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RESEARCH ARTICLE

Up-regulation of Adenosine Receptors in the Frontal Cortex in Alzheimer's Disease

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Abstract

Adenosine receptors are G-protein coupled receptors which modulate neurotransmitter release, mainly glutamate. Adenosine A_1 and A_{2A} receptors were studied in post-mortem human cortex in Alzheimer's disease (AD) and age-matched controls. Total adenosine A_1 receptor number, determined by radioligand binding assay, using [^{3}H]DPCPX, was significantly increased in AD cases in early and advanced stages without differences with the progression of the disease. A significant increase of A_1R (37 kDa) levels was also observed by Western blot in early and advanced stages of AD. In addition, increased numbers of adenosine A_{2A} receptors were observed in AD samples as determined by a binding assay using [^{3}H]ZM 241385 as a radioligand and by Western blot. Increased binding and protein expression levels of adenosine receptors were not associated with increased mRNA levels coding A_1 and A_{2A} receptors. Finally, increased A_1 and A_{2A} receptor-mediated response was observed. These results show up-regulation of adenosine A_1 and A_{2A} receptors in frontal cortex in AD, associated with sensitization of the corresponding transduction pathways.

INTRODUCTION

Adenosine receptors are G-protein coupled receptors which have been classified into A_1, A_{2A}, A_{2B} and A_3 receptors. A_1 and A_3 receptors inhibit adenylyl cyclase through Gi/o proteins, while A_{2A} and A_{2B} receptors stimulate adenylyl cyclase through Gs proteins (16, 25). The endogenous ligand of these receptors is adenosine, which is widely distributed in both the central and the peripheral nervous systems. Adenosine is a neuromodulator and neuroprotective metabolite (13). Adenosine and drugs with effects on adenosine receptors may help to inhibit the progressive neurodegenerative process in dementia (28). However, the implications of adenosine A_1 and A_{2A} receptors in neuroprotection appear to be different. While agonists of A_1 receptors have been widely considered as neuroprotectors, it has recently been observed that pharmacological blockade or gene disruption of adenosine A_{2A} receptors confers neuroprotection. Caffeine, a non-specific antagonist, has shown neuroprotection to β-amyloid (Aβ) neurotoxicity in cultured neurons of rats (11) and mice (5, 12).

Alzheimer's disease (AD) is the major cause of dementia in the elderly. It is characterized by a progressive deterioration in memory and cognitive functions. The characteristic hallmarks of AD include senile plaques, mainly composed of Aβ peptide, neurofibrillary tangles, and selective synaptic and neuronal loss in several brain regions, including the cerebral cortex (7, 29). Previous studies have shown modifications of Adenosine A_1 and A_{2A} receptors in AD. Most of these studies were carried out in the hippocampus and striatum, and they have shown reduced adenosine A_1 receptors in these areas (14, 18, 20, 32). However, little is known about adenosine A_1, and even less about A_{2A} receptors in the frontal cortex in AD in relation to the progressive stages of AD pathology. Therefore, the aim of the present work was to analyze adenosine A_1 and A_2 receptors and their inhibitory and stimulatory coupling, respectively, to adenylyl cyclase activity in brains from AD patients. Results have shown up-regulation and sensitization of A_1 and A_{2A} receptors in the frontal cortex in AD as an early event of disease progression.

MATERIALS AND METHODS

Materials

Cyclopentyl-1,3-dypropylxanthine,8-[dipropy-2,3-^{3}H(N)] ([^{3}H]DPCPX 120 Ci/mmol) and [^{3}H]adenosine 3′, 5′-cyclic phosphate ([^{3}H]cAMP 27.4 Ci/mmol) were from PerkinElmer (Madrid, Spain) and [2-^{3}H](4-(2-[7-amino-2(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl]phenol) ([^{3}H]ZM 241385 27.4 Ci/mmol) from Tocris (Bristol, UK). Anti-A_1 antibody was from

Oncogene (Cambridge, MA) and anti-adenylyl cyclase I (ACI) antibody was from Santa Cruz (Madrid, Spain). Guanosin triphosphate was purchased from Roche (Barcelona, Spain). Calf intestine adenosine deaminase (ADA), forskolin, 2-[4-[(2-carboxyethyl) phenyl]ethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680), N^6-cyclohexyladenosine (CHA), theophylline and N^6-cyclopentyladenosine (CPA) were from Sigma (Madrid, Spain). All other reagents were of analytical grade and obtained from commercial sources.

Tissue samples

Brain samples were obtained from the brain banks of the Institute of Neuropathology and the University of Barcelona-Clinic Hospital following the guidelines of the local ethics committees. The brains of patients with AD and age-matched controls were obtained from 3 to 24 h after death and were immediately prepared for morphological and biochemical studies. Although agonal state seems not to affect adenylyl cyclase measurements (8), cases with prolonged agonal state were not considered suitable for the present study. At autopsy, half of the brain was fixed in formalin, while the other half was cut in coronal sections 1 cm thick, frozen on dry ice and stored at −80°C until use. The neuropathological study was carried out on formalin-fixed, de-waxed 4-μm thick paraffin sections of the frontal (area 8), primary motor, primary sensory, parietal and temporal superior, temporal inferior, anterior cingulate, anterior insular, and primary and associative visual cortices; entorhinal cortex and hippocampus; caudate, putamen and pallidum; medial and posterior thalamus; subthalamus; Meynert nucleus; amygdala; midbrain (two levels) pons and medulla oblongata; and cerebellar cortex and dentate nucleus. The sections were stained with hematoxylin and eosin, Luxol fast blue-Klüver Barrera, and for immunohistochemistry to glial fibrillary acidic protein, CD68 and *Licopericum esculentum* lectin for microglia, Aβ, tau, αB-crystallin, α-synuclein and ubiquitin. AD-related pathology was categorized following the proposal of Braak and Braak (7). The cases from the present study were used for the study of metabotropic glutamate receptors in cerebral cortex in AD (2). The main clinical and neuropathological characteristics are summarized in Table 1. No neurological or neuropathological abnormalities were observed in control cases.

Plasma membrane isolation

Plasma membranes from brain samples were isolated as described previously (10). Samples were homogenized in 20 volumes of isolation buffer (50 mM Tris-HCl, pH 7.4 containing 10 mM $MgCl_2$ and protease inhibitors) in Dounce homogenizer (10 × A, 10 × B). After homogenization, brain preparations were centrifuged for 5 minutes at 1000*g* in a Beckman JA 21 centrifuge. Supernatant was centrifuged for 20 minutes at 27 000*g* and the pellet was finally resuspended in isolation buffer. Protein concentration was measured by the method of Lowry *et al* (23), using bovine serum albumin as a standard.

[^{3}H]DPCPX binding assays to plasma membranes

Binding assays to plasma membranes were performed as described previously (22). Plasma membranes were incubated with 5 U/mg ADA in 50 mM Tris, 2 mM $MgCl_2$, pH 7.4, for 30 minutes at 25°C, in order to eliminate endogenous adenosine from membrane preparations. Then, plasma membranes (50–75 μg of protein) were incubated with [^{3}H]DPCPX for 2 h at 25°C. Saturation assays were carried out at different [^{3}H]DPCPX concentrations (0.1–20 nM) using CPA at a concentration 10^5 times higher than the radioligand, in order to obtain non-specific binding. Binding assays were stopped by rapid filtration through Whatman GF/B filters, preincubated with 0.3% polyethylenimine, and then immediately washed three times with 4 mL ice-cold buffer. Filters were then counted in a Microbeta Trilux (Perkin Elmer) liquid scintillation counter.

[^{3}H]ZM 241385 binding assays to plasma membranes

Binding assays were performed as previously described (3). Plasma membranes were incubated with 5 U/mg ADA in 50 mM Tris, 2 mM $MgCl_2$, 100 mM NaCl, pH 7.4, for 30 minutes at 25°C, in order to eliminate endogenous adenosine from membrane preparations. Then, plasma membranes (100 μg of protein) were incubated with [^{3}H]ZM 241385 for 2 h at 25°C. Saturation assays were carried out at different [^{3}H]ZM 241385 concentrations (0.5–50 nM) using 5 mM theophylline to obtain non-specific binding. As in the case of A_1R, binding assays were stopped by rapid filtration through Whatman GF/B filters, and then immediately washed and transferred to vials to count the radioactivity.

Adenylyl cyclase activity assay and determination of cAMP levels

Adenylyl cyclase activity was determined in brain plasma membranes as previously described (3) with several modifications. The assay was performed with 15–20 μg of protein in a final volume of 0.25 mL of 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mg/mL BSA, 1 mg/mL creatine kinase, 10 mM creatine phosphate and 0.1 mM Ro 20-1724 (specific phosphodiesterase inhibitor). Plasma membranes preincubated with ADA (5 U/mg protein), in order to remove endogenous adenosine, were incubated at 37°C for 15 minutes with 5 μM guanosine-5′-O(3-thiotriphosphate) tetralithium salt (GTP?S), 50 μM forskolin, 1 mM CHA or 1 mM CGS21680. The reaction was started by the addition of 200 μM ATP and incubation at 37°C for 10 minutes. The reaction was stopped by boiling the samples which were then centrifuged at 12 000*g* for 4 minutes. Twenty microliters of supernatant was used to determine cAMP accumulation. Samples were incubated with 0.25 pmol [^{3}H]cAMP and 6.25 μg PKA in a final volume of 200 μl of buffer assay (50 mM Tris-HCl pH 7.4, 4 mM EDTA) for 2–8 h at 4°C. Standard samples (0–16 pmol) were prepared in the same buffer. The reaction was stopped by rapid filtration through Whatman GF/B filters, followed by washing with ice-cold buffer. Filters were then counted in a Microbeta Trilux (Perkin Elmer) liquid scintillation counter.

Western blot assays

For Western blot assays, 30 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 minutes. Sodium dodecylsulphate-

Table 1. Summary of the main clinical and neuropathological data in the present series. Abbreviations: M = male; F = female.

Case number	Gender	Age	p-m delay	Lewy stage	NFT stage	β-amyloid stage
1	M	82	11	0	0	0
2	F	63	7	0	0	0
3	M	76	10	0	0	0
4	M	80	11	0	0	0
5	F	79	7	0	0	0
6	F	80	3	0	0	0
7	F	65	4	0	0	0
8	M	71	12	0	0	0
9	M	78	2	0	0	0
10	M	80	13	0	I	A
11	F	85	12	0	I	B
12	M	59	7	0	I	B
13	F	73	15	0	I	0
14	M	78	6	0	II	B
15	F	76	9	0	II	B
16	F	81	6	0	II	B
17	M	74	24	0	II	B
18	M	74	4	0	III	B
19	F	74	5	0	III	0
20	M	72	3	0	III	0
21	F	81	14	0	III	B
22	M	85	14	0	IV	B
23	F	82	5	0	IV	C
24	M	79	5	0	V	B
25	M	93	7	0	V	C
26	F	78	19	0	V	C
27	M	69	6	0	V	C
28	F	86	10	0	V	C
29	M	69	20	0	V	C
30	F	82	10	0	V	C
31	M	85	8	0	VI	C

Lewy stage corresponds to staging of brain pathology related to sporadic Parkinson's disease. NFT stage refers to neurofibrillary degeneration staging in Alzheimer's-disease-related (AD) pathology, whereas βA-amyloid stage refers to AD amyloid deposition following the classification of Braak and Braak (7). Stages I–II correspond to entorhinal, III–IV to limbic and V–VI to isocortical NFTs. Stage A indicates amyloid deposition in the basal neocortex, stage B indicates cortical association areas affected, and stage C indicates involvement of the whole isocortex.

polyacrylamide gel electrophoresis (10% SDS-PAGE) was carried out using a mini-protean system (Bio-Rad, Madrid, Spain) with molecular weight standards (Bio-Rad) with molecular weight standards. Proteins were transferred to nitrocellulose membranes which were washed with TTBS containing 10 mM Tris-HCl (pH 7.4), 140 mM NaCl and 0.1% Tween-20, blocked with TTBS containing 5% skimmed milk, and then incubated with the primary antibodies at 4°C overnight. The rabbit polyclonal anti-A_1R and anti-A_{2A}R (Oncogene, Barcelona, Spain) were used at a dilution of 1:500. The rabbit polyclonal anti-ACI (Santa Cruz Biotechnology, Madrid, Spain) was used at a dilution of 1:1000. After rinsing, the membranes were incubated with the corresponding anti-rabbit secondary antibody (Dako, Madrid, Spain) at a dilution of 1:1000. The immunoreaction was visualized with the enhanced chemiluminescence detection Kit (Amersham, Madrid, Spain), and the specific bands were quantified by densitometry using Total Laboratory v2.01 software (Amersham, Madrid, Spain). The monoclonal antibody to β-actin (Sigma, Madrid, Spain), at a dilution of 1:30 000, was used as a control of protein loading. Densitometric studies were normalized with β-actin.

mRNA isolation

mRNA isolation was carried out using the Qiagen RNeasy lipid tissue (Qiagen) mini Kit following the instructions provided by the manufacturer. The concentration of each sample was obtained from A_{260} measurements. RNA integrity was tested by the Agilent 2100 BioAnalyzer (Agilent). Only RNAs with a RIN number above 6.5 were selected for TaqMan PCR assay.

cDNA synthesis

The retrotranscriptase reaction (100 ng RNA/μL) was carried out by using the high capacity cDNA Archive kit (Applied Biosystems) following the protocol provided by the supplier. Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the degree of contaminating genomic DNA.

TaqMan probes

Human β-glucuronidase (GUS) (Hs99999908_m1, TaqMan probe 5′-GACTGAACAGTCACCGACGAGAGTG-3′), human β-actin (Hs99999903_m1, TaqMan probe 5′-TCGCCTTTGCCGATCC GCCGCCCGT-3′), human A_1R (Hs00379752_m1, TaqMan probe 5′-GATCCCTCTCCGGTACAAGATGGTG-3′) and human $A_{2A}R$ (Hs00169123_m1, TaqMan probe 5′-CCGCTCCGGTACAATGG CTTGGTGA-3′) were examined in the present study. The TaqMan assay for GUS is located between 11 and 12 exon boundary, at position 1816 of NM_000181.1 transcript sequence. The predicted amplicon size is about 81 base pairs. The TaqMan assay for β-actin is located in the 5′ UTR region at position 36 of NM_001101.2 transcript generating an amplicon of 171 base pairs. The TaqMan assay for A_1R is located between 5 and 6 exon boundary, at position 752 of NM_000674.1 transcript, generating an amplicon of 111 base pairs. The TaqMan assay for $A_{2A}R$ is located between 1 and 2 exon boundary, at position 614 of NM_000675.3 transcript, generating an amplicon of 66 base pairs.

TaqMan PCR

TaqMan PCR assays for every gene were performed in duplicate on cDNA samples in 384-well optical plates using an ABI Prism 7900 Sequence Detection system (Applied Biosystems). The plates were capped using optical caps (Applied Biosystems). The ABI Prism 7900 measures the fluorescent accumulation of the PCR product by continuously monitoring cycle threshold (Ct), which is an arbitrary value assigned manually to a level somewhere above the baseline but in the exponential phase of PCR where there are no rate-limiting components. The Ct value sets the point at which the sample amplification plot crosses the threshold. The Ct values correlate with the initial amount of specific template. For each 20 µL TaqMan reaction, 9 µL cDNA (diluted 1/20, which corresponds approximately to the cDNA from 45 ng of RNA) was mixed with 1 µL 20× TaqMan Gene Expression Assays and 10 µL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Parallel assays for each sample were carried out using primers and probes with β-actin and GUS for normalization. The reactions were carried out using the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 s and 60°C for 1 minute. Standard curves were prepared for A_1R, $A_{2A}R$, β-actin and GUS using serial dilutions of control human brain RNA. Finally, all TaqMan PCR data were captured using the Sequence Detector Software (SDS version 1.9, Applied Biosystems, Madrid, Spain).

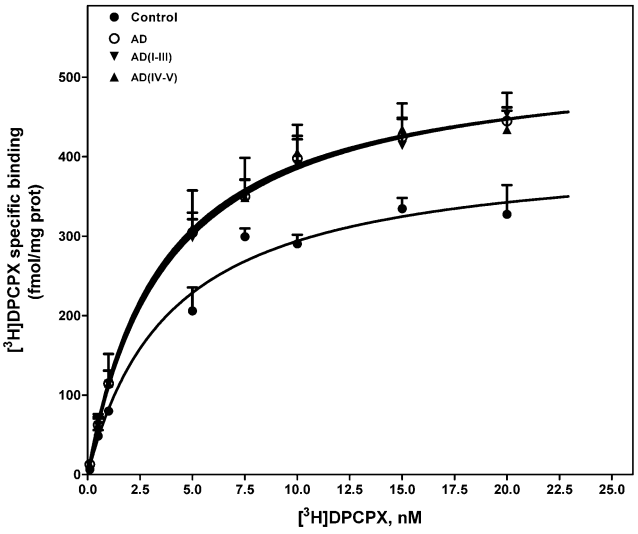
Statistical and data analysis

The binding data were analyzed with the GraphPad Prism 4.0 program (GraphPad Software, San Diego, CA, USA). The numerical data obtained from diseased cases and the corresponding controls were statistically analyzed using STATGRAPHICS plus 5.0 software from ANOVA and the LSD statistical tests. Differences between mean values were considered statistically significant at $P < 0.05$.

RESULTS

Adenosine A_1 receptors in AD

Adenosine A_1 receptors were studied by radioligand binding assay using [^{3}H]DPCPX, a selective A_1 receptor antagonist, as radioligand. [^{3}H]DPCPX binding to human brain was saturable and well-adjusted to a single binding site model in all cases. As shown in Figure 1, the total adenosine A_1 receptor number was significantly increased in AD (318.4% of controls, $P < 0.001$). The increase was observed in the early stages of the disease (stages I-IV of Braak and Braak) without differences with respect to advanced stages (stages V and VI). However, no significant differences were observed in their corresponding receptor affinity (K_d) value, thus showing that receptor affinity was similar in control and AD cases. Figure 2 illustrates total receptor number values in different AD stages. An increased adenosine A_1 receptor number is already observed at stage I, suggesting early involvement of this receptor. These results were confirmed by Western blot using specific A_1 receptor antibodies in which a significant increase in the corresponding band of 37 kDa was observed in both early and advanced stages of AD when compared with controls (Figure 3).



	B_{max} (fmol/mg prot)	K_d (pM)
Control	412.4 ± 11.6 (5)	4299.1 ± 492.7 (5)
AD	533.9 ± 18.6 (14) ***	3957.1 ± 384.6 (14)
AD(I-III)	538.9 ± 24.2 (7) ***	4074.5 ± 586.6 (7)
AD(IV-V)	529.0 ± 30.2 (7) **	3839.7 ± 540.7 (7)

Figure 1. Adenosine A_1 receptor detection in frontal cortex brain from Alzheimer's disease (AD). Saturation curves of [^{3}H]DPCPX binding to plasma membranes were performed by incubation of 50–75 µg of membranes from control and AD brains with increasing concentrations of the radioligand, as described in *Materials and methods*. Total receptor number (B_{max}) and receptor affinity (K_d) determined by Scatchard and non-lineal regression analysis are shown in the inset. Data are mean ± SEM of control and AD cases performed in triplicate using different plasma membrane isolations. ** $P < 0.01$ and *** $P < 0.001$ significantly different from control value.

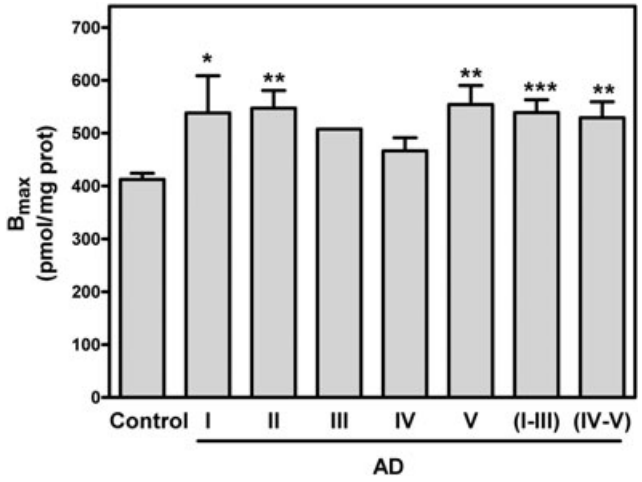


Figure 2. Total receptor number (B_{max}) values of control and different stages of Alzheimer's disease (AD). Data were extracted from saturation assays performed at different stage of AD classified in accord with Braak and Braak, 1999. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ significantly different from control value.

Adenosine A_{2A} receptors in AD

Adenosine A_{2A} receptors were determined by binding assay using [^{3}H]ZM 241385, a selective A_{2A} receptor antagonist, as radioligand. [^{3}H]ZM 241385 specific binding was increased in AD. A significant increase in total A_{2A} receptors was observed in early and advanced stages of AD. However, a significant increase in K_d values was observed in association with the increase in A_{2A} receptors, suggesting a lower receptor affinity (Figure 4). The increase in A_{2A} protein level, although not significant, was also detected by Western blot assays (Figure 5).

Adenylyl cyclase I in AD

Western blot to ACI did not reveal significant differences in the expression levels of ACI between control and AD cases at any stage of the disease (Figure 6).

mRNA levels of A_1, A_{2A} and ACI determined by real time PCR

We also analyzed adenosine A_1R and A_{2A}R mRNA levels by TaqMan PCR using human post-mortem frontal cortex from patients suffering ADI, ADIII and ADV compared with age-matched controls. For each experimental sample, the amount of target and endogenous references was determined from the appropriate standard curve which was plotted showing the cycle threshold, Ct (y), vs. the log of ng total control RNA (Figure 7A). Then the amount of each target was divided by the endogenous references β-glucuronidase (GUSB) and β-actin amount to obtain a normalized target value which permits determination of the relative

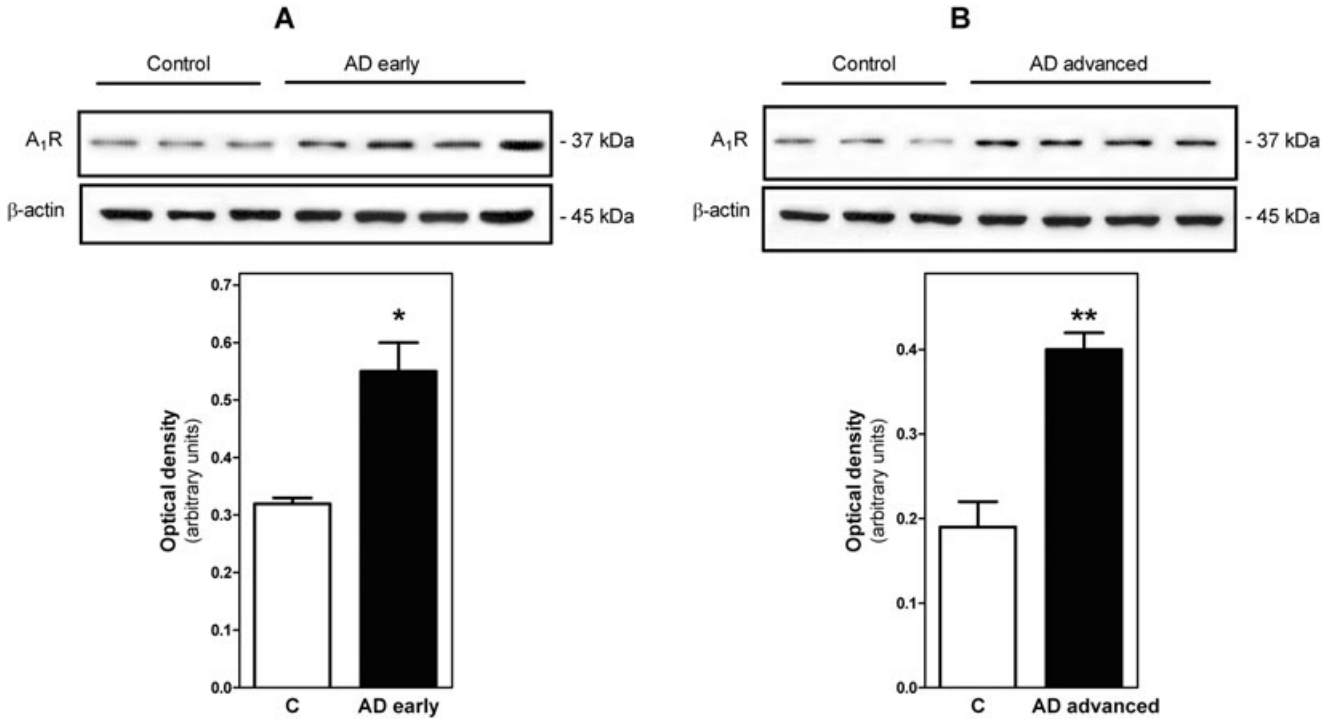
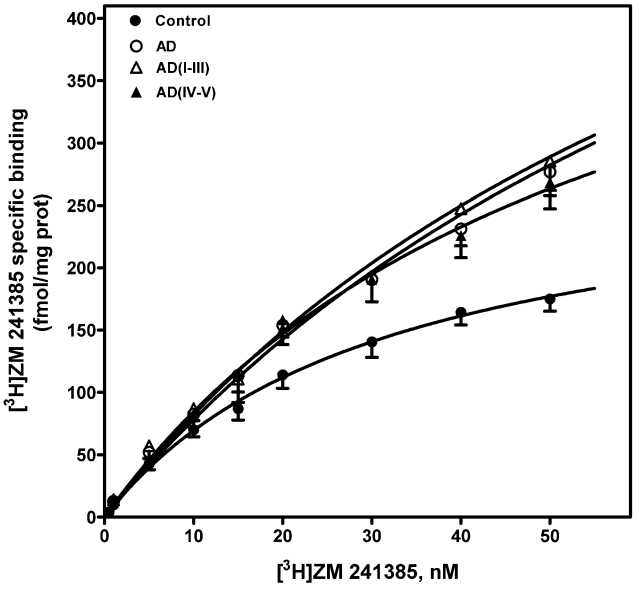


Figure 3. Detection of A_1R by Western blot. Thirty micrograms of protein was subjected to SDS/PAGE, transferred electrophoretically to nitrocellulose and probed with antisera anti-A_1, as described in *Materials and methods*. For the control of protein, loading membranes were incubated with anti-β-actin. Panel **A** represent assays from early stages and Panel **B** from advanced stages of Alzheimer's disease (AD). Data are means ± SEM of represented cases. Inset shows A_1 and β-actin bands corresponding to a representative experiment. $*P < 0.05$ and $**P < 0.01$ significantly different from control value.



	B_{max} (fmol/mg prot)	K_d (nM)
Control	259.4 ± 18.6 (8)	24.24 ± 1.35 (7)
AD(I-VI)	455.0 ± 33.7 (5)***	36.79 ± 1.25 (5)***
AD(I-III)	491.5 ± 55.7 (2)**	38.07 ± 0.97 (2)***
AD(IV-VI)	430.7 ± 45.0 (3)**	35.93 ± 2.01 (3)***

Figure 4. Adenosine A_{2A} receptor detection in frontal cortex brain from Alzheimer's disease (AD). Saturation curves of [^{3}H]ZM 241385 binding to plasma membranes were performed by incubation of 50–75 μg of membranes from control and AD brains as described in *Materials and methods*. Total receptor number (B_{max}) and receptor affinity (K_d) determined by Scatchard and non-lineal regression analysis are shown in the inset. Data are mean ± SEM of control and AD cases performed in triplicate using different plasma membranes isolations. $^{**}P < 0.01$ and $^{***}P < 0.001$ significantly different from control value.

mRNA levels of A_1R and A_{2A}R in control and diseased samples. The use of both endogenous control genes has been previously established by our group (6). Relative A_1R (Figure 7B) and A_{2A}R (Figure 7C) mRNA levels were not modified in the frontal cortex in early and advanced stages of AD when compared with control samples. The same results were obtained when normalization was performed with β-actin (data not shown). mRNA coding ACI was not different between control (1.00 ± 0.20, n = 5) and diseased cases (1.14 ± 0.48, n = 4).

Adenylyl cyclase activity in AD

Basal, GTPγS- and Forskolin-stimulated adenylyl cyclase activity were not altered, suggesting no differences in the G-protein coupling to adenylyl cyclase (AC) in frontal cortex in AD (Figure 8). The inhibitory effect of CHA, a selective A_1 agonist, on Forskolin-stimulated activity was increased in frontal cortex AD (Table 2). The stimulatory effect of CGS 21680, a selective A_{2A} agonist, was also enhanced in frontal cortex in AD (Table 2), suggesting a sensitization of adenosine receptors/adenylyl cyclase systems.

DISCUSSION

Brain adenosine receptors have been suggested as targets for therapeutic intervention in neurodegenerative diseases. A_1 receptor has long been known to mediate neuroprotection, mostly by blockade of calcium influx, which results in inhibition of glutamate release and reduction of its excitatory effects at a postsynaptic level (1, 15, 17, 26, 30). However, the role of A_{2A} receptor in neuroprotection is not so clear, probably because of its lack of abundance in brain regions other than the striatum. Beneficial effects evoked by A_{2A} receptor antagonists may be caused by blockade of presynaptic A_{2A} receptors which are stimulatory on glutamate release (1). Blocking A_{2A} receptor function using antagonists or deleting the A_{2A} receptor gene results in a decrease in the extent of neuronal damage in adult animals. However, the mechanisms of this neuroprotection remain unknown (9).

Adenosine A_1 and A_{2A} receptors have been detected in postmortem human brain (24, 31). A_1 receptors are vulnerable to AD. However, the results are controversial. Previous autoradiography and binding experiments have shown that A_1 receptors were significantly reduced, with receptor affinity being higher in the hippocampus of AD patients as compared with controls (14, 32). In contrast, an increase in Adenosine A_1 receptor immunoreactivity was found in neurons with neurofibrillary tangles, dystrofic neurites of senile plaques in the hippocampus and the frontal cortex of AD patients (4).

Recently, we described how metabotropic glutamate receptors are significantly decreased in AD and noted that the loss of mGlu receptors was more pronounced with disease progression (2). The results presented herein provide evidence that adenosine A_1 and A_{2A} receptors are up-regulated in the frontal cortex in the same series of cases with AD, thus indicating different responses between metabotropic glutamate receptors and adenosine receptors in the frontal cortex in AD. This up-regulation is accompanied by the sensitization of inhibitory and stimulatory transduction pathways mediated by adenosine receptors.

Discrepancies between previous studies and the present findings regarding expression of A_1 receptors in AD cases can be explained

Table 2. Adenosine receptors mediated Adenylyl cyclase activity in Alzheimer's disease (AD).

	Adenylyl cyclase activity			
	A_1-mediated (% of inhibition of Fsk-stimulated)		A_2-mediated (% of basal)	
Control	23.3 ± 6.0	(8)	179.1 ± 18.9	(8)
AD	40.1 ± 3.3	(11)*	253.7 ± 35.7	(9)*

Inhibitory effect of N^6-cyclohexyladenosine, selective A_1 receptor agonist, on Forkolin-stimulated adenylyl cyclase (AC), and stimulatory effect of CGS21680, selective A_{2A} receptor agonist, on basal AC were analyzed in samples from frontal cortex of AD as compared with control. Data are means ± SEM of experiments performed in triplicate with membrane preparations from different cases. $^{*}P < 0.05$ significantly different from control value.

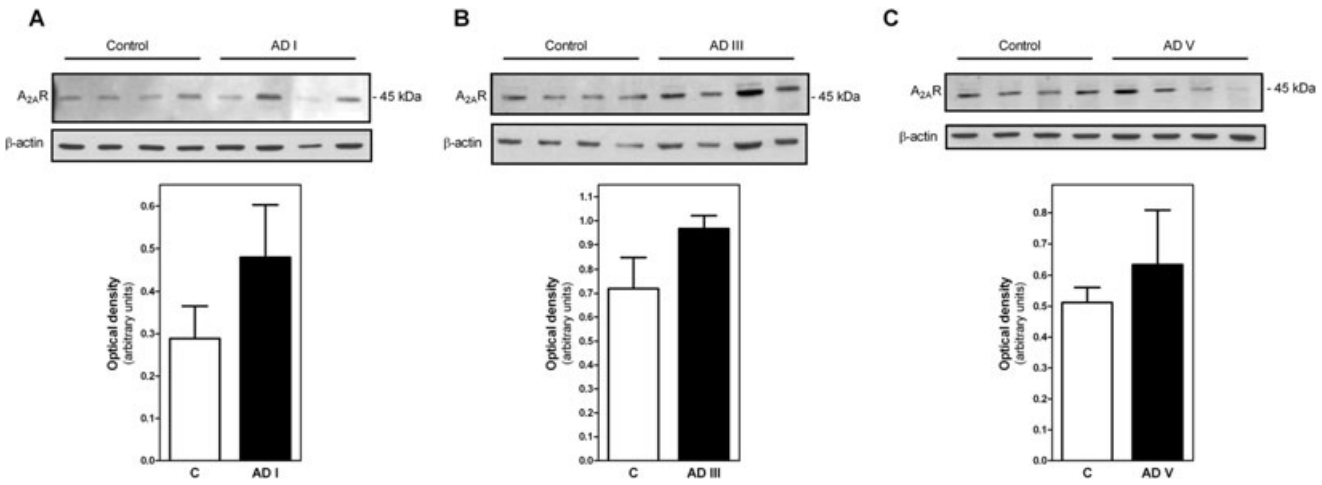


Figure 5. Detection of $A_{2A}R$ by Western blot. Thirty micrograms of protein was subjected to SDS/PAGE, transferred electrophoretically to nitrocellulose and probed with antisera anti-A_{2A}, as described in *Materials and methods*. For the control of protein, loading membranes were incubated with anti-β-actin. A_{2A} was analyzed separately from Alzheimer's disease (AD) stage I (**A**), stage III (**B**) or stage V (**C**). Data are means ± SEM of represented cases. Inset shows A_{2A} and β-actin bands corresponding to a representative experiment.

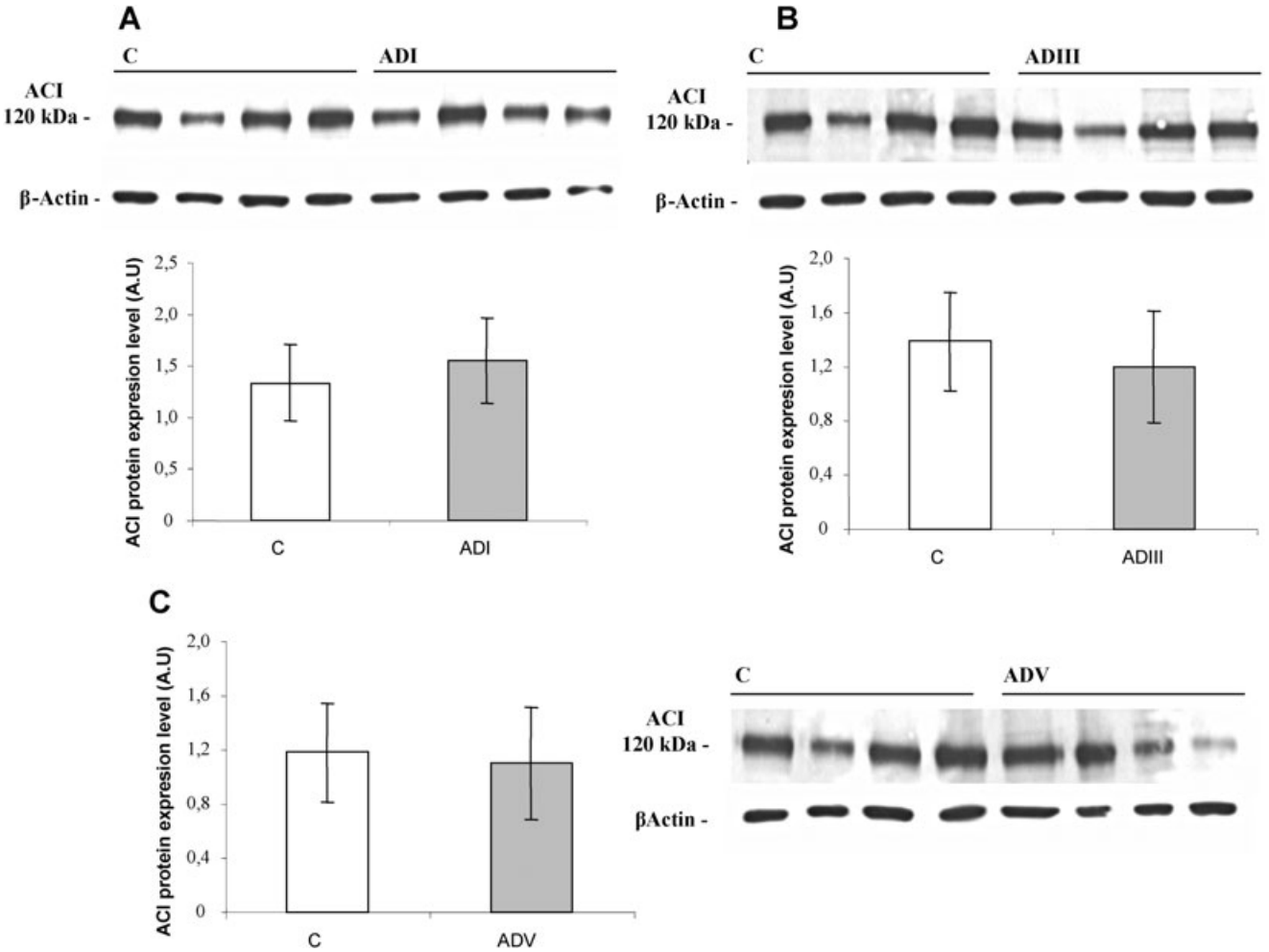


Figure 6. Detection of adenylyl cyclase I (ACI) by Western blot. Assays were performed as described in Methods and in the legend of Figure 3. ACI was analyzed separately from Alzheimer's disease (AD) stage I (**A**), stage III (**B**) or stage V (**C**). Data are means ± SD of represented cases. Inset shows ACI and β-actin bands corresponding to a representative experiment.

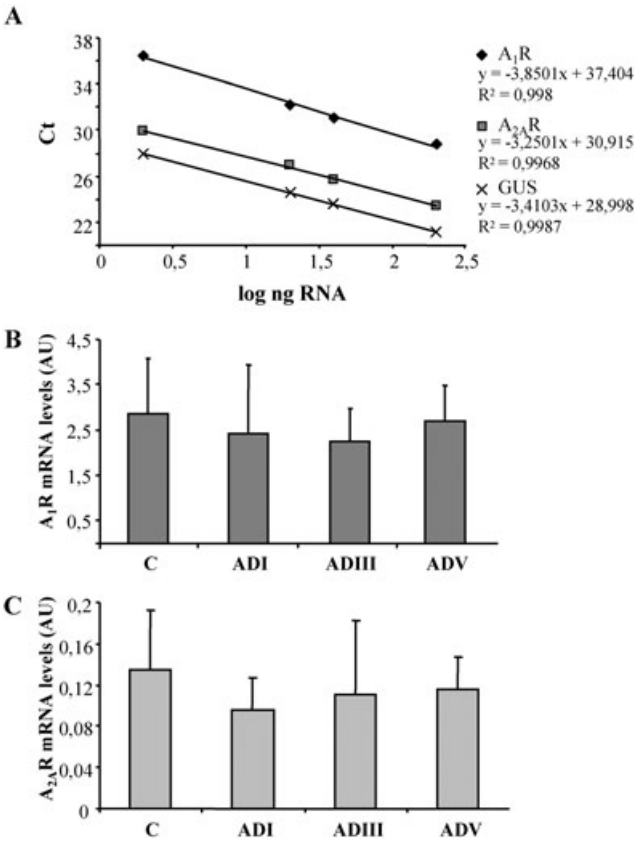


Figure 7. *mRNA levels detected by real time PCR.* **A.** Representative standard curves for A_1R, $A_{2A}R$ and GUSB constructed from several concentrations of control human brain RNA. C_t values (y-axis) vs. log of several RNA concentrations of control samples (x-axis) show a reverse linear correlation. **B.** Relative A_1R and C, $A_{2A}R$ expression levels (mean ± SD) normalized with GUSB in the frontal cortex of controls (C, n = 6) and different stages of Braak and Braak in Alzheimer's disease (AD) samples (ADI, n = 3; ADIII, n = 4; ADV, n = 6).

on the basis of the different methods used. Yet the convergence of results, using various approaches such as binding assays, RT-PCR and gel electrophoresis and Western blotting to analyze binding, mRNA levels and protein levels, respectively, provides a robust combination of techniques in support of the present observations.

In agreement with our results, significantly higher levels of cortical A_1 and hippocampal A_{2A} receptors have been found in a transgenic mice model of AD bearing the APP Swedish mutation when compared with non-transgenic mice (5).

Apart from striatum, adenosine A_{2A} receptors have been detected in other human brain areas such as cortex (3, 19, 31). These receptors are involved in neurodegenerative diseases involving the cerebral cortex, as a significant increase in A_{2A} receptors has been detected in frontal cortex in Pick's disease (3). Moreover, cAMP signaling, as mediated by A_{2A} receptors, increases cellular levels of amyloid precursor protein by stimulating APP gene expression in astrocytes (21). Finally, caffeine, an A_{2A} receptor antagonist, prevents Aβ toxicity in mice (12) and rat cultured cerebellar granule neurons (11).

The possible role of adenosine receptors in processes involved in the pathophysiology of AD is unknown. Location of A_1R in neurodegenerative structures of AD has been described. A marked increase in Adenosine A_1 receptor immunoreactivity was found in degenerating neurons with neurofibrillary tangles and in dystrophic neurites of senile plaques in frontal cortex and hippocampus of AD. A high degree of co-localization of A_1R and Aβ in senile plaques and of A_1R and tau in neurons with tau deposition, but without tangles, has been shown. A_1R are involved in APP processing and in tau phosphorylation and in its translocation toward cytoskeleton in a human neuroblastoma cell line (4). Furthermore, increased A_1R levels have also been detected in the cerebral cortex in Creutzfeldt-Jakob disease and in the murine bovine spongiform encephalopathy model at advanced stages of the disease and coincidental with the appearance of PrP expression in brain from CJD (27). Therefore, changes in adenosine receptors seem not to be limited to AD neurodegeneration.

In summary, the present results show that adenosine A_1 and A_{2A} receptors are up-regulated and sensitized in the AD frontal cortex, suggesting involvement of these receptors in the pathogenesis of AD, and opening new perspectives to therapeutic strategies geared to promote A_1R and antagonize $A_{2A}R$ in AD.

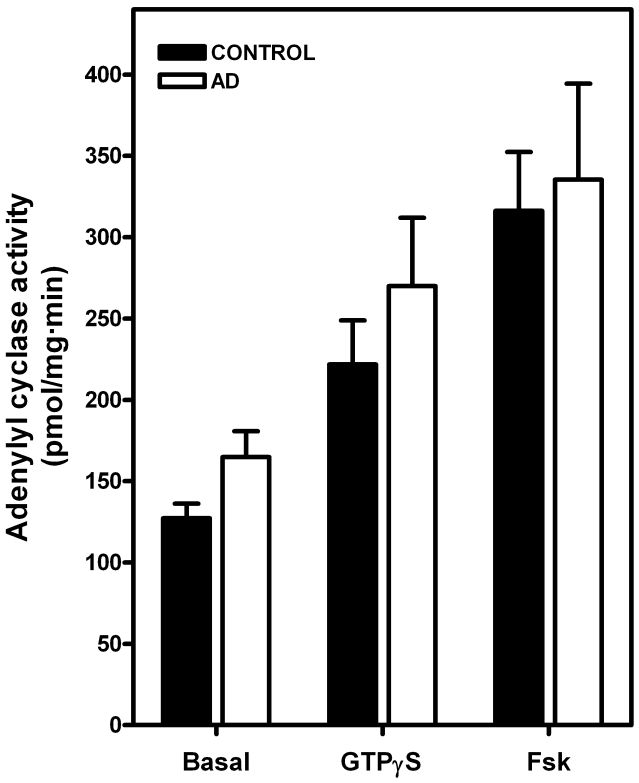


Figure 8. Adenylyl cyclase activity in brain plasma membranes from control and Alzheimer's disease (AD) cases. Fifteen to twenty micrograms of plasma membranes, previously treated with adenosine deaminase, were used to determine basal cAMP level and forskolin- or GTPγS-stimulated in frontal cortex from AD (n = 12) and control (n = 8) cases. Data are means ± SEM of n cases performed in triplicate using different membrane preparations.

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