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

Nelson Rebola,1 Ana M. Sebastião,2 Alexandre de Mendonça,2 Catarina R. Oliveira,1 J. A. Ribeiro,2 and Rodrigo A. Cunha1

1Center for Neurosciences of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra; and 2Laboratory of Neurosciences, Faculty of Medicine, University of Lisbon, 1649-028 Lisboa, Portugal

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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 $A_1$ 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 $A_1$ receptors (Lopes et al. 1999a). Thus the control by adenosine of neurotransmitter release should be conceived as a balance between inhibitory $A_1$ 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 $A_1$ 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 $A_1$ receptors is accompanied by a decreased ability of $A_1$ 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 $A_1$ 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 $A_1$ receptor-mediated inhibition of synaptic transmission (Lopes et al. 2002) and involves activation of protein kinase C (Cunha and Ribeiro 2000). Thus we now
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 $A_1$ 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-oxophenyl]xanthine (XAC), and bisindolylmaleimide were from RBI (Natick, MA); chelerythrine and N-[2-(p-bromocinnamyl)amino]ethyl]-5-isouquinolinesulfonylamine (H-89) were from Calbiochem (Darmstadt, Germany); adenosine, 2-chloroadenosine and H-89 were from Calbiochem (Darmstadt, Germany); adenosine, 2-chloroadenosine and H-89 were from Calbiochem (Darmstadt, Germany); adenosine, 2-cloro-adenosine, and isoproterenol were from Sigma (Reagente 5, Portugal); 4-[(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); [H]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’-cyclopentolyl-4’-methoxyphenyl)-2-pyrrolidone (4-RS)] was a gift of Schering AG; and [H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine ([H]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).

**Animals**

Male Wistar rats from the Gulsbenkian 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 there was 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]SCH8261 (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 membranes 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
Western blot analysis of A2A receptor immunoreactivity in hippocampal nerve terminals

The analysis of adenosine A2A 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 diithiothreitol, 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% concentration 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 A2A 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 A2A 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 A2A receptors to modulate hippocampal synaptic transmission in aged rats. A single concentration (10 nM) of the A2A 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 in Fig. 1C, this effect of CGS21680 was prevented by the adenosine A2A 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 A2A receptor antagonist commercially available, it is not an ideal tool to probe the role of tonic A2A receptor activation by endogenous adenosine since it also interferes with A1 receptor-mediated responses (Lopes et al. 1999c), described (Lopes et al. 1999b), and protein was measured according to Peterson (1977).
although it only interferes with $A_1$ receptor binding in micro-
molar concentrations (Cunha et al. 1997).

Although in young adult rats it is possible to record facili-
tory $A_{2A}$ 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, super-
fusion of hippocampal slices from young adult rats with adeno-
sine 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 $\mu$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 aden-
sine receptors and is less prone to metabolism, 2-chloro-
adenosine (10 $\mu$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 respon-
sible 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 $A_1$ recep-
tors further supports a greater functional impact of $A_{2A}$ recep-
tor 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 transmis-
sion 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 depen-
dent, but not protein kinase A dependent (Cunha and Ribeiro
2000). However, in several other systems, $A_{2A}$ receptors facil-
itate neurotransmitter release on increasing cAMP levels in
nerve terminals (Correia-de-Sá and Ribeiro 1994; Gubitz et al.
1996; Okada et al. 2001). Activation of $A_{2A}$ receptors causes a
greater accumulation of cAMP in cortical slices of aged com-
pared 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 transmis-
sion 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-cyclopentyl-
adenosine (DPCPX; 50 nM), in a manner sensitive to the adenosine $A_{2A}$
receptor antagonist, ZM241385 (20 nM). A and B: time courses of represen-
tative 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 $\mu$M, labeled as 1) and
2-chloroadenosine (10 $\mu$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 $\mu$M) and 2-chloroadenosine (10 $\mu$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 repres-
ent the average percentage increase of fEPSP slope caused by adenosine (50
$\mu$M) or by 2-chloroadenosine (10 $\mu$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 $\pm$ 0.04 mV/ms); *$P < 0.05$ vs. 0%; **$P < 0.05$ between
indicated bars.**
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 \(\beta\)-adrenergic receptor agonist, isoproterenol, to increase cAMP levels in hippocampal nerve terminals of young adult and aged rats, since \(\beta\)-receptor activation increases cAMP in rat cortical nerve terminals (Herrero and Sanchez-Prieto 1996). As shown in Fig. 3B, isoproterenol (30 \(\mu\)M) caused a nearly similar increase of cAMP levels in young adult (58.2 \(\pm\) 5.2 pmol/mg protein, \(n = 4\)) and in aged rat hippocampal nerve terminals (46.6 \(\pm\) 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 \(A_1\) 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 \(\mu\)M), to prevent the facilitatory effects of CGS21680 (10 nM), and the ability of the protein kinase C inhibitor, chelerythrine (6 \(\mu\)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 \(\mu\)M) and by 81.2 \(\pm\) 2.1% by H-89 (1 \(\mu\)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 \(\mu\)M) and only by 16% by another protein kinase C inhibitor, bisindolylmaleimide (1 \(\mu\)M). By themselves, chelerythrine (6 \(\mu\)M) inhibited by 16 \(\pm\) 3% fEPSP slope in aged rats, whereas H-89 (1 \(\mu\)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, \([\text{H}]\text{CGS21680}\) (60 nM) and \([\text{H}]\text{ZM241385}\) (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 \([\text{H}]\text{CGS21680}\) (60 nM) and \([\text{H}]\text{ZM241385}\) (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.
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% 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 ($P >$
The main conclusion of the present work is that in aged rats there is an increased ability of adenosine A2A 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 A2A receptors in hippocampal nerve terminals as well as to a difference in the main transducing system operated by hippocampal A2A receptors. Thus in aged rats, A2A 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 A2A 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 A2A receptors in the hippocampus of aged rats in comparison to young adult rats, the study of the changes on aging of A2A 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 A2A receptors were initially described as being coupled to the Gs/adenylate cyclase/cAMP/protein kinase A pathway (Fredholm et al. 1994). However, several studies have shown that A2A receptors can couple to different transducing systems in different preparations, making it clear that A2A 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 A2A receptors. One would rely on a change in the density of Gs proteins in aged hippocampal nerve terminals, whereas the second would rely on an increase in the number of A2A receptors. There is a slight decrease in the density of Gs 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 A2A 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 A2A 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 A2A receptors (reviewed in Fredholm et al. 2002), it is possible that the small number of A2A 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 A2A receptors in hippocampal nerve terminals of aged rats would allow their more generally recognized coupling to the adenylate cyclase/cAMP/protein kinase A pathway. Furthermore, one needs to consider the possibility that covalent modifications of the A2A receptor, which has the longest C-terminal tail among adeno-
sine receptors (Svenningsson et al. 1999), may control the G protein coupling of A2A 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 A2A 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 A2A receptors is mainly to control A1 receptor function, in the aged rats, activation of A2A receptors causes a direct facilitation of glutamatergic transmission. Interestingly, this occurs in parallel with a decreased density of A1 receptors (Cunha et al. 1995a), resulting in a decreased potency of A1 receptor agonists to modulate hippocampal synaptic transmission (Sebastião et al. 2000). Thus the adenosine neuromodulatory system, which results from a balanced activation of inhibitory A1 and facilitatory A2A receptors (Cunha 2001), appears to be unbalanced toward facilitatory A2A 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 A2A 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 A2A receptors are different from extra-striatal A2A receptors (Fredholm et al. 2002). In fact, whereas in extra-striatal areas the role of neuronal A2A receptors is mostly a facilitation of neurotransmitter release (reviewed in Cunha 2001), striatal A2A receptors have a main postsynaptic location controlling the function of D2 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 A2A receptors appears to be in opposite directions. Indeed, binding studies revealed a decreased binding density of A2A receptors in the striatum of aged rats (Cunha et al. 1995a; Popoli et al. 1998b) that is accompanied by a decreased expression of A2A receptor mRNA (Schiffmann and Vanderhaegen 1993), precisely the opposite of what occurs for A1 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 A2A receptors in the striatum and in the cortex also raises some concern on the therapeutic use of A2A 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 A2A receptors is roughly equivalent to blockade of striatal A2A receptors given the high density of striatal versus extra-striatal A2A 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 A2A 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 A2A receptor antagonists in the elderly, namely in view of the observed effects of A2A receptors in the control of hippocampal synaptic plasticity (de Mendonça and Ribeiro 1994), sleep (Satoh et al. 1999), and psychosis (Ferré et al. 1994; Sills et al. 2001).

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