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Research report

Effects of salient environmental stimuli on extracellular adenosine levels in the rat nucleus accumbens measured by in vivo microdialysis

J. Nagel, W. Hauber *

Department of Animal Physiology, Institute of Biology, University of Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany

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Abstract

In the nucleus accumbens (NAc), the neuromodulator adenosine plays a major role in control of behaviour. The NAc subserves behaviour governed by salient stimuli in the environment, however, it is unknown whether such stimuli and the behavioural effects elicited are associated with changes in NAc extracellular adenosine levels. In order to further characterise the neuromodulatory actions of adenosine, the present study investigated for the first time the effects of four prototypical stimuli known to involve NAc processing on extracellular levels of adenosine in the NAc. Using in vivo microdialysis, the following stimuli were examined: (1) an appetitive, unfamiliar stimulus (palatable food), (2) an appetitive, familiar stimulus (standard laboratory food), (3) an aversive stimulus (handling) and (4) a novelty stimulus (cage change). Results revealed that neither of these stimuli significantly changed extracellular adenosine levels in the NAc. These findings demonstrate that NAc extracellular adenosine is not responsive to a number of prototypical salient stimuli in the environment. Thus the data provide no clues to suggest that transient changes of extracellular adenosine in the NAc modulate behavioural responses governed by these stimuli. © 2002 Published by Elsevier Science B.V.

Keywords: Neuromodulation; Novelty; Feeding; Handling; Adenosine–dopamine interactions

1. Introduction

The nucleoside adenosine plays an important role as modulator of neuronal activity through actions on distinct cell-surface receptors coupled to G-proteins which are termed as A_1, A_{2A}, A_{2B} and A_3 receptors [28]. In the basal ganglia, a group of interconnected forebrain nuclei, neuromodulation by adenosine plays a crucial role in motor control, cognition and reward [27]. The striatum, a major component of the basal ganglia, shows the highest density of A_{2A} receptors in the brain [46] and intermediate densities of A_1 receptors [45,52]. Adenosine regulates the release of several neurotransmitters by acting on striatal A_1 receptors [27,28]. Furthermore, it modulates dopamine transmission through interactions of adenosine A_1/dopamine D_1 receptors and adenosine A_{2A} receptors /dopamine D_2 receptors [29]. In addition, adenosine A_{2A} receptor activation might mediate physiological and behavioural effects through dopamine receptor-independent mechanisms [3,59].

In functional terms, adenosine plays a role opposite to dopamine as demonstrated in studies on the cellular, network and behavioural level [27,30,35]. In the nucleus accumbens (NAc), a subregion of the ventral striatum, these antagonistic adenosine–dopamine interactions play a prominent role in control of behaviour [26,32,33]. The NAc subserves behaviour governed by motivationally significant stimuli in the environment [31] and most of these stimuli produced significant increases in NAc dopamine efflux [12,36,38]. However, it is largely unknown whether salient environmental stimuli and behavioural effects induced by these stimuli are also associated with changes in NAc extracellular adenosine levels. It has been suggested that neuromodulation by adenosine through intra-membrane A_{2A}/D_2 and A_1/D_1 receptor–receptor interactions could substantially increase the computational potential of neuronal networks [29]. If so, co-incident changes in the extracellular concentrations of adenosine and dopamine

* Corresponding author. Tel.: +49-711-685-5003; fax: +49-711-685-5090.

E-mail address: wolfgang.hauber@po.uni-stuttgart.de (W. Hauber).

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might represent a particularly effective way to use this neuromodulatory potential. For a deeper understanding of the neuromodulatory actions of adenosine, it is important to know whether prototypical stimuli known to elevate mesolimbic dopamine also produce changes of extracellular adenosine in the NAc. To this end we tested in the present study the effects of an appetitive, unfamiliar stimulus (palatable food), an appetitive, familiar stimulus (standard laboratory food), an aversive stimulus (handling) and a novelty stimulus (cage change) on extracellular adenosine levels using in vivo microdialysis in freely moving rats. All these stimuli have been shown to stimulate dopamine efflux in the NAc [7,23,24,41,54,60].

2. Materials and methods

Experiments were performed according to the current version of the German Law on Animal Protection and were approved by the proper authorities in Stuttgart, Germany. All efforts were made to minimize the number of animals used and their suffering.

2.1. Subjects

Thirteen male Sprague–Dawley rats (220–280 g; Charles-River, Sulzfeld, Germany) were housed in standard Macrolon® type IV cages ($55 \times 35 \times 10$ cm; Ebeco, Castrop-Rauxel, Germany) in groups up to five animals until stereotaxic surgery. After surgery they were housed individually in Macrolon® type III cages ($37 \times 21 \times 30$ cm; Ebeco, Castrop-Rauxel, Germany) with raised solid walled lids. In the animal house temperature (20 ± 2 °C) and humidity ($50 \pm 10\%$) were kept constant and a 12-h light:12-h dark schedule was used with lights on between 06:00 and 18:00 h. All rats were given ad libitum access to water and standard laboratory maintenance chow (Altromin, Lage, Germany).

2.2. Surgery

The rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) (Sigma, Deisenhofen Germany) after atropine sulfate pretreatment (0.5 mg/kg i.p.) (Research Biochemicals Inc., Natick, USA) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, USA) with the incisor bar set 5 mm above the interaural line. Siliconized guide cannula (CMA/12; outer diameter: 0.9 mm) (CMA, Stockhom, Sweden) were aimed at the NAc and implanted unilaterally using standard stereotaxic procedures. The co-ordinates were AP: +3.4 mm, L: ± 1.5 mm relative to bregma according to the atlas of Pellegrino et al. [48]. Each rat was given at least 5 days to recover from surgery before starting microdialysis experiments.

2.3. Microdialysis

Before the onset of the experiments an animal was transferred from the animal house to the behavioural laboratory. The lid of the home cage was replaced by an open top (height: 20 cm). The microdialysis probes (CMA/12, 2 mm exposed membrane length, 0.5 mm membrane o.d.) (CMA, Stockholm, Sweden) were inserted into the NAc through the guide cannula (ventral position of the probe tip with reference to the skull: −8.0 mm) at 17:00, i.e. 1 h before the lights were turned off. The probes were perfused with artificial cerebrospinal fluid (aCSF) at a flow rate of 2 μl/min (CMA 102; CMA, Stockholm, Sweden). 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). The recovery of the microdialysis probes for adenosine was between 7 and 17%. Twenty minutes samples (40 μl) were collected (CMA/142; CMA, Stockholm, Sweden). Animals were mounted with a head block tether system (Instech, Plymouth Meeting, USA) to a two-channel swivel (Instech, Plymouth Meeting, USA). All inlet and outlet tubing was made of FEP tubing (i.d. 0.12 mm) and tubing adapters (CMA, Stockholm, Sweden). Sample collection started 14 h after the insertion of the probe.

2.4. Chemical assays

Adenosine was quantified by HPLC with fluorometric detection as a fluorescent derivative (1,N6-ethenoadenosine) after derivatisation with chloroacetaldehyd [56]. Zinc acetate (5.3 μl; 0.01 mM) and 7.5 μl chloracetaldehyd (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 and Kontron 565 autosampler, Biotek Kontron, Neufahrn, Germany) with Nucleosil 100-5-C18 column (5 μm particles, length × i.d. 125×3 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 octanesulfonic acid (Sigma, Deisenhofen, Germany) 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 [42]. Ethenoadenosine peaks were identified and quantified by comparison with known 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). The detection limit at a signal-to-noise ratio of 3:1 was lower than 20 fmol (5.3 pg).

2.5. Behavioural experiments

At the beginning of the behavioural experiments (7:00 h) rats were food-deprived for 20 h. At that time microdialysis probes were in position for 14 h and baseline adenosine levels in the NAc were stable. Pilot experiments revealed that there were high dialysate adenosine values of about 330 nM (90 pg/μl) ($N = 3$) immediately after probe insertion which decreased within 8–10 h reaching a stable baseline confirming previous data [6,49]. The effects of four physiological stimuli known to activate the dopamine release in the NAc were studied: (1) unfamiliar, highly palatable food (2.5 g; Fonzie®, kindly provided by KP Snack Food, Donauwoerth, Germany) [8] given for 20 min in the home cage; (2) familiar standard laboratory chow (2.5 g; Altromin, Lage, Germany) given for 20 min in the home cage [60]; (3) handling, i.e. the rat was picked up from the home cage, handled gently for 20 min and put back again in the home cage [23,24]; (4) exposure to novelty, i.e. the rat was picked up from its home cage, placed into an identical, but novel and clean, cage without food and water and was put back into the home cage 20 min later [23,24]. The order of the stimuli was pseudo-randomised for each rat according to the following rule: the unfamiliar food stimulus always preceded the familiar food stimulus and both stimuli were separated by at least one other nonfood stimulus. All stimuli were presented 2 h apart from each other.

2.6. Histology

The animals were killed after the experiment by an overdose of sodium pentobarbital. The 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 (40 μm) stained by cresyl violet (Fig. 5).

2.7. Data analysis

Neurochemical data were transformed into percent changes from 100% baseline, where 100% represented the average concentration of three samples preceding stimulus presentation. Normally distributed data were analysed using a one-way analysis of variance (ANOVA) for repeated measurements followed by a Dunnett's test for multiple comparisons with the last baseline sample before presentation of a stimulus serving as respective baseline value. Data not normally distributed were analysed using a nonparametric one-way Friedman ANOVA for repeated measurements with the last baseline sample before presentation of a stimulus serving as respective baseline value. The level of statistical significance was set at $P < 0.05$. All results are presented as means ± standard error of the mean (S.E.M.). Statistical analysis was performed using SIGMASTAT Version 2.0 (Jandel, Erkrath, Germany).

3. Results

3.1. Basal dialysate adenosine

Basal dialysate values of adenosine at the beginning of the behavioural experiments before presentation of the first stimulus was 10.51 ± 2.61 nM (2.81 ± 0.7 pg/μl) (last baseline sample). The baseline value before presentation of the following stimuli were 10.17 ± 1.72 nM (2.72 ± 0.46 pg/μl) (stimulus 2), 10.92 ± 1.94 nM (2.92 ± 0.52 pg/μl) (stimulus 3), 8.68 ± 2.02 nM (2.32 ± 0.54 pg/μl) (stimulus 4) (last baseline sample, respectively). The baseline value measured before termination of the experiment was 10.17 ± 1.74 nM (2.72 ± 0.48 pg/μl). There were no significant differences between respective pre-stimulus basal dialysate values and the final basal dialysate value ($F_{4/128} = 0.39$, $P = 0.81$).

3.2. Effects of unfamiliar food

Presentation of the unfamiliar, palatable food (Fonzies) to food-deprived rats induced arousal followed by food consumption. Fonzies feeding produced a transient and mild increase in dialysate adenosine to 120% (Friedman ANOVA: $\chi^2 = 5.26$; $P = 0.39$; $N = 10$)(Fig. 1). There was some within-group variability as several animals exhibited an immediate, moderate increase of

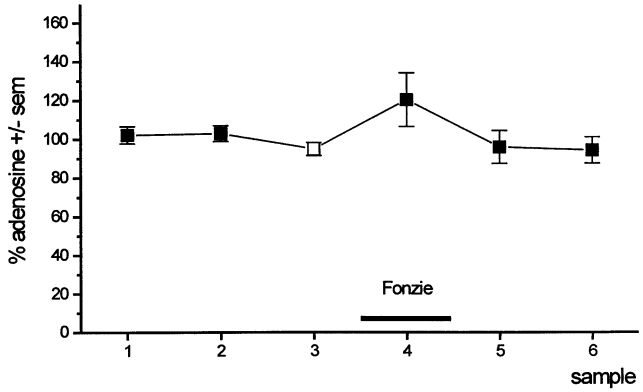

Fig. 1. The effects of Fonzies feeding for one sample period (20 min) on extracellular adenosine in the NAc. Results are mean ± S.E.M. ($N = 10$) (Friedman ANOVA: $P > 0.05$). The last baseline value is indicated with an open symbol.

dialysate adenosine to 140% of baseline, while others showed a delayed, minimal increase to 110% of baseline.

3.3. Effects of familiar food

Presentation of familiar standard laboratory chow in rats pre-exposed at that time to Fonzies, resulted in an immediate feeding response in all animals. Feeding of standard laboratory chow failed to change dialysate adenosine (Friedman ANOVA: $\chi^2 = 4.71$; $P = 0.45$; $N = 8$) as depicted in Fig. 2.

3.4. Effects of handling

Behavioural responses to handling were variable and ranged from intense activity to sleeping. The applied procedure comprised gentle handling using manual restraint only if animals tried to jump off. Handling did not change dialysate adenosine in the NAc (ANOVA: $F_{5,25} = 0.27$; $P = 0.91$; $N = 6$) (Fig. 3).

3.5. Effects of cage change

Placing animals into a novel cage produced arousal followed by explorative locomotor activity for about 10 min in all rats. As shown in Figs. 4 and 5, cage change induced a transient and insignificant increase in dialysate adenosine following cage change (Friedman ANOVA: $\chi^2 = 3.06$; $P = 0.69$; $N = 5$). The increased variance in the sample during stimulus presentation was due to one animal with a three-fold increase of dialysate adenosine.

3.6. Effects of stimulus order

The different behavioural stimuli were presented in a pseudo-randomised order to each rat during the course of the experiment. To check for habituation stimulus

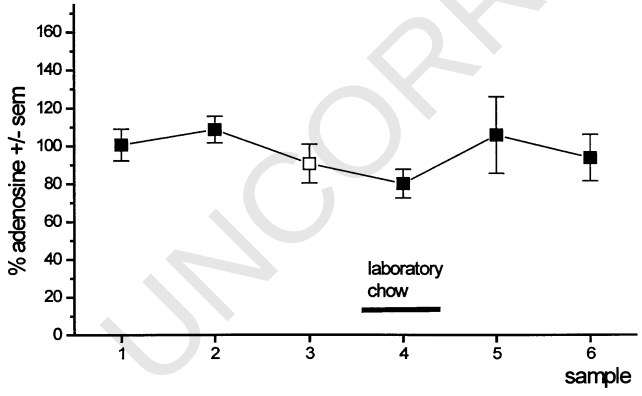

Fig. 2. The effects of feeding with familiar standard lab chow for one sample period (20 min) on extracellular adenosine in the NAc. Results are mean ± S.E.M. ($N = 8$) (Friedman ANOVA: $P > 0.05$). The last baseline value is indicated with an open symbol.

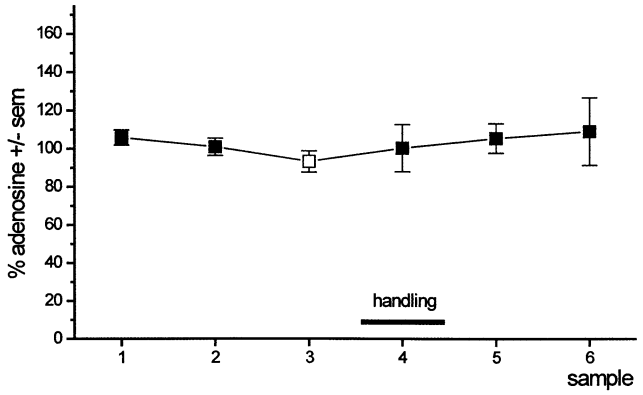

Fig. 3. The effects of handling for one sample period (20 min) on extracellular adenosine in the NAc. Results are mean ± S.E.M. ($N = 6$) (ANOVA: $P > 0.05$). The last baseline value is indicated with an open symbol.

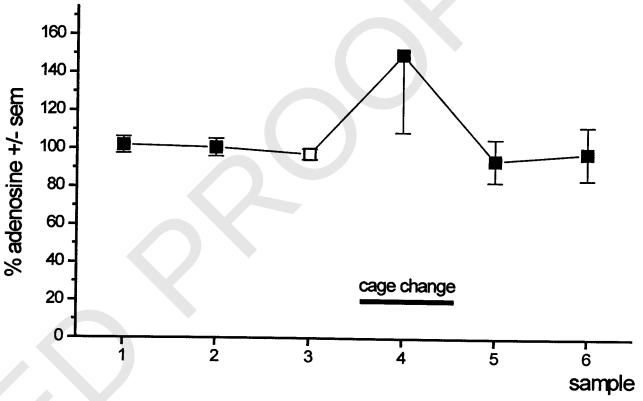

Fig. 4. The effects of exposure to novelty (cage change) for one sample period (20 min) on extracellular adenosine in the NAc. Results are the mean ± S.E.M. ($N = 5$) (Friedman ANOVA: $P > 0.05$). The last baseline value is indicated with an open symbol.

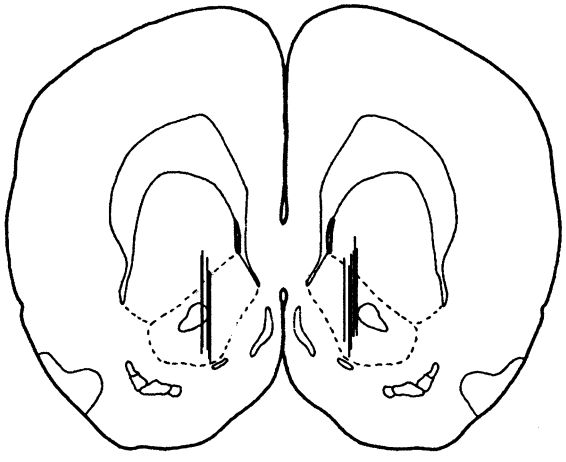

Fig. 5. Location of the microdialysis probes in the NAc. Vertical lines represent the 2 mm dialysing length of the probes. Drawing of the coronal section was adapted from the atlas of Pellegrino (1981). The given section is 3.4 mm anterior bregma.

effects on dialysate adenosine were analysed additionally by grouping stimuli according to their order and not according to their character (as described above). Results (data not shown) reveal that the first stimulus, respectively, given to rats did not increase dialysate adenosine (Friedman ANOVA: $\chi^2 = 4.05$; $P = 0.54$; $N = 9$). Likewise, the second (Friedman ANOVA: $\chi^2 = 4.64$; $P = 0.46$; $N = 8$), third (Friedman ANOVA: $\chi^2 = 5.64$; $P = 0.34$; $N = 8$) and last stimulus (FriedmanANOVA: $\chi^2 = 8.38$; $P = 0.14$; $N = 7$), respectively, given to rats did not change dialysate adenosine in the NAc.

4. Discussion

The present study demonstrates that an unfamiliar appetitive stimulus (palatable food), familiar appetitive stimulus (laboratory food), aversive (handling) or behaviourally arousing stimuli (exposure to novelty) did not significantly change extracellular adenosine levels in the NAc.

4.1. Baseline adenosine levels

The dialysate concentrations of adenosine in baseline samples were between 8.7 and 10.6 nM starting 14 h after probe insertion. These values correspond well with those of other studies in un-anaesthetised rats which reported dialysate adenosine concentrations of 12 [5] and 13 nM [42] in the striatum, but considerably higher levels in the cortex (25–100 nM) [10]; see [39] for review. Based on pilot experiments we used a delay of 14 h between probe insertion and onset of the behavioural experiments to allow for baseline stabilisation. Thereafter, the four pre-stimulus baseline values (2 h apart from one another) and the final baseline value before completion of the experiment did not differ significantly. This indicates that the time window used for experiments provided reliable baseline stability. Parameters as time-of-day which might produce baseline shifts are thus unlikely to infer with our results. These findings are in keeping with recent data showing that adenosine levels in cats declined within 6–9 h after probe insertion into the thalamus and basal forebrain, and remained thereafter constant for 4 days [49]. Also, in rats, extrastriatal adenosine levels were constant, although at a higher level, already 3 h after probe insertion into the hippocampus [11].

4.2. Origin of extracellular adenosine

The sources of extracellular adenosine measured by in vivo microdialysis and the factors that regulate its levels are not well understood [39,58]. Biochemical and electrophysiological studies have established that one potential source of extracellular adenosine is its rapid and quantitative formation in the extracellular space from adenine nucleotides through the action of ectoenzymes [14,18,20,51,53,63]. Thus, adenosine gains access to the extracellular space in part by degradation of vesicularly released ATP [50]. In line with this notion, in vivo microdialysis studies demonstrated that extracellular adenosine levels in the striatum were partly sensitive to TTX [47]. In addition, the intracellular adenosine concentration is regulated by a number of enzymes and an increased intracellular adenosine concentration results in a transport of adenosine to the extracellular space via equilibrative nucleoside transporters [49].

4.3. Effects of physiological stimuli

Little is yet known on the role of adenosine in modulating behavioural responses to motivationally significant stimuli. Our results revealed that prolonged handling which represents an aversive stimulus [36] did not alter extracellular adenosine in the NAc. Restraint stress provoked in rats large increases in striatal glucose [25] and lactate [19] indicating enhanced excitatory neuronal activity. In addition, handling of rats with a similar procedure as used here elicited a dopamine release in the NAc [23,24]. As striatal glutamate levels were increased after handling stress [43] and reverse microdialysis of NMDA and kainate stimulated striatal adenosine [39] one might expect an increase of extracellular adenosine in our experiment. One explanation for the lack of effect could be the mild handling procedure used here which might not result in massive increases of extrastriatal glutamate. Our findings suggest that an aversive stimulus as handling known to induce changes of the neuronal activity in the NAc does necessarily induce alterations in extracellular adenosine. Moreover, we found that exposure to novelty by placing the animals into a novel cage induced behavioural arousal and locomotor exploration without altering extracellular adenosine levels in the NAc significantly. There is converging evidence that the NAc plays a key role in novelty exploration [13] and novelty-induced behavioural activation [36].

Electrophysiological recordings further demonstrated that the activity of NAc neurons is sensitive to novel stimuli [40] and correlated with behavioural arousal [15]. In addition, exposure to novelty using similar cage change procedures as used here provoked an increase in NAc extracellular dopamine [23,41,54]. These data indicate that a stimulus as novelty exposure which is known to be represented by NAc neurones and to elevate NAc dopamine did not induce corresponding changes in NAc extracellular adenosine. In line with previous studies [37] this finding implicates that motor activation per se is not associated with alterations of NAc adenosine. Furthermore, the NAc is an important

neural substrate mediating feeding behaviour [16,57] and microdialysis studies in behaving rats revealed significant increases in NAc extracellular dopamine during feeding [36,38,60], in particular during unpredicted consumption of unfamiliar, palatable food as Fonzies [1,7–9,61]. Our data revealed feeding of unfamiliar, palatable food and familiar food produced no significant changes of NAc extracellular adenosine.

Taken together, the present findings demonstrate that NAc extracellular adenosine is not responsive to a number of salient stimuli in the environment. This effect is not due habituation to repeated exposure to different salient stimuli, as dialysate adenosine levels did not change as a function of the order of stimulus presentation. Therefore, the data suggest that behavioural responses governed by these stimuli might not be modulated by transient changes in NAc extracellular adenosine. There is consistent evidence that the NAc receives converging input from prefrontal cortex, hippocampus, amygdala and midbrain which together transmit information on motivationally significant stimuli in the environment [22,55,62]. These afferent signals are coded by glutamate and dopamine and synapse on NAc projection neurons [2]. Our data give no clues for a role of adenosine neuromodulation in these processes. As microdialysis has significantly contributed to unravel major physiological and pathophysiological roles of adenosine [21], the time resolution of this technique is unlikely to account for these negative results. Also, an insufficient sensitivity of our system may be ruled out as we were able to measure changes after probe implantation in the present study as well as more subtle effects after pharmacological stimulation [44]. Recent studies revealed that A_{2A} receptor blockade had no effects on the rewarding effects of electrical brain stimulation in otherwise intact animals indicating that the neural substrate mediating the rewarding effects of this procedure is not controlled by endogenous adenosine acting on A_{2A} receptor subtype [4]. The failure of rewarding stimuli to produce a transient change in NAc extracellular adenosine as measured here also point to this notion. However, there is evidence that a tonic stimulation of adenosine receptors is involved in regulation of striatal glutamate [17] and striatally mediated behaviour [34]. Thus one can not exclude that a tonic stimulation of striatal adenosine receptors is a prerequisite for the rewarding and other stimuli tested here to become effective. This latter possibility could be only tested by intra-NAc blockade of adenosine receptor subtypes.

Another implication of the present data refers to theoretical models on the physiological significance of adenosine–dopamine interactions. If adenosine modulates dopamine neurotransmission through antagonistic intra-membrane receptor–receptor interactions on a short time scale [29] one might expect that at least some of the stimuli tested should alter extracellular adenosine in the NAc as they have been consistently shown to stimulate mesolimbic dopamine. Our negative results do not support the notion of an interactive modulation of NAc neuronal responses [29] by transient and co-incident changes of dopamine and adenosine, at least with regard to the categories of salient events tested. However, this hypothesis should be regarded as preliminary awaiting results of our current experiments analysing stimulus effects both on dopamine and adenosine levels in core and shell subregions of the NAc.

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