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# Functional Uncoupling of Adenosine $A_{2A}$ Receptors and Reduced Response to Caffeine in Mice Lacking Dopamine $D_2$ Receptors

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Dopamine $D_2$ receptors (Rs) and adenosine $A_{2A}$Rs are coexpressed on striatopallidal neurons, where they mediate opposing actions. In agreement with the idea that $D_2$Rs tonically inhibit GABA release from these neurons, stimulation-evoked GABA release was significantly greater from striatal/pallidal slices from $D_2R$ null mutant ($D_2R^{-/-}$) than from wild-type ($D_2R^{+/+}$) mice. Release from heterozygous ($D_2R^{+/-}$) slices was intermediate. However, contrary to predictions that $A_{2A}$R effects would be enhanced in $D_2$R-deficient mice, the $A_{2A}$R agonist CGS 21680 significantly increased GABA release only from $D_2R^{+/+}$ slices. CGS 21680 modulation was observed when $D_2$Rs were antagonized by raclopride, suggesting that an acute absence of $D_2$Rs cannot explain the results. The lack of CGS 21680 modulation in the $D_2$R-deficient mice was also not caused by a compensatory downregulation of $A_{2A}$Rs in the striatum or globus pallidus. However, CGS 21680 significantly stimulated cAMP production only in $D_2R^{+/+}$ striatal/pallidal slices. This functional uncoupling of $A_{2A}$Rs in the $D_2$R-deficient mice was not explained by reduced expression of $G_s$, $G_{olf}$, or type VI adenylyl cyclase. Locomotor activity induced by the adenosine receptor antagonist caffeine was significantly less pronounced in $D_2R^{-/-}$ mice than in $D_2R^{+/+}$ and $D_2R^{+/-}$ mice, further supporting the idea that $D_2$Rs are required for caffeine activation. Caffeine increased c-*fos* only in $D_2R^{-/-}$ globus pallidus. The present results show that a targeted disruption of the $D_2$R reduces coupling of $A_{2A}$Rs on striatopallidal neurons and thereby responses to drugs that act on adenosine receptors. They also reinforce the ideas that $D_2$Rs and $A_{2A}$Rs are functionally opposed and that $D_2$R-mediated effects normally predominate.

*Key words: adenosine $A_{2A}$ receptor; dopamine $D_2$ receptor; $D_2$ receptor knock-out mouse; CGS 21680; mRNA; [$^3$H]SCH 58261; [$^3$H]CGS 21680; caffeine; striatopallidal pathway; GABA release; cAMP stimulation; $G_s$; $G_{olf}$; type VI adenylyl cyclase; locomotor activity; c-*fos

Efferent neurons from rodent striatum project either directly to the substantia nigra or indirectly via the globus pallidus (for review, see Gerfen, 1992). Both of these striatal projections are GABAergic. However, striatonigral and striatopallidal neurons express different combinations of peptides and receptors. Striatopallidal neurons are distinguished from striatonigral neurons by expression of the preproenkephalin gene and a high density of dopamine $D_2$ and adenosine $A_{2A}$ receptors (Rs) (Schiffmann et al., 1991; Fink et al., 1992; Schiffmann and Vanderhaeghen, 1993; Svenningsson et al., 1997b).

The coexpression of $A_{2A}$Rs and $D_2$Rs on striatopallidal neurons provides an anatomical basis for the opposing interaction that exists between these receptors. Opposing $A_{2A}R/D_2R$ effects have been shown at several different levels, including behavior, neurotransmitter release, receptor binding, and gene expression (for review, see Ferré et al., 1992, 1997). For example, $A_{2A}$R stimulation reduces $D_2$R-mediated locomotor activity (Ferré et al., 1991). Likewise, $A_{2A}$R activation antagonizes the $D_2$R agonist-induced decrease in GABA release from globus pallidus (Ferré et al., 1993; Mayfield et al., 1996).

Two lines of $D_2R$ null mutant ($D_2R^{-/-}$) mice have been generated (Baik et al., 1995; Kelly et al., 1997). The striatal $D_2$R density in heterozygous ($D_2R^{+/-}$) mice is ~50% of that in wild-type ($D_2R^{+/+}$) mice, whereas no specific striatal $D_2$R binding is detectable in $D_2R^{-/-}$ mice. As anticipated, $D_2R^{-/-}$ mice show some impairments in spontaneous locomotor activity (Baik et al., 1995; Kelly et al., 1998). $D_2$Rs are inhibitory modulators of neurotransmitter release in the striatum. $D_2$ autoreceptor inhibition of dopamine release is abolished in striatal synaptosomes from $D_2R^{-/-}$ mice (L'hirondel et al., 1998). Interestingly, however, basal and potassium-evoked extracellular dopamine levels, measured with *in vivo* microdialysis, are similar in $D_2R^{+/+}$ and $D_2R^{-/-}$ mouse striata (Dickinson et al., 1999).

$D_2$R deficiency may alter release of other striatal neurotransmitters, e.g., GABA. Indeed, Baik et al. (1995) observed an increase in mRNA expression of the GABA synthetic enzyme glutamic acid decarboxylase (GAD) in the striatum of $D_2R^{-/-}$ mice, suggesting that GABA release from striatal projection neurons is increased. Enhanced GABA release from striatopallidal neurons in $D_2R^{-/-}$ mice would be predicted whether GABA release is tonically inhibited by $D_2$Rs and/or the action of $A_{2A}$Rs is unopposed by $D_2$Rs. The adenosine receptor antagonist caffeine induces locomotor activation, and this behavioral hyperactivity has been shown to involve $A_{2A}$Rs, $D_2$Rs, and GABA (Mukhopadhyay and Poddar, 1995; Svenningsson et al., 1997a; Khisti et al., 2000). A better understanding of the interactions between $A_{2A}$Rs and $D_2$Rs may lead to novel therapies to treat basal ganglia movement disorders, such as Parkinson's disease and Huntington's disease (for review, see Ongini and Fredholm, 1996; Sebastião and Ribeiro, 1996; Ferré et al., 1997; Moreau and Huber, 1999). Therefore, we have further examined the significance of $A_{2A}R/D_2R$ interactions using $D_2$R-deficient animals. We determined whether the ability of the selective $A_{2A}$R agonist CGS 21680 to modulate GABA release and

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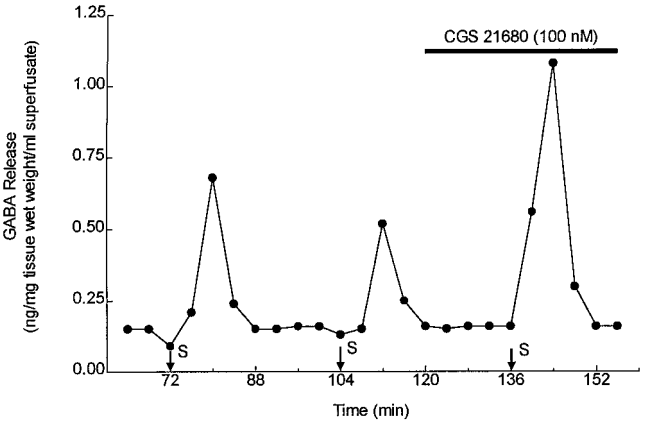


*Figure 1.* Time course of a representative endogenous GABA release experiment showing the adenosine $A_{2A}R$ agonist CGS 21680-induced augmentation of evoked release from a $D_2R^{+/+}$ mouse striatal/pallidal slice. The GABA uptake blocker NO-711 (10 $\mu$M) was present throughout the experiment. Three periods of electrical field stimulation (*S*; 12 Hz, 2 msec; 30 mA, 1 min) were applied as indicated by the *arrows*. CGS 21680 (100 nM) was included in the superfusion buffer from 120 min through the end of the experiment (*horizontal bar*). Drug effects were determined from the S3/S2 ratio, the ratio of GABA release evoked above baseline in response to the third stimulation compared with that in response to the second stimulation.

induce cAMP production, as well as the ability of the adenosine receptor antagonist caffeine to induce locomotor activity and c-*fos* expression, differed among $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mice.

## MATERIALS AND METHODS

*Subjects.* Male and female $N_5$ congenic mice (20–35 gm; Vollum Institute, Portland, OR) were used in most of the experiments. Detailed methods by which these mice were produced have been reported (Kelly et al., 1997, 1998). Briefly, a $D_2R$-genomic clone from a 129/SvEv library was isolated and used for the construction of a replacement-type targeting vector. Homologous recombination in D3 embryonic stem cells produced a mutant $D_2R$ allele, which has a deletion of exon 8 sequences encoding the sixth and seventh putative transmembrane domains, the third extracellular loop, and the cytoplasmic C-terminal tail. Germ-line transmitting chimeras derived from targeted embryonic stem cells were mated to wild-type females to generate F1 heterozygous mice on the mixed 129S2/SvPas × C57BL/6J background. The mutated $D_2R$ allele was then backcrossed an additional five generations by successive matings of heterozygous mice to wild-type C57BL/6J mice. The congenic $N_5$ mice used here were derived from intercrosses of heterozygous mice that yielded all three possible genotypes in normal Mendelian proportions: $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$. The genotypes of all mice were confirmed by Southern blotting. Male C57BL/6J mice (20–30 gm) obtained from The Jackson Laboratory (Bar Harbor, ME) were used only in the experiments testing the effects of raclopride alone and in combination with CGS 21680 on GABA release. Mice were housed in groups of two to five under a 12 hr light/dark cycle with food and water available *ad libitum*. All animal-use procedures were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center.

In vitro *GABA release.* Mice were killed by cervical dislocation, and 400 $\mu$m coronal brain slices were cut before dissecting out the region containing the striatum/globus pallidus (Franklin and Paxinos, 1997). During preparation the tissue was maintained in ice-cold modified Krebs' buffer (118 mM NaCl, 4.7 mM KCl, 11.1 mM D-glucose, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.0 mM $NaH_2PO_4$, 2.6 mM $CaCl_2$, and 4.0 $\mu$M $Na_2$-EDTA, saturated with 95% $O_2$/5% $CO_2$, pH 7.4, at 34°C). The slices were equilibrated in a metabolic shaker at 34°C for 30 min before being transferred to the superfusion chambers.

The GABA release method has been described previously (Mayfield and Zahniser, 1993). Briefly, striatal/pallidal slices were superfused at a rate of 0.25 ml/min with modified Krebs' buffer (34°C) containing 1-(2-((((diphenylmethylene)imino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid hydrochloride (NO-711) (10 $\mu$M) to inhibit GABA uptake. Three trains of monophasic rectangular pulses (12 Hz, 2 msec; 30 mA, 1 min) were applied at $t$ = 72, 104, and 136 min after the start of superfusion, with the collection of 24 consecutive 1 ml superfusate fractions beginning at $t$ = 60 min (Fig. 1). CGS 21680 (100 nM; stock solution made up in DMSO), raclopride (1 $\mu$M), or vehicle was included in the buffer at $t$ = 120 min, 16 min before the third stimulation.

GABA was quantified by HPLC with electrochemical detection after precolumn derivatization with *o*-pthaldialdehyde (Mayfield and Zahniser, 1993). The mobile phase was 1 M disodium phosphate, pH 6.4 (0.5 M final concentration), 0.86 mM NaCl, and 37.5% acetonitrile. The lower sensitivity limit was < 25 pg of GABA per 50 $\mu$l injection. Spontaneous GABA outflow, designated PS1, PS2, or PS3, was defined as the mean concentration of GABA in the three fractions immediately preceding each of the three periods of stimulation, respectively. Stimulation-evoked GABA overflow, designated S1, S2, or S3, was determined from the summed amount of GABA release that exceeded PS1, PS2, or PS3, respectively.

*cAMP levels.* Striatal/pallidal slices were prepared as outlined above and incubated at 34°C in multiwell plates in a metabolic shaker, with a change in modified Krebs' buffer at 30 and 45 min during a 1 hr equilibration period. Slices were then incubated in 0.5 ml of fresh buffer containing either no addition, 1 $\mu$M CGS 21680, 10 $\mu$M CGS 21680, or 1 $\mu$M CGS 21680 + 150 $\mu$M 8-(*p*-sulfophenyl)theophylline (8-*p*-SPT) at 34°C for 15 min. The incubation was terminated by the addition of 0.25 ml of 2.4% perchloric acid, sonication, and centrifugation at 30,000 × *g* for 15 min (Lu and Ordway, 1997). Pellets were dissolved in 0.2 ml of 0.1N NaOH for protein determination (Bradford, 1976) using bovine serum albumin as the standard. The supernatant was neutralized with excess (~20 mg) $CaCO_3$ (Thion et al., 1977). After centrifugation, the supernatant was evaporated to dryness and reconstituted in 0.25 ml of 50 mM sodium acetate buffer, pH 6.2, and cAMP was measured in duplicate 0.1 ml samples by radioimmunoassay (NEN Life Science Products, Boston, MA).

*Locomotor activity.* Mice were allowed to acclimate in individual transfer cages to the behavioral testing room for 30 min. Saline or caffeine (15 mg/kg) was then injected intraperitoneally in a volume of 1 ml/100 gm. Each mouse received only a single injection. Immediately after injection, mice were placed into individual activity chambers (San Diego Instruments, San Diego, CA). The room lights were turned off, and locomotor activity was recorded as the total distance traveled during 5 min periods for 120 min. Caffeine-induced locomotor activity was determined at each time point as a "difference score" by subtracting the mean activity of each genotype after saline injection from the activity of each mouse of that same genotype after caffeine injection.

*Quantitative receptor autoradiography.* Mice were killed 4 hr after saline or caffeine injection. Brains were frozen in powdered dry ice and stored at −70°C. Coronal sections (10 $\mu$m) were cut at −20°C at the levels of the rostral striatum/nucleus accumbens and the caudal striatum/globus pallidus. Binding of [$^3$H]SCH 58261 and [$^3$H]CGS 21680 was assayed using the published methods of Fredholm et al. (1998). Briefly, specific binding of 0.3 nM [$^3$H]SCH 58261, defined with 50 $\mu$M 5′-*N*-ethylcarboxamidoadenosine (NECA), was measured in 170 mM Tris-HCl buffer, pH 7.4, containing 2 U/ml adenosine deaminase. For [$^3$H]CGS 21680, the assay buffer also contained 10 mM $MgCl_2$. Indirect saturation curves were generated with [$^3$H]CGS 21680 (2.5 nM) by incubating slide-mounted brain sections with either no drug (total binding), one of nine concentrations of unlabeled CGS 21680 (1 nM–10 $\mu$M), or 2-chloroadenosine (20 $\mu$M; nonspecific binding). [$^3$H]raclopride binding was measured as described by Johansson et al. (1997). Specific binding of 2 nM [$^3$H]raclopride, defined with 1 $\mu$M (+)-butaclamol, was measured in 170 mM Tris-HCl buffer, pH 7.6, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.001% ascorbic acid. Slides and tritium standards were apposed to tritium-sensitive film for either 3 weeks ([$^3$H]SCH 58261), 6 weeks ([$^3$H]CGS 21680), or 8 weeks ([$^3$H]raclopride).

Films were analyzed using computer-based imaging systems (Imaging Research, St. Catherines, Ontario, Canada). Inplot software (Graph Pad, San Diego, CA) was used to fit the indirect saturation curves. The affinity ($K_d$) and number of receptors ($B_{max}$) were determined from the equations published by DeBlasi et al. (1989). For statistical analyses, data from saline- and caffeine-treated mice of the same genotype were pooled on the basis of the observations that (1) caffeine does not interfere in binding assays because it is easily dissociated from $A_{2A}$Rs by washing (Johansson et al., 1996) and (2) no differences between the saline- and caffeine-treated groups were detected when these data sets were analyzed separately.

In situ *hybridization.* Mice were killed 4 hr after saline or caffeine injection. Previously published methods were used to measure mRNA for $A_{2A}$Rs and c-*fos* (Svenningsson et al., 1997a,b, 1999); these papers also describe the specificity of the probes. Briefly, consecutive coronal brain sections (14 $\mu$m) were cut with a cryostat and thaw-mounted onto poly-L-lysine-coated slides. The $A_{2A}R$ probe was a riboprobe, 208 bases long, encoding amino acids 1196–1405 of the rat $A_{2A}R$ protein (Svenningsson et al., 1997b) and was $^{35}$S labeled by *in vitro* transcription using $^{35}$S-labeled UTP. The c-*fos* probe was an oligodeoxynucleotide probe, 48 bases long, encoding amino acids 137–152 of the rat c-Fos protein. The oligodeoxynucleotide probe (Scandinavian Gene Synthesis AB, Köping, Sweden) was labeled using terminal deoxynucleotidyl transferase (Pharmacia, Uppsala, Sweden) and $^{35}$S-labeled $\alpha$-dATP (NEN Life Science Products, Stockholm, Sweden) to a specific activity of ~$10^9$ cpm/$\mu$g. Sections were hybridized in 50% deionized formamide (Fluka, Buchs, Switzerland), 4× standard sodium citrate, 1× Denhardt's solution, 1% sarcosyl, 0.02 M $NaPO_4$, pH 7.0, 10% dextran sulfate, 0.5 mg/ml yeast tRNA (Sigma Labkemi, Stockholm, Sweden), 0.06 M dithiothreitol, 0.1 mg/ml sheared salmon sperm DNA, and $10^7$ cpm/ml probe. After hybridization for 15 hr at 42°C, the sections were washed four times, for 15 min each, in 1× standard sodium citrate at 55°C; dipped briefly in water and 70, 95, and 99.5% ethanol; and air-dried. The sections were exposed to tritium-sensitive film for 2–5 weeks. The films were analyzed as in the autoradiographic studies above.

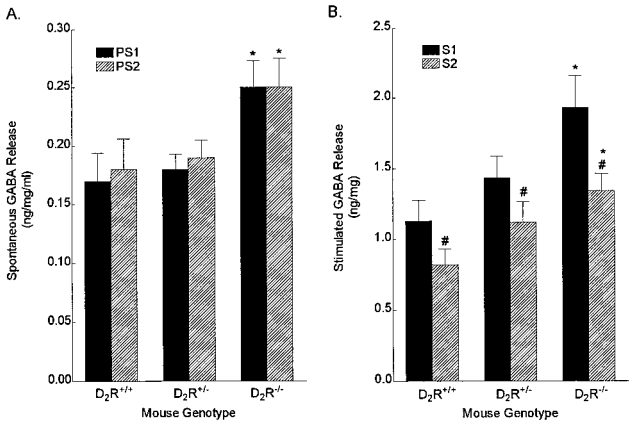


*Figure 2.* GABA release was higher from $D_2R^{-/-}$ than from $D_2R^{+/+}$ mouse striatal/pallidal slices. *A,* Spontaneous GABA release before the first (*PS1*) and second (*PS2*) periods of stimulation. See Figure 1 for experimental details. Mean values ± SEM are shown for $n = 15$ mice ($D_2R^{+/+}$), $n = 22$ mice ($D_2R^{+/-}$), or $n = 18$ mice ($D_2R^{-/-}$). Two-factor ANOVA followed by Newman–Keuls *post hoc* comparisons: $^*p < 0.05$, $D_2R^{-/-}$ versus $D_2R^{+/+}$ or $D_2R^{+/-}$. *B,* Stimulated GABA release evoked by the first (*S1*) and second (*S2*) periods of stimulation. Mean values ± SEM are shown for $n = 15$ mice ($D_2R^{+/+}$), $n = 18$ mice ($D_2R^{+/-}$), or $n = 20$ mice ($D_2R^{-/-}$). Two-factor ANOVA with repeated measures (stimulation period) followed by Newman–Keuls *post hoc* comparisons: $\#p < 0.05$, within genotype *S2* versus *S1*; $^*p < 0.05$, $D_2R^{-/-}$ versus $D_2R^{+/+}$.

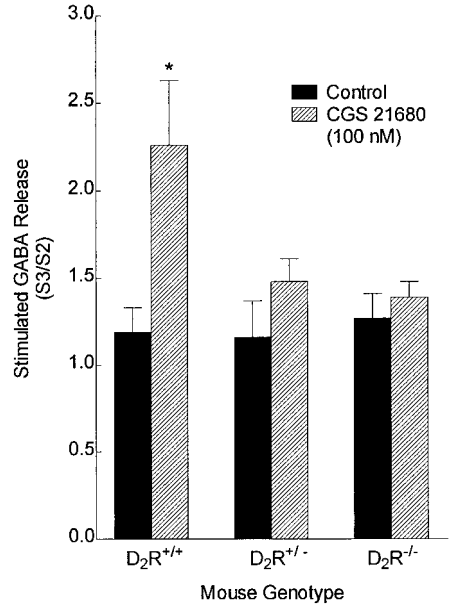


*Figure 3.* CGS 21680 (100 nM) significantly enhanced stimulation-evoked GABA release from striatal/pallidal slices from $D_2R^{+/+}$ mice but not from $D_2R^{+/-}$ or $D_2R^{-/-}$ mice. See Figure 1 for experimental details. Mean values ± SEM are shown for $n = 6$ mice ($D_2R^{+/+}$, $D_2R^{-/-}$) or $n = 8$ mice ($D_2R^{+/-}$). Two-factor ANOVA with repeated measures (drug) followed by Newman–Keuls *post hoc* comparisons: $^*p < 0.05$, $D_2R^{+/+}$ control versus CGS 21680.

The expression of mRNA for the $\alpha$ subunits of $G_s$ and $G_{olf}$, as well as for type VI adenylyl cyclase (AC VI), was examined using cRNA $^{35}$S-riboprobes essentially as described previously (Le Moine et al., 1997; Svenningsson et al., 1997b) using sense and antisense probes for the corresponding rat proteins (Jones and Reed, 1987, 1989; Glatt and Snyder, 1993). The transcription was performed using MAXI-script *in vitro* transcription kits according to the manufacturer's protocol (Ambion, Austin, TX). The probes were separated from unincorporated ribonucleotides using Sephadex G-50 chromatography. No signals were detected with the sense probes.

*Drugs.* [$^3$H]SCH 58261 was a gift from Dr. Ennio Ongini (Schering-Plough, Milan, Italy); [$^3$H]raclopride and [$^3$H]CGS 21680 were obtained from NEN Life Science Products (Boston, MA, or Stockholm, Sweden); and NO-711, CGS 21680, raclopride, 8-*p*-SPT, caffeine, NECA, 2-chloroadenosine, and (+)-butaclamol were obtained from Sigma/RBI (St. Louis, MO).

## RESULTS

### GABA release

Endogenous GABA release was evoked from striatal/pallidal slices by three periods of electrical stimulation (Fig. 1). The GABA uptake inhibitor NO-711 (10 $\mu$M) was present throughout the entire experiment. Control release was assessed in response to the first two stimuli. Spontaneous GABA outflow was measured before each of these stimuli and equaled ~0.18 ng of GABA per mg wet weight of tissue per ml in the $D_2R^{+/+}$ and $D_2R^{+/-}$ mice (Fig. 2*A*). In comparison with both the $D_2R^{+/+}$ and $D_2R^{+/-}$ mice, spontaneous GABA release was significantly elevated by ~40% in the $D_2R^{-/-}$ mice (Fig. 2*A*). In response to each of the two stimuli, GABA overflow was significantly increased in all three genotypes. However, as seen in rat striatal/pallidal slices (Mayfield and Zahniser, 1993; Mayfield et al., 1996), the second stimulation evoked consistently lower GABA release than did the first stimulation in all of the mice (Fig. 2*B*). This is not caused by depletion of releasable pools of GABA but rather involves activation of $GABA_BRs$ (Mayfield and Zahniser, 1993). Nonetheless, both the first and second stimuli increased GABA overflow to a significantly greater extent, by 71 and 63%, respectively, in the $D_2R^{-/-}$ versus the $D_2R^{+/+}$ mice (Fig. 2*B*). Stimulated GABA overflow in the $D_2R^{+/-}$ mice was intermediate and did not differ statistically from that of either of the other two genotypes (Fig. 2*B*). Because the S2/S1 ratios were similar in all three genotypes, the data indicate that $GABA_BR$-mediated autoinhibition is unaltered by elimination of $D_2Rs$.

The $A_{2A}R$ agonist CGS 21680 was introduced before the third period of stimulation (Fig. 1) because preliminary experiments showed a more consistent $A_{2A}R$-mediated modulation of GABA release when the effects of CGS 21680 were tested during the third, rather than the second, stimulation. The more consistent results could reflect the fact that the second and third stimuli released similar amounts of GABA (Fig. 3; control *S3*/*S2* ratios = ~1) whereas the second stimulus evoked less release than did the first stimulus (Fig. 2*B*; *S2*/*S1* ratios < 1). The inclusion of 100 nM CGS 21680 in the superfusion buffer 16 min before the third stimulation did not alter spontaneous GABA outflow from striatal/pallidal slices from any of the three genotypes (data not shown). However, in the $D_2R^{+/+}$ mice, exposure to 100 nM CGS 21680 significantly increased stimulation-evoked GABA overflow by 90% (Fig. 3; *S3*/*S2* ratio). In contrast, stimulated release was not significantly altered in the presence of CGS 21680 in either the $D_2R^{+/-}$ or $D_2R^{-/-}$ mice (Fig. 3).

To test the effect of acute blockade of $D_2Rs$ on CGS 21680 modulation during the GABA release assay, we measured release in C57 mouse striatal/pallidal slices in the presence of maximally effective concentrations of CGS 21680 and/or the $D_2R$ antagonist raclopride. Neither drug altered spontaneous GABA outflow (data not shown). In agreement with the results of the $D_2R^{+/+}$ mice, CGS 21680 (100 nM) significantly increased stimulation-evoked GABA overflow from the C57 mouse slices (Fig. 4; 154% above control). Raclopride (1 $\mu$M) also significantly increased evoked release by 119% (Fig. 4). When CGS 21680 and raclopride were combined, overflow was increased to a significantly greater extent (256%) than when raclopride was present alone (Fig. 4). These results demonstrate that in the presence of $D_2R$ blockade, CGS 21680 increased GABA release from mouse striatal/pallidal slices and suggest that an acute absence of $D_2Rs$ cannot explain the lack of $A_{2A}R$ modulation in the $D_2R$-deficient mice.

### $A_{2A}R$ mRNA, $A_{2A}Rs$, and $D_2Rs$

Downregulation of $A_{2A}Rs$ would be one explanation for the lack of CGS 21680-modulated GABA release in the $D_2R$-deficient mice. $A_{2A}R$ mRNA could be quantitated in the striata and nucleus accumbens, areas containing cell bodies of neurons expressing $A_{2A}Rs$, but was not detectable in the globus pallidus, the area

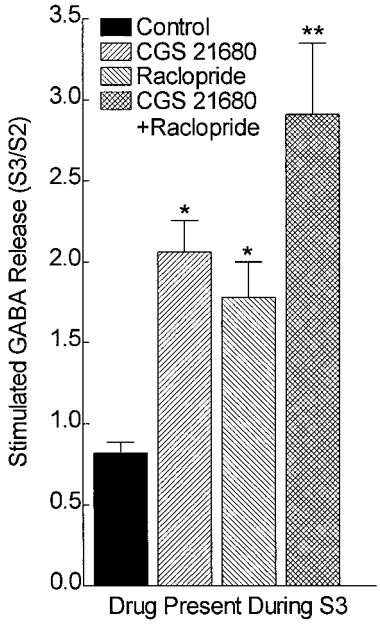


*Figure 4.* CGS 21680 (100 nM) increased evoked GABA release from C57 mouse striatal/pallidal slices whether or not $D_2$Rs were blocked by raclopride (1 $\mu$M). See Figure 1 for experimental details. Mean values ± SEM are shown for $n = 6–8$ mice/condition. One-factor ANOVA followed by Newman–Keuls *post hoc* comparisons: $^*p < 0.05$, CGS 21680 or raclopride versus control; $^{**}p < 0.05$, CGS 21680 + raclopride versus control or raclopride.

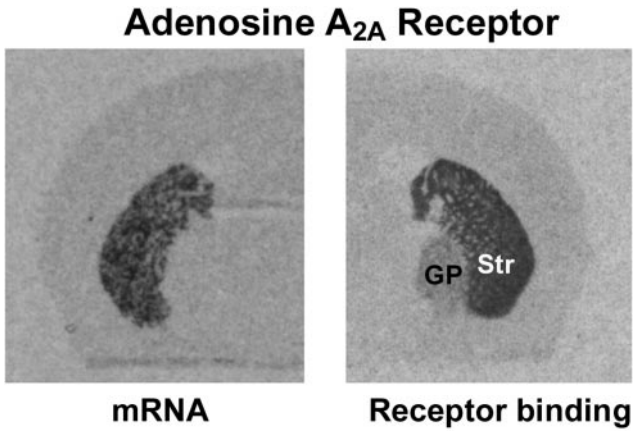


*Figure 5.* mRNA for $A_{2A}$Rs was highly expressed in the caudal striatum, whereas specific $A_{2A}$R binding was detected in both the caudal striatum (*Str*) and globus pallidus (*GP*). Autoradiograms from coronal hemibrain sections of a $D_2R^{-/-}$ mouse are shown; these are representative of all three genotypes (see Table 1). *Left, In situ* hybridization with a $^{35}$S-riboprobe designed against the rat $A_{2A}$R protein. *Right,* Specific binding of the selective $A_{2A}$R antagonist [$^3$H]SCH 58261 (0.3 nM).

containing terminals of neurons expressing $A_{2A}$Rs (Fig. 5). In all three genotypes, lower levels of $A_{2A}$R mRNA were expressed in the nucleus accumbens than in the striatum (Table 1). However, within each brain region, no significant differences among the genotypes were observed in $A_{2A}$R mRNA expression.

[$^3$H]SCH 58261 is a relatively new antagonist that is highly selective for $A_{2A}$Rs versus $A_1$Rs (~800-fold) (Fredholm et al., 1998). Its binding was quantitated by autoradiographic analysis in the striatum, nucleus accumbens, and globus pallidus of the $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mice. An approximate $K_d$ concentration of [$^3$H]SCH 58261 was used (0.3 nM) (Fredholm et al., 1998). In contrast with $A_{2A}$R mRNA, specific $A_{2A}$R antagonist-binding sites were observed in both the striatum and globus pallidus (Fig. 5). Levels of specific binding were approximately threefold higher in the striatum than in the globus pallidus, with that in the nucleus accumbens being intermediate (Table 1). However, in agreement with the $A_{2A}$R mRNA determinations, similar levels of binding were observed within each brain region of the three genotypes (Table 1). Binding of the $D_2$R antagonist [$^3$H]raclopride was also measured in the striatum and nucleus accumbens of these mice. In comparison with the $D_2R^{+/+}$ mice, levels of $D_2$R binding were 50% lower in both brain regions of the $D_2R^{+/-}$ mice and were not detectable in the $D_2R^{-/-}$ mice (Table 1). These results agree with those reported for the $F_2$ generation of these mice (Kelly et al., 1997).

Indirect saturation curves were generated using the $A_{2A}$R agonist [$^3$H]CGS 21680 and quantitative autoradiographic analysis in the striatum, nucleus accumbens, and globus pallidus in the $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mice. Assays contained 10 mM $Mg^{2+}$ to induce the high-affinity agonist-binding state, and the curves were best fit by a single-site model. The affinities, ranging from 23 to 29 nM, were similar in all brain regions of the three genotypes (Table 2). In agreement with the [$^3$H]SCH 58261 results, the receptor densities measured with [$^3$H]CGS 21680 were approximately threefold higher in the striatum than in the globus pallidus, with that in the nucleus accumbens being intermediate (Table 2). Likewise, there were no differences among genotypes in the densities of $A_{2A}$R agonist-binding sites within a single brain region.

## $A_{2A}$R-stimulated cAMP

An alternative explanation to receptor downregulation that could underlie the lack of CGS 21680 modulation of GABA release in the $D_2R^{+/-}$ and $D_2R^{-/-}$ mice would be an uncoupling of $A_{2A}$Rs from adenylyl cyclase. To test this hypothesis, basal and CGS 21680-stimulated cAMP levels were measured in striatal/pallidal slices from the three genotypes. Preliminary experiments confirmed that, similar to previous results in rats (Lupica et al., 1990), maximal stimulation was produced by 1 and 10 $\mu$M CGS 21680. Furthermore, preliminary experiments showed that the adenosine receptor antagonist 8-*p*-SPT (150 $\mu$M) blocked the increases in cAMP induced by 1 $\mu$M CGS 21680, confirming that this is an $A_{2A}$R-mediated response. Basal levels of cAMP were similar in the three genotypes (Fig. 6; legend). CGS 21680 induced a significant 64% increase in cAMP formation in the $D_2R^{+/+}$ mice, whereas cAMP levels in the $D_2R^{-/-}$ mice were not altered by CGS 21680 (Fig. 6). Although there was a trend for CGS 21680 to increase cAMP (34% above basal) in the $D_2R^{+/-}$ mice, this change was not statistically significant (Fig. 6). These results suggest that normal $A_{2A}$R signaling via increased cAMP production is disrupted in the striatopallidal neurons in the $D_2$R-deficient mice.

Levels of mRNA for the stimulatory G-proteins $G_{olf}$ and $G_s$ and for AC VI were measured by *in situ* hybridization in the $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mice. These experiments were conducted as an initial investigation into putative downstream-signaling mechanisms that might explain the compromised ability of $A_{2A}$Rs to stimulate cAMP accumulation in the striatum/globus pallidus of $D_2$R-deficient mice. The isoform(s) of adenylyl cyclase to which striatopallidal $A_{2A}$Rs couple is unknown. However, we focused on AC VI because its mRNA is present in both the striatum and globus pallidus (Liu et al., 1998) and $A_{2A}$Rs are known to activate AC VI in pheochromocytoma 12 cells (Chern et al., 1995). Interestingly, $G_{olf}$ and AC VI mRNAs, but not $G_s$ mRNA, were readily detected in the striatum (Fig. 7). Thus, in this respect the mouse appears similar to the rat (Hervé et al., 1993). However, among the three genotypes, no differences were observed in the levels of expression of any of these mRNAs (Fig. 7).

## Caffeine-induced locomotor activity

Caffeine is known to stimulate locomotion secondarily to blockade of $A_{2A}$Rs (Ledent et al., 1997; Svenningsson et al., 1997a; Hauber et al., 1998). Caffeine-induced locomotor activity was therefore measured as an additional test of $A_{2A}$R function in the $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mice. After acclimatization to the behavioral testing room, the mice were injected with either saline or caffeine (15 mg/kg, i.p.), and locomotor activity was measured for

**Table 1. Levels of $A_{2A}R$ mRNA, specific binding of the $A_{2A}R$ antagonist [$^3$H]SCH 58261, and specific binding of the $D_2R$ antagonist [$^3$H]raclopride in $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mouse brain regions**

| | Mouse genotype | | |
|---|---|---|---|
| | $D_2R^{+/+}$ | $D_2R^{+/-}$ | $D_2R^{-/-}$ |
| Rostral striatum | | | |
| $A_{2A}R$ mRNA (OD units) | 0.484 ± 0.017 | 0.469 ± 0.019 | 0.459 ± 0.012 |
| [$^3$H]SCH 58261 (fmol/mg tissue) | 283 ± 8 | 296 ± 11 | 264 ± 10 |
| [$^3$H]raclopride (fmol/mg tissue) | 109.3 ± 3.7 | 49.0 ± 2.3 | 1.8 ± 0.4 |
| Nucleus accumbens core | | | |
| $A_{2A}R$ mRNA (OD units) | 0.313 ± 0.020 | 0.306 ± 0.016 | 0.322 ± 0.019 |
| [$^3$H]SCH 58261 (fmol/mg tissue) | 169 ± 18 | 216 ± 24 | 188 ± 16 |
| [$^3$H]raclopride (fmol/mg tissue) | 58.4 ± 4.5 | 26.8 ± 2.4 | Not measured |
| Nucleus accumbens shell | | | |
| $A_{2A}R$ mRNA (OD units) | 0.451 ± 0.031 | 0.436 ± 0.021 | 0.447 ± 0.026 |
| [$^3$H]SCH 58261 (fmol/mg tissue) | 232 ± 14 | 287 ± 29 | 215 ± 20 |
| [$^3$H]raclopride (fmol/mg tissue) | 62.0 ± 5.2 | 33.7 ± 4.9 | Not measured |
| Caudal striatum | | | |
| $A_{2A}R$ mRNA (OD units) | 0.499 ± 0.012 | 0.487 ± 0.018 | 0.491 ± 0.012 |
| [$^3$H]SCH 58261 (fmol/mg tissue) | 317 ± 10 | 318 ± 28 | 297 ± 19 |
| [$^3$H]raclopride (fmol/mg tissue) | 111.7 ± 4.0 | 62.8 ± 3.2 | 0.0 ± 0.2 |
| Globus pallidus | | | |
| [$^3$H]SCH 58261 (fmol/mg tissue) | 98 ± 6 | 98 ± 13 | 104 ± 7 |

Mean values ± SEM are shown for $n = 9$ ($D_2R^{+/+}$), $n = 5$–6 ($D_2R^{+/-}$), and $n = 8$ ($D_2R^{-/-}$). Two-factor ANOVAs followed by Tukey *post hoc* comparisons: mRNA, $p < 0.05$, for all brain region comparisons (except rostral vs caudal striatum and nucleus accumbens core vs shell); [$^3$H]SCH 58261, $p < 0.05$, for all brain region comparisons (except rostral vs caudal striatum); and [$^3$H]raclopride, $p < 0.05$, for all brain region comparisons (except rostral vs caudal striatum and nucleus accumbens core vs shell) and all genotype comparisons.

**Table 2. Similar affinities and densities for the $A_{2A}R$ agonist [$^3$H]CGS 21680 in $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mouse brain regions**

| | Mouse genotype | | |
|---|---|---|---|
| | $D_2R^{+/+}$ | $D_2R^{+/-}$ | $D_2R^{-/-}$ |
| Rostral striatum | | | |
| Log $K_d$ | −7.61 ± 0.02 | −7.55 ± 0.04 | −7.62 ± 0.04 |
| $K_d$ (nM) | 24.3 | 28.0 | 24.2 |
| $B_{max}$ (pmol/mg protein) | 1.92 ± 0.08 | 2.14 ± 0.17 | 1.63 ± 0.13 |
| Nucleus accumbens | | | |
| Log $K_d$ | −7.66 ± 0.03 | −7.54 ± 0.02 | −7.58 ± 0.04 |
| $K_d$ (nM) | 21.8 | 28.6 | 26.6 |
| $B_{max}$ (pmol/mg protein) | 1.44 ± 0.10 | 1.65 ± 0.11 | 1.37 ± 0.08 |
| Caudal striatum | | | |
| Log $K_d$ | −7.58 ± 0.03 | −7.56 ± 0.04 | −7.54 ± 0.02 |
| $K_d$ (nM) | 26.6 | 27.6 | 28.8 |
| $B_{max}$ (pmol/mg protein) | 2.17 ± 0.13 | 2.13 ± 0.13 | 2.11 ± 0.09 |
| Globus pallidus | | | |
| Log $K_d$ | −7.63 ± 0.04 | −7.69 ± 0.05 | −7.51 ± 0.05 |
| $K_d$ (nM) | 23.5 | 20.3 | 31.0 |
| $B_{max}$ (pmol/mg protein) | 0.62 ± 0.08 | 0.65 ± 0.16 | 0.70 ± 0.07 |

Mean values ± SEM are shown for $n = 7$–8 ($D_2R^{+/+}$), $n = 6$ ($D_2R^{+/-}$), and $n = 8$ ($D_2R^{-/-}$). Two-factor ANOVAs followed by Tukey *post hoc* comparisons: $B_{max}$, $p < 0.05$, for all brain region comparisons (except rostral vs caudal striatum).

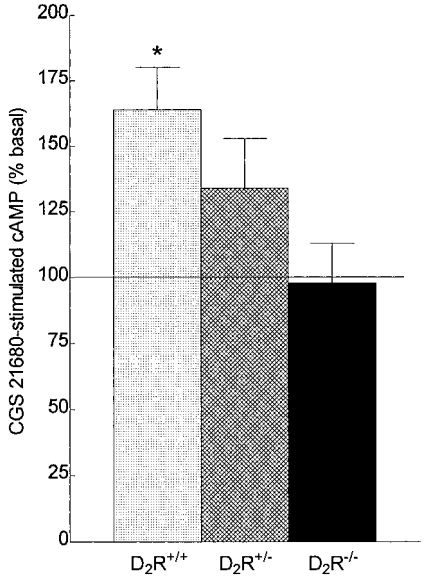


*Figure 6.* CGS 21680 significantly enhanced cAMP formation in striatal/pallidal slices from $D_2R^{+/+}$ mice but not from $D_2R^{+/-}$ or $D_2R^{-/-}$ mice. Basal levels of cAMP were similar in the three genotypes ($D_2R^{+/+}$, 325 ± 92 pmol of cAMP/mg of protein per 15 min; $n = 6$; $D_2R^{+/-}$, 400 ± 134; $n = 6$; $D_2R^{-/-}$, 351 ± 168; $n = 5$). Mean values ± SEM are shown as a percentage of the basal cAMP value for each mouse in the presence of a maximally effective concentration of the agonist CGS 21680 (1–10 μM). One-factor ANOVA followed by Tukey *post hoc* comparisons: $^*p < 0.05$, $D_2R^{+/+}$ versus $D_2R^{-/-}$.

2 hr (Fig. 8*A,B*). During the first 45 min after saline injection, the activity of the $D_2R^{-/-}$ mice was significantly lower than that of the $D_2R^{+/+}$ and/or $D_2R^{+/-}$ mice (Fig. 8*A*). To factor out baseline activity differences, caffeine-induced difference scores were generated (Fig. 8*C*). Mice of all three genotypes injected with caffeine were more active than were the respective controls injected with saline (Fig. 8*C*). Over the 2 hr period after caffeine injection, the total caffeine-induced distance traveled by the $D_2R^{+/+}$ mice was 42100 ± 9750 cm ($n = 5$), that traveled by the $D_2R^{+/-}$ mice was 47200 ± 6970 cm ($n = 5$), and that traveled by the $D_2R^{-/-}$ mice was 24200 ± 3100 cm ($n = 9$). Statistical analysis revealed that the less-pronounced caffeine-induced activation of the $D_2R^{-/-}$ mice reflected the fact that their activity was significantly lower than that of the $D_2R^{+/+}$ and/or $D_2R^{+/-}$ mice from 45 to 100 min after injection (Fig. 8*C*). Direct observation of $D_2R^{-/-}$ mice injected with caffeine revealed that the lower caffeine-induced locomotor

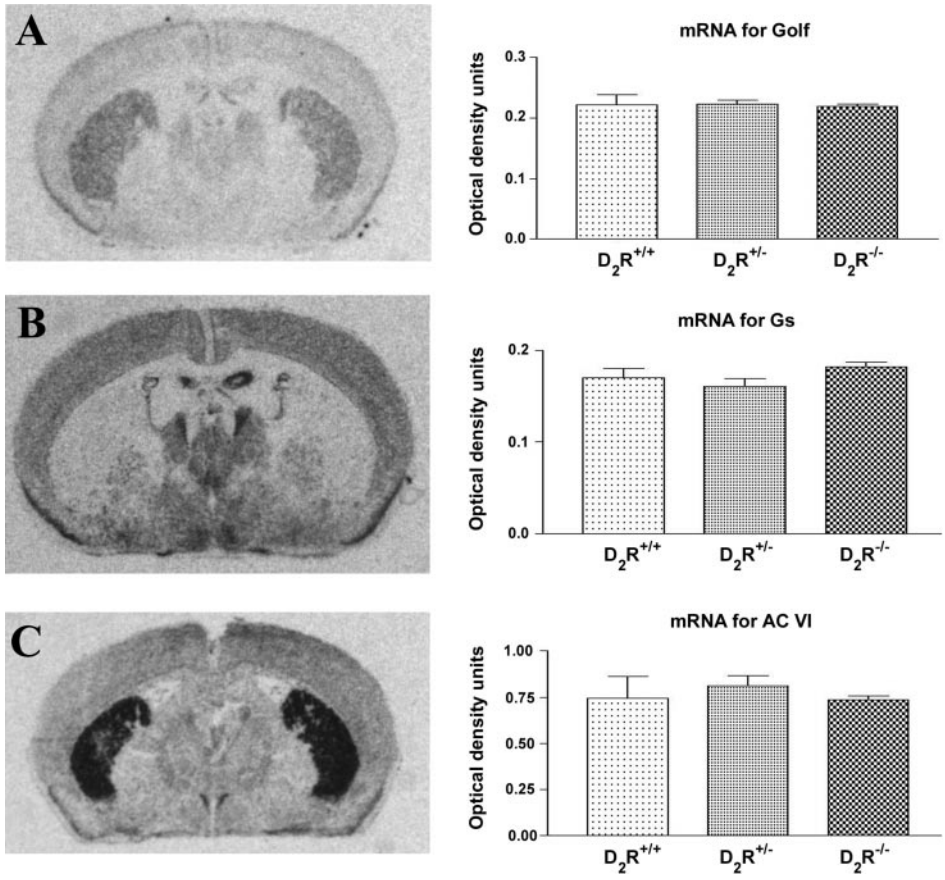


*Figure 7.* Expression of mRNAs for the $\alpha$ subunits of stimulatory G-proteins ($G_{olf}$ and $G_s$) and the catalytic subunit of AC VI was not different in the caudal striata of the $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mice. *Left*, Representative autoradiograms from the *in situ* hybridization assays in coronal brain sections from a $D_2R^{-/-}$ mouse are shown for $G_{olf}$ (*A*), $G_s$ (*B*), and AC VI (*C*). $^{35}$S-riboprobes designed against the respective rat proteins were used. *Right*, Mean values ± SEM from the quantitative analysis of these assays are shown for $n = 9$ ($D_2R^{+/+}$), $n = 5–6$ ($D_2R^{+/-}$), and $n = 8$ ($D_2R^{-/-}$).

activation was not caused by an increase in stereotypic behaviors (data not shown).

### Caffeine-induced c-*fos* expression

The differences in caffeine-induced behavioral activation among the three genotypes might reflect differences in the $D_2R$-mediated activity of intrinsic pallidal neurons. To investigate this possibility, we quantitated the levels of c-*fos* mRNA by *in situ* hybridization 4 hr after injection of either saline or caffeine (15 mg/kg). Expression of c-*fos* in the cerebral cortex, measured as a control, was similar in saline- and caffeine-treated mice of all three genotypes (data not shown). Expression of c-*fos* also did not differ significantly in the globus pallidus of saline- or caffeine-treated $D_2R^{+/+}$ and $D_2R^{+/-}$ mice (Fig. 9). In contrast, c-*fos* expression was significantly increased by 94% in the globus pallidus of the caffeine-treated, versus the saline-treated, $D_2R^{-/-}$ mice (Fig. 9). Caffeine-induced c-*fos* expression in the $D_2R^{-/-}$ mice was also significantly higher than caffeine-induced c-*fos* expression in either the $D_2R^{+/+}$ or $D_2R^{+/-}$ mice (Fig. 9).

## DISCUSSION

### Evidence of tonic $D_2R$ inhibition of striatopallidal GABA release

Activation of $D_2R$s inhibits stimulation-evoked GABA release from striatopallidal neurons. Conversely, in striatal/pallidal slices from control C57 mice, we observed that the $D_2R$ antagonist raclopride increased GABA release. We also hypothesized that GABA release from these striatal projection neurons would be elevated in mice lacking $D_2R$s. In agreement with this hypothesis, we observed significantly greater (40–70%) spontaneous and electrically evoked GABA release from $D_2R^{-/-}$ than from $D_2R^{+/+}$ mouse striatal/pallidal slices. GABA release from $D_2R^{+/-}$ mice was intermediate. Our observations show the essential correctness of the surmise of Baik et al. (1995). They found higher levels of striatal GAD mRNA in a different line of $D_2R^{-/-}$ mice and, on the basis of this observation, suggested that GABA release might be increased. Together, these observations strengthen the idea that $D_2R$s tonically inhibit GABA release from striatopallidal neurons.

The reduced baseline locomotor activity observed here in the $N_5$ congenic $D_2R^{-/-}$ mice is compatible with disinhibited pallidal GABA release. During the initial 40 min after saline injection, the $D_2R^{-/-}$ mice were significantly less active than were the other two genotypes. Subtle differences in the initiation of movement were also observed between $D_2R^{+/+}$ and $D_2R^{-/-}$ mice in the $F_2$ generation (Kelly et al., 1998). The lower level of locomotor activity in the $D_2R^{-/-}$ mice is consistent with pharmacological studies showing that increased $GABA_A$ receptor activation, as well as decreased dopamine receptor activation, reduces locomotor activity (Mukhopadhyay and Poddar, 1995).

### Lack of $A_{2A}R$ agonist effects in $D_2R$-deficient mice

An antagonistic interaction between $D_2R$s and $A_{2A}R$s in striatopallidal neurons is well established (for review, see Ferré et al., 1992, 1997). This antagonistic interaction impacts GABA release. The $A_{2A}R$ agonist CGS 21680 not only increases stimulation-evoked GABA release from rat striatal/pallidal slices but also abolishes the $D_2R$ agonist-mediated inhibition of this release (Mayfield et al., 1993, 1996). Likewise, Ferré et al. (1993) observed that CGS 21680 antagonizes the $D_2R$-mediated reduction in extracellular GABA *in vivo* in rat globus pallidus. Here we observed that CGS 21680 also markedly increased stimulation-evoked GABA release from $D_2R^{+/+}$ and C57 mouse striatal/pallidal slices. Thus, we hypothesized that without opposing $D_2R$s, the stimulation of GABA release by $A_{2A}R$s would be potentiated. Unexpectedly, however, CGS 21680 did not affect GABA release in either $D_2R^{+/-}$ or $D_2R^{-/-}$ mice. It is unlikely that the lack of CGS 21680 potentiation in the $D_2R$-deficient mice was caused by an acute absence of $D_2R$s during the assay. Stimulation-evoked GABA release from control mouse striatal/pallidal slices was still augmented by CGS 21680 when the $D_2R$ antagonist raclopride was

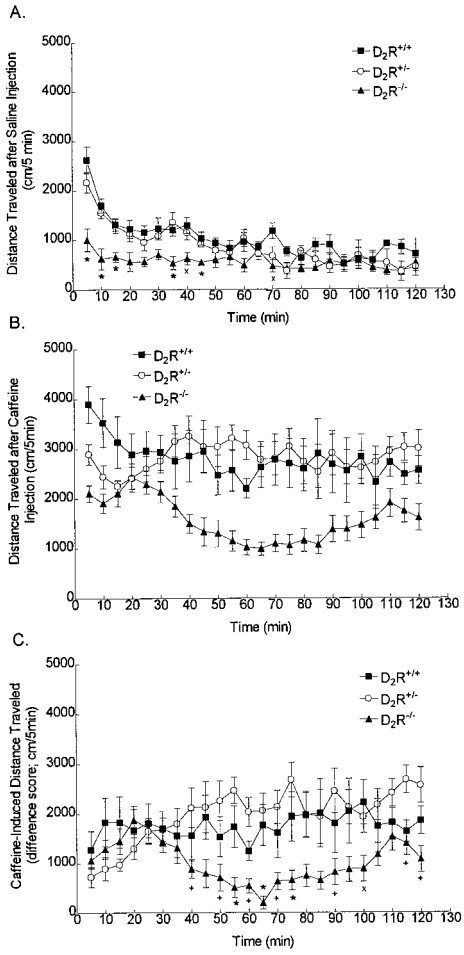


*Figure 8.* Caffeine-induced locomotor activity was reduced in $D_2R^{-/-}$ mice relative to that in $D_2R^{+/+}$ and $D_2R^{+/-}$ mice. Saline or caffeine was administered at time = 0. *A,* Distance traveled after saline injection (1 ml/100 gm, i.p.). Mean values ± SEM are shown for $n = 4$ mice ($D_2R^{-/-}$) or $n = 5$ mice ($D_2R^{+/+}$, $D_2R^{+/-}$). Two-factor ANOVA with repeated measures (time) followed by Tukey *post hoc* comparisons: $^{*}p < 0.05$, $D_2R^{-/-}$ versus $D_2R^{+/+}$ and $D_2R^{+/-}$; $^{x}p < 0.05$, $D_2R^{-/-}$ versus $D_2R^{+/+}$. *B,* Distance traveled after caffeine injection (15 mg/kg, i.p.). Mean values ± SEM are shown for $n = 5$ mice ($D_2R^{+/+}$, $D_2R^{+/-}$) or $n = 9$ mice ($D_2R^{-/-}$). *C,* Caffeine-induced locomotor activity determined at each time point as a difference score by subtracting the mean activity of each genotype after saline injection from the activity of each mouse of that same genotype after caffeine injection. Mean values ± SEM are shown. Two-factor ANOVA with repeated measures (time) followed by Tukey *post hoc* comparisons: $^{*}p < 0.05$, $D_2R^{-/-}$ versus $D_2R^{+/+}$ and $D_2R^{+/-}$; $^{x}p < 0.05$, $D_2R^{-/-}$ versus $D_2R^{+/+}$; $^{+}p < 0.05$, $D_2R^{-/-}$ versus $D_2R^{+/-}$.

included in the assay. It is also unlikely that a ceiling effect, i.e., that GABA release was already maximal, explains the lack of CGS 21680 potentiation because stimulated GABA release from $D_2R^{+/-}$ slices was not statistically higher than that from $D_2R^{+/+}$ slices (Fig. 2*B*).

## Uncoupled $A_{2A}$Rs in $D_2R$-deficient mice

Loss of $A_{2A}R$ effects in the $D_2R^{+/-}$ and $D_2R^{-/-}$ mice likely reflects changes, which may have occurred during development, to compensate for the reduced number of $D_2$Rs. Previous studies (Baik et al., 1995; Kelly et al., 1998) suggest that striatonigral activity is not increased in $D_2R$-deficient animals. Downregulation of $A_{2A}R$ expression was the first potential change that we investigated. Both $A_{2A}$Rs and $D_2$Rs are expressed at very low levels during early striatal development and reach adult levels only at the end of the second postnatal week (Johansson et al., 1997). Nonetheless, levels of $A_{2A}R$ mRNA in the striatum or globus pallidus were similar in the $D_2R^{+/+}$, $D_2R^{+/-}$, and $D_2R^{-/-}$ mice. The antagonist [$^3$H]SCH 58261 has the same high affinity for both

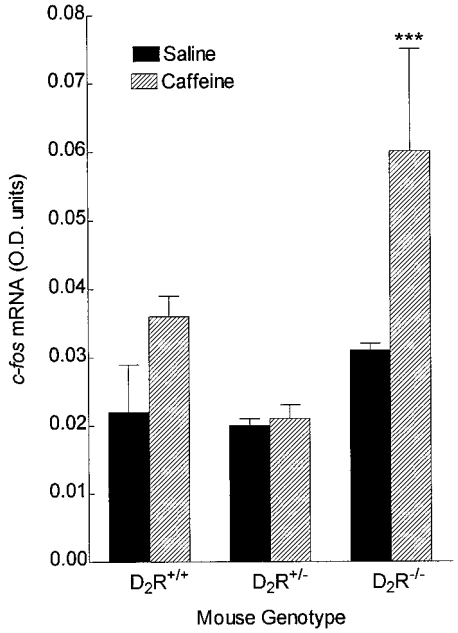


*Figure 9.* Expression of c-*fos* mRNA in the globus pallidus of $D_2R^{-/-}$ mice was increased by caffeine administration. mRNA levels were measured 4 hr after caffeine injection (15 mg/kg, i.p.). Mean values ± SEM are shown for $n = 3$ mice ($D_2R^{+/-}$ saline, $D_2R^{+/-}$ caffeine, $D_2R^{-/-}$ saline), $n = 4$ mice ($D_2R^{+/+}$ caffeine), or $n = 5$ mice ($D_2R^{+/+}$ saline, $D_2R^{-/-}$ caffeine). One-factor ANOVA followed by Newman–Keuls *post hoc* comparisons: $^{***}p < 0.001$, $D_2R^{-/-}$ caffeine versus all other groups. *O.D.*, Optical density.

G-protein-coupled and -uncoupled states of the $A_{2A}R$ (Fredholm et al., 1998). Thus, its binding measures the total complement of $A_{2A}$Rs. In contrast, in the presence of 10 mM $Mg^{2+}$, [$^3$H]CGS 21680 detects primarily the G-protein-coupled state with high affinity for agonists (Johansson et al., 1992). However, we observed similar numbers of $A_{2A}$Rs among the three genotypes in the striatum or globus pallidus with both [$^3$H]SCH 58261 and [$^3$H]CGS 21680. These results indicated that changes in $A_{2A}R$ expression are not the basis for differences in $A_{2A}R$ modulation of GABA release.

A second potential mechanism for reduced $A_{2A}R$ function is the uncoupling of $A_{2A}$Rs from their effector molecule adenylyl cyclase. Whereas CGS 21680 significantly increased cAMP production in striatal/pallidal slices from $D_2R^{+/+}$ mice, it was ineffective in $D_2R^{+/-}$ and $D_2R^{-/-}$ mice. Thus, despite unchanged agonist binding, the cAMP experiments suggested that $A_{2A}$Rs are functionally uncoupled in the $D_2R$-deficient mice. Furthermore, the ability of CGS 21680 to increase GABA release likely depends on increases in cAMP (Wang and Johnson, 1995). The uncoupling does not appear to be caused by lower expression of the $\alpha$ subunits of stimulatory G-proteins ($G_{olf}$ or $G_s$) or the catalytic subunit of AC VI. Alternatively, the $A_{2A}R$ and G-protein could be kinetically uncoupled, the levels of G-protein and/or AC VI protein could be reduced, and/or the subcellular localization of the $A_{2A}R$ relative to the G-protein and/or AC could be altered in the $D_2R$-deficient mice.

## Altered effects of caffeine in $D_2R^{-/-}$ mice

Dopamine receptors are involved in mediating caffeine-induced locomotion, and the differential results in the $D_2R^{+/+}$ and $D_2R^{-/-}$ mice further support a role for $D_2$Rs. Characteristic of the $D_1R$/$D_2R$ synergy required for many dopamine-mediated behaviors, locomotor stimulation induced by caffeine requires activation of both receptor subtypes and is blocked by either selective $D_1R$ or $D_2R$ antagonists (Garrett and Holtzman, 1994). Moreover, Fenu and Morelli (1998) have demonstrated that caffeine produces motor stimulation in 6-hydroxydopamine-lesioned rats, which have had prolonged compromised $D_1R/D_2R$ stimulation, only when the rats have been primed with a dopamine receptor agonist before caffeine testing. Therefore, differential behavioral effects of caf-

feine were expected in the $D_2R^{+/+}$ and $D_2R^{-/-}$ mice. We observed an inability of the $D_2R^{-/-}$ mice to sustain caffeine-induced activation whereas the $D_2R^{+/+}$ and $D_2R^{+/-}$ mice were activated to similar extents.

Caffeine has similar affinities for $A_1Rs$ and $A_{2A}Rs$ (Daly, 1993; Fredholm, 1995; Fredholm et al., 1999). However, it is $A_{2A}R$ antagonism that is required for locomotor activation (Ledent et al., 1997; Svenningsson et al., 1997a; Hauber et al., 1998). Our observation of uncoupled $A_{2A}Rs$ in the $D_2R$-deficient mice predicts that caffeine activation should have been precluded. This clearly was not the case with the $D_2R^{+/-}$ mice and suggests that $A_{2A}Rs$ in these mice must be at least partially functional. We did observe a trend for CGS 21680 to increase cAMP in the $D_2R^{+/-}$ striatum/pallidum (Fig. 6). In any case, it is clear that only half the normal complement of $D_2Rs$ is sufficient to allow normal caffeine activation.

Although locomotor stimulation by caffeine was markedly reduced in the $D_2R^{-/-}$ mice, it was not totally absent. It is possible that blockade of $A_1Rs$ could have also contributed. Indeed, from several rodent studies, combined blockade of $A_1Rs$ and $A_{2A}Rs$ is known to produce larger locomotor responses than is blockade of only one receptor subtype (see Daly, 1993; Fredholm et al., 1999). Because the $D_2R^{-/-}$ mice are functionally devoid of $A_{2A}R$ signaling, blockade of $A_1Rs$ may produce greater effects than in intact animals. $A_1Rs$ can negatively influence $D_1R$ signaling by blocking dopamine release in the striatum (Jin et al., 1993; Harvey and Lacey, 1997), by increasing the firing of dopaminergic neurons (Stoner et al., 1988), and by direct interactions at striatonigral neurons (Ferré et al., 1998). Thus, blockade of $A_1Rs$, by antagonizing any or all of these mechanisms, can increase activity in striatonigral neurons (Ferré et al., 1996). Furthermore, activation of $D_1Rs$ leads to a larger locomotor response in $D_2R^{-/-}$ mice than in $D_2R^{+/+}$ or $D_2R^{+/-}$ mice (Kelly et al., 1998).

In rodents, c-*fos* expression in the globus pallidus is increased when higher doses of caffeine, which induce locomotor depression, are administered (Svenningsson et al., 1995; Svenningsson and Fredholm, 1997; Bennett and Semba, 1998). The induction of c-*fos* in the globus pallidus appears to require simultaneous inhibition of striatopallidal neurons and activation of striatonigral neurons (Le Moine et al., 1997). Indeed, inhibition of $A_{2A}Rs$, combined with activation of $D_1Rs$, is very effective. Hence, our data showing pallidal c-*fos* induction after caffeine in the $D_2R^{-/-}$ animals might be explained if in these animals striatonigral activity is increased by caffeine, even though basal activity is not elevated.

## Summary

The present results show that a targeted disruption of the $D_2R$ influences responses to drugs that act on adenosine receptors. Thus, enhancement of GABA release induced by an $A_{2A}R$ agonist was completely eliminated when $D_2R$ expression was reduced. This likely involves some adaptive change(s), because acute treatment with a $D_2R$ antagonist does not produce the same result. The adaptation is not simply a change in $A_{2A}R$ expression or a loss of any key signaling component. However, the exact mechanism remains unclear. The motor stimulatory effect of caffeine, which depends on $A_{2A}R$ blockade, was substantially reduced in mice lacking $D_2Rs$. These findings support the idea that some level of $D_2R$ activity is required for the action of caffeine, as has been postulated previously on the basis of results with antagonists. The results also show that animals with a targeted disruption of the $D_2R$ have a functional uncoupling of the $A_{2A}R$. This underscores that not all of the phenotypic changes reported for such mice can be necessarily attributed to the $D_2R$ loss.

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