

# Caffeine Regulates Neuronal Expression of the Dopamine 2 Receptor Gene

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## ABSTRACT

The psychoactive drug caffeine influences neuronal physiology; however, it is unknown whether it can dynamically alter the expression of genes that influence neurotransmission. Here, we report that caffeine stimulates transcription of the dopamine 2 receptor (D2R) gene in PC-12 cells and primary striatal cultures and increases D2R protein expression in the striatum. Physiological doses of caffeine and the specific adenosine 2A receptor antagonist 8-(3-chlorostyryl) caffeine both increased the activity of a D2R/luciferase reporter construct within 24 h, and simultaneous treatment with 2-[*p*-(2-carboxyethyl)phenethylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS 21680), a specific adenosine 2A receptor agonist, eliminated this effect. Tests of additional constructs revealed that specific regions of the D2R promoter (−117/−75) and 5'-untranslated region (+22/+317) were required for activation of D2R gene expres-

sion by caffeine. In primary striatal cultures, caffeine increased spontaneous firing of neurons between 12 and 80 min after treatment, whereas it increased D2R mRNA expression after only 4 h. These results indicate that regulation of D2R gene expression by caffeine occurs after the initial physiological response has subsided. In vivo, female mice treated with a dose of caffeine (50 mg/kg) showed 1.94- and 2.07-fold increases in D2R mRNA and protein expression, respectively. In contrast, male mice exhibited a 31% decrease in D2R mRNA expression and showed no changes in D2R protein expression. Collectively, these results demonstrate for the first time that caffeine alters D2R expression in neurons. They also suggest that caffeine consumption can lead to sexually dimorphic patterns of gene expression in the brain.

Dopamine is a major modulatory neurotransmitter that controls a number of important physiological activities and behavioral states of the mammalian brain, including motor control, motivation, and mood (Gingrich and Caron, 1993). Deficits in dopaminergic neurotransmission are associated with neurodegenerative conditions, including Parkinson's disease (McGeer et al., 1988), and neuropsychiatric disorders, such as schizophrenia (Lewis and Lieberman, 2000). Because dopamine transmits signals in neurons via its receptors, elucidating how the expression of dopamine receptors is regulated may be central to understanding both normal and aberrant dopaminergic neurotransmission.

Approximately 80% of the dopamine found in the brain is localized in the striatum, where it binds to dopamine receptors on medium-sized spiny projection neurons (Carlsson,

1959). These neurons specifically express the dopamine receptors D1R and D2R, which are seven transmembrane G-protein-coupled receptors (Gingrich and Caron, 1993) that signal via cAMP and protein kinase A (PKA) pathways (Stoof and Keibarian, 1984). Signaling via D1R and D2R controls GABA release from striatal projection neurons; this response can be modified by inputs from other neuromodulators such as adenosine (Ferre et al., 1991a, 1997; Ferre and Fuxe, 1992). Indeed, it has been shown that D2R and the adenosine receptor A2AR are coexpressed in striatopallidal projection neurons (Schiffmann and Vanderhaeghen, 1993; Aizman et al., 2000) and that these receptors can form functional molecular complexes that influence the physiology of striatal neurons (Ferre et al., 1991b; Diaz-Cabiale et al., 2001). For example, stimulation or blockade of A2AR has been shown to attenuate and potentiate, respectively, D2R-mediated locomotor activity (Fuxe and Ungerstedt, 1974). Given these considerations, it is important to determine whether dopa-

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**ABBREVIATIONS:** D1R, dopamine 1 receptor; D2R, dopamine 2 receptor; PKA, protein kinase A; A2AR, adenosine 2A receptor; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; RT-PCR, reverse transcription-polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; UTR, untranslated region; CGS 21680, 2-[*p*-(2-carboxyethyl)phenethylamino]-5'-*N*-ethylcarboxamidoadenosine; GAP3DH, glyceraldehyde-3-phosphate dehydrogenase; MEP, multielectrode plate; CSC, 8-(3-chlorostyryl) caffeine; DRRF, dopamine receptor regulating factor; PD, Parkinson's disease; RE, retinoic acid response element; SCH 58261, 5-amino-7(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidine; KW 6002, istradefylline; KF 18446, 8-(2,3-dimethyl-4-methoxystyryl)-1,3,7-trimethylxanthine.

mine receptor genes are regulated by pharmacological agents that modulate signaling by dopamine and adenosine receptors.

Caffeine is the most widely used psychoactive drug and stimulates motor activity by altering the biochemistry and enhancing the activity of striatal projection neurons. Physiological doses of caffeine range between 0.01 and 0.25 mM, and the LD<sub>50</sub> in rats is 0.8 mM (Fredholm et al., 1999). Binding of caffeine to A2AR has recently been found to change the phosphorylation state of the dopamine- and cAMP-regulated phosphoprotein (DARPP-32) (Lindskog et al., 2002) and to up-regulate the expression of immediate-early genes such as *c-fos* in striatal neurons (Johansson et al., 1994), providing new insights into the mechanism of caffeine action in neurons. However, whether caffeine administration can directly alter the expression of genes involved in dopaminergic neurotransmission is currently unknown.

In the present study, we found that caffeine increases D2R mRNA and reporter gene expression in PC-12 cells. To investigate regulation of the D2R gene by caffeine in greater detail, we prepared additional D2R luciferase reporter constructs and examined their activity in response to caffeine in PC-12 cells. We found that a region of the D2R promoter (−117/−75) and a segment of the 5′ untranslated region (+22/+317) were both necessary for regulation of D2R gene expression by caffeine. Caffeine also stimulated the D2R mRNA expression in striatal neuronal cultures between 4 and 24 h after drug treatment. In contrast, caffeine was found to increase the firing rates of striatal neurons only within the first 80 min of drug treatment, indicating that the increase in D2R mRNA expression in striatal neurons occurs after the initial physiological response to the drug has subsided. Treatment of mice for 24 h with caffeine (50 mg/kg, i.p.) up-regulated D2R mRNA and protein expression in the striata of female mice but not male mice. Similarly, short-term treatment of mice with caffeine (50 mg/kg, i.p.) or the specific A2AR antagonist CSC (5 mg/kg, i.p.) resulted in up-regulation of striatal D2R protein expression in female mice but not male mice, suggesting that the caffeine effect is mediated, in part, via the A2A receptor. These results provide a basis for understanding the mechanisms underlying changes in the expression of dopamine receptors by psychoactive drugs. In addition, the sexually dimorphic nature of the response to short-term caffeine administration in mice suggests that drugs that affect dopaminergic neurotransmission may have sex-specific effects on gene and protein expression in the basal ganglia.

## Materials and Methods

**Generation of D2R Reporter Constructs.** Luciferase reporter constructs containing different regions of the promoter and 5′-UTR from the rat gene encoding D2R (accession number: U79717) were generated by 30 cycles of PCR amplification with a thermocycler (PerkinElmer Life and Analytical Sciences, Boston, MA) using rat genomic DNA as a template (BD Biosciences Clontech, Palo Alto, CA). PCR products were then subcloned into the pGL3basic vector (Promega, Madison, WI). All constructs were subjected to automated DNA sequencing (Applied Biosystems, Foster City, CA).

**PC-12 Cell Culture, Transfection, and Drug Treatment.** Rat PC-12 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum, 5% fetal calf serum, and

1× penicillin/streptomycin/glutamine (Invitrogen). Cells were dissociated in 1× trypsin/EDTA (Invitrogen), washed in serum-free DMEM, and resuspended in media. For transfection,  $6 \times 10^5$  PC-12 cells were seeded in each well of a six-well plate (Costar, Tustin, CA). Twenty-four hours after seeding, cells were transfected with 1 μg of the promoterless luciferase reporter vector, pGL3basic (Promega) to examine the background luciferase activity or with 1 μg of D2R luciferase reporter construct as described previously (Copertino et al., 1997). In all experiments, cells were cotransfected with 100 ng of the plasmid pCMVβ (BD Biosciences Clontech) to assess transfection efficiency and to normalize luciferase activity values to an internal reference standard of β-galactosidase activity. PC-12 cells were transiently transfected using FuGENE 6 (Roche, Indianapolis, IN) and Opti-MEM I media (Invitrogen). After 24 h, cells were treated with the concentrations of caffeine, 8-(3-chlorostyryl) caffeine, or CGS 21680 that are indicated in each of the figures. After an additional 24 h, cells were harvested, washed in phosphate-buffered saline (Invitrogen), resuspended in 150 μl of 1× reporter lysis buffer (Promega), and assayed for β-galactosidase activity using the FluoReporter kit (Molecular Probes, Eugene, OR), and then for luciferase activity using a Microlumat LB968 luminometer, as described previously (Copertino et al., 1997). The data presented in Figs. 2, 3, and 4 were derived from at least four independent experiments, performed in triplicate.

**Mouse Primary Striatal Cultures and in Vivo Studies.** Embryos from CD-1 mice (Harlan Sprague-Dawley, Indianapolis, IN) pregnant for 14 days were sacrificed, and the striata were collected in dissection buffer (135 mM NaCl, 5 mM KCl, 0.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.2 mM KH<sub>2</sub>PO<sub>4</sub>, 22 mM sucrose, 16.5 mM glucose, 9.8 mM, and HEPES, pH 7.0) and mechanically dissociated using a Sichel microdissection knife. Dissociated cells from striata were recovered in serum-containing media [DMEM (Invitrogen), 5% horse serum, 5% fetal calf serum (Omega Scientific, Tarzana, CA), B-27 supplement, GlutaMAX I (Invitrogen), and 1 μM sodium pyruvate] and incubated with 50 μg/ml DNase (Sigma, St Louis, MO) for 15 min at 37°C. The digestion media were replaced and the striata were triturated 20× using a fine-tip plastic pipette. The neurons were counted and aliquoted onto coverslips (Carolina Biological, Burlington, NC) that were pretreated with polyethylenimine (Sigma) and laminin (Roche). For quantitative RT-PCR,  $1 \times 10^6$  cells were aliquoted onto each coverslip.

Cells, cultured in six-well plates (Costar) were incubated in serum-containing media at 5% CO<sub>2</sub> and 37°C. After 24 h, media were replaced with serum-free media [DMEM, N-2 supplement (Invitrogen), and 0.1% bovine albumin]. After a further 24 h of incubation, 0.5 μM cytosine β-D-arabinofuranoside hydrochloride (Sigma) was administered to inhibit glial proliferation. This drug was removed by serum-free media replacement after a further 24 h. After 6 days in vitro, primary striatal cultures were treated with drugs. Caffeine (Sigma Aldrich, Milwaukee, WI) was dissolved in Krebs-Ringers-Henseleit buffer (117 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 24.8 mM NaHCO<sub>3</sub>, and 11.1 mM glucose in H<sub>2</sub>O) and the adenosine 2A receptor agonist, CGS 21680 (RBI/Sigma, Natick, MA) was dissolved in dimethyl sulfoxide (Sigma Aldrich). Drugs were administered at the appropriate concentrations as indicated under *Results* section.

In short-term in vivo studies, three male and three female (quantitative RT-PCR), or four male and four female (quantitative Western blot) CD-1 mice aged 3 to 6 months (Harlan Sprague-Dawley) were administered either 50 mg/kg caffeine or 5 mg/kg CSC (dissolved in 0.5% methyl cellulose, Sigma) each by i.p. injection. In sham mice, an appropriate volume of 0.5% methyl cellulose (~400 μl) was injected. Twenty-four hours later, all mice, including untreated, naive controls, were sacrificed and the striata were dissected. RNA and protein were then extracted for quantitative RT-PCR (D2R, A2AR, and glyceraldehyde-3-phosphate dehydrogenase (GAP3DH) mRNA expression) or quantitative Western blotting (D2R protein expression) as described below.

**Quantitative RT-PCR.** Total RNA was extracted from PC-12 cells, mouse striata, and striatal neuronal cultures using TRIzol reagent (Invitrogen). One microgram of RNA was reverse transcribed using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was then carried out using either D2R (5'-AAGACGATGAGCCGCAGAA-3' and 5'-AGCAGATGATGAACACACCGA-3'), A2AR in PC-12 cells (5'-CGTGGTGC-CATGAATTACA-3' and 5'-GGCAGTAACACGAACGCAAAG-3'), A2AR in mouse striatum (5'-GCCTACAGGATCCGGGAGTT-3' and 5'-AGGACGTGGGTTCGGATGA-3'), or GAP3DH gene-specific primers (5'-GGCAAGTCAACGGCACAG-3' and 5'-TCGCTCTG-GAAGATGGTG-3'). PCR products were labeled by incorporating fluorescent dNTPs using the SYBR Green PCR master mix (Applied Biosystems). Quantitation of fluorescent PCR products was performed using the GeneAmp 5700 sequence detector (Applied Biosystems). GAP3DH PCR products generated after 15 to 18 cycles were used to normalize quantities of cDNA, allowing quantification of D2R and A2AR PCR products generated after 25 to 28 and 18 to 20 cycles per sample, respectively. D2R, A2AR, and GAP3DH expression levels were derived from at least three independent experiments in which nine separate PCR reactions were performed on each of the cDNA samples.

**Quantitative Western Blotting.** Striata were dissected from mouse brains and original wet weights were measured. The striata were homogenized with a Polytron PT 1200 homogenizer (Brinkmann Instruments, Westbury, NY) in chilled homogenization buffer (50 mM Tris-HCl, 5 mM KCl, 2 mM MgCl<sub>2</sub>, and 2 mM CaCl<sub>2</sub>, pH 7.4) as described by Levant et al. (1992), and centrifuged for 15 min at 48,000g at 4°C. The pellets were resuspended and washed twice in homogenization buffer. The final pellets were resuspended in homogenization buffer and protein concentrations were determined using a detergent-compatible protein assay (Bio-Rad, Hercules, CA). Protein samples (30 µg/lane) were separated either on 10% Tris-glycine or 4 to 12% Bis-Tris-Nu-PAGE gels (Invitrogen) and then electro-transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were incubated with 1:1000 primary antibody [rabbit polyclonal anti-D2R IgG (Chemicon, Temecula, CA), followed by an alkaline phosphatase conjugated anti-rabbit IgG (Western Breeze; Invitrogen)]. Enhanced chemiluminescent detection of the membranes was carried out using CDP-Star (WesternBreeze). Immunoblot analyses on 10% Tris-glycine gels revealed a predominant band at approximately 47 kDa (data not shown), whereas similar analyses on 4 to 12% Bis-Tris gels detected a single band between 60 and 65 kDa. The 47- and 60- to 65-kDa bands detected on these two different gels are consistent with the size of the denatured form of the D2R protein in rodents and compare favorably with that observed for human D2R protein, which was assayed as a control (Sigma). In addition, we observed an additional faint band at 110 kDa on 4 to 12% Bis-Tris gels, which is likely to correspond to the size of the native D2R protein. The intensities of D2R bands were quantified using Scion Image (Scion Corporation, Frederick, MD) and normalized by multiplying the concentration of recovered protein (micrograms per milliliter) by the total amount of original wet weight brain tissue (milligrams).

**Analyses of Striatal Neuronal Physiology in Multielectrode Plates.** Basic procedures for experiments using multielectrode plates (MEPs) were similar to those described previously (Mistry et al., 2002). MEPs (CNNS, Denton, TX) were pretreated with poly-DL-lysine and then laminin (Sigma). Striatal neurons ( $3 \times 10^5$  cells) were applied to each MEP, and cultures were maintained for 21 days before drug treatment and electrophysiological recordings. MEPs were assembled in autoclaved stainless steel recording chambers (CNNS) with 1 ml of culturing media, transferred to the recording apparatus (CNNS), and maintained at 37°C with a steady stream of humidified 10% CO<sub>2</sub>. Electrical activity was amplified, recorded, and discriminated on-line using a 64-channel amplifier (Plexon, Dallas, TX) and digitized simultaneously with an Optiplex GxPro computer (Dell Computer, Round Rock, TX). Units with a 2.5:1 signal-to-noise

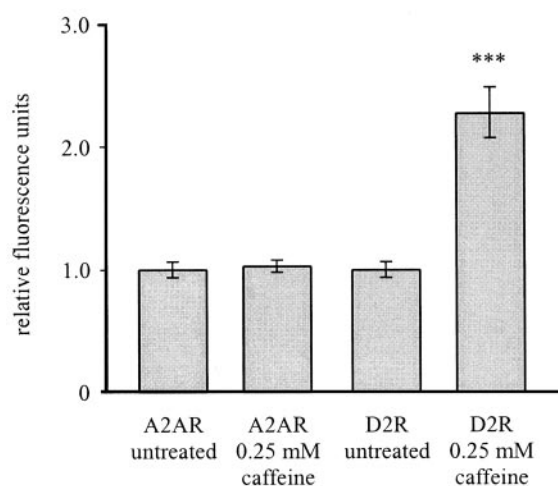
ratio were selected. Drugs were administered to the recording chamber after a baseline recording had been achieved. Firing rate histograms were generated using Neuroexplorer (Plexon).

## Results

**Caffeine Activates the Expression of D2R mRNA in PC-12 Cells.** PC-12 cells express D2R and A2AR (Zhu et al., 1997; Arslan et al., 1999), and are thus a suitable model system to study whether caffeine alters the expression of these genes. To investigate whether a physiological dose of caffeine (Fredholm et al., 1999) produced changes in the expression of the genes encoding D2R and A2AR, PC-12 cells were treated with 0.25 mM caffeine for 24 h, and the expression of the mRNAs corresponding to these neurotransmitter receptors was measured using quantitative RT-PCR. As shown in Fig. 1, caffeine treatment of PC-12 cells resulted in a 2.3-fold increase in the expression of D2R mRNA after 24 h, whereas the level of A2AR mRNA expression did not change.

**Caffeine Activates the Expression of a D2R Reporter Construct.** To examine further the up-regulation of D2R gene expression in response to caffeine, we prepared a luciferase reporter construct containing the 5' end of the rat D2R gene, and examined its activity in response to different concentrations of caffeine. This reporter construct contained 1515 base pairs of the rat D2R promoter sequence and the entire first exon (329 base pairs) of the D2R gene and is hereafter designated -1515/+329.

To determine whether the activity of this reporter was affected by caffeine, the -1515/+329 D2R reporter construct was transiently transfected into PC-12 cells. Twenty-four hours later, cells were treated with different physiological concentrations of caffeine that have been established in previous studies (Fredholm et al., 1999). After an additional 24 h, cells were harvested and assayed for luciferase activity. As shown in Fig. 2A, a range of caffeine concentrations between 0.01 and 0.25 mM increased the activity of the D2R reporter an average of 1.4-fold; however, a concentration of 2 mM had no effect.



**Fig. 1.** Quantitative RT-PCR analysis of the expression of A2AR and D2R mRNAs in PC-12 cells 24 h after caffeine treatment. PC-12 cells treated with 0.25 mM caffeine resulted in a statistically insignificant 1.03-fold increase in A2AR mRNA expression. In contrast, 0.25 mM caffeine resulted in a 2.28-fold (\*\*\*) ( $P \leq 0.001$ ; post hoc test) increase in the level of D2R mRNA expression after 24 h. Error bars represent S.E.M. The data were derived from three independent experiments.



To determine how rapidly caffeine affected the activity of the D2R reporter construct, luciferase activity was examined at 30 min and 4, 8, and 24 h after treatment with an activating (0.03 mM) dose of caffeine. As shown in Fig. 2B, 0.03 mM caffeine activated D2R gene expression between 8 and 24 h after treatment.

**Activation of D2R Promoter Activity by the A2AR Antagonist, CSC Is Eliminated by the A2AR Agonist CGS 21680.** In PC-12 cells and in striatal neurons, it is known that the G protein-coupled receptor A2AR functionally interacts with D2R (Ferre et al., 1991b; Diaz-Cabiale et al., 2001). The interaction of these receptors and signals transduced by them are modulated by dopamine and other drugs such as caffeine. Given these considerations, we examined whether CGS 21680, a potent A2AR agonist, altered the expression of the D2R gene in PC-12 cells and whether CGS 21680 could eliminate activation of D2R gene expression caused by caffeine and 8-(3-chlorostyryl) caffeine (CSC), a specific A2AR antagonist.

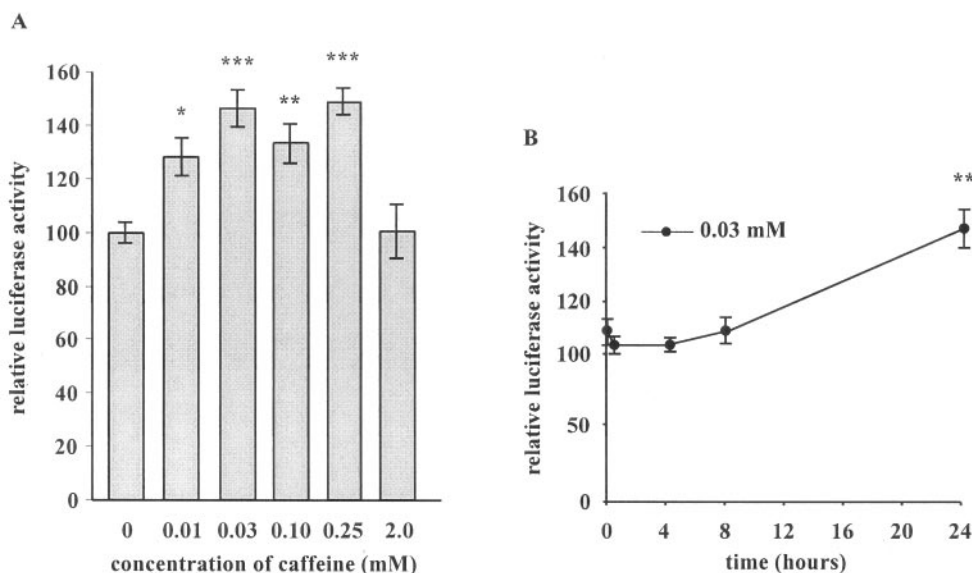
To first examine how different concentrations of CGS 21680 affected D2R promoter activity, we conducted transfection experiments using the D2R/luciferase reporter in the presence of either 10, 1, 0.1, or 0.01  $\mu$ M CGS 21680. As shown in Fig. 3A, 10  $\mu$ M CGS 21680 suppressed the expression of the D2R/luciferase reporter in PC-12 cells by 20%. This level of suppression of D2R luciferase reporter activity was also observed over a large concentration range (5–200  $\mu$ M) of CGS 21680 (data not shown). However, lower concentrations of CGS 21680 (1, 0.1, and 0.01  $\mu$ M) had little or no effect on D2R promoter activity.

We next examined whether CGS 21680 could override the activation of D2R reporter expression by caffeine and CSC. As shown in Fig. 3B, 0.25 mM caffeine and 5  $\mu$ M CSC activated expression of the D2R/luciferase reporter in PC-12 cells, and this response was eliminated by simultaneous administration of 10, 1, or 0.1  $\mu$ M CGS 21680, and was reduced by 0.01  $\mu$ M CGS 21680. These results indicate that a concentration range between 0.01 and 10  $\mu$ M of CGS 21680 is effective at eliminating the activation of D2R gene expression by caffeine. With respect to activation by CSC (Fig. 3C), it was observed that 10, 1, and 0.1  $\mu$ M CGS 21680 were effective

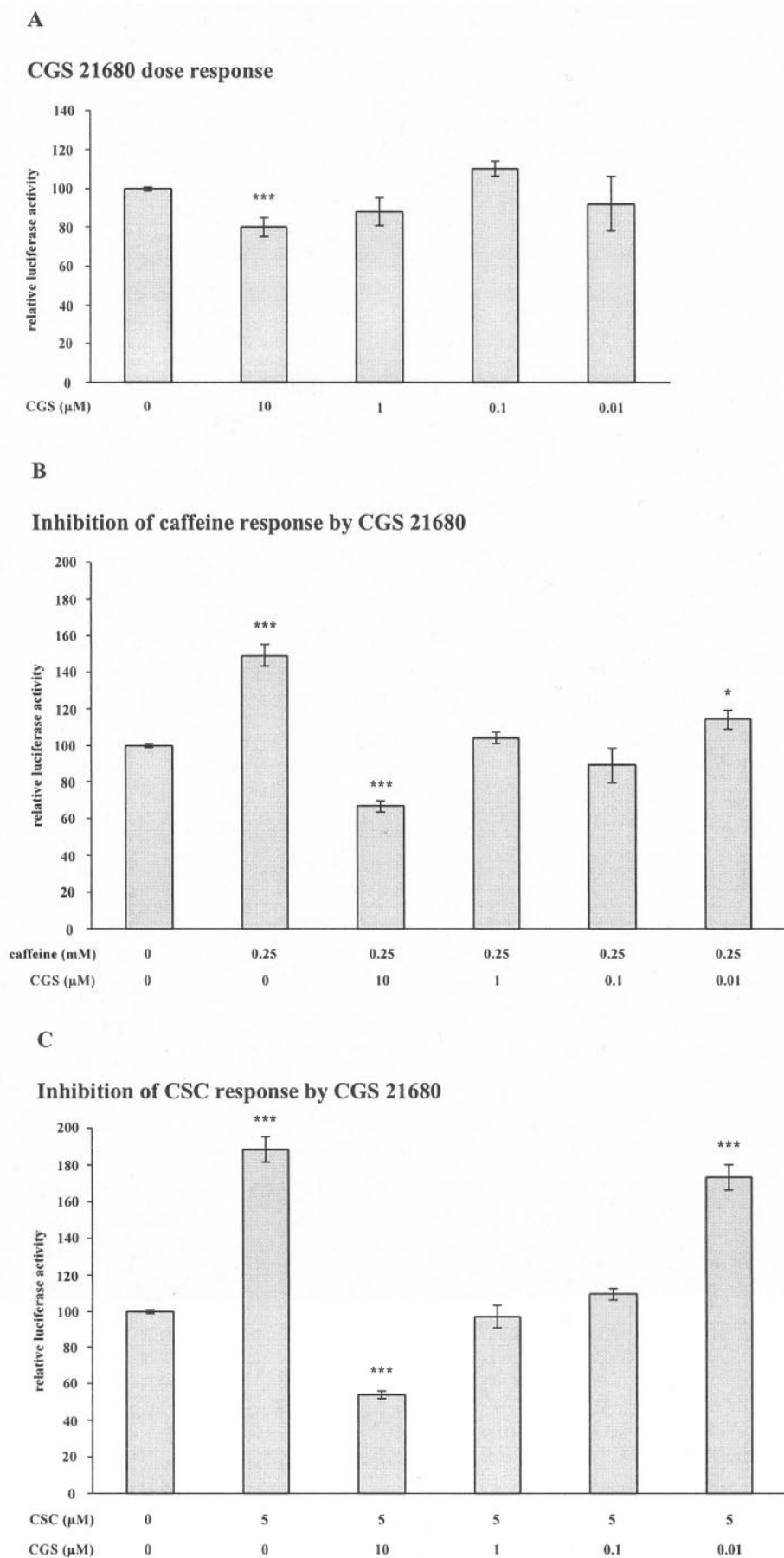
in eliminating the activation of D2R promoter activity by CSC. However, 0.01  $\mu$ M CGS 21680 did not reduce this activation. These experiments indicate that the threshold of reversal of D2R promoter activation by CSC occurs between 0.01 and 0.1  $\mu$ M CGS 21680.

**A Region of the Promoter (–117 to –75) and a Segment of the 5'-Untranslated Region (+22/+317) Are Involved in the Regulation of D2R Gene Expression by Caffeine.** Previous studies on the regulation of the D2R gene have identified several regulatory elements within the promoter that regulate its expression in a variety of neuronal cell lines. These elements include several binding sites for transcription factors (Fig. 4A), including an SP1 motif (which binds to SP1 and SP3 proteins) as well a series of TGGG repeats that interact with the dopamine receptor regulatory factor (DRRF) (Minowa et al., 1994; Hwang et al., 2001). To determine whether the D2R promoter region containing these transcription factor binding sites contributed to regulation of D2R gene expression by caffeine, we generated a D2R reporter construct lacking this region (–117/–75) and examined its activity and responsiveness to caffeine in PC-12 cells after transient transfection (Fig. 4B). In addition, two additional reporter constructs were generated to assess whether 5'-untranslated sequences within the first exon of the D2R gene (+22/+317) also contributed to the regulation of D2R gene expression by caffeine. These two constructs either lacked this 5'-UTR region (–117/+22) alone or lacked both the 5'-UTR region as well as the –117/–75 promoter region (–75/+22).

To determine caffeine-responsive regions in the D2R gene, the activities of the four D2R/luciferase reporters (–117/+317, –75/+317, –117/+22, and –75/+22; Fig. 4A) were compared in PC-12 cells after 24 h of treatment with 0.25 mM caffeine. PC-12 cells treated with 0.25 mM caffeine elevated the activity of the –117/+317, –75/+317, –117/+22, and –75/+22 constructs 1.90, 1.24, 1.30, and 1.01-fold, respectively, compared with the activities produced by these constructs in untreated PC-12 cells. Only the –117/+317 construct showed statistically significant up-regulation in activity by caffeine, indicating that both the –117/–75 and



**Fig. 2.** Dose-response and time course response of D2R gene expression to caffeine after transfection of PC-12 cells with the full-length D2R (–1515/+329) luciferase reporter construct. A, treatment of cells with 0.01 mM, 0.03 mM, 0.10 mM, and 0.25 mM caffeine activated expression of the D2R/luciferase reporter construct 1.28-fold (\*,  $P \leq 0.031$ ), 1.45-fold (\*\*\*,  $P \leq 0.001$ ), 1.33-fold (\*\*,  $P \leq 0.002$ ), and 1.49-fold (\*\*\*,  $P \leq 0.001$ ; post hoc test), respectively, whereas a concentration of 2.0 mM caffeine produced baseline activity. B, the physiological concentration of caffeine (0.03 mM) activated expression of the D2R/luciferase reporter construct by 1.50-fold (\*\*,  $P \leq 0.003$ ; post hoc test) after 24 h. Error bars represent S.E.M. The data presented in Fig. 2A were derived from six independent experiments performed in triplicate, and the data in Fig. 2B were derived from three experiments performed in duplicate.



**Fig. 3.** CGS 21680 overrides caffeine and CSC activation of D2R reporter expression. Levels of expression in PC-12 cells of the -1515/+329 D2R/luciferase reporter construct in response to the A2AR agonist (A), and mixtures of either CGS 21680 (B) and caffeine or CGS 21680 and CSC (C). A, treatment of PC-12 cells with 10 μM CGS 21680 reduced D2R/luciferase reporter activity by 20% (\*\*\*,  $P \leq 0.001$ ) from untreated control cells, whereas 1, 0.1, and 0.01 μM had little effect on promoter activity. B, D2R/luciferase reporter expression is increased by 0.25 mM caffeine by 1.49-fold (\*\*\*,  $P \leq 0.001$ ). However, this activation by caffeine is overridden by simultaneous administration of 10 μM CGS 21680 and is further reduced to a level that is 67% of untreated controls (\*\*\*,  $P \leq 0.001$ ). Concentrations of 1, 0.1, and 0.01 μM (1.14-fold change; \*,  $P \leq 0.02$ ) also eliminated D2R reporter activation by caffeine. C, the D2R reporter is activated by the A2AR-specific antagonist CSC by 1.88-fold (\*\*\*,  $P \leq 0.001$ ). This activation is overridden by simultaneous administration of 10 μM CGS 21680 and is further reduced to 54% of untreated control cells (\*\*\*,  $P \leq 0.001$ ; post hoc test). Doses of 1 and 0.1 μM CGS 21680 also eliminate CSC activation of the D2R reporter and bring luciferase activity back to baseline. However, 0.01 μM CGS 21680 has no effect on the activation of D2R reporter activity by CSC (1.73-fold change; \*\*\*,  $P \leq 0.001$ ). Error bars represent S.E.M. The data were derived from seven independent experiments performed in triplicate.

the +22/+317 regions were required for up-regulation of D2R reporter expression by caffeine.

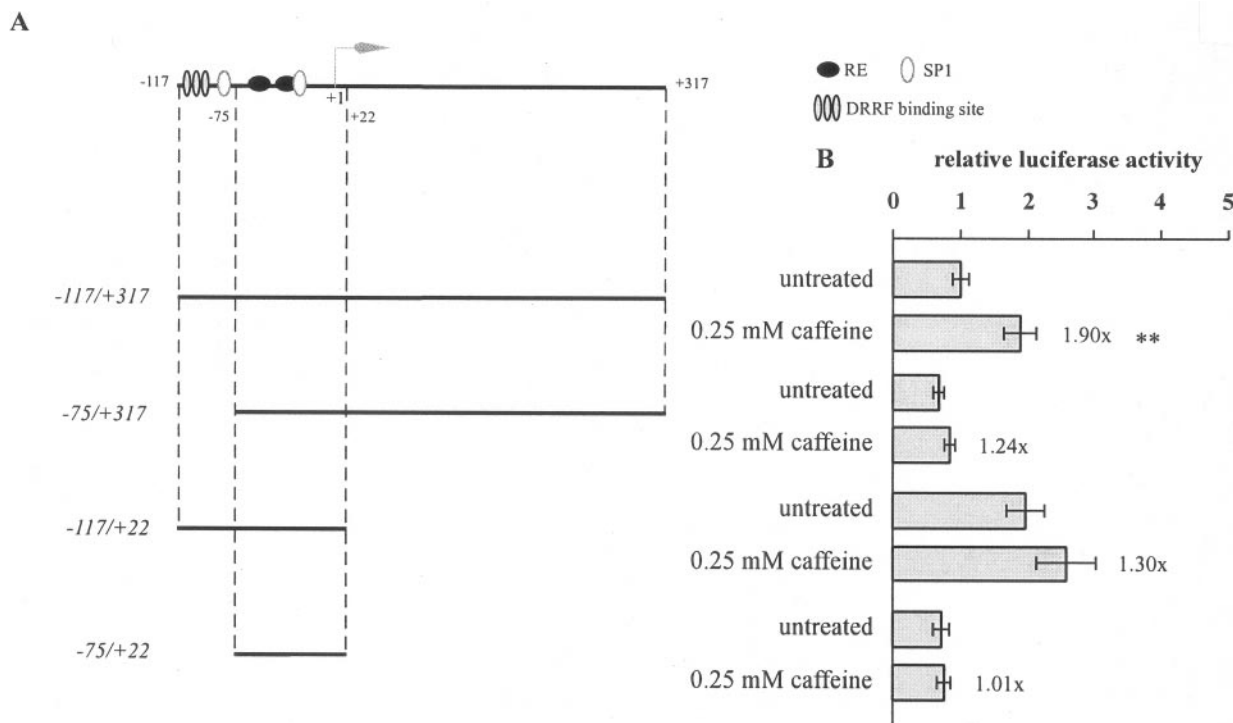
**Caffeine Treatment Increases the Firing Rates of Striatal Neurons in Culture.** To examine whether caffeine could alter the physiology of primary striatal neurons and whether this required the A2A receptor, spontaneous network activity was recorded from cultured striatal neurons grown on MEPs. MEPs have been used successfully to analyze the changes in firing rates that occur in a number of neuronal cell types after administration of current and various pharmacological agents (Gross, 1979; Mistry et al., 2002). For these experiments, primary embryonic mouse striatal neurons were cultured on plates containing an array of 64 electrodes (Fig. 5A). By 21 days in culture, the striatal neurons developed spontaneous spiking activity that varied according to the number of active units on each MEP. Upon treatment with a physiological concentration of caffeine (0.2 mM), striatal neurons showed a dramatic increase in spontaneous activity. A 4-fold increase in firing rate was observed 12 min after drug treatment (Fig. 5B). Increased firing persisted for a total of 80 min, when the activity of neurons returned to baseline levels.

**Caffeine Activates D2R mRNA Expression in Mouse Primary Striatal Cultures.** Having established that striatal neurons treated with caffeine show an increase in firing rates, we examined whether caffeine (0.25 mM) could alter striatal D2R mRNA expression. For these experiments, striatal neurons were treated for 4, 8, or 24 h with 0.25 mM caffeine and changes in D2R mRNA expression were measured using quantitative RT-PCR. As shown in Fig. 6, caffeine did not produce any noticeable change in D2R mRNA

expression after 4 h of caffeine treatment, but after 8 h, caffeine treatment resulted in increased D2R mRNA expression. This induction dramatically increased between 8 and 24 h (Fig. 6). When taken together with the physiological results presented in Fig. 5, these data indicate that up-regulation of embryonic striatal D2R gene expression in response to caffeine occurs after the initial increase in neuronal firing rates has subsided.

**Caffeine Increases D2R mRNA Expression in Striata of Female but Not Male Mice.** To examine whether caffeine could regulate striatal expression of the A2AR and D2R genes in vivo, a single dose of 50 mg/kg caffeine (Chen et al., 2001) was administered to male and female mice, and levels of A2AR and D2R mRNA expression in the striatum were examined 24 h later using quantitative RT-PCR. As shown in Fig. 7, caffeine produced no changes in A2AR mRNA expression in male and female mice, and caffeine led to a 31% decrease and 1.94-fold increase in striatal D2R mRNA expression in male and female mice, respectively, compared with sham-treated mice as well as to naive controls (data not shown). These results indicate that caffeine regulates the striatal expression of the D2R gene in a sex-specific manner.

**Caffeine and CSC Increase D2R Protein Expression in Striata of Female but Not Male Mice.** Having established that caffeine regulates striatal D2R gene expression in vivo, additional experiments were conducted to determine whether administration of caffeine or CSC could alter levels of D2R protein expression in the striatum. For these experiments, single doses of caffeine (50 mg/kg, i.p.) or CSC (5

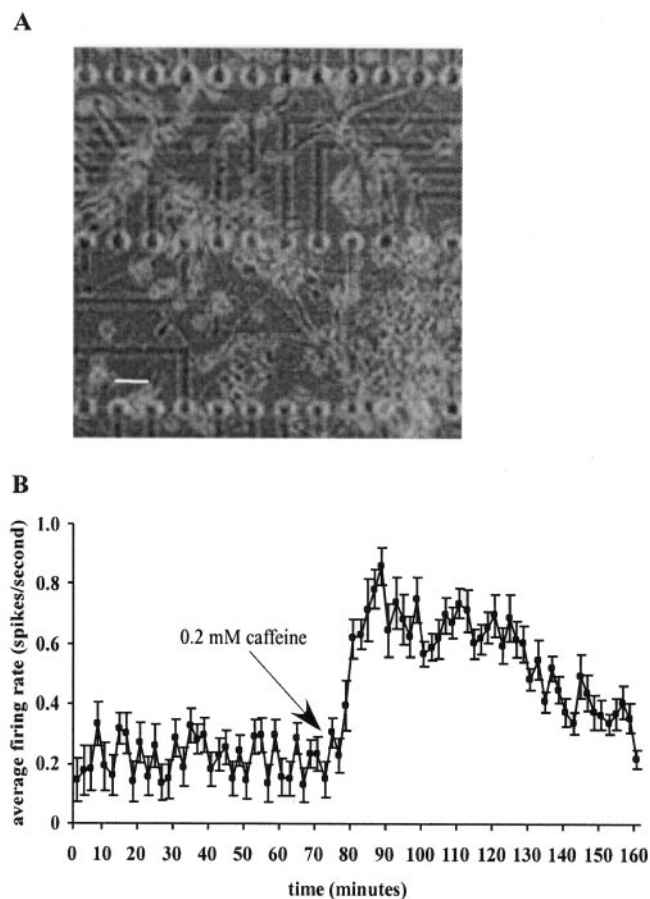


**Fig. 4.** A, map of the 5' end of the rat D2R gene and schematic of four different D2R/luciferase reporters used in caffeine activation experiments. Locations are shown of the binding sites for transcription factors of the SP1 family, the DRRF, and REs. The transcriptional start site is indicated with a rightward-pointing arrow, and 5'-untranslated sequences are located to right of this arrow. B, activities of the four D2R luciferase reporter constructs were assessed for activation by 0.25 mM caffeine after their transient transfection into PC-12 cells. Both the -117/-75 D2R promoter region and the +22/+317 segment of the 5'-UTR are required for the statistically significant activation of D2R reporter activity by caffeine of 1.90-fold (\*\*,  $P \leq 0.002$ ; post hoc test). Error bars represent S.E.M. The data presented were derived from four independent experiments performed in triplicate.

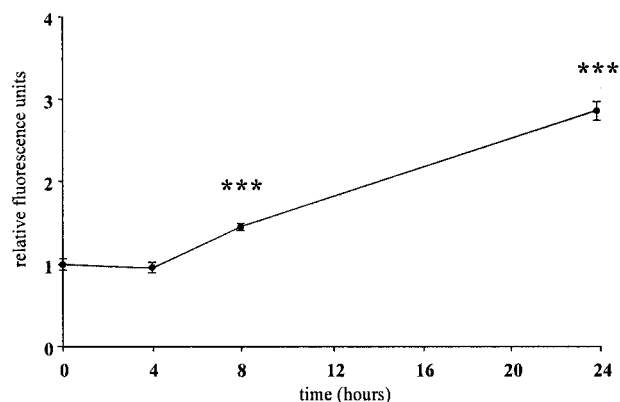


mg/kg, i.p.) were administered to male and female mice. The striata from these mice were collected 24 h later and were homogenized, and membrane fractions were analyzed for

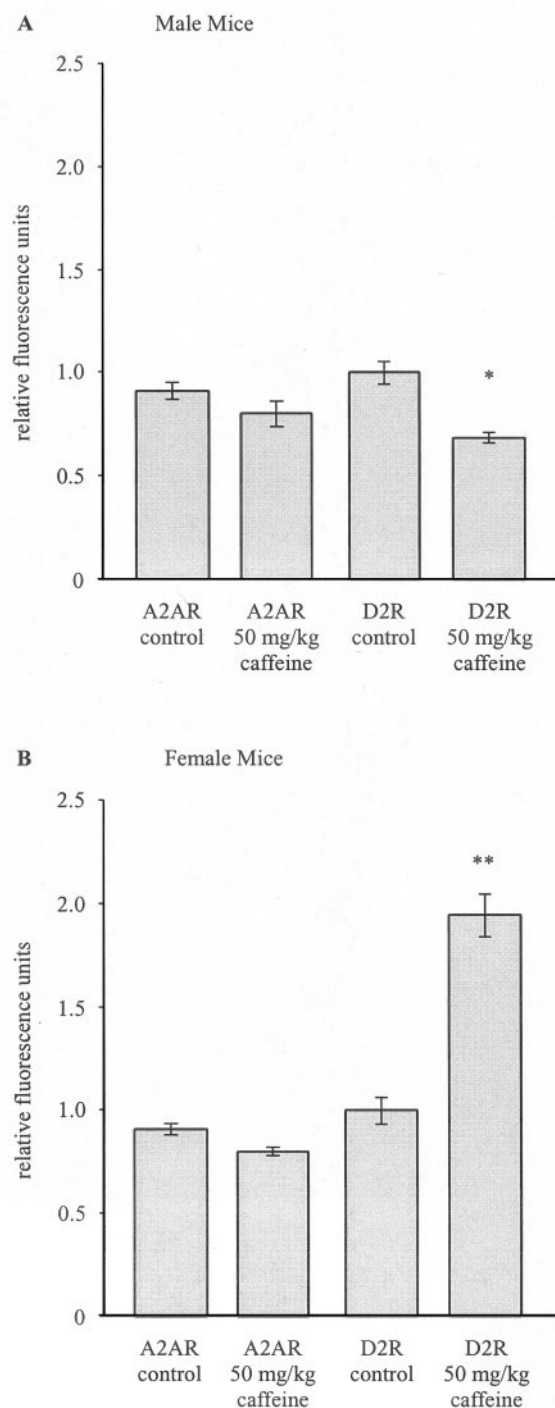
D2R protein expression using quantitative Western blotting. A representative Western blot showing D2R protein levels upon treatment of a single group of male and female mice



**Fig. 5.** Striatal cultures grown on multi-electrode plates showed enhanced neuronal firing in response to 0.2 mM caffeine. A, multi-electrode plate showing mouse primary striatal culture with recording electrodes. Scale bar, 40  $\mu$ m. B, 0.2 mM caffeine increased neuronal firing rates by 4-fold within 12 min of drug treatment; this elevated rate returned to baseline after 80 min. Representative experiment, repeated three times ( $n = 8$  units). Nonresponding units were omitted from the analysis. Error bars represent S.E.M.



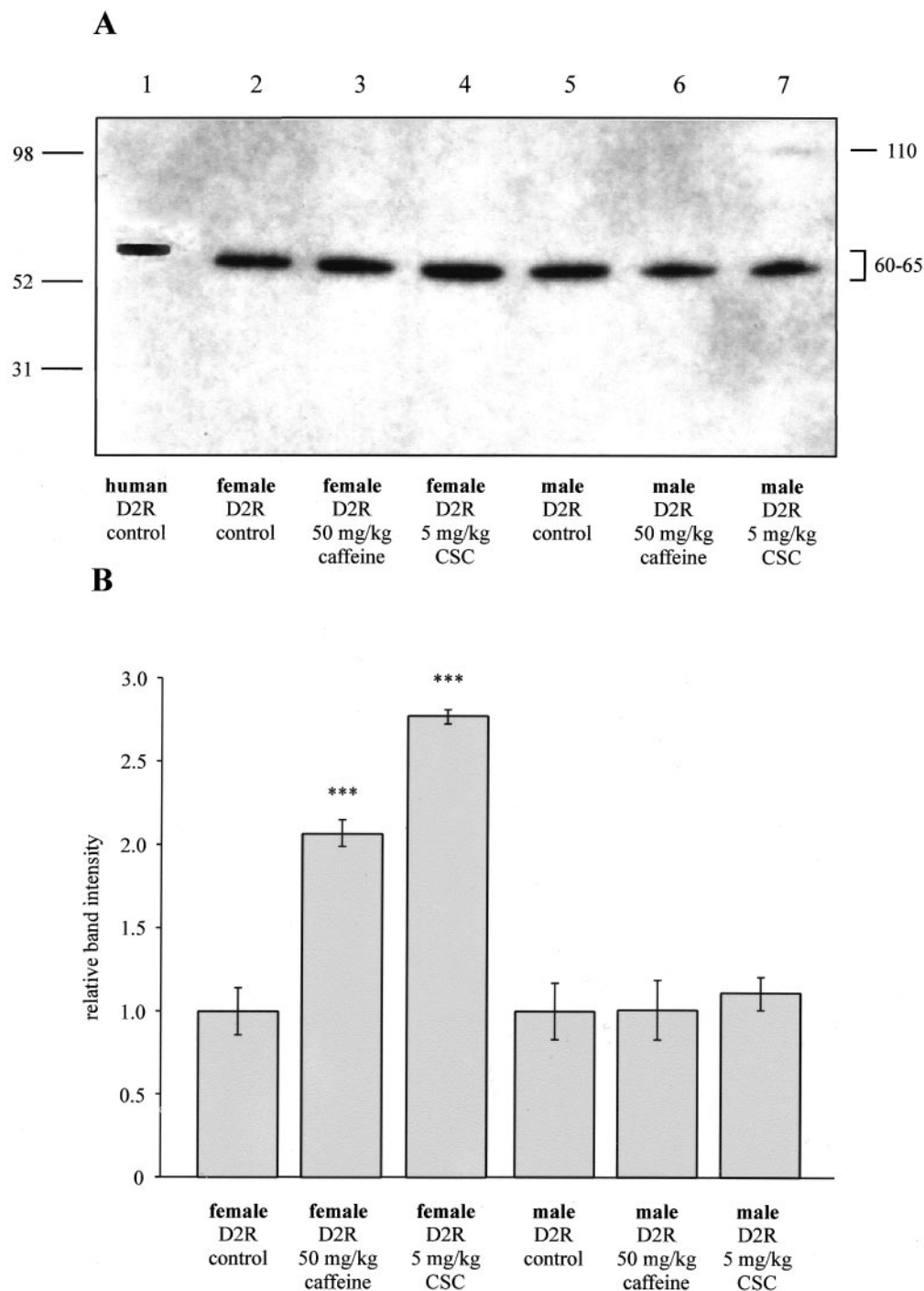
**Fig. 6.** Quantitative RT-PCR analysis of the expression of D2R mRNA in primary cultures of mouse striatal neurons. 0.25 mM caffeine increased D2R mRNA expression by 1.45-fold (\*\*\*) ( $P \leq 0.001$ ) after 8 h and 2.90-fold (\*\*\*) ( $P \leq 0.001$ , post hoc test) after 24 h. Error bars represent S.E.M. The data were derived from striatal cultures prepared from 10 mouse embryos (embryonic day 14); each RNA sample obtained from these cultures was subjected to nine separate PCR reactions.



**Fig. 7.** Quantitative RT-PCR analysis of the expression of A2AR and D2R mRNAs in male and female mouse striata, 24 h after caffeine administration (50 mg/kg, i.p.). A, in male mice, caffeine decreased A2AR mRNA expression by a statistically insignificant 12% ( $P = 0.061$ ). In contrast, caffeine decreased D2R mRNA expression by 31% (\*,  $P \leq 0.017$ ). B, in female mice, caffeine also decreased A2AR mRNA expression by a statistically insignificant 12% ( $P = 0.071$ ). However, caffeine increased D2R mRNA expression by 1.94-fold (\*\*,  $P \leq 0.006$ , post hoc test). Error bars represent S.E.M. The data presented were derived from striatal RNAs obtained from three male and three female mice per treatment. Overall nine separate PCR reactions were carried out for each of the RNA samples.

with caffeine and CSC is shown in Fig. 8A. A single band of approximately 60 to 65 kDa was the major species detected using the rodent D2R antibody (Fig. 8A, lanes 2–7). This size agrees well with the reported size of the denatured form of rodent D2R protein (Zhen Meng et al., 1998; Maltais et al., 2000) and is nearly identical to that of the human D2R protein, which was detected alongside the mouse striatal samples (Fig. 8A, lane 1). In addition, a larger band at 110 kDa was also detected in some samples (Fig. 8A, lane 7), which corresponds to the size of the native D2R protein (as communicated by the manufacturer of the antibody (Chemicon)).

To determine whether caffeine or CSC increased D2R protein expression in the striata of male and female mice, the signal intensities of D2R protein bands on Western blots were quantified from four male and four female mice per treatment. As shown in Fig. 8B, caffeine and CSC both increased striatal D2R protein expression in female mice (2.07- and 2.77-fold, respectively). In contrast, caffeine and CSC showed no increase in D2R protein expression levels in male mice. These results indicate that caffeine up-regulation of striatal D2R mRNA expression are accompanied by similar changes in D2R protein expression in female mice, but not male mice, after 24 h. Moreover, the observation that CSC



**Fig. 8.** Western blot analysis of the expression of D2R protein in male and female mouse striata, 24 h after caffeine (50 mg/kg, i.p.) or CSC (5 mg/kg, i.p.) administration. A, representative Western blot showing levels of D2R protein expression in either female (lanes 2–4) or male (lanes 5–7) mice. A control showing the D2R antibody reactivity with a sample of human D2R protein is shown in lane 1. The sizes of reference standard proteins are indicated on the left side of the blot (in kilodaltons) and sizes corresponding to the native (110 kDa) and denatured (60–65 kDa) forms of D2R are indicated on the right. B, quantitative Western analyses of four different male and female mice per treatment. Caffeine and CSC treatments both produced statistically significant increases in D2R protein expression of 2.07- (\*\*\*,  $P \leq 0.001$ ) and 2.77-fold (\*\*\*,  $P = 0.001$ ; post hoc test), respectively, in female mice. In male mice, however, caffeine and CSC treatments resulted in statistically insignificant changes in D2R protein expression of 1.01- ( $P = 0.938$ ) and 1.11-fold ( $P = 0.515$ ), respectively. Error bars represent S.E.M. The data presented were derived from striatal proteins obtained from four male and four female mice per treatment, with samples from each mouse blotted between six and eight times.



also increases D2R protein expression in female mice provides additional support for the conclusion that up-regulation of D2R expression by caffeine is mediated, at least in part, by signaling via the A2A receptor.

## Discussion

This study examined regulation of D2R gene expression by caffeine in PC-12 cells and the physiology and expression of the D2R gene in response to caffeine in striatal neurons. We found that D2R mRNA and the activities of luciferase reporter constructs driven by the 5' end of the rat D2R gene are up-regulated by caffeine in PC-12 cells. In addition, the increased D2R reporter expression in PC-12 cells in response to caffeine and the specific A2AR antagonist CSC was eliminated by simultaneous treatment with the specific A2AR agonist CGS 21680, indicating that up-regulation of D2R gene expression by caffeine and CSC is dependent, at least in part, on signaling by the A2AR. By testing different D2R reporter constructs, we found that a region of the D2R promoter and a segment of the 5'-UTR were both required for activation of D2R reporter expression by caffeine. Embryonic striatal neurons increased their firing rates for up to 80 min when a physiological concentration of caffeine was added. Caffeine also increased D2R mRNA expression in striatal neuronal cultures within 24 h. This increase began after 4 h of constant caffeine exposure. Taken together, these data support the conclusion that enhanced D2R expression by caffeine occurs after the immediate physiological response to the drug. In vivo, short-term caffeine treatment resulted in a sexually dimorphic response, leading to a 1.94-fold increase and a 31% decrease in striatal D2R mRNA expression in female and male mice, respectively. Similarly, both caffeine and CSC led to 2.07- and 2.77-fold increases in striatal D2R protein expression in female mice, but produced no significant changes in D2R protein expression in male mice. The increase in striatal D2R mRNA and protein expression by caffeine in female mice was similar in magnitude to that observed in PC-12 cells and in primary cultures of embryonic striatal neurons. It was also similar to the level of induction of D2R reporter expression observed in transfection experiments.

**D2R Gene Regulatory Regions.** This study provides evidence for the first time that D2R gene expression is dynamically regulated by caffeine. One of the regulatory regions found to respond to caffeine is bases -117 to -75 of the D2R promoter, which contains a binding site for the transcription factor DRRF. DRRF is known to differentially regulate D2R gene expression by preventing either SP1 (a positive factor) or SP3 (a positive or a negative factor) from binding to SP1 motifs in the D2R promoter (Hwang et al., 2001; Takeuchi et al., 2002). The antipsychotic drug haloperidol and the psychostimulant drug cocaine have both been found to reduce DRRF expression in the striatum, whereas caffeine did not affect DRRF mRNA expression (Hwang et al., 2001). These data suggest that reducing DRRF expression is associated with up-regulation of dopamine receptors and that DRRF may repress D2R gene expression in response to drugs (Hwang et al., 2001). Therefore, a plausible hypothesis is that up-regulation of D2R expression by caffeine might involve derepression of D2R expression in part by mitigating the repressive effect of DRRF. This hypothesis could be ad-

ressed by examining D2R gene regulation by caffeine in striatal neurons from DRRF knockout mice.

However, we conclude from the present study that activation of D2R gene expression by caffeine also requires a region of the 5'-UTR (+22/+317). The 5'-UTRs of the rat and human D2R genes have a high degree of sequence homology, supporting the idea that the caffeine-response elements within the 5'-UTR identified in this study are likely to function in humans. Moreover, the entire 5'-UTR contains a high degree of sequence complementarity (60% overall) with the 28S ribosomal RNA, and previous studies have indicated 5'-UTR regions that are complementary to ribosomal RNA can affect mRNA translation (Mauro and Edelman, 2002). These observations prompt future investigations of the 5'-UTR in regulating D2R gene expression at the level of translation.

**Adenosine Antagonists and CGS 21680 Have Opposing Effects on D2R Reporter Construct Expression.** In transfection experiments, we found that CGS 21680, a potent agonist of the A2AR suppressed the activation of D2R reporter expression by nonspecific and specific antagonists of A2AR (caffeine and CSC, respectively). These results suggest that signal transduction via the A2AR can dynamically regulate the expression of the D2R gene. CGS 21680 and other agonists of the A2AR are known to activate PKA pathways in neurons, and earlier investigations have implicated PKA signaling pathway in physiological responses of neurons to drugs that bind to dopamine and adenosine receptors. PKA also regulates the activities of other downstream messengers, including DARPP-32, an important regulator of neuronal physiology in response to caffeine (Svenningsson et al., 2000). In a recent study, it has been proposed that caffeine, by antagonizing the A2A receptor, decreases cAMP formation, PKA activation, and protein phosphatase 2A phosphorylation, thereby increasing phosphorylation of DARPP-32 at threonine 75 (Lindskog et al., 2002). However, it remains an open question whether PKA pathways and DARPP-32 directly regulate gene expression downstream of caffeine. To address this, it will be informative to compare regulation of D2R and other striatal genes in neurons from DARPP-32 knockout mice.

Other signaling pathways, including those involving S6 and extracellular signal-regulated kinase/mitogen-activated protein kinases, also influence dopaminergic neurotransmission (Welsh et al., 1998; Oak et al., 2001). Furthermore, caffeine releases calcium from intracellular stores, resulting in the activation of PKC pathways (Koizumi and Inoue, 1998). Therefore, it will be revealing to investigate the contribution of these pathways in the regulation of gene expression downstream of caffeine. It is currently unclear whether electrophysiological responses are a prerequisite for up-regulation of D2R expression by caffeine. It is well known that immediate-early genes downstream of PKA, such as *c-fos*, are induced within minutes of depolarization events in several neuronal cell types (Johansson et al., 1994). However, because activation of D2R gene expression by caffeine in striatal neurons occurs between 4 and 24 h, whether neuronal depolarization and expression of immediate-early genes are required for enhanced D2R expression by caffeine is an open question.

**Caffeine As a Neuroprotective Agent in Parkinson's Disease.** Parkinson's disease (PD) is a hypokinetic disorder caused by dopaminergic cell death in the substantia nigra

leading to reduced dopamine input into the striatum (McGeer et al., 1988). Recent work has shown that the expression of dopamine receptors is significantly reduced in patients with PD (Greengard, 2001; Schwarzschild et al., 2002) and that long-term use of levodopa as part of dopamine replacement therapy in PD patients results in down-regulation of striatal dopamine receptors and dyskinesia (Greengard, 2001). These findings suggest that movement disorders in PD might be exacerbated by down-regulation of dopamine receptors in the striatum as a consequence of disrupting the natural dopamine levels or by increasing dopamine by replacement therapy.

Recently, a large epidemiological study concluded that caffeine is neuroprotective against PD, and increasing caffeine intake is correlated with reduced incidence of PD (Ascherio et al., 2001). Moreover, reduced dopamine and dopamine transporter expression in dopamine depletion models of PD is attenuated by prior administration of caffeine (Chen et al., 2001; Xu et al., 2002). A2AR antagonist drugs, including SCH 58261, KW 6002, and KF 18446, are also neuroprotective in rodent models of PD and do not cause dyskinesia (Chen et al., 2001; Ikeda et al., 2002). The mechanisms underlying the neuroprotective effect of caffeine and other A2AR antagonist drugs on PD are unknown. Our finding that striatal D2R gene and protein expression are up-regulated by caffeine may explain part of the therapeutic effects of caffeine and other A2A receptor antagonists on PD. A thorough understanding of mechanisms controlling expression of D2R and other striatal genes by caffeine may aid in the development of better innovative therapies for the treatment of PD.

**Sexually Dimorphic Regulation of Striatal D2R Gene and Protein Expression by Caffeine.** So far, very little is known concerning how drugs that affect the physiology of the basal ganglia alter gene expression. One study has revealed that short-term treatment of rats with morphine resulted in differential patterns of striatal *c-fos* expression in male and female rats, and these sexually dimorphic responses were attenuated by *N*-methyl-D-aspartate receptor antagonists (D'Souza et al., 1999). In addition, these sexually dimorphic responses were found to depend on both sex steroid-dependent and -independent mechanisms (D'Souza et al., 2002). In the present study, we found that short-term administration of caffeine or the specific A2AR antagonist CSC in mice has sexually dimorphic effects on striatal D2R mRNA and protein expression. Because regulation of D2R expression is likely to be an important mechanism in regulating striatal physiology, it will be of interest to investigate whether enhanced D2R expression in female mice and a lack of response in male mice is correlated with sex-specific behavioral responses to caffeine and other A2AR antagonists. In this light, it will be important to determine whether drugs that affect dopaminergic neurotransmission lead to sex-specific expression of genes that regulate striatal physiology and whether certain drug therapeutic regimens are more effective in male or females.

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