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Enhanced Adenosine A_{2A} Receptor Facilitation of Synaptic Transmission in the Hippocampus of Aged Rats

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Rebola, Nelson, Ana M. Sebastião, Alexandre de Mendonça, Catarina R. Oliveira, J. A. Ribeiro, and Rodrigo A. Cunha. Enhanced adenosine A_{2A} receptor facilitation of synaptic transmission in the hippocampus of aged rats. *J Neurophysiol* 90: 1295–1303, 2003; 10.1152/jn.00896.2002. Adenosine either inhibits or facilitates synaptic transmission through A₁ or A_{2A} receptors, respectively. Since A_{2A} receptor density increases in the limbic cortex of aged (24 mo) compared with young adult rats (2 mo), we tested if A_{2A} receptor modulation of synaptic transmission was also increased in aged rats. The A_{2A} receptor agonist, CGS21680 (10 nM), caused a larger facilitation of the field excitatory postsynaptic potential (fEPSP) slope in hippocampal slices of aged (38%) than in young rats (19%), an effect prevented by the A_{2A} receptor antagonist, ZM241385 (20 nM). In contrast to young rats, where CGS21680 facilitation of fEPSPs is prevented by the protein kinase C inhibitor, chelerythrine (6 μM), but not by the protein kinase A inhibitor, H-89 (1 μM), the CGS21680-induced facilitation of fEPSP slope in aged rats was prevented by H-89 (1 μM) but not by chelerythrine (6 μM). Also, in contrast to the β-receptor agonist, isoproterenol (30 μM), CGS21680 (100–1,000 nM) enhanced cAMP levels in hippocampal nerve terminals of aged but not young rats. Finally, we observed a significant increase of both the binding density of [³H]CGS 21680 and the [³H]ZM241385 as well as of the anti-A_{2A} receptor immunoreactivity in hippocampal nerve terminal membranes from aged compared with young rats. This shows that A_{2A} receptor-mediated facilitation of hippocampal synaptic transmission is larger in aged than young rats due to increased A_{2A} receptor density in nerve terminals and to the modified transducing system operated by A_{2A} receptors, from a protein kinase C mediated control of A₁ receptors into a direct protein kinase A dependent facilitation of synaptic transmission.

INTRODUCTION

Maintenance of CNS homeostasis with aging represents a major challenge, since neuronal cells cannot generally be replaced during the lifetime of an individual. Thus the nervous system has to find strategies to cope with the decreased efficiency of neuronal performance, similar to that occurring in CA1 hippocampal circuits (Barnes 1994; Barnes et al. 2000) that results from the general age-induced compromise of cellular function (Dani et al. 1997). The more direct approach to enhance excitatory transmission would be to use analogues of the main excitatory neurotransmitter in the brain, but glutamate analogues can also produce excitotoxic effects (Rothman

1984). An indirect approach is to interfere with neuromodulatory systems of glutamatergic transmission since they fine-tune transmission rather than support information processing.

One ubiquitous neuromodulatory system in the CNS is operated by adenosine. Adenosine neuromodulation is mostly conceived as an inhibitory system that restrains excitatory transmission through activation of inhibitory A₁ receptors (Dunwiddie and Masino 2001). However, neurons are equipped with other adenosine receptors, mainly A_{2A} receptors, whose activation results in a facilitation of neurotransmitter release (Cunha 2001). The search of the physiological relevance of these A_{2A} receptors has given rise to the idea that they mostly act to fine-tune other neuromodulatory systems (Sebastião and Ribeiro 2000) and, among others, to control the tonic inhibitory action of A₁ receptors (Lopes et al. 1999a). Thus the control by adenosine of neurotransmitter release should be conceived as a balance between inhibitory A₁ and facilitatory A_{2A} receptor-mediated actions (Cunha 2001).

This potential for adenosine to either inhibit or facilitate synaptic transmission makes this neuromodulatory system a likely candidate to be reset to compensate the age-related changes in neuronal performance. In fact, previous studies have shown a decrease in the density of A₁ receptors and an increase in the density of A_{2A} receptors in the limbic cortex and neocortex of aged rats (Cunha et al. 1995a). This decrease in the density of A₁ receptors is accompanied by a decreased ability of A₁ receptor agonists to inhibit synaptic transmission in the hippocampus of aged rats (Sebastião et al. 2000). Also, the increased density of A_{2A} receptors is accompanied by a greater efficiency of A_{2A} receptors to enhance acetylcholine release from hippocampal preparations (Lopes et al. 1999b). However, the role of A_{2A} receptors in the control of acetylcholine release appears to be different from the A_{2A} receptor-mediated facilitation of glutamatergic transmission in young adult rats. Thus the A_{2A} receptor-mediated facilitation of acetylcholine release is independent of A₁ receptor function (Cunha et al. 1995b) and involves activation of protein kinase A (Rebola et al. 2002), whereas the A_{2A} receptor-mediated facilitation of glutamatergic transmission results from a desensitization of tonic A₁ receptor-mediated inhibition of synaptic transmission (Lopes et al. 2002) and involves activation of protein kinase C (Cunha and Ribeiro 2000). Thus we now

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investigated if the A_{2A} receptor-mediated modulation of synaptic transmission is modified in hippocampal slices of aged rats.

We found that there is an increased ability of A_{2A} receptors to facilitate synaptic transmission in the hippocampus of aged rats and that this effect of A_{2A} receptors in aged rats is no longer due to a protein kinase C mediated attenuation of A₁ receptor tonic inhibition (Cunha and Ribeiro 2000; Lopes et al. 1999a, 2002) but rather to a protein kinase A mediated direct facilitation of synaptic transmission.

METHODS

1,3-Dipropyl-8-cyclopentyladenosine (DPCPX), 2-[4-(2-*p*-carboxyethyl)phenylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS21680), 8-[4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl]xanthine (XAC), and bisindolylmaleimide were from RBI (Natick, MA); chelerythrine and N-[2-(*p*-bromocinnamyl)amino]ethyl]5-isoquinolinesulfonamide (H-89) were from Calbiochem (Darmstadt, Germany); adenosine, 2-chloroadenosine, and isoproterenol were from Sigma (Reagente 5, Portugal); 4-(2-[7-amino-2-(2-furyl)(1,2,4)triazolo(2,3-*a*)(1,3,5)triazin-5-ylamino]ethyl)phenol (ZM241385) and [³H]ZM241385 (specific activity, 17 Ci/mmol) were from Tocris Cookson (Bristol, UK); [³H]CGS21680 (specific activity, 37.5 Ci/mmol) was from New England Nuclear (Anagene, Portugal); goat purified IgG anti-adenosine A_{2A} receptor antibody (200 μg/ml) was from Santa Cruz Biotechnology-Europe; rolipram [4-(3'-cyclopentyl-4'-methoxyphenyl)-2-pyrrolidone (4-RS)] was a gift of Schering AG; and [³H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([³H]SCH58261, specific activity, 77 Ci/mmol) was a generous gift of Dr. Ennio Ongini (Shering-Plough, Milan). Adenosine deaminase (1914 U/ml, EC 3.5.4.4) was purchased from Boehringer (Dorset, UK), in a suspension in 50% (vol/vol) glycerol in potassium phosphate (pH 6.0) and dilutions of this suspension were used, together with appropriate corrections of glycerol and KH₂PO₄ content of all control solutions.

Adenosine, 2-chloroadenosine, CGS21680, chelerythrine, H-89, and ZM241385 were made up to a 5 mM stock solution in dimethylsulfoxide; rolipram was made up in a 50 mM stock solution in dimethylsulfoxide and DPCPX was made up into 5 mM stock in 99% dimethylsulfoxide and 1% NaOH 1 M. These stock solutions were aliquoted and stored at -20°C and aqueous dilution of these stock solutions was made daily.

Animals

Male Wistar rats from the Gulbenkian Institute animal house or from Harlan Iberica were used throughout this study. Young adult rats were 6 weeks old (140–160 g), whereas aged rats were 21–24 mo old (950–1,080 g). In some experiments, 18-mo-old rats were used (960–1,020 g). Most studies in purinergic modulation have been performed in 6-weeks-aged rats, which are considered juvenile or young adult rats, whereas 18- to 24-mo-old rats are considered aged rats (i.e., close to the limit of life expectancy; Masoro 1991; Miller and Nadon 2000). Indeed, around 30% of the initial population of rats allocated for this study did not reach this age range, confirming by operational criteria that rats 18–24 mo old are aged rats (see Masoro 1991). In neither group of aged rats was there any evidence of gross anatomical lesions in the brain. The handling and use of these animals was according the EU guidelines for use of experimental animals, the animals being anesthetized under halothane atmosphere before being killed by decapitation. The number of animals used was kept to the absolute minimum, with care not to use more animals than these required to obtain statistically significant results.

Electrophysiological recordings of hippocampal synaptic transmission

One 400-μm hippocampal slice, obtained as previously described (e.g., Lopes et al. 1999a), was transferred to a 1-ml recording chamber for submerged slices and continuously superfused, at a flow rate of 3 ml/min, with gassed (95% O₂-5% CO₂) Krebs solution, kept at 30°C, of the following composition (in mM): 125 NaCl, 3 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1 MgSO₄, and glucose 10, pH 7.4. Drugs were added to this superfusion solution. Electrophysiological recordings of field excitatory postsynaptic potentials (fEPSP) were obtained as previously described (e.g., Sebastião et al. 2000). Stimulation (rectangular pulses of 0.1 ms applied once every 15 s) was delivered through a bipolar concentric electrode placed on the Schaffer fibers, in the stratum radiatum near the CA3/CA1 border. Orthodromically evoked fEPSPs were recorded through an extracellular microelectrode (4 M NaCl, 2–5 MΩ resistance) placed in the stratum radiatum of the CA1 area. The intensity of the stimulus [which was similar in hippocampal slices from young adult (220 ± 40 μA, *n* = 7) and aged rats (290 ± 30 μA, *n* = 14)] was adjusted to evoke a fEPSP with an amplitude of 0.7–1 mV without appreciable population spike contamination. Recordings were obtained with an Axoclamp 2B amplifier coupled to a DigiData 1200 interface (Axon Instruments, Foster City, CA) and averages of eight consecutive responses were continuously monitored on a personal computer with the LTP 1.01 software (Anderson and Collingridge 1997). Responses were quantified as the initial slope of the averaged fEPSPs and the effect of drugs, added to this superfusion solution, was estimated by changes in the fEPSP slope compared with baseline.

A_{2A} receptor binding to hippocampal nerve terminals membranes

[³H]CGS21680 or [³H]ZM241385 binding studies were performed as previously described (e.g., Cunha et al. 1995a, 1997) using membranes from hippocampal nerve terminals (see e.g., Lopes et al. 1999a). Briefly, [³H]CGS21680 (60 nM) binding was for 4 h; [³H]ZM241385 (2 nM) binding was for 60 min, and [³H]SCH58261 (10 nM) was for 60 min at room temperature (23–25°C), with 282–384 μg of membrane protein in a final volume of 300 μl in an incubation solution containing 50 mM Tris-HCl and 10 mM MgCl₂, pH 7.4, with 5 U/ml adenosine deaminase. Specific binding was determined by subtraction of the nonspecific binding, which was measured in the presence of 2 μM XAC. The binding reactions were stopped by vacuum filtration through Whatman GF/C glass fiber filters, followed by washing of the filters and reaction tubes with 10 ml of the incubation solution, kept at 4°C. The filters were then placed in scintillation vials, and 5 ml of scintillation liquid (Scintran Cocktail T, Wallac, Pharmacia-Portugal) was added. Radioactivity bound to the filters was determined after 12 h with an efficiency of 55–60% for 2 min. Binding assays were performed in duplicate. Membrane protein was determined according to Peterson (1977).

cAMP assays in hippocampal nerve terminals

Hippocampal synaptosomes were prepared as previously described (e.g., Cunha et al. 1995b) and resuspended in 1 ml gassed Krebs solution also containing 2 U/ml adenosine deaminase and 50 μM rolipram. A 90-μl synaptosomal aliquot was warmed at 37°C for 15 min and then incubated at 37°C for 4 min with gassed Krebs containing adenosine deaminase and rolipram (control) or with this modified Krebs containing isoproterenol (30 μM) or CGS21680 (30–1,000 nM) without or with ZM241385 (20 nM). The treated synaptosomes were then inactivated by boiling for 10 min in 1 ml of 50 mM Tris/4 mM EDTA, pH 7.6. The levels of cAMP in the supernatants obtained after sonication and centrifugation (14,000 *g*, 10 min, 4°C) were quantified with a radioimmunoassay kit (Amersham), as previously

described (Lopes et al. 1999b), and protein was measured according to Peterson (1977).

Western blot analysis of A_{2A} receptor immunoreactivity in hippocampal nerve terminals

The analysis of adenosine A_{2A} receptor immunoreactivity was carried out as previously described (Rebola et al. 2002) in membranes from a Percoll-purified synaptosomal fraction of the hippocampus (e.g., Lopes et al. 1999a). Briefly, after determining the amount of protein (Spector 1978), each sample was diluted with two volumes of a solution containing 8 M urea, 100 mM dithiothreitol, 2% (wt/vol) sodium dodecyl sulfate, and 375 mM Tris-HCl pH 6.8 and incubated for 2 h at 37°C. These diluted samples and the prestained molecular weight markers (Amersham) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% with a 4% concentrating gel) under reducing conditions and electro-transferred to polyvinylidene difluoride membranes (0.45 μm from Amersham). After blocking for 2 h at room temperature with 5% milk in Tris-buffered saline, pH 7.6 containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4°C with the goat IgG anti-adenosine A_{2A} receptor antibody (1:100 dilution). After four washing periods for 10 min with TBS-T containing 0.5% milk, the membranes were incubated with the alkaline phosphatase conjugated rabbit anti-goat IgG secondary antibody (1:2,000 dilution from Calbiochem) in TBS-T containing 1% milk for 90 min at room temperature. After five 10-min washes in TBS-T containing 0.5% milk, the membranes were incubated with Enhanced Chemi-Fluorescence for 5 min and then analyzed with a Storm (Molecular Devices).

Statistics

The values presented are mean ± SE of *n* experiments. To test the significance of the effect of a drug versus control, a paired Student's *t*-test was used. When making comparisons from different sets of experiments with control, ANOVA was used, followed by Dunnett's test. *P* ≤ 0.05 was considered to represent a significant difference.

RESULTS

Modification of adenosine A_{2A} receptor modulation of synaptic transmission in the hippocampus of aged rats

Since there is an increase in the number of CGS21680 binding sites in the hippocampus of aged rats (Cunha et al. 1995a), we investigated whether there was also an increased efficiency of A_{2A} receptors to modulate hippocampal synaptic transmission in aged rats. A single concentration (10 nM) of the A_{2A} receptor agonist, CGS21680, was tested, since it produces a maximal facilitation of synaptic transmission in young adult animals (Cunha et al. 1997). In the present set of experiments, CGS21680 (10 nM) caused a facilitation of 19 ± 2% (*n* = 5) in young adult rats and a greater facilitation (38 ± 6%, *n* = 8, *P* < 0.05) of fEPSP slope in aged rats (Fig. 1, *A* and *B*). To compare the effect of CGS21680 between the two age groups, a similar intensity of stimulation was used, which caused a smaller fEPSP slope and amplitude in aged rats, as previously observed (e.g., Bauman et al. 1992; Sebastião et al. 2000); and, although input/output curves were not performed in the two age groups, previous studies in young adult rats suggested that the CGS21680-induced facilitation of synaptic transmission is apparently not related to the strength of stimulation (see Cunha et al. 1997; Cunha and Ribeiro 2000; Lopes et al. 1999a, 2002). In 18-mo-old rats, CGS21680 (10 nM) caused a 41 ± 4% (*n* = 4) facilitation of fEPSP slope, and, as illustrated

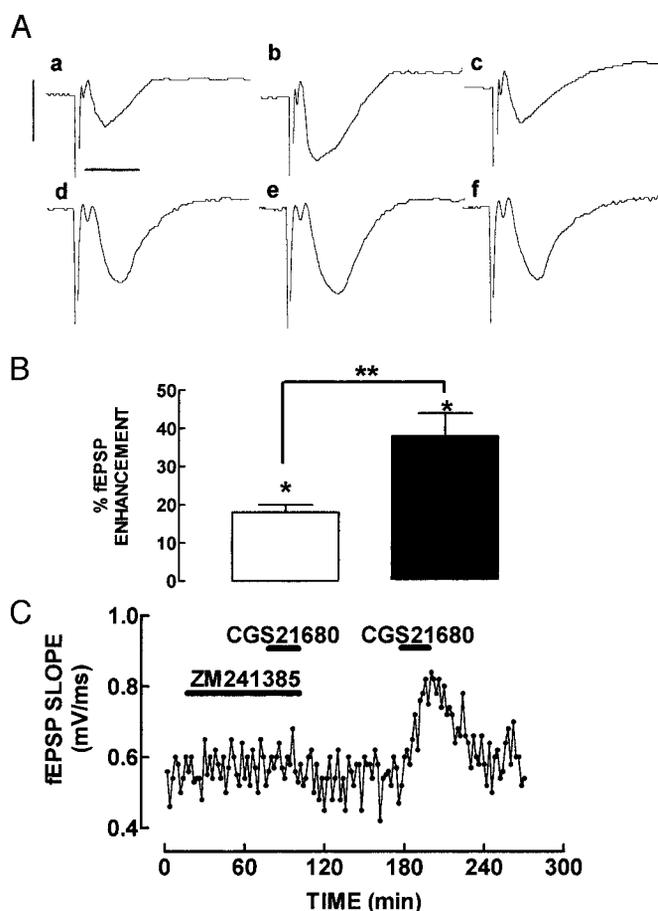


FIG. 1. Comparison of the effects of the adenosine A_{2A} receptor agonist, CGS21680 (10 nM), on glutamatergic transmission in hippocampal slices of young adult (6 wk) and aged (24 mo) rats (*A* and *B*) and prevention of the effect of CGS21680 (10 nM) by the A_{2A} receptor antagonist, 4-(2-[7-amino-2-(2-furyl)(1,2,4)triazolo(2,3-a)(1,3,5)triazin-5-ylamino]ethyl)phenol (ZM241385; 20 nM), in aged (18 mo) rats (*C*). In *A* are shown averages of 8 consecutive field excitatory postsynaptic potentials (fEPSPs) recorded from the CA1 area of hippocampal slices from aged (*a*, *b*, and *c*) and young rats (*d*, *e*, and *f*). The fEPSPs were obtained in *a* and *d* before adding any drug to the superfusion solution, in *b* and *e* 20–24 min after application of CGS21680 (10 nM), and in *c* and *f* 40–42 min after washing out the A_{2A} receptor agonist. Calibration bars: 500 μV, 10 ms. Note that the slope and amplitude of the fEPSPs in the aged rats were lower than those recorded from young rats, as previously reported (e.g., Bauman et al. 1992). In *B*, the ordinates represent the average percentage increase of fEPSP slope caused by CGS21680 (10 nM) in slices from aged (filled bar, *n* = 8, control fEPSP slope of 0.33 ± 0.03 mV/ms) and from young adult rats (open bar, *n* = 5, control fEPSP slope of 0.41 ± 0.02 mV/ms). **P* < 0.05 vs. baseline; ***P* < 0.05 between the 2 age groups. In (*C*) is shown a time course of a representative experiment in a hippocampal slice from an aged rat (18 mo) which was first superfused with ZM241385 (20 nM); then with CGS21680 (10 nM) in the presence of ZM241385 (20 nM), the 2 drugs were washed out and CGS21680 (10 nM) was re-applied, as shown in the horizontal bars.

in Fig. 1*C*, this effect of CGS21680 was prevented by the adenosine A_{2A} receptor antagonist, ZM241385 (20 nM, *n* = 3), as has been shown to occur in young adult animals (Cunha et al. 1997). ZM241385 (20 nM) was devoid of effects on synaptic transmission in young rats, as well as in 18-mo-old rats (*n* = 3). It should be pointed out that although ZM241385 is the best A_{2A} receptor antagonist commercially available, it is not an ideal tool to probe the role of tonic A_{2A} receptor activation by endogenous adenosine since it also interferes with A₁ receptor-mediated responses (Lopes et al. 1999c),

although it only interferes with A_1 receptor binding in micromolar concentrations (Cunha et al. 1997).

Although in young adult rats it is possible to record facilitatory A_{2A} receptor-mediated effects on synaptic transmission, these facilitatory effects are not due to a direct facilitation of synaptic transmission, but rather to an A_{2A} receptor-induced decrease of tonic A_1 receptor-mediated inhibition of synaptic transmission (Lopes et al. 1999a, 2002). Accordingly, superfusion of hippocampal slices from young adult rats with adenosine causes a concentration-dependent inhibition of synaptic transmission that is blocked by effective concentrations of A_1 receptor antagonists, such as DPCPX (e.g., Dunwiddie and Diao 1994). As occurs in young adult rats, superfusion of hippocampal slices of aged rats with adenosine (50 μ M) also produced a nearly complete blockade of synaptic transmission (Fig. 2). However, in contrast with what occurs in young adult rats (Fig. 2A), in the presence of DPCPX (50 nM), adenosine now increased the fEPSP slope in hippocampal slices of aged rats ($57.4 \pm 3.9\%$, $n = 4$; $P < 0.05$) (Fig. 2B). A close chemical analogue of adenosine that is an agonist of all adenosine receptors and is less prone to metabolism, 2-chloroadenosine (10 μ M), also facilitated ($66.9 \pm 7.4\%$, $n = 3$, $P < 0.05$) fEPSP slope in the presence of DPCPX (50 nM) in aged rats, excluding the possibility that modifications of adenosine metabolism in aged rats (Cunha et al. 2001) might be responsible for this effect (Fig. 2B). These facilitatory effects of adenosine and 2-chloroadenosine recorded in the presence of DPCPX (50 nM) in aged rats were attenuated by ZM241385 (20 nM), indicating the involvement of A_{2A} receptor activation (Fig. 2, B and C).

This observation that adenosine is able to facilitate synaptic transmission in aged rats on blockade of inhibitory A_1 receptors further supports a greater functional impact of A_{2A} receptor activation in aged rats. Also, it indicates that the mechanism by which A_{2A} receptors facilitate synaptic transmission is different in aged and in young adult rats. In fact, in young adult rats, the A_{2A} receptor-induced facilitation of synaptic transmission is blocked on removal of tonic A_1 receptor-mediated inhibition of synaptic transmission (Lopes et al. 1999a, 2002), whereas in aged rats the A_{2A} receptor-mediated facilitation of synaptic transmission is independent of A_1 receptor function. Since A_{2A} receptors can couple to different G proteins in different systems (Cunha 2001), we explored the possibility that there was a difference in the transducing systems operated by A_{2A} receptors in young adult and in aged rats.

Effect of A_{2A} receptor activation on cAMP levels in hippocampal nerve terminals

In young adult rats, A_{2A} receptor-mediated facilitation of hippocampal synaptic transmission is protein kinase C dependent, but not protein kinase A dependent (Cunha and Ribeiro 2000). However, in several other systems, A_{2A} receptors facilitate neurotransmitter release on increasing cAMP levels in nerve terminals (Correia-de-Sá and Ribeiro 1994; Gubitza et al. 1996; Okada et al. 2001). Activation of A_{2A} receptors causes a greater accumulation of cAMP in cortical slices of aged compared with young adult rats (Lopes et al. 1999b), but it is not known if this also occurs in hippocampal nerve terminals.

Since we had previously observed that the concentration range required to detect CGS21680-induced cAMP increases is

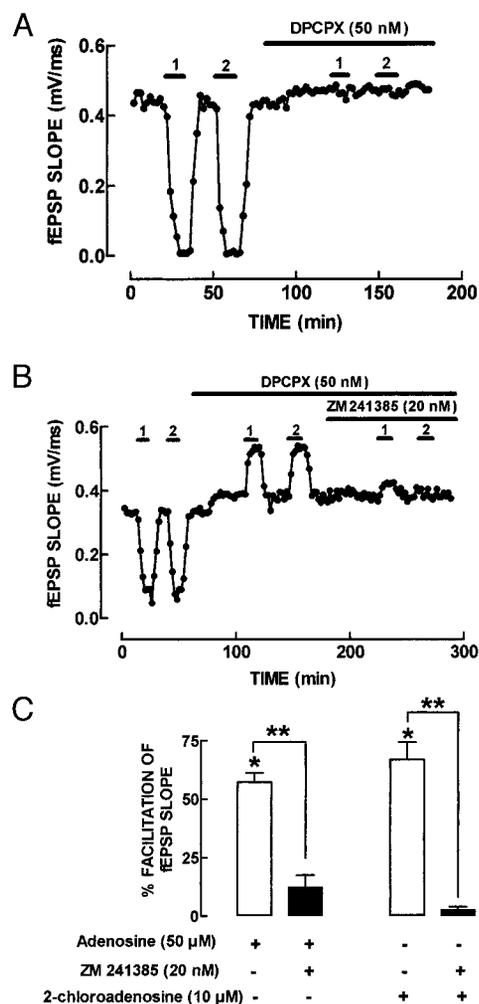


FIG. 2. Adenosine and 2-chloroadenosine facilitate glutamatergic transmission in hippocampal slices of aged (22 mo), but not young adult (6 wk), rats on blockade of adenosine A_1 receptors with 1,3-dipropyl-8-cyclopentyladenosine (DPCPX; 50 nM), in a manner sensitive to the adenosine A_{2A} receptor antagonist, ZM241385 (20 nM). *A* and *B*: time courses of representative experiments presenting the averages of the slopes of 8 consecutive fEPSPs recorded from the CA1 area of hippocampal slices from young adult (*A*) and aged rats (*B*) showing that adenosine (50 μ M, labeled as 1) and 2-chloroadenosine (10 μ M, labeled as 2) nearly block the fEPSP in both young adult and aged rats. However, in the presence of DPCPX (50 nM), the effect of adenosine (50 μ M) and 2-chloroadenosine (10 μ M) is abolished in young adult rats (*A*) but is converted into a facilitatory effect in aged rats (*B*) that is prevented in the presence of ZM241385 (20 nM). Each drug was present when indicated by the upper bars in *A* and *B* (1 indicates the presence of adenosine and 2 indicates the presence of 2-chloroadenosine). In *C*, the ordinates represent the average percentage increase of fEPSP slope caused by adenosine (50 μ M) or by 2-chloroadenosine (10 μ M) in slices from aged rats (19–22 mo) always in the presence of DPCPX (50 nM) and in the absence or in the presence of ZM241385 (20 nM) as indicated under each bar (control fEPSP slope of 0.36 ± 0.04 mV/ms). * $P < 0.05$ vs. 0%; ** $P < 0.05$ between indicated bars.

nearly 10-fold greater than that required to facilitate neurotransmitter release in other CNS preparations (Lopes et al. 1999b), we tested the effect of higher concentrations (30–1,000 nM) of CGS21680 on cAMP levels. As shown in Fig. 3A, CGS21680 (30–1,000 nM) failed to modify cAMP levels in rat hippocampal nerve terminals of young adult rats. In contrast, in aged rats, CGS21680 enhanced cAMP levels in hippocampal nerve terminals from a basal level of 5.2 ± 0.9 pmol/mg protein ($n = 4$) to a maximal level of 12.2 ± 1.1

pmol/mg protein ($n = 4$) at 300 nM CGS21680 (Fig. 3A). This stimulatory effect of CGS21680 was mediated by A_{2A} receptor activation since ZM241385 (20 nM) attenuated the stimulatory effect of CGS21680 (300 nM) on cAMP levels in hippocampal nerve terminals of aged rats (Fig. 3A). It should be noted that the basal levels of cAMP were lower in nerve terminals of aged compared with young adult rats, as previously observed in different brain preparations (Lopes et al. 1999b; Zimmerman and Berg 1974). This is an indication that the tonic activation of A_{2A} receptors is not a receptor system responsible for the control of basal cAMP levels in hippocampal nerve terminals.

To test if this A_{2A} receptor-induced increase in cAMP was due to an increased efficiency of A_{2A} receptors rather than to a general responsiveness of adenylate cyclase-coupled receptor in hippocampal nerve terminals from aged rats, we compared the ability of the β -adrenergic receptor agonist, isoproterenol, to increase cAMP levels in hippocampal nerve terminals of young adult and aged rats, since β -receptor activation increases cAMP in rat cortical nerve terminals (Herrero and

Sanchez-Prieto 1996). As shown in Fig. 3B, isoproterenol (30 μ M) caused a nearly similar increase of cAMP levels in young adult (58.2 ± 5.2 pmol/mg protein, $n = 4$) and in aged rat hippocampal nerve terminals (46.6 ± 4.1 pmol/mg protein, $n = 4$).

Transducing system operated by A_{2A} receptors to facilitate synaptic transmission in aged rats

The findings that A_{2A} receptor-mediated facilitation of hippocampal synaptic transmission in aged rats occurred independently of A₁ receptors and that A_{2A} receptors triggered cAMP accumulation in aged hippocampal nerve terminals led us to test if A_{2A} receptors facilitated hippocampal synaptic transmission in aged rats via an adenylate cyclase/cAMP/protein kinase A pathway, or through a protein kinase C pathway, as has been observed in young adult rats (Cunha and Ribeiro 2000).

Figure 4A shows time-course recordings of synaptic transmission in hippocampal slices from aged rats that illustrate the ability of the protein kinase A inhibitor, H-89 (1 μ M), to prevent the facilitatory effects of CGS21680 (10 nM), and the ability of the protein kinase C inhibitor, chelerythrine (6 μ M), only to attenuate the facilitatory effect of CGS21680 (10 nM). In the average of three experiments, CGS21680 (10 nM) caused a $67.6 \pm 18.7\%$ facilitation of fEPSP slope and this effect was attenuated by $26.5 \pm 9.5\%$ by chelerythrine (6 μ M) and by $81.2 \pm 2.1\%$ by H-89 (1 μ M) (Fig. 4B). In a fourth experiment in aged rats, CGS21680 increased fEPSP slope by 48% and this facilitatory effect was attenuated by 91% by H-89 (1 μ M) and only by 16% by another protein kinase C inhibitor, bisindolylmaleimide (1 μ M). By themselves, chelerythrine (6 μ M) inhibited by $16 \pm 3\%$ fEPSP slope in aged rats, whereas H-89 (1 μ M) was virtually devoid of effects ($5 \pm 4\%$ inhibition of fEPSP slope, $n = 3$).

Increase in the density of A_{2A} receptors in hippocampal nerve terminals of aged rats

The initial reason that led us to test if there was an increase facilitatory effect of A_{2A} receptor activation on hippocampal synaptic transmission was the observation that there was an increased density of A_{2A} receptors in the hippocampus of aged rats (Cunha et al. 1995a). This increase was measured in whole membranes, whereas the A_{2A} receptor facilitation of fEPSPs and of cAMP levels in aged rats results from activation of presynaptic A_{2A} receptors. Thus it remained to be established if there was also a proportionally greater increase in the density of A_{2A} receptors in hippocampal nerve terminals of aged rats. For that purpose, we initially carried out binding studies with an agonist and antagonist of A_{2A} receptors, [³H]CGS21680 (60 nM) and [³H]ZM241385 (2 nM) (Cunha et al. 1997), in membranes derived from synaptosomes prepared from the hippocampus of young adult and aged rats. The data summarized in Fig. 5, A and B, show that there was an increased density of both [³H]CGS21680 (60 nM) and [³H]ZM241385 (2 nM) binding to hippocampal nerve terminal membranes of aged compared with young adult rats ($n = 4$), which is proportionally greater than that occurring in whole hippocampal membranes (Cunha et al. 1995a).

Finally, we confirmed this increased density of A_{2A} receptors in membranes of hippocampal nerve terminals of aged rats

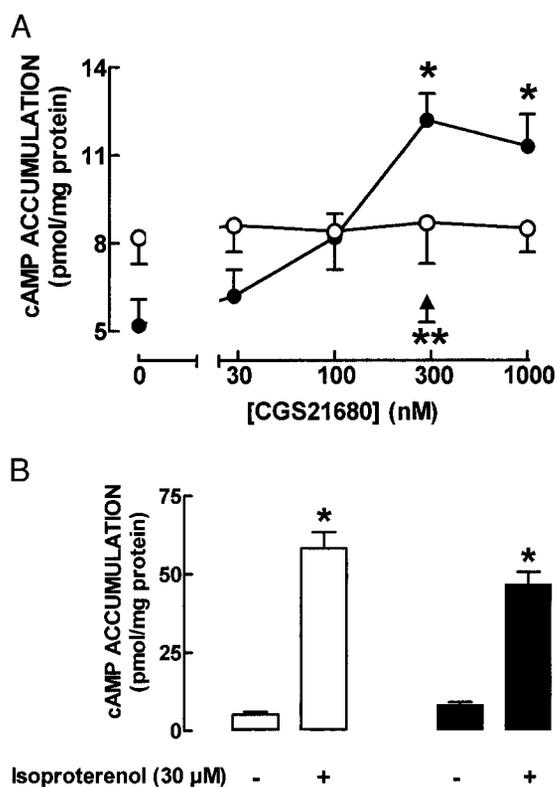


FIG. 3. Activation of A_{2A} receptors increase the levels of cAMP in hippocampal nerve terminals in aged but not in young adult rats, whereas the activation of β adrenergic receptors leads to a similar increase in cAMP levels in young adult and aged hippocampal nerve terminals. Rat hippocampal synaptosomes (342–612 μ g protein) from young adult rats (6 wk, open symbols and bars) or aged rats (19–22 mo, filled symbols and bars) were incubated for 4 min with Krebs containing 2 U/ml adenosine deaminase and 50 μ M rolipram without or with the adenosine A_{2A} receptor agonist, CGS21680 (30–1,000 nM), in the absence (circles) or in the presence of the A_{2A} receptor antagonist ZM241385 (20 nM, triangles) in (A) or with the β -adrenergic receptor agonist, isoproterenol (30 μ M) in (B). The treated synaptosomes were then inactivated and sonicated; their cAMP levels were quantified with a radioimmunoassay kit and the values were normalized per mg of protein. In (A) and (B) the ordinates represent the amounts of cAMP in nerve terminals. The values are mean \pm SE of 4 experiments performed in duplicate in both (A) and (B). * $P < 0.05$ vs. basal value; ** $P < 0.05$ versus absence of ZM241385 (20 nM) in (A).

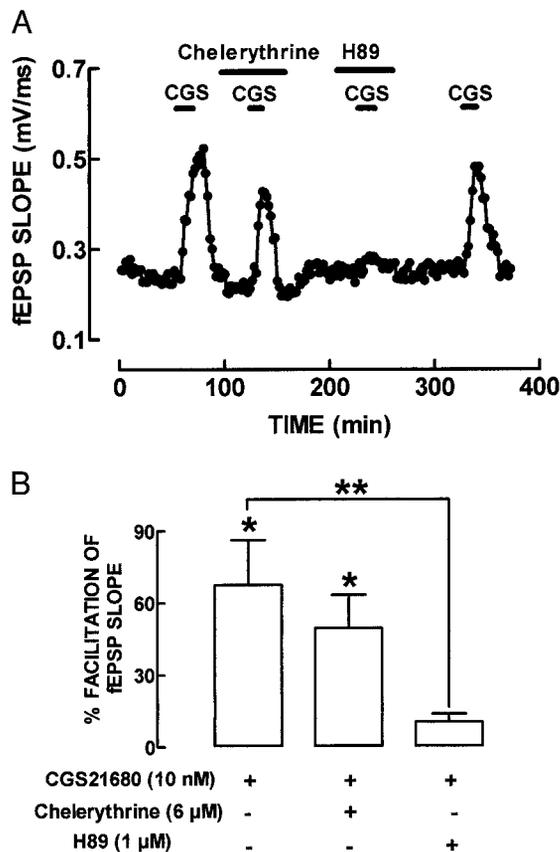


FIG. 4. Comparison of the ability of the protein kinase A inhibitor, H-89 (1 μ M), and of the protein kinase C inhibitor, chelerythrine (6 μ M), to modify the facilitatory effect of the adenosine A_{2A} receptor agonist, CGS21680 (10 nM), on synaptic transmission in rat hippocampal slices of aged rats. In (A) are shown averages of the slopes of 8 consecutive fEPSPs recorded from the CA1 area of an hippocampal slice of an aged rat (21-mo-old) which was superfused with CGS21680 (10 nM) in the absence and in the presence of either the protein kinase A inhibitor, H-89 (1 μ M), or the protein kinase C inhibitor, chelerythrine (6 μ M), as indicated by the upper bars. In (B), the ordinates represent the average percentage increase of fEPSP slope in hippocampal slices from aged rats (19–21 mo) caused by CGS21680 (10 nM) in the absence (control fEPSP slope of 0.31 ± 0.06 mV/ms) and in the presence of either H-89 (1 μ M) or chelerythrine (6 μ M). The results are mean \pm SE of 3 experiments. * $P < 0.05$ vs. 0%; ** $P < 0.05$ between the indicated bars.

using Western blot analysis. As illustrated in Fig. 5C, there was a more intense immuno-reactivity of the anti-adenosine A_{2A} receptor antibody in hippocampal nerve terminals from aged compared with young adult rats. In three Western blots carried out with membranes from three different groups of young adult and aged rats, we observed a $76 \pm 6\%$ increase in the densitometrically measured immuno-reactivity in aged compared with young adult rats.

Age-dependent increase in A_{2A} receptor binding and A_{2A} receptor-induced cAMP accumulation

The results presented so far indicate that there is an increased density of A_{2A} receptors and a change in the transducing system operated by A_{2A} receptors in the hippocampus of aged rats. However, given that 20 mo is a relatively long time window in a rat, it is not clear if the changes observed for the neuromodulatory system operated by A_{2A} receptors result from a gradual change over life span or to a switch occurring

essentially in the aged rats. To address this question, we investigated the changes in the density of A_{2A} receptors and in the ability of A_{2A} receptor activation to increase cAMP levels in nerve terminals from the hippocampus of rats at 0.5, 2, 6, 12, 18, and 24 mo.

As illustrated in Fig. 6A, the binding A_{2A} receptor antagonist, [3 H]SCH58261 (10 nM), was not statistically different ($P > 0.05$) in hippocampal nerve terminals membranes derived from animals with ages ranging from 0.5 to 12 mo. However, the specific binding of [3 H]SCH58261 (10 nM) was significantly ($P < 0.05$) larger in hippocampal nerve terminals membranes from 18-mo-old rats and did not further increase in rats at 24 mo (Fig. 6A). Likewise, there was no significant ($P >$

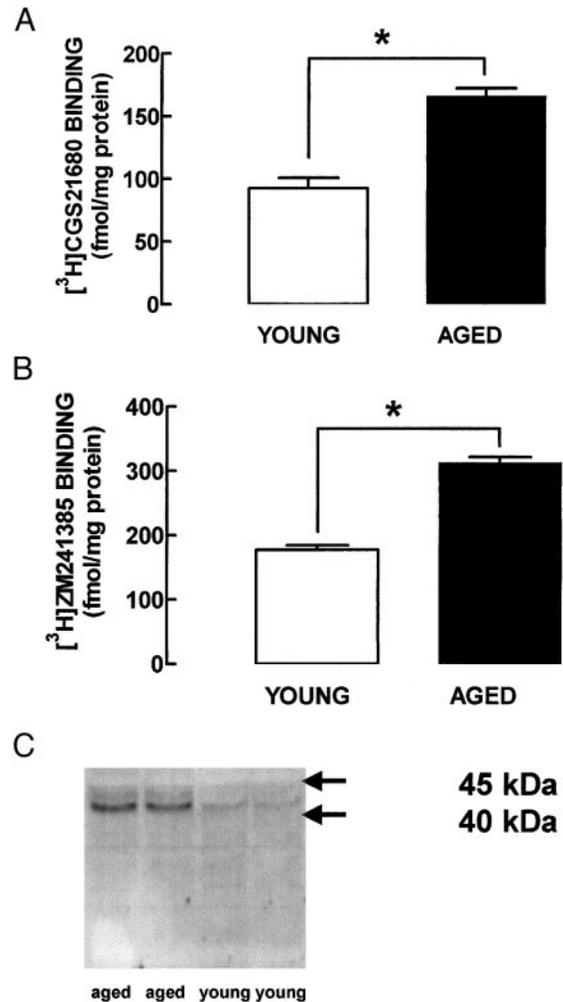


FIG. 5. Comparison of the specific binding of the A_{2A} receptor agonist, [3 H]CGS21680 (60 nM, in A), of the A_{2A} receptor antagonist, [3 H]ZM241385 (2 nM, in B), and of A_{2A} receptor immuno-reactivity (C) to membranes prepared from synaptosomes derived from the hippocampus of young adult (6 wk, open bars in A and B) and aged rats (19- to 21-mo-old, filled bars in A and B). In A and B, the ordinates represent the specific binding of either [3 H]CGS21680 (A) or [3 H]ZM241385 (B) obtained on subtraction of the nonspecific binding, determined in the presence of 2 μ M 8-[4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl]xanthine (XAC), from total binding. Results are mean \pm SE of 4 experiments performed in duplicate. C: Western blot comparing the immuno-reactivity obtained with an anti- A_{2A} receptor antibody in hippocampal synaptosomal membranes from aged (22-mo-old; first 2 lanes from left labeled as aged) and young adult rats (6-wk-old; first 2 lanes from right labeled as young), with 120 μ g of membrane protein being applied in each lane.

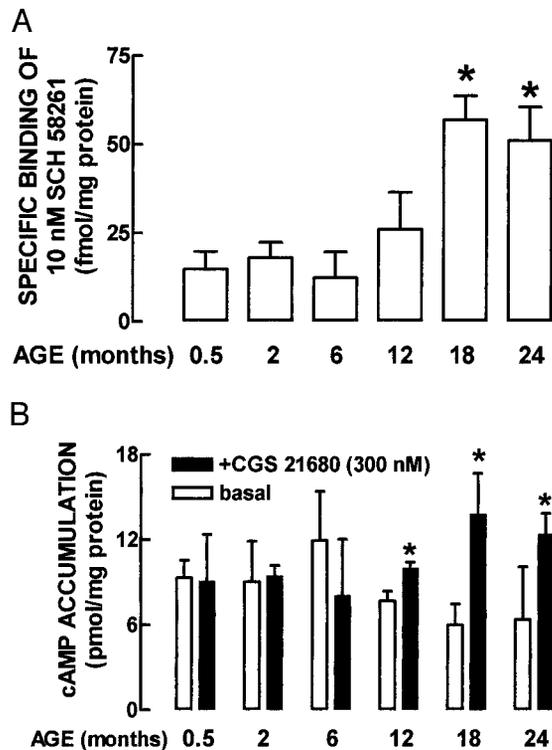


FIG. 6. Changes in the binding density of A_{2A} receptors and in their ability to increase cAMP levels in hippocampal nerve terminals. *A*: the average specific binding of the A_{2A} receptor antagonist, [³H]SCH58261, to membranes prepared from synaptosomes derived from the hippocampus of rats at 0.5, 2, 6, 12, 18 and 24 mo, as indicated below each bar. The ordinates represent the specific binding of [³H]SCH58261 obtained on subtraction of the nonspecific binding, determined in the presence of 2 μM XAC, from total binding. Results are mean ± SE of 3–4 experiments performed in duplicate. **P* < 0.05 when compared with value obtained for 2-mo-old rats. *B*: the average levels of cAMP measured in nerve terminals from rats at 0.5, 2, 6, 12, 18 and 24 mo (as indicated below each bar) when incubated for 4 min with 2 U/ml adenosine deaminase and 50 μM rolipram without (basal, open bars) or with 300 nM CGS21680 (filled bars). The treated synaptosomes were then inactivated and sonicated; their cAMP levels were quantified with a radioimmunoassay kit and the values were normalized per mg of protein. The values are mean ± SE of 3–4 experiments. **P* < 0.05 vs. basal value within each age group.

0.05) change induced by CGS21680 (300 nM) in the levels of cAMP in nerve terminals from 0.5- to 6-mo-old rats. In 12-mo-old rats, CGS21680 (300 nM) caused a small but significant (*P* < 0.05) increase in the levels of cAMP (Fig. 6*B*). However, this CGS21680 (300 nM)-induced increase in the levels of cAMP was considerably greater in hippocampal nerve terminals derived from 18- and 24-mo-old rats (Fig. 6*B*).

DISCUSSION

The main conclusion of the present work is that in aged rats there is an increased ability of adenosine A_{2A} receptors to facilitate hippocampal synaptic transmission when compared with young adult rats and that this is due both to an increase in the binding density of A_{2A} receptors in hippocampal nerve terminals as well as to a difference in the main transducing system operated by hippocampal A_{2A} receptors. Thus in aged rats, A_{2A} receptors no longer facilitate hippocampal synaptic transmission indirectly by attenuating tonic A₁ receptor-mediated inhibition, but they directly facilitate glutamatergic transmission in a protein kinase A dependent manner on stimulation

of cAMP levels in nerve terminals. Although the conclusion that there is a different pharmacology and transducing system operated by A_{2A} receptors mostly stems from comparison of results now reported in aged rats with previously published results in young adult rats (Cunha and Ribeiro 2000; Lopes et al. 2002), all the experiments were carried out by the same experimenter and in the same set-up with a 3- to 7-mo time span, thus limiting possible bias of such cross-experiment comparisons. It is of particular interest to note that, although most of the study was designed to investigate the status of the neuromodulatory system operated by A_{2A} receptors in the hippocampus of aged rats in comparison to young adult rats, the study of the changes on aging of A_{2A} receptor density and of its efficiency to trigger cAMP accumulation in hippocampal nerve terminals suggests that these changes might essentially be a feature of the aging process rather than a slow continuous process that evolves through maturation of the animals.

Adenosine A_{2A} receptors were initially described as being coupled to the G_s/adenylate cyclase/cAMP/protein kinase A pathway (Fredholm et al. 1994). However, several studies have shown that A_{2A} receptors can couple to different transducing systems in different preparations, making it clear that A_{2A} receptors are potentially pleiotropic receptors (reviewed in Cunha 2001). Two possibilities can be considered to understand the age-related change in the transducing system operated by limbic cortical A_{2A} receptors. One would rely on a change in the density of G_s proteins in aged hippocampal nerve terminals, whereas the second would rely on an increase in the number of A_{2A} receptors. There is a slight decrease in the density of G_s proteins in the cortex of aged individuals (Young et al. 1991), but there is a general trend for a preservation of the efficiency to trigger cAMP formation in the cortex or hippocampus of aged animals (Sugawa and May 1993; Zimmerman and Berg 1975). Also, the first hypothesis is not supported by the observation that the β-receptor agonist, isoproterenol, caused a similar increase in cAMP levels in hippocampal nerve terminals of aged compared with young adult rats, in accordance with the preserved function of β-receptors in the cortex or hippocampus of aged animals (Dierssen et al. 1992). In contrast, the present observation that there was a nearly doubling of the binding density of A_{2A} receptors in hippocampal nerve terminals of aged compared with young adult rats makes it possible that the change in receptor number could be responsible for the change in the main transducing system operated by A_{2A} receptors in aged animals. In fact, manipulation of the levels of different receptors in heterologous expression systems has shown that different transducing systems can be recruited according to the density and level of activation of these receptors (reviewed in Gudermann et al. 1997). Although little is known on the trafficking and subcellular location of A_{2A} receptors (reviewed in Fredholm et al. 2002), it is possible that the small number of A_{2A} receptors in hippocampal nerve terminals of young adult rats may be scaffolded with pathways directed for a protein kinase C mediated attenuation of A₁ receptor function (Cunha and Ribeiro 2000; Lopes et al. 1999a, 2002), whereas the increase in the number of A_{2A} receptors in hippocampal nerve terminals of aged rats would allow their more generally recognized coupling to the adenylylase/cAMP/protein kinase A pathway. Furthermore, one needs to consider the possibility that covalent modifications of the A_{2A} receptor, which has the longest C-terminal tail among adeno-

sine receptors (Svenningsson et al. 1999), may control the G protein coupling of A_{2A} receptors, an issue that has not yet been experimentally tested.

Of direct relevance for the functional importance of the changes in the adenosine neuromodulatory system in aged rats is the conclusion that A_{2A} receptors appear to be a direct facilitatory system in aged rats compared with their predominant fine-tuning role in young adult rats (Sebastião and Ribeiro 2000). Thus whereas in young adult rats the role of hippocampal A_{2A} receptors is mainly to control A_1 receptor function, in the aged rats, activation of A_{2A} receptors causes a direct facilitation of glutamatergic transmission. Interestingly, this occurs in parallel with a decreased density of A_1 receptors (Cunha et al. 1995a), resulting in a decreased potency of A_1 receptor agonists to modulate hippocampal synaptic transmission (Sebastião et al. 2000). Thus the adenosine neuromodulatory system, which results from a balanced activation of inhibitory A_1 and facilitatory A_{2A} receptors (Cunha 2001), appears to be unbalanced toward facilitatory A_{2A} receptors in aged animals so as to compensate the lower neuronal efficiency in the CA1 area of the rat hippocampus (Barnes 1994; Barnes et al. 2000). This raises the suggestion that the adenosine neuromodulatory system may be an adaptation system to compensate for the changes in synaptic transmission that occur on aging.

A modified A_{2A} receptor function has also been proposed to occur in the striatum (Corsi et al. 1999, 2000; Popoli et al. 1998a). It is important to keep in mind that the main subcellular localization and functional role of striatal A_{2A} receptors are different from extra-striatal A_{2A} receptors (Fredholm et al. 2002). In fact, whereas in extra-striatal areas the role of neuronal A_{2A} receptors is mostly a facilitation of neurotransmitter release (reviewed in Cunha 2001), striatal A_{2A} receptors have a main postsynaptic location controlling the function of D_2 receptors (Svenningsson et al. 1999) and also of NMDA receptors (Nash and Brotchie 2000). Overall, the evolution on aging of striatal and hippocampal or cortical A_{2A} receptors appears to be in opposite directions. Indeed, binding studies revealed a decreased binding density of A_{2A} receptors in the striatum of aged rats (Cunha et al. 1995a; Popoli et al. 1998b) that is accompanied by a decreased expression of A_{2A} receptor mRNA (Schiffmann and Vanderhaeghen 1993), precisely the opposite of what occurs for A_{2A} receptors in the hippocampus and cortex of aged rats (Cunha et al. 1995a; Lopes et al. 1999b). This opposite age-related change in the density of A_{2A} receptors in the striatum and in the cortex also raises some concern on the therapeutic use of A_{2A} receptor antagonists as anti-Parkinsonian drugs (e.g., Fredholm et al. 2002). In fact, in young adult rats that are used as models for this striatal dysfunction, blockade of central A_{2A} receptors is roughly equivalent to blockade of striatal A_{2A} receptors given the high density of striatal versus extra-striatal A_{2A} receptors (Svenningsson et al. 1999). However, Parkinson's disease is prevalent in aged individuals and we presently observed that there is a marked increase in the density and functional impact of A_{2A} receptors in the hippocampus and in the cortex (see also Lopes et al. 1999b), whereas the opposite is observed in the striatum (Cunha et al. 1995a; Popoli et al. 1998b). This brings to stage the possible more evident side effects of long-term administration of A_{2A} receptor antagonists in the elderly, namely in view of the observed effects of A_{2A} receptors in the control of

hippocampal synaptic plasticity (de Mendonça and Ribeiro 1994), sleep (Sato et al. 1999), and psychosis (Ferré et al. 1994; Sills et al. 2001).

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DISCLOSURES

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