitization protocol (Figure 6a). Student—Newman–Keuls *post hoc* analysis following a

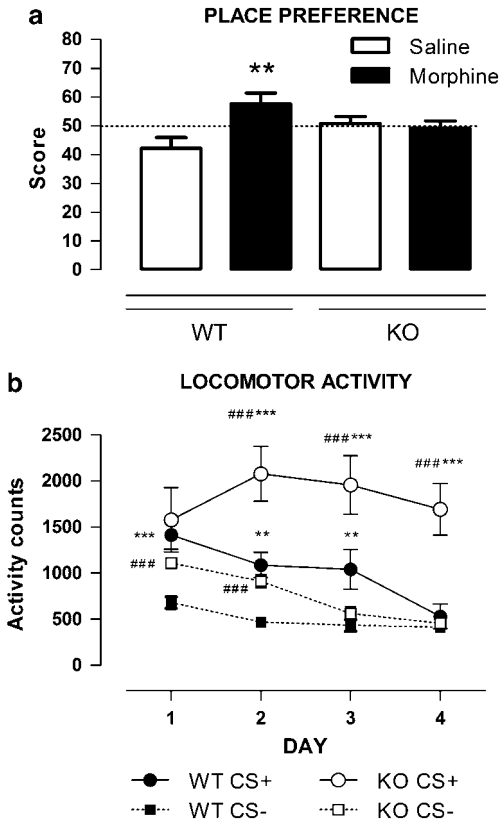

Figure 5 Conditioned place preference to morphine (10 mg/kg, i.p.) in A_{2A} receptor knockout (KO) and wild-type (WT) mice ($n = 12$ per genotype). The data are expressed as mean (± SEM) of the preference score of the specified morphine- or saline-paired compartment. The preference score is the time spent in the specified compartment divided by the total time spent in the both the drug- and saline-paired compartments, multiplied by 100. Time spent in the neutral zone is disregarded. CS− relates to saline pairing whereas CS + relates to morphine pairing. (a) Time spent in the morphine-paired compartment, compared to the saline-paired compartment during the post-conditioning session; $^{**}p < 0.01$ (one-way ANOVA). (b) Locomotor activity over the conditioning period. Data are expressed as the mean for each day (± SEM); $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to CS− for that day (two-way ANOVA); $^{\#\#\#}p < 0.001$ as compared to WT for that day (two-way ANOVA).

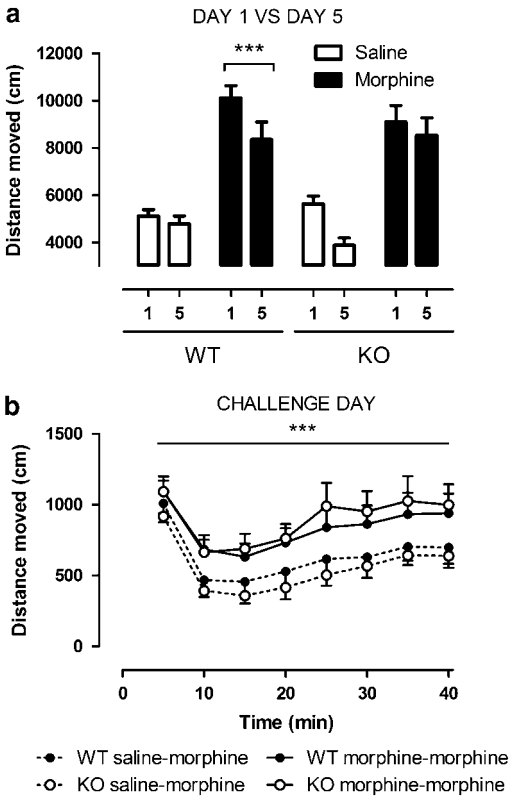

Figure 6 (a) Locomotor activity totals in response to morphine administration (10 mg/kg, i.p.) or saline administration on days 1 and 5 in A_{2A} knockout (KO) and wild-type (WT) mice. Data are expressed as the mean distance moved in cm (± SEM); $^{***}p < 0.001$, compared to day 1 (two-way ANOVA with Student—Newman–Keuls post-tests, $n = 18$–40 per group). (b) Challenge day in A_{2A} KO and WT mice: data are expressed as the mean distance moved in cm measured in 5 min time bins (± SEM); $^{***}p < 0.001$ for treatment as a factor (three-way ANOVA, with Student—Newman–Keuls post-tests, $n = 10$ per group).

two-way ANOVA revealed that the locomotor activity on the last day of morphine treatment was significantly decreased in wild-type mice compared to the first day ($q = 6.235$, $p < 0.001$). This was not the case for A_{2A} receptor knockout mice ($q = 1.192$, $p > 0.05$).

A_{2A} Receptor Deletion does not Alter the Expression of Behavioral Sensitization to Morphine

Behavioral sensitization to morphine was observed in both knockout and wild-type mice. As can be seen in Figure 6b, there was an increase in the locomotor response on challenge day in the mice pretreated with morphine compared to mice pretreated with saline. Three-way ANOVA revealed a significant effect of treatment ($F_{(1,288)} = 43.918$, $p < 0.001$) and time bin ($F_{(7,288)} = 9.430$, $p < 0.001$), but not genotype ($F_{(1,288)} = 0.556$, $p = 0.457$).

DISCUSSION

The current study demonstrates a key role for the adenosine A_{2A} receptor in the centrally mediated effects of morphine. We have shown that deletion of the adenosine A_{2A} receptor results in decreased self-administration of morphine as well as a decreased breakpoint on a PR schedule. In addition, at the dose and conditions tested, A_{2A} knockout mice did not acquire a conditioned place preference to morphine, nor develop tolerance to the locomotor-activating properties of morphine. Despite this pronounced reward-related phenotype in mice devoid of the A_{2A} receptor, this receptor is not necessary for the expression of behavioral sensitization to morphine or cue-conditioned initial drug-seeking after a period of withdrawal, as both of these behaviors were intact in A_{2A} knockout mice.

Potential differences in the 'hedonic' value of morphine were evaluated using conditioned place preference. Rewarding effects of morphine were revealed in wild-type mice, as previously reported (Pchelintsev *et al*, 1991), but these effects were absent in mice lacking the A_{2A} receptor, under the current experimental paradigm. The possibility of a learning impairment is unlikely, as both A_{2A} knockout and

wild-type mice readily acquired the instrumental learning task for sucrose self-administration. In addition, it has been reported that mice lacking the A_{2A} receptor actually display enhanced spatial recognition memory (Wang *et al*, 2003), and should presumably have no learning impairment in a conditioned place preference paradigm on this basis. Hence, these results concur with a decreased reward value of morphine in A_{2A} knockout mice. The possibility that the dose–response curve to morphine has shifted in the opposite direction in A_{2A} knockout mice cannot be completely ruled out however. A decreased place preference in A_{2A} receptor knockout mice has also been observed for THC (Soria *et al*, 2004) and nicotine (Castane *et al*, 2006), but not for cocaine (Soria *et al*, 2006), indicating a differential role for the A_{2A} receptor with regard to different drugs of abuse.

A_{2A} knockout mice reliably maintained self-administration of morphine, though the level of self-administration was markedly reduced when compared to wild-type mice. This decrease in morphine self-administration was observed for both doses tested. The effect of genotype appears to be specific to drug reinforcers, as no genotype difference in sucrose self-administration was observed over a range of concentrations. Decreased self-administration of morphine following A_{2A} receptor deletion is in agreement with previous pharmacological studies. The adenosine A_2 receptor antagonist DMPX reduces morphine self-administration in rats whereas CGS21680, a selective adenosine A_2 receptor agonist, increases morphine self-administration (Sahraei *et al*, 1999). Our data suggest a possible decreased 'reward value' of morphine in A_{2A} knockout mice and implicate the A_{2A} receptor in the reinforcing properties of morphine. An inability for A_{2A} receptor knockout mice to develop a conditioned place preference for the morphine-paired chamber is consistent with this finding, as is the reduced breakpoint on the PR schedule observed in the operant responding paradigm. Over half of the A_{2A} knockout mice assessed reached a true breakpoint during the 2 h session. This figure was only 12.5% for wild-type mice which suggests that the true breakpoint for wild-type mice may be even higher than that reported here. A lower breakpoint in A_{2A} knockout mice implies decreased motivation to obtain a reward and therefore further implicates the A_{2A} receptor in the incentive/motivational properties of morphine. Inherent issues exist in the interpretation of FR1 data with respect to 'reinforcing efficacy' of drugs (Arnold and Roberts, 1997). In contrast, PR schedule data in the form of breakpoint, response records, and the number of infusions administered during a session provide invaluable information regarding the motivational drive to obtain a drug reward. The fact that A_{2A} knockout mice displayed a reduction in all of these parameters certainly supports the hypothesis of impaired morphine reward as a result of A_{2A} receptor deletion. The possibility of the inverse occurring—that the reward value of morphine is increased in A_{2A} knockout mice, hence causing a reduction in morphine self-administration due to an increased sensitivity—cannot be ruled out, but is unlikely, as one would not expect in this scenario to observe such a poor performance by the knockout mice on a PR schedule compared to wild type. In addition, a place preference to morphine was absent in A_{2A} knockout mice, and FR1 event records for self-administration showed similar bunching of infusions between genotypes.

A decrease in the reward value of morphine as a result of A_{2A} receptor deletion is consistent with findings with other drugs of abuse. Soria *et al* (2006) observed decreased self-administration of cocaine and a reduced breakpoint on a PR in the same line of A_{2A} knockout mice. Our findings support the hypothesis proposed by these authors, that A_{2A} knockout mice represent a phenotype of low vulnerability to drug addiction and that absence of the A_{2A} receptor could provide resistance against the addictive properties of drugs of abuse such as cocaine, and now opiates.

The rewarding effects of morphine are primarily due to actions on μ-opioid receptors (Contet *et al*, 2004), in part by an increase in firing rate of midbrain dopaminergic neurons, mediated by inhibition of GABAergic interneurons within the ventral tegmental area (VTA), enhancing release of dopamine in the NAc (Johnson and North, 1992). A functional hypodopaminergic state exists in A_{2A} knockout mice (Dassesse *et al*, 2001) and two studies have demonstrated increased expression of D_1 and D_2 mRNA in the striatum of these mice (Dassesse *et al*, 2001; Short *et al*, 2006b), a finding consistent with models of decreased dopaminergic function (Fang *et al*, 1997; Smith *et al*, 1997). A decreased dopaminergic state would be in line with decreased reward in A_{2A} receptor knockout mice. A_{2A} receptors are colocalized with D_2 receptors on striatopallidal neurons, can form heterodimers (Canals *et al*, 2003), and also interact in an antagonistic fashion. A direct effect in the form of removal of the A_{2A} receptor-mediated antagonism of D_2 receptor-mediated effects is unlikely to be the mechanism by which A_{2A} receptor deletion modulates morphine reward. If this were the case one would expect an increased reward value of morphine in A_{2A} knockout mice, and not the opposite, as we and other researchers have observed.

Interactions between A_{2A} and D_2 receptors may be more complex than as described above, though, as for example, a synergistic relationship between D_2 and A_{2A} receptors was observed in relation to protein kinase A signaling in ethanol consumption (Yao *et al*, 2002). In support of D_2–A_{2A} receptor synergy, with respect to opioids, Yao *et al* (2003) demonstrated that A_{2A} receptor antagonists abolish synergistic actions between opioids and dopamine D_2 receptors. Dopamine- and cyclic AMP (cAMP)-regulated phosphoprotein of 32 kDa (DARPP-32) is a downstream effector molecule of dopamine receptors and it has been shown that the A_{2A} antagonist, SCH 58261, counteracts the increase in Thr^{34}-DARPP-32 phosphorylation observed following treatment with selective D_2 receptor antagonists (Svenningsson *et al*, 2000). This is particularly relevant in the context of morphine reward, as DARPP-32 phosphorylation at Thr^{34} is increased following morphine administration (Lindskog *et al*, 1999). In addition, despite segregation on a separate striatal output pathway, dopamine D_1 receptors have also been shown to interact with A_{2A} receptors at a 'network level', therefore influences of A_{2A} receptors on morphine reward by interactions with D_1 receptors cannot be dismissed (Short *et al*, 2006a).

Administration of moderate doses of morphine to rats produces a biphasic locomotor response, consisting of an initial reduction followed by an increase in locomotor

activity (Babbini and Davis, 1972). A similar acute locomotor response to morphine was observed in both A_{2A} knockout and wild-type mice, indicating the A_{2A} receptor is not involved in mediating the acute psychomotor response to morphine. Activation of dopamine D_1 receptors located on striatal medium spiny neurons is involved in the acute psychomotor effect of morphine. Thus, the increase in locomotion elicited by morphine can be prevented by systemic administration of SCH23390, a dopamine D_1 receptor antagonist (Jeziorski and White, 1995; Serrano *et al*, 2002), and is absent in D_1 receptor knockout mice (Becker *et al*, 2001). In contrast, the increase in motor activity produced by morphine in dopamine D_2 receptor knockout mice does not differ from that observed in wild-type littermates (Maldonado *et al*, 1997). The D_1 receptor-mediated effect is dependent on activation of the cAMP/PKA/DARPP-32 pathway (Borgkvist and Fisone, 2007). Despite the decrease in locomotor response elicited in DARPP-32 mutant mice following morphine administration, conditioned place preference and locomotor sensitization to morphine were observed. These data suggest that morphine reward and psychomotor stimulation are separate neuronal processes, the latter being dependent on activation of D_1 receptors. Adenosine A_{2A} receptors are not expressed on striatonigral neurons with D_1 receptors (Rosin *et al*, 2003). This may explain the absence of A_{2A} receptor involvement in the mediation of the acute locomotor-activating properties of morphine.

Tolerance to the locomotor-activating properties of morphine was observed in wild-type but not A_{2A} knockout mice, suggesting a perturbation of the cellular and molecular processes underlying tolerance as a result of A_{2A} receptor deletion. Tolerance to opiates is proposed to be due to upregulation of the adenylate cyclase/cAMP/PKA pathway (Nestler, 1996). Adenosine is formed from cAMP both inside and outside the cell (Hack and Christie, 2003). Morphine dose dependently increases adenosine release (Sandner-Kiesling *et al*, 2001), perhaps by inhibition of adenosine uptake (Halimi *et al*, 2000). Sensitivity to exogenous adenosine is increased at excitatory synapses in the NAc following chronic morphine treatment (Brundege and Williams, 2002), and chronic morphine treatment results in an upregulation of adenosine transporters in the striatum (Kaplan and Leite-Morris, 1997). Furthermore, an increased severity in some withdrawal symptoms has been observed in A_{2A} knockout mice (Bailey *et al*, 2004), and this is confined to withdrawal symptoms that are locomotor in nature (paw shakes and withdrawal jumping).

Repeated exposure to morphine causes an increase in motor stimulation induced by a subsequent morphine challenge, an effect known as sensitization (Babbini and Davis, 1972; Lett, 1989). This behavioral (and presumably incentive) sensitization is believed to reflect some of the motivational aspects of drug addiction, such as craving and drug-seeking (Robinson and Berridge, 1993). Previous work has suggested a role for adenosine in morphine sensitization (Weisberg and Kaplan, 1999). Adenosine A_{2A} knockout mice sensitized to morphine in the current study indicating that the A_{2A} receptor is not necessary for the development or expression of sensitization to morphine. This is consistent with the finding that selective A_1 receptor antagonists inhibit the development of morphine sensitization in mice (Weisberg and Kaplan, 1999), suggesting that any role that adenosine may play in morphine sensitization is likely mediated by the A_1 receptor.

Previous work has demonstrated a definitive role for glutamate, but not necessarily dopamine, in behavioral sensitization to morphine (Vanderschuren and Kalivas, 2000). In addition, the VTA appears to be the site important for the development of sensitization to opiate agonists (Vanderschuren and Kalivas, 2000). Thus, opioid receptor antagonist administration to the VTA, but not the NAc, blocks the development of sensitization (Kalivas and Duffy, 1987), and morphine administration to the VTA, but not the NAc, causes sensitization (Vezina *et al*, 1987). Involvement of the VTA as opposed to the NAc supports the lack of involvement of the A_{2A} receptor in induction of morphine sensitization as the A_{2A} receptor is not localized within the VTA (Rosin *et al*., 1998). Thus, it is afferents projecting to the VTA (such as corticotegmental inputs) that would be presumably the anatomical substrates for this process, as opposed to the medium spiny neurons of the striatopallidal neurons where A_{2A} receptors are predominantly located.

In contrast, the expression of behavioral sensitization to morphine does appear to involve the NAc (Vanderschuren and Kalivas, 2000). There are presynaptic A_{2A} receptors located on cortical glutamatergic afferents to the NAc and these have been shown to form heterodimers with A_1 receptors able to regulate glutamate release (Ciruela *et al*, 2006). Despite a role for the A_{2A} receptor in presynaptic regulation of glutamatergic input to the NAc, deletion of the A_{2A} receptor does not prevent the expression of behavioral sensitization to morphine. This suggests the drug-induced plasticity resulting from repeated morphine administration can occur in the absence of the A_{2A} receptor, though adaptive compensation in A_{2A} knockout mice cannot be ruled out.

In humans, drug-associated stimuli is important in drug relapse after prolonged abstinence (Kalivas and Volkow, 2005). In animals, cue-conditioned drug-seeking is said to model the formation of contextual/cued associations with drugs of abuse in humans (Shaham *et al*, 2003). Significant studies have shown that cue-induced drug-seeking increases over time during abstinence, leading to the concept of 'incubation of craving' (Grimm *et al*, 2001). This is a robust phenomenon that has been observed across a number of different drug classes in rats (Di Ciano and Everitt, 2004; Grimm *et al*, 2002; Shalev *et al*, 2001; Lu *et al*, 2007). Moreover, this type of delayed onset craving can be present in human cocaine users (Kosten *et al*, 2005). Both wild-type and A_{2A} knockout mice exhibited robust cue-induced drug-seeking behavior, under extinction conditions (no drug delivery) following a 3-week withdrawal period. These data are consistent with the hypothesis that incubation of craving for an opiate occurs in mice. Increased drug-seeking behavior is most likely a result of neuroadaptations that occur over time with repeated drug administration. Absence of the A_{2A} receptor does not affect cue-induced drug-seeking behavior following a period of withdrawal. This suggests that the lack of A_{2A} receptors does not prevent the pathophysiological changes that contribute to compulsive drug-seeking behavior, despite mutant mice showing a reduced level of morphine self-administration. Importantly, although a number of brain nuclei have been implicated in

cue-induced drug-seeking following extinction, there appears to be a critical role for the basolateral amygdala in this process (Fuchs and See, 2002), an area essentially devoid of A_{2A} receptors (Rosin *et al*, 1998). A recent study (Yao *et al*, 2006) found that intra-accumbal administration of the A_{2A} receptor antagonist DMPX reduced heroin-primed reinstatement of heroin-seeking in rats, confirming the involvement of multiple brain nuclei in reinstatement of drug-seeking. Importantly, there are network differences between drug-primed drug-seeking and cue-induced drug-seeking that could contribute to these apparently opposing results (Shaham *et al*, 2003). Clearly, further studies are warranted to explore in detail the role of the adenosine A_{2A} receptor in the process of extinction and subsequent reinstatement. Moreover, our data suggest that mice are a suitable species to probe the genetics and mechanism(s) behind incubation of craving.

In conclusion, under the conditions of our experiments, our data are consistent with a putative dislocation in the role of the A_{2A} receptor in the acute rewarding effects of morphine and the drug-induced plasticity occurring as a result of repeated morphine administration. This is demonstrated by decreased morphine reward in A_{2A} knockout mice, but intact psychomotor sensitization and cue-conditioned initial drug-seeking following a period of withdrawal.

ACKNOWLEDGEMENTS

This work was supported by a program grant (236805) from the National Health and Medical Research Council of Australia of which AJL is a senior research fellow. An equipment grant from ANZ Trustees (William Buckland Foundation) contributed toward the purchase of mouse operant chambers.

DISCLOSURE/CONFLICT OF INTEREST

None.

REFERENCES

Arnold JM, Roberts DC (1997). A critique of fixed and progressive ratio schedules used to examine the neural substrates of drug reinforcement. *Pharmacol Biochem Behav* **57**: 441–447.

Arolfo MP, Yao L, Gordon AS, Diamond I, Janak PH (2004). Ethanol operant self-administration in rats is regulated by adenosine A2 receptors. *Alcohol Clin Exp Res* **28**: 1308–1316.

Babbini M, Davis WM (1972). Time–dose relationships for locomotor activity effects of morphine after acute or repeated treatment. *Br J Pharmacol* **46**: 213–224.

Bailey A, Davis L, Lesscher HM, Kelly MD, Ledent C, Hourani SM *et al* (2004). Enhanced morphine withdrawal and mu-opioid receptor G-protein coupling in A2a adenosine receptor knockout mice. *J Neurochem* **88**: 827–834.

Becker A, Grecksch G, Kraus J, Peters B, Schroeder H, Schulz S *et al* (2001). Loss of locomotor sensitisation in response to morphine in D1 receptor deficient mice. *Naunyn Schmiedebergs Arch Pharmacol* **363**: 562–568.

Berrendero F, Castane A, Ledent C, Parmentier M, Maldonado R, Valverde O (2003). Increase of morphine withdrawal in mice lacking A2a receptors and no changes in CB1/A2a double knockout mice. *Eur J Neurosci* **17**: 315–324.

Borgkvist A, Fisone G (2007). Psychoactive drugs and regulation of the camp/pka/darpp-32 cascade in striatal medium spiny neurons. *Neurosci Biobehav Rev* **31**: 79–88.

Brundege JM, Dunwiddie TV (1997). Role of adenosine as a modulator of synaptic activity in the central nervous system. *Adv Pharmacol* **39**: 353–391.

Brundege JM, Williams JT (2002). Increase in adenosine sensitivity in the nucleus accumbens following chronic morphine treatment. *J Neurophysiol* **87**: 1369–1375.

Canals M, Marcellino D, Fanelli F, Ciruela F, de Benedetti P, Goldberg SR *et al* (2003). Adenosine A2a-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J Biol Chem* **278**: 46741–46749.

Castane A, Soria G, Ledent C, Maldonado R, Valverde O (2006). Attenuation of nicotine-induced rewarding effects in A2a knockout mice. *Neuropharmacology* **51**: 631–640.

Chen JF, Beilstein M, Xu YH, Turner TJ, Moratalla R, Standaert DG *et al* (2000). Selective attenuation of psychostimulant-induced behavioral responses in mice lacking A2a adenosine receptors. *Neuroscience* **97**: 195–204.

Ciruela F, Casado V, Rodrigues RJ, Lujan R, Burgueno J, Canals M *et al* (2006). Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2a receptor heteromers. *J Neurosci* **26**: 2080–2087.

Clemens KJ, Cornish JL, Hunt GE, McGregor IS (2006). Intravenous methamphetamine self-administration in rats: effects of intravenous or intraperitoneal MDMA co-administration. *Pharmacol Biochem Behav* **85**: 454–463.

Contet C, Kieffer BL, Befort K (2004). Mu opioid receptor: a gateway to drug addiction. *Curr Opin Neurobiol* **14**: 370–378.

Cornish JL, Clemens KJ, Thompson MR, Callaghan PD, Dawson B, McGregor IS (2008). High ambient temperature increases intravenous methamphetamine self-administration on fixed and progressive ratio schedules in rats. *J Psychopharmacol* **22**: 100–110.

Dassesse D, Massie A, Ferrari R, Ledent C, Parmentier M, Arckens L *et al* (2001). Functional striatal hypodopaminergic activity in mice lacking adenosine A2a receptors. *J Neurochem* **78**: 183–198.

Di Chiara G, Imperato A (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci USA* **85**: 5274–5278.

Di Ciano P, Everitt BJ (2004). Conditioned reinforcing properties of stimuli paired with self-administered cocaine, heroin or sucrose: implications for the persistence of addictive behaviour. *Neuropharmacology* **47**(Suppl 1): 202–213.

Dunwiddie TV (1985). The physiological role of adenosine in the central nervous system. *Int Rev Neurobiol* **27**: 63–139.

Fang Y, Janowsky A, Ronnekleiv OK (1997). Cocaine exposure in fetal rhesus monkey: consequences for dopamine D1- and D2-like receptor binding densities. *Brain Res Dev Brain Res* **104**: 163–174.

Ferre S, Fredholm BB, Morelli M, Popoli P, Fuxe K (1997). Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. *Trends Neurosci* **20**: 482–487.

Ferre S, Herrera-Marschitz M, Grabowska-Anden M, Casas M, Ungerstedt U, Anden NE (1991a). Postsynaptic dopamine/adenosine interaction: II. Postsynaptic dopamine agonism and adenosine antagonism of methylxanthines in short-term reserpinized mice. *Eur J Pharmacol* **192**: 31–37.

Ferre S, Rubio A, Fuxe K (1991b). Stimulation of adenosine A2 receptors induces catalepsy. *Neurosci Lett* **130**: 162–164.

Ferre S, Snaprud P, Fuxe K (1993). Opposing actions of an adenosine a2 receptor agonist and a gtp analogue on the regulation of dopamine D2 receptors in rat neostriatal membranes. *Eur J Pharmacol* **244**: 311–315.

Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM *et al* (1992). Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Brain Res Mol Brain Res* **14**: 186–195.

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA *et al* (1994). VI. Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46**: 143–156.

Fredholm BB, AP IJ, Jacobson KA, Klotz KN, Linden J (2001a). International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53**: 527–552.

Fredholm BB, Irenius E, Kull B, Schulte G (2001b). Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem Pharmacol* **61**: 443–448.

Fuchs RA, See RE (2002). Basolateral amygdala inactivation abolishes conditioned stimulus- and heroin-induced reinstatement of extinguished heroin-seeking behavior in rats. *Psychopharmacology (Berl)* **160**: 425–433.

Griffin III WC, Middaugh LD (2003). Acquisition of lever pressing for cocaine in C57Bl/6J mice: effects of prior Pavlovian conditioning. *Pharmacol Biochem Behav* **76**: 543–549.

Grimm JW, Hope BT, Wise RA, Shaham Y (2001). Neuroadaptation. Incubation of cocaine craving after withdrawal. *Nature* **412**: 141–142.

Grimm JW, Shaham Y, Hope BT (2002). Effect of cocaine and sucrose withdrawal period on extinction behavior, cue-induced reinstatement, and protein levels of the dopamine transporter and tyrosine hydroxylase in limbic and cortical areas in rats. *Behav Pharmacol* **13**: 379–388.

Hack SP, Christie MJ (2003). Adaptations in adenosine signaling in drug dependence: therapeutic implications. *Crit Rev Neurobiol* **15**: 235–274.

Halimi G, Devaux C, Clot-Faybesse O, Sampol J, Legof L, Rochat H *et al* (2000). Modulation of adenosine concentration by opioid receptor agonists in rat striatum. *Eur J Pharmacol* **398**: 217–224.

Hettinger BD, Lee A, Linden J, Rosin DL (2001). Ultrastructural localization of adenosine A2a receptors suggests multiple cellular sites for modulation of GABAergic neurons in rat striatum. *J Comp Neurol* **431**: 331–346.

Jeziorski M, White FJ (1995). Dopamine receptor antagonists prevent expression, but not development, of morphine sensitization. *Eur J Pharmacol* **275**: 235–244.

Johnson SW, North RA (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J Neurosci* **12**: 483–488.

Kalivas PW, Duffy P (1987). Sensitization to repeated morphine injection in the rat: possible involvement of A10 dopamine neurons. *J Pharmacol Exp Ther* **241**: 204–212.

Kalivas PW, Volkow ND (2005). The neural basis of addiction: a pathology of motivation and choice. *Am J Psychiatry* **162**: 1403–1413.

Kaplan GB, Coyle TS (1998). Adenosine kinase inhibitors attenuate opiate withdrawal via adenosine receptor activation. *Eur J Pharmacol* **362**: 1–8.

Kaplan GB, Leite-Morris KA (1997). Up-regulation of adenosine transporter-binding sites in striatum and hypothalamus of opiate tolerant mice. *Brain Res* **763**: 215–220.

Kaplan GB, Sears MT (1996). Adenosine receptor agonists attenuate and adenosine receptor antagonists exacerbate opiate withdrawal signs. *Psychopharmacology (Berl)* **123**: 64–70.

Kosten T, Sofuoglu M, Poling J, Gonsai K, Oliveto A (2005). Desipramine treatment for cocaine dependence in buprenorphine- or methadone-treated patients: baseline urine results as predictor of response. *Am J Addict* **14**: 8–17.

Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ *et al* (1997). Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* **388**: 674–678.

Lett BT (1989). Repeated exposures intensify rather than diminish the rewarding effects of amphetamine, morphine, and cocaine. *Psychopharmacology (Berl)* **98**: 357–362.

Lindskog M, Svenningsson P, Fredholm B, Greengard P, Fisone G (1999). Mu- and delta-opioid receptor agonists inhibit DARPP-32 phosphorylation in distinct populations of striatal projection neurons. *Eur J Neurosci* **11**: 2182–2186.

Lu L, Uejima JL, Gray SM, Bossert JM, Shaham Y (2007). Systemic and central amygdala injections of the mGlur(2/3) agonist LY379268 attenuate the expression of incubation of cocaine craving. *Biol Psychiatry* **61**: 591–598.

Maldonado R, Saiardi A, Valverde O, Samad TA, Roques BP, Borrelli E (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature* **388**: 586–589.

McPherson CS, Lawrence AJ (2006). Exposure to amphetamine in rats during periadolescence establishes behavioural and extrastriatal neural sensitization in adulthood. *Int J Neuropsychopharmacol* **9**: 377–392.

Nestler EJ (1996). Under siege: the brain on opiates. *Neuron* **16**: 897–900.

Pchelintsev MV, Gorbacheva EN, Zvartau EE (1991). Simple methodology of assessment of analgesics' addictive potential in mice. *Pharmacol Biochem Behav* **39**: 873–876.

Pinna A, di Chiara G, Wardas J, Morelli M (1996). Blockade of A2a adenosine receptors positively modulates turning behaviour and c-fos expression induced by D1 agonists in dopamine-denervated rats. *Eur J Neurosci* **8**: 1176–1181.

Pollack AE, Fink JS (1996). Synergistic interaction between an adenosine antagonist and a D1 dopamine agonist on rotational behavior and striatal c-fos induction in 6-hydroxydopamine-lesioned rats. *Brain Res* **743**: 124–130.

Robinson TE, Berridge KC (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev* **18**: 247–291.

Rosin DL, Hettinger BD, Lee A, Linden J (2003). Anatomy of adenosine A2a receptors in brain: morphological substrates for integration of striatal function. *Neurology* **61**: S12–S18.

Rosin DL, Robeva A, Woodard RL, Guyenet PG, Linden J (1998). Immunohistochemical localization of adenosine A2a receptors in the rat central nervous system. *J Comp Neurol* **401**: 163–186.

Sahraei H, Motamedi F, Khoshbaten A, Zarrindast MR (1999). Adenosine A2 receptors inhibit morphine self-administration in rats. *Eur J Pharmacol* **383**: 107–113.

Salem A, Hope W (1997). Effect of adenosine receptor agonists and antagonists on the expression of opiate withdrawal in rats. *Pharmacol Biochem Behav* **57**: 671–679.

Sandner-Kiesling A, Li X, Eisenach JC (2001). Morphine-induced spinal release of adenosine is reduced in neuropathic rats. *Anesthesiology* **95**: 1455–1459.

Schiffmann SN, Libert F, Vassart G, Vanderhaeghen JJ (1991). Distribution of adenosine A2 receptor mRNA in the human brain. *Neurosci Lett* **130**: 177–181.

Serrano A, Aguilar MA, Manzanedo C, Rodriguez-Arias M, Minarro J (2002). Effects of DA D1 and D2 antagonists on the sensitisation to the motor effects of morphine in mice. *Prog Neuropsychopharmacol Biol Psychiatry* **26**: 1263–1271.

Shaham Y, Shalev U, Lu L, De Wit H, Stewart J (2003). The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology (Berl)* **168**: 3–20.

Shalev U, Morales M, Hope B, Yap J, Shaham Y (2001). Time-dependent changes in extinction behavior and stress-induced reinstatement of drug seeking following withdrawal from heroin in rats. *Psychopharmacology (Berl)* **156**: 98–107.

Short JL, Drago J, Lawrence AJ (2006a). Comparison of ethanol preference and neurochemical measures of mesolimbic

dopamine and adenosine systems across different strains of mice. *Alcohol Clin Exp Res* **30**: 606–620.

Short JL, Ledent C, Drago J, Lawrence AJ (2006b). Receptor crosstalk: characterization of mice deficient in dopamine D1 and adenosine A2a receptors. *Neuropsychopharmacology* **31**: 525–534.

Smith TS, Trimmer PA, Khan SM, Tinklepaugh DL, Bennett Jr JP (1997). Mitochondrial toxins in models of neurodegenerative diseases. II: elevated zif268 transcription and independent temporal regulation of striatal D1 and D2 receptor mRNAs and D1 and D2 receptor-binding sites in C57Bl/6 mice during MPTP treatment. *Brain Res* **765**: 189–197.

Snell BJ, Short JL, Drago J, Ledent C, Lawrence AJ (2000). Characterisation of central adenosine A1 receptors and adenosine transporters in mice lacking the adenosine A2a receptor. *Brain Res* **877**: 160–169.

Snyder SH (1985). Adenosine as a neuromodulator. *Annu Rev Neurosci* **8**: 103–124.

Soria G, Castane A, Berrendero F, Ledent C, Parmentier M, Maldonado R *et al* (2004). Adenosine A2a receptors are involved in physical dependence and place conditioning induced by THC. *Eur J Neurosci* **20**: 2203–2213.

Soria G, Castane A, Ledent C, Parmentier M, Maldonado R, Valverde O (2006). The lack of A2a adenosine receptors diminishes the reinforcing efficacy of cocaine. *Neuropsychopharmacology* **31**: 978–987.

Svenningsson P, Le Moine C, Kull B, Sunahara R, Bloch B, Fredholm BB (1997). Cellular expression of adenosine A2a receptor messenger RNA in the rat central nervous system with special reference to dopamine innervated areas. *Neuroscience* **80**: 1171–1185.

Svenningsson P, Lindskog M, Ledent C, Parmentier M, Greengard P, Fredholm BB *et al* (2000). Regulation of the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa *in vivo* by dopamine D1, dopamine D2, and adenosine A2a receptors. *Proc Natl Acad Sci USA* **97**: 1856–1860.

Svenningsson P, Lindskog M, Rognoni F, Fredholm BB, Greengard P, Fisone G (1998). Activation of adenosine A2a and dopamine D1 receptors stimulates cyclic AMP-dependent phosphorylation of DARPP-32 in distinct populations of striatal projection neurons. *Neuroscience* **84**: 223–228.

Thomsen M, Woldbye DP, Wortwein G, Fink-Jensen A, Wess J, Caine SB (2005). Reduced cocaine self-administration in muscarinic M5 acetylcholine receptor-deficient mice. *J Neurosci* **25**: 8141–8149.

Trigo JM, Panayi F, Soria G, Maldonado R, Robledo P (2006). A reliable model of intravenous MDMA self-administration in naive mice. *Psychopharmacology (Berl)* **184**: 212–220.

Vanderschuren LJ, Kalivas PW (2000). Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: A critical review of preclinical studies. *Psychopharmacology (Berl)* **151**: 99–120.

Vezina P, Kalivas PW, Stewart J (1987). Sensitization occurs to the locomotor effects of morphine and the specific mu opioid receptor agonist, DAGO, administered repeatedly to the ventral tegmental area but not to the nucleus accumbens. *Brain Res* **417**: 51–58.

Wang JH, Short J, Ledent C, Lawrence AJ, van den Buuse M (2003). Reduced startle habituation and prepulse inhibition in mice lacking the adenosine A2a receptor. *Behav Brain Res* **143**: 201–207.

Weisberg SP, Kaplan GB (1999). Adenosine receptor antagonists inhibit the development of morphine sensitization in the C57Bl/6 mouse. *Neurosci Lett* **264**: 89–92.

Yang M, Soohoo D, Soelaiman S, Kalla R, Zablocki J, Chu N *et al* (2007). Characterization of the potency, selectivity, and pharmacokinetic profile for six adenosine A2a receptor antagonists. *Naunyn Schmiedebergs Arch Pharmacol* **375**: 133–144.

Yao L, Arolfo MP, Dohrman DP, Jiang Z, Fan P, Fuchs S *et al* (2002). $\beta\gamma$ dimers mediate synergy of dopamine D2 and adenosine A2 receptor-stimulated PKA signaling and regulate ethanol consumption. *Cell* **109**: 733–743.

Yao L, Fan P, Jiang Z, Mailliard WS, Gordon AS, Diamond I (2003). Addicting drugs utilize a synergistic molecular mechanism in common requiring adenosine and Gi- $\beta\gamma$ dimers. *Proc Natl Acad Sci USA* **100**: 14379–14384.

Yao L, McFarland K, Fan P, Jiang Z, Ueda T, Diamond I (2006). Adenosine A2a blockade prevents synergy between mu-opiate and cannabinoid CB1 receptors and eliminates heroin-seeking behavior in addicted rats. *Proc Natl Acad Sci USA* **103**: 7877–7882.