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Marti'N

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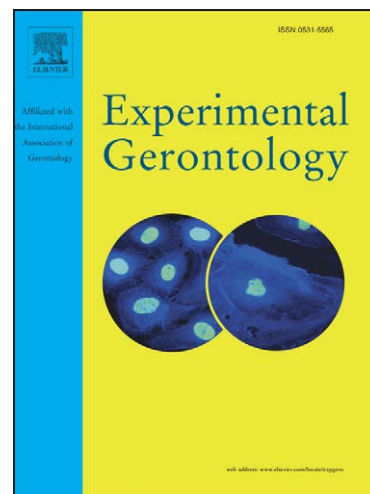
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**AGE-RELATED EXPRESSION OF ADENOSINE RECEPTORS IN BRAIN  
FROM THE SENESENCE-ACCELERATED MOUSE**

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**Keywords:** Senescence-accelerated mouse, adenosine receptors, aging.

## Abstract

Senescence-accelerated mice (SAM) are used as a model of aging and age-associated diseases. SAMP8 are prone strains that show shortened life span and deficits in learning and memory processes, while SAMR1 are strains of accelerated senescence-resistant, long-lived mice. Due to their abnormal APP (amyloid precursor protein) metabolism in brain, SAMP8 may be an Alzheimer-type model. Adenosine receptors are G-protein coupled receptors which are altered in brain from Alzheimer Disease (AD) cases. The analysis of adenosine receptors in brain from young (21 days old) and middle-aged (180 days old) SAMP8 as compared with SAMR1 mice revealed differences between these strains associated with age. The age-related increase in mRNA coding  $A_1$  and  $A_{2B}$  receptors observed in SAMR1 was absent in SAMP8.  $A_1$  receptors were significantly decreased with age in SAMR1, while no differences were observed in SAMP8. However, the levels of  $A_1$  receptors in young SAMP8 were even lower than those obtained in middle-aged SAMR1. In addition,  $A_{2A}$  receptors were significantly increased only in aged SAMR1. A similar age-related decrease in  $A_1$  receptors level was also observed in brain from male Wistar rats. These results suggest different age-related effects on adenosine receptors in SAMR1 and SAMP8 strains. Since  $A_1$  receptors are mainly neuroprotective, their important loss in very young SAMP8 strain suggests the involvement of these receptors in the pathogenesis of neurodegenerative diseases associated with aging.

# 1. Introduction

Senescence-accelerated prone mouse strains (SAMP) and resistant strains (SAMR) were established by Takeda and colleagues at Kyoto University by selective breeding from their AKR/J colony in the early 1970s (Takeda et al., 1981). Prone strains of SAM (SAMP8) show shortened life span and early manifestation of senescence with characteristic pathological phenotypes similar to those often observed in elderly humans. These include senile osteoporosis, osteoarthritis, age-related deficits in learning and memory with/without forebrain atrophy, senile amyloidosis, et al. SAMR1 are strains of accelerated senescence-resistant, long-lived mice. The mean life span in the P series is about 9.7 months, 40% shorter than that of the R series (16.3 months). Senescence accelerated mouse prone strain SAMP8 is one of the most appropriate models to study aging and age-associated diseases because it shows an age-related deterioration of learning and memory at an earlier age, compared with the control mouse R1 strain in senescence-accelerated resistant mouse (SAMR1) (Miyamoto et al., 1986; Miyamoto, 1997; Nomura and Okuma, 1999; Butterfield and Poom, 2005). Alteration of learning and memory of SAMP8 have been associated with a decline in neurotransmitter activity as well as decreased serotonin, increased GABA and decreased cholinergic activity (Morley et al., 2002b). In addition, cognitive defect in these animals can be due to overproduction of  $\beta$ -amyloid peptide (A $\beta$ P) being reversed by specific antibodies to  $\beta$ -amyloid. Furthermore, SAMP8 mice present lower mRNA levels of apolipoprotein E and increased levels of presenilin-2 when compared to SAMR1 (Wei et al., 1999). Therefore, these animals have been suggested as a good model for the study of Alzheimer's disease (Morley, 2002; Morley et al., 2002a; Banks et al., 2007; Pallas et al., 2008).

SAMP8 mice also present other characteristics that contribute to their pathological phenotype. SAMP8 mice present a lower expression of neurotrophic genes as glial cell derived neurotrophic factor (GDNF) (Miyazaki et al., 2003), neurotrophin-3 (NT-3) and nerve growth factor (NGF) (Kaisho et al., 1994). Furthermore, the expression of proteins involved in reactive oxygen species (ROS) metabolism is altered in SAMP8 mice, which may be involved in the increased oxidative stress found in SAMP8 mouse brain. Compared to SAMR1 mice, the activity of enzymes involved in antioxidant processes are decreased in the cerebral cortex of aged SAMP8. This has been reported for manganese superoxide dismutase (Mn-SOD), glutamine synthase (GS), glutathione peroxidase (GPx) and peroxidase activities (Kurokawa et al., 2001; Sato et al., 1996; Okatani et al., 2002). Moreover, other enzymes which contribute to ROS generation, such as nitric oxide synthase (NOS), are increased in aged SAMP8 mice (Inada et al., 1996).

Adenosine is a nucleoside widely distributed in central and peripheral nervous system that exerts its actions through four types of receptors named A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>, all of them being G-protein coupled receptors (GPCR). A<sub>1</sub> and A<sub>3</sub> receptors are coupled, through Gi/o proteins, to adenylyl cyclase activity inhibition, while A<sub>2A</sub> and A<sub>2B</sub> receptors are coupled to stimulation of the enzymatic activity, through Gs protein (Ralevic and Burnstock, 1998, Fredholm et al., 2001; 2005). Out of the four adenosine receptors, the A<sub>1</sub> subtype is the most abundant and widespread in the brain, where it plays a neuroprotective role because of its capacity to decrease the release of excitatory neurotransmitters, mainly glutamate (Dunwiddie and Masino, 2001). A<sub>2A</sub> receptors are concentrated in the basal ganglia but they are also present throughout the brain, albeit in a considerably lower density. A<sub>2B</sub> and A<sub>3</sub> receptors are the least abundant in the brain (Cunha, 2005).

Changes in adenosine receptors during aging have been demonstrated in several animals (Cunha, 2005) and in humans (Meyer et al., 2007). Decreased expression and density of A<sub>1</sub> in the cortical and hippocampal regions has been observed (Cheng et al., 2000; Cunha et al., 1995, 2001a, b; Paganopoulou et al., 1992) whereas the density of A<sub>2A</sub> receptors increases with age (Cunha et al., 1995; Cunha, 2005; Lopes et al., 1999). However, these age-related changes in adenosine receptor levels observed in some brain areas have not been detected in the striatum, where these levels might be only slightly affected by ageing (Cunha et al., 1995). Beyond this, adenosine receptors have not been analyzed in SAM strains until now. The SAMP8 mouse has been proposed as an excellent model for studying the pathogenesis of learning and memory disturbances associated with AβP overproduction. The identification of senile plaques and the increased expression of AβP in this mouse model suggest it may be an acceptable model for Alzheimer's disease (Morley, 2002; Morley et al., 2002 a,b; Banks et al., 2007; Pallas et al., 2008). We have previously reported that adenosine A<sub>1</sub> and A<sub>2A</sub> receptors are altered in frontal cortex brain from AD patients (Albasanz et al., 2008). The aim of the present work was to determine the expression of different adenosine receptors, mainly A<sub>1</sub> and A<sub>2A</sub>, and their possible age-related changes in brains from SAMR1 and SAMP8 strains, in order to use these animals as models to study the mechanism involved in the neuropathogenesis of Alzheimer's disease.

## 2. Materials and Methods

### 2.1. Materials

Cyclopentyl-1,3-dipropylxanthine,8-[dipropyl-2,3-<sup>3</sup>H(N)] ([<sup>3</sup>H]DPCPX) 120 Ci/mmol) and adenosine 3', 5'-cyclic phosphate [2,8-<sup>3</sup>H] ([<sup>3</sup>H]cAMP) 27.4 Ci/ mmol) were

purchased from PerkinElmer (Madrid, Spain). Anti-A<sub>1</sub> antibody was purchased from Calbiochem (Bionova, Madrid, Spain), anti-A<sub>2A</sub> antibody from Upstate (Millipore, Madrid, Spain) and anti-β-actin from Abcam (Cambridge, UK). Guanosine-5'-O(3-thiotriphosphate) tetralithium salt (GTPγS) and calf intestine adenosine deaminase (ADA) were purchased from Roche (Barcelona, Spain). Creatine kinase, Creatine phosphate, Protein kinase A, 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro 20-1724), Forskolin, N<sup>6</sup>-cyclopentyladenosine (CPA), N<sup>6</sup>-cyclohexyladenosine (CHA), Adenosine 5'-triphosphate (ATP) and Guanosine 5'-[β,γ-imido]triphosphate (Gpp(NH)p) were from Sigma (Madrid, Spain). All other reagents were of analytical grade and obtained from commercial sources.

## 2.2. Animals

Young (21 days old) and middle-aged (180 days old) SAMR1 and SAMP8 mice or young male (3 months) and aged male (24 months) Wistar rats were kept at constant temperature (25 ± 1 °C) and humidity, maintained on a 12 h light/12 h dark cycle with *ad libitum* access to rodent food and water. All experiments followed the European Union regulations about animal experimentation and those of the Experimentation Animal Committee of the Castilla-La Mancha University. Special care was taken to minimize animal suffering and to reduce the number of animals used.

## 2.3. Plasma membrane isolation

Plasma membranes from mouse and rat brain were isolated as described previously (León et al., 2002). Samples were homogenized in 20 volumes of isolation buffer (50 mM Tris-HCl, pH 7.4 containing 10 mM MgCl<sub>2</sub> and protease inhibitors) in Dounce homogenizer (10xA, 10xB). After homogenization, whole brain preparations



(excluding cerebellum and spinal cord) were centrifuged for 5 min at 1000 x g in a Beckman JA 21 centrifuge. Supernatant was centrifuged for 20 min at 27000 x g and the pellet was finally resuspended in isolation buffer.

## 2.4. [<sup>3</sup>H]DPCPX binding assays to plasma membranes

Binding assays to plasma membranes from mouse and rat brain were performed as described previously (León et al., 2002). Plasma membranes were incubated with 5 U/mg adenosine deaminase (ADA) in 50 mM Tris, 2 mM MgCl<sub>2</sub>, pH 7.4, for 30 min at 25°C, in order to eliminate endogenous adenosine from membrane preparations. Then, plasma membranes (50-75 µg of protein) were incubated with [<sup>3</sup>H]DPCPX for 2 h at 25°C. Saturation assays were carried out at different [<sup>3</sup>H]DPCPX concentrations (0.1-20 nM) using CPA at a concentration 10<sup>5</sup> times higher than the radioligand, in order to obtain non-specific binding. Binding assays were stopped by rapid filtration through Whatman GF/B filters, previously pre-incubated with 0.3% polyethylenimine using a FilterMate Harvester (Perkin-Elmer). Scintillation liquid mixture was added in order to measure radioactivity in a Microbeta Trilux (Perkin Elmer) liquid scintillation counter.

When the effect of Gpp(NH)p was studied [<sup>3</sup>H]DPCPX binding assays were performed at saturation concentration using the same methodology with minor modifications. Briefly, the buffer employed was 50 mM Tris, pH 7.4, containing 10 µM Gpp(NH)p, and the final concentration of [<sup>3</sup>H]DPCPX used was 20 nM.

## 2.5. Western blot assays

For Western blot assays, 50 µg of protein was mixed with loading buffer containing 0.125 M Tris (pH 6.8), 20% glycerol, 10% β-mercaptoethanol, 4% SDS and 0.002% bromophenol blue, and heated at 95°C for 5 min. Protein was electrophoresed

on a 10% SDS-PAGE gel using a mini-protean system (Bio-Rad) with molecular weight standards (Bio-Rad). Protein transfer to nitrocellulose membranes was carried out in iBlot™ Dry Blotting System (Invitrogen). Membranes were washed with PBS-tween-20, blocked with PBS containing 5% skimmed milk, and then incubated with the primary antibodies at 4°C overnight (1:1000 dilution for anti-A<sub>1</sub>R, 1:500 dilution for anti-A<sub>2A</sub>R and 1:5000 dilution for anti-β-actin). After rinsing, the membranes were incubated with the corresponding secondary antibody (Bio-Rad) at a dilution of 1:5000 in PBS containing 5% skimmed milk for 30 minutes. Antigen was visualized using the ECL chemiluminescence detection kit (Amersham), and specific bands were quantified by densitometry using Multianalyst 1.0 software (Bio-Rad). Membranes were stripped for a second probe by incubating with 0.15% glycine, 0.1% SDS, and 1% tween-20, pH 2.2, for 30 minutes at room temperature. After removing the stripping buffer and washing them twice with PBS, membranes were ready for the blocking stage.

## 2.6. Preparation of Total RNA and cDNA

Total RNA was extracted from whole brain (excluding cerebellum and spinal cord) using an ABI 6100 Nucleic Acid PrepStation according to the manufacturer's protocol. All chemicals for the ABI 6100 were purchased from Applied Biosystems (Foster City, CA). Total RNA from mice was isolated and stored individually at -80°C. Ratio of A<sub>260</sub>/A<sub>280</sub> (purity of RNA) was in the range 1.9–2.1. RNA concentrations were determined from the A<sub>260</sub>. One microgram of total RNA was reverse transcribed using Applied Biosystems High-Capacity cDNA Archive Kit.

## 2.7. Quantitative Real Time RT-PCR Analysis

Quantitative real time RT-PCR analysis (Higuchi et al, 1993) was performed with an Applied Biosystems Prism 7500 Fast Sequence Detection System using TaqMan® universal PCR master mix according to the manufacturer's specifications (Applied Biosystems Inc., Foster City, CA). The validated TaqMan probes and primers for A<sub>1</sub> (assay ID: Rn00567668\_m1), A<sub>2A</sub> (assay ID: Rn00583935\_m1), A<sub>2B</sub> (assay ID: Rn00567697\_m1), A<sub>3</sub> (assay ID: Rn00563680\_m1) and β-actin (Rn00667869\_m1) were assay-on-demand gene expression products (Applied Biosystems). The TaqMan® primer and probe sequences are packaged together in a 20x solution. Rat β-actin gene was used as endogenous control. The thermal cycler conditions were as follows: hold for 20 s at 95 °C, followed by two-step PCR for 40 cycles of 95 °C for 3 s, followed by 60 °C for 30 s. Levels of RNA expression were determined using the 7500 Fast System SDS software version 1.3.1 (Applied Biosystems) according to the 2<sup>-ΔΔCt</sup> method. Briefly, expression results for a gene were normalized to internal control β-actin relative to a calibrator, consisting of the mean expression level of the corresponding gene in control samples as follows:  $2^{-\Delta\Delta C_t} = 2^{-((C_t \text{ receptor gene} - C_t \text{ actin})_{\text{sample}} - (C_t \text{ receptor gene} - C_t \text{ actin})_{\text{calibrator}})}$ . The results from three independent repeat assays, performed on different plates each using different cDNAs from the animals analyzed, were averaged to produce a single mean quantity value for each mRNA for each animal.

## 2.8. Adenylyl cyclase activity determination

Adenylyl cyclase activity was determined in brain plasma membranes as previously described (León et al., 2005). Assays were performed with 10-20 μg of protein in a final volume of 0.25 mL of 50 mM Tris-HCl pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM 1,4- Dithiothreitol, 1 mg/mL bovine serum albumin, 1 mg/mL creatine kinase, 10 mM creatine phosphate, and 0.1 mM Ro 20-1724 (a specific phosphodiesterase inhibitor).

Plasma membranes, previously incubated with adenosine deaminase (0.2 U/mg protein) at 37°C for 30 min to remove endogenous adenosine, were incubated for 6 min at 30°C in the absence or presence of 5  $\mu$ M GTP $\gamma$ S, 10  $\mu$ M forskolin, or 1 mM CHA, followed by a further incubation with 200  $\mu$ M ATP at 30°C for 10 min. Reaction was stopped by the boiling of samples and then centrifugation at 12000 x g for 4 min. Fifty microliters of supernatant were used to determine cAMP accumulation. Samples were incubated with 0.25 pmol of [ $^3$ H]cAMP and 6.25  $\mu$ g protein kinase A in a final volume of 200  $\mu$ L of buffer assay (Tris-HCl 50 mM, pH 7.4, EDTA 4 mM) for 2 h at 4°C. Standard samples were prepared in the same buffer in the range of concentration of 0-16 pmol. Reaction was stopped by the addition of 100  $\mu$ L of active charcoal solution containing bovine serum albumin and then centrifugation of the samples at 12000 x g for 4 min. Radioactivity was measured in a Microbeta Trilux (Perkin Elmer) liquid scintillation counter.

## 2.9. Protein determination

Protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

## 2.10. Statistical and data analysis

Data are mean  $\pm$  SEM. Statistical analysis was performed using Student t-test. Differences between mean values were considered statistically significant at  $p < 0.05$ . Non-linear regression curve fit (one site binding hyperbola) was performed for binding data using the GraphPad Prism 5.0 program for Windows (GraphPad Software, San Diego, CA, USA).

### 3. Results

#### 3.1. Expression of genes coding adenosine receptor types in SAMR1 and SAMP8 mice

To determine the age-related changes in expression of gene coding adenosine receptors in SAMP8 and SAMR1 mice, we isolated total RNA from both strains and performed quantitative real time PCR using selective oligonucleotides to adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> adenosine receptors. The ages of animals were three weeks (21d) and six months (180d). Results show that mRNA coding adenosine A<sub>1</sub> receptors are significantly increased in middle-aged versus young SAMR1 animals, suggesting an age-associated up-regulation in resistant control mice. However, the level of A<sub>1</sub> mRNA in young and middle-aged SAMP8 animals was not significantly different—of the same order as that obtained in young SAMR1 (Figure 1, panel A). Similar results were obtained for A<sub>2B</sub> receptors whose level was increased in middle-aged versus young resistant mice but was not altered in SAMP8 (Figure 1, panel D). Concerning A<sub>2A</sub> receptors, no significant differences were obtained between young and middle-aged animals, either in SAMP8 or in SAMR1. However, the level of A<sub>2A</sub> mRNA expression was significant lower in SAMP8 versus SAMR1 (Figure 1 panel C). Finally, an increase in mRNA coding A<sub>3</sub> receptor was observed in both middle-aged SAMR1 and SAMP8 animals without significant differences between strains (Figure 1, panel B).

#### 3.2. Adenosine A<sub>1</sub> receptor in SAMR1 and SAMP8 mice

Adenosine A<sub>1</sub> receptors are widely distributed in the brain and they are involved in neuroprotection. We measured adenosine A<sub>1</sub> receptors in plasma membranes from SAMR1 and SAMP8 mice by binding assay using [<sup>3</sup>H]DPCPX, a selective A<sub>1</sub> receptor antagonist, as radioligand. In all cases assayed saturation curves were better fitted to one

binding site model. Results from saturation curves showed a significant decrease in total adenosine A<sub>1</sub> receptor numbers in middle-aged SAMR1 (38%), suggesting a loss of receptors associated with aging (Figure 2, panels A and C). This decrease was not observed in the SAMP8 strain (Figure 2, panels B and C). However, the total adenosine A<sub>1</sub> receptor number in SAMP8 was significantly lower than in SAMR1, with even fewer receptors detected than in middle-aged SAMR1 mice, suggesting that A<sub>1</sub> receptors are already altered at a very early age in SAMP8. No significant age-associated differences in K<sub>d</sub> values were observed in any SAM strain. However, K<sub>d</sub> values in SAMP8 strain were lower than in SAMR1, suggesting a higher receptor affinity in the former (Figure 2, panel D).

Due to the discrepancy observed in data obtained by real time PCR and by radioligand binding assays in these samples, additional binding assays were carried out in the presence of Gpp(NH)p, a non-hydrolyzable GTP analogue, to rule out possible [<sup>3</sup>H]DPCPX activity as an inverse agonist (Finlayson et al., 2003). Results obtained (Figure 3) showed a significant decrease in [<sup>3</sup>H]DPCPX binding in middle-aged SAMR1, young SAMP8 and middle-aged SAMP8 as compared to [<sup>3</sup>H]DPCPX binding levels in young SAMR1 strain. These results correlate with binding data obtained in the absence of Gpp(NH)p and confirm [<sup>3</sup>H]DPCPX as a selective antagonist.

To measure adenosine A<sub>2A</sub> receptors, Western blot experiments were performed with membranes from SAM animals. Results showed a significant age-related increase in the level of A<sub>2A</sub> receptors in SAMR1, while no significant difference was observed in SAMP8 related to age. Moreover, levels of A<sub>2A</sub> receptors observed in both young and middle-aged SAMP8 were of the same order as those obtained from young SAMR1 (Figure 4). In addition, to confirm the variation of A<sub>1</sub> receptors detected by radioligand binding assay, Western blot experiments were also performed using anti-A<sub>1</sub> receptor

antibody. Results show an aged-associated loss of adenosine A<sub>1</sub> receptors in SAMR1 mice (Figure 5). In addition, the level of A<sub>1</sub> receptors in very young SAMP8 mice was of the same order as that observed in middle-aged SAMR1 animals, confirming results obtained by saturation binding assays.

### 3.3. Adenosine A<sub>1</sub> receptors/AC activity in Wistar rats

To compare and confirm the effect of aging on adenosine A<sub>1</sub> receptor levels we also analyzed the status of these receptors in brain from Wistar rats. Two groups of animals were used, young (3 months) and old (24 months) male rats. Saturation curves obtained by radioligand binding assays showed a significant decrease in adenosine A<sub>1</sub> receptors in brain from old versus young rats (49.3% of young rats) without significant variation in receptor affinity (Figure 6).

To evaluate whether age-related variation in adenosine receptors from Wistar rats modulates the transduction pathway mediated by these receptors, we analyzed the status of adenylyl cyclase activity in young and old rats. Results show that basal and forskolin or forskolin plus GTP $\gamma$ S-stimulated adenylyl cyclase activity were significantly lower in old than in young animals (Figure 7, panel A). Moreover, the ability of CHA, a selective A<sub>1</sub> receptor agonist, to inhibit this enzymatic activity, was also decreased, suggesting a desensitization of the response system associated with the loss of receptors in the plasma membrane (Figure 7, panel B).

## 4. Discussion

To our knowledge, this study is the first to deal with the expression of mRNA coding different adenosine receptors and to quantify adenosine A<sub>1</sub> and A<sub>2A</sub> receptors in

1 brain from SAMR1 and SAMP8 mice. Results presented herein show an age-related  
2 loss of adenosine A<sub>1</sub> receptors in SAMR1, associated with an increase in the rate of  
3 synthesis of this receptor, probably as a compensatory mechanism to prevent the  
4 important loss of receptor detected at the membrane surface. In contrast, an age-related  
5 increase in adenosine A<sub>2A</sub> receptors was observed in SAMR1 with no variation in  
6 SAMP8. However, the most important finding in our study is that adenosine A<sub>1</sub>  
7 receptors, which have been described as being neuroprotective, are already severely  
8 decreased in very young SAMP8 mice (three weeks old) suggesting a great alteration of  
9 these receptors in this aging model.  
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24 Morphological changes in the aging brain have been described and they depend  
25 on the species, the strains and the ages included in the study, as well as on individual  
26 differences. Changes in A<sub>1</sub> and A<sub>2A</sub> receptors and their function during aging have been  
27 demonstrated, with A<sub>1</sub> being decreased and A<sub>2A</sub> increased, respectively (Cunha, 2005;  
28 Rodrigues et al., 2008). In spite of the lack of anatomical resolution in our study, our  
29 findings of age-related changes in A<sub>1</sub> receptors in normal aging mice (SAMR1) are  
30 consistent with previously published data. Age-related changes in adenosine A<sub>1</sub> receptor  
31 binding have been described in mouse brain using the selective agonist CHA. In the  
32 cortex, hippocampus, and cerebellum of aged mice (28 months old), a significant  
33 decrease of about 44%, 50% and 12%, respectively, was observed in CHA binding  
34 compared to young animals (3 months old), without changes in K<sub>d</sub> values and not  
35 dependant on a general cell degeneration in old age (Pagonopoulou and Angelatou,  
36 1992). Also in mice, there has been confirmed to be widespread age-related loss of A<sub>1</sub>  
37 receptors in various cortical and subcortical structures involved in the neuromodulatory  
38 role of adenosine (Ekonomou et al., 2000).  
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The methodology employed to quantify A<sub>1</sub> receptors in SAM mice was validated by our results in male Wistar rats, which agree with those published by Cunha and co-workers in which the density of A<sub>1</sub> binding sites in aged rats (24 months) was reduced by 42% in whole brain membranes (Cunha et al., 2001b) by 33% in the hippocampus, and by 60% in the cortex, when compared with young adult rats (6 weeks), with no changes in K<sub>d</sub> (Cunha et al., 1995). A significant decrease in DPCPX binding to hippocampal membranes of aged versus young rats has also been observed (Sperlágh et al., 1997). By immunoblotting analysis there was shown to be an age-related decrease in A<sub>1</sub> receptor in cerebral cortex from Wistar rats. Compared to preparations from 2-month-old animals, the levels of A<sub>1</sub> in 6, 12 and 24-month-old rats were reduced by 14, 32 and 28% respectively (Cheng et al., 2000). In addition, the pattern of binding of the A<sub>1</sub> receptor antagonist [<sup>3</sup>H]DPCPX decreased with aging in membranes from whole brain of male Wistar rats while K<sub>d</sub> values were not different among age groups (Cunha et al., 2001b).

Loss of A<sub>1</sub> receptors in plasma membrane has been associated with a down-regulation of adenosine receptor by ligand overexposure. Down-regulation and desensitization of adenosine A<sub>1</sub> receptors in vivo has been suggested as being induced by high adenosine levels (León et al., 2002; León et al., 2004; 2005) and it comprises the classical GPCR response when exposed chronically to agonist (Hanyaloglu and von Zastrow, 2008). In agreement, upon chronic noxious brain conditions, and also upon aging, adenosine modulation is modified, with increased levels of adenosine, which modifies the density of adenosine receptors (Lopes et al., 1999; reviewed in Cunha, 2005). Previous studies have shown that endogenous extracellular adenosine levels are

larger in hippocampal preparations (Cunha et al., 2001a) and basal forebrain (Murillo-Rodriguez et al., 2004) from aged compared to young adult rats. This increase could be related to increased activity of 5'-nucleotidases with age (Cunha et al., 2001a; Mackiewicz et al., 2006), which transforms ATP released into adenosine at the synaptic level (Zimmermann, 1996; Cunha, 2001). Other authors lend support to the idea that adenosine is released as such, with only a minor contribution of extracellular ATP-derived adenosine (Pedata et al., 1993), and the existence of non-concentrative bidirectional adenosine transporters allows balancing of changes in the intracellular and extracellular adenosine concentrations (Plagemann and Wohlhueter, 1984). It has been described that the greater levels of extracellular adenosine in aged animals cause a greater tonic A<sub>1</sub>-mediated inhibition (Sebastião et al., 2000). Moreover, during aging, the level of adenosine in the extracellular fluid is increased in the hippocampus, associated with a down-regulation and reduced responsiveness of presynaptic adenosine A<sub>1</sub> receptors (Spelágh et al., 1997). Further, in previous studies we have shown that adenosine release during hypoxic conditions is responsible for adenosine A<sub>1</sub> receptor down-regulation and adenosine A<sub>2A</sub> receptor up-regulation in C6 cells (Castillo et al., 2008). Therefore, we hypothesize that the age-related adenosine level increase could be responsible for the down-regulation of A<sub>1</sub> receptors in SAMR1 mice.

A significant decrease in the A<sub>1</sub> mRNA level was obtained using Northern blotting analysis, associated with age in male Wistar rats, probably due to a decrease in transcription of the receptor gene and/or decrease in the stability of mRNA (Cheng et al., 2000). This result contrasts with the significant increase in mRNA level coding A<sub>1</sub> receptor we detected by quantitative real time PCR in old SAMR1 animals, probably as a compensatory mechanism for the loss of the adenosine A<sub>1</sub> receptor detected at the

membrane surface. This compensatory mechanism is absent in old SAMP8 mice, in agreement with the non-variation in  $A_1$  associated with aging.

SAMP8 have been proposed as a model for Alzheimer disease (AD). It has been demonstrated that SAMP8 mice overproduce  $\beta$ -amyloid in the hippocampus, and late in life they develop amyloid plaques (Morley et al., 2000). This is associated with an increase in mRNA for the Amyloid Precursor Protein (APP). In addition, it has been postulated that aged SAMP8 mice have higher levels of  $A\beta$ P (Banks et al., 2007). The 12-month-old SAMP8 mouse has APP and  $A\beta$ P levels that are about twice those of the 4-month-old SAMP8, and it has developed severe learning and memory problems (Kumar et al., 2000; Morley et al., 2000), which can be reversed by treatment with either antibody or antisense directed against  $A\beta$ P (Kumar et al., 2000; Morley et al., 2002a). Thus, the SAMP8 strain represents a model of age-related and  $A\beta$ P-dependent cognitive decline.

Loss of adenosine receptors in SAMP8 mice observed in the present study could be associated with the increased  $A\beta$ P level detected in these animals, which is thereafter related to degeneration characteristics of AD. However, there is no evidence supporting increased  $A\beta$ P levels in SAMP8 as young as three weeks old, at which time point we observed an important loss of adenosine  $A_1$  receptors. In addition, results obtained previously by our group in frontal cortex brain from AD patients show a significant increase in adenosine  $A_1$  receptors already in early stages of the disease and then maintained with the progression of neurodegeneration (Albasanz et al., 2008). Moreover, up-regulation of  $A_1$  receptors has also been observed in brain from patients with other dementias such as Pick disease or argyrophilic grain disease, in which there is no significant  $A\beta$ P accumulation (Albasanz et al., 2007; Perez-Buira et al., 2007).

Therefore, variation in adenosine receptors seems not to be specific for neurodegenerative diseases associated with A $\beta$ P accumulation. In other words,  $\beta$ -amyloid seems not to be responsible for variation in A<sub>1</sub> receptors. As postulated above, an increase in endogenous adenosine level could be responsible for the lower adenosine A<sub>1</sub> receptors observed in SAMP8 versus young SAMR1 animals. In agreement, the increase in adenosine receptors in human brain from AD patients has been related with the lower endogenous adenosine level, as detected in plasma (Selly, 2004). Moreover, adenosine and pharmaceutical agents that raise the extracellular adenosine concentration have been suggested as reinforcements to conventional treatment with acetylcholinesterase inhibitors in AD (Ferroni et al., 2002; Schubert et al., 2001). Furthermore, A<sub>2A</sub> receptor blockade is also nowadays considered a promising target in AD treatment (Dall'Igna et al., 2007; Cunha et al., 2008).

Although no references are available about adenosine content in SAM brain, at least to our knowledge, changes in neurotransmitter levels in SAM animals have been reported as increasing in GABA and decreasing in acetylcholine and serotonin (Morley, 2002). In the hippocampus and/or cerebral cortex of P8 strain at early ages, the contents of Glutamate (Glu) and Glutamine (Gln) were higher than in R1, while the aspartate (Asp) and Alanine (Ala) content was lower, which suggests that a metabolic pathway from alphaketoglutarate to Glu predominates in SAMP8 brain (Kitamura et al., 1992; Nomura et al., 1991). Local accumulation of excitatory amino acids can lead to neuronal degeneration because of the neurotoxic effects at high concentrations of Glu and analogues in Glu receptive areas (Olney et al., 1990). Increased glutamate levels have been found in several brain areas in AD and in cerebrospinal fluid (CSF) from AD patients (Procter et al., 1988; Pomara et al., 1992). This excess of endogenous glutamate

would contribute to cell death in AD, possibly as the result of the failure to remove glutamate from synapse (Greenamyre and Young, 1989). In agreement, a down-regulation and desensitization of metabotropic glutamate receptors in the frontal cortex in common forms of dementia with Lewy bodies (DLB) and in Alzheimer disease cases have been detected (Albasanz et al., 2005). The neuroprotective role of adenosine is mainly due to inhibition of glutamate release through adenosine A<sub>1</sub> receptor activation. Therefore, the loss of A<sub>1</sub> receptors could be responsible for the increase in the glutamate level in SAM mice.

In summary, in SAMR1 mice used here as controls, it was shown that there is an age-related loss of adenosine A<sub>1</sub> receptor, as also observed in rats, but at early stages. Moreover, the most important result in this work is that in brain from very young SAMP8 mice there is a reduced A<sub>1</sub> receptor level similar to that obtained in aged SAMR1, suggesting an important alteration of these receptors at only three weeks of life which is maintained thereafter. In vivo PET study detected an age-dependent A<sub>1</sub> receptor loss in humans that may be of pathophysiological importance in various neurological diseases associated with aging (Meyer et al., 2006). Therefore, SAM mice may represent an important model in the study of alteration of neurotransmitter receptors related to neurodegenerative diseases.

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## FIGURE LEGENDS

**Figure 1. Adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptor gene expression assayed by quantitative real time RT-PCR.** After RNA isolation from 21-day-old SAMR1 (R1 21d), 180-day-old SAMR1 (R1 180d), 21-day-old SAMP8 (P8 21d) and 180-day-old SAMP8 (P8 180d) animals, quantitative real time RT-PCR assays for the determination of A<sub>1</sub> (panel A), A<sub>3</sub> (panel B) A<sub>2A</sub> (panel C) and A<sub>2B</sub> (panel D) gene expression levels were carried out using TaqMan® universal PCR following the protocol indicated in Materials and Methods. Histograms represent mean  $\pm$  SEM values obtained from four separate experiments using different preparations. \*  $p < 0.05$  and \*\*  $p < 0.01$  significantly different from indicated value.

**Figure 2. Adenosine A<sub>1</sub> receptor detection in SAM mice by radioligand binding assay.** Saturation curves of [<sup>3</sup>H]DPCPX binding to plasma membranes were performed by incubation of 50-75 µg of protein from SAMR1 (panel A) or SAMP8 (panel B) mice with increasing concentrations of the radioligand, as described in Materials and Methods, after pre-incubation with adenosine deaminase in order to remove endogenous adenosine. Total receptor number (B<sub>max</sub>) and receptor affinity (K<sub>d</sub>) determined by Scatchard and nonlinear regression analysis are shown in panels C and D, respectively. Data are mean ± SEM of independent experiments performed in triplicate using different membranes isolated from four SAMR1 and four SAMP8 animals. \*p<0.05 and \*\*\*p<0.001 significantly different from indicated value.



**Figure 3. Gpp(NH)p effect on radioligand binding assay in SAM mice. [<sup>3</sup>H]DPCPX**

binding assays were performed in plasma membranes (50-75 µg of protein) from SAMR1 or SAMP8 mice. After pre-incubation with adenosine deaminase, binding assays were performed with 20 nM [<sup>3</sup>H]DPCPX in the presence of 10 µM Gpp(NH)p, without MgCl<sub>2</sub>, and in the presence of MgCl<sub>2</sub>, without Gpp(NH)p. CPA was used to obtain nonspecific binding. Data are mean ± SEM of independent experiments performed in duplicate using different membranes isolated from four animals of each condition. No statistical differences were observed between binding data obtained in the presence or the absence of Gpp(NH)p. \*p < 0.05, \*\*p < 0.01 significantly different from control young SAMR1 mice in the absence of Gpp(NH)p; #p < 0.05 significantly different from control young SAMR1 mice in the presence of Gpp(NH)p.

**Figure 4. Western-blot of A<sub>2A</sub>R in plasma membranes from SAM mice.** Fifty micrograms of protein were subjected to SDS/PAGE, transferred electrophoretically to nitrocellulose and probed with antisera anti-A<sub>2A</sub>, as described in Materials and Methods. Upper panel shows a representative autoradiography, showing the bands corresponding to A<sub>2A</sub>R and  $\beta$ -actin, which was used as gel loading control. Lower panel shows the obtained histogram. Data is expressed as mean  $\pm$  SEM using arbitrary units. \*\*p < 0.01 significantly different from control young SAMR1 mice.

**Figure 5. Western-blot of A<sub>1</sub>R in plasma membranes from SAM mice.** Fifty micrograms of protein were subjected to SDS/PAGE, transferred electrophoretically to nitrocellulose and probed with antisera anti-A<sub>1</sub>, as described in Materials and Methods. Upper panel shows a representative autoradiography, showing the bands corresponding to A<sub>1</sub>R and  $\beta$ -actin, which was used as gel loading control. Lower panel shows the obtained histogram from densitometric analysis of bands. Data is expressed as mean  $\pm$  SEM using arbitrary units. \* $p < 0.05$  and \*\*\* $p < 0.001$  significantly different from control young SAMR1 mice.

**Figure 6. Adenosine A<sub>1</sub> receptor detection in Wistar rats by radioligand binding assay.** Saturation curves of [<sup>3</sup>H]DPCPX binding to plasma membranes were performed by incubation of 50-75 µg of protein from young and old rats with increasing concentrations of the radioligand, as described in Materials and Methods, after pre-incubation with adenosine deaminase in order to remove endogenous adenosine. Total receptor number (B<sub>max</sub>) and receptor affinity (K<sub>d</sub>) determined by Scatchard and nonlinear regression analysis are shown in the inset. Data are mean ± SEM of number of experiments in brackets performed in triplicate using different membrane isolations from different animals. \* p<0.01 significantly different from young rats.

**Figure 7. Adenylyl cyclase activity in brain plasma membranes from Wistar rats.**

**Panel A.** Ten to twenty micrograms of plasma membranes, previously incubated with adenosine deaminase, were used to determine basal cAMP level and stimulation after incubation with 10  $\mu$ M forskolin (Fsk) or 10  $\mu$ M Fsk plus 5  $\mu$ M GTP $\gamma$ S. **Panel B.** Ten to twenty micrograms of plasma membranes were incubated with Fsk plus GTP $\gamma$ S in the presence and in the absence of CHA, a selective A<sub>1</sub>R agonist. cAMP accumulation was measured as described in Materials and Methods. Data are means  $\pm$  SEM of at least four experiments performed in triplicate with different membrane preparations from different rats. \*  $p < 0.01$  significantly different from young rats.

Figure 1

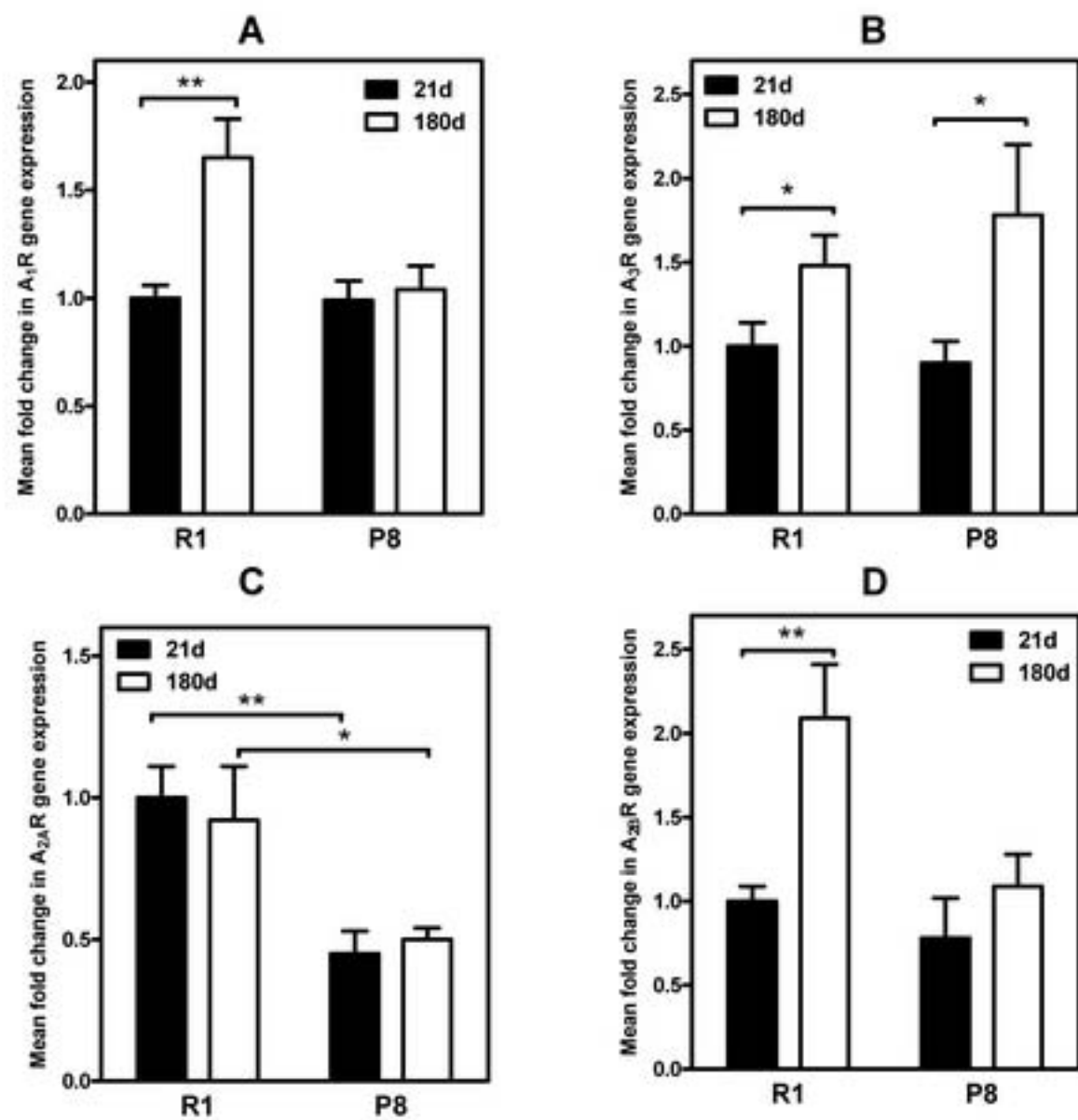


Figure 2

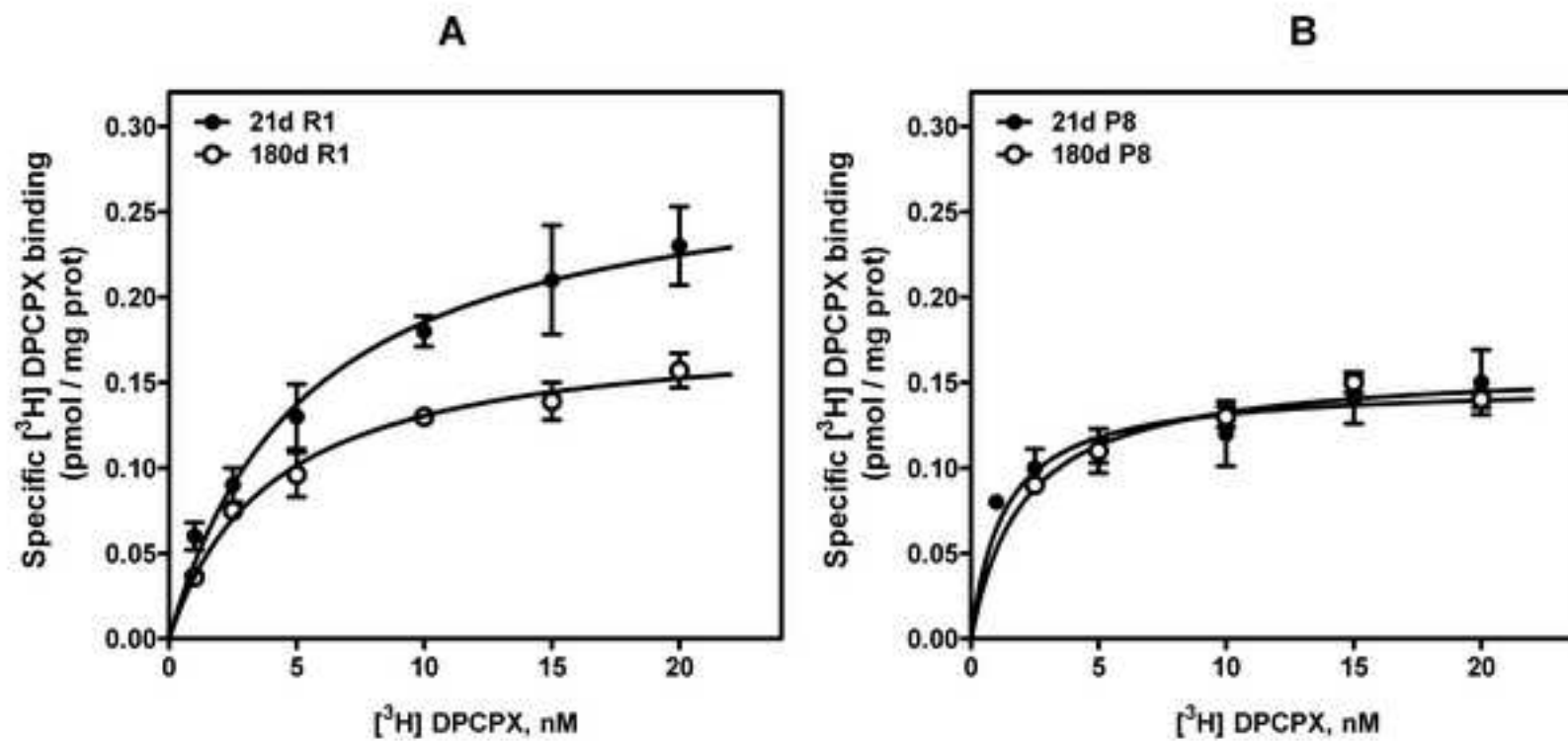


Figure 2

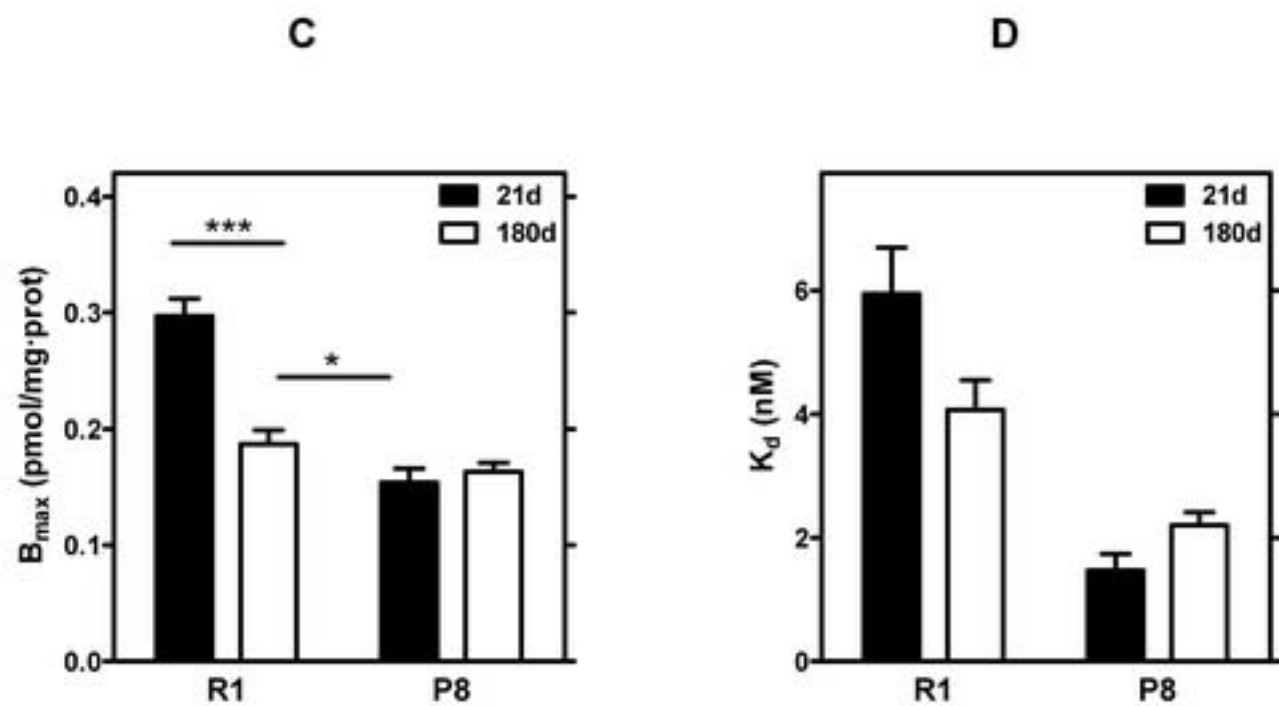




Figure 3

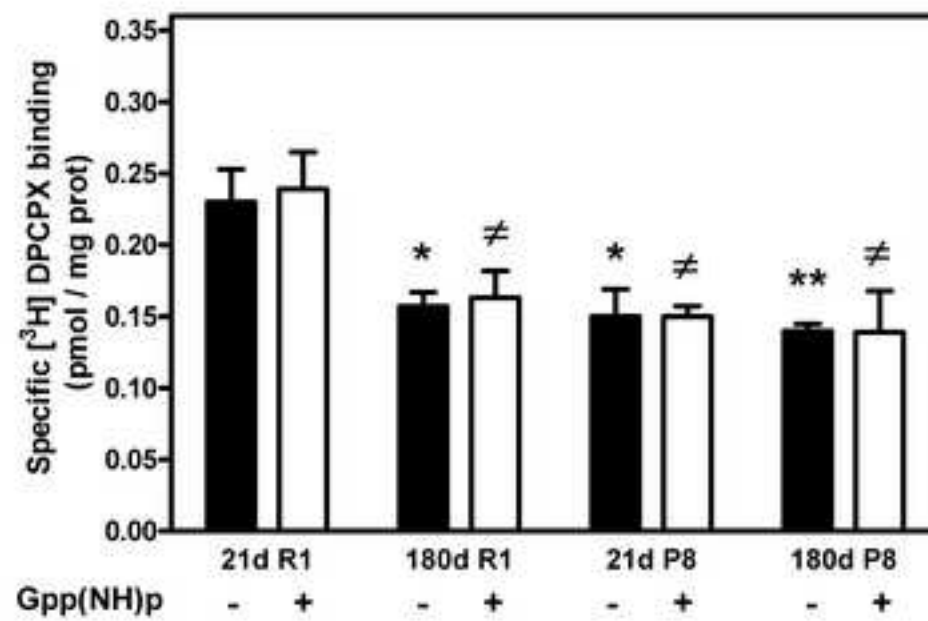


Figure 4

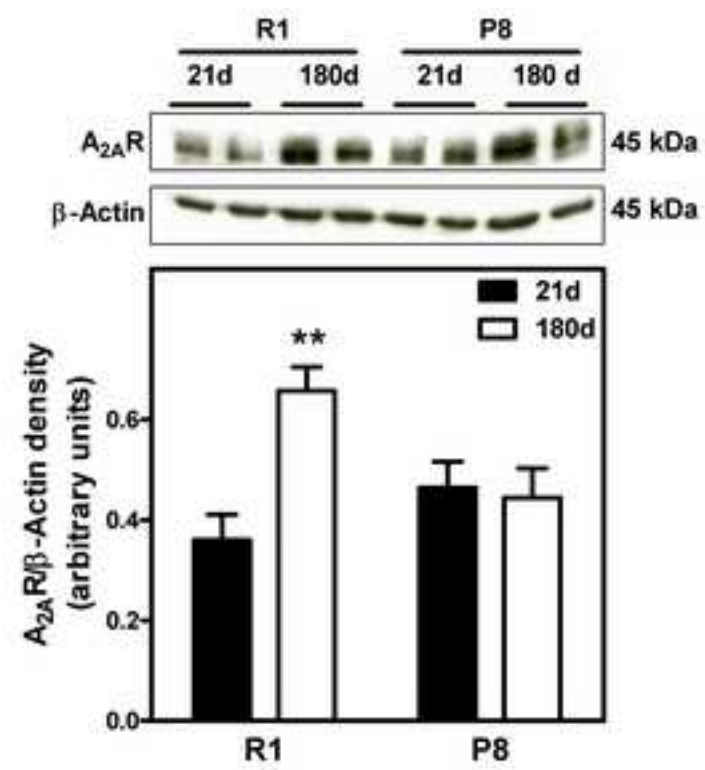


Figure 5

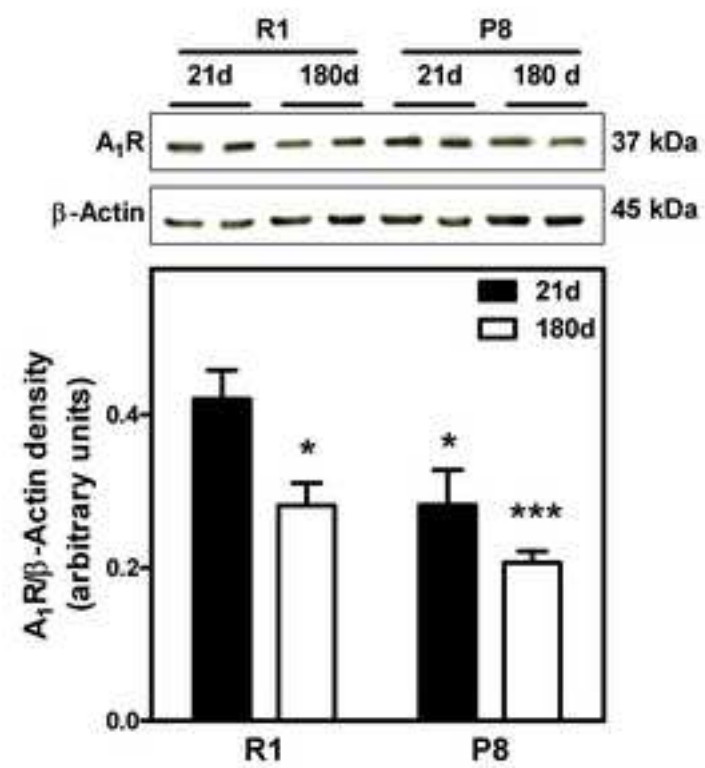
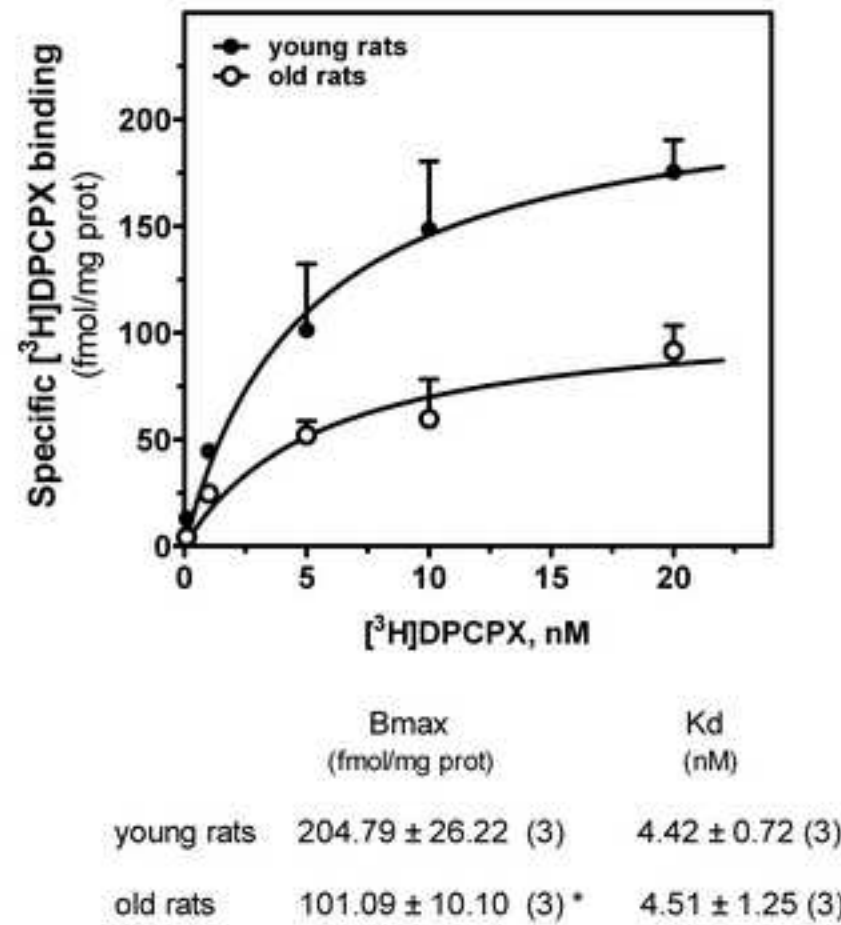


Figure 6



**Figure 7**

