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Stimulation of Adenosine Receptors Selectively Activates Gene Expression in Striatal Enkephalinergic Neurons

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In the striatum, adenosine A_{2A} and dopamine D_2 receptors exert reciprocal antagonistic interactions that modulate the function of GABAergic enkephalinergic neurons. We have previously shown that stimulation of adenosine A_1 receptors allows the stimulation of A_{2A} receptors to overcome a tonic inhibitory effect of D_2 receptors and induce striatal expression of *c-fos*. In the present work, by studying co-localization of c-Fos immunoreactivity and *preproenkephalin* and *preprodynorphin* transcripts, we show that co-administration of the A_1 receptor agonist CPA and the A_{2A} receptor agonist CGS 21680 increases the striatal expression of *c-fos* in GABAergic enkephalinergic but not in GABAergic dynorphinergic neurons. Co-administration of CPA and CGS 21680 also induced a significant increase in the striatal expression of *preproenkephalin*. The results underscore the role of adenosine in the activation of gene expression in the GABAergic enkephalinergic neuron.

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INTRODUCTION

Adenosine and dopamine modulate the activity of the striatal γ-aminobutyric acid (GABA)ergic efferent neurons, which constitute more than 90% of the neuronal population in the striatum. Striatal GABAergic efferent neurons can be classified into two major classes according to their peptide expression. GABAergic enkephalinergic neurons express dopamine and adenosine receptors predominantly of the D_2 and A_{2A} subtype, respectively, while GABAergic dynorphinergic neurons predominantly express dopamine D_1 and adenosine A_1 receptors (for reviews, see Ferré *et al*, 1997; Gerfen, 2004).

Studies on the expression of immediate-early genes, such as *c-fos*, and of the expression of the peptide genes, such as *preproenkephalin* and *preprodynorphin* (precursors of enkephalin and dynorphin, respectively), have consistently shown a differential effect of dopamine in the two striatal GABAergic efferent neurons (Gerfen, 2004). This is due to the segregation of D_1 and D_2 receptors, whose activation stimulates and inhibits the function of GABAergic dynorphinergic and GABAergic enkephalinergic neurons, respectively (Gerfen, 2000). A_{2A} and D_2 receptors exert reciprocal antagonistic interactions that modulate the function of GABAergic enkephalinergic neurons (Ferré *et al*, 1993, 1997, 2003; Agnati *et al*, 2003) and recent studies have demonstrated the ability of A_{2A} and D_2 receptors to heteromerize (Canals *et al*, 2003; Kamiya *et al*, 2003; Ciruela *et al*, 2004; Woods and Ferré, 2005). A_{2A} and D_2 receptors are prototypical G_s- and G_i-coupled receptors, respectively, which play opposite effects on adenylyl-cyclase (Kull *et al*, 1999; Hillion *et al*, 2002; Lee *et al*, 2002). Stimulation of A_{2A} receptors can potentially stimulate striatal adenylyl-cyclase, with the consequent activation of the cAMP-PKA signaling pathway and the induction of the expression of different genes, such as *c-fos* and *preproenkephalin* (Agnati *et al*, 2003; Ferré *et al*, 2003, 2004). However, under normal conditions, the ability of A_{2A} receptors to activate the cAMP-PKA signaling pathway is restrained by the tonic inhibitory effect of D_2 receptors, which potently inhibit A_{2A}-receptor mediated adenylyl-cyclase activation (Agnati *et al*, 2003; Ferré *et al*, 2003, 2004). We have shown that activation of adenosine A_1 receptors allows A_{2A} receptors to overcome the inhibitory effect of D_2 receptors and induce striatal expression of *c-fos* (Karcz-Kubicha *et al*, 2003). However, it remained to be demonstrated if this increase in *c-fos* expression takes place in GABAergic enkephalinergic neurons.

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In the present work, we used a combination of immunohistochemistry, to detect c-Fos (a cellular marker for neuronal activation), and *in situ* hybridization to determine the phenotype (enkephalinergic or dynorphinergic) of striatal neurons activated by co-administration of A_1 and A_{2A} receptor agonists.

METHODS

Subjects and Drugs

Male Sprague–Dawley rats (Charles River Laboratory, Wilmington, MA, USA), weighing 300–350 g were used in all experiments. Animals were maintained in accordance with guidelines of the Institutional Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse, NIH. The A_1 receptor agonist N^6-cyclopentyladenosine (CPA) and the adenosine A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) were purchased from Sigma Chemical Company (St Louis, MO, USA). Both drugs were dissolved in sterile saline and administered intraperitoneally (i.p.) in a volume of 2 ml/kg of body weight.

In Situ Hybridization and Immunocytochemistry

At 2 h following the administration of drugs, rats were anesthetized with Equithesin (NIDA Pharmacy, Baltimore, MD, USA) and perfused transcardially with a solution of 4% (W/V) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. The brains were postfixed for 2 h, immersed in 20% sucrose in PB overnight and frozen in powdered dry ice. Sections were cut at 30 μm thickness and stored frozen in cryoprotectant (Morales *et al*, 1998). Phenotypic characterization of enkephalinergic and dynorphinergic neurons was carried out by *in situ* hybridization as previously described (Backman *et al*, 2001). Sections were washed three times in PB, incubated for 10 min in PB containing 0.5% Triton X-100, rinsed 2 × 5 min with PB, treated with 0.2 N HCl for 10 min, rinsed 2 × 5 min with PB, and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min. Sections were rinsed 2 × 5 min with PB, postfixed with 4% paraformaldehyde for 10 min, and after a final rinse with PB were hybridized at 55°C for 16 h in hybridization buffer (50% formamide; 10% dextran sulfate; 5 × Denhardt's solution; 0.62 M NaCl; 50 mM DTT; 10 mM EDTA; 20 mM PIPES, pH 6.8; 0.2% SDS; 250 μg/ml ssDNA; 250 μg/ml tRNA) containing 10^7 cpm/ml of each [^{35}S]- and [^{33}P]*preproenkephalin* or *preprodynorphin* riboprobes (original plasmids were kindly provided by Dr Sabol, NIH). A 422 bp cDNA fragment (nucleotides 314–736, Accession # NM 001002927) was transcribed with T3 RNA polymerase to generate a radioactive antisense *preproenkephalin* riboprobe. A 1.4 kbp fragment (nucleotides 2700–1300, Accession # NM-018863) was transcribed with SP6 to generate a radioactive antisense *preprodynorphin* riboprobe. At 1 μg of linearized cDNA template was transcribed with their respective RNA polymerase (20 U, Roche, Indianapolis, IN) in 1 × transcription buffer containing 10 mM dithiothreitol (DTT), 40 U ribonuclease inhibitor with 100 μCi ^{35}S-labeled UTP, or 125 μCi ^{33}P-labeled UTP (^{35}S-labeled specific activity 1250 Ci/mM; ^{33}P-labeled specific activity 3000 Ci/mM, Perkin-Elmer, Boston, MA), 1.5 mmol each of rATP, rCTP, and rGTP in a volume of 20 μl. Transcription reactions were carried out at 37°C for 120 min with the addition of 20 U of RNA polymerase at 60 min. Complementary RNA probes were purified by gel filtration column chromatography (Roche, Indianapolis, IN).

Sections were rinsed in 2 × SSC before incubation with RNase A at 4 μg/ml at 37°C for 1 h, washed with 1 × SSC, 50% formamide at 55°C for 1 h, and with 0.1 × SSC at 68°C for 1 h. After this last SSC wash, sections were rinsed with PB buffer and then incubated in 4% bovine serum albumin supplemented with 0.3% Triton-X-100 in PB for 1 h followed by incubation with a polyclonal anti c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution for 24 h at 4°C. Sections were processed with an ABC kit (Vector) and peroxidase activity was revealed with 0.003% H_2O_2 and 0.05% 3,3-diaminobenzidine-4 HCl. Brain sections were mounted on coated slides, dehydrated in ethanol, treated with Histoclear, rinsed in 100% ethanol and exposed to BAS-MS 2025 imaging plates (Fujifilm, Stamford, CT) for multiple exposure times to determine the linear range of detection and produce clear images using a BAS-5000 imaging plate scanner (Fujifilm, Stamford, CT). Thereafter, sections were dipped in nuclear track emulsion and exposed for several weeks prior to development. Two sets of experiments were performed and analyzed independently. The first set included a group treated with saline (control) and a group treated with CGS 21680 (0.5 mg/kg, i.p.) plus CPA (0.3 mg/kg, i.p.). The second set included three groups, treated with either saline (control), CGS 21680 (0.5 mg/kg, i.p.) or CPA (0.3 mg/kg, i.p.).

Image Analysis

Two types of analysis, at two different levels of magnification, were performed. At the lower magnification level, the total number of c-Fos positive nuclei and the averaged hybridization signal were measured from the areas of the Nucleus Accumbens (NAc) and Caudate-Putamen (CPu) shown in Figure 1. For quantification of c-Fos immuno-

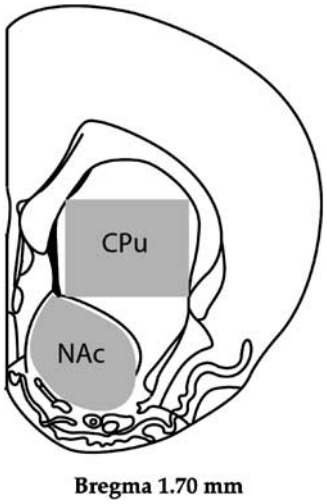

Figure 1 Scheme of rat brain section showing the level of the brain areas analyzed for c-Fos immunohistochemistry and *preproenkephalin* or *preprodynorphin* mRNA *in situ* hybridization. CPu, caudate-putamen; NAc, nucleus accumbens.

reactivity, positive nuclei were counted by using IPLab (version 3.5) software (Scanalytics, Billerica, MA). Phosphoimages were quantified using Image Gauge software (v3.46, Fujifilm, Stamford, CT). Hybridization signal was measured bilaterally for each rat by tracing 4–6 sections and calculating an averaged photo stimulated luminescence (PSL) over the 4–6 sections for each animal group. At the higher magnification level, the number of cell profiles per field ($200 \times 200\,\mu m$) containing *preproenkephalin* or *preprodynorphin* mRNA and c-Fos immunoreactivity was calculated. A neuron was considered double labeled when its nucleus was brown and contained an aggregation of silver particles clearly above the immediately surrounding background. Background was evaluated from slides hybridized with sense probes. Double-labeled cells were counted in a total of 2–3 nonoverlapping fields per four nonadjacent sections per rat (with a separation of at least $100\,\mu m$ between the sections, to avoid repeated counting of the same cells). Material was analyzed and photographed under bright field (for c-Fos immunoreactivity) or epiluminescence (for mRNA signal) microscopy using a Nikon Eclipse E 800 microscope with $20\times$ objective lenses. Images were opened and processed with a Photoshop 5.5 program (Adobe, Seattle, WA). Figure 1 shows a schematic drawing of rat brain section at the level of brain areas analyzed for c-Fos immunohistochemistry and *preproenkephalin* or *preprodynorphin* mRNA *in situ* hybridization. Non-paired Student's *t*-test was used for statistical analysis.

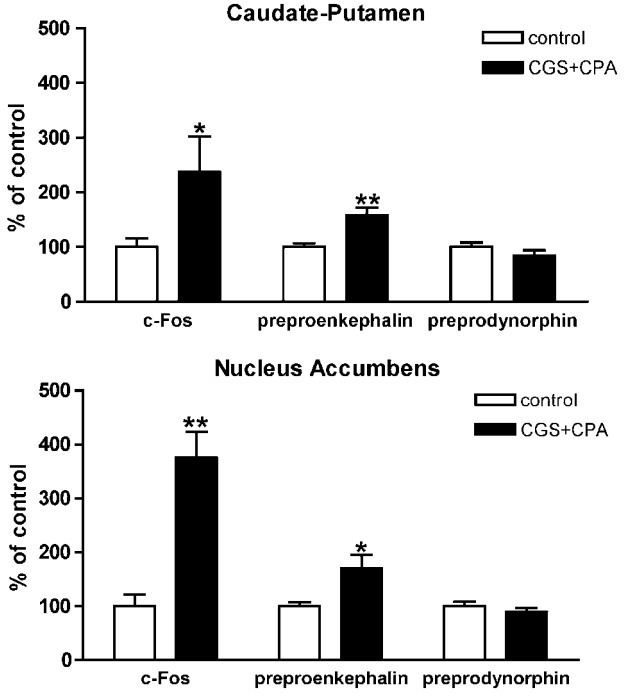

Figure 2 Quantification of C-Fos positive nuclei and the expression of *preproenkephalin* and *preprodynorphin* mRNA in the caudate-putamen and nucleus accumbens after administration of saline (control) or the A_{2A} receptor agonist CGS 21680 (0.5 mg/kg i.p.) plus the A_1 receptor agonist CPA (0.3 mg/kg i.p.) (CGS + CPA). The results are expressed as means + SEM ($n = 5$–6/group) of the percentage values from control animals. * and **: significantly different compared with controls (nonpaired Student's *t*-test: $p < 0.05$ and < 0.01, respectively).

RESULTS

Co-Administration of A_1 and A_{2A} Receptor Agonists Induces Striatal *c-fos* and *Preproenkephalin* Expression

As previously reported (Karcz-Kubicha *et al*, 2003), co-administration of CGS 21680 (0.5 mg/kg i.p.) and CPA (0.3 mg/kg i.p.) induced a significant increase in the number of c-Fos positive nuclei both in the NAc (above threefold increase) and the CPu (above twofold increase), compared to control, saline-treated animals (non-paired Student's '*t*-test: $p < 0.01$ and < 0.05, respectively; $n = 6$/group; Figure 2). The number of c-Fos positive nuclei in the NAc and CPu of the saline-treated animals was (in means $\pm$ SEM) 91 ± 7 and 76 ± 5, respectively. Furthermore, co-administration of CGS 21680 and CPA induced a 50% increase of *preproenkephalin* mRNA expression in the NAc and CPu (non-paired Student's '*t*'-test: $p < 0.05$ and < 0.01, respectively; $n = 6$/group; Figures 2 and 3). On the other hand, no significant differences of striatal *preprodynorphin* mRNA expression were observed between animals treated with CGS 21680 plus CPA and controls (Figures 2 and 3). Administration of either CGS 21680 (0.5 mg/kg i.p.) or CPA (0.3 mg/kg i.p.) did not induce any significant change in the striatal expression of *c-fos*, *preproenkephalin* or *preprodynorphin* (Figure 4).

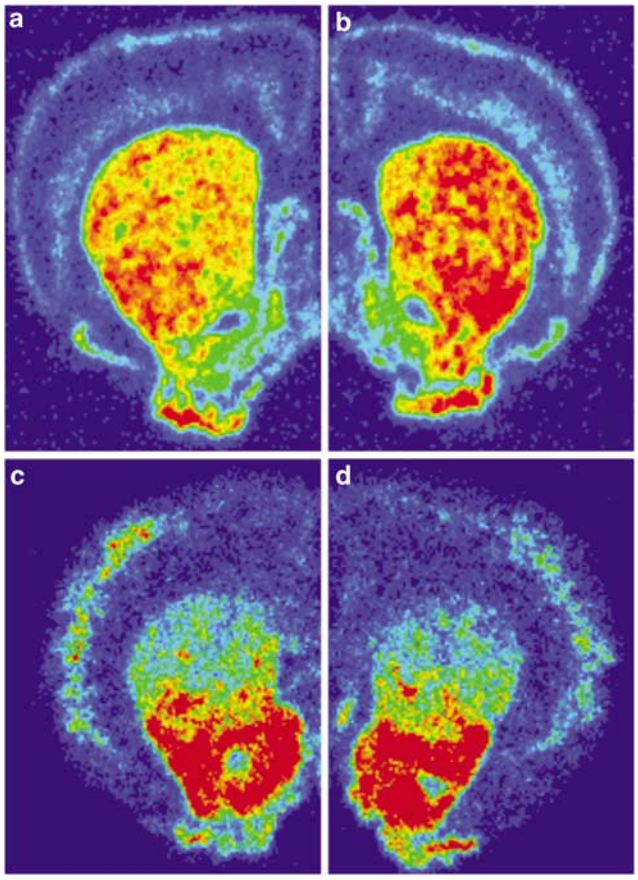

Figure 3 Expression of *preproenkephalin* (a, b) and *preprodynorphin* (c, d) mRNA in striatum after administration of saline (a, c) or the A_{2A} receptor agonist CGS 21680 (0.5 mg/kg i.p.) plus the A_1 receptor agonist CPA (0.3 mg/kg i.p.) (b, d). The intensity of the *in situ* hybridization signal increases with the following color sequence: blue–green–yellow–red.

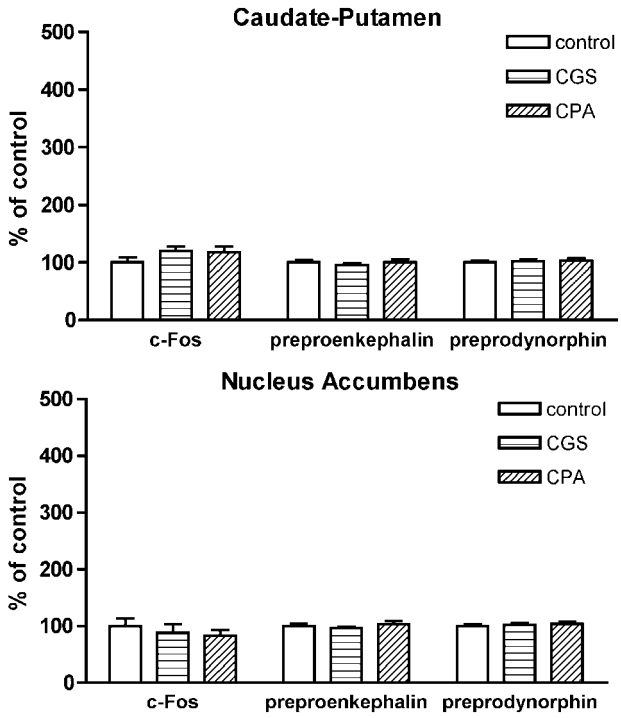

Figure 4 Quantification of c-Fos positive nuclei and the expression of *preproenkephalin* and *preprodynorphin* mRNA in the Caudate-Putamen (CPu) and Nucleus Accumbens (NAc) after administration of saline (control), the A_{2A} receptor agonist CGS 21680 (0.5 mg/kg i.p.) or the A_1 receptor agonist CPA (0.3 mg/kg i.p.). The results are expressed as means + SEM ($n = 5$–6/group) of the percentage values from control animals.

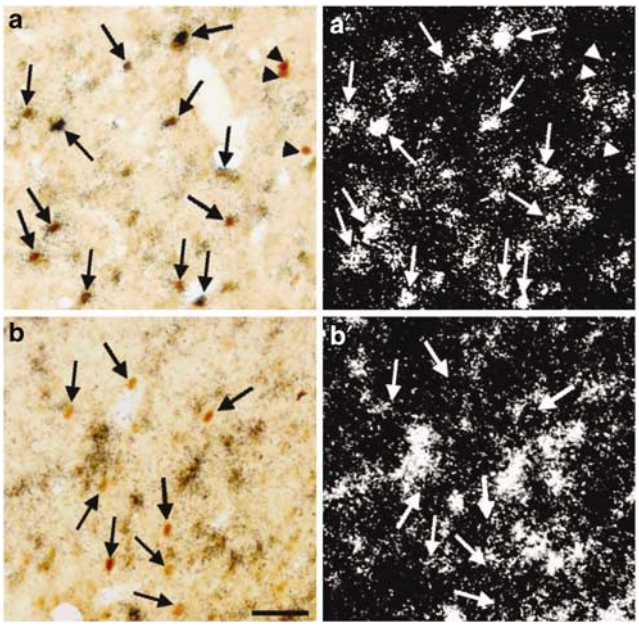

Figure 5 Simultaneous detection of c-Fos immunoreactivity (a, b) and mRNA encoding preproenkephalin (a′) or preprodynorphin (b′) in NAc after treatment with the A_{2A} receptor agonist CGS 21680 (0.5 mg/kg i.p.) plus the A_1 receptor agonist CPA (0.3 mg/kg i.p.). Bright field (a, b) and epiluminescence (a′, b′) microscopy. (a, a′) Pairs of micrographs showing c-Fos immunoreactivity (a) and *preproenkephalin* mRNA (a′); arrows indicate examples of neurons containing c-Fos and *preproenkephalin* mRNA and arrow-heads indicate c-Fos immunoreactive neurons lacking *preproenkephalin* mRNA. (b, b′) Pairs of micrographs showing c-Fos immunoreactivity (b) and *preprodynorphin* mRNA (b′); arrows indicate examples of neurons containing c-Fos immunoreactivity but lacking *preprodynorphin* mRNA. Scale bar = 40 μm.

Co-Administration of A_1 and A_{2A} Receptor Agonists Induces *c-fos* Expression in Striatal Enkephalinergic Neurons

The NAc was chosen for the co-localization analysis since the present and previous studies (Karcz-Kubicha *et al*, 2003) showed that this is the striatal region with the largest increase in *c-fos* expression after co-administration of adenosine A_{2A} and A_1 receptor agonists. The simultaneous detection of c-Fos immunoreactivity and *preproenkephalin* or *preprodynorphin* transcripts in the NAc showed a significant increase in the number of c-Fos immunoreactive cells that co-express *preproenkephalin* in animals treated with CGS 21680 (0.5 mg/kg) plus CPA (0.3 mg/kg) when compared with control saline-treated animals (non-paired Student's *t*-test: $p < 0.001$; $n = 70$ and 32 fields for the groups treated with CGS 21680 plus CPA and saline, respectively). The same analysis showed no significant differences between the animals treated with CGS 21680 plus CPA and controls in the number of c-Fos immunoreactive cells co-expressing *preprodynorphin* or those lacking *preproenkephalin* and *preprodynorphin* expression (Figures 5 and 6).

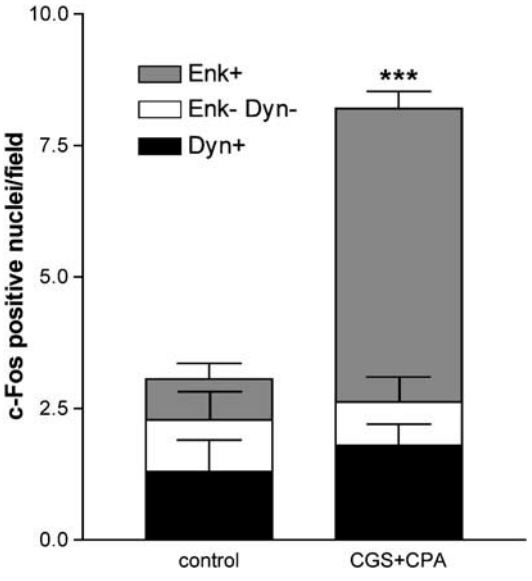

Figure 6 Quantification of c-Fos immunoreactivity from the nucleus accumbens of rats treated with the A_{2A} receptor agonist CGS 21680 (0.5 mg/kg i.p.) plus the A_1 receptor agonist CPA (0.3 mg/kg i.p.) (CGS + CPA) or with saline (control). The results are expressed as means + SEM ($n = 6$/group) of the number of c-Fos positive nuclei per field. Enk + and Dyn +: c-Fos positive cells expressing *preproenkephalin* and *preprodynorphin*, respectively; Enk- Dyn-: c-Fos positive cells expressing neither transcript. ***: significant increase in Enk + with CGS + CPA treatment compared to control (nonpaired Student's *t*-test: $p < 0.001$).

DISCUSSION

In a previous study it was shown that co-administration of the A_1 receptor agonist CPA and the A_{2A} receptor agonist CGS 21680 increases the expression of the immediate-early

gene *c-fos* in the striatum (Karcz-Kubicha *et al*, 2003). In the present study, we demonstrate that this increase is selective for the GABAergic enkephalinergic neurons. Furthermore, A_1–A_{2A} receptor co-stimulation induced an increase in the expression of the neuropeptide gene *preproenkephalin*, but not *preprodynorphin*. These were expected findings given the fact that GABAergic enkephalinergic neurons show the highest expression of A_{2A} receptors in the brain (Schiffmann *et al*, 1991; Hettinger *et al*, 2001).

Adenosine A_{2A} receptors are Gs-olf protein-coupled receptors whose main signaling pathway is adenylyl-cyclase activation, the cAMP-PKA cascade (Kull *et al*, 1999, 2000). Important PKA substrates include the dopamine and cyclic adenosine 3′,5′-monophosphate-regulated phophoprotein, 32 kDa (DARPP-32) and the nuclear constitutive transcription factor cAMP response element binding protein (CREB) (Greengard *et al*, 1999). DARPP-32 acts as an amplificatory mechanism of the cAMP-PKA cascade and it has been suggested to be essential for striatal A_{2A} receptor signaling (Svenningsson *et al*, 1998; Lindskog *et al*, 2002). The catalytic subunits of PKA can diffuse into the nucleus and induce cellular gene expression by phosphorylating CREB. The immediate-early gene *c-fos* and the *preproenkephalin* gene are very well-studied target genes the promoters of which contain consensus sites for pCREB binding (Borsook and Hyman, 1995; Herdegen and Leah, 1998). Thus, A_{2A} receptor stimulation can potentially activate the cAMP-PKA cascade and increase the expression of immediate-early genes and the *preproenkephalin* gene, which codes for the precursor of enkephalin.

A_{2A} and D_2 receptors form heteromeric complexes with reciprocal antagonistic interactions that regulate the function of the GABAergic enkephalinergic neurons (Ferré *et al*, 1991, 1993, 1997, 2003; Agnati *et al*, 2003; Canals *et al*, 2003; Kamiya *et al*, 2003; Ciruela *et al*, 2004; Woods and Ferré, 2005). Stimulation of A_{2A} receptors decreases the affinity of D_2 receptors for agonists by means of an intramembrane interaction (Ferré *et al*, 1991), while stimulation of D_2 receptors inhibits A_{2A} receptor-induced activation of adenylyl-cyclase (Kull *et al*, 1999; Hillion *et al*, 2002). By means of the strong antagonistic D_2–A_{2A} receptor interaction at the adenylyl cyclase level, and due to the existence of a tonic effect of dopamine on D_2 receptors, under normal conditions A_{2A} receptors are practically unable to activate the cAMP-PKA signaling pathway (Agnati *et al*, 2003; Ferré *et al*, 2003, 2004). In fact, the systemic administration of A_{2A} receptor antagonists produces either no effect or a modest decrease in the striatal expression of *c-fos* or *preproenkephalin* (Le Moine *et al*, 1997; Pinna *et al*, 1997; Aoyama *et al*, 2002; Carta *et al*, 2002; Karcz-Kubicha *et al*, 2003; Wardas *et al*, 2003). It must also be pointed out that some studies suggest that A_{2A} and D_2 receptor can interact synergistically, with stimulation of D_2 receptors potentiating the effects of A_{2A} receptor stimulation. These conditions seem to depend on the isoform of adenylyl cyclase involved or on the interruption of a previous long-term exposure to D_2 receptor agonists (Yao *et al*, 2002; Kudlacek *et al*, 2003; Vortherms and Watts, 2004). In any case, the main isoform of adenylyl cyclase in the striatum is AC5, and co-stimulation of G_s- and G_i-coupled receptors shows antagonistic interactions at the AC5 level (Chern, 2000; Defer *et al*, 2000). In fact, D_2 receptor stimulation has been shown to counteract cAMP accumulation induced by A_{2A} receptor stimulation in membrane preparations from mouse striatum (Lee *et al*, 2002).

The effects on the striatal expression of *c-fos* and *preproenkephalin* observed in the present study depend on the concomitant administration of A_1 and A_{2A} agonists, since neither agonist produced any effect when administered alone. Nevertheless, in our previous study, the A_1 receptor agonist CPA alone induced a significant increase in *c-fos* expression in the medial part of the NAc (Karcz-Kubicha *et al*, 2003). The simultaneous analysis of the medial and lateral parts of the NAc might have diluted such a local effect of CPA. We have previously suggested that the inability of the A_{2A} receptor agonist CGS 21680 to induce an increase in the striatal expression of *c-fos* depends on its ability to produce striatal dopamine release (Karcz-Kubicha *et al*, 2003), which further stimulates D_2 receptors and, therefore, antagonizes the effects of A_{2A} receptor stimulation by the exogenous agonist. CPA administration was shown to decrease the striatal extracellular concentration of dopamine and to counteract CGS 21680-induced dopamine release (Karcz-Kubicha *et al*, 2003). Therefore, this local-circuit mechanism can explain why activation of adenosine A_1 receptors allows A_{2A} receptors to overcome the inhibitory effect of D_2 receptors and induce striatal expression of *c-fos* and *preproenkephalin*. We have recently studied the mechanisms involved in the A_{2A} receptor-mediated striatal dopamine release and suggested that it depends on glutamate neurotransmission (facilitation of glutamate release by the stimulation of A_{2A} receptor localized in striatal glutamatergic terminals) and NMDA receptor stimulation (facilitation of dopamine release by spillover of glutamate and stimulation of NMDA receptors localized in dopaminergic terminals) (Quarta *et al*, 2004). Concomitant administration of an A_1 receptor agonist counteracts CGS 21680-induced dopamine release (Karcz-Kubicha *et al*, 2003) most probably by indirect inhibitory effects on glutamate release (Quarta *et al*, 2004) and direct inhibitory effects on dopamine release (Borycz *et al*, in preparation). Whatever the mechanisms involved, concomitant A_1 receptor stimulation, which is actually what occurs with endogenous adenosine release, enables A_{2A} receptor stimulation to produce a significant increase in the striatal expression of *c-fos* and *preproenkephalin*.

The present results underscore the role of adenosine in the activation of gene expression in the GABAergic enkephalinergic neuron. Previous studies showed evidence for an opposite modulatory role of adenosine in the other major striatal neuronal element, the GABAergic dynorphinergic neuron, where activation of A_1 receptors attenuates dopamine D_1 receptor-mediated release of GABA and increase in the striatal expression of the immediate-early genes *NGFI-A*, *c-fos* and *jun-B* (Ferré *et al*, 1996, 1999). Therefore, under physiological conditions of increased adenosine release, a preferential activation of GABAergic enkephalinergic *vs* GABAergic dynorphinergic neurons must take place. Since this activation has consequences at the gene level, adenosine could selectively facilitate plastic changes in the synapses of the GABAergic enkephalinergic neurons, which can have implications for drug therapy in neuropsychiatric disorders and drug abuse. For instance, there is experimental evidence for the existence of selective

gene expression in GABAergic enkephalinergic neurons during sensitization to the behavioral effects of psychostimulants (Uslaner *et al*, 2001). In fact, genetic inactivation of A_{2A} receptors has been reported to attenuate amphetamine-induced behavioral sensitization (Chen *et al*, 2003).

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