The role of the D_2 dopamine receptor (D_2R) in A_{2A} adenosine receptor ($A_{2A}R$)-mediated behavioral and cellular responses as revealed by A_{2A} and D_2 receptor knockout mice

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The $A_{2A}R$ is largely coexpressed with D_2Rs and enkephalin mRNA in the striatum where it modulates dopaminergic activity. Activation of the $A_{2A}R$ antagonizes D_2R-mediated behavioral and neurochemical effects in the basal ganglia through a mechanism that may involve direct $A_{2A}R$–D_2R interaction. However, whether the D_2R is required for the $A_{2A}R$ to exert its neural function is an open question. In this study, we examined the role of D_2Rs in $A_{2A}R$-induced behavioral and cellular responses, by using genetic knockout (KO) models (mice deficient in $A_{2A}Rs$ or D_2Rs or both). Behavioral analysis shows that the $A_{2A}R$ agonist 2–4-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine reduced spontaneous as well as amphetamine-induced locomotion in both D_2 KO and wild-type mice. Conversely, the nonselective adenosine antagonist caffeine and the $A_{2A}R$ antagonist 8-(3-chlorostyryl)caffeine produced motor stimulation in mice lacking the D_2R, although the stimulation was significantly attenuated. At the cellular level, $A_{2A}R$ inactivation counteracted the increase in enkephalin expression in striatopallidal neurons caused by D_2R deficiency. Consistent with the D_2 KO phenotype, $A_{2A}R$ inactivation partially reversed both acute D_2R antagonist (haloperidol)-induced catalepsy and chronic haloperidol-induced enkephalin mRNA expression. Together, these results demonstrate that $A_{2A}Rs$ elicit behavioral and cellular responses despite either the genetic deficiency or pharmacological blockade of D_2Rs. Thus, $A_{2A}R$-mediated neural functions are partially independent of D_2Rs. Moreover, endogenous adenosine acting at striatal $A_{2A}Rs$ may be most accurately viewed as a facilitative modulator of striatal neuronal activity rather than simply as an inhibitory modulator of D_2R neurotransmission.

$A_{2A}Rs$ are highly concentrated in the basal ganglia where they modulate dopaminergic activity (1–3). Within the striatum, $A_{2A}R$ mRNA is largely coexpressed with D_2R as well as enkephalin mRNA in striatopallidal neurons (4, 5) (although the expression of $A_{2A}R$ mRNA also has been detected in striatal cholinergic interneurons; ref. 6). For example, *in situ* hybridization studies reveal that 93% of D_2R mRNA-bearing cells contain $A_{2A}R$ mRNA, and 95% of $A_{2A}R$ mRNA-bearing cells have D_2R mRNA in striatum (4, 5). This colocalization of $A_{2A}R$ and D_2R mRNAs suggests that the striatal efferent system is an important site for the integration of adenosine and dopamine signaling in brain. Indeed, behavioral analyses show that the nonselective adenosine antagonists caffeine and theophylline as well as the more selective $A_{2A}R$ antagonists SCH58261 {7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-c]-1,2,4-triazolo-[1,5,-c]-pyrimidine} and KW6002 [(E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione] potentiate dopamine-mediated psychomotor stimulant effects (2, 7, 8) whereas the $A_{2A}R$ agonists 2–4-(2-carboxyethyl)-phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS21680) and APEC inhibit the psychomotor effects induced by dopamine agonists (9, 10). This antagonism between A_{2A} and D_2 receptors is further supported by the neurochemical demonstration that activation of the $A_{2A}R$ antagonizes the D_2R agonist-mediated inhibition of acetylcholine release in the striatum (11, 12) and γ-aminobutyric acid (GABA) release in the striatum and globus pallidus (13), and potentiates D_2R antagonist-induced expression of the immediate early gene c-*fos* in striatum (1, 3, 14).

The antagonistic interaction between A_{2A} and D_2 receptors has been explained by a model of receptor–receptor interaction, i.e., postsynaptic inhibition of D_2Rs by $A_{2A}Rs$ in striatum (15). This model is based not only on the colocalization of $A_{2A}Rs$ and D_2Rs in striatopallidal neurons, but also on pharmacological findings that some psychomotor effects of adenosine agonists and antagonists depend on an intact nigrostriatal dopaminergic system (1). In addition, neurochemical studies have shown that activation of $A_{2A}Rs$ reduces the binding affinity of D_2 agonists to their receptors. This A_{2A}–D_2 receptor–receptor interaction has been demonstrated in striatal membrane preparations of rats (16) as well as in fibroblast cell lines after cotransfection with $A_{2A}R$ and D_2R cDNAs (17, 18). In agreement with an intramembrane interaction, A_{2A}–D_2 receptor interactions have been demonstrated in membrane preparations without ATP addition and in transfected cell lines without cotransfection of adenylyl cyclase (1, 15). Furthermore, $A_{2A}R$-mediated direct inhibition of D_2Rs also has been suggested to contribute to $A_{2A}R$ modulation of GABA release in the striatum and globus pallidus (6).

However, the direct receptor–receptor antagonistic model does not adequately explain recent findings that activation of the $A_{2A}R$ exerts a tonic excitatory effect on c-*fos* expression in dopamine-depleted animals and on D_2R antagonist-(haloperidol)-induced phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) in striatum (19). For example, the $A_{2A}R$ agonist CGS21680 induced c-*fos* expression in the 6-hydroxy-

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Abbreviations: $A_{2A}R$, A_{2A} adenosine receptor; CGS21680, 2–4-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine; CSC, 8-(3-chlorostyryl)caffeine; D_2R, D_2 dopamine receptor; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; DPCPX, 8-cyclopentyl-1,3-dipropylxanthyne; KO, knockout; PD, Parkinson's disease; WT, wild type; GABA, γ-aminobutyric acid.

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dopamine (6-OHDA)-lesioned striatum, but failed (at doses up to 50-fold higher) to stimulate c-*fos* expression in normal striatum (20). Also, the $A_{2A}R$ antagonist 8-(3-chlorostyryl)caffeine (CSC) has been shown to inhibit D_2R antagonist-induced c-Fos immunoreactivity in reserpinized rats (21). Furthermore, the D_2R antagonist eticlopride induces DARPP-32 phosphorylation in the striatum of wild-type (WT) mice but not A_{2A} knockout (KO) mice (22), suggesting that DARPP-32 phosphorylation requires tonic stimulation of $A_{2A}Rs$, independent of D_2R blockade. These results may be best explained by a proposed model of opposing, independent A_{2A} and D_2 receptor modulation of cellular responses, i.e., $A_{2A}R$ activation by endogenous adenosine may exert an excitatory influence on striatopallidal neurons by a D_2R-independent mechanism (19, 23).

Thus, whether or not striatal $A_{2A}R$ functions depend, in part or entirely, on D_2Rs is a central but open question. This is critical to our understanding not only of the cellular mechanisms underlying adenosine–dopamine interaction, but also of the physiology of endogenous adenosine at $A_{2A}Rs$: Endogenous adenosine may act at $A_{2A}Rs$ not only as an inhibitory modulator of dopaminergic neurotransmission (as proposed by $A_{2A}R$–D_2R direct interaction model) but also as a tonic excitatory modulator of striatopallidal neurons (opposing D_2R function through its independent cellular actions). The current evidence for D_2R-independent effects of striatal $A_{2A}Rs$ is based on persistent $A_{2A}R$ actions in dopamine-depleted animals or the presence of D_2R antagonists as described above. However, these pharmacological studies cannot exclude the possibility of partial depletion of dopamine or partial inhibition of D_2Rs, and therefore residual D_2R function may account for the observed persistence of $A_{2A}R$ actions. The recent development of KO mice deficient in D_2Rs and $A_{2A}Rs$ provides excellent models to address the D_2R requirement for $A_{2A}R$-mediated neural function *in vivo*. In the present study, we have used the approach of genetic inactivation of D_2Rs and $A_{2A}Rs$ (in D_2 KO, A_{2A} KO, and A_{2A}–D_2 double KO mice) as well as pharmacological manipulations of these receptors to clarify the role of D_2Rs in the behavioral and cellular actions of endogenous adenosine acting at $A_{2A}Rs$ in the striatum.

Materials and Methods

Breeding and Genotyping of A_{2A} and D_2 Receptor KO Mice. *Generation of A_{2A}-D_2 double KO mice.* A_{2A} KO mice were generated by homologous recombination by using a standard replacement targeting vector as described (24). Chimeric A_{2A} KO mice (F_0) which were derived from 129-Steel embryonic stem cells (25) were bred to C57BL/6 mice (Taconic Farms), resulting in mice of hybrid C57BL/6 × 129-Steel background. The generation of D_2 KO mice has been described (26). Heterozygous D_2 KO mice (derived from an N6 near congenic line in C57BL/6 background) were bred to generate D_2 KO and their WT littermates. To generate double homozygous mice ($A_{2A}-/-$, $D_2-/-$), we first obtained mice heterozygous for either the A_{2A} or D_2 receptor gene mutation [i.e., ($A_{2A}+/-$, $D_2+/+$) and ($A_{2A}+/+$, $D_2+/-$) mice]. These mice were then crossbred to generate double heterozygous mice. These double heterozygous mice were then crossed to produce double homozygous mutant mice ($A_{2A}-/-$, $D_2-/-$), D_2R-deficient mice ($A_{2A}+/+$, $D_2-/-$), $A_{2A}R$-deficient mice ($A_{2A}-/-$, $D_2+/+$), and WT ($A_{2A}+/+$, $D_2+/+$) mice, all from the same litters.

Genotyping of mutant mice. The genotype of each mouse was determined by genomic Southern blot analysis as described (24). Briefly, mouse tail DNA was isolated and digested with *Bam*HI (for the $A_{2A}R$ gene) or *Sac*I/*Not*I (for the D_2R gene). The genomic DNA was hybridized to radiolabeled probes (a 560-bp cDNA fragment for the $A_{2A}R$ gene or a 600-bp cDNA fragment for the D_2R gene) as described (24, 26).

Behavioral Assessments. *Animal and drug treatments.* The animals were maintained in temperature- and humidity-controlled rooms with a 12-h light/12-h dark cycle. Before drug treatment, all mice were habituated to the testing environment and basal spontaneous locomotion was recorded for 120 min. Mice were monitored during the light phase of the light/dark cycle to obtain low baseline locomotor activity in the studies with $A_{2A}R$ antagonists and dopaminergic agents, or conversely, in the dark phase to obtain high baseline locomotor activity in the studies with $A_{2A}R$ agonists. All drugs were administered i.p. in a volume of 0.1 ml/10 g of body weight, and locomotor behavior was monitored for 120–480 min. WT, A_{2A} KO, D_2 KO, and A_{2A}-D_2 double KO mice (male and female littermates from 3–8 months old) were used for this study.

Locomotor activity. Horizontal locomotor activity was assessed in standard polypropylene cages (15 × 25 cm) that were placed into adjustable frames equipped with seven infrared photocell beams (San Diego Instruments, San Diego). Ambulation (sequential breaks in two adjacent beams) were recorded and analyzed on a computer as described (24, 27).

Catalepsy score. Catalepsy behavior was induced by the D_2R antagonist haloperidol (1.5 mg/kg s.c.). Thirty minutes after haloperidol treatment, mice underwent a habituation session (pretest) and then 150 min later, the extent of catalepsy was evaluated by the vertical grid test. Mice were allowed to climb a vertical metal-wire grid (1.3-cm squares). Duration of immobility (descent latency) was taken as the dependent measurement, with an arbitrary maximal cut-off time set at 180 s.

Neurochemical Assessments. *Receptor autoradiography.* Receptor autoradiography of dopamine and adenosine receptors was performed as described (24, 27–30). For adenosine receptors, mouse brain sections were preincubated in Tris buffer containing 2.0 units/ml adenosine deaminase for 30 min and then incubated at room temperature for 60 min with the same buffer containing either an A_1R ligand {1.0 nM [^{3}H]cyclohexyladenosine or 1.0 nM [^{3}H]8-cyclopentyl-1,3-dipropylxanthyne (DPCPX) in the presence of 1 μM GTP} or an $A_{2A}R$ ligand (2.0 nM [^{3}H]CGS21680 or 3.0 nM [^{3}H]SCH58261 in the presence of adenosine deaminase. Nonspecific binding of A_1 and A_{2A} receptors were determined by coincubated [^{3}H]ligands with 25 μM 2-chloroadenosine. For dopamine receptors, striatal sections were preincubated with ice-cold 50 mM Tris·HCl buffer (pH 7.7) for 30 min, and then incubated at room temperature for 60 min with 0.8 or 2.0 nM [^{3}H]2,3,4,5-Tetrahydro-3-methyl-5-phenyl-1H-3-benzapin-7-olhydrochloride (SCH23390), 1.0 nM [^{3}H]quinpirole, or 2.0 nM [^{3}H]raclopride. To define nonspecific binding for the D_1- and D_2-like receptors, 2.0 μM SCH23390 or 10 μM eticopride, respectively, was coincubated in adjacent sections.

In situ *hybridization histochemistry. In situ* hybridization histochemistry with oligonucleotide and cRNA probes was performed according to protocols described (27, 28, 31, 32). Mouse brain sections were postfixed in buffered 4% paraformaldehyde, acetylated in acetic anhydride, and dehydrated in graded ethanols. The sections were then hybridized with about 0.4 nM [^{35}S]oligonucleotide probe (about 1.5 × 10^6 cpm per 300 μl per slide) in hybridization buffer at 37°C overnight. The slides were washed to a final stringency of 0.5× SSC at 48°C, or the sections were hybridized with a ^{35}S-labeled 423-bp riboprobe for preproenkephalin gene (gift from S. L. Sabol, National Institute of Mental Health, Bethesda, MD) following described protocols (32). Posthybridization treatment included three washes in 0.1× SSC at 70°C and 100 μg/ml RNase A at 37°C.

Receptor autoradiography and *in situ* hybridization histochemistry were quantified by using the MULTIANALYST program (Bio-Rad) by an observer blind to treatment assignments as described (27). Receptor-binding densities were expressed as fmol/mg tissue after subtracting the nonspecific binding and calibrating with a [^{3}H]receptor-binding standard (24, 28).

Statistical Analysis. Single statistical comparisons between two groups were performed by using a nonpaired Student's *t* test.

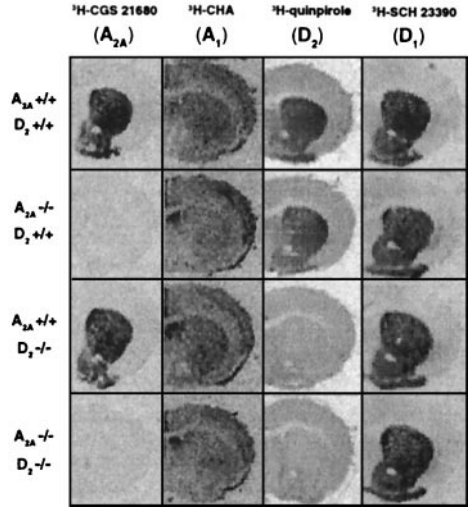

Fig. 1. Effects of genetic inactivation of A_{2A} and D_2 receptors on the expression of adenosine and dopamine receptors in the brain. A_{2A}-D_2 double heterozygous mice ($A_{2A}+/-$ $D_2+/-$) were crossbred to generate WT, A_{2A} KO, D_2 KO, and A_{2A}-D_2 double KO mice, as described in *Materials and Methods*. Receptor-binding densities for adenosine and dopamine receptors were determined by receptor autoradiography by using specific ligands in coronal brain sections of drug-naive WT ($A_{2A}+/+$ $D_2+/+$), A_{2A} KO ($A_{2A}-/-$ $D_2+/+$), D_2 KO ($A_{2A}+/+$ $D_2-/-$), and A_{2A}-D_2 double KO ($A_{2A}-/-$ $D_2-/-$) mice. Representative autoradiograms show receptor-binding densities for D_1-like ([^{3}H]SCH23390), D_2-like ([^{3}H]quinpirole), A_1 ([^{3}H]cyclohexyladenosine), and A_{2A} ([^{3}H]CGS21680) receptor in mouse brains of the four different genotypes.

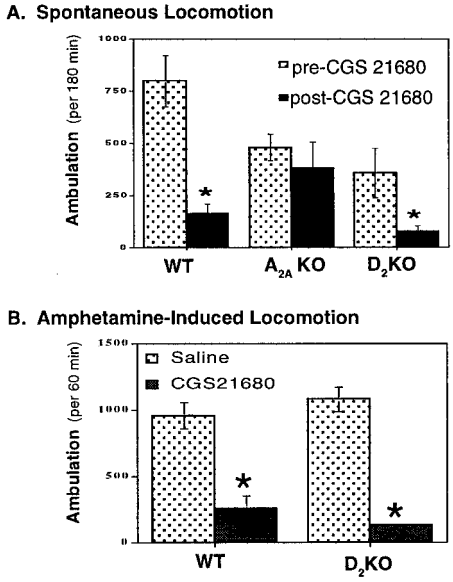

Fig. 2. Effects of D_2R inactivation on A_{2A}R agonist-induced motor depression in naive and amphetamine-treated mice. (*A*) Effects of CGS21680 on spontaneous locomotion of WT, A_{2A} KO, and D_2 KO mice. Mice were treated with CGS21680 (0.5 μg/kg i.p.) and their locomotor activities were recorded for 180 min as described in *Materials and Methods*. (Bars = cumulative ambulation for 180 min before or after CGS21680 treatment.) * indicates a significant reduction by CGS21680 when compared with basal ambulation for the corresponding genotypes (n = 9–10, $P < 0.05$, Tukey's test after two-way ANOVA). (*B*) Effects of CGS21680 on amphetamine-induced locomotion of WT and D_2 KO mice. Mice were pretreated with CGS21680 (0.5 mg/kg i.p.) or saline 5 min before amphetamine treatment (2.5 mg/kg i.p.). * indicates a significant reduction compared with saline-pretreated group. $P < 0.05$, Tukey's test after two-way ANOVA.

Analysis of receptor-binding densities or enkephalin mRNA levels of four different genotype groups were performed by one-way ANOVA followed by Tukey's post hoc comparisons. For behavioral analysis, we performed two-way ANOVA followed by Tukey's post hoc comparison to determine the effect of genotype, drug treatment, and their interaction.

Results

Effects of Genetic Inactivation of A_{2A} and D_2 Receptors on the Expression of Adenosine and Dopamine Receptors in Striatum. As the first step in characterizing the A_{2A} KO, D_2 KO, and A_{2A}-D_2 double KO mice, we determined the effects of genetic deletion of A_{2A} and D_2 receptors on the expression of adenosine (A_1 and A_{2A}) and dopamine (D_1- and D_2-like) receptors in striatum of drug-naive adult mice by receptor autoradiography. Specific agonist ligands ([^{3}H]quinpirole for D_2R, [^{3}H]cyclohexyladenosine for A_1R, and [^{3}H]CGS21680 for A_{2A}R; Fig. 1) as well as antagonist ligands {[^{3}H]SCH23390 for D_1R (Fig. 1), [^{3}H]raclopride for D_2R, [^{3}H]DPCPX for A_1R, and [^{3}H]SCH58261 for A_{2A}R; data not shown} were used to determine binding densities for dopamine and adenosine receptors in striatum. A_{2A}R deficiency completely abolished A_{2A}R binding but did not alter binding density for D_1- or D_2-like dopamine receptors in the striatum (Fig. 1) (24, 27) as well as D_1- and D_2-like-induced behaviors (27). Consistent with previous results (24), D_2R deficiency almost completely abolished D_2R binding in the striatum (Fig. 1), and D_2 antagonist-induced catalepsy (data not shown). However, D_2R deficiency did not alter binding densities for A_1Rs or A_{2A}Rs in striatum. A_{2A}R and D_2R deficiency, nevertheless, produced small but significant opposing effects on striatal A_1R binding density [< 8% reduction and increase, respectively, with [^{3}H]cyclohexyladenosine {but not [^{3}H]DPCPX; $n = 7$, $P < 0.05$, Student's t test}. A similarly small reduction in D_1R binding densities also was observed in D_2 KO and A_{2A}-D_2 double KO mice compared with their WT littermates ($n = 7$, $P < 0.05$, Student's t test), in agreement with previous findings by Kelly *et al.* (33). However, the functional significance of these modest changes in A_1R- and D_1R-binding densities in the striatum is not clear.

Effects of D_2R Inactivation on A_{2A} Agonist-Induced Motor Depression in Naive and Amphetamine-Treated Mice. To determine whether or not D_2Rs are required for A_{2A}R-mediated motor function, we compared the motor depressant effect of the A_{2A}R agonist CGS21680 on spontaneous as well as amphetamine-induced motor activity in mice lacking the D_2R with that in WT mice. Mice were treated with CGS21680 (0.5 mg/kg i.p.) and motor activity was monitored for the 60 min before and after treatment. As expected, CGS21680 produced a significant motor depressant effect in WT mice (Fig. 2*A*; $n = 9$; $P < 0.05$, Tukey's test after two-way ANOVA), but not in A_{2A} KO mice ($n = 10$), confirming the specificity of CGS21680 for A_{2A}Rs. However, D_2 KO mice (whose basal locomotion is also lower than that of WT) still displayed significant motor depression in response to CGS21680 ($n = 9$; $P < 0.05$, Tukey's test after two-way ANOVA). Two-way ANOVA analysis grouped on genotype and treatment showed that there was genotype–treatment interaction ($F_{(2,56)} = 4.20$, $P = 0.021$).

To further examine the role of the D_2R in A_{2A}R agonist-induced motor effect, we studied the motor depressant effect of CGS21680 on amphetamine-induced locomotion in D_2 KO mice. Amphetamine (2.5 mg/kg) produced marked locomotion in both WT and D_2 KO mice. However, pretreatment with CGS21680 (0.5 mg/kg) almost completely abolished the amphetamine-induced motor stimulation in both WT and D_2 KO mice (Fig. 2*B*; $n = 5$, $P < 0.05$, compared with the saline pretreatment group, Student's t test). These results clearly demonstrate that A_{2A} agonist-induced motor-depressant effects on spontaneous as well as amphetamine-induced locomotion can occur in the absence of D_2Rs.

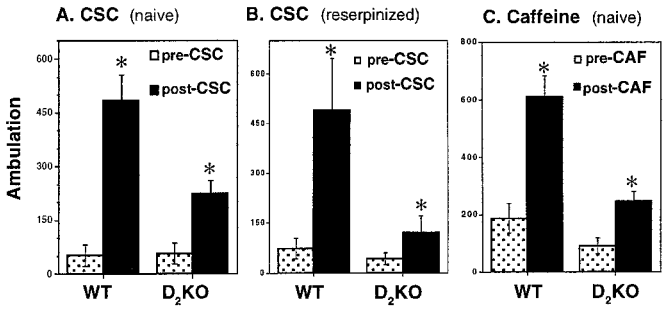

Fig. 3. Effects of the D_2R inactivation on A_{2A} antagonist-induced motor stimulation in naive and reserpinized mice. (*A*) Effect of the A_{2A} antagonist CSC (5 mg/kg i.p.) on locomotor activity was measured in naive WT and D_2 KO mice. (*B*) Effect of CSC (5 mg/kg i.p.) was measured in WT and D2 KO mice after pretreatment with reserpine (5 mg/kg i.p.). Reserpine was administered 20 h before CSC to reduce basal locomotor activity to a similar low baseline level in both groups. (*C*) WT and D_2 KO mice were treated with caffeine (20 mg/kg i.p.). [Bars in *A* (n = 6–10) and *C* (n = 7–8) represent the cumulative ambulation for 60 min before or after antagonist treatment; bars in *B* represent cumulative ambulation over 180-min periods which was used here because of the reduced motor activity in reserpinized mice (n = 8).] * indicates a significant increase after CSC/caffeine when compared with prior basal locomotion ($P < 0.05$, Tukey's test after two-way ANOVA).

Effects of D_2R Inactivation on A_{2A} Antagonist-Induced Motor Stimulation in Naive and Reserpinized Mice. To assess the role of D_2Rs in the function of $A_{2A}Rs$ stimulated by endogenous adenosine, we also compared the motor stimulant effect of the A_{2A} antagonist CSC and the nonselective adenosine antagonist caffeine on locomotion in D_2 KO mice. At the dose of CSC used here, its motor-stimulating effect completely depended on the $A_{2A}R$, because 5 mg/kg CSC was devoid of activity in A_{2A} KO mice (data not shown). CSC significantly stimulated locomotor activity in WT as well as D_2 KO mice, although the absolute level of CSC-induced activity was attenuated in D_2 KO mice compared with WT mice (Fig. 3*A*; n = 7–8, $P < 0.05$, Tukey's test after two-way ANOVA). Genotype-treatment interaction was found [$F_{(2,36)} = 4.78$, $P = 0.016$]. To assess the reliance of the motor stimulant effect of CSC on D_2Rs under conditions of dopamine depletion, we treated WT and D_2 KO mice with reserpine (5 mg/kg) 20 h before CSC administration. After reserpine treatment, CSC still produced significant motor stimulation in the D_2 KO mice, although CSC-induced motor stimulation was again significantly attenuated in D_2 KO mice compared with their WT littermates (Fig. 3*B*; $n = 8$, $P < 0.05$, two-way ANOVA followed by Tukey's post hoc comparison). Again, genotype-treatment interaction was found [$F_{(1,36)} = 5.98$, $P = 0.021$].

Caffeine has been shown to produce motor stimulation in WT mice through $A_{2A}R$ blockade (7, 34). We also tested the effects of caffeine-induced motor stimulation in A_{2A} KO and D_2 KO mice. Caffeine (20 mg/kg) produced motor stimulation in WT but not A_{2A} KO mice (data not shown), confirming that the motor-stimulant effect of caffeine at this dose is mediated by $A_{2A}Rs$. Caffeine-induced motor stimulation persisted in D_2 KO mice, although the absolute level of caffeine-induced motor activity was lower in the D_2 KO compared with their WT mice (Fig. 3*C*; n = 6–10, $P < 0.05$, Tukey's test after two-way ANOVA). These results demonstrate that adenosine antagonists acting specifically at the $A_{2A}R$ produce motor stimulation in a manner partially independent of D_2R function.

$A_{2A}R$ Inactivation Partially Reverses D_2R Inactivation-Induced Enkephalin Expression in A_{2A}-D_2 Double KO Mice. We also investigated the D_2R requirement for $A_{2A}R$ action at the cellular level by using striatal enkephalin mRNA levels as a marker of striatopallidal neuron activity (Fig. 4). *In situ* hybridization histochemistry showed

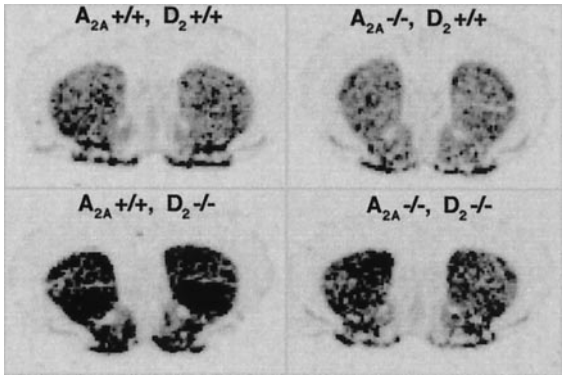

Fig. 4. $A_{2A}R$ inactivation partially reverses D_2R inactivation-induced enkephalin mRNA expression in A_{2A}-D_2 double KO mice. Striatal enkephalin mRNA levels were determined by *in situ* hybridization histochemistry as described in *Materials and Methods* and quantified in the text. Representative *in situ* hybridization autoradiograms illustrating enkephalin mRNA expression in coronal sections of drug-naive WT, A_{2A} KO, D_2 KO, and A_{2A}-D_2 double KO mice.

that whereas $A_{2A}R$ deficiency did not significantly reduce enkephalin mRNA expression [comparing $A_{2A}-/-$ $D_2+/+$ (means ± SEM; OD = 0.526 ± 0.014) vs. $A_{2A}+/+$ $D_2+/+$ (OD = 0.481 ± 0.019; $P > 0.05$, $n = 7$], D_2R inactivation markedly increased enkephalin mRNA levels in striatum by about 33% [comparing $A_{2A}+/+$ $D_2-/-$ (OD = 0.640 ± 0.031) vs. $A_{2A}+/+$ $D_2+/+$; n = 7, $P < 0.016$, Tukey's test after one-way ANOVA]. This D_2 receptor KO-induced increase in striatal enkephalin mRNA levels was largely reversed (by about 70%) by $A_{2A}R$ inactivation as seen in A_{2A}-D_2 double KO mice [comparing $A_{2A}-/-$ $D_2-/-$ (OD = 0.534 ± 0.015) vs. $A_{2A}+/+$ $D_2-/-$, $n = 7$, $P < 0.016$, Tukey's test after one-way ANOVA]. These results suggest that $A_{2A}Rs$ and D_2Rs exert opposing effects on enkephalin mRNA expression in striatum, with the stimulatory effect of $A_{2A}R$ most apparent when D_2R-mediated inhibitory tone is removed.

$A_{2A}R$ Inactivation Counteracts D_2R Antagonist-Induced Catalepsy and Striatal Enkephalin mRNA Expression. Finally, we also determined the effects of $A_{2A}R$ inactivation on catalepsy and enkephalin mRNA levels in the setting of pharmacological blockade (rather than genetic inactivation) of D_2 receptors as induced by using the haloperidol. Catalepsy was scored by the vertical grid test in A_{2A} KO mice and their WT littermates 3 h after haloperidol treatment (1.5 mg/kg s.c.) as described in *Materials and Methods*. A_{2A} KO mice exhibited significantly less catalepsy compared with their WT littermates (Fig. 5*A*; n = 9–10; $P < 0.01$, Student's t test). Selective blockade of D_2R by this dose of haloperidol was confirmed by showing that the drug treatment did not produce catalepsy in D_2 KO mice (data not shown).

Enkephalin mRNA levels also were determined in striatum of WT and A_{2A} KO mice after pharmacological blockade of D_2Rs with haloperidol (5 mg/kg s.c.) or saline for 7 days. Chronic treatment with haloperidol increased the expression of striatal enkephalin mRNA in WT mice (Fig. 5*B*, $n = 4$, $P < 0.05$, compared with the saline-treated WT group, Student's t test). Although $A_{2A}R$ deficiency again did not alter basal striatal enkephalin mRNA levels, it did partially reverse the haloperidol-induced enkephalin mRNA levels (Fig. 5*B*, $n = 4$, $P < 0.05$ compared with the haloperidol-treated WT group, Student's t test). These results demonstrate that the $A_{2A}R$ inactivation-induced reductions of both catalepsy and enkephalin mRNA levels can occur in the presence of pharmacological blockade of the D_2R, consistent with the genetic demonstration that $A_{2A}R$-mediated motor and cellular effects are at least partially independent of D_2Rs.

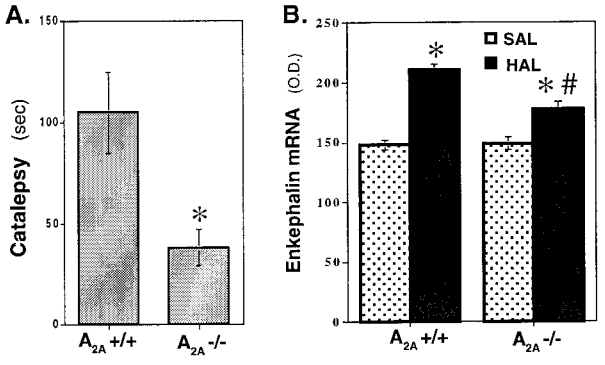

Fig. 5. $A_{2A}R$ inactivation decreases acute haloperidol-induced catalepsy and chronic haloperidol-induced enkephalin mRNA in striatum. (*A*) Effects of $A_{2A}R$ inactivation on acute haloperidol-induced catalepsy. Mice were treated with haloperidol (1.5 mg/kg s.c.) 180 min before testing. Catalepsy was scored by the vertical grid test (see *Materials and Methods*) in WT and A_{2A} KO mice. A_{2A} KO mice exhibited significantly less catalepsy compared with their WT littermates (n = 9–10; *, $P < 0.01$, Student's t test). (*B*) Effects of $A_{2A}R$ inactivation on the striatal enkephalin mRNA levels induced by the chronic treatment with haloperidol. WT and A_{2A} KO mice were treated with saline or haloperidol (1 mg/kg i.p.) daily for 7 days. Mice were killed 24 h after the last treatment. Striatal enkephalin mRNA levels were determined by *in situ* hybridization histochemistry and quantified by densitometric analysis as described in *Materials and Methods*. * indicates significant difference ($n = 4$, $P < 0.05$, Student's t test) when comparing haloperidol group to saline controls of the same genotype. # indicates significant difference ($n = 4$, $P < 0.05$, Student's t test) when comparing the haloperidol-treated KO group to its WT counterpart.

Discussion

The A_{2A} Adenosine Receptor Exerts Its Neural Effects at Least Partially Independently of D_2 Receptors. The basis for the antagonistic relationship between A_{2A} and D_2 receptor function in striatum has not been established. A widely adopted model proposes a direct A_{2A}-D_2 receptor–receptor interaction in striatopallidal neurons, and holds that $A_{2A}R$-mediated effects are based on the inhibition of D_2R function (1, 3, 15). This model has been widely used to explain how $A_{2A}Rs$ modulate locomotor activity, GABA release, and c-*fos* expression in the basal ganglia (3). Fuxe *et al.* (15) have further proposed A_{2A}-D_2 receptor heterodimers as a potential mechanism for direct functional intramembrane interactions, a concept that received experimental support from the recent demonstration of heterodimeric interaction between D_2 and somatostatin (SST5) receptors in striatal neurons (35).

Implicit in this model that $A_{2A}Rs$ exert their effects by modulating D_2R activity is the dependence of $A_{2A}R$ actions on the presence and integrity of the D_2R. However, the data presented here clearly demonstrate that the $A_{2A}R$ can modulate motor function and striatal cellular activity in a manner that is partly independent of the D_2R. The demonstration of $A_{2A}R$-induced locomotor behavioral and enkephalin mRNA responses in mice lacking D_2Rs argues strongly for D_2R-independent mechanisms contributing to $A_{2A}R$ actions in the brain. This result is consistent with a very recent finding that the A_{2A} antagonist KW6002 reverses locomotor impairment in D_2R KO mice (36). Furthermore, genetic inactivation of the $A_{2A}R$ did not alter enkephalin mRNA expression, but partially reversed the D_2 KO-induced enkephalin mRNA in A_{2A}-D_2 double KO mice, suggesting that an $A_{2A}R$-mediated facilitation of enkephalin mRNA expression is best manifested when D_2R-mediated inhibitory tone is removed. The demonstration of $A_{2A}R$ modulation of motor activity and enkephalin expression occurring despite pharmacological blockade of D_2Rs (as well as genetic inactivation of D_2Rs) suggests that D_2R-independent effects of $A_{2A}Rs$ do not result from developmental adaptations to D_2R inactivation.

However, in each of the varied behavioral and cellular experiments performed here, $A_{2A}R$ functions were not completely independent of the D_2R. For example, whereas the $A_{2A}R$ agonist CGS21680-induced motor depression in D_2 KO mice was comparable to that of their WT littermates, the $A_{2A}R$ antagonists CSC- and caffeine-induced motor stimulation were greatly attenuated in D_2 KO mice (Fig. 3; see also ref. 37), indicating the partial D_2R dependency of endogenous adenosine acting on the $A_{2A}R$. Alternatively, attenuation of $A_{2A}R$ antagonist-induced locomotor activity in D_2 KO mice may result from adaptive change(s) leading to functional uncoupling of $A_{2A}Rs$ in D_2 KO mice, as suggested by Zahniser *et al.* (37). Interestingly, in their study, CGS21680 failed to increase either GABA release or cAMP accumulation in striatopallidal slices from D_2 KO mice, despite normal expression of $A_{2A}R$ and its coupled signaling molecules (G_s, G_{olf}, and adenylyl cyclase type 6) (37). In contrast to the behavioral data, their neurochemical results suggest a functional uncoupling of $A_{2A}Rs$ in D_2 KO mice and indicate a critical role for D_2Rs in mediating $A_{2A}R$-induced GABA release in the striatum. The difference in CGS21680-induced GABA release and motor depression observed by their group and ours, respectively, may be caused by the different preparations (slices versus intact animals) and the different readouts (GABA release versus locomotor behavior). Because $A_{2A}R$-mediated motor effects may involve multiple neurotransmitter systems (e.g., dopamine, GABA, and acetylcholine), it is possible that the D_2R may be critical in $A_{2A}R$ modulation of GABA release but not as essential in $A_{2A}R$ modulation of motor activity or striatal enkephalin mRNA expression. However, electrophysiological and pharmacological studies support GABAergic involvement in $A_{2A}R$-mediated motor regulation (6, 38). Further studies are needed to determine the exact role of GABA neurotransmission in the $A_{2A}R$ modulation of motor behavior.

The demonstration of D_2R-independent effects of $A_{2A}Rs$ indicates that neural pathways not associated with striatal D_2Rs may contribute to $A_{2A}R$-mediated behavioral and cellular responses *in vivo*. In this regard, the $A_{2A}R$ has been shown to interact with D_1Rs at a network level (3, 39). Although $A_{2A}Rs$ and D_1Rs localize to different striatal projection neurons, $A_{2A}Rs$ have been shown to indirectly interact with D_1Rs to modulate D_1R-mediated locomotor activity and c-*fos* expression (39, 40). This network level interaction has recently been found to involve a synergistic contribution from the $A_{2A}R$ and D_1R in their regulation of DARPP-32 phosphorylation and cAMP accumulation in a striatal slice preparation (23). Thus, multiple mechanisms are likely involved in A_{2A}–dopamine receptor interactions.

Endogenous Adenosine Acting at $A_{2A}Rs$ Exerts a Tonic Facilitative Influence on the Expression of Enkephalin mRNA in Striatum Independent of D_2Rs. The fundamental aspect of the direct receptor–receptor model is that activation of the $A_{2A}R$ exerts its inhibitory influence on D_2Rs, which, in turn, have inhibitory effects on striatopallidal neurons (1, 15, 19). Thus, the $A_{2A}R$ may exert its neuronal function by releasing the inhibitory D_2R influence on these neurons, i.e., by disinhibiting them. An alternative model focuses on cellular actions of $A_{2A}Rs$ and proposes that $A_{2A}R$ activation exerts an excitatory influence on striatopallidal neurons, independent of D_2Rs (19, 41). Consistent with the second model, we noted that D_2 KO-induced enkephalin mRNA expression in striatum was reduced in A_{2A}-D_2 double KO mice. These results would agree with pharmacological studies showing that $A_{2A}R$ inactivation (Fig. 5) or repeated treatment with the $A_{2A}R$ antagonist KF17387 (6) partially reverses the elevation of enkephalin mRNA expression induced by the repeated treatment with the D_2R antagonist eticlopride. The notion of $A_{2A}R$-mediated facilitation on the striatal cell is supported by a recent study showing that the D_2R antagonist haloperidol induces phosphorylation of DARPP-32 in the striatum of WT but not A_{2A} KO mice (22). This result indicates a critical, independent role of $A_{2A}R$ facilitation on DARPP-32 phosphorylation in the striatum. Together, these results

strongly support the view that endogenous adenosine acting at the A_{2A}Rs exerts a facilitative influence on striatal cellular activity, manifesting best when D_2R-mediated inhibitory tone is removed.

The facilitative influence of A_{2A}R on striatal cellular activity may in part be explained by the fact that A_{2A}Rs positively couple with G_s protein to stimulate adenylyl cyclase and increase production of cAMP and consequent DARPP-32 phosphorylation (19). Several cAMP responsive elements in the promotor regions of the enkephalin gene (42), and their role in regulating enkephalin gene expression by the cAMP pathway have been demonstrated (42, 43). Thus, the regulation of enkephalin mRNA may result from A_{2A}R inactivation and associated decreased activity of the cAMP-signaling pathway. The demonstration of reversal of D_2 KO-induced enkephalin mRNA by A_{2A}R inactivation has implications for the development of A_{2A}R antagonists as a potential therapeutic intervention for Parkinson's disease (PD). It is interesting to note that neither genetic nor pharmacological inactivation of A_{2A}Rs alters enkephalin mRNA in naive mice, but both reverse the increase in enkephalin mRNA induced by chronic blockade of D_2Rs. The fact that an A_{2A}R-mediated facilitative effect on enkephalin mRNA expression is best observed when the strong inhibitory tone of the D_2R is removed (such as in the D_2 KO mice) suggests that A_{2A}R antagonists may more efficiently improve PD symptoms when the dopaminergic degeneration is more advanced. This notion is consistent with the previous demonstration that the A_{2A}-D_2 receptor interaction is enhanced in the dopamine-depleted animals (1, 3, 19). Furthermore, L-dopa, the mainstay treatment of PD, has been shown to reverse the decrease in substance P but fails to counteract the increase in enkephalin mRNA noted in animal models of PD (44, 45). It has been suggested that one of the reasons that L-dopa fails to fully alleviate the symptoms of PD may be related to its inability to reverse the induction of enkephalin mRNA (44, 45). Thus, the ability of A_{2A}R antagonists to reduce the levels of enkephalin mRNA induced by D_2R blockade may prove advantageous in PD treatment.

In summary, we have complemented standard pharmacological methods with a set of genetic KO models to demonstrate that the A_{2A}R exerts its neuronal activity in the striatum in a manner partially independent of D_2Rs. The D_2R-independent component of A_{2A}R function is demonstrable at the behavioral (motor activity) as well as cellular (enkephalin mRNA expression) levels. Furthermore, A_{2A} and D_2 receptors produce opposite effects on enkephalin mRNA expression, with A_{2A}R-mediated stimulation of enkephalin mRNA manifesting best when D_2R-mediated inhibition is removed. These results argue strongly for D_2R-dependent as well as D_2R-independent mechanisms of A_{2A}R neural functions *in vivo*. Furthermore, they suggest that endogenous adenosine acting at striatal A_{2A}Rs may be most accurately viewed as a facilitative modulator of striatal neuronal activity rather than simply as an inhibitory modulator of D_2R neurotransmission.

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