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SYNAPTIC MECHANISMS

Enhanced role of adenosine A_{2A} receptors in the modulation of LTP in the rat hippocampus upon ageing

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Abstract

Adenosine neuromodulation depends on a balanced activation of inhibitory A_1 (A_1R) and facilitatory A_{2A} receptors ($A_{2A}R$). Both A_1R and $A_{2A}R$ modulate hippocampal glutamate release and NMDA-dependent long-term potentiation (LTP) but ageing affects the density of both A_1R and $A_{2A}R$. We tested the effects of selective A_1R and $A_{2A}R$ antagonists in the modulation of synaptic transmission and plasticity in rat hippocampal slices from three age groups (young adults, 2–3 month; middle-aged adults, 6–8 months; aged, 18–20 months). The selective $A_{2A}R$ antagonist SCH58261 (50 nM) attenuated LTP in all age groups, with a larger effect in aged (−63 ± 7%) than in middle-aged adults (−36 ± 9%) or young adult rats (−36 ± 9%). In contrast, the selective A_1R antagonist DPCPX (50 nM) increased LTP magnitude in young adult rats (+42 ± 6%), but failed to affect LTP magnitude in the other age groups. Finally, in the continuous presence of DPCPX, SCH58261 caused a significantly larger inhibition of LTP amplitude in aged (−71 ± 45%) than middle-aged (−28 ± 9%) or young rats (−11 ± 2%). Accordingly, aged rats displayed an increased expression of $A_{2A}R$ mRNA in the hippocampus and a higher number of glutamatergic nerve terminals equipped with $A_{2A}R$ in aged (67 ± 6%) compared with middle-aged (34 ± 7%) and young rats (25 ± 5%). The results show an enhanced $A_{2A}R$-mediated modulation of LTP in aged rats, in accordance with the age-associated increased expression and density of $A_{2A}R$ in glutamatergic terminals. This age-associated gain of function of $A_{2A}R$ modulating synaptic plasticity may underlie the ability of $A_{2A}R$ antagonists to prevent memory dysfunction in aged animals.

Introduction

Adenosine is a neuromodulator in the central nervous system by mainly activating inhibitory A_1 receptors (A_1R) and facilitatory A_{2A} receptors ($A_{2A}R$) (reviewed in Fredholm *et al.*, 2005). Adenosine mainly acts at the synaptic level, inhibiting the release of glutamate and postsynaptic responsiveness and predominantly modulates excitatory rather than inhibitory synapses (Fredholm *et al.*, 2005). There seems to be a segregation of the role of A_1R and $A_{2A}R$ in excitatory synapses, which seems to depend on the source of extracellular adenosine (reviewed in Cunha, 2008) – A_1R are activated by purines originated from astrocytes (Pascual *et al.*, 2005) and efficiently inhibit basal synaptic transmission and synaptic plasticity phenomena that are dependent on more integrated properties of the neuronal network (Stäubli & Chun, 1996; Fujii *et al.*, 1999; Huang *et al.*, 1999; de Mendonça & Ribeiro, 2001; Izumi & Zorumski, 2008). In contrast, $A_{2A}R$ play a prominent facilitatory role in homo-synaptic plasticity phenomena (Rebola *et al.*, 2008; Fontinha *et al.*, 2009). This ability of $A_{2A}R$ to enhance the activity-dependent efficiency of excitatory synapses has been argued to result from an enhanced release of neurotransmitters (Lopes *et al.*, 2002; Rebola *et al.*, 2003b), from a localized desensitization of A_1R-mediated inhibition (Lopes *et al.*, 1999; reviewed in Cunha, 2008), from a facilitation of brain-derived neurotrophic factor-induced signalling (Fontinha *et al.*, 2008) and from an enhanced responsiveness of *N*-methyl-D-aspartate (NMDA) receptors (Rebola *et al.*, 2008). It is likely that all these mechanisms contribute to the modulation of synaptic plasticity by $A_{2A}R$, under different functional conditions or at different excitatory synapses.

Interest in the modulation of synaptic plasticity by adenosine is prompted by the combined arguments that synaptic plasticity phenomena may be a neurophysiological correlate of learning and memory (Martin *et al.*, 2000; Govindarajan *et al.*, 2006) and that caffeine (an antagonist of adenosine receptors, see Fredholm *et al.*, 1999) can modify cognitive function (reviewed in Cunha & Agostinho, 2010). In

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fact, the chronic consumption of caffeine is particularly effective in preventing cognitive dysfunction caused by different noxious insults (de Mendonça & Cunha, 2010), in a manner mimicked by $A_{2A}R$ but not A_1R antagonists (e.g. Prediger *et al.*, 2005; Dall'Igna *et al.*, 2007). Although it has not been clarified how caffeine and $A_{2A}R$ antagonists control memory dysfunction, it is hypothesized that this might result from an $A_{2A}R$-mediated modulation of synaptic function, given that both A_1R and $A_{2A}R$ are most abundantly located in excitatory synapses in cortical regions (Rebola *et al.*, 2003a; Rebola *et al.*, 2005a,b).

It is not only under pathological conditions that caffeine and $A_{2A}R$ antagonists have the ability to normalize memory function. Ageing is also accompanied by cognitive deterioration, which we have found to be attenuated by caffeine consumption (Ritchie *et al.*, 2007). This is also observed in experimental animals, where both caffeine and $A_{2A}R$ antagonists prevent age-related memory deficits (Prediger *et al.*, 2005; Costa *et al.*, 2008). Interestingly, we also observed an imbalance between markers of excitatory and inhibitory terminals in the hippocampus of aged rats (Canas *et al.*, 2009), which is accompanied by an up-regulation of $A_{2A}R$ and a down-regulation of A_1R (e.g. Cunha *et al.*, 1995; Canas *et al.*, 2009). We have previously found that this corresponds to a modified modulation of hippocampal synaptic transmission by A_1R and $A_{2A}R$ (e.g. Sebastião *et al.*, 2000; Rebola *et al.*, 2003b). However, it is not known if there is also a different ability of adenosine receptors to modulate synaptic plasticity phenomena in aged rodents (see Costenla *et al.*, 1999). This is particularly relevant in view of the distinctive ability of A_1R and $A_{2A}R$ to modulate basal synaptic transmission and synaptic plasticity (reviewed in Cunha, 2008). In this study, we investigated in Schaffer fibre–CA1 pyramid synapses if the activation of A_1R and $A_{2A}R$ by endogenous adenosine differently modulates synaptic plasticity in young adult, middle-aged and aged rats.

Materials and methods

Animals

Experiments were performed in male Wistar rats (Harlan Interfauna Iberica, Barcelona, Spain) divided into three age groups: young adult rats (2–3 months, an age at which reproductive behaviour is fully established), middle-aged adult rats (6–8 months) and aged rats (18–20 months) (see Havenaar *et al.*, 1993). The animals were handled according to EU guidelines for the use of experimental animals (86/609/EEC), the rats being anaesthetized under halothane atmosphere before being killed by decapitation.

Drugs

As the goal of this study was to characterize the modification with ageing of the activation of A_1R and $A_{2A}R$ by endogenous adenosine, we used a selective antagonist of each of these receptors, namely DPCPX (1,3-dipropyl-8-cyclopentylxanthine; Sigma) and SCH58261 {7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazol-ol[1,5c] pyrimidine; a generous gift of S. Weiss, Vernalis, UK}. We have previously characterized the efficacy and selectivity of each of these antagonists for A_1R and $A_{2A}R$ in the hippocampus (Sebastião *et al.*, 1990; Rebola *et al.*, 2003b; Lopes *et al.*, 2004). We used a supra-maximal but selective concentration of DPCPX (50 nM) and of SCH58261 (50 nM) to define the role of each receptor in mediating the effects of endogenous extracellular adenosine. A 5 mM stock solution of these antagonists was prepared in dimethylsulfoxide, and dissolved in Krebs solution to a concentration of 50 nM.

Electrophysiological recordings

One 400-μm hippocampal slice, obtained as previously described (e.g. Costenla *et al.*, 2001), 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% O_2 and 5% CO_2) Krebs solution, kept at 30 °C, with the following composition (in mM): 125 NaCl, 3 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$ and glucose 10 (pH 7.4). Electrophysiological recordings of field excitatory postsynaptic potentials (fEPSPs) were obtained as previously described (e.g. Costenla *et al.*, 1999). Two bipolar concentric electrodes placed on two separate sets of the Schaffer collateral/commissural pathway (S1 and S2) were alternately stimulated every 20 s with rectangular pulses of 0.1 ms. 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 was adjusted to evoke an fEPSP with an amplitude of 0.5–1 mV without appreciable population spike contamination at the beginning of the experiment, although population spike contamination often occurred during the experiment as a result of modification of transmission and excitability. Recordings were obtained with an Axoclamp 2B amplifier and digitized using a DigiData 1200 interface (Axon Instruments, Foster City, CA, USA). Averages of eight consecutive responses from each pathway were continuously monitored on a personal computer with LTP 1.01 software (Anderson & Collingridge, 2001). Responses were quantified as the initial slope of the averaged fEPSPs and the effects of drugs (DPCPX and/or SCH58261), added to the superfusion solution, were estimated based on changes in the fEPSP slope compared with baseline. The independence of the pathways was tested by studying paired-pulse facilitation across both pathways, < 10% facilitation being usually observed (Costenla *et al.*, 1999).

Long-term potentiation (LTP) was induced by a high-frequency stimulation pattern (HFS, one train of 100 Hz, 100 stimuli). The intensity of the stimulus was not changed during these stimulation protocols, which were applied after reaching a stable baseline for at least 30 min. LTP was quantified as the percentage change between two values: the average slope of the five potentials taken between 50 and 60 min after the induction protocol in relation to the average slope of the fEPSP measured during 15 min that preceded the induction protocol. LTP was elicited in one pathway under control conditions, and afterwards in the other pathway in the presence of the drugs to be tested. We have previously optimized the experimental conditions ensuring similar amplitudes of LTP in these two pathways recorded in the same hippocampal slice (e.g. Costenla *et al.*, 1999, 2001). Thus, the effects of SCH58261 or DPCPX on LTP were evaluated by comparing the magnitude of LTP in the first pathway in the absence of drugs (control pathway) with the magnitude of LTP in the second pathway in the presence of the adenosine receptor antagonists (test pathway). The drugs were added at least 30 min before applying the HFS train to the test pathway and were present throughout the rest of the experiment. When we tested if the effect of SCH58261 was modified by blockade of A_1R, DPCPX was present during both LTP-inducing periods (i.e. throughout all the experiment), whereas SCH58261 was added only 30 min before the induction of LTP in the test pathway. This type of protocol allows direct comparison of the effect of a tested drug with an internal control in the same slice in the absence of this tested drug. Care was always taken to change the control and test pathways on alternate days to avoid any bias. Clearly the aim of this study was to explore differences of adenosine receptor modulation on LTP across different aged groups rather than exploring whether this

specific LTP protocol triggers a different pattern of LTP in the different age groups.

Input/output curves, in the different age groups, were obtained to ensure that modifications of LTP amplitude were not due to changes in baseline synaptic efficiency. After obtaining a stable baseline for at least 15 min, the input delivered to the slice was decreased until the slope of the fEPSP was virtually zero. Then, the current delivered to the slice was increased by steps of 20 μV and three data points were collected at each stimulation amplitude (each data point being the average of eight individual fEPSPs). The range of all the inputs delivered to the slice was typically from 60 μV to a supramaximal stimulation amplitude of 300 μV. The input/output curves were plotted as the relationship of fEPSP slope versus stimulus intensity (i.e. fiber volley amplitude), which provides a measure of synaptic efficiency.

Analysis of A_1 and A_{2A} receptor expression

One hippocampus per rat was used to extract total RNA with a MagNA Lyser Instrument and a MagNA Pure Compact RNA Isolation kit (Roche, Amadora, Portugal), according to the manufacturer's instructions. The integrity, quantity and purity of the RNA yields were checked by electrophoresis and spectrophotometry. Reverse transcription for first-strand cDNA synthesis from each sample was performed using a random hexamer primer with the Transcriptor First Strand cDNA Synthesis kit (Roche), according to the manufacturer's instructions. The resulting cDNAs were used as templates for real-time polymerase chain reaction (PCR), which was carried out on a LightCycler instrument (Roche) using the FastStart DNA Master SYBR Green I kit (Roche). A_{2A}R and A_1R mRNA expression was calculated relative to β-actin mRNA expression, using the following primers (obtained from Tib MolBiol, Berlin, Germany): A_{2A}R (forward: 5′-AGTCAGAAAGACGGGAAC-3′; reverse: 5′-CAGTAACACGAACGCAA-3′), A_1R (forward: 5′-GGATCGATACCTCCGAGTCA-3′; reverse: 5′-GAGAATCCAGCAGCCAGCTA-3′). Quantification was carried out based on standard curves run simultaneously with the test samples, with A_{2A}R, A_1R and β-actin standards being generated by conventional PCR amplification, as previously described (Duarte *et al.*, 2007). The PCR products were run in a 3% agarose gel electrophoresis to verify fragment size and the absence of other contaminating fragments, estimated by absorbance at 260 nm, and serially diluted to produce the standard curve (90–112 copies/μL). Each real-time PCR reaction was run in triplicate and contained 2 μL of cDNA template, 0.3 μM of each primer, and 3.5 mM $MgCl_2$ in a reaction volume of 20 μL. Cycling parameters were: 95 °C for 10 min to activate DNA polymerase, followed by 40 cycles at 95 °C for 10 s, annealing at 60 or 65 °C for 10 s (for A_{2A}R and A_1R, respectively), and a final extension step at 72 °C for 10 s, in which fluorescence was acquired. The purity and specificity of the resulting PCR products were assessed by melting curve analysis and electrophoresis. Control reactions were performed to verify that no amplification occurred without cDNA.

Immunocytochemical analysis of A_1R and A_{2A}R in glutamatergic nerve terminals

Hippocampal nerve terminals were purified through a discontinuous Percoll gradient and plated over poly-L-lysine-coated cover-slips for immunocytochemical analysis, using previously validated antibodies (e.g. Rodrigues *et al.*, 2008). Permeabilized nerve terminals were incubated for 1 h with guinea pig anti-vesicular glutamate transporters (vGluT1) (1 : 1000; Chemicon, Temecula, CA, USA) and either rabbit anti-A_1R (1 : 200; Affinity Bioreagent, Rockford, IL, USA) or goat anti-A_{2A}R (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by 1 h of incubation with AlexaFluor-labelled secondary antibodies (1 : 2000; Molecular Probes, Leiden, the Netherlands). We also checked that the antibodies against A_1R or against A_{2A}R did not yield any signal in the preparation from each adenosine receptor knockout mouse and the antibody against vGluT1 did not label any element labelled by markers of GABAergic, cholinergic or dopaminergic markers in hippocampal nerve terminals, thus suggesting their selective ability to recognize their purported targets in rat hippocampal nerve terminals. After washing and mounting onto slides with Prolong Gold Antifading (Invitrogen, Eugene, OR, USA), preparations were visualized in a Zeiss Axiovert 200 inverted fluorescence microscope. Digital photomicrographs were obtained using a PlanNeofluar oil objective with 100 × magnification and 1.30 numerical aperture and acquired with a cooled CCD digital camera. As the fluorophores linked to secondary antibodies emitted at different wavelengths, different images of the same field are acquired for each fluorophore by rotating the filter wheel of the microscope. The following filter sets were used for the acquisition: set 38 (excitation – BP 470/40, beam splitter FT 495, emission – BP525/50) for Alexa Fluor 488 and set 31 (excitation – BP 565/30, beam splitter FT 585, emission – BP620/60) for Alexa Fluor 594. It was confirmed that none of the secondary antibodies produced any signal in preparations in which the addition of the corresponding primary antibody was omitted. Most importantly, it was confirmed that individual signals in double-labelled fields are not enhanced over the signals under single-labelling conditions. The exposure time for vGluT1 ranged between 100 and 125 ms and between 150 and 200 ms for A_1R and A_{2A}R, depending on the density of the targeted protein in the observed coverslip. The images, acquired in TIFF format, had the following characteristics: dimensions of 1024 × 767 pixels, resolution of 150 pixels per inch with 32 RGB bits per pixel and a depth/colour pixel relation of 24/16 million. Each coverslip was analysed by counting three different fields and in each field a total of 500 individualized elements, as previously described (Rodrigues *et al.*, 2008). It should be noted that we can never guarantee to achieve plating similar amounts of nerve terminals in each experiment. This is minimized by gently suspending (rather than homogenizing) the nerve terminals until reaching an optical density of 0.055, which provides the best relationship between the number of nerve terminals per coverslip and a minimum number of nerve terminal aggregates, which are discarded from analysis. Hence, no conclusion should be drawn by comparing the number of plated nerve terminals in each experiment.

The acquired images were quantified using a Java-based image processing program, ImageJ 1.37v (NIH, Bethesda, MD, USA), to determine the co-localization of the different fluorophores in the plated nerve terminals using a macro developed by us and validated through manual counting (Rodrigues *et al.*, 2008). Briefly, the recorded images of the same field were grouped according to the fluorophore/colour and converted to black and white images. Thresholding was performed using Otsu's method and the 'rolling ball' algorithm was applied when the background was heterogeneous, both applied equally in all images from the same field. We then applied a counting mask, corresponding to an 8-bit black-and-white analysis, with characteristics adapted to the expected morphological characteristics of nerve terminals, namely to include only particles with a minimum dot size of 4 pixels, a maximum dot size of 25 pixels and a circularity of 0.0–1.0. The individual images from each field were treated with the same settings and the total number of particles labelled with each fluorophore counted using the same mask parameters; as the coordinates of each

particle are recorded, this enables us to detect the co-localization of each fluorophore in the same field. To ensure that putative co-localizations do not result from superimposed nerve terminals, we repeated this same analysis, but now using images acquired using a Zeiss LSM Meta confocal microscope and we concluded that superimposition of particles accounts for < 3% of the estimated co-localization of different fluorophores.

Statistics

Values are presented as mean ± SEM of *n* different animals. As the values for fEPSP slope and LTP magnitude were obtained in the same slices, in the absence and in the presence of a specific drug, a mixed repeated-measures analysis of variance (ANOVA) was the most appropriate statistical test, using control solution and drug as the within-subjects condition, and the age group as the between-subjects condition; if a drug × age group interaction was found significant, *post-hoc* comparisons for the three age groups were done with Fisher's least-significant difference (LSD) test. For the analysis of the impact of ageing on the expression of adenosine receptors and of the association of adenosine receptors to glutamatergic nerve terminals, we first carried out a one-way ANOVA and compared the mean values of the each group using the Newman–Keuls test. Unless otherwise specified, values of $P \leq 0.05$ were considered to be statistically significant. All statistical analyses were performed with SPSS (v. 19.0; IBM SPSS Inc., Chicago, IL, USA).

Results

Input/output curves determined in two age groups (2–3 and 18–20 months old) were super-imposable (Fig. 1), indicating that changes in synaptic strength were unlikely to be responsible for eventual LTP differences among the age groups.

Effects of the A_{2A}R antagonist, SCH58261, on LTP

We used a selective antagonist of A_{2A}R, SCH58261, to probe the role of the tonic activation by endogenous adenosine of A_{2A}R in the modulation of synaptic transmission and plasticity. The supra-maximal concentration of 50 nM was used to block virtually all adenosine A_{2A}R, but maintaining the selectivity towards other adenosine receptor subtypes (see Lopes *et al.*, 2004). Under low-frequency stimulation (0.05 Hz), SCH58261 (50 nM) slightly increased the slope of fEPSPs in hippocampal slices from young adult rats by 11.9 ± 4.3% (n = 8), in middle-aged adult rats by 8.0 ± 4.0% (n = 6) and in aged rats by 6.0 ± 5.1% (n = 5) (non-significant effects, $F_{1,16} = 3.19$, $P > 0.05$, repeated-measures ANOVA) (Fig. 2A).

In contrast to the lack of significant effects under low-frequency stimulation, SCH58261 (50 nM) consistently decreased the magnitude of HFS-induced (100 Hz for 1 s) LTP in all age groups (Fig. 2). In young adult rats, the magnitude of LTP was decreased from 51.4 ± 7.9% in control conditions to 32.0 ± 1.9% in the presence of SCH58261 (n = 8, Fig. 2B and C), which corresponds to a 37.7 ± 8.3% reduction of LTP amplitude by SCH58261. In middle-aged adult rats, the magnitude of LTP was decreased from 73.4 ± 3.4% in control conditions to 45.4 ± 6.0% in the presence of SCH58261 (n = 6, Fig. 2B and D), which corresponds to a 36.2 ± 9.1% reduction of LTP amplitude by SCH58261. In aged rats, the magnitude of LTP was decreased from 80.9 ± 7.4% in control conditions to 28.1 ± 4.4% in the presence of SCH58261 (n = 5, Fig. 2B and E), which corresponds to a 63.4 ± 6.9% reduction of LTP amplitude by SCH58261. Thus, the selective A_{2A}R antagonist SCH58261 decreased LTP ($F_{1,16} = 41.2$, $P < 0.001$, repeated-measures ANOVA), an effect that was marginally dependent upon age (interaction between age group and drug effect, $F_{2,16} = 3.54$, $P = 0.05$), being more pronounced in aged rats than young rats ($P = 0.04$, *post-hoc* LSD test), and in middle-aged rats than young rats ($P = 0.005$, *post-hoc* LSD test).

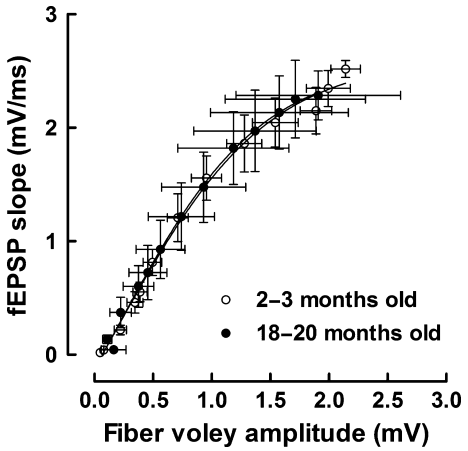

FIG. 1. Input–output curves obtained in hippocampal slices of young adult rats (2–3 months old) and aged rats (18–20 months old) are nearly super-imposable. Input/output curves are displayed as the relationship between fEPSP slope (ordinates) and stimulus intensity (measured as the amplitude of the presynaptic volley, in the abscissa) in the two age groups. After obtaining a stable baseline for at least 15 min, the input delivered to the slice was decreased until the slope of the fEPSP was virtually zero. Afterwards, the current delivered to the slice was increased in steps of 20 μV, with three data points collected at each stimulation amplitude (each data point being the average of eight individual fEPSPs). The range of all the inputs delivered to the slice was typically from 60 μV to a supramaximum stimulation amplitude of 300 μV. Data are mean ± SEM (both of fibre volley and fEPSP slope in each data point) of n = 3 rats.

It should be noted that fEPSPs recorded from slices of old rats tended to be more contaminated with population spikes that fEPSPs recorded from slices of young adult rats, and this was particularly evident after LTP induction (e.g. Fig. 2C). However, none of the slices displayed epileptogenic-like activity before LTP induction. The surgical disconnection between the CA3 and CA1 regions failed to affect either the magnitude of LTP or the degree of contamination of the fEPSPs after LTP induction both in young adult and in aged rats (not shown); this suggests that this experimentally evoked process is independent of spontaneous activity emerging from the CA3 area circuitry. Thus, the greater excitability observed after LTP induction in aged rats might result from different intrinsic neuronal properties or local circuit modifications, such as rheobase properties of CA1 pyramids (Potier *et al.*, 1992; see Barnes, 1994) or lower GABAergic inhibition (Billard *et al.*, 1995) in aged rats.

Effects of the selective A_1R antagonist, DPCPX, on LTP

As the A_{2A}R signalling mechanism changes upon ageing from mainly counteracting A_1R-mediated inhibition in young adults (Lopes *et al.*, 1999, 2002) to a direct facilitatory effect independent of A_1R in aged animals (Rebola *et al.*, 2003b), we next tested if the inhibitory effects of SCH58261 on LTP might result from a modified tonic activation of A_1R.

We used the selective antagonist of A_1R, DPCPX, to probe the role of the tonic activation by endogenous adenosine of A_1R in the modulation of synaptic transmission and plasticity; a supra-maximal concentration of 50 nM of DPCPX was used to block virtually all A_1R, but maintaining the selectivity towards other adenosine receptor

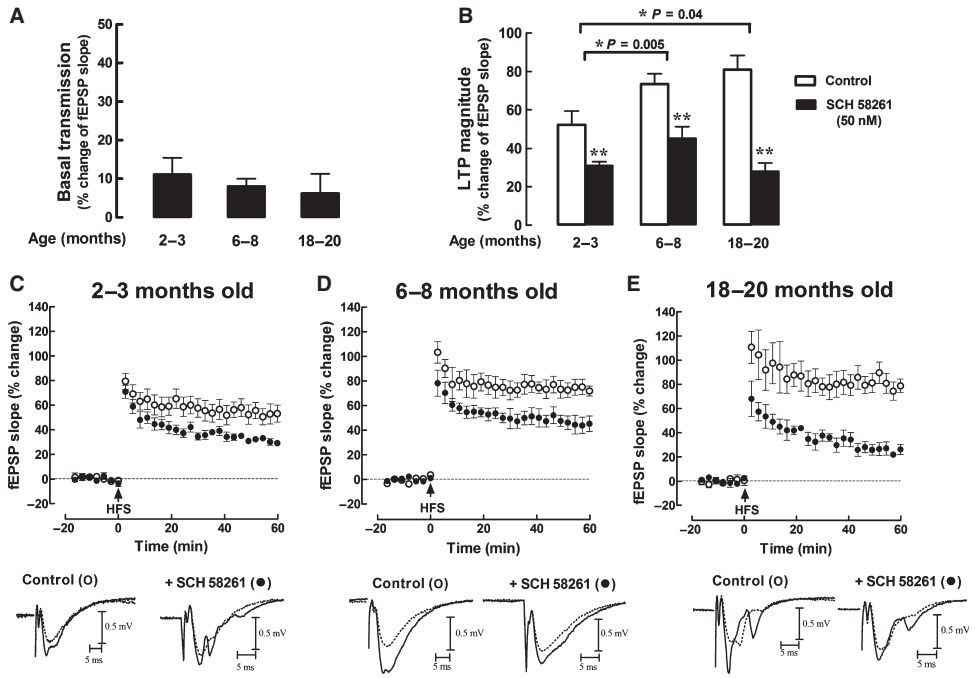

FIG. 2. Adenosine A_{2A} receptors ($A_{2A}R$) modulate synaptic plasticity throughout the age of the animals. (A) The effect of the selective $A_{2A}R$ antagonist SCH 58261 (50 nM) on basal fEPSP amplitude; (B) the magnitude of LTP (change in fEPSP slope at 50–60 min) induced by a high-frequency stimulation (HFS) train in relation to pre-HFS values (0%) in the absence (control) or in the presence of SCH 58261 (50 nM). All values are mean ± SEM; $^{**}P < 0.05$ repeated-measures ANOVA for the effect of SCH 58261 (50 nM); there was a marginally significant interaction between age group and drug effect; **post-hoc* comparisons of the effects of SCH 58261 (50 nM) on LTP in different age groups using Fisher's LSD test. (C–E) Averaged time course changes of fEPSP slope induced by HFS in the absence or in the presence of SCH 58261 (50 nM) in hippocampal slices taken from 2–3 (A, $n = 8$), 6–8 (B, $n = 6$) and 18–20 (C, $n = 5$) month-old rats. SCH 58261 (50 nM) was applied 30 min before the LTP induction in the second pathway and remained in the bath up to the end of the experiment. The ordinates represent normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 15 min before the HFS and the abscissa represents the time of each recording. Recordings obtained in representative experiments are shown below each panel; each recording is the average of eight consecutive responses obtained before (dotted line) and 50–60 min after (filled line) LTP induction in the presence or in the absence (control) of SCH 58261 (50 nM); each trace comprises the stimulus artefact, followed by the presynaptic volley and the fEPSP.

subtypes (see Sebastião *et al.*, 2000). In hippocampal slices from young adult rats, DPCPX (50 nM) increased the slope of fEPSPs by 35.0 ± 6.7% ($n = 7$), in middle-aged adult rats by 30.7 ± 5.4% ($n = 5$) and in aged rats by 27.5 ± 8.1% ($n = 4$). The excitatory effect of DPCPX (50 nM) on fEPSPs slope was statistically significant ($F_{1,11} = 93.95$, $P < 0.001$, repeated-measures ANOVA), but was not modified by the age of the rats (interaction between age group and drug effect, $F_{2,11} = 0.33$, $P > 0.05$) (Fig. 3A).

The effect of DPCPX (50 nM) on the magnitude of LTP was studied in the three age groups. In young adult rats, the magnitude of LTP was 46.8 ± 5.2% in control conditions and increased to 80.9 ± 4.0% in the presence of DPCPX ($n = 6$, Fig. 3B and C). In middle-aged adult rats, the magnitude of LTP was 67.4 ± 5.4% in control conditions and 74.1 ± 8.8% in the presence of DPCPX ($n = 5$, Fig. 3B and D), and in aged rats the magnitude of LTP was 86.7 ± 5.5% in control conditions and 89.9 ± 9.7% in the presence of DPCPX ($n = 4$; Fig. 3B and E). Thus, the selective A_1R antagonist DPCPX (50 nM) increased LTP ($F_{1,14} = 11.93$, $P = 0.004$, repeated-measures ANOVA), an effect that was dependent upon age (interaction between age group and drug effect, $F_{2,14} = 6.33$, $P = 0.01$), tending to be more pronounced in young than in aged rats ($P = 0.06$, *post-hoc* LSD test).

Effects of the $A_{2A}R$ antagonist, SCH58261, on LTP under A_1R blockade

Finally, to determine if the effects of $A_{2A}R$ on LTP might result from an interaction with A_1R, we tested the effect of SCH58261 (50 nM) on the magnitude of LTP in the presence of DPCPX (50 nM) to block A_1R. In these experiments, control LTP was elicited in the presence of DPCPX (50 nM), whereas the test LTP was carried out in the simultaneous presence of DPCPX (50 nM) and SCH58261 (50 nM). As occurred in the absence of A_1R blockade, SCH58261 (50 nM) had only slight effects on basal synaptic transmission in the presence of DPCPX (50 nM) in hippocampal slices from either young adult (4.7 ± 2.9%, $n = 5$), middle-aged adult (0.6 ± 7.3%, $n = 6$) or aged rats (6.7 ± 3.8%, $n = 6$) (non-significant effects, $F_{1,14} = 0.71$, $P > 0.05$, repeated-measures ANOVA) (Fig. 4A).

Upon blockade of A_1R with DPCPX (50 nM), we also observed an inhibitory effect of SCH58261 (50 nM) on the magnitude of LTP (Fig. 4). In young adult rats, the magnitude of LTP was 83.3 ± 12.8% in the presence of DPCPX and 73.9 ± 8.9% in the simultaneous presence of DPCPX and SCH58261 ($n = 5$, Fig. 4B and C). In middle-aged adult rats, the magnitude of LTP was 81.1 ± 8.1% in the presence of DPCPX and 58.2 ± 10.6% in the simultaneous presence of DPCPX and SCH58261 ($n = 6$, Fig. 4B and D), which corresponds to a 27.8 ± 9.4% reduction of LTP magnitude by SCH58261 in the presence of DPCPX. In aged rats, the magnitude of LTP was 90.2.0 ± 6.8% in control conditions and 24.8 ± 2.7% in the presence of SCH58261 ($n = 4$, Fig. 4B and E), which corresponds to a 71.1 ± 4.4% reduction of LTP magnitude by SCH58261 in the presence of DPCPX. Thus, the selective $A_{2A}R$ antagonist SCH58261 (50 nM) decreased LTP in the presence of DPCPX (50 nM) ($F_{1,13} = 18.54$, $P = 0.001$, repeated-measures ANOVA), an effect that was dependent upon age (interaction between age group and drug effect, $F_{2,13} = 3.86$, $P = 0.048$), being more

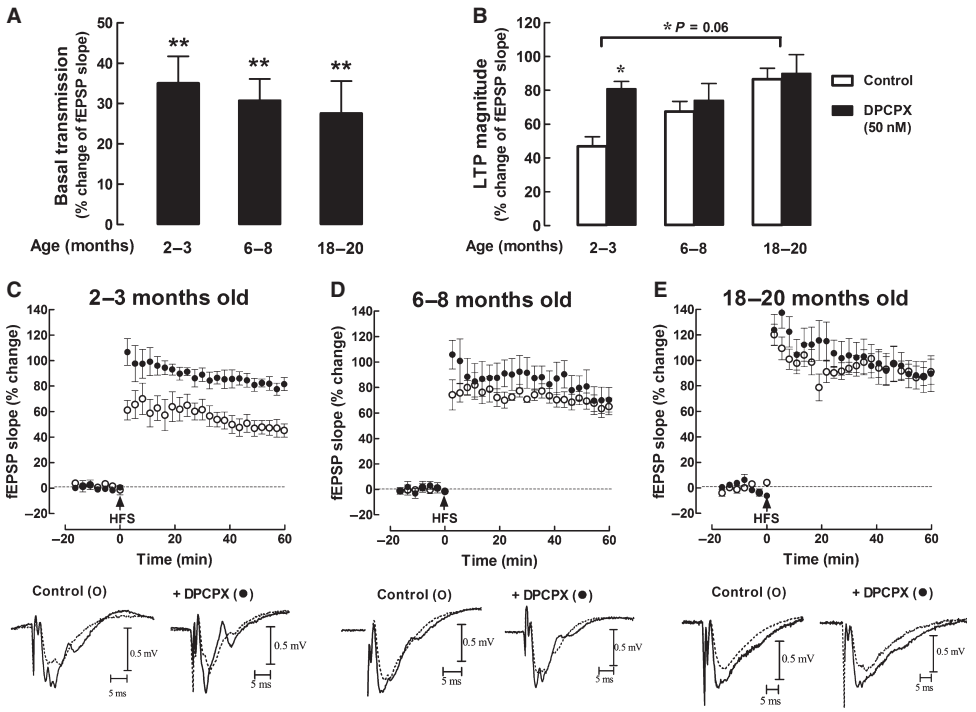

FIG. 3. Adenosine A_1 receptors (A_1R) only modulate synaptic plasticity in hippocampal slices taken from young adult rats. (A) The effect of the selective A_1R antagonist DPCPX (50 nM) on basal fEPSP amplitude; (B) the magnitude of LTP (change in fEPSP slope at 50–60 min) induced by a high-frequency stimulation (HFS) train in relation to pre-HFS values (0%) in the absence (control), or in the presence of DPCPX (50 nM). All values are mean ± SEM; $^{**}P < 0.05$ repeated-measures ANOVA for the effect of DPCPX (50 nM); the interaction between age group and drug effect on LTP was significant; *post-hoc* comparisons of the effect of DPCPX (50 nM) on LTP in the different age groups using Fisher's LSD test. (C–E) Averaged time course changes of fEPSP slope induced by an HFS in the absence or in the presence of DPCPX (50 nM) in hippocampal slices taken from 2–3 (A, $n = 12$), 6–8 (B, $n = 16$) and 18–20 (C, $n = 16$) month-old rats. DPCPX (50 nM) was applied 30 min before the LTP induction in the second pathway and remained in the bath up to the end of the experiment. The ordinates represent normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 15 min before the HFS and the abscissa represents the time of each recording. The recordings obtained in representative experiments are shown below each panel; each recording is the average of eight consecutive responses obtained before (dotted line) and 50–60 min after (filled line) LTP induction in the presence or in the absence (control) of DPCPX (50 nM); each trace comprises the stimulus artefact, followed by the presynaptic volley and the fEPSP.

pronounced in aged than in young rats ($P = 0.04$, *post-hoc* LSD test).

Age-dependent modification of the expression and density of A_1R and $A_{2A}R$ in glutamatergic terminals

In accordance with the previously observed age-associated decrease of the density of A_1R and increase of the density of $A_{2A}R$ in the hippocampus (Cunha *et al.*, 1995; Canas *et al.*, 2009), we now report that ageing caused a reduced expression of A_1R mRNA ($F_{2,12} = 4.718$, $P = 0.0308$, one-way ANOVA), with aged rats displaying less A_1R mRNA than young adult rats ($P < 0.05$, *post-hoc* Newman–Keuls test with $q = 4.323$, corresponding to a 24.3 ± 2.6% decrease, $n = 5$). In contrast, ageing enhanced the expression of $A_{2A}R$ mRNA in aged rats ($F_{2,12} = 23.45$, $P < 0.0001$, one-way ANOVA), with aged rats displaying more $A_{2A}R$ mRNA than middle-aged ($P < 0.05$, *post-hoc* Newman–Keuls test with $q = 8.786$) or young adult rats ($P < 0.05$, *post-hoc* Newman–Keuls test with $q = 7.923$, corresponding to a 103.4 ± 10.9% increase, $n = 5$) (Fig. 5A and B).

We next investigated if aged animals displayed a different association of A_1R and $A_{2A}R$ with glutamatergic nerve terminals of the hippocampus that could explain the observed age-related changes in the efficiency of A_1R and $A_{2A}R$ modulating the functioning of glutamatergic synapses. As shown in Fig. 5C–F, the association of $A_{2A}R$, but not of A_1R, with glutamatergic synapses was modified in aged rats. Thus, the number of glutamatergic terminals (identified as immunopositive for vGluT1) endowed with A_1R was similar ($F_{2,9} = 0.1797$, $P = 0.8384$, one-way ANOVA comparing the different age groups) in young adult rats (71.2 ± 12.7%, $n = 4$), in middle-aged rats (59.6 ± 15.2%, $n = 4$) and in aged rats (63.1 ± 13.5%, $n = 4$). In contrast, there was an age-related increase of $A_{2A}R$ immunoreactivity in vGluT1-positive terminals ($F_{2,15} = 17.41$, $P < 0.0001$, one-way ANOVA): in young adult rats 25.3 ± 3.9% ($n = 6$) of vGluT1-positive terminals were endowed with $A_{2A}R$ and a similar number was present in middle-aged rats (33.7 ± 6.9%, $n = 6$, $P > 0.05$, $q = 1.559$, *post-hoc* Newman–Keuls test), whereas in aged rats the number of glutamatergic terminals endowed with $A_{2A}R$ was increased to 66.8 ± 5.8% ($n = 6$), which was larger than for the middle-aged ($P < 0.05$, $q = 6.321$, *post-hoc* Newman–Keuls test) and young adult groups ($P < 0.05$, $q = 7.880$, *post-hoc* Newman–Keuls test).

Discussion

The main conclusion of this study is that the ability of adenosine to modulate hippocampal LTP through activation of adenosine $A_{2A}R$ is observed from young to aged animals and is more pronounced in aged animals. This is accompanied by an age-related increase of the expression of $A_{2A}R$ mRNA in the hippocampus, in agreement with the previously reported increased density of $A_{2A}R$ in the hippocampus of aged rats (Cunha *et al.*, 1995; Canas *et al.*, 2009); furthermore, we now report that there is an increased number of glutamatergic nerve

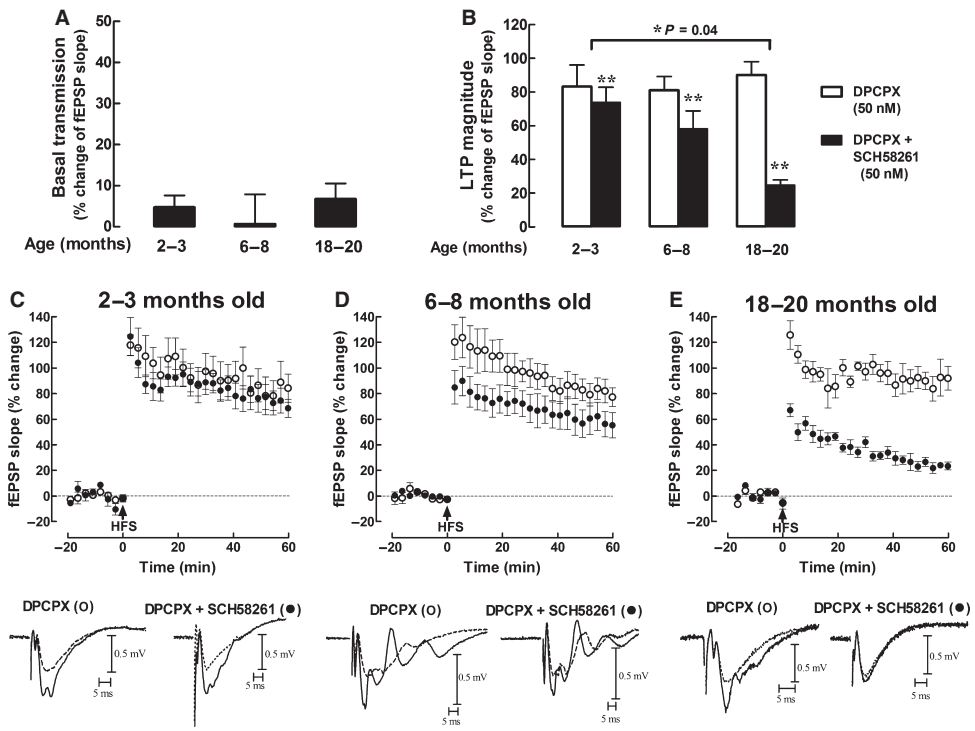

FIG. 4. Adenosine A_{2A} receptors ($A_{2A}R$) modulate synaptic plasticity throughout the age of the animals independently of adenosine A_1 receptors (A_1R). (A) The effect of the selective $A_{2A}R$ antagonist SCH58261 (50 nM) on basal fEPSP amplitude, under the continuous blockade of the A_1R with DPCPX (50 nM); (B) the magnitude of LTP (change in fEPSP slope at 50–60 min) induced by high-frequency stimulation (HFS) train in relation to pre-HFS values (0%) in the presence of DPCPX (50 nM), in the absence (control), or in the presence of SCH 58261 (50 nM). All values are mean ± SEM; $^{**}P < 0.05$ repeated-measures ANOVA for the effect of SCH 58261 (50 nM); there was a significant interaction between age group and drug effect, **post-hoc* comparisons of the effects of SCH58261 (50 nM) on LTP in the different age groups using Fisher's LSD test. (C–E) Averaged time course changes of fEPSP slope induced by HFS in the presence of DPCPX (50 nM) or in the simultaneous presence of DPCPX (50 nM) and SCH 58261 (50 nM), in hippocampal slices taken from 2–3 (A, $n = 5$), 6–8 (B, $n = 6$) and 18–20 (C, $n = 4$) month-old rats. DPCPX (50 nM) was applied 30 min before the LTP induction in the first pathway and remained in the bath up to the end of the experiment; SCH 58261 (50 nM) was applied 30 min before LTP induction in the second pathway and remained in the bath up to the end of the experiment. The ordinates represent normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 15 min before HFS and the abscissa represents the time of each recording. The recordings obtained in representative experiments are shown below each panel; each recording is the average of eight consecutive responses obtained before (dotted line) and 50–60 min after (filled line) LTP induction in the presence of DPCPX (50 nM) or in the presence of DPCPX (50 nM) plus SCH 58261 (50 nM); each trace comprises the stimulus artefact, followed by the presynaptic volley and the fEPSP.

terminals endowed with $A_{2A}R$ in the hippocampus of aged rats, which might explain the enhanced ability of $A_{2A}R$ to modulate LTP in aged rats. Although these results favour the hypothesis that the enhanced ability of $A_{2A}R$ to modulate LTP in aged rats might result from an enhanced efficiency of the $A_{2A}R$ neuromodulation system, it cannot be excluded that age-related changes in the dynamic range of LTP might also contribute to an enhanced impact of $A_{2A}R$ on LTP with ageing. In fact, there was a tendency (which did not reach statistical significance) for a large LTP magnitude as well as an increased excitability after LTP in aged rats, which could contribute to an increased efficiency of the $A_{2A}R$ neuromodulation system.

In contrast, we observed that adenosine modulates hippocampal LTP through activation of adenosine A_1R only in young animals. This is in agreement with our previous observations that LTP magnitude is more affected by A_1R blockade in young adult than in aged animals (Costenla *et al.*, 1999), although we have not detailed possible regional differences in age-dependent modulation by A_1R of LTP magnitude (Rex *et al.*, 2005). Interestingly, there is a selective decrease of A_1R modulation of LTP in aged rats, as the A_1R-mediated modulation by endogenous adenosine of basal synaptic transmission was not modified with ageing. This probably results from the previously reported decreased efficiency of A_1R in aged rats (Sebastião *et al.*, 2000), which is compensated for by a different extracellular metabolism of adenosine in aged rats (Cunha *et al.*, 2001a) leading to different bioavailability of extracellular adenosine to activate A_1R and $A_{2A}R$ in hippocampal synapses of aged rats. This decreased ability of A_1R to modulate LTP magnitude in aged rats is also in agreement with the presently observed decreased expression of A_1R mRNA in the hippocampus of aged rats. A similar decrease of A_1R mRNA expression has also been reported to occur in the cerebral cortex (Cheng *et al.*, 2000) and is in accordance with the decreased density of A_1R in both the hippocampus and cerebral cortex of aged animals (Cunha *et al.*, 1995; Canas *et al.*, 2009). We also observed that the number of glutamatergic nerve terminals endowed with A_1R in aged rats was maintained, indicating that the decreased ability of A_1R in the control of LTP in aged rats may be related mostly to a possible reduction of the density of A_1R (Cunha *et al.*, 1995, 2001b), probably also occurring in glutamatergic terminals. It cannot also be completely discarded that the different impact of A_1R on LTP at different ages may result from their known influence on circuits in the CA3 area (Moore *et al.*, 2003), which drive the recorded CA1 synaptic activity; however, the likeliness of such a scenario is reduced by the observation that the surgical disconnection between the CA3 and CA1 has a limited impact on the magnitude of LTP in slices from young adults or aged rats.

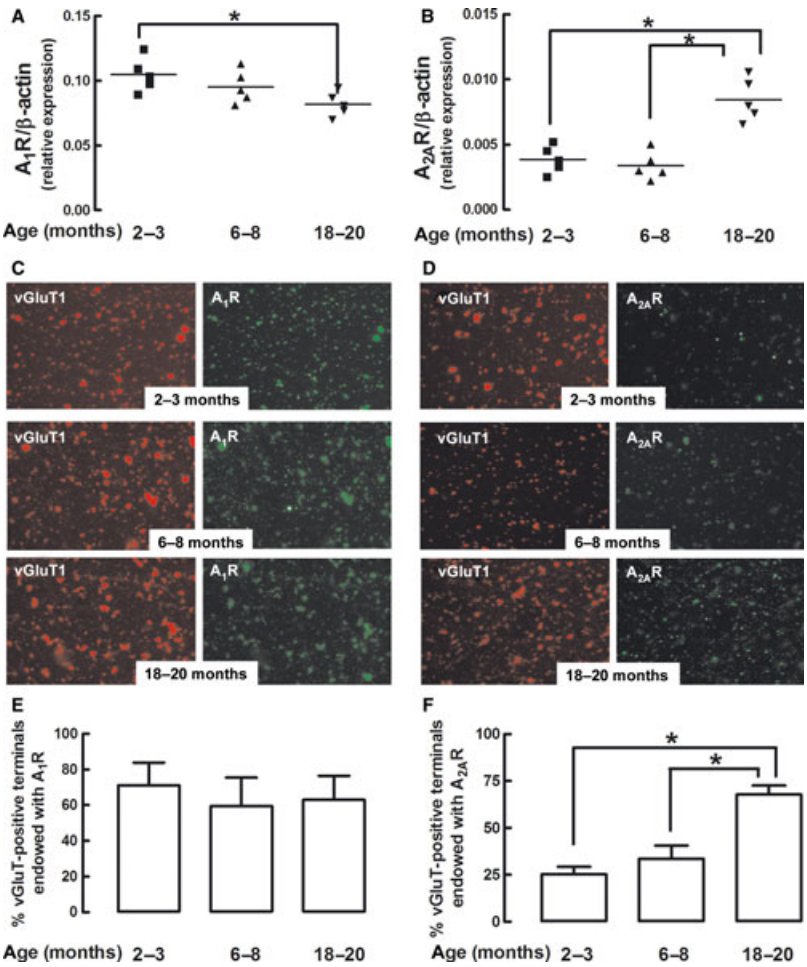

FIG. 5. Increased expression of A_{2A} receptor ($A_{2A}R$) mRNA and increased association of $A_{2A}R$ to glutamatergic nerve terminals in the hippocampus of aged rats. (A, B) Expression of A_1R (A) and $A_{2A}R$ (B) in the hippocampus of rats (2–3, 6–8 and 18–20 months old). A_1R and $A_{2A}R$ mRNA levels (copies/μL) were determined with qRT-PCR and normalized to the level of β-actin mRNA. Bars represent mean ± SEM from five different rats from each age group, run in triplicate. (C, D) Double immunocytochemical staining of A_1R (C) or $A_{2A}R$ (D) together with a glutamatergic marker (vesicular glutamatergic transporter type 1, vGluT1) in hippocampal nerve terminals obtained from 2–3-month-old (top photographs in each panel), 6–8-month-old (middle photographs in each panel) or 18–20-month-old rats (bottom photographs in each panel). (E, F) The fraction of glutamatergic terminals (i.e. vGluT1-positive) containing A_1R (E) or $A_{2A}R$ (F). Data are mean ± SEM of n = 4–6 rats in each age group. *$P < 0.05$ between the indicated bars using the Newman–Keuls test applied after ANOVA. Note that there is always a different density of plated nerve terminals in different experiments (each pair of pictures corresponds to a different experiment) and, hence, attention should be focused on the co-localization of the two fluorophores rather than the absolute value of each fluorophore.

It thus seems that ageing impacts differently on $A_{2A}R$ and A_1R in terms of their expression and density, which may underline their different functional roles in the modulation of glutamatergic synaptic transmission in the hippocampus of aged rats. The molecular mechanisms of these age-related changes are still poorly understood, as a consequence of our limited knowledge on the mechanisms controlling the expression of A_1R and $A_{2A}R$ and their subcellular targeting. However, the present data further emphasize the different functional roles of A_1R and $A_{2A}R$ in the modulation of synaptic transmission and synaptic plasticity (reviewed in Cunha, 2008), which seem to be modified upon ageing. In fact, as previously shown (Dunwiddie, 1980), the tonic activation of A_1R by endogenous adenosine modulates basal synaptic transmission; however, there does not seem to be sufficient adenosine to tonically activate $A_{2A}R$ under conditions of a lower frequency of stimulation (basal conditions) (see Cunha *et al.*, 1997). In contrast, under conditions of HFS able to trigger LTP, there is now a tonic activation of $A_{2A}R$ by endogenous adenosine that facilitates LTP. This probably results from the particular pool of extracellular adenosine that activates $A_{2A}R$, which is formed from synaptically released ATP (Cunha *et al.*, 1996a; Rebola *et al.*, 2008) that is released in a frequency-dependent manner (Cunha *et al.*, 1996b; Pankratov *et al.*, 2007; reviewed in Cunha, 2008). Thus, as previously found in a different set of hippocampal synapses (Rebola *et al.*, 2008), the results presented in this study show that $A_{2A}R$ are selectively engaged during synaptic plasticity, whereas the activation of A_1R could instead act as a general threshold barrier that tonically restrains synaptic transmission and, to a lower extent, LTP (reviewed in Cunha, 2008). Notably, these different roles of A_1R and $A_{2A}R$ in the modulation of synaptic transmission and plasticity are increased with ageing. In the case of $A_{2A}R$, their tonic activation by endogenous adenosine becomes increasingly evident upon ageing only upon LTP and not under basal stimulation conditions. In contrast, A_1R seem to be selectively involved in the modulation of basal synaptic transmission at all tested ages but lose their tonic effect on LTP, which is only observed in young animals. Hence, in aged rats there is a more evident functional segregation of the roles

played by A_1R and $A_{2A}R$: whereas the former selectively modulate basal transmission, the latter selectively modulate LTP. Further studies will be required to determine if these changes in the density and function of the adenosine neuromodulation system contribute to age-related dysfunction or represent an adaptive mechanism to counteract the different and heterogeneous modifications of neuronal circuits on ageing (e.g. Barnes, 1994). Further studies should also be directed to explore if these age-related changes in adenosine neuromodulation are also found at other synapses, for instance at CA3 synapses, where both A_1R and $A_{2A}R$ are also known to modulate synaptic plasticity (Moore *et al.*, 2003; Rebola *et al.*, 2008).

It is interesting to note that this age-related segregation of the roles of A_1R and $A_{2A}R$ is also accompanied by a disappearance of a close interplay between A_1R and $A_{2A}R$. Thus, we observed that in aged rats the enhanced tonic activation of $A_{2A}R$ by endogenous adenosine was independent of A_1R function (i.e. SCH58261 decreased the magnitude of LTP irrespective of the presence of DPCPX), in accordance with previous neurochemical data (Lopes *et al.*, 1999) indicating that the role of $A_{2A}R$ in the modulation of hippocampal physiology changes upon ageing from a modulator of other modulators (i.e. A_1R in adult rats) to a direct modulator of synaptic function in aged rats. Furthermore, it should be noted that the modifications of $A_{2A}R$ seem to be more evident in aged rats. Thus, it is in aged rats that we observe an enhanced expression of $A_{2A}R$ mRNA, a greater association of $A_{2A}R$ with glutamatergic nerve terminals and a greater impact of $A_{2A}R$ to modulate LTP. This is in accordance with previous studies showing that the ontogenic modifications of $A_{2A}R$ and A_1R in the hippocampus seem to differ: whereas the enhanced density of $A_{2A}R$ was only observed in aged rats (Rebola *et al.*, 2003b), there was an age-dependent continuous decrease of the density of A_1R (Cunha *et al.*, 2001b).

The presently observed greater effects of tonic $A_{2A}R$ activation on the modulation of LTP in hippocampal slices of aged rats are particularly relevant in view of the ability of $A_{2A}R$ antagonists to recover and normalize age-related memory deficits (Prediger *et al.*, 2005). In fact, $A_{2A}R$ antagonists recovered the deficient social recognition memory of aged rats to levels of performance similar to those recorded in adult rats, whereas $A_{2A}R$ antagonists did not modify memory performance in adult rats (Prediger *et al.*, 2005). However, the relationship between the presently observed effects of $A_{2A}R$ antagonists on LTP and the ability of $A_{2A}R$ antagonists to normalize memory performance should be considered with care for two reasons: (i) the effects of $A_{2A}R$ on memory were observed upon prolonged administration of $A_{2A}R$ antagonists, whereas we now report acute effects of $A_{2A}R$ antagonists on LTP; and (ii) the ability of $A_{2A}R$ to control memory performance was observed in whole animals, whereas we now report effects of $A_{2A}R$ in isolated hippocampal slices, which only partially (at best) recapitulate the functioning of brain circuits. Furthermore, the relationship between LTP and memory is certainly complex, because it involves the correct processing of memory-related information, which is dependent on defining the salience of information to be encoded, a process that requires not only potentiation of synaptic strength but also depression and depotentiation (e.g. Martin *et al.*, 2000; Govindarajan *et al.*, 2006; Kemp & Manahan-Vaughan, 2007), both homosynaptic and heterosynaptic (e.g. Remondes & Schuman, 2004; Izumi & Zorumski, 2008). In this context, $A_{2A}R$ were proposed to have a normalizing, rather than a purely facilitatory, role on synaptic plasticity and memory (discussed in Cunha & Agostinho, 2010).

Recently, great interest was raised about a possible protective effect of caffeine, the most widely consumed psychotropic drug in the world, against cognitive decline and dementia, based on both animal studies and epidemiological data (de Mendonça & Cunha, 2010). Caffeine is a non-selective antagonist for both adenosine A_1R and $A_{2A}R$ and its impact on synaptic plasticity is qualitatively similar to that caused by $A_{2A}R$ blockade (Costenla *et al.*, 2010). The increased role for $A_{2A}R$ in the modulation of synaptic plasticity in aged subjects could help to explain the reported benefits for caffeine in memory and cognition specifically in aged animals (Costa *et al.*, 2008) and old people (e.g. Ritchie *et al.*, 2007).

In conclusion, the present results show that the role of adenosine receptors is modified upon ageing. The observed predominant effect of adenosine $A_{2A}R$ to modulate LTP in aged rats is in notable agreement with the ability of $A_{2A}R$ antagonists to normalize the age-related decline of memory performance, and should encourage testing a possible effect of adenosine $A_{2A}R$ antagonist to prevent or ameliorate cognitive deficits in the elderly.

Acknowledgements

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Abbreviations

A_1R, A_1 receptor; $A_{2A}R$, A_{2A} receptor; ANOVA, analysis of variance; DPCPX, 1,3-dipropyl-8-cyclopentyl xanthine; fEPSPs, field excitatory postsynaptic potentials; HFS, high-frequency stimulation; LTP, long-term potentiation; SCH58261, 5-amino-7-2-phenylethyl.-2-2-furyl-pyrazolo[4,3-ex-1,2,4-triazol-o-1,5-9]pyrimidine; vGluT1, vesicular glutamate transporters.

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