

Differential glutamate-dependent and glutamate-independent adenosine A₁ receptor-mediated modulation of dopamine release in different striatal compartments

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Abstract

Adenosine and dopamine are two important modulators of glutamatergic neurotransmission in the striatum. However, conflicting reports exist about the role of adenosine and adenosine receptors in the modulation of striatal dopamine release. It has been previously suggested that adenosine A₁ receptors localized in glutamatergic nerve terminals indirectly modulate dopamine release, by their ability to modulate glutamate release. In the present study, using *in vivo* microdialysis, we provide evidence for the existence of a significant glutamate-independent tonic modulation of dopamine release in most of the analyzed striatal compartments. In the dorsal, but not in the ventral, part of the shell of the nucleus accumbens (NAc), blockade of A₁ receptors by local

perfusion with the selective A₁ receptor antagonist 8-cyclopentyl-1,3-dimethyl-xanthine or by systemic administration of the non-selective adenosine antagonist caffeine induced a glutamate-dependent release of dopamine. On the contrary, A₁ receptor blockade induced a glutamate-independent dopamine release in the core of the NAc and the nucleus caudate–putamen. Furthermore, using immunocytochemical and functional studies in rat striatal synaptosomes, we demonstrate that a fraction of striatal dopaminergic terminals contains adenosine A₁ receptors, which directly inhibit dopamine release independently of glutamatergic transmission.

Keywords: adenosine A₁ receptor, caffeine, dopamine, glutamate, rat, striatum.

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Adenosine plays a key modulatory role in the control of motor function, mainly by acting on striatal circuits. Striatal circuits are triggered by cortico-limbic-thalamic glutamatergic inputs and the flow of information is under tight mesencephalic dopaminergic control (Gerfen 2004). Both striatal dopamine and glutamate inputs are under the inhibitory control of adenosine acting through adenosine A₁ receptors. In fact, endogenous adenosine exerts a tonic A₁ receptor-mediated inhibition of glutamate and dopamine release in the ventral striatum, particularly in the shell of the nucleus accumbens (NAc) (Solinas *et al.* 2002; Quarta *et al.* 2004a,b). Blockade of this tonic A₁ receptor-mediated inhibition is involved in the increase in extracellular levels of dopamine and glutamate in the NAc after the systemic administration of caffeine (Solinas *et al.* 2002; Quarta *et al.* 2004a,b).

We have recently shown that the majority of glutamatergic terminals in the striatum contain A₁ receptors (Ciruela *et al.* 2006), which are responsible for the A₁ receptor-mediated inhibition of striatal glutamatergic neurotransmission and

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Abbreviations used: ANOVA, analysis of variance; APV, DL-2-amino-5-phosphonovaleric acid; CPA, N⁶-cyclopentyladenosine; CPT, 8-cyclopentyl-1,3-dimethyl-xanthine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; HPLC, high-performance liquid chromatography; NAc, nucleus accumbens; PBS, phosphate-buffered saline.

glutamate release that has been demonstrated with *in vitro* and *in vivo* experiments (Malenka and Kocsis 1988; Lovinger and Coi 1995; Calabresi *et al.* 1997; Flaggmeyer *et al.* 1997; Manzoni *et al.* 1998; Solinas *et al.* 2002; Quarta *et al.* 2004a,b; Ciruela *et al.* 2006). However, the possible localization of A₁ receptors in dopaminergic terminals remains unclear (Wojcik and Neff 1983; Alexander and Reddington 1989; Moser *et al.* 1991). As A₁ receptors are located in glutamatergic terminals, it has been suggested that the ability of A₁ receptors to modulate dopamine release is secondary to their ability to decrease glutamate release and, thus, decrease the activation of ionotropic glutamate receptors localized in dopaminergic terminals (Gracy and Pickel 1996; Tarazi *et al.* 1998). In fact, dopamine release induced by the direct application of an A₁ receptor antagonist or caffeine in the NAc was counteracted by NMDA receptor blockade (Quarta *et al.* 2004b).

The A₁ receptors localized in the striatal glutamatergic terminals form heteromeric complexes with A_{2A} receptors. In the A₁–A_{2A} receptor heteromer, the A_{2A} receptor exerts a strong inhibitory modulation of A₁ receptor function and stimulation of A_{2A} receptors overrides the inhibitory effects of A₁ receptors and induces glutamate release (Ciruela *et al.* 2006). As A₁ receptors have a higher affinity for adenosine than A_{2A} receptors, the A₁–A_{2A} receptor heteromer provides a switch mechanism by which low and high concentrations of adenosine inhibit and stimulate glutamate release, respectively (Ciruela *et al.* 2006). In a previous study, we found that the dopamine release in the NAc induced by an A_{2A} receptor agonist could be counteracted by co-administration of an A₁ receptor agonist (Karcz-Kubicha *et al.* 2003). In view of the predominant effects of the A_{2A} receptor in the A₁–A_{2A} receptor heteromer of the striatal glutamatergic terminals, those results suggested the existence of a glutamate-independent modulation of dopamine release, possibly by A₁ receptors localized in striatal dopaminergic nerve terminals.

In the present study, using *in vivo* microdialysis, we provide additional evidence for the existence of a significant glutamate-independent tonic modulation of dopamine release in most of the analyzed striatal compartments and, using immunocytochemical and functional studies in rat striatal synaptosomes, we demonstrate that a fraction of striatal dopaminergic terminals contains adenosine A₁ receptors, which directly inhibit dopamine release independently of glutamatergic transmission.

Materials and methods

In vivo microdialysis

Male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA, USA), weighing 300–350 g were used. Animals were maintained in accordance with the guidelines of the Institutional

Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse, NIH. Concentric microdialysis probes with 2-mm long dialysis membranes were prepared as described previously (Pontieri *et al.* 1995). Animals were anesthetized with Equithesin (NIDA Pharmacy, Baltimore, MD, USA) and probes were implanted in six different striatal areas about 1.7 mm anterior to bregma and in the medial prefrontal cortex, about 2.2 mm anterior to bregma. Figure 1 shows the limits of the positions of the dialysis probes (superimposed rectangles), with overlapping of the positions for 'shell v' and 'shell d' and the positions 'core m' and 'cpu m'. The striatal areas included the ventral and dorsal portions of the shell of the NAc ('shell v' and 'shell d', respectively), lateral and medial cores of the NAc ('core l' and 'core m', respectively) and lateral and medial caudate–putamen ('cpu l' and 'cpu m', respectively). Lateral and ventral coordinates with respect to bregma were, respectively: 'shell v' = +0.9 and –8.4; 'shell d' = +0.9 and –7.4; 'core l' = +1.7 and –7.6; 'core m' = +1.2 and –7.6; 'cpu l' = +2.5 and –6.2; 'cpu m' = +1.2 and –6.0; cortex = +0.7 and –4.9. The experiments were performed on freely moving rats 24 h after the probe implantation. A Ringer solution (in mmol/L) of 147 NaCl, 4 KCl, and 2.2 CaCl₂ was pumped through the dialysis probe at a constant rate of 1 µL/min. After a washout period of 90 min, samples were collected at 20-min intervals and split into two fractions of 10 µL, to separately measure glutamate and dopamine contents. Additional experiments were performed to measure adenosine content. Each animal was used to study the effect of one treatment by local administration (perfusion by reverse dialysis) of the A₁ receptor antagonist 8-cyclopentyl-1,3-dimethyl-xanthine (CPT; Sigma, St Louis, MO, USA), with or without the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV; Tocris, Ellisville, MO, USA), or systemic administration of caffeine (Sigma). The concentration of CPT used (1 mmol/L) was previously shown to be selective for the A₁ receptor (Quarta *et al.* 2004b). In addition, the concentration of APV used (0.1 mmol/L) was previously found to be optimal for counteracting NMDA-induced striatal dopamine release (Quarta *et al.* 2004b). At the end of the experiment, rats were killed with an overdose of Equithesin and methylene blue was perfused through the probe. The brain was removed and placed in a 10% formaldehyde solution, and coronal sections were cut to verify the probe location. Dopamine content was measured by reverse high-performance liquid chromatography (HPLC) coupled to an electrochemical detector, as described in detail previously (Pontieri *et al.* 1995). Glutamate content was measured by HPLC coupled to a fluorimetric detector, as described before (Quarta *et al.* 2004a). Adenosine content was analyzed by HPLC coupled to a spectrofluorimetric detector, as described elsewhere (Melani *et al.* 1999). In the microdialysis experiments, 'n' corresponds to the number of animals per group. One value per animal (basal value previous to any drug administration) was used to study differences between striatal compartments on the basal extracellular levels of dopamine, glutamate and adenosine and statistical comparisons were made with one-way analysis of variance (ANOVA) followed by Newman–Keuls tests. In the experiments with different drug administrations (either intracerebral perfusion or systemic administration), the statistical analysis consisted of a multi-level analysis with maximum likelihood estimation with SAS software (SAS Institute, Cary, NC, USA) using Proc Mixed procedure (Singer 1998). This

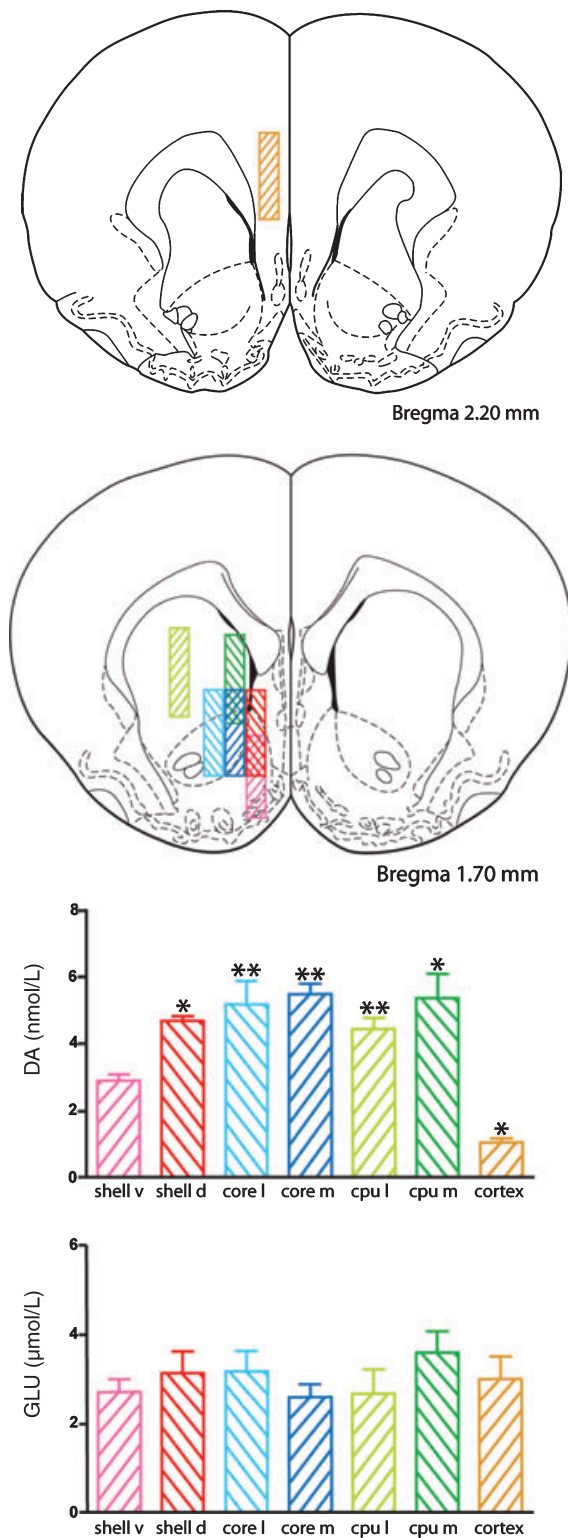


Fig. 1 Extracellular levels of dopamine and glutamate in the ventral and dorsal parts of the shell of the NAc ('shell v' and 'shell d', respectively) lateral and medial parts of the core of the NAc ('core l' and 'core m', respectively), lateral and medial parts of the nucleus caudate-putamen ('cpu m' and 'cpu l', respectively) and the medial prefrontal cortex. Data represent means \pm SEM ($n = 10$ – 16 /group); * and **: $p < 0.05$ and $p < 0.01$ compared with 'shell v', respectively (one-way ANOVA followed by Newman–Keuls tests).

Striatal synaptosomes

Male Wistar rats (6–8 weeks old, 140–160 g, obtained from Harlan Ibérica, Barcelona, Spain) were used and were handled according to EU guidelines for use of experimental animals, the rats being anesthetized under halothane atmosphere before being killed by decapitation. The synaptosomes were prepared by centrifugation of homogenized striatal tissue (dissected striatum corresponded mostly to the nucleus caudate-putamen) in sucrose medium (see Rodrigues *et al.* 2005). For immunochemical analysis (see Rodrigues *et al.* 2005), the striatal synaptosomes were placed onto coverslips previously coated with poly-L-lysine, fixed with 4% paraformaldehyde for 15 min and washed twice with phosphate-buffered saline (PBS). The synaptosomes were permeabilized in PBS with 0.2% Triton X-100 (Sigma) for 10 min and then blocked for 1 h in PBS with 3% bovine serum albumin and 5% normal rat serum. The synaptosomes were then washed twice with PBS and incubated with either rabbit anti-adenosine A₁ receptor (1 : 500; from Upstate Biotechnology, Golden, CO, USA) or rabbit anti-synaptophysin antibodies (1 : 200; from Zymed Laboratories, Lisbon, Portugal) together with either rat anti-dopamine transporter (1 : 500; from Chemicon, Southampton, UK) or mouse anti-tyrosine hydroxylase antibodies (1 : 500; from Chemicon) for 1 h at 20°C. The synaptosomes were then washed three times with PBS with 3% bovine serum albumin and incubated for 1 h at 20°C with AlexaFluor-488 (green)-labeled goat anti-rabbit and either AlexaFluor-594 (red)-labeled goat anti-rat or AlexaFluor-594 (red)-labeled goat anti-mouse antibodies (1 : 200 for all, from Molecular Probes, Leiden, The Netherlands). The selectivity of the A₁ receptor antibody was confirmed by the lack of signal obtained in synaptosomes derived from A₁ receptor knockout mice tissue (generously supplied by Bertil B. Fredholm, Karolinska Institutet, Sweden). We also confirmed that none of the secondary antibodies produced any signal in preparations to which the addition of the corresponding primary antibody was omitted. After washing and mounting on slides with Prolong Antifade, the preparations were visualized in a Zeiss Axiovert 200 inverted fluorescence microscope (Zeiss, Göttingen, Germany) equipped with a cooled CCD camera and analyzed with MetaFluor 5.0 software (Universal Imaging Co., Downingtown, PA, USA). Data represent means \pm SEM of three experiments and in each experiment, using different synaptosomal preparation from different animals, four different fields acquired from two different coverslips were analyzed. Each coverslip was analyzed by counting two different fields and in each field, a total amount of 500 individualized elements. For the release experiments (see Köfalvi *et al.* 2005), the synaptosomes were labeled with 5 μ Ci of [7,8-³H]-dopamine (41.0 Ci/mmol from Amersham, Buckinghamshire, UK) for 5 min at 37°C, layered over Whatman GF/C filters and superfused (flow rate: 0.7 mL/min) with Krebs solution for 20 min before starting collection of the superfusate every 2 min.

procedure has the flexibility to handle repeated-measures data sets in which some subjects were not tested under all conditions. The Tukey–Kramer procedure was used to conduct *post hoc* pairwise comparisons.

The synaptosomes were stimulated for 1 min with 20 mmol/L K^+ (isomolar substitution of NaCl by KCl in the Krebs solution) at 4 and 16 min (first and second stimulation periods, S_1 and S_2 , respectively) after starting sample collection, triggering a release of tritium in a Ca^{2+} -dependent manner that was mostly 3H -dopamine, gauged by HPLC (data not shown). The A_1 receptor agonist N^6 -cyclopentyladenosine (CPA; Sigma) was added 6 min before the start of S_2 and its effect was quantified by the modification of S_2/S_1 ratio versus control (i.e., absence of drugs), whereas the A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; Sigma) was added 15 min before starting sample collection and did not modify the S_2/S_1 ratio versus control. The concentrations of A_1 receptor agonists and antagonists used in synaptosomal experiments were previously shown to be selective for the A_1 receptor (Ciruela *et al.* 2006). Radioactivity was expressed in terms of fractional release, i.e., percentage of tritium released as a function of the total amount of tritium retained in each chamber. A paired Student's *t*-test was used to test the significance of the effect of a drug versus control with 'n' representing the number of experiments carried out using different animals, always in duplicate. When making comparisons from different sets of experiments with control, one-way ANOVA was used followed by Dunnett's test.

Results

Differential effects of adenosine A_1 receptor blockade on dopamine and glutamate release in different striatal compartments

Basal extracellular levels of dopamine were significantly lower (about 40%) in the ventral portion of the NAC compared with other striatal areas (one-way ANOVA followed by Newman–Keuls tests; see Fig. 1 for statistical significance). Extracellular dopamine levels were even lower in the medial prefrontal cortex (about 60% lower than that in the ventral shell of the NAC; see Fig. 1 for statistical significance). On the contrary, no significant differences were observed between the basal extracellular levels of glutamate in all the areas analyzed (Fig. 1). The extracellular levels of adenosine were also measured in three striatal areas, the dorsal and ventral portions of the shell and the lateral portion of the core of the NAC, and their values (in means \pm SEM: 28 ± 2 , 29 ± 3 and 28 ± 3 nmol/L, respectively; $n = 8$ –12) were not significantly different (one-way ANOVA followed by Newman–Keuls tests; $p = 0.8$).

Perfusion with the A_1 receptor antagonist CPT (1 mmol/L) by reverse dialysis produced different qualitative and quantitative effects in the different analyzed areas. In the dorsal portion of the shell of the NAC, but not in the ventral portion of the shell of the NAC, CPT significantly increased both dopamine and glutamate levels (with a maximum increase of about 200% vs. basal levels; SAS Proc Mixed analysis followed by Tukey–Kramer tests; see Figs 2 and 3 for statistical significance). Taking into account the overlapping positions of the microdialysis probes implanted in the shell of

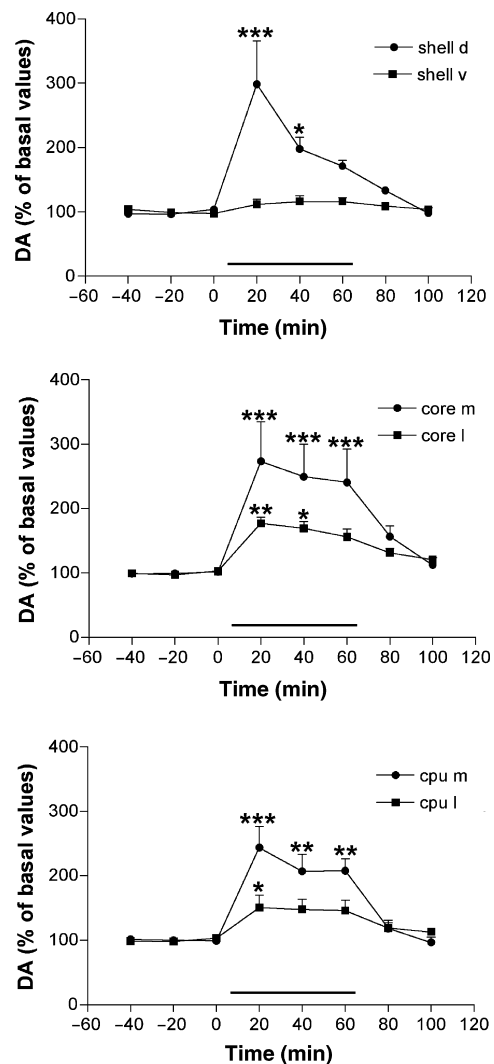


Fig. 2 Differential effect of the local perfusion with the A_1 receptor antagonist CPT on the extracellular levels of dopamine in different striatal compartments (see Fig. 1 legend for abbreviations). Horizontal bars show the period of CPT perfusion. Data represent means \pm SEM ($n = 5$ –8/group); *, ** and ***: $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared with the values previous to CPT perfusion, respectively (SAS Proc Mixed analysis followed by Tukey–Kramer tests).

the NAC (Fig. 1), these results indicate that only the most dorsal part of the shell of the NAC (which is excluded in the 'shell v' position) responded to the A_1 receptor antagonist. Perfusion of the core of the NAC and the nucleus caudate–putamen with CPT produced strikingly different results than that in the dorsal shell of the NAC. Levels of dopamine, but not of glutamate, were significantly increased in the core of the NAC and nucleus caudate–putamen (SAS Proc Mixed analysis followed by Tukey–Kramer tests; see Figs 2 and 3 for statistical significance). Increases in dopamine levels were significantly larger in the medial compared with the lateral portion of the core of the NAC (SAS Proc Mixed:

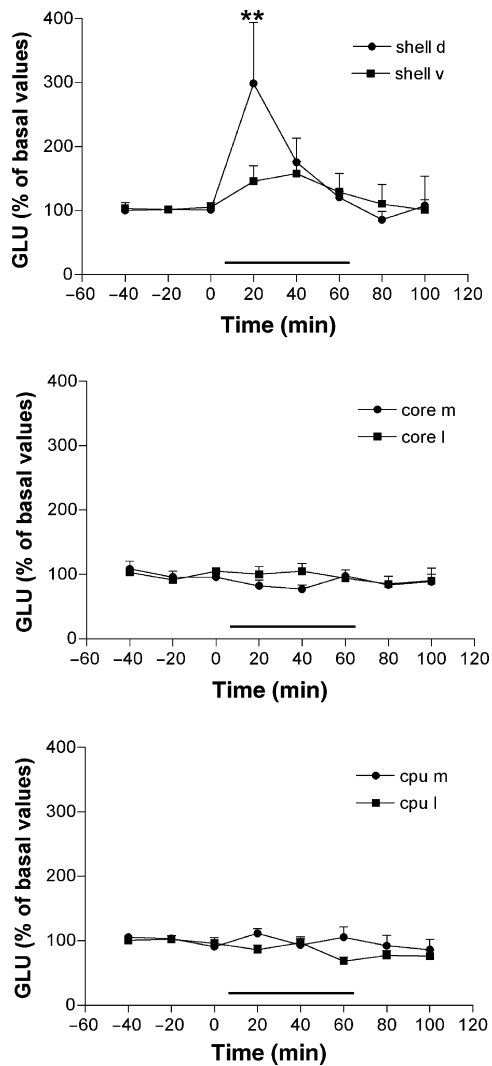


Fig. 3 Differential effect of the local perfusion with the A₁ receptor antagonist CPT on the extracellular levels of glutamate in different striatal compartments (see Fig. 1 legend for abbreviations). Horizontal bars show the period of CPT perfusion. Data represent means \pm SEM ($n = 5\text{--}8/\text{group}$); **: $p < 0.01$ compared with the values previous to CPT perfusion (SAS Proc Mixed analysis followed by Tukey–Kramer tests).

$p < 0.05$), with maximum increases of about 180% and 80%, respectively, versus basal levels. Similarly, increases in dopamine levels were significantly larger in the medial compared with the lateral portion of the nucleus caudate–putamen (SAS Proc Mixed: $p < 0.05$), with maximum increases of about 100% and 40%, respectively, versus basal levels. Intracortical perfusion with CPT did not significantly modify the extracellular concentrations of dopamine or glutamate in the medial prefrontal cortex (Fig. 4).

It was previously reported that dopamine release produced by CPT in the shell of the NAc was secondary to glutamate release and NMDA receptor stimulation (Quarta *et al.*

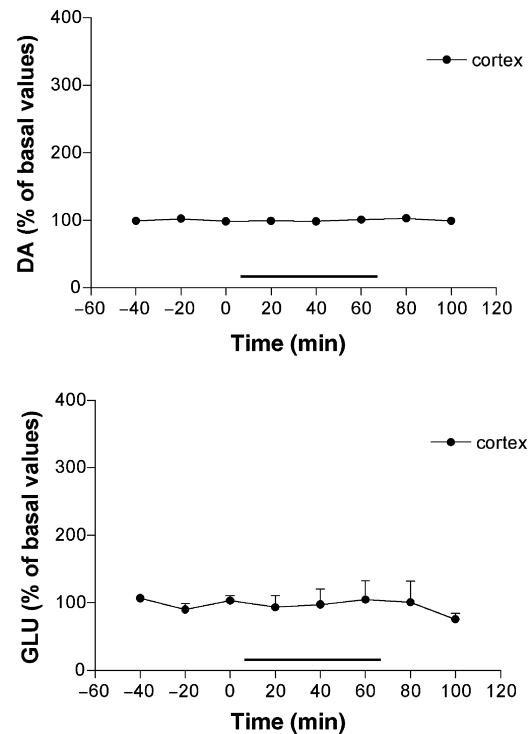


Fig. 4 Lack of effect of the local perfusion with the A₁ receptor antagonist CPT on the extracellular levels of dopamine and glutamate in the medial prefrontal cortex. Horizontal bars show the period of CPT perfusion. Data represent means \pm SEM ($n = 5\text{--}7/\text{group}$).

2004b). In the present study, co-perfusion with the competitive NMDA receptor antagonist APV (0.1 mmol/L) blocked the increase in dopamine release induced by CPT in the dorsal portion of the shell, but not in the medial portion of the core of the NAc (SAS Proc Mixed analysis followed by Tukey–Kramer tests; see Fig. 5 for statistical significance). Therefore, the present results not only confirm the existence of a glutamate-dependent A₁ receptor-mediated modulation of dopamine levels in the dorsal portion of the shell of the NAc, but also demonstrate the existence of a glutamate-independent modulation of dopamine levels by A₁ receptors in the core of the NAc and nucleus caudate–putamen.

It was also previously shown that the effect of local perfusion of CPT on dopamine and glutamate release in the shell of the NAc was mimicked by caffeine, but not by an A_{2A} receptor antagonist (Quarta *et al.* 2004b). Furthermore, systemic administration of either CPT or caffeine, but not an A_{2A} receptor antagonist, induced dopamine and glutamate release in the shell of the NAc (Solinas *et al.* 2002; Quarta *et al.* 2004a). In the present study, the systemic administration of caffeine (30 mg/kg, i.p.) significantly increased extracellular levels of dopamine and glutamate (with maximum increases of about 50% and 100%, respectively, vs. basal values), in the dorsal, but not the ventral shell of the NAc (SAS Proc Mixed analysis followed by Tukey–Kramer

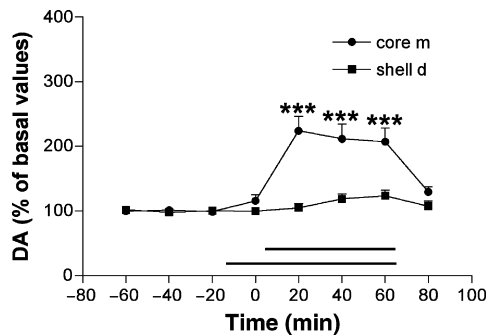


Fig. 5 Counteraction by the NMDA receptor antagonist APV on the effects of the A_1 receptor antagonist CPT on the extracellular levels of dopamine in the dorsal part of the shell ('shell d') but not in the medial part of the core ('core m') of the NAc. Horizontal bars show the period of APV and CPT perfusion (long and short bars, respectively). Data represent means \pm SEM ($n = 5$ –6/group); ***: $p < 0.001$ compared with the values previous to APV perfusion (SAS Proc Mixed analysis followed by Tukey–Kramer tests).

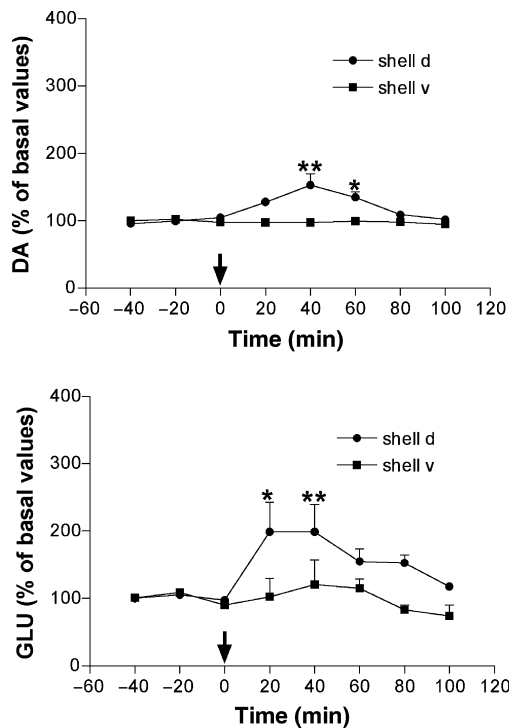


Fig. 6 Differential effect of the systemic administration of caffeine (30 mg/kg, i.p.) on the extracellular levels of dopamine and glutamate in the dorsal and ventral parts of the shell of the NAc. Arrows show the time of administration. Data represent means \pm SEM ($n = 5$ –6/group); * and **: $p < 0.05$ and $p < 0.01$ compared with the values previous to caffeine administration (SAS Proc Mixed analysis followed by Tukey–Kramer tests).

tests; see Fig. 6 for statistical significance). These results provide a possible explanation for previous contradictory findings about the ability of caffeine to increase dopamine

levels in the shell of the NAc (Acquas *et al.* 2002; Solinas *et al.* 2002).

Identification of A_1 receptors in striatal dopaminergic nerve terminals

The glutamate-independent effects of CPT on dopamine levels in the core of the NAc and the caudate–putamen observed in the microdialysis experiments support the hypothesis that there are functional adenosine A_1 receptors in dopaminergic terminals. Double immunocytochemical studies were carried out with striatal synaptosomes to confirm the presence of A_1 receptors in striatal dopaminergic nerve terminals (labeled with either tyrosine hydroxylase or dopamine transporter immunoreactivity). We first determined that only $25\% \pm 1\%$ and $22\% \pm 1\%$ of the striatal nerve terminals (immunopositive for synaptophysin) were labeled with either anti-tyrosine hydroxylase or anti-dopamine transporter antibodies, respectively ($n = 3$; data not shown). As shown in Fig. 7, we determined that 20–25% of the

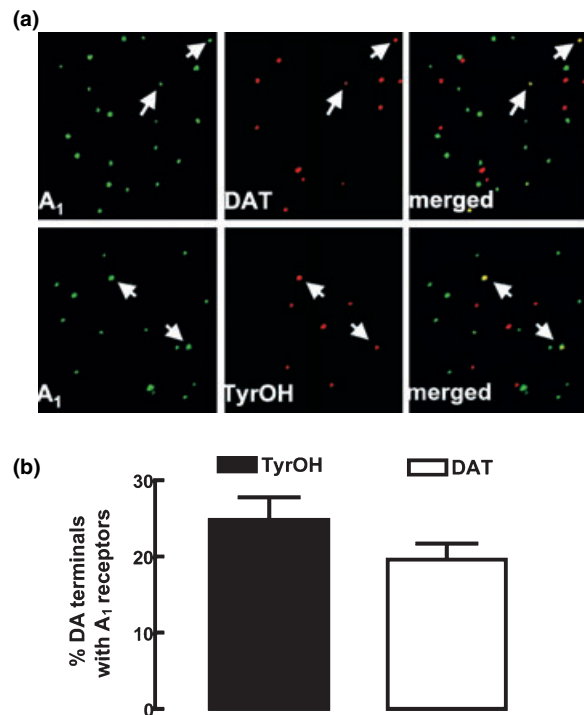


Fig. 7 Identification of the presence of A_1 receptors in a subset of dopaminergic nerve terminals by double immunocytochemical analysis of rat striatal single nerve terminals. (a) Immunocytochemical identification of A_1 receptors in dopaminergic terminals identified as immunopositive for either vesicular dopamine transporter (DAT, first row) or tyrosine hydroxylase (TyrOH, second row), which comprise 20–25% of the total synaptosomal population. The right panels show the co-localization of A_1 receptors with DAT or TyrOH in yellow (arrows). (b) Quantification of the percentage of dopaminergic nerve terminals endowed with A_1 receptors. Data represent means \pm SEM of three experiments and, each experiment obtained using different synaptosomal preparation from different animals.

putative dopaminergic nerve terminals contained A₁ receptor immunoreactivity (24.8% ± 2.9% of tyrosine hydroxylase-positive terminals endowed with A₁ receptors and 19.6% ± 2.1% of dopamine transporter-positive terminals endowed with A₁ receptors, $n = 3$).

A₁ receptor-mediated modulation of dopamine release in striatal dopaminergic nerve terminals

To demonstrate that the adenosine A₁ receptors localized in striatal dopaminergic terminals were functional, depolarization-induced dopamine release experiments were performed in preparations of striatal nerve terminals. In control conditions, striatal nerve terminals were superfused with 20 mmol/L K⁺ for 1 min, during two stimulation periods (S₁ and S₂) separated by 12 min. There was a release of ³H-dopamine with a constant S₂/S₁ ratio of 0.61 ± 0.01 (see open symbols of Fig. 8a). When the A₁ receptor agonist, CPA (100 nmol/L), was added 6 min before the second stimulation period (S₂), the amount of ³H-dopamine released during S₂ was systematically lower than control (compare the open symbols with the filled symbols, corresponding to the presence of 100 nmol/L CPA in Fig. 8a). In four similar experiments, it was found that 100 nmol/L CPA inhibited the stimulation-induced release of ³H-dopamine from striatal synaptosomes by 22% ± 5% ($p < 0.05$). Higher (300 nmol/L, $n = 4$), but not lower (30 nmol/L, $n = 5$), concentrations of CPA also significantly ($p < 0.05$) inhibited the stimulation-induced release of ³H-dopamine (Fig. 8b). In accordance with the involvement of A₁ receptors, the ability of CPA (100 nmol/L) to inhibit the release of ³H-dopamine from striatal synaptosomes was blocked by the selective A₁

receptor antagonist DPCPX (100 nmol/L, $n = 4$) (closed square in Fig. 8b).

Discussion

In the present study, we demonstrate that functional A₁ receptors exist in striatal dopaminergic nerve terminals using a combination of immunological and pharmacological techniques. Immunocytochemical analysis of striatal synaptosomes demonstrates that at least one fourth of striatal dopaminergic terminals contain adenosine A₁ receptors. Activation of these A₁ receptors directly inhibits depolarization-induced dopamine release. In previous studies, the non-selective adenosine agonist 2-chloroadenosine (van Galen *et al.* 1994) was also found to inhibit depolarization-induced dopamine release in striatal synaptosomes (Michaelis *et al.* 1979; Ebstein and Daly 1982). However, the adenosine receptor subtype involved in this modulation of dopamine release was not determined, as the non-selective adenosine receptor ligand used had an atypical pharmacological profile (Michaelis *et al.* 1979; Ebstein and Daly 1982). We were able to demonstrate the involvement of A₁ receptors in the present study using selective adenosine A₁ receptor ligands. The selective A₁ receptor antagonist DPCPX counteracted the inhibition of dopamine release induced by the selective A₁ receptor agonist CPA. In superfused synaptosomes, there is no possibility for bioactive molecules to accumulate in the biophase around terminals, because of their very small thickness and because of the efficient removal of substances by superfusion (e.g., Raiteri and Raiteri 2000). As a result, the effect of the A₁ receptor agonist can only be interpreted as an activation of A₁ receptors in dopaminergic terminals directly inhibiting the release of dopamine. Previous neurochemical studies using integral tissue preparations, such as brain slices (Jin *et al.* 1993) or *in vivo* microdialysis (Zetterström and Fillenz 1990; Ballarin *et al.* 1995; Okada *et al.* 1996), did not allow establishing this A₁ receptor-mediated pre-synaptic effect. Thus, the present studies provide the first clear demonstration that adenosine A₁ receptors are present in a fraction of dopaminergic terminals of the rat striatum, and that activation of these receptors directly inhibits the release of dopamine.

In a previous study using *in vivo* microdialysis techniques in awake, freely moving rats, we found that the selective A₁ receptor antagonist CPT produced a glutamate-dependent elevation in dopamine levels in the shell of the NAc (Quarta *et al.* 2004b). In the present study, we further demonstrate a differential adenosine A₁ receptor-mediated modulation of dopamine release in different compartments of the shell of the NAc. Blockade of A₁ receptors produced a glutamate-dependent elevation in dopamine levels in the dorsal part of the shell of the NAc, but there were no significant changes in dopamine or glutamate extracellular levels in the ventral part of the shell of the NAc. In addition, blockade of A₁ receptors

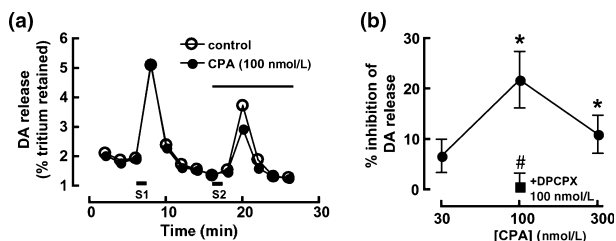


Fig. 8 Stimulation of A₁ receptors inhibits the evoked release of ³H-dopamine (DA) from rat striatal nerve terminals. (a) Super-imposed time course of ³H-DA release from superfused synaptosomes labeled with ³H-DA in control conditions (open symbols) and when the selective A₁ receptor agonist CPA (100 nmol/L) was added 6 min before the second stimulation period with 20 mmol/L K⁺, as indicated by the upper bar. The lower bars indicated the two periods of stimulation (S₁ and S₂) with 20 mmol/L K⁺. (b) Concentration-response curve for the ability of CPA to inhibit the evoked release of ³H-DA (filled circles) and the ability of the selective A₁ receptor antagonist DPCPX (100 nmol/L) (added from 15 min before starting sample collection onwards) to abolish the effect of CPA 100 nmol/L (filled square). Data represent means ± SEM ($n = 4-5$ /group); * and #: $p < 0.05$ compared with control or with 100 nmol/L CPA, respectively.

produced glutamate-independent dopamine release in the core of the NAc and the nucleus caudate–putamen. Again, there were differential effects, with significantly greater increases in dopamine in the medial portions of the core of the NAc and the nucleus caudate–putamen compared with the lateral portions. These regional differences in adenosine A₁ receptor-mediated modulation of striatal dopamine release fit with recent functional subdivisions of striatal compartments, which consider a more mediolateral instead of a dorsomedial functional gradient (Voorn *et al.* 2004).

The present study also sheds light on the mechanisms by which caffeine controls levels of dopamine in striatal structures (reviewed in Cauli and Morelli 2005). We previously reported that either systemic or local (reverse dialysis) administration of caffeine produces elevated dopamine levels in the shell of the NAc and that this is related to caffeine's actions as an adenosine A₁ receptor antagonist (Solinas *et al.* 2002; Quarta *et al.* 2004a,b). This finding could not be reproduced by another research group (Acquas *et al.* 2002). Di Chiara *et al.* (2004) subsequently suggested that the increases in extracellular levels of dopamine in the shell of the NAc after systemic administration of caffeine in our study might be related to sampling from the adjacent medial prefrontal cortex (infralimbic and prelimbic), where caffeine was reported to increase extracellular dopamine (Acquas *et al.* 2002). The present study does not support this hypothesis, as adenosine A₁ receptor blockade did not alter extracellular levels of dopamine or glutamate in the medial prefrontal cortex. Furthermore, as extracellular levels of dopamine in the prefrontal cortex were about five times lower than that in the adjacent (dorsal) part of the NAc, caffeine would have had to produce exceptionally large increases in dopamine levels in the medial prefrontal cortex (a fivefold increase vs. basal levels) to sufficiently contaminate samples from the probes implanted in the NAc shell and thus account for findings in our study. The present findings suggest, instead, that the precise localization of the microdialysis probe may account for these conflicting findings. Both the local perfusion with the adenosine A₁ receptor antagonist and the systemic administration of caffeine only produced dopamine and glutamate release in the present study when the area being sampled included the most dorsal portion of the shell of the NAc.

The present *in vivo* results showing a significant effect of the A₁ receptor antagonist on glutamate release in only one of the striatal areas analyzed (the dorsal part of the shell of the NAc) might seem at odds with the current knowledge of the important role of A₁ receptors in the modulation of striatal glutamate release (Lovinger and Coi 1995; Calabresi *et al.* 1997; Flagmeyer *et al.* 1997; Malenka and Kocsis, 1988; Manzoni *et al.* 1998), including our recent observation that most striatal glutamatergic terminals from striatal synaptosomal preparations contain A₁ receptors, which on stimulation inhibit glutamate release (Ciruela *et al.* 2006).

However, this would only indicate a weak tonic activation of A₁ receptors localized in the glutamatergic terminals of most striatal areas under basal conditions. In fact, most *in vitro* studies demonstrate a preferential effect of adenosine A₁ receptor agonists versus antagonists upon stimulated corticostriatal synaptic transmission (Lovinger and Coi 1995; Calabresi *et al.* 1997; Flagmeyer *et al.* 1997; Malenka and Kocsis, 1988; Manzoni *et al.* 1998).

The regional differences in the effects of A₁ receptor blockade on glutamate and dopamine release can be better explained by considering that different A₁ receptor-containing glutamatergic and dopaminergic synapses from different striatal regions are under different tonic adenosinergic control. We attempted to provide direct evidence for the presence of different levels of endogenous extracellular adenosine in different striatal compartments with *in vivo* microdialysis, but found no significant differences. As microdialysis only allows a direct estimate of global extracellular levels of adenosine outside synapses, this supports the hypothesis that the control of the release of neurotransmitters by endogenous adenosine is predominantly a synaptic event, depending on the levels of intrasynaptic adenosine, which cannot be estimated with currently available microdialysis probes. Recent electrophysiological experiments in slices from the NAc agree with this interpretation. The level of tonic inhibition by endogenous adenosine mediated by A₁ receptors was found to differ in different synapses, by analyzing glutamatergic excitatory and GABAergic inhibitory post-synaptic currents in the core and shell of the NAc (Brundege and Williams 2002).

In conclusion, the present results show a regional difference in the A₁ receptor-mediated control of glutamate and dopamine release in different limbic structures. This emphasizes the care required when drawing general conclusions on the role of a particular neuromodulatory system in a given brain area when only a limited set of synapses are studied or when global non-synaptic end points are evaluated.

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