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A Crucial Role for Forebrain Adenosine A_{2A} Receptors in Amphetamine Sensitization

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Adenosine A_{2A} receptors (A_{2A}Rs) are well positioned to influence the maladaptive CNS responses to repeated dopaminergic stimulation in psychostimulant addiction. Expression of A_{2A}Rs in brain is largely restricted to the nucleus accumbens and striatum, where molecular adaptations mediate chronic effects of psychostimulants such as behavioral sensitization. Using a novel forebrain-specific conditional (Cre/*loxP* system) knockout of the A_{2A}R in coordination with classical pharmacological approaches, we investigated the involvement of brain A_{2A}Rs in amphetamine-induced behavioral sensitization. Tissue-specific, functional disruption of the receptor was confirmed by autoradiography, PCR, and the loss of A_{2A} antagonist-induced motor stimulation. Daily treatment with amphetamine for 1 week markedly enhanced locomotor responses on day 8 in control mice and the sensitization remained robust after a week of washout. Their conditional knockout littermates however showed no sensitization to amphetamine on day 8 and only a modest sensitization following the washout. Pharmacological blockade of adenosine A_{2A}Rs also was able to block the development (but not the expression) of sensitization in multiple mouse strains. Thus activation of brain A_{2A}Rs plays a critical role in developing augmented psychomotor responses to repeated psychostimulant exposure.

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INTRODUCTION

The behavioral effects of psychostimulants such as amphetamine and cocaine are mediated by their indirect activation of dopamine receptors in the nucleus accumbens and caudate-putamen (Koob, 1996). Psychostimulants are thought to be addictive due to neuronal and molecular adaptations both within and outside the mesoaccumbens circuitry (Vanderschuren and Kalivas 2000). Behavioral sensitization, which is described as a progressive augmentation of responses to repeated drug administration, is an expression of neuroadaptations. In humans, sensitization to psychostimulant drugs may appear as craving behavior or paranoia (Robinson and Berridge 1993; Kalivas *et al*, 1998). Critical roles have been established for dopaminergic and subsequently glutamatergic transmission in psychostimulant-induced behavioral sensitization (Wolf, 1998). Recently, the adenosine A_{2A} receptor (A_{2A}R) has also emerged as a potential modulator of psychostimulant sensitization and as an attractive therapeutic target. A_{2A}Rs are ideally located to modulate neuronal pathways involved in psychostimulant sensitization, given that their brain expression is largely restricted to the nucleus accumbens, striatum, and olfactory tubercle (Rosin *et al*, 2003). Furthermore, A_{2A} adenosinergic and D_2 dopaminergic systems interact at the membrane (Ferre *et al*, 1994; Canals *et al*, 2003), intracellular (Morelli *et al*, 1995), and behavioral levels (Ferre *et al*, 1993; Fenu *et al*, 1997). In addition, activation of A_{2A}Rs enhances the release of several brain neurotransmitters including dopamine and glutamate, which contribute to the development of psychostimulant behavioral sensitization (Quarta *et al*, 2004).

Recent pharmacological data provide direct evidence for an important role of adenosine A_{2A}Rs in the long-term adaptive responses to repeated dopaminergic stimulation both in rats (Bove *et al*, 2002; Bibbiani *et al*, 2003) and non-human primates (Bibbiani *et al*, 2003). In addition, using A_{2A}R knockout (A_{2A} KO) mice, our laboratory has shown that behavioral sensitization to repeated treatment either with L-dopa in hemiparkinsonian mice or with amphetamine in unlesioned mice does not develop in the absence of

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the A_{2A}R (Fredduzzi *et al*, 2002; Chen *et al*, 2003). However, a facilitative role of A_{2A}Rs in sensitization is controversial and other reports have shown that the A_{2A} agonist CGS21680 attenuates the development of behavioral sensitization induced by methamphetamine (Shimazoe *et al*, 2000). This discrepancy between genetic and pharmacological studies of A_{2A}Rs functions may reflect particular limitations of either of these approaches, for example, developmental or chronic inactivation of the A_{2A}R in the KO model, or exogenous *vs* endogenous modulation of the adenosinergic system using a pharmacological agonist.

In order to clarify the role for A_{2A}Rs in behavioral adaptations induced by repeated psychostimulant exposure, we used a brain-specific conditional KO of the A_{2A}R in coordination with a classical pharmacological approach. The genetic model allows us to specifically explore the A_{2A}R in forebrain areas and to avoid possible compensatory developmental responses of other genes, given the fact that the deletion of the A_{2A}R is postnatal. Moreover, we took advantage of newly available A_{2A} antagonists, which offer improved specificity despite persistent problems of solubility and stability, to test the role of the A_{2A}R in the development of amphetamine sensitization and its discrete phases (induction and expression).

MATERIALS AND METHODS

Generation and Genotyping of Postnatal Forebrain-Specific A_{2A} Conditional KO Mice (Cre/*loxP* System)

The *L7ag13* line of *CaMKIIα-cre* transgenic mice (in a C57Bl/6 background) expresses the Cre recombinase under the direction of the *CaMKIIα* gene promoter in postnatal neurons of the striatum as well as other forebrain structures and in germline cells (Dragatsis *et al*, 2000; Dragatsis and Zeitlin 2000; Morozov *et al*, 2003) and was kindly provided by WT Dauer, A Morozov, R Hen, and ER Kandel. Mice with a 'floxed' adenosine A_{2A}R gene were generated by insertion of *loxP* sequences within the introns flanking a critical exon (2) of the A_{2A}R gene (YJ Day and J Linden, unpublished results). Homozygous floxed ($A_{2A}{}^{flox/flox}$) mice (F5 generation in a mixed 129-Steel and C57Bl/6 genetic background) were crossed with *L7ag13 cre*(+) mice, and female *cre*(+) ($A_{2A}{}^{flox/+}$) offspring were then crossed with $A_{2A}{}^{flox/+}$ males. Their *cre*(+) $A_{2A}{}^{flox/flox}$ and *cre*(−) $A_{2A}{}^{flox/flox}$ offspring were compared in an initial autoradiographic and pharmacological assessment (Figure 1a, c, and d). A_{2A} genotypes for several brain regions and peripheral tissues from (4-month-old) adult $A_{2A}{}^{flox/+}$ (and *cre*(−) $A_{2A}{}^{flox/+}$) littermates were compared (Figure 1b) to test for tissue-specific expression of the *cre* transgene and consequent Cre-mediated recombination of the floxed A_{2A} allele.

Initial efforts to expand the numbers of *cre*(+) $A_{2A}{}^{flox/flox}$ and *cre*(−) $A_{2A}{}^{flox/flox}$ littermates were complicated by unexpected germline *cre* transgene expression and recombination events in some female *cre*(+) $A_{2A}{}^{flox/+}$ breeder mice (in addition to those expected in male *cre*(+) $A_{2A}{}^{flox/+}$ mice; Dragatsis *et al*, 2000; Dragatsis and Zeitlin 2000; Zakharenko *et al*, 2003). This newly identified *CaMKIIα* promoter-driven recombination in female as well as male gametes may reflect differences in Cre-mediated recombination in germline cells seen with different floxed genes—presumably due to differences in local chromatin structure effects on *loxP* site accessibility (Morozov *et al*, 2003). Thus, the expected $A_{2A}{}^{flox/flox}$ offspring resulting from crossing a female *cre*(+) $A_{2A}{}^{flox/+}$ with male *cre*(−) $A_{2A}{}^{flox/+}$ mice were found to have one recombined (−) as well as one floxed A_{2A} allele (ie to be $A_{2A}{}^{flox/-}$). The *cre*(+) $A_{2A}{}^{flox/-}$ mice among these offspring were then crossed with their *cre*(−) $A_{2A}{}^{flox/-}$ littermates in multiple matings to produce the 16 male $A_{2A}{}^{flox/-}$ (half that were *cre*(+) and half that were *cre*(−)) employed in this study of amphetamine sensitization (Figure 2).

Genotyping for the presence of the *cre* transgene and separately for the presence of the wild-type (WT), floxed, or recombined (inactivated) alleles of the A_{2A}R gene was conducted by polymerase chain reaction (PCR) analysis of tail DNA unless otherwise indicated. The three-probe PCR strategy employed for A_{2A} genotyping is schematized in Figure 1b, and is based on the location of *loxP* inserts within the gene (Day and Linden, unpublished results).

Animals and Drug Treatments

All experiments were performed in accordance with the Massachusetts General Hospital and NIH guidelines on the ethical use of animals. Both colony and commercial (129-Steel and C57Bl/6) mice (Charles River Laboratories) were habituated to the testing environment for 120 min prior to behavioral testing. In the dose–response studies (Figure 3a), mice were treated intraperitoneally (i.p.) with the A_{2A} antagonist SCH58261, KW-6002, or vehicle (10% DMSO, 15% ethoxylated castor oil, 75% distilled water). In the amphetamine-induced sensitization studies (Figure 3b–d), as schematized in Figure 4, mice received daily injections (in their test cage) of amphetamine (2.5 mg/kg, i.p.) combined with vehicle, SCH58261 (0.03 mg/kg, i.p.), or KW-6002 (0.03 mg/kg, i.p.) for 8 consecutive days. A_{2A} antagonists were injected 1–2 min before amphetamine. Locomotor activity was recorded (using an automated open field system) for 120 min following drug injection on days 1 and 8 and 1 and 2 weeks after the cessation of the treatment (days 15 and 22). In the induction study of amphetamine sensitization (Figure 5a), drug treatments are identical to those of the amphetamine sensitization studies up to day 15. On day 22, the locomotor activity was monitored upon challenge with amphetamine (2.5 mg/kg, i.p.) alone in all mice. In the expression study (Figure 5b), all mice were treated for 8 consecutive days with amphetamine alone (2.5 mg/kg, i.p.). A week after the last treatment (day 15), mice were treated either with amphetamine (2.5 mg/kg, i.p.) alone or paired with SCH58261 (0.03 mg/kg, i.p.), and their locomotor activity was recorded on days 1, 8, and 15. When using the A_{2A} conditional KO mice in the amphetamine sensitization study (Figure 2), animals were treated with amphetamine (2.5 mg/kg, i.p.) alone.

Locomotor and Fine Motor Activity

Horizontal locomotor and fine motor activity were primarily assessed by an automated recording system (San Diego Instruments) in standard polypropylene cages (15 × 25 cm)

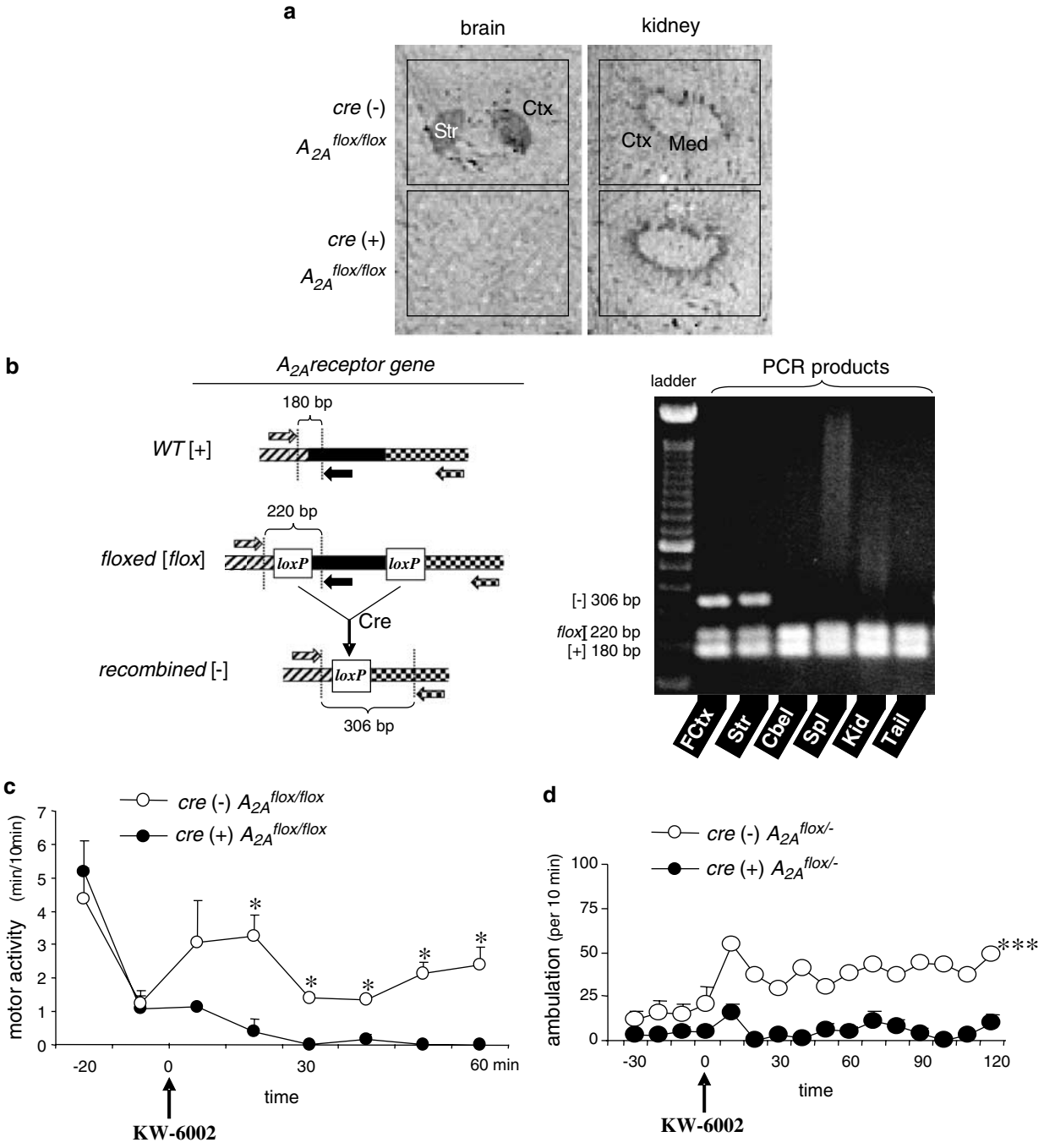

Figure 1 Tissue-specific, functional disruption of the adenosine A_{2A}R. (a) A_{2A}R autoradiography (^{3}H-CGS21680 binding) of coronal brain and sagittal kidney sections from a 3-month-old *cre*(−) $A_{2A}^{flox/flox}$ control mouse and its *cre*(+) $A_{2A}^{flox/flox}$ conditional KO littermate qualitatively shows striatum-specific depletion of A_{2A}Rs. st: striatum; ctx: cortex; med: medulla. (b) Assessing A_{2A}R genotypes of CNS and peripheral tissues at 4 months in *cre*(+) $A_{2A}^{flox/+}$ mice using oligonucleotide primers designed (as schematized on the left) to produce distinct PCR products for WT [+], floxed, and recombined [−] A_{2A} alleles. Frontal cortex (FCtx), striatum (Str), cerebellum (Cbel), spleen (Spn), kidney (Kid), and Tail. (c) Motor response to KW-6002 (3 mg/kg, i.p.) in *cre*(+) $A_{2A}^{flox/flox}$ conditional KO and their littermate controls. *$p<0.001$, $n=3$. (d) Locomotor response to KW-6002 (3 mg/kg, i.p.) in *cre*(+) $A_{2A}^{flox/-}$ conditional KO mice and their littermate controls. ***$p<0.001$, $n=8$.

placed into adjustable frames equipped with five infrared photocell beams that traverse each cage in a plane above the floor. Locomotor activity ('ambulation') was measured as number of sequential breaks in two adjacent beams, and fine motor activity was measured as number of sequential breaks in a single beam. For the functional characterization of *cre*(+) $A_{2A}^{flox/flox}$ mice (Figure 1c), a manual blinded recording method for locomotor activity (time spent in horizontal motion during each 10 min period) was used.

Receptor Autoradiography

Qualitative receptor autoradiography for detecting A_{2A}Rs using the specific ligand ^{3}H-CGS21680 (46.0 Ci/mmol; NEN,

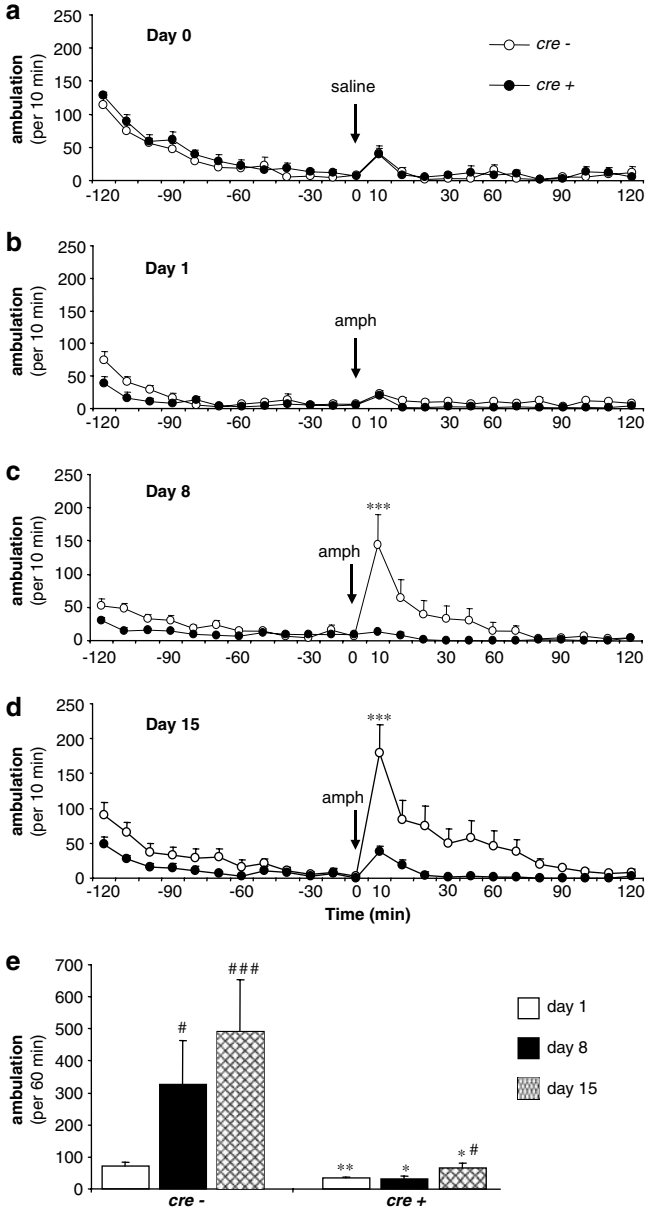

Figure 2 Absence of amphetamine-induced behavioral sensitization in postnatal brain-specific A_{2A} KO mice. Conditional A_{2A} KO (*cre*(+)) mice and littermate (*cre*(−)) controls were habituated to test cages and injected with saline (day 0, panel a), treated with amphetamine (2.5 mg/kg, i.p.) daily for 8 days, and then rechallenged with the same dose of amphetamine after a week-long washout period. Locomotor responses to amphetamine were recorded on the first day of treatment (panel b), the 8th day of daily treatment (panel c), and upon rechallenge on day 15 (panel d). ***$p<0.001$. Cumulative locomotion for the 60 min after amphetamine injection is compared between genotypes for days 1, 8, and 15 in panel e ($^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$ *vs* day 1 of *cre*(−); *$p<0.05$, **$p<0.01$ *vs* the same day of treatment of *cre*(−); $^{\ddagger}p<0.05$ *vs* day 1 of *cre*(+); $n=7$–8).

Boston, MA) was performed as described previously (Chen *et al*, 1999). Coronal brain and tissue sections were preincubated at room temperature with 50 mM Tris-HCl buffer, pH 7.5, and 1 U of adenosine deaminase for 30 min and then incubated with the Tris buffer containing 5 nM ^{3}H-CGS21680 for 60 min. To define nonspecific binding, 2.5 μM of 2-chloroadenosine was coincubated in adjacent sections.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using Prism3 software. The effects of genotype and chronic treatment (treatment days 1, 8, and 15) were analyzed by two-way ANOVA followed by post-test using the Bonferroni method. For all the other behavioral studies, one-way ANOVA followed by Dunnett's test was applied.

RESULTS

Complete, Specific Inactivation of Adult Brain A_{2A}Rs in Conditional A_{2A} KO Mice

To clarify the neurobiology of A_{2A}Rs, we generated conditional A_{2A} KO mice using a forebrain-specific Cre/*loxP* system (Morozov *et al*, 2003). Transgenic mice expressing the Cre recombinase under the direction of the *CaMKIIα* gene promoter in postnatal forebrain neurons (including those of the striatum) were crossed with mice whose *A_{2A}R* gene contained *loxP* excision sequences inserted on either side of (ie flanking) a critical exon, yielding so-called 'floxed' *A_{2A}R* alleles. Successful forebrain-specific recombination was confirmed by autoradiographic, genetic, and behavioral assessments.

Receptor autoradiography with the A_{2A} agonist ^{3}H-CGS21680 demonstrates characteristic ligand binding to A_{2A}Rs in the striatum of adult nontransgenic (ie *cre*(−)) $A_{2A}{}^{flox/flox}$ mice but complete absence of detectable binding in the striatum of *cre*(+) $A_{2A}{}^{flox/flox}$ littermates (Figure 1a). By contrast, A_{2A}R binding sites in the kidneys (specifically in the renal medulla, where A_{2A}R expression is known to be enriched; Weaver and Reppert 1992) of the same mice appear indistinguishable in the *cre*(+) and *cre*(−) ($A_{2A}{}^{flox/flox}$) mice (Figure 1a). Together with genetic evidence against Cre-mediated recombination in all other peripheral somatic tissues tested (heart, spleen, tail; Figure 1b and data not shown) in *CaMKIIα-cre*(+) mice, these anatomical findings demonstrate the brain specificity of this conditional A_{2A} KO approach. It should be noted however that disruption of the *A_{2A}R* gene also occurred in some gonadal cells (due to variable germline expression of *cre* as expected with the *CaMKIIα* promoter; Dragatsis *et al*, 2000; Morozov *et al*, 2003), complicating breeding strategies to generate *cre*(+) $A_{2A}{}^{flox/flox}$ mice (see Materials and methods).

In addition, within the CNS, the predicted further restriction of *A_{2A}R* gene recombination to the forebrain was confirmed by the genetic (PCR) analysis, showing prominent recombination of the floxed *A_{2A}R* allele in the frontal cortex and striatum but no detectable recombination in the cerebellum (as well as peripheral tissues) (Figure 1b). Note that the relatively small amount of residual floxed (nonrecombined) *A_{2A}R* allele likely reflects the lack of *cre* expression in glial cells, since forebrain neurons rather than glia are primarily targeted in the *CaMKIIα-cre*(+) mice used here (Dragatsis and Zeitlin 2000; Morozov *et al*, 2003). On the other hand, incomplete recombination in striatal neurons could be excluded as a contributor to the residual floxed *A_{2A}R* gene in the adult striatum.

In initially assessing the functional effects of eliminating A_{2A}Rs in adult striatal neurons, we examined behavioral

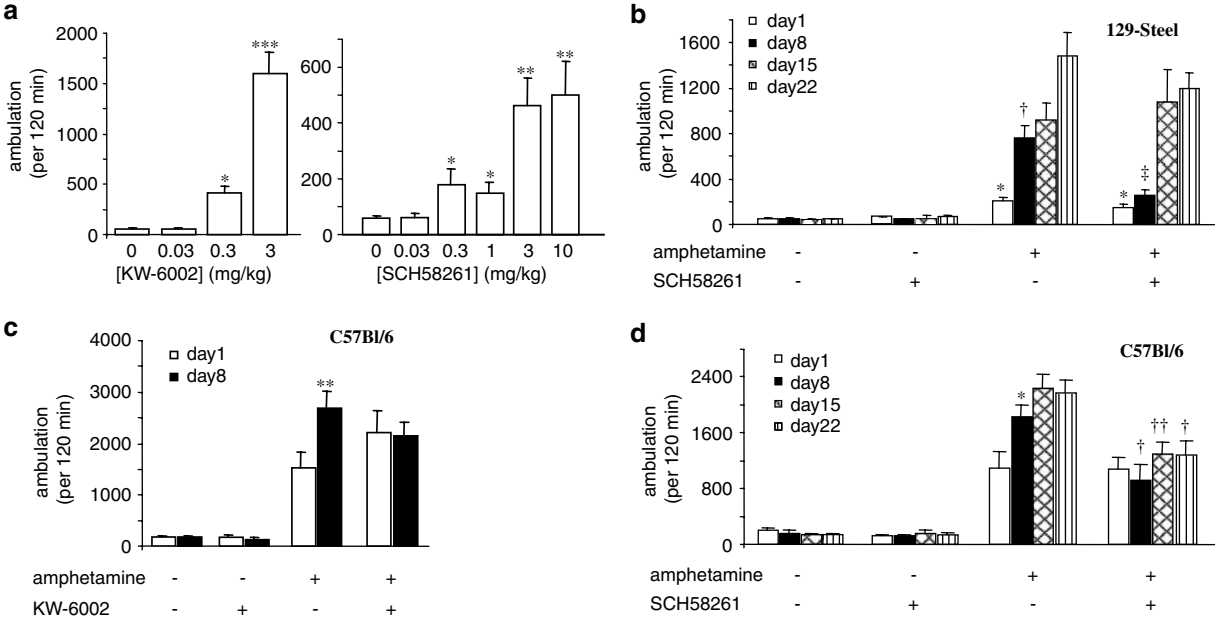

Figure 3 A_{2A} antagonists attenuate amphetamine-induced behavioral sensitization. (a) In 129-Steel mice, A_{2A} antagonists enhance locomotor activity at concentrations $\geqslant 0.3$ mg/kg ($n = 8$–10). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. (b) 129-Steel mice ($n = 10$–18) were treated with amphetamine (2.5 mg/kg or saline) and SCH58261 (0.03 mg/kg or vehicle) daily for 8 days. After a washout period of 1 week, they were rechallenged (with the same treatments) on day 15 and again on day 22. $^{*}p < 0.01$ *vs* day 1 of the vehicle-treated mice; $^{\dagger}p < 0.01$ *vs* amphetamine day 1; $^{\ddagger}p < 0.05$ *vs* amphetamine at the same day of treatment. (c) C57Bl/6 mice were treated with amphetamine (2.5 mg/kg) and KW-6002 (0.03 mg/kg) daily for 8 days ($n = 8$). $^{**}p < 0.01$ *vs* amphetamine at day 1. (d) C57Bl/6 mice ($n = 7$–9) were treated with amphetamine (2.5 mg/kg) and SCH58261 (0.03 mg/kg) as in panel b. $^{*}p < 0.05$ *vs* amphetamine at day 1; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ *vs* amphetamine treatment at the same day.

responses to the selective A_{2A} antagonist KW-6002 (3 mg/kg, i.p.) in conditional A_{2A} KO mice. Although basal locomotor activity did not differ between conditional A_{2A} KO (*cre*(+) $A_{2A}^{flox/flox}$ or *cre*(+) $A_{2A}^{flox/-}$) mice and their respective control (*cre*(−)) littermates, KW-6002 induced locomotion only in the *cre*(−) controls (Figure 1c and d). In control mice from the *CaMKIIα-cre* line (expressing the *cre* transgene in the forebrain without a floxed A_{2A} gene target), KW-6002 stimulated locomotion to the same extent as in their nontransgenic littermates, ruling out the possibility that the absence of A_{2A} antagonist-induced locomotion in the conditional KO is simply due to the expression of *cre* alone. It is to be noted that these locomotor data provide the strongest evidence yet that KW-6002 and other relatively specific A_{2A} antagonists enhance movement in Parkinson's disease patients (Bara-Jimenez *et al*, 2003; Hauser *et al*, 2003) as well as laboratory animals (Jenner 2003) through blockade of neuronal CNS A_{2A}Rs rather than non-neuronal or peripheral A_{2A}Rs. Together, these behavioral, anatomical, and genetic features of the Cre/*loxP* conditional A_{2A} KO approach confirm that it selectively eliminates the A_{2A}R from the forebrain of adult mice.

Amphetamine-Induced Sensitization Requires Brain A_{2A}R Activation

We compared the effects of brain A_{2A}R inactivation on locomotor responses to daily treatment with a low dose of amphetamine (2.5 mg/kg) in *cre*(+) $A_{2A}^{flox/-}$ (conditional KO) mice and their *cre*(−) $A_{2A}^{flox/-}$ (control) littermates. Although forebrain-specific A_{2A}R depletion had no effect on the locomotor response to a novel environment or to a habituating saline injection on day 0 (Figure 2a), locomotor activity after the first dose of amphetamine was slightly greater in control mice (on day 1; Figure 2b and e). This is consistent with a partial A_{2A}R dependence of the acute stimulant action of amphetamine, which we had observed with a global A_{2A}R KO line (Chen *et al*, 2000). Continued daily treatment with amphetamine markedly enhanced (sensitized) locomotor responses in control mice ($p < 0.05$ day 8 *vs* 1), whereas no sensitization to amphetamine occurred in their conditional KO littermates on day 8 (Figure 2c and e). Similarly, 1 week after discontinuation of daily amphetamine exposure (day 15), robust locomotor sensitization persisted in control ($p < 0.001$ *vs* day 1) but was not seen in conditional A_{2A} KO mice (Figure 2d and e), although at this point a slightly enhanced locomotor activity appeared to be present in the conditional KO group compared to day 1 ($p < 0.05$) (Figure 2e). Given this result and the fact that on day 1 the amphetamine response in the conditional A_{2A} KO was lower compared to the WT mice, we cannot exclude the possibility that the absence of sensitization on day 8 in the conditional A_{2A} KO is a reflection of subthreshold motor responses at the dose of amphetamine used in this study. We have however noted that the absence of sensitization we observed in global A_{2A} KO mice (Chen *et al*, 2003) was independent of the amphetamine dose used and was not attributable to a threshold effect on sensitization.

The possibility that the absence of sensitization in conditional A_{2A} KO mice is due to the expression of the *cre* gene rather than the selective deletion of the A_{2A}R was excluded by the finding that (*L7ag13*) control (A_{2A} WT) mice expressing *cre* (ie in the absence of a floxed $A_{2A}R$ gene)

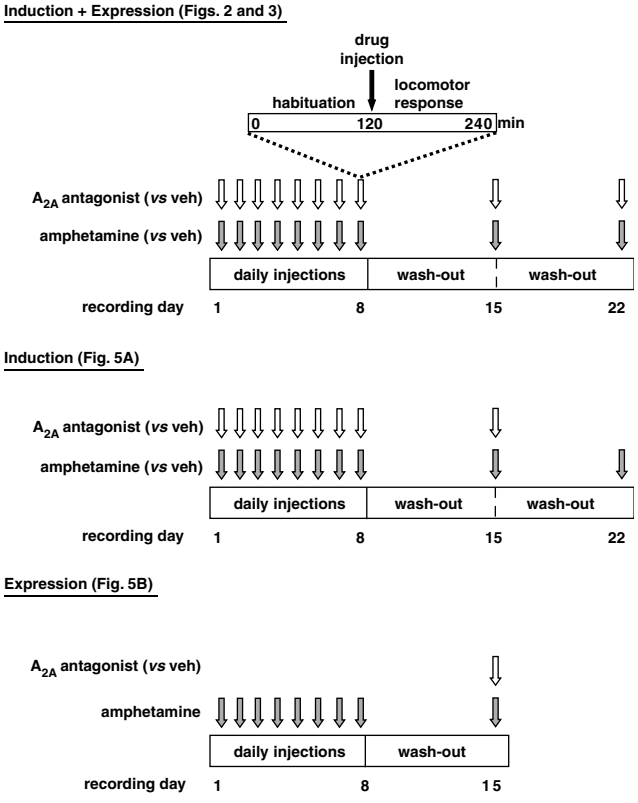

Figure 4 Schematics of paradigms for assessing A_{2A}R involvement in different phases of amphetamine-induced locomotor sensitization.

showed the same level of amphetamine sensitization as their nontransgenic (ie *cre*(−), fully WT) littermates (ambulations for 120 min after the eighth amphetamine dose: 4154 ± 844 and 3568 ± 854, respectively). Since amphetamine is known to induce stereotyped stationary behaviors as well as locomotion, we considered the possibility that the lack of locomotor sensitization in conditional KO mice could be due to immobility associated with increased stereotypes in these mice. However, an enhancement of amphetamine-induced fine movement behavior in the control mice on days 8 and 15 ($p < 0.05$) was also blocked in their A_{2A} conditional KO littermates—with increases from day 1 (182 ± 40 repetitive single photobeam breaks) to day 8 (391 ± 86) to day 15 (456 ± 101) in *cre*(−) $A_{2A}^{flox/-}$ controls, *vs* no significant change from day 1 (123 ± 22) to day 8 (220 ± 69) to day 15 (251 ± 69) in *cre*(+) $A_{2A}^{flox/-}$ mice. Together, these data demonstrate that brain A_{2A}Rs play an important role in amphetamine-induced sensitization of both locomotor and stereotyped behaviors.

A_{2A} Antagonists Attenuate Amphetamine-Induced Locomotor Sensitization

To investigate whether pharmacological blockade of A_{2A}Rs can also attenuate amphetamine sensitization, we first determined the dose of A_{2A} antagonist to be paired with amphetamine administration. To avoid the confound of the motor stimulant effects of adenosine A_{2A} antagonists (Popoli *et al*, 1998; El Yacoubi *et al*, 2000; Halldner *et al*, 2000), and given the evidence that doses of an A_{2A} antagonist below its threshold for motor stimulation are

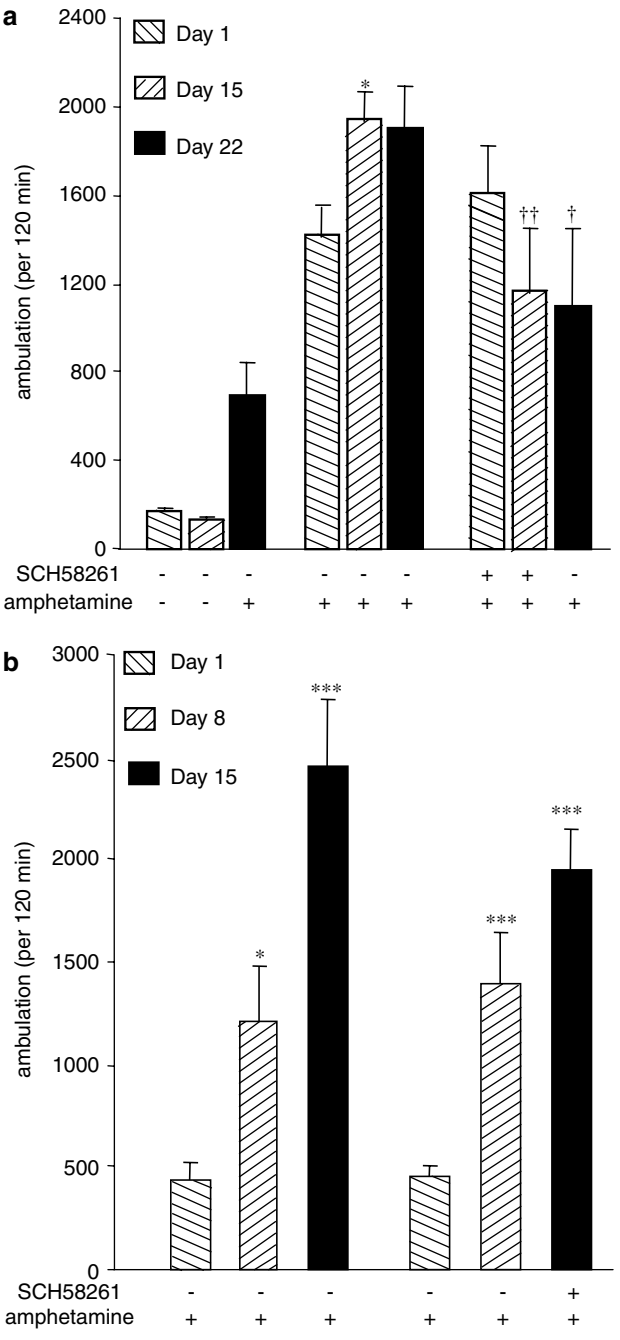

Figure 5 A_{2A}Rs are required for the induction of sensitization by amphetamine. (a) C57Bl/6 mice ($n = 8$) were treated for 8 consecutive days with amphetamine (2.5 mg/kg) alone or paired to SCH58261 (0.03 mg/kg). On day 22, all mice were challenged with amphetamine (2.5 mg/kg) alone. $^{*}p < 0.05$ *vs* amphetamine at day 1; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ *vs* amphetamine treatment at the same day. (b) C57Bl/6 mice ($n = 8$) were treated with amphetamine (2.5 mg/kg) daily for 8 days. On day 15, mice were challenged with amphetamine paired to SCH58261 (0.03 mg/kg) or vehicle. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs* amphetamine at day 1. Note that although several fold variability in acute motor response to amphetamine is routinely observed between experiments (eg a *vs* b for day 1), the amphetamine-induced sensitization phenomenon occurs consistently.

capable of modulating striatal physiology (Monopoli *et al*, 1998; Popoli *et al*, 2002), we tested several doses of two different A_{2A} antagonists, SCH58261 and KW-6002. Both

dose-dependently stimulated locomotor activity, with 0.3 mg/kg being the lowest dose that stimulated locomotion (Figure 3a). Accordingly, we selected 0.03 mg/kg as a subthreshold dose (ie that does not enhance locomotor activity) for each antagonist to be coadministered with amphetamine in sensitization experiments.

Pairing daily amphetamine doses with an A_{2A} antagonist (SCH58261 or KW-6002) prevented locomotor sensitization on day 8 in both 129-Steel and C57Bl/6 strains of mice ($p<0.05$) (Figure 3b–d). Upon rechallenge with amphetamine plus SCH58261 on days 15 and 22, C57Bl/6 mice still did not show sensitization, while sensitization remained undiminished in the amphetamine plus vehicle group (Figure 3d). In contrast, 129-Steel mice developed a sensitized response upon delayed rechallenge with amphetamine plus SCH58261 on days 15 and 22 (Figure 3b). Fine movements were also monitored and they paralleled the results with locomotor activity (data not shown). These data suggest that A_{2A}R blockade prevents or delays the development of locomotor sensitization to amphetamine, and that A_{2A}Rs facilitate neuroadaptive changes induced by repeated dopaminergic stimulation.

A_{2A}Rs are Required for the Induction (but not Expression) of Amphetamine Sensitization

We also explored the effects of adenosine A_{2A}Rs on discrete phases of sensitization. In particular, we challenged with amphetamine alone mice that have been daily treated with amphetamine or amphetamine plus the A_{2A} antagonist SCH58261 (0.03 mg/kg). Upon amphetamine challenge, locomotor activity of amphetamine plus SCH58261 chronically treated mice was still not sensitized and remained significantly lower than that of the amphetamine chronically treated mice ($p<0.05$) (Figure 5a). We also addressed possible A_{2A}R involvement in the expression phase of amphetamine sensitization. After having induced sensitization with daily administration of amphetamine alone, mice were challenged either with amphetamine or amphetamine paired with SCH58261 (0.03 mg/kg). Amphetamine-treated mice as well as SCH58261-treated mice still showed sensitization ($p<0.001$ *vs* amphetamine day 1) (Figure 5b). The data suggest that A_{2A}Rs play a role in the induction or maintenance of amphetamine-induced sensitization rather than its expression.

DISCUSSION

We have shown that postnatal inactivation of brain adenosine A_{2A}Rs dramatically attenuates sensitized behavioral responses to repeated amphetamine administration using a conditional gene depletion technique in combination with classical pharmacology. We previously showed that a global KO of the A_{2A}R (ie in all cells and at all times from conception onward) prevents amphetamine sensitization. However, our earlier study could not distinguish between a developmental, chronic, or acute inactivation of the A_{2A}R as the basis for this phenotype. Given that the A_{2A}R is expressed in brain as early as E-15 in rats (Weaver, 1993), it was possible that altered development of dopaminergic, glutamatergic, GABAergic, or other CNS signaling systems in A_{2A} KO mice could lead to an alteration in their amphetamine-induced behavioral sensitization. Similarly, the global KO study could not distinguish between the effects of A_{2A}R inactivation in brain and its many effects in the periphery. The absence of amphetamine sensitization in the conditional KO in the present study argues strongly against compensatory developmental modifications by A_{2A}R depletion as the cause of altered amphetamine-induced sensitization because the particular (*L7ag13*) line of *CaMKIIa-cre* mice used here has been shown to reduce forebrain 'floxed' gene expression somewhere between postnatal days 6 and 60 (Dragatsis and Zeitlin, 2000). In addition, the conditional KO phenotype also largely excludes the possibility of non-CNS (or non-neuronal) A_{2A}Rs contributing to amphetamine sensitization, as we (Figure 1) and others (Dragatsis *et al*, 2000; Dragatsis and Zeitlin 2000) have found no evidence of Cre recombinase activity outside the brain (or neurons) in male *CaMKIIa-cre* mice except for that in testes, which is unlikely to be a major contributor to psychostimulant-induced sensitization.

Although the postnatal conditional KO strategy helps eliminate a role for developmental actions of A_{2A}Rs, the absence of forebrain A_{2A}Rs for weeks to months prior to repeated amphetamine administration in the *cre*(+), floxed A_{2A} mice precludes a distinction between effects of chronic receptor depletion and the effects of acute inactivation just at the times of the amphetamine exposure. To address A_{2A}R involvement in amphetamine sensitization with an even greater temporal resolution than afforded by the conditional KO, we turned to complementary pharmacological antagonists of the A_{2A}R that were administered acutely together with the individual amphetamine doses. Pairing of A_{2A} antagonists with amphetamine also prevented locomotor sensitization after eight daily drug injections in both the 129-Steel and C57Bl/6 mouse strains, but prevented persistent sensitization weeks later only in C57Bl/6 mice. The different durations of sensitization blockade in the two mouse strains might be related to their differences in the metabolism of the drugs as well as to different drug sensitivities in the CNS. It also might be possible that the phases of sensitization (eg induction and maintenance) are affected differently in different mouse strains. In any event, the recapitulation of the global and conditional A_{2A} KO phenotype of attenuated amphetamine sensitization in the antagonist-treated mice strengthens further the evidence against developmental or prolonged actions of the A_{2A}R as the basis for its facilitative role in psychostimulant sensitization. Thus, from the present findings, we can now conclude that postnatal forebrain A_{2A}Rs—probably on neurons—play a critical role in behavioral sensitization to repeated amphetamine administration.

The absence of behavioral sensitization to repeated amphetamine treatment in A_{2A} KO and antagonist-treated mice may reflect a broader phenotype of attenuated adaptive motor responses to intermittent dopaminergic stimulation. Fredduzzi *et al* (2002) showed that in unilaterally 6-OHDA-lesioned (global) A_{2A} KO mice, daily treatment with L-dopa did not produce progressively sensitized behaviors (contralateral rotations and grooming) compared to their WT littermates. In analogous pharmacological studies of A_{2A}R involvement in neuroplasticity induced by L-dopa in hemiparkinsonian rodents, Bibbiani

et al (2003) have recently shown that oral KW-6002 coadministered with L-dopa daily prevented the characteristic shortening of motor response to acute L-dopa challenge. Together, these studies raise the possibility that the maladaptive involuntary movements (known as dyskinesias) that develop after chronic L-dopa treatment in Parkinson's disease may be reduced or prevented by antagonist coadministration. This hypothesis was strongly supported by a study of parkinsonian non-human primates in which chronic oral administration of KW-6002 with a dopaminergic agonist completely prevented the delayed development of dyskinesias (Bibbiani *et al*, 2003). Furthermore, A_{2A}R involvement in neural adaptations may extend beyond those induced by direct dopaminergic stimulation. For example, El Yacoubi *et al* (2001) recently reported that classical genetic deletion of A_{2A}Rs also attenuates a withdrawal syndrome after chronic alcohol administration.

On the other hand, not all pharmacological studies have supported a facilitative role for A_{2A}Rs in the neural adaptations that underlie sensitization. Shimazoe *et al* (2000) found that the A_{2A} agonist CGS21680 attenuates sensitization to repeated methamphetamine administration in rats. Their use of a different psychostimulant drug and paradigm of sensitization, as well as an A_{2A} agonist (which may be less relevant to endogenous adenosine actions on CNS A_{2A}Rs than are A_{2A} antagonists) could account for the difference in results. Moreover, although Lundblad *et al* (2003) have confirmed that treatment of 6-OHDA-lesioned rats with an adenosine A_{2A} antagonist alone did not elicit any abnormal involuntary movements while relieving motor disabilities, they did not observe any effect of KW-6002 on the severity of dyskinesias when it was coadministered with L-dopa. Another study of unilaterally 6-OHDA-lesioned rats found that an A_{2A} antagonist reversed but did not prevent L-dopa-induced motor alterations (Bove *et al*, 2002). In general, all these studies have suggested an A_{2A}R role in behavioral sensitization despite some differences in the nature of its role. The present study greatly strengthens the evidence that in the case of the brain A_{2A}R, its role in psychostimulant sensitization is facilitative.

Our finding that pharmacological blockade of A_{2A}Rs can be as effective as their genetic depletion in preventing amphetamine sensitization adds to the therapeutic potential of A_{2A} antagonists for neuropsychiatric diseases. Several A_{2A} antagonists (eg KW-6002) are already in various phases of clinical trials as a novel symptomatic treatment for Parkinson's disease. Our findings support the possibility that brain A_{2A} blockade may help prevent or delay the development of maladaptive dyskinetic motor responses to chronic dopaminergic stimulation (Pinna *et al*, 2001; Fredduzzi *et al*, 2002; Bibbiani *et al*, 2003). Moreover, our data raise the possibility that A_{2A} antagonists could provide a rational pharmacological intervention for the treatment of addictive disorders. In support of A_{2A} antagonists as therapy in neuropsychiatric disorders is the efficacy of very low doses, which are subthreshold for stimulatory motor effects. The development of sensitization may result from a series of transient neural adaptations that occur with each drug administration, ultimately establishing enduring changes in the response of the brain to subsequent drug administration. Our results implicating CNS A_{2A}Rs in the development rather than the expression of amphetamine sensitization indicate not only that CNS A_{2A}Rs play a critical role in sensitized psychostimulant responses, but also that they could be targeted to prevent or delay the maladaptive neuroplasticity that contributes to the induction or maintenance phases of some addictive behaviors.

The neurochemical mechanisms by which basal ganglia A_{2A}Rs may facilitate behavioral sensitization are unknown. A_{2A}R inactivation may prevent behavioral sensitization by modulating presynaptic dopamine release (Zetterstrom and Fillenz 1990; Okada *et al*, 1996; Dassesse *et al*, 2001). Since there is no evidence of A_{2A}Rs expression on nigrostriatal neurons (Rosin *et al*, 2003), it has been suggested that A_{2A}R-mediated facilitation of dopamine release may arise indirectly, that is, through regulation of glutamate and GABA release (Sebastiao and Ribeiro, 1996; Wolf, 1998; Corsi *et al*, 1999). Alternatively, a direct postsynaptic interaction between A_{2A} and dopamine D_2 receptor may facilitate amphetamine sensitization. In addition, the interaction among A_{2A} and mGluR5 metabotropic glutamate receptors in the basal ganglia could also modulate psychostimulant-induced sensitization (Chiamulera *et al*, 2001). Changes in the expression of presumably postsynaptic A_{2A}Rs after repeated dopaminergic exposures might also play a functional role in the nucleus accumbens or dorsal striatum (Zeng *et al*, 2000; Calon *et al*, 2004; Tomiyama *et al*, 2004). Potential downstream postsynaptic mediators of sensitization that are also known to be regulated by the A_{2A}R include cytoplasmic signal transducers (eg dopamine- and cAMP-regulated phosphoprotein of 32 kDa or DARPP-32) and nuclear transcriptional targets (eg ΔFosB, enkephalin, and dynorphin gene expression in striatal neurons; Fienberg *et al*, 1998; Canals *et al*, 2003; Lundblad *et al*, 2003; Hakansson *et al*, 2004).

In summary, by complementing classical pharmacology with a powerful new conditional KO approach to brain receptor inactivation, we have demonstrated that antagonists of the A_{2A}R and its genetic disruption in the postnatal forebrain markedly attenuate behavioral sensitization to repeated amphetamine exposure. Furthermore, the findings indicate a critical if not requisite role for brain A_{2A}Rs in an early phase of psychostimulant-induced neuroplasticity. Thus, targeting the A_{2A}R in the basal ganglia may provide a novel therapeutic strategy to prevent or reduce maladaptive biochemical and behavioral responses to repeated drug administration in human psychostimulant addiction.

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