Journal of Neurochemistry, 2005, **93**, 595–604 doi:10.1111/j.1471-4159.2005.03071.x

Adenosine A_{2A} receptors control the extracellular levels of adenosine through modulation of nucleoside transporters activity in the rat hippocampus

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Abstract

Adenosine, a neuromodulator of the CNS, activates inhibitory-A_1 receptors and facilitatory-A_{2A} receptors; its synaptic levels are controlled by the activity of bi-directional equilibrative nucleoside transporters. To study the relationship between the extracellular formation/inactivation of adenosine and the activation of adenosine receptors, we investigated how A_1 and A_{2A} receptor activation modifies adenosine transport in hippocampal synaptosomes. The A_{2A} receptor agonist, CGS 21680 (30 nM), facilitated adenosine uptake through a PKC-dependent mechanism, but A_1 receptor activation had no effect. CGS 21680 (30 nM) also increased depolarization-induced release of adenosine. Both effects were prevented by A_{2A} receptor blockade. A_{2A} receptor-mediated enhancement of adenosine transport system is important for formatting adenosine neuromodulation according to the stimulation frequency, as: (1) A_1 receptor antagonist, DPCPX (250 nM), facilitated the evoked release of [^{3}H]acetylcholine under low-frequency stimulation (2 Hz) from CA3 hippocampal slices, but had no effect under high-frequency stimulation (50 Hz); (2) either nucleoside transporter or A_{2A} receptor blockade revealed the facilitatory effect of DPCPX (250 nM) on [^{3}H]acetylcholine evoked-release triggered by high-frequency stimulation. These results indicate that A_{2A} receptor activation facilitates the activity of nucleoside transporters, which have a preponderant role in modulating the extracellular adenosine levels available to activate A_1 receptors.

Keywords: A_{2A} receptors, acetylcholine, adenosine, hippocampus, nucleoside transporters, stimulation frequency.
J. Neurochem. (2005) **93,** 595–604.

Adenosine is a modulator that exerts its action through four types of metabotropic receptors – A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al*. 2001). In particular, the neuromodulatory role of adenosine depends mostly on a balanced activation of inhibitory A_1 receptors and facilitatory A_{2A} receptors (see Sebastião and Ribeiro 2000). There are two main sources of extracellular adenosine in the nervous system: adenosine release through bi-directional equilibrative nucleoside transporters (ENTs) and extracellular conversion of adenine nucleotides into adenosine by ecto-nucleotidases (for review see Latini and Pedata 2001). Whereas ecto-nucleotidases can only form adenosine, nucleoside transporters fulfil a dual

Received September 24, 2004; revised manuscript received November 19, 2004; accepted December 7, 2004.

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Abbreviations used: ACh, acetylcholine; CGS 21680, 2-p-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride; CNT, concentrative transporter; CPA, N^6-cyclopentyladenosine; dbcAMP, N^6,2′-*o*-dibutyryladenosine-3′,5′-cyclic monophosphate sodium salt; DMSO, dimethylsulfoxide; DPCPX, 1,3-dipropyl,8-cyclopentylxanthine; ENT, equilibrative nucleoside transporters; GF-109203X, 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide; H-89, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; HC-3, hemicholinium-3; HFS, high-frequency stimulation; HPLC, high-performance liquid chromatography; LFS, low-frequency stimulation; NBTI, *S*-(p-nitrobenzyl)-6-thioinosine; PDD, phorbol-12,13-didecanoate; SCH 58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; ZM 241385, 4-(2-[7-Amino-2-(2-furyl) [1,2,4] triazolo [2,3-a] [1,3,5] triazin-5-ylamino]ethyl)phenol.

role, because blockade of adenosine transport can inhibit either adenosine release or adenosine uptake in the hippocampus, depending upon its intra- and extracellular levels (Gu *et al*. 1995).

The activity of ENTs is controlled by G proteins (Sweeney 1996) and protein kinases (Sen *et al*. 1999). Adenosine receptors, which are coupled to G proteins and protein kinase-dependent transducing systems, appear therefore to be good candidates to modify the levels of their endogenous ligand, adenosine. It has been shown that A_2 receptor activation can increase adenosine uptake in chromaffin cells (Delicado *et al*. 1990), an issue that remains to be confirmed in neurones. This control of the levels of extracellular adenosine by adenosine A_2 receptors might be particularly important in brain areas such as the hippocampus under high-frequency neuronal firing, which favours the activation of adenosine receptors of the A_{2A} subtype (Cunha *et al*. 1996a), as well as a predominant formation of extracellular adenosine through the ecto-nucleotidase pathway (Cunha *et al*. 1996b).

Thus, we now directly tested if the activation of adenosine A_{2A} receptors could control the release and uptake of adenosine through nucleoside transporters in hippocampal preparations. This was tested in nerve terminals where ENTs play an important role in the metabolism of adenosine (e.g. Gu *et al*. 1995). Because it was previously shown that the evoked release of acetylcholine (ACh) from rat hippocampal slices is under the control of endogenous extracellular adenosine operating mainly A_1 receptors (Jackisch *et al*. 1984), but also A_{2A} receptors (Cunha *et al*. 1994), the modulation of electrically evoked [^{3}H]ACh release by tonic A_1 receptor activation was subsequently used in the present work as a 'model' to evaluate how the A_{2A} receptor-mediated enhancement of nucleoside transporters could affect tonic neuromodulation by endogenous adenosine.

Materials and methods

Materials

4-(2-[7-Amino-2-(2-furyl) [1,2,4] triazolo [2,3-a] [1,3,5] triazin-5-ylamino]ethyl)phenol (ZM 241385), 2-p-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21680) and 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide (GF-109203X) were purchased from Tocris Cookson (Ballwin, MO, USA). N^6-cyclopentyladenosine (CPA), 1,3-dipropyl,8-cyclopentylxanthine (DPCPX), dipyridamol, hemicholinium-3 (HC-3), S-(p-nitrobenzyl)-6-thioinosine (NBTI), N^6,2′-*o*-dibutyryladenosine-3′,5′-cyclic monophosphate sodium salt (dbcAMP), N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), phorbol-12,13-didecanoate (PDD) and adenosine were from Sigma (St Louis, MO, USA). 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) was a gift from Dr Scott Weiss (Vernalis, UK). [Methyl-^{3}H]choline chloride (specific activity 76–86.3 Ci/mmol), and [2–^{3}H]adenosine (22–28 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, USA). DPCPX was made up as a 5-mM stock solution in dimethylsulfoxide (DMSO) with 1% NaOH 1 M (v/v). The following stock solutions were prepared in DMSO: CGS 21680 (5 mM), SCH 58261 (5 mM), CPA (5 mM), adenosine (100 mM), dipyridamol (2.5 mM), NBTI (10 mM), PDD (5 mM), GF-109203X (5 mM) and H-89 (5 mM). HC-3 (10 mM) and dbcAMP (10 mM) were prepared in water. Aliquots of all stock solutions were kept frozen at −20°C until used.

Preparation of CA3 subslices and synaptosomes from rat hippocampus

The experiments were performed on Wistar rats (6–8 weeks old) from Harlan Interfauna Iberica, SL (Barcelona, Spain). The animals were handled according to the European Community guidelines and Portuguese law on Animal Care and were anaesthetized with halothane before decapitation. The hippocampus was dissected free within ice-cold Krebs' solution of the following composition: 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM glucose, pH 7.4.

When preparing hippocampal slices, the hippocampi were transversely cut 400 μm thick, the CA3 subslices manually dissected and allowed to recover for 1 h in gassed Krebs solution (95% O_2 and 5% CO_2) kept at room temperature (20–22°C).

The synaptosomal P2 fraction and purified nerve terminals (synaptosomes) were isolated as previously (e.g. Cunha *et al*. 2000).

[^{3}H]Adenosine uptake assay

Synaptosomes were resuspended in 1 mL of Krebs/HEPES solution with the following composition: NaCl 124 mM, KCl 3 mM, NaH_2PO_4 1.25 mM, $MgSO_4$ 1 mM, $CaCl_2$ 2 mM, HEPES 26 mM, glucose 10 mM, pH 7.4) and equilibrated at 37°C. All adenosine transport assays were conducted at 37°C in a total volume of 300 μL, containing 150–200 μg of protein (see Cunha *et al*. 2000). Transport was initiated by addition of 1 μM [^{3}H]adenosine, added at least 10 min after exposing synaptosomes to the tested drugs, and was terminated 15 s after initiating its uptake by the addition of 5 mL of an ice-cold transport inhibitor mixture composed by dipyridamol (20 μM), NBTI (10 μM) and adenosine (1 mM), in Krebs/H followed by low-pressure filtration through 0.45-μm filters (Millipore LCWP-047, Millipore Corporation, Bedford, MA, USA) loaded in a Millipore holder. The reaction tube was washed off with further 5 mL of the same solution. The filters were analysed by liquid scintillation counting for determination of tritium retained by synaptosomes after addition of 5 mL of scintillation cocktail (Optiphase Hi-Safe 2, Perkin-Elmer, Foster City, CA, USA). Adenosine transport was calculated as the difference between the total amount of adenosine taken up by synaptosomes and the non-specific component of [^{3}H]adenosine fixation by synaptosomes, determined in the presence of dipyridamol (20 μM), NBTI (10 μM) and adenosine (1 mM).

Adenosine release experiments

Because considerable amounts of protein are required for these assays, the P2 synaptosomal fraction was used. Perfusion chambers (90 μL) fitted with Whatman (Brentford, Middlesex, U.K.) GF/C filters were loaded with the synaptosomal fraction and a period of 45 min was allowed for equilibrium, before starting sample collection. Perfusion was at 0.6 mL/min with a 95% O_2/5% CO_2 gassed Krebs solution at 32°C throughout the experiment. Drugs were added

10 min prior to starting sample collection, and were present onwards. Synaptosomes were stimulated from minute 6 to minute 11 with a high-K^+ (28 mM) solution. Each individual experiment comprised a control chamber and one different chamber for each drug condition. The amount of adenosine in each collected sample (3 min) was assessed after derivatization to N^6-ethenoadenosine, and then quantified by high-performance liquid chromatography [HPLC; 100 mM K_2HPO_4 in 15% (v/v) methanol/water, pH 6.5, 1.75 mL/min flow rate] with fluorescence detection (see Zhang *et al*. 1991). The retention time of N^6-ethenoadenosine was of 2.9 min, identified by comparison with an injection of a N^6-ethenoadenosine standard. All injections were performed in duplicate. Adenosine release evoked by high-K^+ stimulation, i.e. the evoked release (expressed in nmol/mg protein), was calculated by integration of the area of the peak on subtraction of the estimated basal outflow of endogenous adenosine, with GraphPad Software (Prism, version 4.02 for Windows, GraphPad Software Inc., San Diego, CA, USA).

[^{3}H]Ach-evoked release experiments

The release of [^{3}H]ACh from CA3 subslices was performed as previously (e.g. Cunha *et al*. 1994). Briefly, after incubation with [methyl-^{3}H]choline (12.3 μCi/mL, 0.15 μM) for 30 min at 32°C and washout, the subslices were placed in 100 μL Perspex chambers (three subslices per chamber) and superfused with gassed Krebs solution at 32°C (flow rate of 0.6 mL/min). From this moment onwards hemicholinium-3 (10 μM) was present in all solutions used. A washout period of 45–60 min was allowed before starting sample (3 min) collection. The preparations were then stimulated at min 6 (S1) and at min 36 (S2) with supramaximal square-wave pulses (1 ms, with an amplitude of approximately 15 V). Stimuli were delivered either with low-frequency (LFS, 2 Hz for 2 min; total number of pulses: 240) or with high-frequency (HFS, 50 Hz). HFS paradigm consisted of four bursts (1.2 s each) of 60 pulses (total number of pulses: 240), applied with 200 ms interburst periods, except otherwise indicated.

Four perfusion chambers were used per experiment, two for controls (absence of drugs, or same drugs during S1 and S2) and two for test conditions (test drug before S2). When the effects of either the A_1 antagonist, DPCPX (250 nM), or the A_1 agonist, CPA (300 nM), were investigated, these test drugs were added to the perfusion medium 21 min before S2, i.e. 15 min after starting sample collection, and remained in the superfused solution up to the end of the experiment. The effects of each of these test drugs on the evoked release of [^{3}H]ACh were expressed by changes of the ratios between the evoked release in S2 (in the presence of the drug) and the evoked release in S1 (in the absence of drugs; S2/S1 ratio), by comparison with the S2/S1 ratios obtained in control conditions in the same experiment (i.e. in the absence of drugs). When evaluating the effects of DPCPX (250 nM) in the presence of the ENT inhibitor, NBTI (10 μM), or the A_{2A} receptor antagonist, SCH 58261 (250 nM), these drugs were applied 15 min before starting sample collection and were present in all superfused solutions from that moment onwards; because both stimulation periods were performed under the effects of those drugs, the S2/S1 ratios remained virtually unaffected. In these experiments, DPCPX (250 nM) was added to the superfused conditions 15 min before S2, as before, and its effect expressed by changes in the S2/S1 ratio, as compared it with the S2/S1 ratios obtained in control conditions in the same experiments (i.e. in the presence of either NBTI or SCH 58261).

At the end of the experiments the slices were homogenized (sonicated in 500 μL of 3 M perchloric acid and 20% Triton X-100) and sampled. All samples were analysed by scintillation counting. The evoked release was calculated by integration of the area of the peak on subtraction of the estimated basal tritium outflow from the total outflow due to electrical stimulation. Field-electrically evoked tritium outflow from hippocampal slices loaded with [^{3}H]choline is Ca^{2+}-dependent and tetrodotoxin-sensitive (Fredholm and Dúner-Engstrom 1989) and is mainly due to [^{3}H]ACh release (Cunha *et al*. 1994), which makes tritium quantification a good measure of the evoked release of [^{3}H]ACh.

Statistical analyses

Data are presented as the mean results ± SEM from n experiments. When comparing two-groups of results, statistical significance was assessed using Student's t-test. When doing multiple comparisons, statistical significance was assessed by one-way ANOVA followed by the Bonferroni correction, using GraphPad Software (Prism, version 4.02 for Windows). Values of $p < 0.05$ were considered statistically significant.

Results

Activation of A_{2A} receptor enhances adenosine uptake

In the experiments evaluating adenosine uptake, [^{3}H]adenosine was applied at a concentration near the nucleoside transporters K_m value for adenosine, 1 μM, as first calculated by Bender *et al*. (1981) for cortical synaptosomes, and found to be similar for nucleoside transporters in the hippocampus (Cunha *et al*. 2000). Thus, we compared the ability of hippocampal nerve terminals to take up adenosine at a concentration of 1 μM in the absence and in the presence of the selective A_{2A} receptor agonist CGS 21680. In a control situation, synaptosomes incorporated 0.27 ± 0.06 pmol of [^{3}H]adenosine per mg of protein. As Fig. 1 shows, CGS 21680 (30 nM) significantly ($p < 0.05$) increased the uptake of adenosine by 66 ± 17% ($n = 7$). This CGS 21680-induced enhancement of adenosine uptake was totally prevented by the selective A_{2A} receptor antagonist, SCH 58261 (250 nM, $n = 7$), which by itself did not appreciably modify [^{3}H]adenosine uptake. Activation of A_1 receptors with the selective A_1 receptor agonist, CPA (100 nM), failed to modify ($n = 7$, $p > 0.05$) the uptake of [^{3}H]adenosine (Fig. 1).

A_{2A} receptor-mediated enhancement of adenosine uptake occurs via PKC activation

It is consensual that pre-synaptic A_{2A} receptor activation triggers intracellular cAMP accumulation leading to the subsequent activation of PKA. However, it is also accepted that A_{2A} receptors operate a parallel signalling transducing mechanisms, via PKC activation (for review see Cunha 2001). Accordingly, we next investigated which intracellular

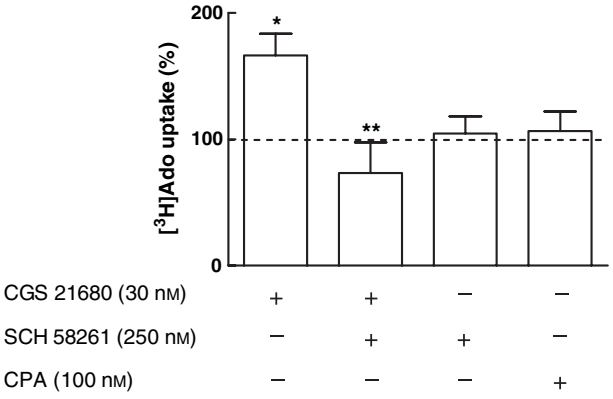

Fig. 1 Effect of A_1 and A_{2A} receptor activation on [^{3}H]adenosine (1 μM) uptake by hippocampal nerve terminals. A total of 100% in the ordinates represents the amount of [^{3}H]adenosine taken up in control conditions (0.27 ± 0.06 pmol). Drugs added to the incubation medium are indicated below each bar. Data are mean ± SEM. Note that the A_{2A} receptor agonist CGS 21680 (30 nM) increased [^{3}H]adenosine uptake ($n = 7$), an effect prevented by the A_{2A} antagonist SCH 58261(250 nM; $n = 7$). On the other hand, the A_1 receptor agonist, CPA (100 nM), did not modify ($n = 6$, $p > 0.05$) [^{3}H]adenosine uptake by hippocampal synaptosomes. When applied in the absence of CGS 21680, SCH 58261 (250 nM) did not significantly modify ($n = 6$, $p > 0.05$) [^{3}H]adenosine uptake. *$p < 0.05$ as compared to control, **$p < 0.05$ as compared to the effect of CGS 21680 (30 nM) alone.

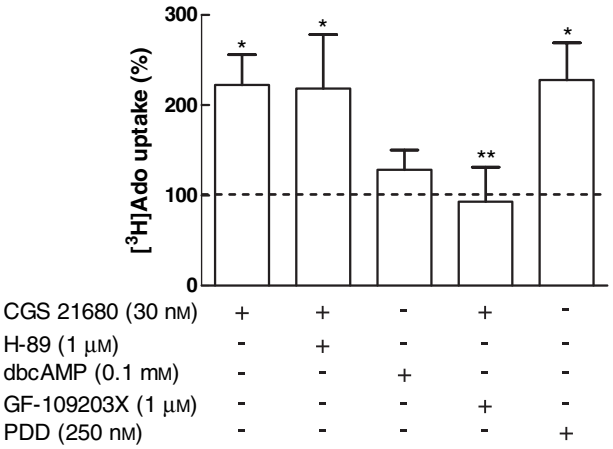

Fig. 2 Influence of PKA and PKC activities upon adenosine uptake and its modulation by A_{2A} receptor activation. A total of 100% in the ordinates represents the amount of [^{3}H]adenosine taken up in control conditions (0.30 ± 0.06 pmol). Synaptosomes pre-incubation for 10 min with the PKC inhibitor, GF-109203X (1 μM), fully prevented the CGS 21680-induced facilitation of [^{3}H]adenosine uptake ($n = 4$). Furthermore, PKC activation with phorbol-12–13-didecanoate (PDD, 250 nM) mimicked the CGS 21680-induced facilitation of [^{3}H]adenosine uptake ($n = 4$, $p < 0.05$). On the other hand, synaptosomes pre-incubation for 10 min with the selective PKA inhibitor, H-89 (1 μM), did not modify the facilitatory effect of CGS 21680 (30 nM; $n = 5$). Also, the enhancement of cAMP levels with dbcAMP (0.1 mM) did not significantly increase [^{3}H]adenosine uptake ($n = 6$). Neither H-89 (1 μM) nor GF-109203X (1 μM), when applied in the absence of CGS 21680, significantly modified ($n = 3$–4, $p > 0.05$) [^{3}H]adenosine uptake (not shown in the Figure). *$p < 0.05$ as compared to control, ** $p < 0.05$ as compared to the effect of CGS 21680 (30 nM) alone.

messengers were involved in the A_{2A} receptor-mediated facilitation of adenosine uptake by studying the effect of CGS 21680 in the presence and in the absence of protein kinases inhibitors. We also compared the effect of CGS 21680 on [^{3}H]adenosine uptake with those of the cAMP analogue, dbcAMP (0.1 mM) and the PKC activator, PDD (250 nM; Castagna *et al*. 1982). In this new set of experiments, synaptosomes took up 0.30 ± 0.06 pmol of [^{3}H]adenosine per mg of protein ($n = 7$) in a control situation. Figure 2 shows that, as before, CGS 21680 (30 nM) enhanced the amount of [^{3}H]adenosine retained by the synaptosomes by 122 ± 34% ($n = 6$, $p < 0.05$). However, when CGS 21680 (30 nM) was added in the presence of the PKC inhibitor, GF-109203X (1 μM; Toullec *et al*. 1991), the A_{2A} receptor-mediated facilitation of [^{3}H]adenosine uptake was fully prevented ($p < 0.05$ as compared to CGS 21680 alone, $n = 4$). Furthermore, direct PKC activation with PDD (250 nM) also increased [^{3}H]adenosine uptake by 128 ± 41% ($n = 4$, $p < 0.05$), mimicking the effect of CGS 21680 (30 nM). On the other hand, the PKA selective inhibitor H-89 (1 μM; Chijiwa *et al*. 1990), failed to significantly modify the facilitatory effect of CGS 21680 on [^{3}H]adenosine uptake ($n = 5$, $p > 0.05$). The enhancement of cAMP levels with dbcAMP (0.1 mM) also did not significantly increase [^{3}H]adenosine uptake ($n = 6$, $p > 0.05$; Fig. 1). When applied in the absence of CGS 21680, neither H-89 (1 μM) nor GF-109203X (1 μM), significantly modified [^{3}H]adenosine uptake ($n = 3$–4, $p > 0.05$).

Activation of A_{2A} receptor enhances adenosine release

The observation that the activation of A_{2A} receptors enhanced the uptake of adenosine does not allow to discriminate between the possibilities that A_{2A} receptor activation was either directly interfering with the activity of nucleoside transporters or indirectly conditioning their function by modifying the gradient of adenosine across the plasma membrane, i.e. changing intrasynaptosomal metabolism (see e.g. Hammer *et al*. 2001). To check the first possibility we took advantage of the bi-directionality of the nucleoside transporters, and evaluated if the activation of A_{2A} receptors would modulate the release of adenosine from hippocampal nerve terminals. Figure 3 shows that CGS 21680 (30 nM) significantly ($n = 6$, $p < 0.05$) increased by 91 ± 12% the extracellular accumulation of adenosine evoked by a pulse of high potassium, when compared to the same depolarizing conditions in the absence of CGS 21680. This effect was totally prevented by the selective A_{2A} receptor antagonist, ZM 241385 (50 nM).

Under the experimental conditions used, the evoked-release of adenosine was in great part due to the activity of ENTs as the inhibitors of nucleoside transporters, NBTI

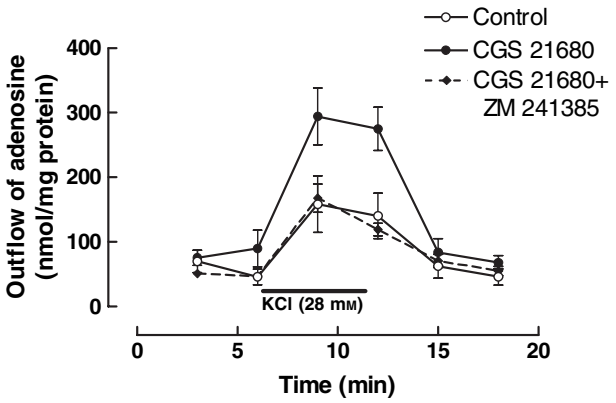

Fig. 3 Time course of the outflow of adenosine from superfused hippocampal nerve terminals stimulated with KCl (28 mM) for 5 min as indicated by the bar above the abscissa. A_{2A} receptor agonist CGS 21680 (30 nM; ●), or a combination of both CGS 21680 (30 nM) and the A_{2A} receptor antagonist, ZM 241385 (20 nM; ◆) were added 10 min before starting sample collection. Each point is mean ± SEM. Note that CGS 21680 (30 nM) increased the evoked outflow of adenosine (●), as compared to control conditions (○; $n = 7$, $p < 0.05$), an effect prevented by ZM 241385 (30 nM; ◆; $n = 4$, $p < 0.05$).

(10 μM) plus dipyridamol (20 μM), reduced the evoked release of adenosine by 63 ± 9% ($n = 3$, $p < 0.05$). Furthermore, the ability of A_{2A} receptors to facilitate the evoked release of adenosine from nerve terminals was also significantly attenuated (69 ± 18%, $n = 4$, $p < 0.05$) in the presence of dipyridamol (20 μM) plus NBTI (10 μM), indicating that the facilitatory effect of CGS 21680 mostly depends on the activity of ENTs.

Differential modulation of [^{3}H]ACh by adenosine release under two stimulation paradigms: an example of the functional relevance of the activation of ENTs by A_{2A} receptors

To evaluate the potential implications of the control of ENTs by A_{2A} receptor activation, we investigated a functional situation in which the control of nucleoside transporters by A_{2A} receptors could crucially affect adenosine neuromodulation. We hypothesized that the well-known A_1 receptor-mediated tonic inhibition of [^{3}H]ACh-evoked release could be affected if the stimulation protocol was changed from LFS to HFS conditions. Indeed, HFS paradigms favour A_{2A} receptor activation by endogenous adenosine (Correia-de-Sa *et al.* 1996), and any influence of A_{2A} receptor activation upon the transport of adenosine across the plasma membrane should result in differences in the modulation of evoked [^{3}H]ACh release by endogenous adenosine. We performed these experiments in the CA3 area of the hippocampus because it was previously shown that, in this area, endogenous adenosine tonically activates both inhibitory-A_1 and facilitatory-A_{2A} receptors, with the overall effect being a tonic inhibition of [^{3}H]ACh release (Cunha *et al.* 1994).

The relative amount of tritium released after depolarizing the subslices with either LFS (2 Hz, 2 min) or HFS (four bursts of 60 pulses at 50 Hz, with an interburst interval of 200 ms) was similar for both stimulation paradigms (Fig. 4a). As expected, the selective A_1 receptor antagonist, DPCPX (250 nM; Cunha *et al.* 1994), facilitated the release of [^{3}H]ACh triggered by LFS (14.5 ± 2.8% increase; $n = 4$, $p < 0.05$). In contrast, when CA3 hippocampal subslices were stimulated with the HFS paradigm, DPCPX (250 nM) failed significantly ($n = 5$, $p > 0.05$) to modify the evoked release of [^{3}H]ACh (Fig. 4b). To avoid the feed-forward inhibition of ecto-5′-nucleotidase by ATP released during stimulation (e.g. James and Richardson 1993), which could occlude endogenous neuromodulation by adenosine during HFS trains, we performed experiments in which the CA3 hippocampal subslices were also stimulated at 50 Hz (five bursts of 150 pulses for 3 s; total number of pulses 240, as for the LFS) but with longer (17 s) interburst intervals (HFS_L). This protocol might temporally bypass inhibition of ecto-5′-nucleotidase and create conditions for the formation of adenosine from released adenine nucleotides (see Correia-de-Sá *et al.* 1996). However, under this HFS_L protocol, DPCPX (250 nM) also failed to modify ($n = 5$, $p > 0.05$) the evoked release of [^{3}H]ACh.

This modification of the effect of DPCPX on [^{3}H]ACh release according to the frequency of stimulation could be due to: (i) a modification of the source and/or metabolism of released adenosine available to activate A_1 receptors or (ii) to a desensitization and/or modification of efficacy of A_1 receptors. To distinguish between these two possibilities, we directly activated A_1 receptors with its selective agonist, CPA. The evoked release of [^{3}H]ACh under both paradigms of stimulation was inhibited by CPA (300 nM) in a similar manner (LFS: 26.3 ± 1.8% inhibition, $n = 3$; HFS: 23.2 ± 2.9% inhibition, $n = 3$), which seems to exclude the hypothesis of occurring a HFS-induced desensitization of A_1 receptors and favours the possibility of existing a different extracellular metabolism of adenosine reaching A_1 receptors under HFS.

Our first working hypothesis predicted that HFS would trigger a preferential formation of ATP-derived adenosine, potentiating A_{2A} receptor activation and enhancing the uptake activity of nucleoside transporters, thus largely decreasing A_1 receptor tonic activation. Some readily testable strings of this hypothesis were considered: (i) the blockade of nucleoside transporters would allow a greater accumulation of adenosine, which would now be able to activate A_1 receptors; (ii) the blockade of A_{2A} receptors would prevent the enhancement of nucleoside transporters efficiency and hence adenosine would accumulate and activate A_1 receptors. As predicted, upon blockade of ENTs with NBTI (10 μM, present in S1 and S2), DPCPX (250 nM, added before S2) was now able to increase by 15.6 ± 0.9% ($n = 5$, $p < 0.05$) the evoked release of [^{3}H]ACh triggered by HFS,

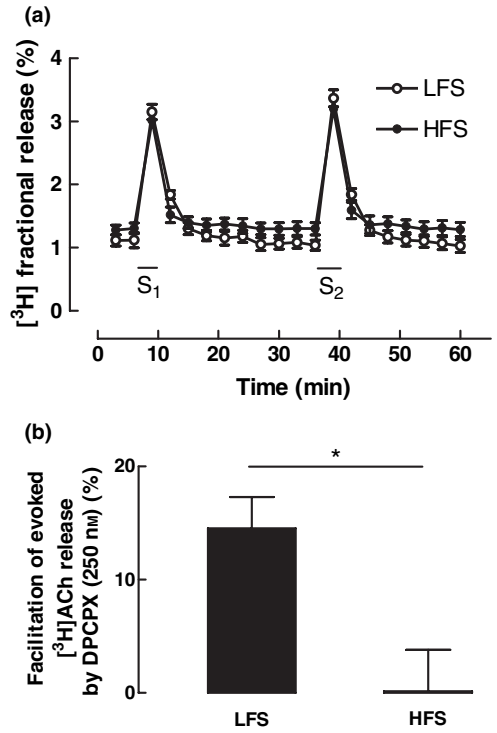

Fig. 4 [^{3}H]ACh release under high-frequency stimulation (HFS: four bursts of 1.2 s at 50 Hz, separated by 200 ms) and low-frequency stimulation (LFS: 2 Hz, 2 min) in the absence and in the presence of the A_1 selective antagonist, DPCPX (250 nM). (a) Time course of the tritium outflow from hippocampal slices under LFS (○) and under HFS (●) in control conditions, i.e. in the absence of drugs. Fractional release is the relative amount of tritium present in each collected sample in comparison to the total amount of tritium present in the tissue at the time of sample collection. The stimulation periods, S1 and S2, were applied at min 6 (S1) and min 36 (S2). Note that the relative amount of tritium released after stimulation is similar for both stimulation paradigms. The $S2/S_1$ ratios obtained under HFS (1.15 ± 0.02, $n = 8$) and LFS (1.12 ± 0.04, $n = 7$) were also not significantly different ($p > 0.05$). Each point is mean ± SEM (b) Comparison of the facilitatory effect of the selective A_1 receptor antagonist, DPCPX (250 nM), on the field electrically evoked release of [^{3}H]ACh from CA3 hippocampal slices under LFS and HFS, as indicated below each bar. DPCPX (250 nM) application 15 min before S2 significantly increased the S2/S1 ratios under LFS ($n = 4$, $p < 0.05$), but not under HFS ($n = 5$, $p > 0.05$). As indicated (*$p < 0.05$), the effects of DPCPX under both stimulation paradigms were statistically different. Each bar is mean ± SEM.

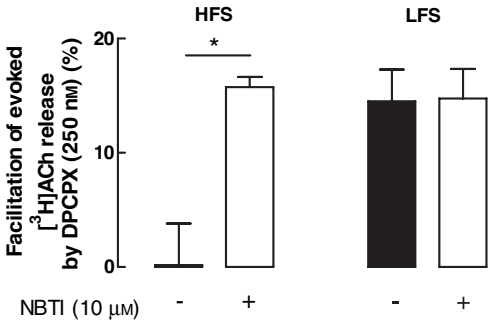

Fig. 5 Effect of the nucleoside transport blocker, NBTI (10 μM) on the facilitation caused by the selective A_1 antagonist, DPCPX (250 nM), of the evoked release of [^{3}H]ACh under high-frequency-stimulation (HFS: four bursts of 1.2 s at 50 Hz, separated by 200 ms) and low-frequency-stimulation (LFS, 2 Hz, 2 min) paradigms. The absence (–) or presence (+) of NBTI (10 μM) is indicated below each bar. NBTI (10 μM) was added before starting sample collection and was present thereafter during S1 and S2; DPCPX (250 nM) was added 15 min before S2. The action of DPCPX in the presence of NBTI (+) was calculated by comparing the S2/S1 ratio obtained in the same experiments in parallel chambers where only NBTI was added. The effect of DPCPX in the absence of NBTI (–) was calculated by taking as control the S2/S1 ratios obtained in the same experiments in parallel chambers with no added drugs. Note that the presence of NBTI (10 μM) enhanced the facilitatory effect of DPCPX (250 nM), under HFS ($n = 5$, *$p < 0.05$) but not under LFS ($n = 3$, $p > 0.05$), as compared to the absence of NBTI. Each bar is mean ± SEM.

in marked contrast with the lack of effect of DPCPX (250 nM) in the absence of the adenosine transport inhibitor (Fig. 5). Using the LFS paradigm, no significant ($n = 3$, $p > 0.05$) differences in the DPCPX-induced facilitation of [^{3}H]ACh-evoked release were encountered when looking at its effects in the absence or in presence of NBTI (10 μM; Fig. 5). We then tested if the blockade of A_{2A} receptor would increase A_1 receptor-mediated tonic inhibition of [^{3}H]ACh-evoked release. Also as predicted, in the presence of SCH 58261 (250 nM, present in S1 and S_2), DPCPX (250 nM, added before S2) significantly ($n = 4$, $p < 0.05$) increased HFS-evoked [^{3}H]ACh release by 32.7 ± 10.0%, in marked contrast with the lack of effect of DPCPX (250 nM) in the absence of the A_{2A} receptor antagonist (Fig. 6). SCH 58261 (250 nM) also tended to increase the effects of DPCPX (250 nM) under LFS stimulation but this did not reach statistical significance ($n = 4$, $p > 0.05$; Fig. 5).

From the results shown in Figs 5 and 6 it also emerged that the action of DPCPX upon HFS and LFS is similar, providing that adenosine transporters are inhibited (Fig. 5, 2nd and 4th bar) or A_{2A} receptors blocked (Fig. 6, 2nd and 4th bar).

Discussion

The main finding of the present work was that A_{2A} receptors enhance the activity of nucleoside transporters in rat hippocampal nerve terminals. Thus, activation of A_{2A} receptors with their selective agonist, CGS 21680, facilitated the uptake of adenosine and enhanced the evoked release of adenosine, which points to a direct effect of A_{2A} receptors on nucleoside transporters, rather than an indirect action resulting from a modification of the adenosine gradient of concentrations across the plasma membrane (i.e. metabolic

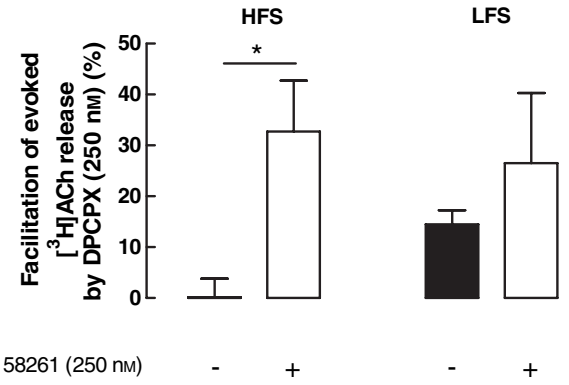

Fig. 6 Effect of the A_{2A} receptor agonist, SCH 58261 (250 nM) on the facilitation caused by the selective A_1 antagonist, DPCPX (250 nM), of the evoked release of [^{3}H]ACh under high-frequency-stimulation (HFS: four bursts of 1.2 s at 50 Hz, separated by 200 ms) and low-frequency-stimulation (LFS, 2 Hz, 2 min) paradigms. The absence or presence of SCH 58261 (250 nM) is indicated below each bar. SCH 58261 (250 nM) was added before starting sample collection and was present thereafter during S1 and S2; DPCPX (250 nM) was added 15 min before S2. The action of DPCPX in the presence of SCH 58261 (+) was calculated by comparing the S2/S1 ratio obtained in the same experiments in parallel chambers where only SCH 58261 was present. The effect of DPCPX in the absence of SCH 58261 (–) was calculated by taking as control the S2/S1 ratios obtained in the same experiments in parallel chambers with no added drugs. Note that the presence of SCH 58261 (250 nM) significantly increased the facilitatory effect of DPCPX (250 nM) under HFS ($n = 4$, $^*p < 0.05$) but not under LFS ($n = 4$, $p > 0.05$), as compared to the absence of SCH 58261. Each bar is mean ± SEM.

effect). Furthermore, as for the exogenously added A_{2A} receptor agonist, endogenous adenosine released upon HFS was able to activate A_{2A} receptors and enhance the activity of nucleoside transporters. The main consequence of this A_{2A} receptor-mediated enhancement of the activity of nucleoside transporters was the marked reduction of the tonic activation of inhibitory A_1 receptors upon high-frequency firing. This modulatory action of A_{2A} receptors on the activity of the adenosine transporters constitutes a clear demonstration that a neuromodulatory receptor is able to control the extracellular levels of its endogenous ligand and, hence, to influence its ability to control neurotransmitter release.

Nucleoside transporters are a family of membrane proteins with different pharmacological and kinetic properties that have in common their ability to transport nucleosides, namely adenosine, through the plasma membrane (reviewed in Cabrita *et al*. 2002). Two main groups of nucleoside transporters have been identified: (i) concentrative transporters (CNTs; Gray *et al*. 2004), which use sodium gradient to transport nucleosides against their concentration gradient and (ii) equilibrative bi-directional transporters (ENTs; Baldwin *et al*. 2004). Low nanomolar concentrations (50 nM) of NBTI block the ENT1 subtype (also called *es* from *e*quilibrative-*s*ensitive), whereas blockade of the *e*quilibrative *i*nsensitive (*ei*) ENT2 subtype is attained with micromolar (10 μM) concentrations of NBTI or dipyridamole (Archer *et al*. 2004). Therefore, in our experimental conditions, the NBTI (10 μM)-sensitive adenosine uptake and release might occur through ENTs, either of the ENT1 or ENT2 subtypes, which are widely expressed in the brain, including in hippocampal neurones (Anderson *et al*. 1999a, 1999b) and play the major role in controlling the extracellular levels of adenosine in neuronal cells. Evidence for moderate levels of CNT2 transcripts in adult rat brain including hippocampal neurones has been recently reported (Guillén-Gómez *et al*. 2004). It was also recently shown that the activity of CNT2 in non-excitable cells is enhanced by activation of A_1 receptors (Duflot *et al*. 2004), which in our experimental conditions did not influence adenosine uptake by nerve terminals. The relative contribution, if any, of CNTs for adenosine transport in the hippocampus and how they are regulated by selective activation of adenosine receptors remains to be investigated and is outside the scope of the present work.

In spite of the evidence available to support a predominant role of ENTs in controlling the levels of extracellular adenosine in the nervous system, little is known about the modulation of the activity of ENTs. They are known to display a peculiar mnemonic-like activity (Casillas *et al*. 1993) and some evidence has been gathered indicating that their activity can be modulated by G proteins (Sweeney 1996) and protein kinases (Sen *et al*. 1999). A_{2A} receptors are coupled to several G proteins and their activation influences at least the activities of two types of protein kinases, PKA and PKC. The findings that a PKC inhibitor, but not a PKA inhibitor, prevents the enhancement of adenosine transport induced by the A_{2A} receptor agonist, and that PKC activators *per se* influence adenosine transport in a similar way to CGS 21680, suggest that A_{2A} receptors control ENTs activity in a PKC-dependent manner.

The function of A_{2A} receptors in the hippocampus still remains controversial. In fact, the most evident role of endogenous extracellular adenosine in the hippocampus is an inhibition of synaptic transmission and neuronal excitability through the activation of the more abundant A_1 receptors (reviewed in Dunwiddie and Masino 2001). However, the activation of A_{2A} receptors causes a discrete facilitation of synaptic transmission and neurotransmitter release, which is in part due to their ability to decrease A_1 receptor function (Lopes *et al*. 2002). Also, A_{2A} receptors effectively control, in a permissive manner, the action of other neuromodulatory systems, leading to the proposal that A_{2A} receptors are mostly devoted to the fine-tuning of other neuromodulatory systems, rather than to directly controlling the release of neurotransmitters (Sebastião and Ribeiro 2000). The current observation that A_{2A} receptors can also control the activity of ENTs further widens the scope of the potential impact of A_{2A}

receptors in controlling the function of nerve terminals and challenges further investigations on other cell types in the brain that also possess A_{2A} receptors and ENTs, such as microglia (e.g. Küst *et al*. 1999; Hong *et al*. 2000) and astrocytes (e.g. Gu *et al*. 1996; Nishizaki *et al*. 2002). Interestingly, the fine-tuning neuromodulatory actions mediated by A_{2A} receptor activation may involve the PKA/cAMP transducing system (e.g. Diógenes *et al*. 2004) but in some cases, as observed in the present work, may be independent of PKA and involve PKC (e.g. Lopes *et al*. 2002).

One important aspect in the realm of the physiological function of the observed modulation of ENTs activity by A_{2A} receptor activation is to understand if this will lead to a greater ability to release or remove synaptic adenosine. In fact, the seminal work of Geiger's group has clearly established the bi-directional activity of ENTs (see Gu *et al*. 1995). When studying the release of adenosine from nerve terminals, inhibition of ENTs decreases the evoked release of adenosine (MacDonald and White 1985), which indicates that, at least in these structures, ENTs contribute to the release of the nucleoside. Nonetheless, in more integrated preparations, such as electrically stimulated brain slices, it is most commonly observed that inhibitors of ENTs actually enhance the extracellular levels of adenosine (reviewed in Latini and Pedata 2001), implying that the net result of ENT activity is to take up the nucleoside. Indeed, extracellular adenosine concentrations are usually higher than the intracellular ones because (i) neuronal stimulation leads to the release of adenine nucleotides that are converted to adenosine through the ecto-nucleotidase pathway (see Zimmermann 2000) and (ii) cytoplasmatic adenosine is quickly converted into AMP, through adenosine kinase (see e.g. Latini and Pedata 2001), which may further favour the uptake of adenosine in order to re-equilibrate its gradient across the cell membrane. In accordance with the predominant role of ENTs to take up, rather than to release, adenosine in hippocampal preparations, one should expect that the A_{2A} receptor-mediated enhancement of the activity of ENTs would result in an enhancement of the uptake of extracellular adenosine and therefore, to a lower degree of tonic activation of the predominant adenosine receptors in the hippocampus, the A_1 inhibitory receptors. This was directly confirmed when comparing adenosine modulation of the electrically evoked release of ACh at high and low frequencies of stimulation.

The different patterns of adenosine modulation of ACh-evoked release according to the frequency of stimulation observed in the present work, may be combined with what is known about frequency–dependency of adenosine metabolism, as summarized in Fig. 7. Upon HFS of hippocampal preparations, there is a predominant release of ATP and a predominant formation of adenosine from this released ATP (Cunha *et al*. 1996b). Thus, under conditions of HFS it is expected that the gradient of adenosine concentrations across

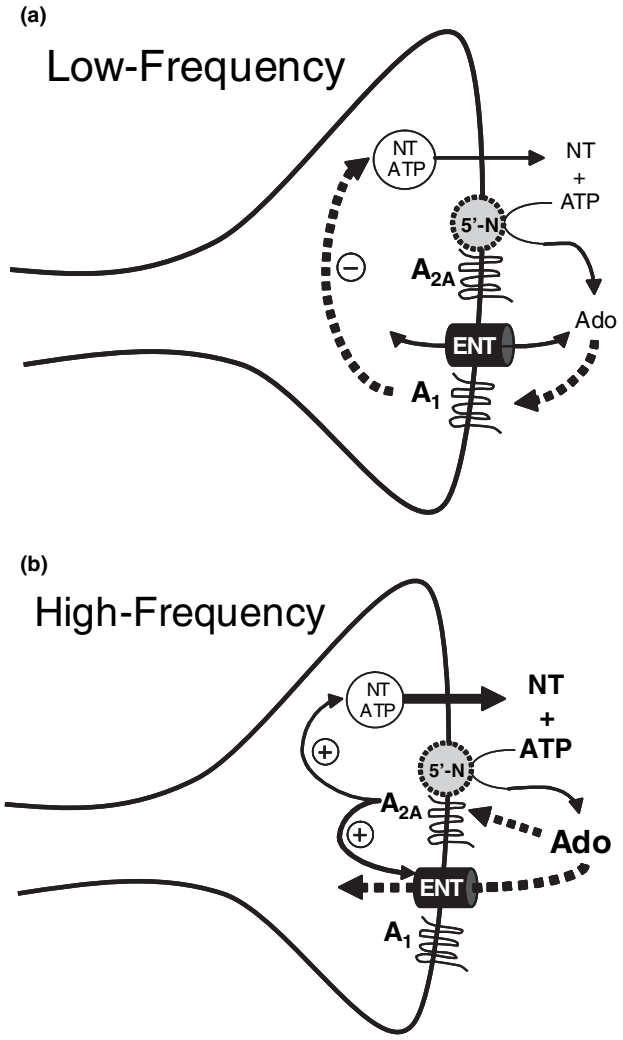

Fig. 7 Model for the role of A_{2A} receptors in the modulation of synaptic adenosine levels. Upon low frequency stimulation (a), the build-up of extracellular adenosine is moderate and dependent on both ENT's activity and the ecto-5′-nucleotidase (5′-N) pathway derived adenosine. In these conditions adenosine reaches A_1 receptors in the active zone, resulting in a tonic inhibitory effect of neurotransmitter (NT) release. During high frequency stimulation (b) the increased formation of ATP-derived adenosine leads to a greater activation of A_{2A} receptors that in turn increase the activity of nucleoside transporters to avoid a spread of extracellular adenosine, limiting its availability to activate A_1 receptors.

the plasma membrane will direct ENTs to take up adenosine. Also, it was previously shown that ATP-derived adenosine preferentially activates A_{2A} receptors (Cunha *et al*. 1996a) and, consequently, stimulation at high frequencies leads to a preferential activation of A_{2A} receptors. This is consistent with the hypothesis displayed in Fig. 7(b) that the greater activation of A_{2A} receptors by the increased formation of ATP-derived adenosine upon HFS might be increasing the activity of nucleoside transporters to avoid a spread of

extracellular adenosine sufficient to activate A_1 receptors. This means that there is a tight interplay between the extracellular metabolism of adenosine and the activation of adenosine receptors in the synaptic cleft to define the outcome of adenosine neuromodulation. Accordingly, under low-frequency firing there is a moderate build-up of extracellular adenosine that reach A_1 receptors because of the moderate activity of ENTs to take up adenosine (Fig. 7a). This hypothetical scenario, which successfully explains the data observed, takes into account the tight association between ecto-nucleotidases, namely ecto-5′-nucleotidase, and A_{2A} receptors (Cunha *et al*. 1996a; Napieralski *et al*. 2003) and re-enforces the likely close association of A_1 receptors with ENTs. However, it is obviously based on some key concepts that await experimental confirmation. Namely, it assumes that the different players involved in adenosine neuromodulation have a highly organized localization in a synapse and that there is a gradient of synaptic adenosine concentrations that varies according to the site of formation and removal of adenosine from the synapse.

In conclusion, the present work indicates that synaptic A_{2A} receptors in the hippocampus enhance the activity of ENTs. The study of the evoked release of ACh at different frequencies of stimulation allowed documenting a physiological situation where this A_{2A} receptor-mediated enhancement of ENTs activity plays a pivotal role in re-setting adenosine neuromodulation. Overall, these findings show a close interplay between adenosine production/inactivation and the activity of adenosine receptors to define adenosine neuromodulation.

Acknowledgements

Work supported by Fundação para a Ciência e a Tecnologia (FCT). APD and JEC were receiving FCT fellowships (POCTI/37332/FCB/2001 and SFRH/BD/937/2000). The authors wish to thank Catarina C. Fernandes for her collaboration in the final steps of the manuscript and Dr Scott Weiss (Vernalis, UK) for the kind gift of SCH 58261. The animal housing facilities of the Institute of Physiology of the Faculty of Medicine, University of Lisbon, are also acknowledged.

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