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Reverse microdialysis of a dopamine D_2 receptor antagonist alters extracellular adenosine levels in the rat nucleus accumbens

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Abstract

Recent evidence suggests that modulation of dopaminergic transmission alters striatal levels of extracellular adenosine. The present study used reverse microdialysis of the selective dopamine D_2 receptor antagonist raclopride to investigate whether a blockade of dopamine D_2 receptors modifies extracellular adenosine concentrations in the nucleus accumbens. Results reveal that perfusion of raclopride produced an increase of dialysate adenosine which was significant with a high (10 mM) and intermediate (1 mM) drug concentration, but not with lower drug concentrations (10 and 100 μM). Thus, the present study demonstrates that a selective blockade of dopamine D_2 receptors in the nucleus accumbens produced a pronounced increase of extracellular adenosine. The cellular mechanisms underlying this effect are yet unknown. It is suggested that the increase of extracellular adenosine might be related to a homeostatic modulatory mechanism proposed to be a key function of adenosine in response to neuronal metabolic challenges.
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1. Introduction

The purine nucleoside adenosine is an important modulator of neuronal activity through actions on four distinct G-protein coupled cell-surface receptors termed as A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm et al., 1994; Ralevic and Burnstock, 1998). Neuromodulation by adenosine plays a crucial role in the nucleus accumbens (NAC), a subregion of the ventral striatum involved in motivation and reward (Robbins and Everitt, 1996; Kelley, 1999). In the NAC, A_{2A} receptors are expressed with high density (Ongini and Fredholm, 1996; Rosin et al., 1998) and have been implicated in selection and execution of adaptive behaviour (Ferre, 1997). For instance, stimulation of A_{2A} receptors in the NAC inhibited locomotor activity in rats (Barraco et al., 1994; Turgeon et al., 1996, Hauber and Munkle, 1997) and reversed reduced prepulse inhibition induced by apomorphine (Hauber and Koch, 1997). In turn, intra-NAC blockade of A_{2A} receptors enhanced locomotor activity and inhibited feeding behaviour (Nagel et al., 2003). A_{2A} receptors have a high affinity for adenosine (Fredholm, 1995) and intra-NAC stimulation of A_{2A} receptors with low doses

of selective agonists induced prominent behavioural effects (e.g. Hauber and Munkle, 1997). Thus, a precise regulation of extracellular levels of adenosine in the NAC is probably essential. Extracellular adenosine originates from degradation of vesicularly released purine nucleotides and from intracellular adenosine released by a transmembrane transport out of the cell (Dunwiddie and Masino, 2001; Latini and Pedata, 2001). A number of pharmacological stimuli have been identified which enhance extracellular adenosine, e.g. NMDA or AMPA receptor activation (Craig and White, 1993; Delaney and Geiger, 1998; Chen et al., 1992; Manzoni et al., 1994). However, it is still unknown whether dopamine receptors of the D_2 subtype are involved in regulation of extracellular adenosine levels. Adenosine plays a role opposite to dopamine in the NAC in control of behaviour (Ferre et al., 1997), as D_2 receptor antagonists and A_{2A} receptor agonists elicit similar behavioural effects, e.g. on locomotor activity (Hauber and Munkle, 1997). Recent evidence suggests that manipulation of dopaminergic neurotransmission altered actions of A_{2A} receptor ligands (Fenu and Morelli, 1998; Fenu et al., 2000) which might be due to changes in the level of extracellular adenosine. Furthermore, a recent microdialysis study revealed that dopamine denervation by 6-hydroxydopamine lesions of the medial forebrain bundle altered striatal levels of extracellular adenosine (Pinna et al., 2002). Taken together, these data suggest that changes of

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dopaminergic transmission might alter extracellular adenosine levels. Using reverse microdialysis of the selective dopamine receptor antagonist raclopride, the present study sought to determine whether a blockade of D_2 receptor modifies extracellular adenosine concentrations in the NAC.

2. Material and methods

All animal experiments were conducted according to the current version of the German Law on the Protection of Animals and approved by the proper authorities in Stuttgart, Germany.

2.1. Subjects

Subjects were 18 male Sprague–Dawley rats (220–280 g, Charles River, Sulzfeld, Germany). Up to five animals were housed in groups until surgery in standard Macrolon® type IV cages (55 cm × 35 cm × 10 cm; Ebeco, Castrop-Rauxel, Germany) in a temperature- and humidity-controlled animal room (20 ± 2 °C, 50 ± 10%). After surgery animals were housed individually in Macrolon® type III cages (37 cm × 21 cm × 30 cm; Ebeco, Castrop-Rauxel, Germany) with raised solid walled lids. A 12:12 h light–dark schedule was used with lights on between 6:00 and 18:00 h. Water and standard laboratory maintenance chow (Altromin, Lage, Germany) was available ad libitum.

2.2. Surgery

Using standard stereotaxic procedures, siliconized guide cannula (CMA/12; outer diameter: 0.9 mm) (CMA, Stockholm, Sweden) were implanted unilaterally in anaesthetised animals (50 mg/kg pentobarbital, i.p., Sigma, Deisenhofen, Germany, after pre-treatment with 0.5 mg/kg atropine sulphate i.p., Research Biochemicals Inc., Natick, USA) aiming the NAC (AP: +3,4 mm, L+ or −1,5 mm relative to bregma) with the incisor bar set 5 mm above the interaural line according to the atlas of Pellegrino et al. (1981). Each rat was given at least 4 days to recover from surgery before starting microdialysis experiments.

2.3. Microdialysis

Microdialysis experiments were performed in the home cage of an animal with the lid replaced by a metal frame bearing a counter-balanced arm with the swivel assembly. A microdialysis probe (CMA/12, 2 mm exposed membrane length, 0.5 mm membrane o.d.; CMA, Stockholm, Sweden) was lowered through the guide cannula to the NAC (ventral position of probe tip with reference to the skull: −8.0 mm) at 17:00 h the day before the experiment. Six hours before the insertion of the microdialysis probes food pellets were removed, water was available throughout the experiment. The probes were perfused with artificial cerebrospinal fluid

(aCSF) at a flow rate of 2 μl/min using a CMA 102 perfusion pump (CMA, Stockholm, Sweden). The in vitro recovery of the microdialysis set-up for adenosine was between 7 and 17%. The composition of the aCSF was 147 mM Na^+, 3 mM K^+, 1.2 mM Ca^{2+}, 1.0 mM Mg^{2+} (pH 6.6). Fourteen hours after probe insertion (7:00 h) three 20 min fractions were taken to obtain baseline values. Thereafter, a drug was applied via reverse microdialysis into the NAC for 20 min followed by 60 min with perfusion fluid alone. The samples (40 μl) were collected automatically with a fraction collector (CMA/142; CMA, Stockholm, Sweden) and stored deeply frozen (−70 °C) until analysis. The animals were connected by a head block tether system (Instech, Plymouth Meeting, USA) to a dual channel liquid swivel (Tsumura TCS2-23, Pronexus, Skärholmen, Sweden). FEP tubing (i.d. 0.12 mm, Microbiotech/se, Stockholm, Sweden) and tubing adapters (CMA, Stockholm, Sweden) were used.

2.4. Drug

Raclopride [$S(−)$3,5-dichloro-N-(1-ethyl-2-pyrrolidinyl-methyl)-2-hydroxy-6-methoxy-benz-amid (+) tartrate] (Astra Arcus, Soedertaelje, Sweden), a selective dopamine D_2 receptor antagonist (D_1 receptors: $K_i = 18000$ nM; D_2 receptors: $K_i = 3.4$; Seeman and Ulpian, 1988) was dissolved in four concentrations (10, 100 μM, and 1, 10 mM) in aCSF for application via reverse microdialysis into the NAC. Each animal received one concentration of the drug.

2.5. Chemical assays

All chemicals were obtained from Sigma (Deisenhofen, Germany) except differently stated. Adenosine was quantified by HPLC with fluorometric detection as a fluorescent agent 1,N_6-ethenoadenosine after derivatisation with chloroacetaldehyde (Secrist et al., 1972). Zinc acetate (5.3 μl; 0.01 mM) and chloroacetaldehyde (7.5 μl; 4.5%) were added to the microdialysis sample (40 μl). This solution was kept for incubation at 90 °C for 45 min.

Analysis was performed by using a reversed-phase ion-pair HPLC. An isocratic HPLC system (Kontron 520 pump, Kontron 565 autosampler, Biotek Kontron, Neufahrn, Germany) with Nucleosil 100-5-C18 column (5 μm particles, length × i.d.: 125 mm × 3 mm; Bischoff, Leonberg, Germany) with a column heater (Echotherm C030, Torrey Pines Sci., Santa Florencia, USA) set at 36 °C was employed. The mobile phase consisting of a 30 mM acetate buffer with 11% methanol and 1 mM octanesulphonic acid was adjusted to pH 3.6. The flow rate was 0.3 ml/min. Fluorescence was determined with a detector (RF-10 AXL, Shimadzu, Kyoto, Japan) with fixed excitation (270 nm) and emission (394 nm) wavelengths (Melani et al., 1999b). Ethenoadenosine peaks were identified and quantified by comparison with known adenosine standards which underwent the identical preparation procedure as samples. Furthermore, adenosine was identified by its disappearance

induced by addition of adenosine deaminase to the sample before derivatisation (incubation for 2 min at room temperature) (Wojcik and Neff, 1982). The detection limit of adenosine in a derivated sample was 12.7 fmol (3.4 pg).

2.6. Histology

After microdialysis, animals were killed by an overdose of pentobarbital. Then brains were removed from the skull, fixed for 2.5 h in 10% (v/v) formaldehyde and kept in 30% (w/v) sucrose for at least 2 days. The probe location was verified in frontal sections (60 μm) stained by Cresyl Violet. The locations of microdialysis probes are shown in Fig. 1.

2.7. Data analysis

Data were expressed as percent changes from 100% baseline, in which 100% represented the average concentration of three samples preceding drug perfusion. All results are presented as means + standard deviation (S.D.). Data were not normally distributed and analysed using a nonparametric one-way Friedman ANOVA for repeated measurements followed by Dunnett's test for multiple comparisons with the last baseline sample before presentation of a stimulus serv-
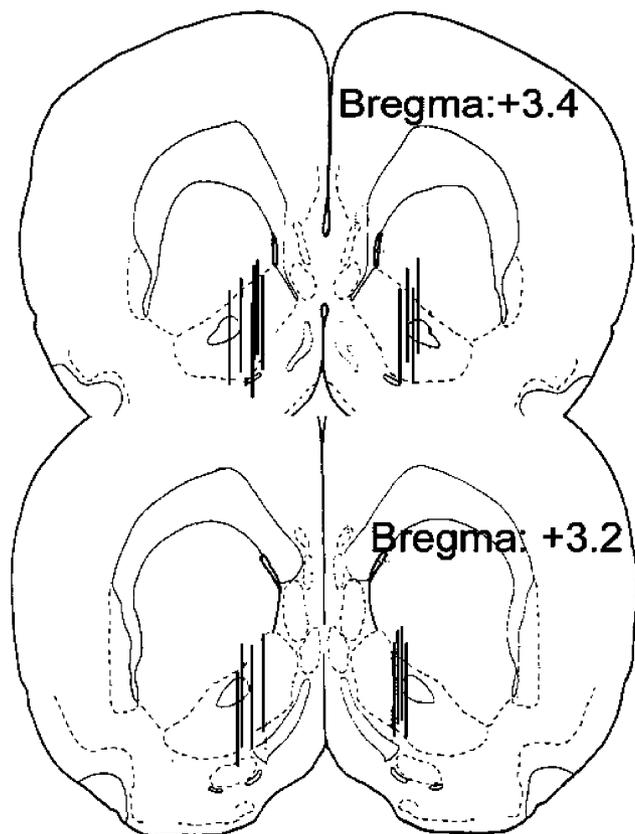

Fig. 1. Location of probes in the NAC reconstructed on the basis of coronal sections according to the atlas of Pellegrino et al. (1981). Only the dialyzing part of the probe is shown in representative planes. The distance from bregma is labelled in each section.

ing as control value. The level of statistical significance was set at $P < 0.05$. Statistical analysis was performed using SigmaStat Version 2.0 (Jandel, Erkrath, Germany).

3. Results

3.1. Basal adenosine levels

The mean basal dialysate concentration ($\pm$S.E.M.) of adenosine of the three samples before the onset of reverse dialysis of drugs were 1.288 ± 0.308 pg/μl (4.809 ± 0.861 nM) ($N = 15$). Data of animals ($N = 3$) with basal values below the detection limit were excluded from further analysis. The data were not corrected for in vitro-recovery.

3.2. Reverse microdialysis of raclopride

Intra-NAc administration of raclopride by reverse microdialysis (10, 100 μM, and 1, 10 mM) produced a dose-dependent increase of dialysate adenosine (Fig. 2). The increase of adenosine induced by perfusion of 10 μM raclopride (ANOVA: $\chi^2 = 6.857$, $P > 0.05$, $N = 5$) and 100 μM raclopride (ANOVA: $\chi^2 = 4.929$, $P > 0.05$, $N = 4$) was not significant. In contrast, perfusion of 1 mM raclopride (ANOVA: $\chi^2 = 12.714$, $P < 0.05$, $N = 3$) and 10 mM raclopride (ANOVA: $\chi^2 = 16.571$, $P < 0.05$, $N = 3$) resulted in a significant increase of adenosine levels in the NAC.

4. Discussion

The present study demonstrates that reverse microdialysis of the selective dopamine D_2 receptor antagonist raclopride (Hall et al., 1988; Seeman and Ulpian, 1988) dose-dependently elevated extracellular adenosine in the NAC.

Perfusion of lower concentrations of raclopride (10 and 100 μM) known to alter the release of glutamate (Saulskaya and Mikhailova, 2002) or dopamine (Rahman and McBride, 2000) in the NAC induced a non-significant, up to twofold increase of dialysate adenosine. The intermediate concentration of raclopride (1 mM) resulted in a significant, about sevenfold increase, the highest concentration (10 mM) in a massive, about 450-fold increase of dialysate adenosine. The peak dialysate adenosine concentrations after perfusion of 10 mM raclopride were about 2.5 μM. Although it is generally difficult to estimate extracellular concentrations of neuroactive compounds from sampled dialysate concentrations (Westerink, 1995; Morrison et al., 1991), it is likely that these maximum adenosine dialysate levels measured here correspond approximately to extracellular adenosine concentrations observed under pathological conditions. For instance, during ischemia rapid increases of adenosine
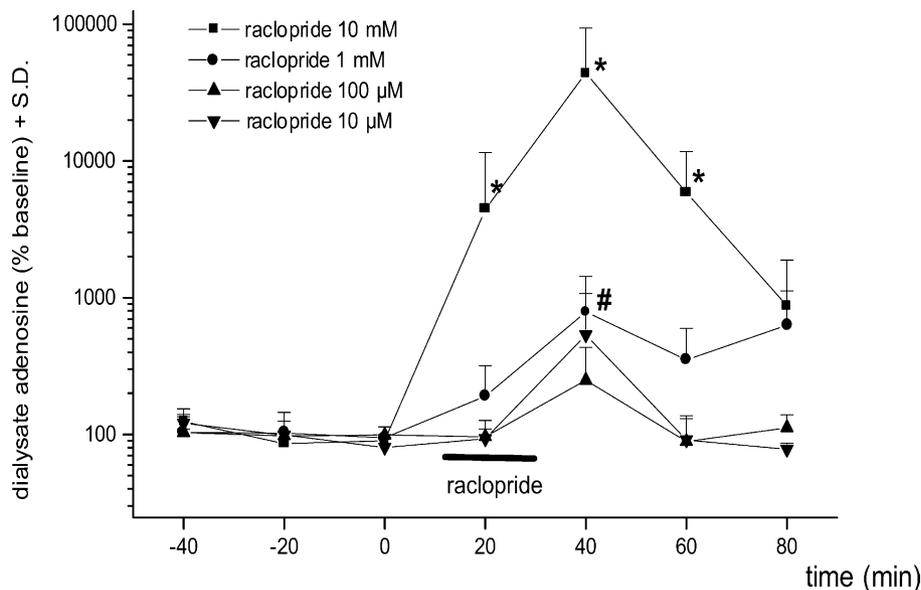
Fig. 2. Effects of perfusion of raclopride (10, 1 mM, and 100, 10 μM; black bar) on dialysate adenosine levels in the NAc. Significant differences ($P < 0.05$) compared to the basal values ($T = 0$ min) are indicated for the doses of 10 mM (∗) and 1 mM (#) raclopride. (ANOVA followed by Dunnett's test with $T = 0$ min as reference).

concentrations have been determined (Rudolphi and Schubert, 1997; Melani et al., 1999b; von Lubitz, 1999) up to 40 μM (Hagberg et al., 1987) probably involving stimulation of A_{2B} and A_3 receptors with their low dissociation constants in the μM range (Dunwiddie and Masino, 2001). Accordingly, the level of dialysate adenosine induced by the highest dose of raclopride (10 mM) in our study might involve unphysiological mechanisms. The reasons for the massive increase (450-fold) of extracellular adenosine induced by the high concentration of raclopride are not entirely clear. It might be in part due to lower basal levels of extracellular adenosine in our study compared to others which did not use a delay after probe implantation to allow baseline stabilisation at lower levels (Ballarin et al., 1991).

Lower doses of raclopride produced two- to sevenfold increases of extracellular adenosine levels which have been also measured in response to a number of pharmacological and other stimuli, e.g. glutamate receptor ligands, in various in vitro and in vivo preparations (Latini and Pedata, 2001). Raclopride is highly selective to dopamine D_2 receptors and nearly devoid of affinity for other receptors, e.g. 5-HT$_1$-, 5-HT$_2$-, α_1-, α_2-, β- and muscarinic receptors, including D_1-receptors (Hall et al., 1988). Therefore, the significant increase of dialysate adenosine levels induced by 1 mM raclopride and the less pronounced increases after perfusion of lower concentrations of raclopride might be brought about by a selective blockade of D_2 receptors.

A number of physiological and pharmacological stimuli have been identified which enhance adenosine release, however, the underlying cellular mechanisms are often not well understood (e.g. Dunwiddie and Masino, 2001). Likewise, there are no data pointing to an involvement of dopamine receptors in one or more of the multiple mechanisms contribut-

ing to the regulation of extracellular adenosine (Strecker et al., 2000; Dunwiddie and Masino, 2001; Latini and Pedata, 2001). One of several potential D_2 receptor-related mechanisms which might account for the observed increase of extracellular adenosine levels in the NAC refers to the suggested homeostatic modulatory role of adenosine and its enhanced formation upon neuronal metabolic challenges (Cunha, 2001). There is consistent evidence that reduced activation of D_2 receptors leads to an enhanced activity of striatal neurons containing D_2 receptors (e.g. LeMoine et al., 1997; Svenningsson et al., 1999; Gerfen, 2000, West and Grace, 2002). Furthermore, it is hypothesised that experimental manipulations which augment cellular energy requirements result in increased extracellular adenosine levels (Cunha, 2001; Dunwiddie and Masino, 2001). Thus, it is conceivable that an intra-NAC D_2 receptor blockade might cause increased activity and energy demand of D_2 receptor-containing neurons resulting in elevated levels of extracellular adenosine.

Enhanced extracellular glutamate levels provide another mechanism by which the extracellular adenosine concentration in the NAC could be augmented. It has been shown that reverse microdialysis of raclopride increased extracellular glutamate in the NAC (Saulskaya and Mikhailova, 2002) and that stimulation of striatal NMDA receptors is a powerful stimulus to rise extracellular adenosine (Manzoni et al., 1994; Jin and Fredholm, 1997; Delaney et al., 1998; Melani et al., 1999a) probably involving an enhanced metabolic demand. In addition, previous data suggest that reverse dialysis of raclopride into the NAC-facilitated dopamine release (Imperato and Di Chiara, 1988; Ferre et al., 1994; Freeman and Tallarida, 1994; Thermos et al., 1996). If extracellular levels of dopamine are enhanced in our experiment, it is

unlikely that dopamine competes effectively with raclopride at postsynaptic D_2 receptors as the high concentrations of raclopride used here should produce a near total receptor blockade. Rather, raclopride-induced dopamine release might stimulate D_1 receptor containing striatal neurons (e.g. Gerfen, 2000; LeMoine et al., 1997; Svenningsson et al., 1999; West and Grace, 2002) thereby promoting their energy demand and, in turn, increasing extracellular adenosine levels. Taken together, one possible mechanism to explain raclopride-induced stimulation of extracellular adenosine in the NAC might be an enhanced energy demand of accumbal neurons mediated in part by dopamine and glutamate-dependent processes.

4.1. Possible functional implications

The present study demonstrates for the first time that a selective intra-NAC blockade of dopamine D_2 receptors produced a significant increase of extracellular adenosine through yet unknown cellular mechanisms. It is suggested that the increase of extracellular adenosine which was significant only with higher doses of raclopride might represent a homeostatic modulatory mechanism proposed to be a main function of adenosine in response to neuronal metabolic challenges (e.g. Cunha, 2001, Dunwiddie and Masino, 2001). The enhanced metabolic challenges to accumbal neurons could be imposed by dopamine and glutamate-dependent mechanisms.

There is compelling evidence that dopamine plays a role opposite to adenosine in the NAC in control of behaviour and that these antagonistic effects are in part mediated by intra-membrane adenosine–dopamine receptor–receptor interactions (Ferre et al., 1997; Fuxe et al., 1998, Zahniser et al., 2000). Our data imply that behavioural or immunohistochemical effects induced by dopamine D_2 receptor antagonists administered either systemically or locally (e.g. Chartoff et al., 1999, Hauber et al., 2001; Ouagazzal and Amalric, 1995; Ögren and Fuxe, 1988; Ozer et al., 1997) involve, at least in higher doses, a secondary increase in extracellular adenosine in the NAC-stimulating accumbal A_1 and A_2 receptors. For instance, 1–$10\,\mu g$ raclopride was microinfused in behavioural studies (Ozer et al., 1997; van den Boss et al., 1988) corresponding to concentrations up to $40\,mM$. Thus, it is conceivable that motor inhibition produced by raclopride after systemic or intra-NAC administration (e.g. Ozer et al., 1997) might also involve a stimulation of accumbal A_{2A} receptors which are known to cause locomotor inhibition (Barraco et al., 1994; Hauber and Munkle, 1997). In addition, increased extracellular adenosine levels induced by D_2 receptors antagonists would explain why A_{2A} receptor antagonists have low motor stimulant effects by its own (Svenningsson and Fredholm, 1997; Hauber et al., 1998, Marston et al., 1998; Popoli et al., 1998), but produce prominent motor stimulant effects when co-administered with D_2 receptor antagonists (Kanda et al., 1994; Kafka and Corbett, 1996; Hauber et al., 1998;

Ward and Dorsa, 1999; Hauber et al., 2001). Therefore, the present data point to the possibility that behavioural effects induced by D_2 receptor antagonists in the NAC are in part mediated by a secondary increase of extracellular adenosine.

Acknowledgements

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