Inactivation of Adenosine A$_{2A}$ Receptors Selectively Attenuates Amphetamine-Induced Behavioral Sensitization

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Repeated treatment with the psychostimulant amphetamine produces behavioral sensitization that may represent the neural adaptations underlying some features of psychosis and addiction in humans. In the present study we investigated the role of adenosine A$_{2A}$ receptors in psychostimulant-induced locomotor sensitization using an A$_{2A}$ receptor knockout (A$_{2A}$ KO) model. Daily treatment with amphetamine for 1 week resulted in an enhanced motor response on day 8 (by two-fold compared to that on day 1), and remained enhanced at day 24 upon rechallenge with amphetamine. By contrast, locomotor sensitization to daily amphetamine did not develop in A$_{2A}$ KO mice on day 8 or 24, and this absence was not the result of a nonspecific threshold effect. The absence of behavioral sensitization was selective for amphetamine since daily treatment with the D$_1$ agonist SKF81297 (2.5 mg/kg) or the D$_2$ agonist quinpirole (1.0 mg/kg) produced similar behavioral sensitization in both WT and A$_{2A}$ KO mice. Furthermore, coinjection of SKF81297 and quinpirole also resulted in indistinguishable locomotor sensitization in A$_{2A}$ KO and WT mice, suggesting normal D$_1$ and D$_2$ receptor responsiveness. Finally, at the cellular level A$_{2A}$ receptor inactivation abolished the increase in striatal dynorphin mRNA induced by repeated amphetamine administration. The selective absence of amphetamine-induced behavioral sensitization in A$_{2A}$ KO mice suggests a critical role of the A$_{2A}$ receptor in the development of psychostimulant-induced behavioral sensitization, and supports the pharmacological potential of A$_{2A}$ adenosinergic agents to modulate adaptive responses to repeated psychostimulant exposure.
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INTRODUCTION

Repeated administration of psychostimulants (such as amphetamine or cocaine) induces an enhanced behavioral response to subsequent drug exposure, a phenomenon known as behavioral sensitization and capable of persisting for months (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). The development of these maintained behavioral adaptations parallels the progressive and sustained enhancement of drug-craving and psychotic behaviors displayed by addicts only after repeated administration (Robinson and Berridge, 1993). Psychostimulant-induced behavioral sensitization in rodents provides a model of the addictive behaviors (such as those associated with craving and relapse) and psychotic complications of psychostimulant abuse (Robinson and Becker, 1986; Robinson and Berridge, 1993). Thus, understanding the neural adaptations associated with psychostimulant-induced behavioral sensitization may be relevant to the pathophysiology of psychostimulant-associated disorders such as drug addiction and psychosis.

The critical role of dopaminergic transmission in psychostimulant-induced behavioral sensitization has been inferred from evidence that psychostimulants enhance the level of dopamine in the synapse either by increasing presynaptic dopamine release (amphetamine) or blocking dopamine reuptake (cocaine) (Koob, 1992; Self and Nestler, 1995; Tan *et al*, 2000). Activation of both dopamine D$_1$-like and D$_2$-like receptors is involved in the behavioral response to amphetamine as antagonists at these receptors attenuate amphetamine-dependent behaviors (Hyman, 1996; Bardo, 1998; Wolf, 1998; Hyman and Malenka, 2001). Glutamatergic transmission has also been implicated in behavioral sensitization either through modulation of dopaminergic transmission or through an independent action (Wolf, 1998; Sripada *et al*, 2001). In spite of intensive studies of psychostimulant action, effective pharmacological strategies for treating chronic psychostimulant-associated disorders

remain limited by side effects of glutamatergic and dopaminergic antagonists (Hyman, 1996; Hyman and Malenka, 2001). Moreover, the involvement of neurotransmitters other than dopamine and glutamate in psychostimulant-induced behavioral sensitization is largely unexplored, and may provide alternative therapeutic opportunities to modify psychostimulant effects.

The brain adenosine A$_{2A}$ receptor is emerging as a promising target site for the modulation of psychostimulant actions. Accumulating evidence supports functional interactions between the A$_{2A}$ adenosinergic and dopaminergic systems in the brain (Ferre et al, 1997; Svenningsson et al, 1999b). Brain A$_{2A}$ receptor mRNA is coexpressed with D$_2$ receptor mRNA in the striatum and nucleus accumbens (Schiffmann et al, 1991; Fink et al, 1992) (Svenningsson et al, 1999a), a critical area involved in behavioral sensitization (Koob, 1992; Pierce and Kalivas, 1997). Activation of A$_{2A}$ receptors antagonizes D$_2$ receptor-mediated behavioral and neurochemical effects, (eg on GABA and acetylcholine release and on the striatal expression of the neuropeptide enkephalin) (Ongini and Fredholm, 1996). Moreover, pharmacological studies show that A$_1$ and A$_{2A}$ agonists inhibit acute amphetamine-induced locomotion in intact animals and amphetamine-induced rotation in rats unilaterally lesioned with 6-OHDA (Turgeon et al, 1996; Ferre et al, 1997; Rimondini et al, 1997; Chen et al, 2000). Similarly, A$_{2A}$ antagonists potentiate the acute motor effects of dopamine agonists, psychostimulants and L-dopa (Fenu et al, 1997; Fenu and Morelli, 1998; Poleszak and Malec, 2000). Thus, A$_{2A}$ receptors profoundly influence central dopaminergic mechanisms and psychostimulant action. This antagonistic interaction between A$_{2A}$ adenosine and dopamine receptors may be mediated by a direct A$_{2A}$-D$_2$ receptor–receptor interaction at an intramembrane level, as well as by an opposing, independent functional antagonism at the levels of postreceptor signaling pathways and of neural networks (Svenningsson et al, 1999a; Chen et al, 2001). In addition, activation of the A$_{2A}$ receptor has been shown to enhance the release of several neurotransmitters in brain including dopamine and glutamate, which contribute to the development of psychostimulant behavioral sensitization (Okada et al, 1996; Sebastiao and Ribeiro, 1996; Golembiowska and Zylewska, 1998). Together these studies suggest that the A$_{2A}$ receptor may represent an ideal site at which psychostimulant actions can be selectively modulated.

While pharmacological studies clearly demonstrate A$_{2A}$ adenosinergic modulation of acute psychostimulant action, relatively little is known about A$_{2A}$ receptor involvement in the development of behavioral sensitization by chronic psychostimulant treatment. Although recent reports showed that the A$_{2A}$ agonist CGS21680 attenuates the development of behavioral sensitization induced by methamphetamine or morphine (Weisberg and Kaplan, 1999; Shimazoe et al, 2000), there is no information available on the effect of A$_{2A}$ receptor blockade on chronic psychostimulant-induced behavioral sensitization. Furthermore, pharmacological investigation of A$_{2A}$ receptor involvement in behavioral sensitization is limited by the intrinsic partial specificity of A$_{2A}$ antagonists. A$_{2A}$ antagonists, generally also have poor solubility and poor CNS penetration. In addition, most A$_{2A}$ antagonists are unstable in solution and undergo rapid

isoform conversion in light, resulting in reduced affinity for the receptor (Dionisotti et al, 1994; Nonaka et al, 1994; Ongini and Fredholm, 1996). All of these limitations make A$_{2A}$ antagonists difficult to use for in vivo pharmacological studies. In this study, we employed an A$_{2A}$ receptor knockout (A$_{2A}$ KO) model that provides specific and complete inactivation of A$_{2A}$ receptors in order to evaluate their role in the adaptive behavioral and neurochemical responses to repeated exposure to dopaminergic stimulation. Specifically, we examined whether the A$_{2A}$ receptor is required for behavioral sensitization in response to repeated treatments with amphetamine or dopaminergic agonists.

MATERIALS AND METHODS

Breeding and Genotyping of A$_{2A}$ KO Mice

The A$_{2A}$ KO mouse line was generated and genotyped as previously described (Chen et al, 1999). Mutant mice in a pure 129-Steel genetic background were used in this study to avoid potential confounding effects of commonly employed mixed genetic backgrounds. A$_{2A}$ KO mice in a pure 129-steel genetic background were produced by crossbreeding chimeric A$_{2A}$ KO mice (derived from embryonic stem cells of 129-steel background) to 129-steel mice (Taconic), and were maintained through breeding by heterozygote intercrosses (Chen et al, 1999). For each of the present experiments WT and KO littermates (both male and female) of the F2–F4 generations were matched for gender, age (2–5 months), and body weight.

Animals and Drug Treatments

All experiments were performed in accordance with Massachusetts General Hospital and NIH guidelines on the ethical use of animals. The homozygous KO ($-/-$) and WT ($+/+$) mice were housed in plastic cages and provided free access to food and water. The animals were maintained in temperature and humidity-controlled rooms with a 12-h light–dark cycle (light from 7:00 am to 7:00 pm). Prior to behavioral testing (which was conducted during the light phase of the light–dark cycle), all mice were habituated to the testing environment for 120 min. The mice were evaluated for spontaneous locomotion, as well as for locomotor activity in response to dopamine agonists. The mice were treated intraperitoneally with amphetamine (2.5 or 5.0 mg/kg), quinpirole (1 mg/kg), and SKF81297 (2.0 mg/kg) for 8–9 days. Locomotor activity was recorded at the first and eighth or ninth days of the treatment, or 2 weeks after the cessation of the treatment (24th day).

Locomotor and Fine Motor Activity

Horizontal locomotor and fine motor activities were assessed in standard polypropylene cages (15×25 cm^2) placed into adjustable frames equipped with five infrared photocell beams (San Diego Instruments) that traverse each cage in a plane above its floor. Ambulation (sequential breaks in two adjacent beam), fine movement (sequential breaks in a single beam), and total locomotion (ambulation plus fine movement) were recorded and analyzed on a computer. On the first and eighth treatment day mice were

weighed and placed in the test cages for a 120-min habituation period before drug injection. On the second through seventh treatment days mice remained in their home cages before and after injections.

6-Hydroxydopamine Lesions and Rotational Behavioral Analysis

Mice were pretreated with desipramine hydrocloride (25 mg/kg, Sigma), to minimize damage to noradrenergic neurons. Under Avertin (2% 2,2,2-tribromoethanol and 1% amyl alcohol) anesthesia (20 ml/kg, i.p.), 10 µg of 6-OHDA (2.5 µg/µl in normal saline containing 0.05% of ascorbic acid) was delivered by a microinfusion pump (1 µl/min) into the left dorsal striatum at the following coordinates (from Bregma point: 1.1 mm anterior, 1.5 mm lateral, 2.0 mm ventral). Seven days following 6-OHDA lesioning, rotational behavior was evaluated by an observer who was blind to the genotype of the animals. WT and A$_{2A}$ KO mice ($n = 9$–12) were treated daily with quinpirole (1.0 mg/kg) for 3 weeks. Contralateral rotation behavior was evaluated in a test cage daily. The intensity and kinetic profile of L-dopa-induced contralateral behavior was established by monitoring the number of complete (360°) rotations ipsilateral and contralateral to the lesion in a 30-min test period immediately after the injection of quinpirole.

In Situ Hybridization Histochemistry

In situ hybridization histochemistry with cRNA probes was performed according to protocols described previously (Moratalla *et al*, 1996a). Mouse brain sections (10 µm thick) were cut in a cryostat and then sequentially postfixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), washed for three times in PBS, acetylated in acetic anhydride and dehydrated in graded ethanol. Sections were hybridized overnight at 55°C in a humid chamber with ^{35}S-labeled cRNA probes (150.000 cpm/µl buffer) specific for the rat prodynorphin cDNA (provided by Dr J Douglass) (Civelli *et al*, 1985). Hybridization buffer contained 50% formamide, 4 × SSC, 1 × Denhardt's solution, 100 µg/ml salmon sperm DNA, 250 µg/ml yeast tRNA, 10% w/v dextran sulfate, and 100 mM dithiothreitol. After hybridization, sections were washed and then treated with RNase A (100 µg/ml), and washed again to final stringency in 0.1 × SSC at 70°C for 30 min. The slides were rinsed, dried, and exposed to BioMax MR films (Amersham) for 10–15 days.

Dynorphin mRNA levels were determined by optical density using a computing densitometer equipped with an image analysis system (Model 300A, Molecular Dynamics). Five to six sections through the striatum (both hemispheres) were analyzed for each mouse.

Statistical Analysis

Statistical analyses of the behavioral data were performed using SAS (8.0) or SPSS (11.0) software. The effects of genotype (WT and KO) and chronic treatment (treatment days 1, 8, or rechallenge at day 24) and postinjection time (0–90 min) were analyzed by three-way ANOVA for repeated measurements. This is followed by *post hoc* comparisons between different treatment days at various postinjection times using Tukey's honestly significant difference test. For behavioral analysis of experiments using amphetamine at 5.0 mg/kg, we adapted a two-way ANOVA, with an unbalanced design for repeated measurements because of an unequal number of animals in the groups for different treatment days. Following two-way ANOVA, the LSD test was used for *post hoc* preplanned comparison.

RESULTS

A$_{2A}$ Receptor Inactivation Prevents Amphetamine-Induced Behavioral Sensitization in Mice

First we examined the effects of A$_{2A}$ receptor inactivation on locomotor responses to repeated amphetamine treatment in WT and A$_{2A}$ KO mice. Mice were treated with amphetamine (2.5 mg/kg, i.p.) daily for 8 days and locomotor response to amphetamine was monitored on the first and eighth day (Figure 1a). Acute treatment with amphetamine induced significant locomotion in both WT and A$_{2A}$ KO mice. Following daily injection of amphetamine for 7 days, WT mice displayed significantly enhanced locomotor response to the same dose of amphetamine on day 8 compared to their response on day 1 (Figure 1a, left panel, $n = 7$–8, $p < 0.05$, two-way ANOVA followed by Tukey's test). By contrast, A$_{2A}$ KO mice did not show any enhancement of locomotion on day 8 compared to day 1 (Figure 1a, right panel, $n = 8$). Three-way ANOVA analysis shows that there were significant effects of treatment day ($F(17,261) = 3.95$, $p = 0.031$) and genotype ($F(1,277) = 16.6$, $p = 0.002$), and an interaction between genotype and treatment ($F(17,251) = 2.94$, $p = 0.057$).

Furthermore, although the number of fine movements recorded in WT mice after the initial amphetamine injection was significantly higher than that in KO mice (WT *vs* KO, $n = 8$, $F(1,31) = 13.4$, $p = 0.003$, two-way ANOVA followed by the LSD test), repeated treatments with amphetamine did not induce a significant increase in fine movements (comparing day 8 *vs* day 1, $n = 8$, $F(1,31) = 0.61$, $p = 0.447$, two-way ANOVA followed by the LSD test) in either WT (mean $\pm$ SE are 1570 ± 151 and 1793 ± 154 for days 1 and 8, respectively, over a 120 min period) or A$_{2A}$ KO mice (1017 ± 154 and 940 ± 148 for days 1 and 8, respectively). This suggests that the lack of amphetamine-induced locomotor sensitization in A$_{2A}$ KO mice is not owing to sensitized stereotyped behavior of the type reflected in horizontal fine movements, which could have masked the appearance of enhanced locomotor activity in these mice.

To distinguish between stress-induced and amphetamine-induced behavioral sensitization in WT mice we also compared amphetamine-stimulated motor activity following 1 week of daily saline injections with amphetamine-stimulated motor activity in naïve mice. Locomotor activity simulated by amphetamine (2.5 mg/kg) in WT mice measured on day 8 (290 ± 42 ambulations per 120 min) following seven daily saline injections was significantly increased over saline-stimulated locomotion on day 1 (62 ± 17, $n = 8$, $p < 0.001$, two-way ANOVA followed by Tukey's test), but was indistinguishable from amphetamine-stimulated locomotor activity on day 1 (202 ± 13; $p > 0.05$, $n = 8$, two-way ANOVA followed by Tukey's test).
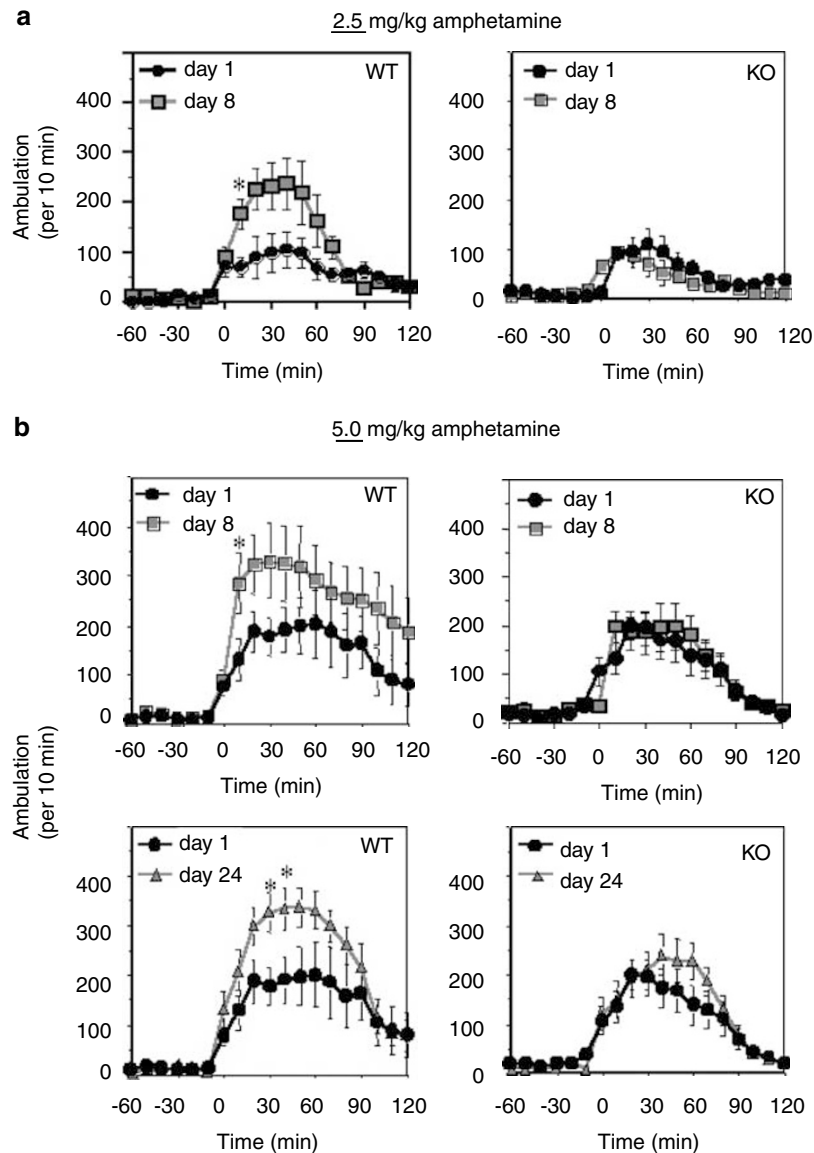
Figure 1 Absence of repeated psychostimulant-induced behavioral sensitization in A$_{2A}$ KO mice. WT and A$_{2A}$ KO mice (3- to 8-week-old, male and female) were treated with amphetamine (a) 2.5 mg/kg, (b) 5.0 mg/kg) daily for 8 or 24days. Ambulation was recorded for 120 min after amphetamine treatment on day 1 and either immediately following (day 8, (a) and (b)) or 2 weeks after (day 24, (b)) 1 week of daily amphetamine injections. WT = wild-type; KO = A$_{2A}$ receptor knockout. All mice are offspring of heterozygote crosses in a pure 129-steel background. *$p < 0.05$, three-way ANOVA with repeated treatments for two factors, followed by Tukey's *post hoc* test, $n = 7$ and 8 (a); $n = 11$, 8, and 6 for days 1, 8, and 24, respectively (b).

Thus, the A$_{2A}$ receptor-dependent sensitization of the motor response to amphetamine primarily reflects amphetamine- (rather than injection stress-) induced behavioral sensitization.

The Absence of Behavioral Sensitization in A$_{2A}$ KO Mice is not Attributable to a Threshold Effect or Delayed Sensitization

The process of sensitization may require that individual locomotor responses to each amphetamine injection achieve a threshold magnitude in order to generate enhanced responses upon repeated administration. Indeed, we found that the small motor stimulation produced by at a

very low dose (1.25 mg/kg) amphetamine did not augment with repeated daily administration under our standard 1-week sensitization paradigm (data not shown). Since the locomotion induced by a single dose of amphetamine was found to be partially attenuated in A$_{2A}$ KO compared to WT mice (Chen *et al*, 2000); and also in Figure 1a, by ∼25% although not significantly, the absence of sensitization observed in KO mice using amphetamine at 2.5 mg/kg (Figure 1a) could have simply reflected subthreshold motor responses at that dose. We addressed this possibility by testing the effects of a higher dose of amphetamine. In order to exceed any such motor response threshold in A$_{2A}$ KO mice, we treated the mice daily for 8 days with the amphetamine dose doubled (to 5 mg/kg), and with loco-

motor responses again recorded in activity cages on days 1 and 8 (Figure 1b).

As expected, on day 1 the higher amphetamine dose induced a greater cumulative locomotor response in KO mice (1405 total ambulations on average over the 2 h after 5 mg/kg in A_{2A} KO mice; Figure 1b, upper right panel) than the lower dose had induced in WT mice (994 after 2.5 mg/kg in WT mice; Figure 1a, left panel). Analysis by two-way ANOVA for repeated measurements shows that there were significant effects of treatment day ($F(1,569) = 7.70$, $p = 0.010$) and the interaction between treatment day and genotype ($F(1,569) = 6.06$, $p = 0.021$). If the lack of sensitization observed in A_{2A} KO mice at 2.5 mg/kg amphetamine were in fact because of a subthreshold motor response, we would now expect a robust sensitization in KO mice treated with double the dose (since their initial motor response of 1405 ambulations would now be well above a possible motor response threshold of $\leqslant 994$). However, the sensitized locomotor response to 5 mg/kg amphetamine observed on day 8 in WT mice ($p < 0.05$, $n = 13–15$, two-way ANOVA followed by Tukey's test) was again completely prevented by A_{2A} receptor inactivation in the KO mice (Figure 1b, upper panels, $n = 11$ and 8 at days 1 and 8, respectively). These data argue strongly against a non-specific motor threshold effect as the basis for attenuated locomotor sensitization in A_{2A} KO mice.

In addition, the locomotor sensitization in WT mice and its absence in A_{2A} KO mice persisted for at least 2 weeks after discontinuation of daily amphetamine injections (Figure 1b, lower panels, $n = 11$ and 6 for days 1 and 24). Two-way ANOVA analysis with an unbalanced design shows that there was a borderline significant effect of treatment day ($F(1,519) = 4.05$, $p = 0.050$). While the extent of sensitization in WT mice appeared to be long lasting (Figure 1b, $n = 10–12$, $p < 0.05$, comparing the day 24 vs day 1, two-way ANOVA followed by Tukey's test) with at least as much expression on day 24 as on day 8 (Figure 1b, lower vs upper panel, $n = 10–12$), delayed rechallenge with amphetamine still did not produce an enhanced motor response in KO mice. These data suggest that A_{2A}R inactivation prevents (rather than delays) locomotor sensitization to amphetamine.

Repeated D₁ or D₂ Agonist Treatment Produced Indistinguishable Behavioral Sensitization in WT and A₂ₐ KO Mice

In contrast to amphetamine, the full D_1 receptor agonist SKF81297 (2.5 mg/kg) produced an identical locomotor sensitization in WT and A_{2A} KO mice, following the same 8-day treatment schedule used for amphetamine (Figure 2a). The peak locomotion induced by SKF81297 in WT mice was (mean $\pm$ SE) 176 ± 18 and 254 ± 26 ambulations per 10 min at days 1 and 8, respectively ($n = 8$, $p < 0.05$, three-way ANOVA followed by Tukey's test). Similarly, peak locomotion by SKF81297 in A_{2A} KO mice were 131 ± 22 and 292 ± 21 ambulations per 10 min at days 1 and 8, respectively ($n = 7$, $p < 0.05$, three-way ANOVA followed by Tukey's test). However, there was no significant difference in motor responses by SKF81297 treatment between WT and A_{2A} KO littermates on either day (Figure 2a, $n = 7$). Three-way ANOVA shows that there were significant effects of treatment day (day 1 vs day 8) ($F(1,215) = 11.15$, $p = 0.075$) and postinjection time ($F(1,215) = 23.76$, $p < 0.001$), and an interaction between treatment day and postinjection time ($F(1,215) = 3.78$, $p = 0.001$), but that there was no significant effect of genotype ($F(1,215) = 0.00$, $p = 0.970$).

Owing to the specific antagonistic interaction between adenosine A_{2A} and dopamine D_2 receptors, we also
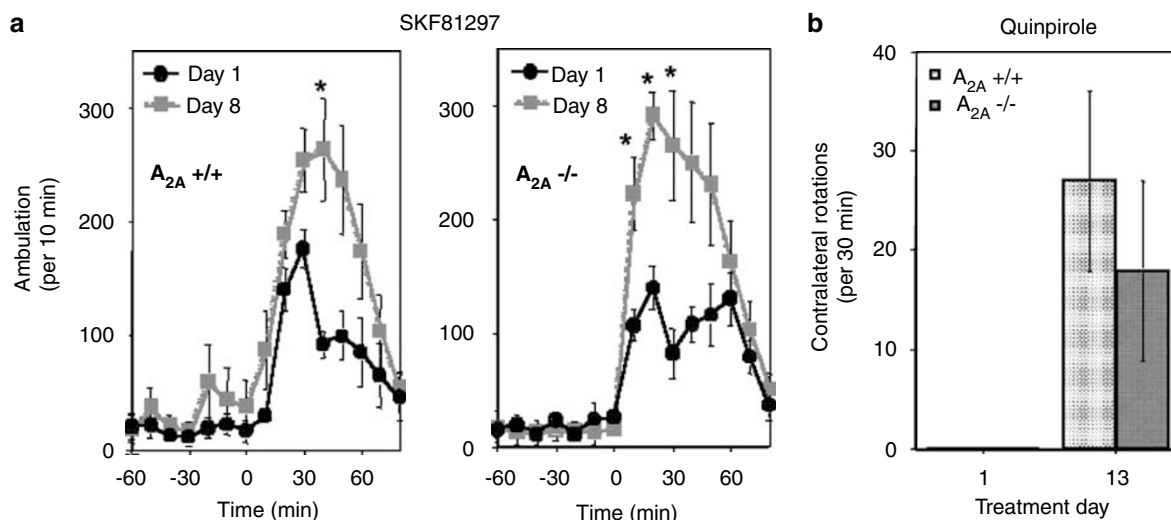

Figure 2 Dopamine D₁ and D₂ agonists induced indistinguishable behavioral sensitization in WT and A₂ₐ KO mice. (a) Mice were treated with D₁ agonist SKF81297 (2.0 mg/kg, i.p.) daily for 8 days, and ambulation was recorded for 120 min after the injection on days 1 and 8 as described in the Methods. Repeated treatments with SKF81297-induced sensitized locomotor responses in both WT and A₂ₐ KO mice ($n = 8$, *$p < 0.05$, three-way ANOVA followed by Tukey's post hoc test). However, the locomotor sensitization was indistinguishable between WT and A₂ₐ KO mice ($n = 7–8$, $F(1,215) = 0.02$, $p = 0.96$, three-way ANOVA comparing WT and A₂ₐ KO mice at days 1 and 8, respectively). (b) To study behavioral sensitization by D₂ agonists, WT, and A₂ₐ KO mice were first unilaterally lesioned by intrastriatal injection of 6-OHDA. Seven days after lesioning, mice were treated with daily injections of quinpirole (1.0 mg/kg, i.p.). Contralateral rotations were recorded for 30 min on days 1 and 13. Repeated treatments with quinpirole apparently increased rotational responses in both WT and A₂ₐ KO mice ($n = 7$).

addressed the role of D$_2$ receptor involvement in A$_{2A}$ receptor modulation of amphetamine-induced behavioral sensitization by comparing the effects of repeated treatments with D$_2$ agonists in WT and A$_{2A}$ KO littermates. Using the same 1-week daily treatment paradigm, we failed to demonstrate locomotor stimulation by several D$_2$ agonists, including quinpirole, bromocriptine and pramipexole at various doses (from 0.1 to 5 mg/kg), and under both context-dependent conditions (ie in which the mice were placed in the test cage after each injection regardless of whether locomotion was measured) and our standard context-independent conditions (data not shown). This prompted us to adopt a parallel paradigm of repeated D$_2$ agonist treatment in which rotational responses to daily i.p. quinpirole administration were measured daily in unilaterally 6-OHDA-lesioned mice for 2 weeks. Acute treatment with quinpirole produced typical stereotyped behavior (freezing, and fixed and stretched postures for several seconds to minutes) without any appreciable locomotion in WT and A$_{2A}$ KO mice. Although repeated treatment with quinpirole for 8 days did not induce locomotor sensitization in either WT or A$_{2A}$ KO mice, continued daily treatment apparently increased rotational behavior by day 13 in both WT and A$_{2A}$ KO mice (Figure 2b), which remained enhanced for the duration of the treatment period (data not shown). This sensitized rotational behavior was observed in both WT and A$_{2A}$ KO mice, ($n = 7$), although there was considerable variation in the rotational response to quinpirole. These results suggest that individual D$_1$- and D$_2$-mediated signaling pathways leading to motor sensitization were not affected by the A$_{2A}$ receptor.

Repeated Combined Administration of D$_1$ and D$_2$ Receptor Agonists also Produced Indistinguishable Sensitization in WT and A$_{2A}$ KO Mice

Since D$_1$–D$_2$ receptor interactions are critical to dopaminergic function, the A$_{2A}$ receptor might contribute to amphetamine-induced sensitization by altering the interplay between its D$_1$ and D$_2$ components (ie without actually altering sensitization produced by these individual components). To address this possibility we also examined the effect of A$_{2A}$ receptor inactivation on locomotor sensitization induced by coactivation of D$_1$ and D$_2$ receptors. Mice received intraperitoneal injections of SKF81297 (2.5 mg/kg) and quinpirole (1 mg/kg) 1 min apart, daily for 8 days. Locomotor responses were recorded at days 1 and 8. Acute treatment with the combination of SKF81297 and quinpirole produced significantly less motor stimulation (compared to SKF81297 alone) in both WT and A$_{2A}$ KO mice (at day 1; see Figure 3, compared to Figure 2a; $F(8,127) = 4.96$, $p = 0.001$, two-way ANOVA). Nevertheless, following 1 week of daily combined treatments, SKF81297 plus quinpirole produced locomotor sensitization in both WT (Figure 3, $n = 7-8$,) and A$_{2A}$ KO mice (Figure 3, $n = 7-8$,). However, there was no difference in locomotor sensitization between WT and A$_{2A}$ KO mice (Figure 3, $n = 6-8$, $p = 0.85$ and 0.32 for days 1 and 8, respectively, LSD test). Three-way ANOVA shows that there were significant effects of treatment day (day 1 *vs* day 8 or 9) ($F(1,251) = 33.15$, $p = 0.001$), but clearly there were no effects of genotype ($F(1,251) = 1.68$, $p = 0.219$). There were also significant effects of postinjection time
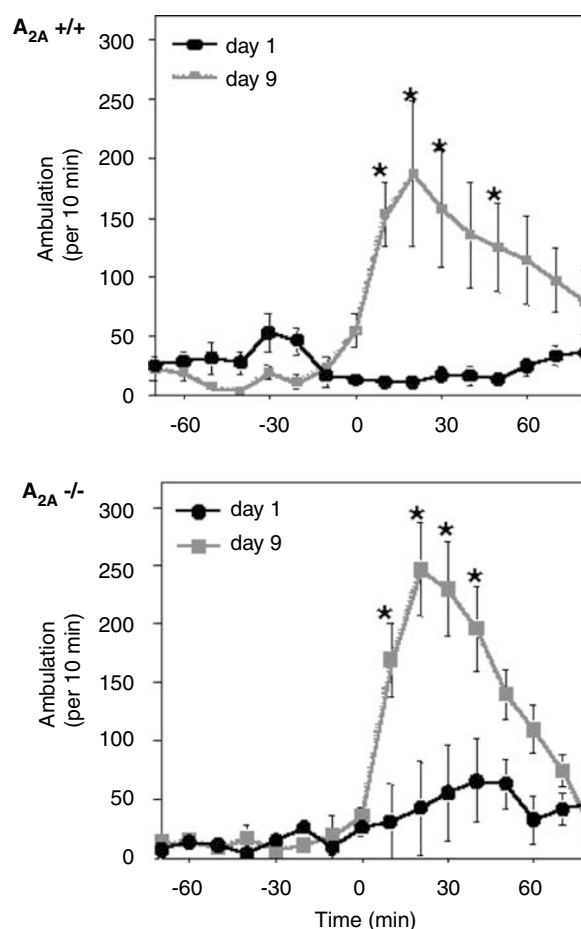

Figure 3 Coinjection of D$_1$ and D$_2$ agonists induced indistinguishable locomotor behavioral sensitization in WT and A$_{2A}$ KO mice. WT ($+/+$) and A$_{2A}$ KO ($-/-$) mice were injected with SKF81297 (2.0 mg/kg, i.p.) and quinpirole (1.0 mg/kg, i.p.) one min apart, daily for 8 days. Locomotor responses to the coinjection of SKF81297 and quinpirole were recorded for 120 min after the treatment on days 1 and 8. Daily coadministration of SKF81297 and quinpirole produced enhanced locomotor activity ($n = 7-8$, *$p < 0.05$, three-way ANOVA followed by Tukey's test, comparing day 8 to day 1 for WT or A$_{2A}$ KO mice), but the locomotor sensitization was indistinguishable between WT and A$_{2A}$ KO mice ($n = 7-8$, $F(1,251) = 1.68$, $p = 0.22$, three-way ANOVA comparing the WT with A$_{2A}$ KO mice at day 8).

($F(1,251) = 23.31$, $p < 0.001$) and a significant interaction between treatment day and postinjection time ($F(1,251) = 29.3$, $p < 0.01$). Thus, inactivation of A$_{2A}$ receptors did not affect locomotor sensitization induced by direct D$_1$ and D$_2$ agonists, either alone or in combination.

Repeated Amphetamine Induces Striatal Dynorphin mRNA in WT but not A$_{2A}$ KO Mice

Finally, we also examined the effect of A$_{2A}$ receptor inactivation on the induction of dynorphin mRNA, a cellular readout of striatal function. Dynorphin mRNA is predominantly coexpressed with dopamine D$_1$ receptor mRNA in striatonigral neurons (of the 'direct' striatal output pathway) (Gerfen *et al*, 1990; Le Moine *et al*, 1991), and has been implicated in neuroadaptive responses to psychostimulants (Xu *et al*, 1994a, b; Moratalla *et al*, 1996b).
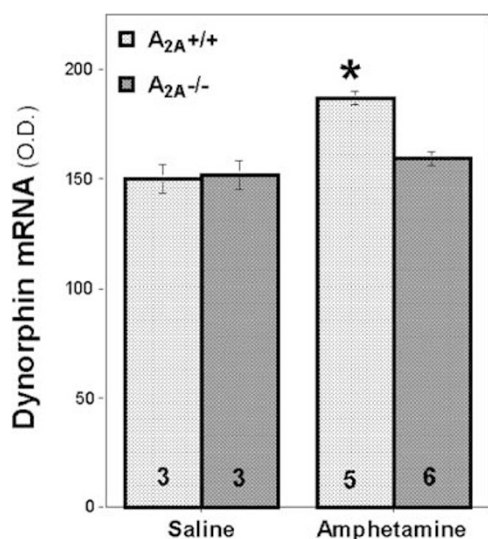
Figure 4 Repeated treatments with amphetamine increased striatal dynorphin mRNA in WT but not in A$_{2A}$ KO mice. WT and A$_{2A}$ KO mice were treated with amphetamine (2.5 mg/kg, i.p.) daily for 8 days. At 120 min after the last injection of amphetamine, mice were killed and whole brains were removed and sectioned through the striatum. Mouse brain sections were hybridized to radiolabeled dynorphin RNA probe as described in the Methods. Striatal dynorphin mRNA levels were quantified by densitometry and expressed in arbitrary optical density units. *$p < 0.05$, t-test, compared to the saline-treated group. The numbers inside the bar represent animal numbers for the corresponding groups.

Consistent with previous studies, repeated treatments with amphetamine significantly enhanced dynorphin mRNA expression in the striatum of WT mice (Figure 4, $p = 0.044$ compared to saline-treated mice, two-way ANOVA followed by the LSD test). However in the absence of the A$_{2A}$ receptor, repeated amphetamine administration had no effect on dynorphin mRNA expression (Figure 4, $p = 0.641$ comparing amphetamine to saline treatments in A$_{2A}$ KO mice by two-way ANOVA followed by the LSD test).

DISCUSSION

Amphetamine-Induced Behavioral Sensitization Requires Adenosine A$_{2A}$ Receptors

The colocalization of A$_{2A}$ and D$_2$ receptor mRNA in the dopamine receptive areas, the antagonistic interaction between adenosine A$_{2A}$ and dopamine receptor systems, and the A$_{2A}$ receptor-mediated facilitation of glutamate and dopamine release, all point to the A$_{2A}$ receptor as an important target for regulating psychostimulant-induced behavioral sensitization. Using the A$_{2A}$ KO model we have unambiguously demonstrated that inactivation of A$_{2A}$ receptors can abolish amphetamine-induced behavioral sensitization. The absence of behavioral sensitization in A$_{2A}$ KO mice was demonstrated following repeated treatments with amphetamine for 1 week as well as after a 2-week washout period (ie upon rechallenge at day 24), indicating a long-lasting effect of A$_{2A}$ receptors in amphetamine sensitization. Furthermore, the absence of amphetamine-induced behavioral sensitization cannot be attributed to a nonspecific threshold effect since a higher dose of amphetamine still failed to produce any locomotor

sensitization in the A$_{2A}$ KO mice (Figure 1b). Finally, at the cellular level, repeated amphetamine induced an increase in dynorphin mRNA in WT but not A$_{2A}$ KO mice. These results strongly suggest that the activation of the adenosine A$_{2A}$ receptor is required for behavioral sensitization induced by repeated psychostimulant treatment.

The absence of behavioral sensitization to repeated amphetamine treatment in A$_{2A}$ KO mice reflects their broader phenotype of attenuated adaptive motor responses to intermittent dopaminergic stimulation. Our recent studies demonstrate that in unilaterally 6-OHDA-lesioned animals, daily L-dopa treatment produced progressively sensitized behaviors (contralateral rotation and grooming) in WT mice, but not in A$_{2A}$ KO mice. Similarly, repeated L-dopa treatment reversed the 6-OHDA-induced reduction of striatal dynorphin mRNA in WT but not in A$_{2A}$ KO mice (Fredduzzi et al, 2002). Furthermore, El Yacoubi et al (2001) recently reported that genetic depletion of A$_{2A}$ receptors also attenuates a withdrawal syndrome after chronic treatment with alcohol. Together these results demonstrate that the A$_{2A}$ receptor plays a critical role in facilitating the neural adaptations that underlie behavioral sensitization.

Although the few available pharmacological studies of adenosine receptors in sensitization phenomena have suggested an important role for adenosine, its effects may differ depending on the particular adenosine receptor subtype and sensitizing stimulus involved (Weisberg and Kaplan, 1999; Shimazoe et al, 2000; El Yacoubi et al, 2001). Our complementary transgenic approach has provided strong evidence that the A$_{2A}$ receptor can in fact play a critical role in sensitization to certain dopaminergic stimuli. Nevertheless, our specific conclusion that the A$_{2A}$ receptor may facilitate the development of sensitization after repeated amphetamine administration seems to differ from that suggested by Shimazoe et al (2000), who found that the A$_{2A}$ agonist CGS21680 attenuates sensitization to repeated methamphetamine administration. The apparent discrepancy between our A$_{2A}$ KO study and their A$_{2A}$ agonist study may result from the intrinsic limitations of adenosinergic pharmacology (particularly when relying on agonists that do not assess the more physiological role of endogenous ligands as do antagonists).

Alternatively, limitations of the standard KO technology employed here may also have contributed to the dissociation between transgenic and pharmacological data on the role of the A$_{2A}$ receptor. The A$_{2A}$ receptor is completely inactivated throughout development as well as during adult life in the KO mice studied here. Given that A$_{2A}$ receptors can be detected at E16 and that the effect of chronic A$_{2A}$ receptor inactivation in adulthood is not known, it is possible that the A$_{2A}$ receptor deficiency may alter the development or maintenance of dopaminergic, glutamatergic, cholinergic, and GABAergic systems, and in turn lead to altered amphetamine-induced behavioral sensitization. The findings that A$_{2A}$ KO mice produced normal behavioral sensitization in response to direct D$_1$ and D$_2$ agonists are, however, not consistent with this generalized, nonspecific alteration in brain, and argue for selective impairment of behavioral sensitization via an apparent presynaptic site. Despite indistinguishable D$_1$ and D$_2$ agonist-induced behavioral sensitization, and despite our neuroanatomical characterization of the striatum in A$_{2A}$ KO mice showing no

evidence for such a developmental effect of A_{2A} receptor deficiency (Chen et al, 1999, 2000, 2001), such a possibility has not been excluded. Future studies of mutant mice with an inducible gene depletion of the A_{2A} receptor may be needed to clarify this potential confounding effect.

Adenosine A_{2A} Receptors may Modulate Psychostimulant Action through Interaction with Dopamine and Glutamate Systems

Extensive adenosine–dopamine interactions in the brain suggest that adenosinergic modulation of psychostimulant effects may result from the regulation of dopaminergic activity. At the postsynaptic level, specific changes in G proteins, adenylyl cyclase, and transcription factors (such as c-Fos, c-Jun and CREB) have also been documented to accompany chronic amphetamine-induced behavioral sensitization (Moratalla et al, 1996a; Nestler and Aghajanian, 1997). Since A_{2A} receptors are known to be positively coupled by G_s to adenylyl cyclase and PKA activity (Svenningsson et al, 1999a), inactivation of A_{2A} receptors may affect psychostimulant sensitization by influencing the cAMP signaling pathway, which in turn affects long-term gene expression associated with psychostimulant-induced behavioral sensitization.

Since A_{2A} and D_2 receptors are colocalized in striatopallidal neurons and have antagonistic interactions in brain, we attempted to evaluate D_2 agonist-induced behavioral sensitization, and its modulation by the A_{2A} receptor. However, instead of a motor stimulation, we consistently observed a motor depressant effect of D_2 agonists, which appears to be stronger and more consistent in mice than that in rats. Similar motor depression by D_2 agonists in mice at the doses used in the current study have been reported by other groups (Xu et al, 1997; Wang et al, 2000). This precluded the direct comparison between amphetamine and D_2 agonist-induced sensitization in identical paradigms, thus limiting the strength of the evidence that A_{2A} receptor involvement in amphetamine sensitization does not involve postsynaptic D_2 receptors. Nevertheless, the demonstration that behavioral sensitization to direct D_1 and D_2 agonists (alone or in combination) is similar in A_{2A} KO and WT littermates argues against significant alteration in postsynaptic D_1 or D_2 receptor-mediated signaling pathways as the basis of attenuated sensitization in the A_{2A} KO mice. The selective absence of amphetamine-induced behavioral sensitization suggests that A_{2A} receptor inactivation may prevent behavioral sensitization by impairing a presynaptic mechanism. A prominent presynaptic contribution to psychostimulant-induced sensitization has long been supported by the findings of enhanced dopamine release in the striatum and nucleus accumbens after chronic amphetamine treatment (Robinson and Becker, 1986; Kalivas and Stewart, 1991; Pierce and Kalivas, 1997). In this context, the findings that A_{2A} agonists generally increase and antagonists decrease dopamine accumulation in striatal microdialysates (Zetterstrom and Fillenz, 1990; Okada et al, 1996; Okada et al, 1997) suggest a presynaptic mechanism through which A_{2A} receptor inactivation could prevent psychostimulant-induced sensitization. In agreement, our preliminary study showed that depolarization-elicited dopamine release was significantly attenuated in striatal synaptosomes from A_{2A} KO mice (Chen et al, 1998). This notion is further supported by recent in vivo data demonstrating that basal dopamine efflux in the striatum, measured by microdialysis, is significantly lower in A_{2A} KO mice compared to their WT littermates (Dassesse et al, 2001). Together these studies suggest that A_{2A} receptors can facilitate dopamine release in the striatum, and inactivation of A_{2A} receptors may attenuate psychostimulant sensitization by attenuating dopamine release.

The notion of A_{2A} receptor-mediated facilitation of dopamine release suggests that A_{2A} receptor regulation of dopaminergic activity may depend on a fine balance between pre- and postsynaptic functions of the A_{2A} receptor. Thus, A_{2A} receptor-mediated presynaptic facilitation of dopamine release may offset the A_{2A} receptor-mediated postsynaptic inhibition of D_2 receptor function to 'fine tune' the final cellular and behavioral output from central dopaminergic pathways.

In addition, A_{2A} receptors may modulate psychostimulant-induced behavioral sensitization through interactions with neurotransmitter systems other than dopamine. For example, both muscarinic (Heidbreder and Shippenberg, 1996) and nicotinic (Schoffelmeer et al, 2002) cholinergic receptors have been implicated in psychostimulant behavioral sensitization, and A_{2A} receptor stimulation is capable of facilitating the release of acetylcholine in the striatum (Kurokawa et al, 1994). Thus, A_{2A} antagonists could attenuate amphetamine behavioral sensitization in part by reducing acetylcholine release in the striatum. Moreover, increasing evidence suggests that activation of A_{2A} receptors enhances glutamate release in the striatum and cortex (Sebastiao and Ribeiro, 1996). Activation of A_{2A} receptors can also modulate NMDA receptor conductance (Norenberg et al, 1998; Wirkner et al, 2000). Both NMDA and non-NMDA glutamate receptor antagonists have been shown to interfere with the development of behavioral sensitization to amphetamine (Wolf, 1998). Thus, inactivation of A_{2A} receptors could block behavioral sensitization by diminishing glutamate release presynaptically or affecting NMDA receptors in neurons directly. Moreover, recent studies have suggested that metabotropic glutamate receptors also regulate psychostimulant-induced neurochemical and behavioral effects (Chiamulera et al, 2001; Swanson et al, 2001). Interestingly, Chiamulera et al (2001) reported that reinforcing and locomotor stimulant effect of cocaine are absent in mice lacking mGluR5. Several studies have demonstrated a close interaction between A_{2A} receptor and mGluR5 receptors in modulating quinpirole-induced turning, D_2 receptor binding, and GABA release (Kearney and Albin, 1995; Kearney et al, 1997, 1998; Popoli et al, 2001; Diaz-Cabiale et al, 2002). Therefore, A_{2A} receptors could either directly or indirectly (eg through glutamatergic pathways) exert a modulatory effect on psychostimulant-induced sensitization.

Finally, d'Alcantara et al (2001) recently reported that genetic depletion or pharmacological blockade of A_{2A} receptors attenuates long-term potentiation in the nucleus accumbens without affecting basal synaptic transmission. Thus, inactivation of A_{2A} receptors may impair LTP and learning processes to influence psychostimulant-induced behavioral sensitization.

In summary, we demonstrated that genetic inactivation of A$_{2A}$ receptors abolishes locomotor behavioral sensitization and dynorphin mRNA expression induced by repeated treatments with amphetamine. The absence of locomotor behavioral sensitization appears selective for amphetamine since D$_1$ and D$_2$ agonists alone or in combination produce identical locomotor sensitization in WT and A$_{2A}$ KO mice, pointing to a presynaptic action of the receptor. These results indicate that behavioral sensitization to repeated psychostimulant administration requires the activation of the adenosine A$_{2A}$ receptor. Thus, targeting the brain A$_{2A}$ receptor may provide a pharmacologically specific strategy to prevent or reverse maladaptive biochemical and behavioral responses to repeated drug administration in human psychostimulant addiction.

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