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Modification of adenosine modulation of synaptic transmission in the hippocampus of aged rats

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1 We compared the modulation of synaptic transmission by adenosine A_1 receptors in the hippocampus of aged (24 months) and young adult rats (6 weeks).

2 The adenosine A_1 receptor agonist, N^6-cyclopentyladenosine, was less potent ($P<0.05$) to inhibit synaptic transmission in aged ($EC_{50}=53$ nM) than young adult ($EC_{50}=14$ nM) hippocampal slices, these effects being prevented by the A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX).

3 In contrast with the lower effect of the A_1 receptor agonist, it was observed that blockade of A_1 receptors with DPCPX (50 nM), or removal of endogenous extracellular adenosine with adenosine deaminase (2 u ml^{-1}), caused a more pronounced disinhibition of synaptic transmission in aged rats. Also consistent with a more intense A_1 receptor-mediated inhibitory tonus by endogenous adenosine in aged rats was the finding that to fully prevent the depression of synaptic transmission induced by 3 min hypoxia, a higher concentration of DPCPX was required in slices from aged (100 nM) than from young (50 nM) rats.

4 It is concluded that in hippocampal slices of aged rats the efficiency of A_1 receptors to modulate synaptic transmission is reduced, but this may be compensated by an enhanced inhibitory tonus by endogenous adenosine.

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Abbreviations: ADA, adenosine deaminase; CPA, N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; fEPSP, field excitatory post-synaptic potentials

Introduction

Adenosine is a neuromodulator that predominantly inhibits synaptic transmission through activation of inhibitory A_1 receptors, but can also facilitate synaptic transmission through adenosine A_{2A} receptor activation (Sebastião & Ribeiro, 1996). The hippocampus is endowed with inhibitory A_1 receptors (Sebastião *et al*., 1990; Cunha *et al*., 1994) and with facilitatory A_{2A} receptors (Sebastião & Ribeiro, 1992; Cunha *et al*. 1994) but the predominant tonus by endogenous adenosine, with regards to its ability to modulate synaptic transmission, is inhibitory (Dunwiddie & Diao, 1994). Moreover, the hippocampus is a brain region particularly vulnerable to ischaemic insults, and adenosine, mainly by activating inhibitory A_1 receptors, plays an important neuroprotective role, reducing hypoxic/ischaemic neuronal damage (see e.g. Rudolphi *et al*., 1992; Fredholm, 1997).

Young adult animals (6–8 weeks) have been used to study the neuroprotective effects of adenosine. Since stroke and other cerebral disorders are prevalent in the elderly, it is important to know if the neuromodulatory role of adenosine is maintained in aged animals. The previously observed decrease of A_1 receptor binding sites in rat hippocampal membranes of aged rats (Cunha *et al*., 1995), is suggestive of a lower ability of adenosine to modulate hippocampal synaptic transmission in aged rats. However, the number of receptors is only one of the parameters that control the response to a substance. Thus, in the present study, we directly investigated whether the neuromodulatory role of A_1 receptors on synaptic transmission in the hippocampus is modified in aged rats. Due to the predominant role of adenosine A_1 receptors in the synaptic responses to hypoxia (e.g. Lucchi *et al*., 1996), we also compared the ability of endogenous adenosine to mediate the hypoxic depression of synaptic transmission in aged and young adult rats. This study was thus designed to evaluate adenosine A_1 receptor-mediated neuromodulation in 24-month-old rats, since disorders such as stroke or memory dysfunction are prevalent in the aged.

Preliminary accounts of some of this work have been presented previously (Sebastião *et al*., 1997; Sebastião & Ribeiro, 1999).

Methods

Animals

Male Wistar rats from the Gulbenkian Institute animal house were used throughout this study. Young adult rats were 6 weeks old (140–160 g), whereas aged rats were 24 months old (950–1080 g). Most studies on purinergic modulation have been performed in 6-week-old rats, which are considered juvenile or young adult rats. Rats at 24 months are considered aged rats, i.e. close to the limit of life expectancy. Indeed, around 40% of the initial population of rats allocated for this study did not reach this age. In neither group of aged rats was any evidence of gross anatomical

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lesions in the brain. The handling and use of these animals was according with the EU guidelines for use of experimental animals.

Electrophysiological recordings from hippocampal slices

Rats were killed by decapitation under halothane anaesthesia, and the brain was rapidly removed into an ice-cold Krebs solution of the following composition (mM): NaCl 124, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10, gassed with 95% O_2/5% CO_2 (pH = 7.4). The brain was cut longitudinally, the two hippocampi removed, and slices transversely cut 400 μm thick with a McIlwain tissue chopper. The slices were then stored immersed in gassed Krebs solution for at least 1 h to allow their energetic and functional recovery. A slice was then transferred to a 1 ml (plus 2 ml dead volume) recording chamber for submerged slices and continuously superfused with gassed solution, at a flow rate of 3 ml min^{-1}. The temperature of the bath was kept constant with an accuracy of ± 0.2°C, with the aid of a temperature controller. Though the action of exogenous (Ribeiro, 1982) and endogenous (Masino & Dunwiddie, 1999) adenosine is higher at 37°C than at lower temperatures, we used lower temperatures to avoid irreversible changes in CA1 pyramidal neurones, which occur at 37°C, but not up to 33°C, and might be attributed to hypoxic injury (see Schiff & Somjen, 1985). As pointed out (Schiff & Somjen, 1985), at 37°C the rate of oxygen consumption may be too high for adequate oxygen delivery into the slice core. So, the bath temperature was set at 30.5°C in all experiments except when testing the role of A_1 receptors upon hypoxia. In the hypoxia experiments, the bath temperature was kept at 32°C throughout the experiment, to enhance the effects of hypoxia (Taylor & Weber, 1993) and to facilitate comparisons with previously published data (see e.g. Lucchi *et al*., 1996). In these experiments, hypoxia was applied by switching the 95%O_2/5%CO_2 saturated perfusion solution (pO_2 in the recording chamber $\approx$600 mmHg) into 95%N_2/5%CO_2 saturated solution, for 3 min (pO_2 in the recording chamber $\approx$250 mmHg). Hypoxia was applied to each slice only once. In order to minimize individual variations between slices, the effects of hypoxia in test conditions and control conditions, i.e. in the presence and in the absence of test drugs, were compared in paired slices morphologically similar and taken from the same hippocampus. The experiments performed in hippocampal slices taken from aged or from young rats were performed in alternating days.

Evoked field excitatory post-synaptic potentials (fEPSP) were recorded through an extracellular microelectrode (4 M NaCl, 2–5 MΩ resistance) placed in the *stratum radiatum* of the CA1 area. Stimulation (rectangular pulses of 0.1 ms) was delivered (once every 10 s, or 15 s in the hypoxia experiments) through a concentric electrode placed on the Schaffer collateral/commissural fibres, in the *stratum radiatum* near the CA3/CA1 border. The intensity of the stimulus (80–400 μA) was initially adjusted to obtain a large fEPSP with a minimal population spike contamination. Recordings were obtained with an Axoclamp 2B amplifier coupled to a DigiData 1200 interface (Axon Instruments), or through a WPI 750 amplifier coupled to a Tektronix digitalizing oscilloscope. Averages of eight consecutive responses were continuously monitored on a personal computer with the LTP program (Anderson & Collingridge, 1997), kindly supplied by W.W. Anderson (Univ. Bristol, U.K.), or with the Signal Processing and Display (SPD, Tektronix) software with local modifications. Responses were quantified as the slope of the initial phase of the averaged fEPSPs, since slope measures are considered a more accurate measure of fEPSP magnitude than the amplitude, due to eventual contamination by the population spike.

Drugs

1,3-Dipropyl-8-cyclopentylxanthine (DPCPX) and N^6-cyclopentyladenosine (CPA) were from RBI (Natik, MA, U.S.A.). Adenosine deaminase (type VI) was from Sigma (St. Louis, MO, U.S.A.) and CPA was made up in 5 mM stock solution in dimethylsulphoxide and DPCPX was made in a 5 mM stock solution in 99% dimethylsulphoxide and 1% NaOH (1 M).

Data analysis

The data are presented as mean $\pm$ s.e.mean, except the EC_{50} values which are presented as mean (95% confidence interval), from n experiments. To calculate the E_{max} (maximal effect) and the EC_{50} values, the log concentration-response curve for a drug was fitted to a sigmoid (variable slope and with a constant bottom value of zero) by non-linear regression analysis using the GraphPad Prism software. The statistical significance of the effect of one drug (i.e. of the averaged percentage effect) was evaluated with the Student's t-test. The comparison among the different drug treatments in the same group of animals (young or aged) was made using one-way repeated-measures ANOVA followed by the Tukey test. The statistical comparisons of the effects of the same drug in young adult and in aged animals were made using the two-tailed Mann-Whitney U-test. Values of $P<0.05$ were considered to represent significant differences.

Results

To test whether the decrease in the number of binding sites of the A_1 receptor antagonist, DPCPX, in the hippocampus of aged rats (Cunha *et al*., 1995) would be reflected in a modified A_1 receptor modulation, we investigated the effect of the selective adenosine A_1 receptor agonist, CPA, on CA1 hippocampal excitatory synaptic transmission. As illustrated in Figure 1a, the A_1 adenosine receptor agonist, CPA, at a concentration of 40 nM, caused only a near 40% inhibition of fEPSP slope (average inhibition of 37$\pm$6%, n=4) in aged rats whereas in young adult rats the same concentration of CPA decreased the fEPSP slope by nearly 80% (average inhibition of 83$\pm$1%, n=5, $P<0.05$ as compared with the effect in aged rats). As expected, the A_1 receptor selective antagonist, DPCPX (50 nM), prevented the inhibitory effect of 30 nM CPA on fEPSPs in aged rats (n=2), which is in accordance with an A_1 receptor mediated effect of this agonist. As shown in Figure 1b, CPA was more potent to inhibit fEPSPs in young than in aged rats. The EC_{50} of CPA inhibition was 14 nM (95% confidence interval: 12–16 nM) in young adult rats and 53 nM (95% confidence interval: 40–70 nM) in aged animals. The maximal inhibition of fEPSP slope caused by CPA (E_{max}), calculated by non-linear regression fit of the data shown in Figure 1b, was similar in young adult (99$\pm$3% inhibition) and aged rats (93$\pm$6% inhibition, $P>0.05$ as compared with young adults). Indeed, the inhibition of the fEPSP slope caused by a supramaximal concentration (100 nM) of CPA in young adult rats (96$\pm$1%, n=2) was not significantly different ($P>0.05$) from that caused by a high concentration (1 μM) of CPA in aged rats

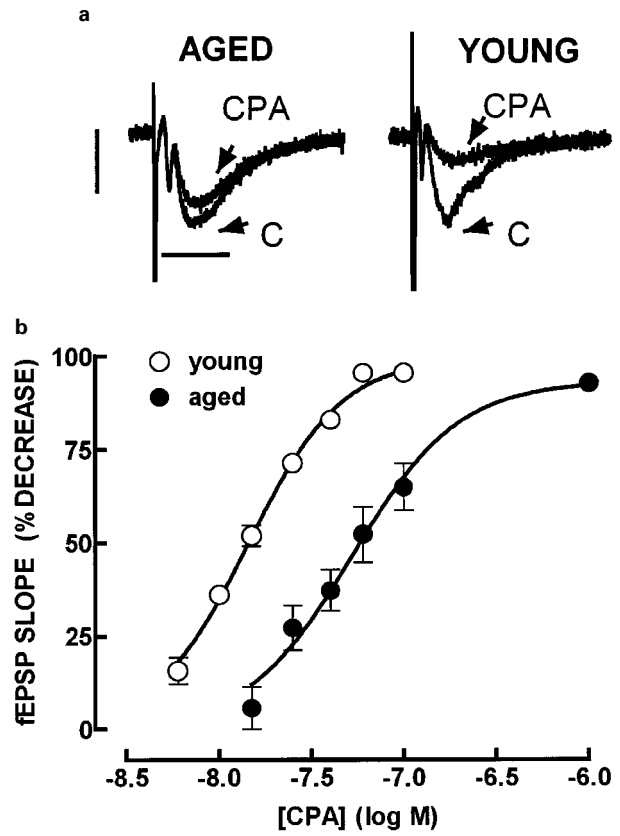

Figure 1 Comparison between the effects of the adenosine A_1 receptor agonist, CPA, on field excitatory post-synaptic potentials (fEPSPs) recorded from the CA1 area of hippocampal slices taken from young adult (6 weeks) and aged (24 months) rats. (a) Shows trace recordings of averaged fEPSPs obtained in one experiment with an aged rat (left), and in another experiment with a young rat (right); in each panel the fEPSPs obtained in the same slice in control conditions (C) and 30–34 min after application of CPA (40 nM) are superimposed; calibration bars: 500 μV, 10 ms. (b) Shows the log concentration-response curves for the inhibitory effects of CPA on the slope of fEPSPs in aged and young adult rats; in the ordinates 0% corresponds to the fEPSP slope before CPA application (0.42 ± 0.06 mV ms^{-1} in young and 0.42 ± 0.10 mV ms^{-1} in aged rats) and 100% represents the complete inhibition of fEPSPs. The data for each curve were obtained in 4–5 experiments, except for saturating concentrations of CPA (60–100 nM) in young animals, which represent results from two experiments; the s.e.mean are shown when they exceed the symbols in size.

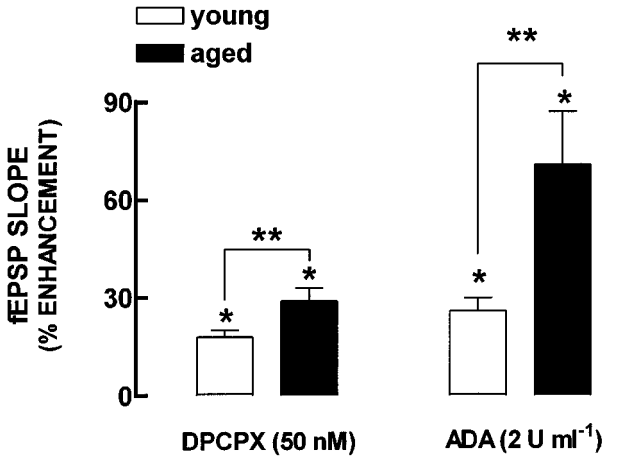

Figure 2 Comparison between the ability of endogenous extracellular adenosine to modulate synaptic transmission in hippocampal slices of young adult (6 weeks) and aged (24 months) rats. The ordinates represent the percentage increases of fEPSP slope caused by the A_1 receptor antagonist, DPCPX (50 nM), and by adenosine deaminase (ADA, 2 u ml^{-1}), in slices from aged ($n=4-7$) and from young adult rats ($n=4-5$). 0% corresponds to the control fEPSP slope which ranged from 0.31 to 0.51 mV ms^{-1}. *$P<0.05$ versus baseline (Student's t-test); **$P<0.05$ between the two age groups (two-tailed Mann-Whitney U-test).

(93 ± 1%, $n=4$), this effect of CPA (1 μM) in aged rats being fully antagonized by DPCPX (200 nM).

In order to test whether the lower effect of CPA in aged than young adult rats could be attributed to different levels of endogenous adenosine, we compared the effect of this A_1 receptor selective agonist in the same slices of aged rats in the absence and in the presence of adenosine deaminase (converts adenosine into its inactive metabolite, inosine), which was applied to the preparations at least 15 min before addition of CPA. It was observed that the inhibitory effect of 25 nM CPA on fEPSP slope in hippocampal slices of aged rats was similar in the absence (32 ± 2%, $n=4$) or in the presence of 2 u ml^{-1} adenosine deaminase (30 ± 3%, $n=4$).

We then evaluated the tonic inhibition by endogenous extracellular adenosine on excitatory synaptic transmission, which was found to be different in hippocampal slices of young adult and aged rats (Figure 2). Thus, blockade of tonic A_1 receptor activation with DPCPX (50 nM) enhanced fEPSP slope by 18 ± 2% ($n=5$) in young adult rats, whereas it caused a greater facilitation in aged rats (29 ± 4%, $n=7$, $P<0.05$ as compared with the effect in young rats). Removal of endogenous extracellular adenosine with adenosine deaminase (2 u ml^{-1}) also caused a greater facilitation of fEPSP slope ($P<0.05$) in aged (71 ± 16%, $n=10$) compared to young adult rats (26 ± 4%, $n=4$) (Figure 2).

The results showing that an A_1 receptor agonist is less potent to depress synaptic transmission in hippocampus from aged than from young rats, suggest a lower functioning of A_1 receptors in aged animals. This contrasts with the observation that removal of tonic A_1 receptor inhibition causes a greater facilitation of synaptic transmission in aged hippocampus, and prompted further investigation of the role of endogenous adenosine in the aged hippocampus. We used an experimental paradigm, hypoxia (3 min), where activation of A_1 receptors by released adenosine fully accounts for the hypoxic depression of synaptic transmission (e.g. Canhão *et al*., 1994; Lucchi *et al*, 1996). As illustrated in Figure 3a,b, hypoxia (3 min) induced a transient decrease (68 ± 8%, $n=4$) in the slope of fEPSPs recorded from hippocampal slices taken from aged rats. A similar decrease (62 ± 9%, $n=4$) of the fEPSP slope was observed upon 3 min hypoxia in young adult hippocampal slices (Figure 3c). In the hippocampal slices taken from both young and old rats, blockade of adenosine A_1 receptors with DPCPX (100 nM) almost completely prevented the depression of synaptic transmission caused by 3 min hypoxia (maximal decrease in fEPSP slope in the presence of 100 nM DPCPX: 12 ± 2%, $n=4$, in young rats and 6 ± 5%, $n=4$, in aged rats). However, a lower concentration (50 nM) of DPCPX was unable to fully prevent the hypoxic depression of synaptic transmission in aged rats, but in young adult rats was as efficient as 100 nM DPCPX to attenuate the decrease in fEPSP slope caused by hypoxia (Figure 3). Thus, the maximal decrease in fEPSP slope caused by 3 min hypoxia in slices from aged hippocampus in the presence of 50 nM DPCPX (30 ± 7%, $n=4$) was significantly different ($P<0.05$) from that observed in the presence of 100 nM DPCPX in slices taken from the same hippocampus; in slices from young adult rats, the maximal decrease in fEPSP slope caused by 3 min hypoxia in the presence of

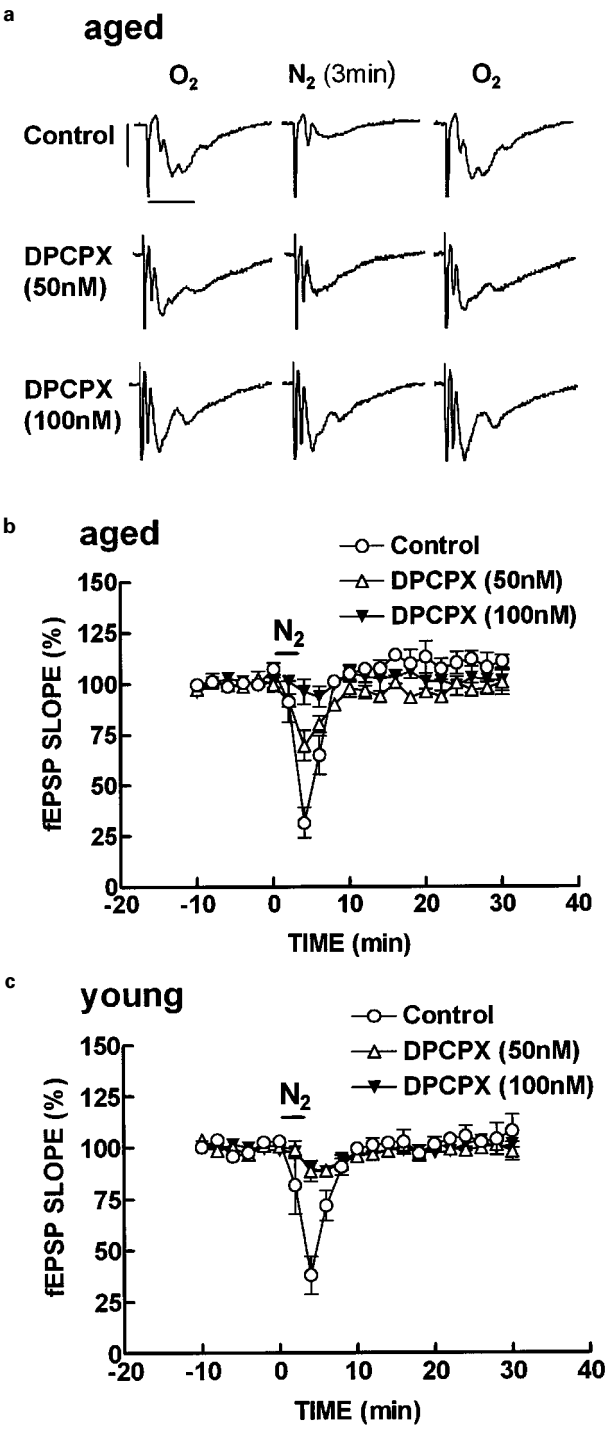

Figure 3 Comparison between the effects of the selective adenosine A_1 receptor antagonist, DPCPX, on the depressions of field excitatory postsynaptic potentials (fEPSPs) induced by 3 min hypoxia in hippocampal slices taken from aged (24 months) and young adult (6 weeks) rats. In (a) are shown averaged fEPSPs recorded from three hippocampal slices taken from one hippocampus of an aged rat; in the upper row are shown responses recorded from a slice in the absence of any drug (control); in the middle row, from a slice in the presence of 50 nM DPCPX and in the lower row from a slice in the presence of 100 nM DPCPX; fEPSPs obtained: before applying hypoxia (left), between 2 and 4 min after starting hypoxia, which corresponds to the maximal depression of the fEPSPs (middle) and upon full recovery of the fEPSPs after starting reoxygenation (right). (b and c) illustrate the averaged time courses of the fEPSP depressions induced by 3 min hypoxia in hippocampal slices from aged (b, $n=4$) and young (c, $n=4$) rats, in the absence and in the presence of 50 or 100 nM DPCPX, as indicated by the symbols. 100% = 0.31 to 0.75 mV ms^{-1}. Note that in slices from aged animals there were statistically significant differences ($P<0.05$) among the maximal depressions of fEPSPs induced by hypoxia in the absence of 50 nM DPCPX ($11 \pm 5\%$, $n=4$) was not significantly different ($P>0.05$) from that observed in the presence of 100 nM DPCPX in slices from the same hippocampus.

Discussion

In the present study we observed a decreased ability of an adenosine A_1 receptor agonist to inhibit synaptic transmission in aged rats. This decreased potency of the A_1 agonist could be due to: (1) higher levels of endogenous extracellular adenosine that would compete with the exogenous agonist (see Jin *et al*., 1993; Sperlagh *et al*., 1997); (2) lower affinity of A_1 receptors for A_1 agonists; (3) less efficient G protein coupling and lower amplification of the transducing system operated by A_1 receptors and (4) reduced number of A_1 receptors. The decreased potency of A_1 receptor agonists in aged rats is apparently not due to a greater inhibitory tonus of endogenous adenosine, since the effect of CPA was not modified by adenosine deaminase; adenosine deaminase, in amounts up to 2 u ml^{-1} selectively and efficiently removes endogenous extracellular adenosine in the hippocampus since its disinhibitory action is prevented by adenosine deaminase inhibitors (Cunha *et al*., 1996) and is able to prevent the inhibitory action of adenosine formed extracellularly from micromolar concentrations of AMP (Lee *et al*., 1981). The observed decrease in the ability of the selective A_1 receptor agonist to decrease synaptic transmission in aged rats is probably not due to a decrease in the affinity of agonists for A_1 receptors, since the affinity of A_1 receptor agonists is nearly identical in young adult and aged animals (Pagonopoulou & Angelatou, 1992). Also, there is an increased G protein coupling (measured as an increased GTP shift) of A_1 receptors in aged rats (Cunha & Ribeiro, 1998), which is the opposite of what would be expected to account for a lower potency of A_1 receptors agonists. It remains to be established whether the transducing system operated by A_1 receptors, which is not yet fully elucidated, is modified in aged rats. Finally, the age-induced decrease in the number of binding sites for A_1 receptor ligands ([^{3}H]-DPCPX and [^{3}H]-N^6-cyclohexyladenosine) in the hippocampus (Pagonopoulou & Angelatou, 1992; Cunha *et al*., 1995; Sperlagh *et al*., 1997), interpreted as a decrease in the number of A_1 receptors, is a likely reason for the decreased potency of A_1 agonists to modulate excitatory neurotransmission in aged rats, which is mainly due to a decrease in the apparent affinity, i.e. to an increased EC_{50}, rather than a change in the maximal inhibition (c.f. Figure 1). Indeed, theoretical studies (Kenakin, 1993) predict that in systems where the receptor is significantly distributed between two different affinity states for the agonists, as is the case for hippocampal A_1 receptors (Fastbom & Fredholm, 1990), a decrease in receptor number may produce a dextral displacement of the concentration-response curve without measurable depression of the maximal response.

DPCPX, in the presence of 50 mM DPCPX and in the presence of 100 nM DPCPX, whereas in young rats there were no statistically significant differences ($P>0.05$) between the maximal fEPSP depressions induced by hypoxia in the presence of 100 or 50 nM DPCPX, both being significantly different ($P<0.05$) from the depression obtained in the absence of DPCPX (one-way repeated-measures ANOVA followed by the Tukey test).

Another finding in the present study was that the disinhibitory effects of the A_1 receptor antagonist, DPCPX, and of adenosine deaminase, on synaptic transmission were greater in hippocampal slices from aged rats than from young adult rats. A greater effect of adenosine inhibitory receptor blockade in aged rats has also been described in the modulation of hippocampal synaptic transmission (Bauman *et al*., 1992), in phenomena of cold tolerance (Wang *et al*., 1992) or in the regulation of lipolysis in fat cells (Hoffman *et al*., 1984), but a decreased (Corsi *et al*., 1997; Sperlagh *et al*., 1997; Giovannelli *et al*., 1988) or maintained (Pereira *et al*., 2000) effect of A_1 receptor antagonists in aged animals has been reported in other preparations. The observed greater effect of DPCPX on synaptic transmission in aged rats may be due to: (1) higher affinity of A_1 receptors for DPCPX; (2) higher efficiency of A_1 receptor activation; (3) greater amount of endogenous adenosine tonically activating A_1 receptors. The first hypothesis is excluded by the observation that no difference in the K_D for DPCPX was observed in hippocampal membranes from aged and young adult rats (Cunha *et al*., 1995), as previously discussed. The observed lower ability of CPA to inhibit synaptic transmission seems to exclude the possibility that the greater disinhibitory action of the A_1 receptor antagonist is due to a higher efficiency of A_1 receptor activation. It is more likely that the greater facilitation of synaptic transmission observed in the presence of DPCPX, as well as in the presence of adenosine deaminase, is a consequence of a greater tonic inhibitory effect of endogenous adenosine at hippocampal synapses, caused by a localized synaptic (Cunha *et al*., 1998) modification of extracellular adenosine metabolism in aged rats (Cunha *et al*., 2001). Accordingly, the evoked release of adenosine from synaptosomes is greater in aged than in young adult hippocampus (Cunha *et al*., 2001). This fits the results now obtained with mild hypoxia, which are also suggestive that greater amounts of adenosine are released at the synaptic level. Indeed, higher concentrations of DPCPX were required to fully prevent the hypoxia-induced depression of synaptic transmission in aged hippocampus. As discussed above, this should not be attributed to a lower affinity of DPCPX for A_1 receptors in the aged hippocampus, and a likely reason is that in slices from aged hippocampus, hypoxia induces the release of higher amounts of adenosine at the synaptic level. This would compensate for the lower efficiency of A_1 receptors to inhibit synaptic transmission, which is in accordance with the similarity of the hypoxia-induced depression of synaptic transmission in the slices taken from aged and from young adult animals. Increased levels of extracellular adenosine together with a lower efficiency of A_1 receptors in the aged allows the maintenance of the role of A_1 receptors during hypoxia in aged (Gribkoff & Bauman, 1992) as in young adult (Canhão *et al*., 1994; Lucchi *et al*., 1996) animals. Interestingly, the role of A_1 receptors in synaptic plasticity phenomena (de Mendonça & Ribeiro, 1997) is also maintained in aged rats (Costenla *et al*. 1999).

In conclusion, the present results provide direct functional evidence of a lower ability of A_1 receptors to inhibit synaptic transmission in the hippocampus of aged rats but, interestingly, this may be compensated with higher amounts of endogenous adenosine at the synaptic level. Further detailed ageing studies are required to establish if these changes in adenosine neuromodulation are a cause or an adaptive consequence in the changes in neuronal functioning occurring during ageing.

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