Neuropsychopharmacology (2006) 31, 978–987

www.neuropsychopharmacology.org

The Lack of A_{2A} Adenosine Receptors Diminishes the Reinforcing Efficacy of Cocaine

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Adenosine is an endogenous purine nucleoside, which acts as a neuromodulator in the central nervous system. A_{2A} adenosine and D_2 dopamine receptors are colocalized in the same neurons in discrete brain areas, and the dopaminergic transmission plays a crucial role in the addictive properties of drugs of abuse, such as cocaine. In the present study, we have investigated the specific role of A_{2A} adenosine receptors in cocaine-induced behavioral responses related to its addictive properties. For this purpose, we have evaluated the acute locomotor effects produced by cocaine and the development of locomotor sensitization by repeated cocaine administration. In addition, we have also examined cocaine acute rewarding properties using the conditioned place preference. Finally, we used the intravenous drug self-administration paradigm to investigate the acquisition of an operant response maintained by cocaine self-administration and the reinforcing efficacy of the drug in these knockout animals. Acute cocaine induced a similar increase of locomotor activity in mice lacking A_{2A} adenosine receptors and wild-type littermates. Cocaine-induced locomotor sensitization and conditioned place preference were also maintained in A_{2A} knockout mice. Nevertheless, these knockout mice showed a lower rate of cocaine self-administration than wild-type mice in both fixed ratio 1 and 3 schedules of reinforcement. Moreover, a reduction in the maximal effort to obtain a cocaine infusion was found in A_{2A} knockout mice under a progressive ratio schedule. In addition, a vertical shift of the cocaine dose–response curve was observed in mice lacking A_{2A} adenosine receptors in comparison with wild-type littermates. Our study demonstrates that A_{2A} adenosine receptors play an important role in cocaine addictive properties, and these receptors seem to be required to develop the addictive effects of this drug.

Neuropsychopharmacology (2006) **31,** 978–987. doi:10.1038/sj.npp.1300876; published online 17 August 2005

Keywords: mouse; knockout; cocaine reinforcement; self-administration; conditioned place preference; reward; sensitization; A_{2A} adenosine receptors

INTRODUCTION

Adenosine is an endogenous purine nucleoside, which acts as a neuromodulator in the central nervous system (CNS). The physiological effects of adenosine are produced through the activation of four receptor types: A_1, A_{2A}, A_{2B}, and A_3. A_{2A} adenosine receptors are found at high concentrations in the olfactory tubercle, the striatum, and the nucleus accumbens (Nac) (Moreau and Huber, 1999). In the striatum, A_{2A} adenosine receptors are particularly expressed in the GABAergic striatopallidal neurons, where they are colocalized with D_2 dopamine receptors (Ferré *et al*, 1997). Adenosine regulates dopamine transmission through antagonistic interactions between adenosine A_1/dopamine D_1 receptors and adenosine A_{2A}/dopamine D_2 receptors (Franco *et al*, 2000). Dopaminergic transmission in the mesocorticolimbic system plays a crucial role in the modulation of reward-related process (Koob, 1996; Di Chiara, 2002), as well as in the addictive properties of drugs of abuse.

There is evidence to support a role for adenosine in mediating different responses induced by several drugs of abuse such as opioids, cannabinoids, and psychostimulants. Thus, adenosine agonists inhibit the expression of morphine withdrawal, while adenosine antagonists increase the incidence of withdrawal signs (Kaplan and Sears, 1996; Salem and Hope, 1997). In agreement, mice lacking A_{2A} adenosine receptors showed an increased morphine withdrawal in comparison with wild-type mice (Berrendero *et al*, 2003). On the other hand, we have recently shown that A_{2A} knockout mice exhibited a significant reduction of Δ^9-tetrahydrocannabinol (THC)-induced rewarding and aversive effects, as well as rimonabant-precipitated THC withdrawal syndrome (Soria *et al*, 2004). However, there are

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Received 1 March 2005; revised and accepted 18 July 2005
Online publication: 19 July 2005 at http://www.acnp.org/citations/Npp07190505014 2/default.pdf

controversial results about the possible involvement of A_{2A} adenosine receptors in the reinforcing effects induced by psychostimulant drugs. It has been demonstrated that the A_{2A} receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) and the nonselective adenosine antagonist caffeine decreased cocaine-induced conditioned place preference in rats (Poleszak and Malec, 2002), and that the A_2 agonist 5′-*N*-ethylcarboxamido-adenosine (NECA) reduced cocaine self-administration in rats (Knapp *et al*, 2001). A role for adenosine in reinstatement of cocaine-seeking behavior has also been suggested since caffeine and CGS15943 reinstated this behavior (Weerts and Griffiths, 2003). In addition, the selective A_{2A} receptor agonist CGS21680 attenuated the rewarding impact of brain stimulation, whereas the A_2 adenosine antagonist DMPX reversed the reward impairment produced by cocaine withdrawal (Baldo *et al*, 1999). Furthermore, CGS21680 also potentiated the discriminative-stimulus actions of cocaine as shown by the leftward shift of the cocaine dose–response curve (Justinova *et al*, 2003). Therefore, the specific role of A_{2A} adenosine receptors in the processes underlying cocaine addiction remains unclear.

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 genetic model to clarify the role of A_{2A} receptors on cocaine pharmacological responses *in vivo*. The aim of the present study was to investigate the specific role of A_{2A} adenosine receptors in cocaine-induced behavioral responses related to its addictive properties. For this purpose, we have evaluated the acute locomotor effects produced by cocaine and the development of locomotor sensitization by repeated cocaine administration. In addition, we have also examined cocaine acute rewarding properties using the conditioned place preference. Finally, we used the intravenous (i.v.) drug self-administration paradigm in order to evaluate the acquisition of an operant response maintained by cocaine self-administration and the reinforcing efficacy of the drug in these knockout animals.

MATERIALS AND METHODS

Animals

Mice lacking A_{2A} adenosine receptors were generated as previously reported (Ledent *et al*, 1997). In order to homogenize the genetic background of the mice, the first generation heterozygotes were bred for 30 generations on a CD1 background (Charles River, France) with selection for the mutant A_{2A} gene at each generation. The A_{2A} receptor knockout mice were derived from the backcrossing of chimeric CD1-A_{2A} receptor knockout mice, developed by Ledent *et al* (1997), with wild-type CD1 females (Charles River, France). Beginning with the 30th generation of backcrossed mice, heterozygote–heterozygote matings of A_{2A} knockout mice produced wild-type and knockout littermates for subsequent experiments. Breeding couples were periodically renovated by crossing heterozygote mice with wild-type CD1 females (Charles River, France) in order to maintain a genetically diverse outbred background.

A_{2A} knockout mice and wild-type littermates (30–35 g) (14-week-old) were housed five per cage in temperature ($21 \pm 1^{\circ}$C)- and humidity ($55 \pm 10\%$)-controlled rooms, with a 12-h light/12-h dark cycle (light between 0800 and 2000). For the self-administration study, mice were exposed to a 12 h light : dark reversed cycle (light on between 2000 and 0800), and the experiments took place during the dark phase. Food and water were available *ad libitum* during all experiments except during the exposure to the different behavioral paradigms. Mice were handled for 1 week before starting the experiments.

Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA-IMAS-UPF). All experiments were performed under blind conditions.

Drugs

Cocaine hydrochloride was obtained from Ministerio Sanidad y Consumo (Spain) and dissolved in sterile 0.9% physiological saline.

Locomotor Effects Induced by Cocaine

Locomotor activity responses induced by an acute injection of cocaine (5, 10, and 20 mg/kg, intraperitoneal (i.p.)) or vehicle were evaluated using locomotor activity boxes ($9 \times 20 \times 11$ cm) (Imetronic, Bordeaux, France). The boxes were provided with two lines of 14 photocells each, one 2 cm above the floor to measure horizontal activity, and the other located 6 cm above the floor to measure vertical activity (rears), in a low luminosity environment (5 lux). Mice were habituated to the locomotor cages during 10 min for 3 consecutive days. On day 4, mice were placed in the locomotor activity boxes immediately after cocaine (5, 10, and 20 mg/kg, i.p.) or vehicle injection, and their locomotor activity was recorded during 10 min. Locomotor activity was measured as number of beam breaks, and two different measures were evaluated: directional or ambulatory movements and small local movements.

'Context-specific' sensitization to the locomotor responses induced by chronic cocaine treatment. Mice treated with 10 mg/kg of cocaine or saline on the acute test received chronically the same treatment during the following 12 days. These animals received cocaine (10 mg/kg, i.p.) or vehicle once daily and were then immediately confined into the locomotor activity boxes to record locomotion during 10 min. After completing chronic cocaine administration on day 12, mice remained without any treatment from day 13 to 18. On day 19, animals received a challenge of cocaine (10 mg/kg, i.p.) or vehicle, and locomotor activity was measured for 10 min in order to evaluate the sensitization maintenance. On day 20, all the mice received a saline injection and locomotor activity was evaluated again for 10 min. As above, locomotor activity was measured as number of beam breaks, and two different measures were evaluated: directional or ambulatory movements and small local movements.

Cocaine-Induced Conditioned Place Preference

The acute rewarding properties of cocaine (5, 10, and 20 mg/kg, i.p.) were measured using a place conditioning

paradigm. The apparatus consisted of two main square conditioning compartments ($15 \times 15 \times 15$ cm), with differences in visual and tactile cues, separated by a triangular central area (Maldonado *et al*, 1997). The light intensity within the conditioning chambers was 30 lux. During the preconditioning phase, drug-naive mice were placed in the middle of the central area and had free access to both compartments of the apparatus for 18 min. The time spent in each compartment was recorded by computerized monitoring software (Smart; Panlab, Barcelone, Spain). During the conditioning phase, mice received alternating injections of cocaine (5, 10, or 20 mg/kg) or vehicle, and were immediately confined into one of the two conditioning compartments during 20 min. Three pairings were performed with cocaine and three pairings with vehicle on alternate days. Treatments were counterbalanced as closely as possible between compartments. Control animals received vehicle every day. The postconditioning phase was conducted exactly as the preconditioning phase, that is, free access to each compartment for 18 min. A score value was calculated for each mouse as the difference between times spent in the drug-paired compartment during the postconditioning and preconditioning phases.

Cocaine Maintained Operant Self-Administration

Apparatus. The self-administration experiments were conducted in mouse operant chambers (Model ENV-307A-CT, Medical Associates, Georgia, VT, USA) equipped with two holes, one was selected as active hole for delivering the reinforcer and the other as inactive hole. Nose-poking on the active hole resulted in a reinforcer (cocaine infusion) while nose-poking on the inactive hole had no consequences. The chambers were housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and white noise. A stimulus light, located above the active hole, was paired contingently with the delivery of the reinforcer.

Surgery for drug self-administration study. Mice were anesthetized under isoflurane anesthesia (1.5–2.0%) and then implanted with indwelling i.v. silastic catheters as described previously (Caine *et al*, 1999) with minor modifications. Briefly, a 6 cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter) (Silastic®, Dow Corning, Houdeng-Goegnies, Belgium) was fitted to a 22-gauge steel cannula (Semat, Herts, England) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing runs subcutaneously to the cannula, which exits at the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline, Spain). After surgery, animals were allowed to recover for 3 days prior to initiation of self-administration sessions. The catheter was flushed daily with a saline solution containing heparin (30 UI/ml) in order to maintain its patency. The patency of i.v. catheters was evaluated periodically (approximately every 6 days) and whenever drug self-administration behavior appeared to deviate dramatically from that observed previously, by infusion of 0.1 ml of tiobarbital (5 mg/ml) through the catheter. If prominent signs of anesthesia were not apparent within 3 s of the infusion, the mouse was removed from the experiment.

Drug self-administration procedure. Cocaine self-administration sessions were performed as described previously (Soria *et al*, 2005). Briefly, sessions started 3 days after surgery. Responding was maintained by cocaine (1 mg/kg per injection) delivered in 23.5 µl over 2 s. Cocaine was infused via a syringe that was mounted on a microinfusion pump (PHM-100A, Med-Associates, Georgia, VT, USA) and connected via Tygon tubing (0.96 mm outer diameter, Portex Fine Bore Polythene Tubing, Portex Limited, Kent, England) to a single-channel liquid swivel (375/25, Instech Laboratories, Plymouth Meeting, PA, USA) and to the mouse i.v. catheters. The swivel was mounted on a counterbalanced arm above the operant chamber. Self-administration sessions (1 h daily) were conducted 6 days per week. The house light was on at the beginning of the session for 3 s and off during the remaining time of the session. Each daily session started with a priming injection of the drug. First, mice were trained under a fixed ratio 1 (FR1) schedule of reinforcement. A 30 s time-out period was established after each reinforcement. During this 30 s period, the cue light was off and no reward was provided on the active hole. Responses on the inactive hole and all the responses during the 30 s time-out period were also recorded. The session was terminated after 50 reinforcers were delivered or after 1 h, whichever occurred first. The stimulus light signaled delivery of the reinforcer. The criteria for the acquisition were achieved when mice maintained a stable responding with less than 20% deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% of stability), with at least 75% responding on the active hole, and a minimum of 10 reinforcers per session. Once mice achieved the acquisition criteria, the reinforcement schedule was changed to fixed ratio 3 (FR3). The same criteria as above were used to move mice from FR3 to a progressive ratio (PR) schedule in which the response requirement to earn an injection escalates according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The PR session lasted for 2 h or until mice did not complete the ratio for delivery of one reinforcer within 1 h, and was performed only once. The breaking point to extinguish self-administration behavior was determined in each animal. After each session, mice were returned to their home-cages.

A cocaine dose–response curve was performed in new group of naive animals that were operated and trained to self-administer cocaine (1 mg/kg/infusion) under an FR1 schedule of reinforcement on the same conditions as described before. After acquisition (same criteria as above), self-administration of various doses of cocaine (0.032, 0.1, 0.32, 1, and 3.2 mg/kg/infusion) was tested during 2 h using a Latin square design: a single dose was presented each session, and the order of presentation was counterbalanced between mice.

Statistical Analysis

Acute effects of cocaine administration and cocaine-induced conditioned place preference scores were com-

pared by two-way ANOVA (genotype and treatment as factors of variation) between subjects, followed by one-way ANOVA and Tukey *post hoc* test when required. Data of sensitization study were compared by three-way ANOVA (genotype and treatment as between factors and day as within-group factor of variation). Tukey *post hoc* test was performed when required. For cocaine maintained operant responding, two-way ANOVA was performed on the mean of nose-pokes performed during the last 3 days required to reach the stability criteria, with hole (active *vs* inactive) and genotype (knockout *vs* wild-type) as factors of variation. This statistical analysis was performed for FR1 and FR3 schedules of reinforcement in cocaine operant responding experiments. One-way ANOVA and Dunnet *post hoc* test were performed when required. The breaking point values obtained on the PR schedule for cocaine self-administration were compared by calculating Mann–Whitney *U*-test between genotypes. For the cocaine dose–response curve, the number of infusions was compared by repeated measures two-way ANOVA (dose as within-subject factor and genotype as between-subject factor). Subsequent one-way ANOVA was performed to calculate differences between genotypes at each dose. Additionally, one-way ANOVA within subjects was performed to discard a day effect for the Latin square design in the dose–response curve. Differences were considered significant if the probability of error was less than 5%.

RESULTS

Acute Locomotor Effects Induced by Cocaine

On days 1–3, animals were exposed to the locomotor activity boxes in order to be habituated to the test environment (data not shown). Acute cocaine administration (5, 10, and 20 mg/kg) increased ambulatory movements in a dose-dependent manner in both genotypes, as shown in Figure 1a. Two-way ANOVA and subsequent one-way ANOVA are shown in Table 1. *Post hoc* analysis revealed a significant increase in locomotor activity in cocaine-treated wild-type mice at the doses of 10 ($p<0.01$) and 20 mg/kg ($p<0.01$) compared to saline-treated mice. Similarly, cocaine-treated knockout mice showed a significant increase in locomotor activity at the doses of 10

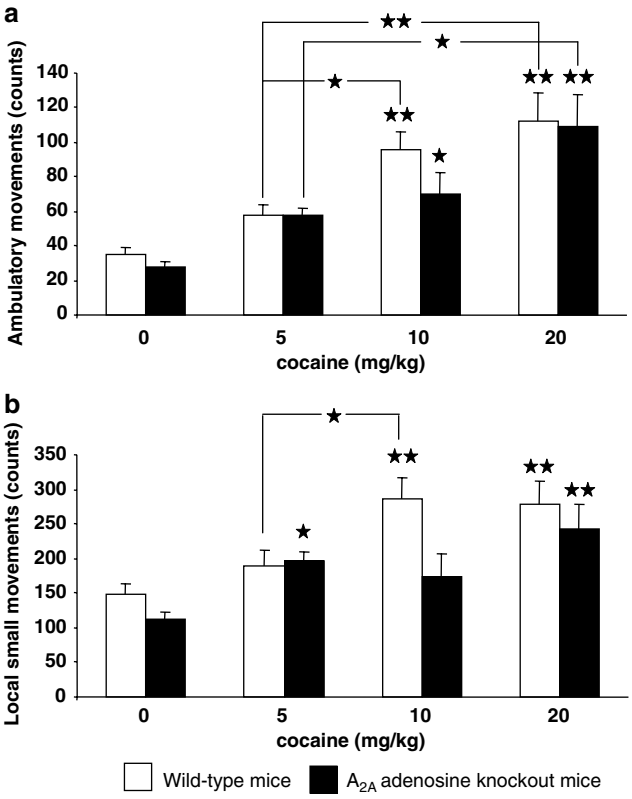

Figure 1 Acute locomotor effects induced by cocaine in A_{2A} knockout and wild-type mice. Mice were exposed to the locomotor activity boxes immediately after cocaine injection (0, 5, 10, and 20 mg/kg, i.p.). Measurements of (a) ambulatory movements and (b) local small movements were performed during 10 min. Data are expressed as mean + SEM of the percentage of increase *vs* last day of habituation ($n=16$ for each group). White columns represent wild-type mice and black columns represent A_{2A} adenosine receptor knockout mice. Stars over the bars mean differences *vs* vehicle group. ★$P<0.05$; ★★$P<0.01$ (Tukey *post hoc* test).

Table 1 Two- and One-Way ANOVAs for Acute Locomotor Effects Induced by Cocaine in A_{2A} Knockout Mice (Ambulatory Movements and Local Small Movements)

	Ambulatory movements		Local small movements	
	***F*-value**	***p*-value**	***F*-value**	***p*-value**
Two-way ANOVA				
Genotype (G)	$F(1,108)=1.412$	n.s.	$F(1,107)=6.127$	<0.01
Treatment (T)	$F(3,108)=19.122$	<0.01	$F(3,107)=10.024$	<0.01
G × T	$F(3,108)=0.567$	n.s.	$F(3,107)=1.984$	n.s.
One-way ANOVA for treatment effect				
Wild type	$F(3,52)=11.070$	<0.01	$F(3,51)=6.824$	<0.01
Knockout	$F(3,55)=8.974$	<0.01	$F(3,55)=5.109$	<0.01

Two-way ANOVA with treatment and genotype as factors of variations, and subsequent one-way ANOVA for treatment effect in each genotype. See Materials and methods for details. n.s.: nonsignificant.

($p<0.05$) and 20 mg/kg ($p<0.01$) compared to saline-treated mice (Figure 1a).

Acute cocaine administration (5, 10, and 20 mg/kg) also increased local small movements in a dose-dependent manner in both genotypes, as shown in Figure 1b. Two-way ANOVA and subsequent one-way ANOVA are shown in Table 1. *Post hoc* analysis revealed a significant increase in local small movements in cocaine-treated wild-type mice at the doses of 10 ($p<0.01$) and 20 mg/kg ($p<0.01$) compared to saline-treated animals. Similarly, cocaine-treated knockout mice increased local small movements at the doses of 5 ($p<0.05$) and 20 mg/kg ($p<0.01$) compared to saline-treated animals (Figure 1b).

Sensitization to the Locomotor Responses Induced by Chronic Cocaine Treatment

Chronic cocaine treatment induced a sensitization to its locomotor effects in both genotypes as shown in Figure 2a. Three-way ANOVA is shown in Table 2. Subsequent *post hoc* analysis showed significant differences between day 1 and days 19 ($p=0.05$) and 20 ($p<0.05$) in cocaine-treated wild-type mice. Significant differences were also observed between days 1 and 19 ($p=0.05$) in cocaine-treated knockout mice (Figure 2a).

Three-way ANOVA calculated for small local movements is shown in Table 2. Subsequent *post hoc* analysis showed significant differences between days 1 and 20 ($p<0.01$) in cocaine-treated wild-type mice (Figure 2b).

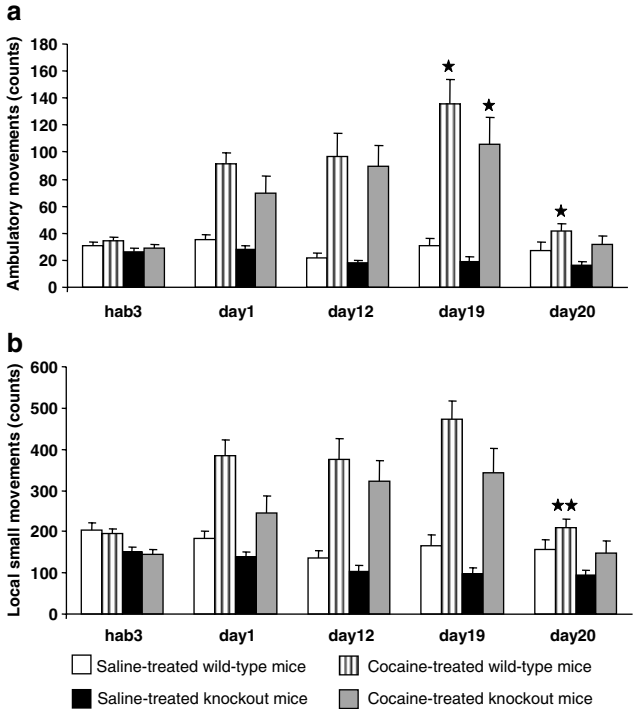

Figure 2 Locomotor sensitization to the effects induced by chronic cocaine administration in A_{2A} knockout and wild-type mice. Mice received chronic cocaine during 12 days and were exposed to the locomotor activity boxes immediately after the injection (10 mg/kg, i.p.). Mice remained without any treatment from day 13 to 18. On day 19, animals received a challenge of cocaine (10 mg/kg, i.p.) or vehicle, and locomotor activity was measured in order to evaluate the sensitization maintenance. On day 20, all the mice received a saline injection and locomotor activity was evaluated again. The figure shows locomotor activity on days 1, 12, 19, and 20. Measurements of (a) ambulatory movements and (b) local small movements were performed during 10 min. Data are expressed as mean ± SEM of the percentage of increase *vs* last day of habituation ($n=15$–16). White columns represent saline-treated mice and black columns represent cocaine-treated mice. ★$P\leqslant0.05$; ★★$P<0.01$ (Tukey *post hoc* test, cocaine-treated group *vs* day 1).

Table 2 Three-way ANOVA for Locomotor Effects of Chronic Cocaine Treatment in A_{2A} Knockout Mice (Ambulatory Movements and Local Small Movements)

	Ambulatory movements		Local small movements	
	F-value	*p*-value	*F*-value	*p*-value
Genotype (G)	$F(1,48)=2.915$	n.s.	$F(1,48)=11.051$	<0.01
Treatment (T)	$F(1,48)=53.635$	<0.01	$F(1,48)=28.075$	<0.01
Day (D)	$F(4,192)=27.406$	<0.01	$F(4,192)=15.247$	<0.01
G × T	$F(1,48)=0.317$	n.s.	$F(1,48)=0.518$	n.s.
G × D	$F(4,192)=0.774$	n.s.	$F(4,192)=1.219$	n.s.
T × D	$F(4,192)=29.167$	<0.01	$F(4,192)=26.787$	<0.01
G × T × D	$F(4,192)=0.341$	n.s.	$F(4,192)=1.243$	n.s.

Three-way ANOVA with treatment and genotype (between subjects) and day (within subjects) as factors of variations. See Materials and methods for details. n.s.: nonsignificant.

Cocaine-Induced Conditioned Place Preference

Acute rewarding responses induced by cocaine were investigated in A_{2A} knockout and wild-type mice using the place conditioning paradigm. A similar conditioned place preference was observed in wild-type and knockout mice treated with cocaine (5, 10, and 20 mg/kg) (Figure 3). Indeed, two-way ANOVA revealed a significant effect of cocaine treatment ($F(3,123)=12.637$; $p<0.01$), but no effect of genotype ($F(1,123)=0.233$; n.s.) nor interaction between these two factors ($F(3,123)=0.399$; n.s.). Subsequent one-way ANOVA calculated for each genotype to compare treatment effects in each dose showed significant effect in both wild-type ($F(3,64)=5.553$; $p<0.01$) and knockout ($F(3,64)=7.081$; $p<0.01$) mice. *Post hoc* analysis revealed significant differences between saline- and cocaine-treated mice at doses of 5, 10, and 20 mg/kg in both genotypes ($p<0.01$ for all the cases) (Figure 3).

Cocaine Maintained Operant Self-Administration

The effects of the A_{2A} receptor mutation on the reinforcing properties of cocaine were evaluated using the operant self-administration procedure. Nose-poke behavior maintained by cocaine infusions (1 mg/kg/infusion) was acquired by both wild-type (86%) and A_{2A} knockout (76.9%) mice. Knockout mice reached the acquisition criteria faster than wild-type mice (7.8 ± 0.88 and 4.4 ± 0.57 days, respectively) ($F(1,13)=10.601$; $p<0.01$). Despite the fact that A_{2A} knockout mice showed a lower cocaine intake ($F(1,13)=9.227$; $p<0.01$), both genotypes maintained

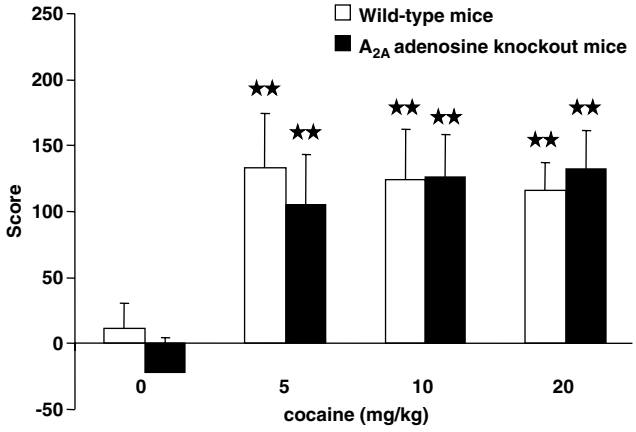

Figure 3 Cocaine (5, 10, and 20 mg/kg, i.p.)-induced conditioned place preference in A_{2A} knockout and wild-type mice. Results are expressed as mean ± SEM of the place preference score, calculated as the time spent in the drug-paired compartment on the test day minus the time spent in the same compartment on the preconditioning day. Wild-type (WT) vehicle ($n=25$), knockout (KO) vehicle ($n=25$), WT cocaine 5 mg/kg ($n=10$), KO cocaine 5 mg/kg ($n=10$), WT cocaine 10 mg/kg ($n=11$), KO cocaine 10 mg/kg ($n=11$), WT cocaine 20 mg/kg ($n=20$), KO cocaine 20 mg/kg ($n=19$) were studied. ★★$P<0.01$ *vs* vehicle (Dunnet test).

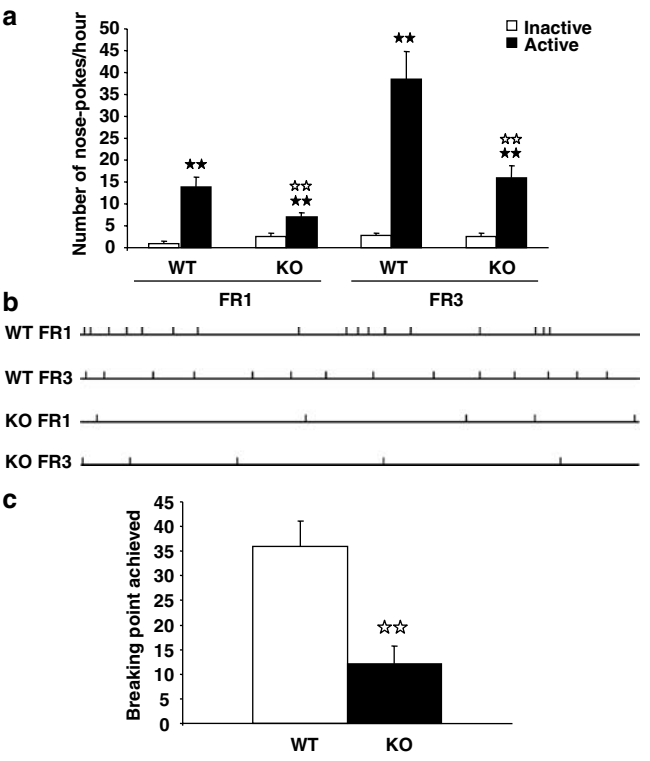

Figure 4 Cocaine self-administration (1 mg/kg/infusion) in A_{2A} knockout and wild-type mice. (a) Average of the number of nose-pokes in both the active and the inactive holes made in the three consecutive sessions (1 h each) required to achieve the acquisition criteria in FR1 and FR3 schedule of reinforcement. (b) Patterns of cocaine self-administration in FR1 and FR3 schedules of reinforcement in A_{2A} knockout (KO) and wild-type (WT) mice. Each vertical line represents a cocaine infusion (1 mg/kg/infusion). (c) Breaking points achieved in PR schedule. Data are expressed as mean ± SEM ($n=7$ per group). ★★$P<0.01$ comparison between either holes (one-way ANOVA). ☆☆$P<0.01$ comparison between genotypes (one-way ANOVA).

active nose-poke preference during the whole experiment, as shown in Figure 4a. Thus, two-way ANOVA showed in FR1 a significant effect of nose-poke preference ($F(1,24)=50.231$; $p<0.01$), genotype ($F(1,24)=4.692$; $p<0.05$), and interaction between genotype and nose-poke ($F(1,24)=12.481$; $p<0.01$). Nose-poke responding by cocaine in FR3 schedule of reinforcement was maintained by both genotypes, with a clear preference for the active *vs* the inactive nose-poke, and no differences between genotypes in the time spent to reach the FR3 criteria ($F(1,13)=0.038$; n.s.). However, cocaine intake was again reduced in knockouts in comparison with wild-type mice ($F(1,13)=10.912$; $p<0.01$). Two-way ANOVA showed a significant effect of nose-poke ($F(1,24)=50.305$; $p<0.01$), genotype ($F(1,24)=10.925$; $p<0.01$), and interaction between these two factors ($F(1,24)=10.474$; $p<0.01$). Subsequent one-way ANOVA revealed significant preference for the active *vs* the inactive nose-poke in wild-type during FR1 ($F(1,13)=35.801$; $p<0.01$) and FR3 ($F(1,13)=32.203$; $p<0.01$) schedules of reinforcement, and also in A_{2A} knockout mice during FR1 ($F(1,13)=14.872$; $p<0.01$) and FR3 ($F(1,13)=21.643$; $p<0.01$) schedules of reinforcement. The infusion pattern of the cocaine self-administration sessions was also evaluated (Figure 4b). A regular and consistent pattern of response was observed in both wild-type and A_{2A} knockout mice during the whole duration of the self-administration session, which excludes a possible random nose-poking behavior. In the case of PR schedule, the breaking point values were significantly reduced in mice lacking the A_{2A} adenosine receptor when compared to wild-type littermates (Mann–Whitney *U*-test, $p<0.01$) (Figure 4c). A limit of 2 h was established to terminate the PR session in both wild-type and knockout mice. The decreased breaking point values observed in knockout mice were not due to the relative short time period of the PR session since the analysis of infusion patterns revealed that most of the knockout mice stopped nose-poking before than wild-type animals (data not shown). Furthermore, 43% of knockout mice showed an interinfusion interval longer than 1 h, while this pattern was not observed in any of wild-type mice.

As shown in Figure 5a, a bell-shaped dose–response curve was obtained when different doses of cocaine were tested in wild-type and in A_{2A} knockout mice. Nevertheless, a vertical shift of this dose–response curve was observed in the knockout group. Two-way ANOVA with repeated measures revealed a main effect of the dose ($F(4,40)=11.552$; $p<0.01$), genotype ($F(1,10)=11.882$; $p<0.01$), but no interaction between these two factors ($F(4,40)=1.607$; n.s.). One-way ANOVA for genotype revealed significant differences at the doses of 0.032 ($F(1,11)=9.012$; $p<0.01$), 0.1 ($F(1,11)=7.592$; $p<0.05$), and 1 ($F(1,11)=12.024$; $p<0.01$). One-way ANOVA revealed no effect of the day in the Latin square design ($F(4,59)=0.534$; n.s.). Figure 5b shows the total cocaine intake received when different doses were tested. Two-way ANOVA revealed main effects of genotype ($F(1,50)=12.627$; $p<0.01$), dose ($F(4,50)=22.174$; $p<0.01$), but no interaction between these two factors ($F(4,50)=2.408$; n.s.). One-way ANOVA revealed significant differences between genotypes at the

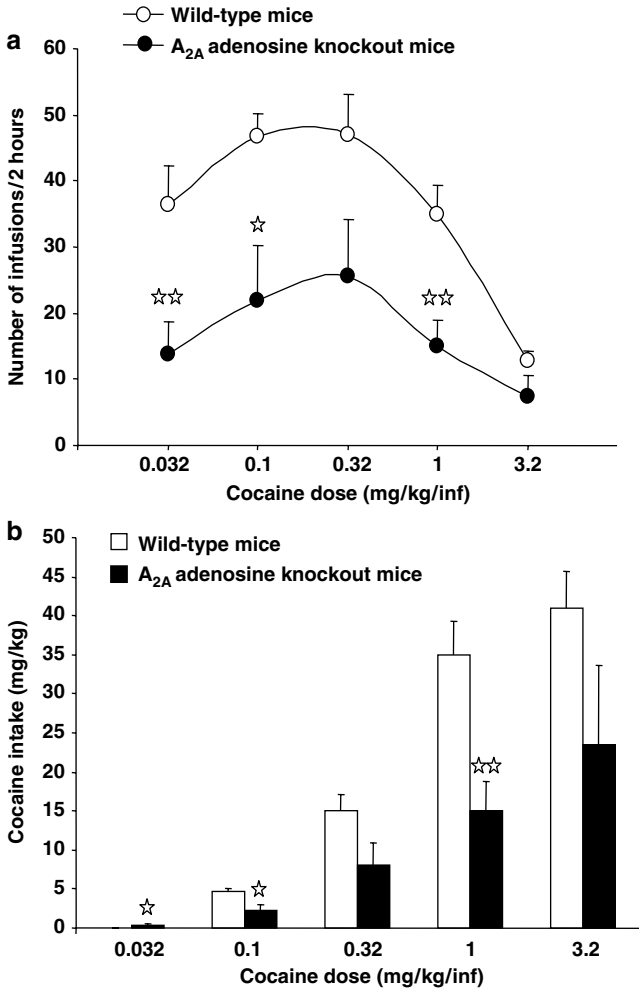

Figure 5 Cocaine self-administration (0.032, 0.1, 0.32, 1, and 3.2 mg/kg/infusion) dose–response curve in A_{2A} knockout and wild-type mice. (a) Number of infusions during the 2-h self-administration session performed at different doses of cocaine (see Materials and methods for details). (b) Cocaine intake (mg/kg) received per session in the dose–response curve. Data are expressed as mean ± SEM ($n = 6$ per group). $^{\star}P < 0.05$; $^{\star\star}P < 0.01$ comparison between genotypes (one-way ANOVA).

doses of 0.032 ($p < 0.05$), 0.1 ($p < 0.05$), and 1 mg/kg ($p < 0.01$).

DISCUSSION

The present study demonstrates the involvement of A_{2A} adenosine receptors in the addictive properties of cocaine. Mice lacking A_{2A} adenosine receptors showed a decreased rate of self-administration and motivation for cocaine, as well as reduced efficacy of cocaine reinforcing effects. Nevertheless, the increased locomotor activity produced by an acute injection of cocaine, the development of locomotor sensitization by repeated cocaine administration, and cocaine-induced conditioned place preference were maintained in these A_{2A} knockout mice.

Deletion of A_{2A} adenosine receptors did not modify acute effects induced by cocaine. Thus, acute cocaine (5, 10, and 20 mg/kg)-induced hyperlocomotion was similar in wild-type and knockout mice, suggesting that A_{2A} adenosine receptors do not mediate these acute effects of cocaine. Our data also confirm the hypolocomotor phenotype described in A_{2A} knockout mice (Ledent *et al*, 1997) since locomotor basal activity of knockout mice was decreased *vs* wild-type littermates. However, this hypolocomotion did not impair the acute effects induced by cocaine administration. On the other hand, a similar locomotor sensitization was observed when A_{2A} adenosine receptor knockout mice and wild-type littermates were chronically treated with cocaine (10 mg/kg). In contrast, other authors have shown that locomotor responses to acute cocaine administration were attenuated in mice lacking A_{2A} receptors (Chen *et al*, 2000). In addition, a recent study demonstrates that conditional A_{2A} knockout mice showed no sensitization to locomotor effects of amphetamine (Bastia *et al*, 2005). Although the discrepancies between these findings could be due to the different genetic background used to generate each line of A_{2A} knockout mice, C57Bl/6 *vs* CD1, the different experimental conditions employed in each study could probably better explain this divergence. Indeed, locomotor activity was measured during a longer period in the previous studies (120–480 min), whereas a 10 min period was used in the present work. Repeated drug exposure is known to induce sensitization to its behavioral stimulant effects (Koob, 1996; Vanderschuren and Kalivas, 2000). The mesocorticolimbic dopaminergic system has been proposed as the neural substrate underlying this phenomenon (Kalivas *et al*, 1992), which is also involved in the reinforcing properties of all drugs of abuse (Koob and Le Moal, 2001; Nestler, 2004). Locomotor sensitization has been proposed to reflect the increase of the expectation or 'wanting' for drug reward produced by the repeated exposure to drugs of abuse and that would be the result of increasing the basic responsiveness of dopaminergic neurons to stimuli (Robinson and Berridge, 1993). The locomotor activity displayed by animals in a novel environment has been positively correlated with the sensitivity to both the locomotor and reinforcing effects of psychostimulants such as cocaine (Piazza *et al*, 1989; Hooks *et al*, 1991a, b). In the present study, A_{2A} knockout mice showed similar acute cocaine locomotor effects and cocaine-induced locomotor sensitization as wild-type mice, although cocaine self-administration behavior was different in both genotypes, suggesting separate neuronal substrates for these behavioral responses induced by cocaine.

Acute rewarding properties of cocaine were indirectly evaluated using the place conditioning procedure. Mice lacking A_{2A} adenosine receptors showed similar conditioned place preference to different doses of cocaine (5, 10, and 20 mg/kg) as wild-type littermates. In agreement with this result, the amount of cocaine self-administered in the first session of the operant self-administration paradigm was similar in both wild-type (9.69 ± 1.70 mg/kg) and knockout mice (11.08 ± 2.03 mg/kg). Although acute rewarding effects are important to initiate the drug addictive process, other factors are also needed to develop this complex behavior (Koob and Le Moal, 2001).

A_{2A} adenosine receptor knockout mice acquired an operant behavior maintained by i.v. cocaine infusions and showed a reliable cocaine self-administration. However, the cocaine intake under FR1 and FR3 schedules was signifi-

cantly lower in A_{2A} knockout mice compared with wild-type littermates (approximately 40–50%). This result is in agreement with the reduction in the maximal effort required to obtain a cocaine infusion in A_{2A} knockout mice under a PR schedule of reinforcement. In animal drug self-administration studies, response rates usually show an inverted U-shaped function of drug dose where rate of responding is inversely related to the injection dose (Meisch and Lemaire, 1993). In the present study, typical inverted U-shaped dose–response curves were obtained in both wild-type and A_{2A} knockout mice. The possibility that A_{2A} knockout mice would experience increased reinforcing effects of cocaine, and therefore a reduction in cocaine self-administration, can be ruled out since knockout mice did not show a leftward shift in the dose–response curve. Indeed, the vertical shift obtained indicates a difference in the efficacy, but not in the potency of the reinforcing effects of cocaine. In agreement, A_{2A} adenosine knockout mice showed a decreased breaking point in the PR schedule and did not exhibit a higher sensitivity to cocaine-induced behavioral effects in the other models evaluated (locomotion, sensitization, and conditioned place preference). There is evidence showing that vertical shifts in self-administration dose–response curve predict a drug-vulnerable phenotype predisposed to addiction (Piazza *et al*, 2000). Thus, it could be hypothesized that A_{2A} knockout mice represent a low-vulnerable phenotype to cocaine addiction and the lack of A_{2A} adenosine receptors could provide resistance against the addictive properties of cocaine. The lower reinforcing efficacy of cocaine found in A_{2A} adenosine knockout mice that have acquired a stable cocaine self-administration behavior was not due to an impairment of the acute rewarding properties of cocaine, since cocaine-induced conditioned place preference and the cocaine intake in the first session of the self-administration paradigm were not modified in these knockouts. Differences in conditioned place preference and self-administration results are not surprising since the behavior responses evaluated in these two paradigms are not equivalent. While conditioned place preference evaluates the expression of indirect reward, self-administration paradigm is used to directly study the reinforcing effects of a drug.

Adenosine and its receptors have been involved in learning and memory processes (de Mendonca and Ribeiro, 1997; Svenningsson *et al*, 1999; Hauber and Bareiss, 2001; Justinova *et al*, 2003). However, the lower rate of cocaine self-administration found in A_{2A} knockout mice does not appear to be a consequence of learning impairment since operant responding for food is maintained in these animals (Soria *et al*, 2004). In addition, A_{2A} adenosine receptor knockout mice achieved the FR1 acquisition criteria for cocaine self-administration faster than wild-type littermates (7.8 ± 0.88 and 4.4 ± 0.57 days, respectively). Moreover, the similar results obtained on cocaine-induced conditioned place preference in both genotypes also support a normal learning and memory response in these knockout mice.

Dopamine D_2-like receptors are known to be particularly important in mediating the abuse-related effects of cocaine (Caine *et al*, 2002). Adenosine A_{2A} receptors are colocalized with dopamine D_2 receptors in striatopallidal GABAergic neurons and stimulation of A_{2A} adenosine receptors decreases the affinity of D_2 dopamine receptors (Ferré *et al*, 1997). Our study does not show this antagonistic interaction since the reinforcing efficacy of cocaine was diminished in A_{2A} knockout mice. However, other indirect interactions between A_{2A} and D_2 receptors have also been reported at intracellular levels, which may explain our results. DARPP-32, a downstream effector molecule of D_2-like receptors, has been reported to play an important role in mediating the pharmacological effects of a variety of drugs of abuse (Nairn *et al*, 2004). In this sense, mice lacking DARPP-32 showed attenuated conditioned place preference to cocaine without involving alteration of dopamine release or reuptake (Zachariou *et al*, 2002). DARPP-32 phosphorylation could also be directly regulated by A_{2A} adenosine receptors (Svenningsson *et al*, 2004). Dopamine D1 receptors are also involved in cocaine effects (Hummel and Unterwald, 2002). In this sense, D1 and A_{2A} adenosine receptors have an additive effect on DARPP-32 phosphorylation (Svenningsson *et al*, 2004), raising the possibility that D1 receptors could compensate the consequences of the absence of A_{2A} on intracellular signals implicated in cocaine reward. On the other hand, extracellular signal-regulated kinase (ERK) pathway activation has been involved in synaptic plasticity related to long-term effects of psychostimulants addiction (Valjent *et al*, 2000, 2003). More recently, the control of ERK pathway by DARPP-32 in the shell of the Nac has been demonstrated, suggesting a critical role for DARPP-32 in long-term effects of psychostimulants (Valjent *et al*, 2005). Interestingly, DARPP-32 has been reported to be altered in mice lacking A_{2A} adenosine receptors (Svenningsson *et al*, 2000). Therefore, a possible explanation for our findings could be that intracellular cascades downstream of dopamine release could be impaired in mice lacking A_{2A} adenosine receptors producing an alteration in the brain reward function. Thus, recent studies reported the existence of a synergy between A_2 and D_2 dopamine receptors in protein kinase A signaling mediated by $\beta\gamma$ dimers of G-proteins (Yao *et al*, 2002, 2003). Therefore, this synergy might be absent in A_{2A} knockout mice, which would have a negative effect in drug reinforcement properties.

In conclusion, the present study demonstrates the important role played by A_{2A} adenosine receptors in cocaine addictive properties, as revealed by the decreased rate of self-administration and motivation for cocaine, and the lower efficacy of cocaine reinforcing effects found in mice lacking A_{2A} adenosine receptors. However, acute locomotor effects of cocaine and repeated cocaine-induced locomotor sensitization and conditioned place preference were preserved in these animals. These findings support the hypothesis that separate neuronal substrates mediate cocaine-induced locomotor effects and the self-administration in an operant behavior paradigm Therefore, pharmacological manipulation of these receptors may be a possible target in the treatment of cocaine addiction.

ACKNOWLEDGEMENTS

This study was supported by grants from Spanish MCYT (SAF 2001/0745, SAF 2004/568, and GEN2003-20651-C06-04), HFSP (RG007/200-B), Redes del Instituto de Salud Carlos III (C03/06 and G03/005), and European Commu-

nities (NEWMOOD OJ2003/C164-05166). GS is a fellowship from Spanish MCYT. AC is a fellowship from DURSI (Generalitat de Catalunya).

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