Modulation of GABA Transport by Adenosine A₁R–A_{2A}R Heteromers, Which Are Coupled to Both G_s- and G_{i/o}-Proteins

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Astrocytes play a key role in modulating synaptic transmission by controlling the available extracellular GABA via the GAT-1 and GAT-3 GABA transporters (GATs). Using primary cultures of rat astrocytes, we show here that an additional level of regulation of GABA uptake occurs via modulation of the GATs by the adenosine A₁ (A₁R) and A_{2A} (A_{2A}R) receptors. This regulation occurs through a complex of heterotetramers (two interacting homodimers) of A₁R–A_{2A}R that signal via two different G-proteins, G_s and G_{i/o}, and either enhances (A_{2A}R) or inhibits (A₁R) GABA uptake. These results provide novel mechanistic insight into how G-protein-coupled receptor heteromers signal. Furthermore, we uncover a previously unknown mechanism in which adenosine, in a concentration-dependent manner, acts via a heterocomplex of adenosine receptors in astrocytes to significantly contribute to neurotransmission at the tripartite (neuron–glia–neuron) synapse.

Introduction

Astrocytes modulate synaptic transmission because they can release and uptake neurotransmitter (Hamilton and Attwell, 2010) and, therefore, fine tune the balance between excitation and inhibition. GABA is the main inhibitory neurotransmitter in the CNS, in which it plays a crucial role in the control of excitability (Krnjević and Scharf, 1967), plasticity (Artola and Singer, 1987), and network synchronization (Blatow et al., 2003). These actions depend on changes in the extracellular concentrations of GABA, which are under control of GABA transporters (GATs) expressed in both neurons and astrocytes (Minelli et al., 1995, 1996). Cortical astrocytes express GAT-1 and GAT-3 subtypes, and it has been estimated that ~20% of extracellular GABA may be taken up into astrocytes (Hertz and Schousboe, 1987).

Astrocytes release large amounts of ATP, which is then hydrolyzed into adenosine by the action of ecto-nucleotidases (Hamilton and Attwell, 2010). Extracellular adenosine operates through G-protein-coupled receptors. In the case of neural cells, the A₁ (A₁R) and A_{2A} (A_{2A}R) receptor subtypes are those that are most likely activated by basal levels of extracellular adenosine. The A₁R is often inhibitory and couple to G_{i/o}-proteins, whereas the A_{2A}R is usually coupled to G_s-proteins, enhancing cAMP accumulation and PKA activity (Fredholm et al., 2001). A₁R and A_{2A}R may closely interact in such a way that activation of A_{2A}Rs can lead to inhibition of A₁R-mediated responses (Correia-de-Sá and Ribeiro, 1994; Cunha et al., 1994; Lopes et al., 1999). Some interactions may occur at the functional and transducing system levels (Sebastião and Ribeiro, 2000), but energy transfer assays in the form of bioluminescence (BRET) and fluorescent (FRET) resonance energy transfer have identified the presence of A₁R–A_{2A}R heteromers in immortalized transfected cells (Ciruela et al., 2006). In addition, the A₁R–A_{2A}R heteromers have been identified in presynaptic membranes via coimmunoprecipitation experiments, and it has been shown that these heteromers modulate glutamate release from presynaptic nerve terminals (Ciruela et al., 2006). Together, these data strongly suggest a putative role of A₁R–A_{2A}R heteromers in neurons. However, direct evidence for A₁R–A_{2A}R heteromerization in neural cells is still lacking.

Because of the role of astrocytes in overall GABA transport, a first aim of the present work was to clarify whether A₁R and A_{2A}R modulate GAT-1- and/or GAT-3-mediated GABA transport into

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astrocytes. We detected a tight interaction between A₁R and A_{2A}R, including evidence for cross-antagonism, a biochemical property often demonstrated for receptor heteromers (Ferré et al., 2010). In addition, we found A₁R–A_{2A}R receptor heteromers in astrocytes. We found that these heteromers are likely in a tetrameric heteromeric complex and couple to two different G-proteins, G_i and G_o, both regulating GABA transport in an opposite way, with the A₁R protomer mediating inhibition of GABA transport and the A_{2A}R protomer mediating facilitation of GABA transport into astrocytes. This A₁R–A_{2A}R receptor functional unit may, therefore, operate as a dual amplifier to control ambient GABA levels at synapses.

Materials and Methods

Drugs. Adenosine deaminase [ADA; E.C. 3.5.4.4; 200 U/mg in 50% glycerol (v/v), 10 mM potassium phosphate] was acquired from Roche. GABA was obtained from Sigma, and the [³H]GABA-specific activity 87.00 Ci/mmol was from PerkinElmer Life and Analytical Sciences. [³H]-labeled R-phenylisopropyladenosine ([³H]R-PIA, 30.5 Ci/mmol) was from Moravsek Biochemicals. CGS 21680 [4-[2-[[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-yl]amino]ethyl]benzenepropanoic acid hydrochloride], SCH 58261 [2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo [4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine], CPA (N⁶-cyclopentyladenosine), DPCPX (8-cyclopentyl-1,3-dipropylxanthine), SKF 89976A hydrochloride [1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride], SNAP 5114 and U73122 [1-[6[[17-β)-3-methoxyestradiol, 3,5(10)-trien-17-yl]amino]hexyl]-1-H-pyrrole-2,5-dione] were obtained from Tocris Bioscience. 2-Chloro-adenosine (CADO), pertussis toxin (PTx), cholera toxin (ChTx), forskolin, Rp-cAMPs (Rp-cyclic 3',5'-hydrogen phosphorothioate adenosine triethylammonium salt), and R-phenylisopropyladenosine were obtained from Sigma.

Cell lines and primary astrocytic cultures. The astrocytes were prepared from the cortex of newborn (P1–P2) Wistar rats of either sex according to the European guidelines (86/609/EEC). Briefly, rat brains were dissected out of pups, cortex was isolated, and the meninges and white matter were removed. Cortex was dissociated gently by grinding in DMEM, filtered through a cell strainer, and centrifuged at 200 × g, 10 min. The pellet was resuspended in DMEM and filtered. The cells were then seeded and kept for 4 weeks in DMEM containing 10% (v/v) fetal bovine serum with antibiotic (Sigma) in a humidified atmosphere (5% CO₂) at 37°C. CHO cells clones expressing A₁R, A_{2A}R, or both were obtained and cultured as indicated previously (Cristóvão-Ferreira et al., 2011). HEK-293T cells were grown in DMEM (Invitrogen) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated fetal bovine serum (all supplements were from Invitrogen). Cells were maintained at 37°C in an atmosphere of 5% CO₂ and were passaged when they were 80–90% confluent (i.e., approximately twice a week).

[³H]GABA uptake. Assays were performed in a non-supplemented low-glucose DMEM. The astrocytes were incubated with ADA for 15 min before the addition of the test drugs; test drugs were added and incubation continued for an additional 20 min. GABA uptake was initiated by the addition of 30 μM [³H]GABA (except otherwise specified). The transport was stopped after 40 s with 2 ml of ice-cold PBS. The amount of [³H]GABA taken up by astrocytes was quantified by liquid scintillation counting. The GAT-1- and GAT-3-mediated transports were calculated through the subtraction of the amount of GABA taken up in the presence of the specific blocker of GAT-1, SKF 89976A (20 μM), or the specific blocker of GAT-3, SNAP 5114 (40 μM), respectively, to the total transport.

Expression vectors and cell transfections. Sequences encoding amino acids residues 1–155 and 155–238 of yellow fluorescent protein (YFP) Venus protein and amino acids residues 1–229 and 230–311 of RLuc8 protein were subcloned in pcDNA3.1 vector to obtain the YFP Venus and RLuc8 hemi-truncated proteins. The human cDNAs for A_{2A}R and A₁R, cloned into pcDNA3.1, were amplified without their stop codons using sense and antisense primers harboring unique EcoRI and BamHI sites to clone A_{2A}R in RLuc8 vector and EcoRI and KpnI to clone A₁R in enhanced YFP (EYFP) vector. The amplified fragments were subcloned to be in-

frame into restriction sites of pcDNA3.1RLuc (pRLuc-N1; PerkinElmer Life and Analytical Sciences) and pEYFP-N1 (enhanced yellow variant of GFP; Clontech) to give the plasmids that express A₁R or A_{2A}R fused to RLuc or YFP on the C-terminal end of the receptor (A_{2A}R–RLuc and A₁R–YFP). The cDNA encoding the serotonin 5-HT_{2B}–YFP fusion protein was kindly provided by Dr. Irma Nardi (University of Pisa, Pisa, Italy). Human cDNA for A₁R was subcloned in pcDNA3.1–nRLuc8 or pcDNA3.1–nVenus to give the plasmids that express A₁R fused to either nRLuc8 or nYFP Venus on the C-terminal end of the receptor (A₁R–nRLuc8 and A₁R–nVenus). Human cDNA for A_{2A}R was subcloned in pcDNA3.1–cRLuc8 or pcDNA3.1–cVenus to give the plasmids that express A_{2A}R fused to either cRLuc8 or cYFP Venus on the C-terminal end of the receptor (A_{2A}R–cRLuc8 and A_{2A}R–cVenus). Expression of constructs was tested by confocal microscopy and the receptor functionality by second messengers, ERK1/2 phosphorylation, and cAMP production as described previously (Sarrió et al., 2000; Canals et al., 2003). HEK-293T cells or 2-week cultured primary astrocytes growing in six-well dishes were transiently transfected with the corresponding fusion protein cDNA by the polyethylenimine (PEI; Sigma) method. Cells were incubated (4 h) with the corresponding cDNA together with PEI (5.47 mM in nitrogen residues) and 100 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium. Forty-eight hours after transfection, cells were washed twice in quick succession in HBSS with 25 mM glucose detached, and resuspended in the same buffer containing 1 mM EDTA. To control the cell number, sample protein concentration was determined using a Bradford assay kit (Bio-Rad) using bovine serum albumin dilutions as standards. Cell suspension (20 μg of protein) was distributed into 96-well microplates; black plates with transparent bottom were used for fluorescence determinations, whereas white plates were used for BRET experiments.

Immunocytochemistry. For immunocytochemistry with primary cultured astrocytes, cells were incubated with ADA (1 U/ml) for 15 min, fixed in 4% paraformaldehyde for 20 min, and washed with PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. Then, after permeabilization with 10% normal goat serum containing 0.3% Triton X-100 for 5 min, cells were treated with PBS containing 1% bovine serum albumin. After 1 h at room temperature, astrocytes were incubated with antibodies rabbit anti-GAT-1 (1:100; kindly provided by N. Brecha, University of California, Los Angeles, Los Angeles, CA) and mouse anti-GFAP (1:800; Sigma) or rabbit anti-GAT-3 (1:200; kindly provided by N. Brecha) and mouse anti-GFAP (1:800; Sigma) antibodies for 3 h at room temperature. After washes, astrocytes were stained with the secondary antibodies for 1.5 h at room temperature [FITC-conjugated anti-rabbit IgG (FI-1000) and TRITC-conjugated anti-mouse IgG (T-2762); Vector Laboratories]. Dishes were then mounted, air dried, and coverslipped using Vectashield mounting medium (H-1000; Vector Laboratories). For immunocytochemistry with transiently transfected HEK-293T cells, cells treated as indicated in figure legends were fixed and permeabilized as indicated above. Cells expressing A_{2A}R–RLuc were labeled with the primary mouse monoclonal anti-RLuc antibody (1:100; Millipore Bioscience Research Reagents) for 1 h, washed, and stained with the secondary antibody Cy3 donkey anti-mouse (1:100; Jackson ImmunoResearch). A₁R–YFP was detected by its fluorescence properties. Samples were rinsed and observed in a Leica SP2 confocal microscope (Leica Microsystems).

Western blot. For A₁R and A_{2A}R detection, primary astrocytes were rinsed with ice-cold PBS and lysed in 8 M urea, 2% SDS, 100 mM DTT, 375 mM Tris, pH 6.8, by heating to 37°C for 2 h and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes using a semidry transfer system and immunoblotted with the primary antibodies mouse anti-A_{2A} antibody (1:1000; Millipore) or rabbit anti-A₁ antibody (1:1000; ABR05). The blots were then incubated with a secondary horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (1:2500) or goat anti-rabbit IgG antibody (1:60,000). The immunoreactive bands were developed using a chemiluminescent detection kit. For GAT-1 and GAT-3 detection, the primary cultures of astrocytes were mechanically lysed with sucrose-containing buffer (0.32 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mg/ml bovine serum albumin, pH 7.4). To clarify, the homogenate was centrifuged (13,000 × g, 10 min), and the

supernatant was collected. After denaturation (by Laemli's buffer heated at 95°C for 5 min), the extracts were run on a 10% acrylamide gel. Protein was transferred to a nitrocellulose membrane by electroblotting. Western blotting was performed using the anti-GAT-1 (1:100) and anti-GAT-3 (1:200) (kindly provided by N. Brecha). After exposure to secondary antibody (peroxidase anti-rabbit at 1:250; Vector Laboratories), bands were visualized by Bio-Rad Chemidoc and Quantity One software.

BRET and BRET with bimolecular fluorescence complementation assays. Primary astrocytes or HEK-293T cells were transiently cotransfected with a constant amount of the cDNA encoding for receptors fused to RLuc, nRLuc8, or cRLuc8 and with increasingly amounts of the cDNA corresponding to receptors fused to YFP, nYFP Venus, or cYFP Venus (see figure legends). To quantify receptor–YFP expression or receptor–reconstituted YFP Venus expression, cells (20 μ g protein) were distributed in 96-well microplates (black plates with a transparent bottom), and fluorescence was read in a Fluo Star Optima Fluorimeter (BMG Lab Technologies) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400 nm reading. Receptor–fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing the BRET donor alone. For BRET or BRET with bimolecular fluorescence complementation (BiLFC) measurements, the equivalent of 20 μ g of cell suspension was distributed in 96-well microplates (Corning 3600, white plates; Sigma), and 5 μ M coelenterazine H (Invitrogen) was added. After 1 min for BRET or after 5 min for BRET with BiLFC of adding coelenterazine H, the readings were collected using a Mithras LB 940 that allows the integration of the signals detected in the short-wavelength filter at 485 nm (440–500 nm) and the long-wavelength filter at 530 nm (510–590 nm). To quantify receptor–RLuc or receptor–reconstituted RLuc8 expression, luminescence readings were also performed after 10 min of adding 5 μ M coelenterazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)] – cf, where cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the donor construct expressed alone in the same experiment. BRET is expressed as net BRET units (mBU = net BRET $\times$ 1000).

Radioligand binding experiments. Four-week cultured primary astrocytes were disrupted with a Polytron homogenizer (FA 2000S rotor, setting 3; Kinematica) for three 5-s periods in 10 ml of 50 mM Tris-HCl buffer, pH 7.4, containing a proteinase inhibitor cocktail (Sigma). Cell debris were eliminated by centrifugation at 1000 $\times$ g, and membranes were obtained by centrifugation at 105,000 $\times$ g (30 min, 4°C). Pellet was resuspended and recentrifuged under the same conditions. Membranes were stored at –80°C and were washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Competition experiments were performed by incubating (120 min) membranes (0.18 mg protein/ml) at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ and 0.2 U/ml adenosine deaminase with 0.8 nM [³H]R-PIA in the absence or presence of increasing concentrations of CGS 21680 or SCH 58261. Nonspecific binding was determined in the presence of 10 μ M R-PIA. Free and membrane-bound ligand were separated by rapid filtration of 500 μ l aliquots in a cell harvester (Brandel) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecocint H scintillation cocktail (National Diagnostics) overnight at room temperature, and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer Life and Analytical Sciences) with an efficiency of 62% (Ciruela et al., 2004). Radioligand displacement curves were analyzed by nonlinear regression using commercial program GRAFIT (Erithacus Software) as indicated previously (Ciruela et al., 2006).

[³⁵S]GTP- γ -S assay. For quantification of GTP activity, GDP (10 μ M) was added to the primary astrocytic membranes and incubated on ice for 10 min. Membranes were incubated at 37°C for 10 min with ADA (1 U/ml) before adding the antagonists. After 10 min, the [³⁵S]GTP- γ -S (1 nM) and the agonists were added and incubated for 30 min at 37°C. Membranes were collected and solubilized, and the antibodies were added: 5 μ g of anti-G_{ai-3} (sc-262), 10 μ g of anti-G_{as} (sc-6766), or 10 μ g of anti-G_{aq/11} (sc-392) for G_i, G_s, and G_q studies, respectively. After an overnight incubation at 4°C, protein G-Sepharose was added and incubated for 90 min at 4°C. The Sepharose was washed five times with the solubilization buffer, and the incorporation of [³⁵S]GTP- γ -S was measured by liquid scintillation.

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Biotinylation assays. Astrocytes were incubated for 30 min without (control) or with the agonists or antagonist of A₁R or A_{2A}R or both. When antagonist and agonist were tested together, the antagonist was added 15 min before. Afterward, they were incubated for 1 h with 1 mg/ml Sulfo-NHS-LC-biotin (Pierce) in PBS–Ca–Mg with gentle shaking. The biotin reaction was quenched with 100 mM glycine. The astrocytes were mechanically lysed with sucrose-containing buffer and centrifuged at 14,000 $\times$ g, 4°C, 10 min. Biotinylated surface proteins were immunoprecipitated with avidin beads (Pierce) overnight at 4°C and centrifuged at 14,000 $\times$ g, 10 min at 4°C. The avidin beads were pelleted by centrifugation at 3000 $\times$ g, 4°C, 10 min. The pellet (biotinylated fraction) was separated from the supernatant (intracellular fraction). Then, 150 μ l of Laemli's buffer (70 mM Tris-HCl, pH 6.8, 6% glycerol, 2% SDS, 120 mM DTT, 0.0024% bromophenol blue) was added to the pellet and heated to 37°C for 30 min. The avidin beads were removed by filtration. Equal volumes of each sample was loaded on gel and resolved by SDS-PAGE.

CellKey label-free assays. The CellKey system provides a universal, label-free, cell-based assay platform that uses cellular dielectric spectroscopy to measure endogenous and transfected receptor activation in real time in live cells (Schröder et al., 2010). Changes in the complex impedance (ΔZ or $\Delta Z'$) of a cell monolayer in response to receptor stimulation were measured. Impedance (Z) is defined by the ratio of voltage/current as described by Ohm's law ($Z = V/I$). CHO cell clones stably expressing A₁R, A_{2A}R, or both were grown to confluence in a CellKey Standard 96-well microplate that contains electrodes at the bottom of each well. For untreated cells or for cells preincubated (overnight at 37°C) with PTx (100 ng/ml) or ChTx (100 ng/ml), medium was replaced by HBSS buffer (Invitrogen) supplemented with 20 mM HEPES for 30 min before running the cell equilibration protocol. A baseline was recorded for 5 min, and then cells were treated with the A₁R agonist CPA (10 nM) or with the A_{2A}R agonist CGS 21680 (10 nM), and data were acquired for the following 10 min. To calculate the impedance, small voltages at 24 different measurement frequencies were applied to treated or nontreated cells. At low frequencies, extracellular currents that pass around individual cells in the layer were induced. At high frequencies, transcellular currents that penetrate the cellular membrane were induced, and the ratio of the applied voltage/measured current for each well is the impedance. The data shown refer to the maximum complex impedance induced extracellular currents response to the ligand addition.

Data analyses and statistics. From the indicated number of experiments/replicates, data are given as mean $\pm$ SEM. To test for statistical significance, the data were analyzed by one-way ANOVA, followed by Bonferroni's correction for multiple comparisons or by Student's *t* test (when only two means are analyzed). Values of *p* < 0.05 were considered to represent statistical significance.

Results

Endogenous adenosine tonically modulates GABA uptake

To assess the role of adenosine during GABA uptake, we first incubated the astrocytes with different concentrations of CADO, an adenosine analog with similar affinity for A₁R and A_{2A}R that is resistant to hydrolysis or uptake by the cells. At a relatively low CADO concentration (0.3 μ M), there was an inhibition of total GABA taken up by astrocytes, whereas at higher concentrations (3–10 μ M), CADO facilitated total GABA uptake (Fig. 1A). This biphasic influence on GABA transport could be either attributable to activation of different adenosine receptors, namely A₁R and A_{2A}R, or a differential influence over the two GATs present in astrocytes, GAT-1 and GAT-3 (Fig. 1F,G). Hence, GAT-1 or GAT-3 activity was independently assayed (see Materials and Methods). The removal of endogenous adenosine with ADA (1 U/ml) led to a decrease in GABA transport, and this decrease was highly significant when transport was mediated by either GAT-1

(Fig. 1*B*) or GAT-3 (Fig. 1*C*), suggesting that extracellular adenosine is tonically facilitating GAT-1 and GAT-3 activity. To avoid occupation of adenosine receptors with the endogenous ligand, all subsequent transport assays were performed in cells preincubated with ADA (1 U/ml) (see Materials and Methods).

A₁R activation decreased and A_{2A}R activation enhanced GABA uptake

Selective agonists of A₁R and A_{2A}R were used to assess the influence of the adenosine receptors on GABA transporters. The selective A₁R agonist CPA (30 nM) decreased maximal velocity (*V*_{max}) of GABA transport mediated by GAT-1 (Fig. 1*D*) or GAT-3 (Fig. 1*E*), whereas the selective agonist for A_{2A}R, CGS 21680 (30 nM), enhanced *V*_{max} for GAT-1 (Fig. 1*D*) and GAT-3 (Fig. 1*E*), without affecting transport *K*_M values (*p* > 0.05, *n* = 6). These data indicate that adenosine receptor activation modified maximum transport capacity rather than in the affinity of the transporters for GABA and that inhibition of GAT-1 and GAT-3 is mediated by A₁R, whereas facilitation requires A_{2A}R activation. To further confirm that A₁R and A_{2A}R affect GABA transport in opposite ways, we used combinations of agonists and antagonists selective for either receptor (Table 1). Results are summarized in Figure 2. CPA and CGS 21680 effect were measured in the presence of the A₁R-selective antagonist DPCPX (50 nM) or the A_{2A}R-selective antagonist SCH 58261 (50 nM). Surprisingly, the effect of the A₁R agonist was fully prevented not only by previous blockade of the A₁R with DPCPX but also by the blockade of A_{2A}R with SCH58261. For these experiments, the concentration of each compound was chosen to act in a selective way. Analogously, facilitation of GABA transport by the A_{2A}R agonist CGS 21680 was completely abolished by the blockade of either A_{2A}R or A₁R. These results strongly indicate that A₁R and A_{2A}R are tightly interacting and represent a clear example of cross antagonism between the two receptors. Such antagonism may be attributable to heteromerization (Ferrada et al., 2009; Moreno et al., 2011); thus, we decided to test whether A₁R and A_{2A}R may form heteromers in astrocytes.

A₁R–A_{2A}R heteromers in astrocytes

The BRET approach was used to evaluate the ability of A₁R to heteromerize with A_{2A}R in astrocytes. First, the endogenous A₁R and A_{2A}R expression in astrocytes was investigated by Western blot (Fig. 3*A,B*). A₁R and A_{2A}R expression is relatively low at 2 weeks of cell culture but increases later. Thus, to avoid competition with endogenous receptors, BRET measurements were performed using 2-week cultured astrocytes transiently cotransfected with a constant amount of A_{2A}R–RLuc (7.5 μg of

Figure 1. Adenosine receptor activation modulates [³H]GABA uptake in astrocytes. Astrocytes were incubated with medium or with increasing CADO concentrations (*a*) or 1 U/ml ADA (*b, c*), and the total [³H]GABA uptake (*a*) or GAT-1-mediated (*b*) or GAT-3-mediated (*c*) uptake was determined. In *d* and *e*, uptake kinetics was determined using increasing [³H]GABA concentrations. The A_{2A}R agonist CGS 21680 (30 nM, squares) enhanced and the A₁R agonist CPA (30 nM, triangles) decreased the GAT-1-mediated (*d*) or GAT-3-mediated (*e*) uptake (control uptake: circles) (*V*_{max} of GAT-1, 25.1 ± 1.7 pmol GABA/min vs 14.9 ± 0.9 pmol GABA/min of control, **p* < 0.01, *n* = 6; and *V*_{max} of GAT-3, 30.9 ± 1.6 pmol GABA/min vs 22.5 ± 1.6 pmol GABA/min of control, **p* < 0.001, *n* = 6) with no changes in *K*_M values (GAT-1, 4.9 ± 0.5 vs 5.0 ± 0.8 μM, *p* > 0.05, *n* = 6; GAT-3, 17.6 ± 2.2 vs 18.2 ± 2.7 μM, *p* > 0.05, *n* = 6). In *f*, for immunohistochemistry analysis of GAT-1 (green, top row) and GAT-3 (green, bottom row) expression by astrocytes, GFAP (red) was used as astrocyte marker. In *g*, solubilized astrocytes were analyzed by SDS-PAGE and immunoblotted using rabbit anti-GAT-1 antibody (1:100) or rabbit anti-GAT-3 antibody (1:200) (M, molecular mass markers). Results in *a–e* are shown as mean ± SEM of four to six independent experiments. Statistical significance was calculated by one-way ANOVA, followed by Bonferroni's multiple comparison test; **p* < 0.01 compared with control (white bars).

Table 1. Binding affinity of agonists and antagonists of adenosine receptors [*K*_i values with 95% confidence intervals (in parentheses) or ± SEM] (adapted from Fredholm et al., 2001)

Drug	A ₁ R	A _{2A} R	A _{2B} R	A ₃ R
CPA	2.3 (1.5–3.4) ^a	790 (470–1360) ^a	34,400 ± 11,100 ^b	43 (30–61) ^a
CGS 21680	290 (230–360) ^a	27 (12–59) ^a	21,000 ± 4300 ^d	67 (50–90) ^a
DPCPX	3.9 (3.5–4.2) ^a	129 (35–260) ^a	50 ± 3.7 ^b	4000 (2600–6000) ^a
SCH 58261	290 (210–410) ^c	0.6 (0.5–0.7) ^c	51 ± 6.1 ^d	>10,000 ^c

^aKlotz et al., 1998.

^bLinden et al., 1999.

^cOngini et al., 1999.

^dJi and Jacobson, 1999.

cDNA) and increasing amounts of A₁R–YFP (4–15 μg of cDNA). Fusion of RLuc to A_{2A}R or YFP to A₁R did not modify receptor function as determined by cAMP assays (Canals et al., 2003 and results not shown). A positive and saturable BRET signal was

Figure 2. Inhibition of [³H]GABA uptake is promoted by A₁R, whereas facilitation is mediated by A_{2A}R. Astrocytes were treated for 15 min with 1 μM CPA (see Materials and Methods) before the addition of medium, the A₁R antagonist DPCPX (1 μM), or the A_{2A}R antagonist SCH 58261 (50 nM). After 20 min, the A₁R agonist CPA (30 nM) (**a–d**) or the A_{2A}R agonist CGS 21680 (30 nM) (**e–h**) were added, and the GAT-1 (**a, e**) or GAT-3 (**c, d, g, h**) mediated [³H]GABA uptake was measured as indicated in Materials and Methods. Results are mean ± SEM of six independent experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test; **p* < 0.001 compared with control (white bar); NS, *p* > 0.05.

found for the pairs A_{2A}R–RLuc and A₁R–YFP (Fig. 3C). From the saturation curve, a BRET_{max} of 94 ± 15 mBU and a BRET₅₀ of 16 ± 2 were calculated. As a negative control, the A_{2A}R–RLuc and serotonin 5-HT_{2B}R–YFP pair was used. As shown in Figure 3C, the negative control gave a linear nonspecific BRET signal, thus confirming the specificity of the interaction between A_{2A}R–RLuc and A₁R–YFP in astrocyte primary cultures.

Ligand binding assays to receptor heteromers in isolated membranes usually reveal a “biochemical fingerprint,” which consists of changes in ligand binding characteristics of one receptor when the partner receptor is occupied by agonist (Ferré et al., 2009). No intracellular crosstalk can occur in disrupted membranes, and therefore it can be assumed that the “fingerprint” results from intramembrane receptor–receptor interactions. Although an indirect approach, it is accepted as identifier of receptor heteromers in native tissues or in cells expressing the natural non-heterologous receptors (Ferré et al., 2009). Therefore, binding experiments were performed to identify native A₁R–A_{2A}R

Figure 3. A₁R–A_{2A}R heteromers in astrocytes. In **a**, the expression of A₁R and A_{2A}R in astrocytes after different weeks of culture was detected by Western blot as indicated in Materials and Methods using α-tubulin as loading control. Averaged (*n* = 3) densitometric analysis of immunoblots is shown in **b**. In **c** and **e–h**, BRET saturation experiments were performed using 2-week cultured astrocytes (**c**) or HEK-293 cells (**e–h**) cotransfected with 1.5 μg (**c**) or 1 μg (**e–h**) cDNA corresponding to A_{2A}R–RLuc and increasing amounts of cDNA corresponding to A₁R–YFP (squares) or 5-HT_{2B}R–YFP (triangles, as negative control) constructs. In **e–h**, cells were treated for 10 min with medium (squares, solid line) or with 30 nM CGS 21680 (**e**), 30 nM CPA (**f**), 50 nM SCH 58261 (**g**), or 50 nM DPCPX (**h**) (circles, dotted lines). The BRET_{max} and BRET₅₀ values are shown in the insets. Both fluorescence and luminescence of each sample were measured before every experiment to confirm similar donor expressions (~100,000 luminescent units) while monitoring the increase acceptor expression (500–10,000 fluorescent units). Data are means ± SD of three different experiments grouped as a function of the amount of BRET acceptor. In **d**, competition experiments of 0.8 nM [³H]R-PIA versus increasing concentrations of the A_{2A}R agonist CGS 21680 (solid line) or the A_{2A}R antagonist SCH 58261 (dotted line) were performed using astrocytic membranes (0.18 mg protein/ml). Data are mean ± SEM of a representative experiment (*n* = 3) performed in triplicate.

heteromers in 4-week cultured astrocytes. As shown in Figure 3D, the displacement of A₁R agonist [³H]R-PIA binding by the A_{2A}R agonist CGS 21680 (but not by the A_{2A}R antagonist SCH 58261) was significantly (*p* < 0.01) better represented by a biphasic than by a monophasic curve. It is not expected that the A_{2A}R agonist, at concentrations lower than 500 nM, would significantly bind to A₁R (<1% binding to A₁R, according to the known K_D value). However, 500 nM CGS 21680 significantly (*p* < 0.05) displaced the binding of the selective A₁R agonist [³H]R-PIA, with an IC₅₀ value of 90 ± 30 nM. Obviously, higher concentrations of CGS

21680 caused an additional displacement of [³H]R-PIA binding that, according to its IC₅₀ value (8 ± 4 μM), reflects the binding of CGS 21680 to the A₁R. As expected, the A_{2A}R antagonist SCH 58261 only displaced A₁R agonist binding (IC₅₀ of 500 ± 120 nM) at concentrations known to lose A_{2A}R selectivity and to bind to A₁R (Fig. 3D). Together, these data indicate that the biphasic [³H]R-PIA binding displacement curve observed in the presence of the A_{2A}R agonist constitutes a fingerprint of the A₁R–A_{2A}R heteromer in nontransfected primary cultured astrocytes.

To evaluate whether A₁R–A_{2A}R heteromerization could be influenced by agonist or antagonist binding, a series of experiments was performed in transiently cotransfected HEK-293T cells using a constant amount of A_{2A}R–RLuc (1.5 μg of cDNA) and increasing amounts of A₁R–YFP (1–8 μg of cDNA). In agreement with previous results (Ciruela et al., 2006), a positive and saturable BRET signal was found. Stimulation (20 min) with the A_{2A}R agonist (CGS 21680, 30 nM) (Fig. 3E) or with the A₁R agonist (CPA, 30 nM) (Fig. 3F) did not promote any consistent (*p* > 0.05) change in BRET_{max} or BRET₅₀ values. Similar BRET values were also obtained in the presence or absence of A_{2A}R (Fig. 3G) or A₁R (Fig. 3H) antagonists, indicating that neither agonist nor antagonist binding affected the receptor oligomerization state.

A₁R or A_{2A}R activation, but not its blockade, leads to internalization of the heteromers

Heteromerized receptors are expected to internalize together. To test this possibility, agonist-mediated internalization of A₁R and A_{2A}R was studied in astrocytes. Western blot data clearly showed that A₁R immunoreactivity at the cell surface did not only increase after incubation of astrocytes with the A₁R agonist but also after incubation with the A_{2A}R agonist (Fig. 4A). This increase was accompanied by an increase in A₁R immunoreactivity in the intracellular fraction (Fig. 4B). No significant changes in surface (Fig. 4C) or intracellular (Fig. 4D) A₁R immunoreactivity were detected during incubation with either A₁R or A_{2A}R antagonists. Interestingly, when the A₁R agonist was added after previous blockade of either A₁R or A_{2A}R by the selective antagonists, it was no longer able to modify A₁R immunoreactivity at the cell surface (Fig. 4E) or in the intracellular fraction (Fig. 4F). Similarly, adding the A_{2A}R agonist after a previous blockade of A₁R or A_{2A}R did not promote any modification of A₁R immunoreactivity at the cell surface (Fig. 4E) or in the intracellular fraction (Fig. 4F). It therefore becomes clear that blockade of either A₁R or A_{2A}R prevents A₁R internalization induced by exposure to A₁R or A_{2A}R agonists.

Cointernalization of A₁R and A_{2A}R after incubation with A₁R or A_{2A}R agonists was also assessed by confocal microscopy analysis of HEK-293T cells coexpressing A₁R–YFP and A_{2A}R–RLuc. After exposure to either A₁R- or A_{2A}R-selective agonists, intracellular A₁R–YFP fluorescence and A_{2A}R–RLuc immunoreactivity markedly increased; a similar phenomenon was observed after exposure to an A₁R agonist but not after exposure to A₁R or A_{2A}R antagonists (Fig. 5). Collectively, these results indicate that A₁R and A_{2A}R do internalize together in response to A₁R- or A_{2A}R-selective agonists.

The A₁R–A_{2A}R heteromer is coupled to G_{i/o}- and G_s-proteins

To figure out which G-proteins are coupled to the A₁R–A_{2A}R heteromer, assays of [³⁵S]GTP-γ binding followed by immunoprecipitation using antibodies against different G-proteins (G_s, G_{i/o}, and G_{q/11}) were performed. The approach is similar to that reported by Rashid et al. (2007) to identify G_q coupling to the dopamine D₁–D₂ receptor heteromer. As illustrated in Figure 6A

Figure 4. A₁R or A_{2A}R activation (but not its blockade) in astrocytes promotes internalization of A₁R and A_{2A}R. Astrocytes were incubated for 30 min with the A₁R agonist CPA (30 nM) or with the A_{2A}R agonist CGS 21680 (30 nM), alone (**a, b**) or in the presence of either the A₁R antagonist DPCPX (50 nM) or the A_{2A}R antagonist SCH 58261 (50 nM) (**e, f**) or only with DPCPX (50 nM) or SCH 58261 (50 nM) (**c, d**), before starting the biotinylation protocol. When testing the action of agonists in the presence of antagonists, the antagonists were added 15 min before the agonists. A₁R expression at surface membranes (left panels) and intracellular fraction (right panels) was determined as indicated in Materials and Methods. Results are mean ± SEM of five independent experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test; **p* < 0.001 compared with control (100%, white bar).

(left), the A₁R-selective agonist CPA (30 nM) but not the A_{2A}R-selective agonist CGS 21680 (30 nM) significantly increased the G_{i/o} activity, an effect unpredictably prevented by the A_{2A}R-selective antagonist. In what concerns G_s activity (Fig. 6A, middle), it was enhanced by the A_{2A}R-selective agonist CGS 21680 (30 nM) but not by the A₁R agonist CPA (30 nM); again, and unpredictably, the effect of the A_{2A}R agonist was fully abolished by the A₁R antagonist DPCPX (50 nM). None of the adenosine receptor agonists affected G_{q/11} activity, which was enhanced by acetylcholine (10 μM), used as a positive control in the same batch of astrocytic membranes (Fig. 6C, right). These data suggest that A₁R–A_{2A}R heteromers are coupled to both G_{i/o}- and G_s-proteins and not to a unique G_{q/11}-protein.

G-protein activity may be permanently modified by the binding of several toxins; thus, these are useful tools to dissect out a

Figure 5. A₁R and A_{2A}R are internalized together during exposure to either A₁R or A_{2A}R agonists. HEK-293 cells were transfected with 1 μ g of cDNA corresponding to A_{2A}R–RLuc (red) or 1 μ g of cDNA corresponding to A₁R–YFP (green) (*a, c, e, g, i*) or both (*b, d, f, h*), and, 48 h after transfection cells, were treated for 60 min with medium (*a, b*), 100 nM A_{2A}R agonist CGS 21680 (*c, d*), 1 μ M A_{2A}R antagonist SCH 58261 (*e, f*), 100 nM A₁R agonist R-PIA (*g, h*), or 1 μ M A₁R antagonist DPCPX (*i, j*). Immunocytochemistry was performed as indicated in Materials and Methods, and A_{2A}R–RLuc was labeled with the anti-RLuc antibody, and A₁R–YFP was detected by its fluorescence properties. Colocalization was shown in yellow. The quantification of receptor internalization after the exposure to ligands was determined by analyzing, for each condition, 40–50 cells from 12 different fields in three independent preparations by confocal microscopy. Values are expressed as mean $\pm$ SEM.

differential receptor–G-protein coupling in intact cells and to evaluate the functional consequences of G-protein-mediated-signaling blockade. GABA uptake assays were therefore performed using ChTx, which uncouples G_s from the receptors as a result of ADP-ribosylation and permanent activation of the α_s subunit (Gill and Meren, 1978), as well as using PTx, which catalyzes the ADP-ribosylation of the G $\alpha_{i/o}$ subunit and locks it in the GDP-bound inactive state, thus preventing G_{i/o}-protein activation (Bokoch and Gilman, 1984). Inhibition of either GAT-1- or GAT-3-mediated GABA uptake induced by the A₁R agonist CPA was fully prevented by PTx, but, interestingly, this toxin also prevented A_{2A}R-mediated facilitation of GAT-1- and GAT-3-

mediated GABA uptake (Fig. 6*B*). Similar results were obtained in the reciprocal experiment using ChTx. In fact, the toxin prevented not only the facilitation of GAT-1- and GAT-3-mediated transport caused by the A_{2A}R agonist CGS 21680 but also the inhibition of GABA transport mediated by the A₁R agonist CPA (Fig. 6*C*). The ChTx and PTx data strongly suggest that the A₁R–A_{2A}R heteromer is coupled to both G_s- and G_i- proteins. The results also indicate that, if one G-protein (G_s or G_i) is blocked or receptor uncoupled, both A₁R and A_{2A}R agonists lose their effect on GABA uptake. It seems that the A₁R–A_{2A}R heteromer is the mediator of both the inhibitory and the excitatory effects triggered by, respectively, CPA and CGS 21680.

As an additional approach, G_{i/o}, G_s, or G₁₂ activity was also measured by the Cytosol label-free assay (see Materials and Methods) in intact CHO cells transfected with A₁R, A_{2A}R, or both. In A₁R-transfected cells, the signaling obtained during A₁R activation with the agonist CPA (10 nM) showed a G_i profile (increases in impedance) that was completely blocked when cells were treated with PTx. Impedance did not significantly change when cells were treated with ChTx (Fig. 6*D*, left). In A_{2A}R-transfected cells, the A_{2A}R agonist CGS 21680 (10 nM) induced a G_s profile (decreases in impedance) that was completely blocked when cells were treated with ChTx but not significantly modified during PTx treatment (Fig. 6*D*, middle). Interestingly, in cells coexpressing A_{2A}R and A₁R, the impedance profiles obtained by activation with CPA (G_i profile) or CGS 21680 (G_s profile) were fully blocked by either PTx or ChTx (Fig. 6*D*, right). These results strongly reinforce the notion that the A₁R–A_{2A}R heteromer is coupled to both G_s- and G_i- proteins.

A₁R–A_{2A}R heterotetramers

Because of the size of G-proteins and of seven-transmembrane domain receptors, space restrictions make it difficult that a

receptor heterodimer couples to two distinct G-proteins. In light of the data described in the previous section, clearly pointing toward the coupling of A₁R–A_{2A}R heteromers to two distinct G-proteins, BRET experiments with complemented luminescent and fluorescent proteins were performed to evaluate whether A₁R–A_{2A}R heteromers may result from the interaction of A₁R and A_{2A}R homodimers. An explanatory diagram showing the luminescence/fluorescence complementation approach (BRET with BiFC assay; see Materials and Methods) is shown in Figure 7*A*. Accordingly, cells were cotransfected with a constant amount of the two cDNAs corresponding to A₁R–nRLuc8 and A_{2A}R–cRLuc8 (equal amounts of the two cDNAs) and with increasing

amounts of the two cDNAs corresponding to A_1 R–nVenus and A_{2A} R–cVenus (equal amounts of the two cDNAs). Specific BRET would only be possible if RLuc is reconstituted by A_1 R–nRLuc8– A_{2A} R–cRLuc8 dimerization and if YFP Venus is reconstituted by A_1 R–nVenus– A_{2A} R–cVenus dimerization. Heterotetramerization was in fact demonstrated by a positive and saturable BRET signal ($BRET_{max} = 35 \pm 2$ mBU; $BRET_{50} = 16 \pm 3$; Fig. 7B). Cells expressing A_1 R–nRLuc8, A_{2A} R–cRLuc8, and A_1 R–nVenus or A_{2A} R–cVenus did not provide any significant fluorescent signal or positive BRET (Fig. 7C). Analogously, cells expressing A_1 R–nVenus, A_{2A} R–cVenus, and A_1 R–nRLuc8 or A_{2A} R–cRLuc8 did not display any significant luminescence or positive BRET (Fig. 7C). As an additional negative control, BRET was not detected in cells expressing A_1 R–nRLuc8, A_{2A} R–cRLuc8, and YFP Venus or expressing A_1 R–nVenus, A_{2A} R–cVenus, and RLuc8 (Fig. 7C). Collectively these results indicate that A_1 – A_{2A} R heteromers seem to be constituted by the interaction of receptor homomers, and the minimal structural unit is the A_1 R– A_1 R– A_{2A} R– A_{2A} R heterotetramer.

A transducing unit constituted by an A_1 R– A_{2A} R heterotetramer

The above-described data strongly support the notion that the heteromer is, at least, in an A_1 R– A_1 R– A_{2A} R– A_{2A} R tetrameric form and is coupled to $G_{i/o}$ and G_s but not to $G_{q/11}$. This arrangement predicts that the transducing system operated by adenosine receptors to modulate GABA transport into astrocytes is centered in the adenylate cyclase (AC)/cAMP/PKA cascade. To address the question of whether a single cAMP/PKA-centered transducing unit is able to both inhibit and facilitate GABA transport, we tested the influence of drugs known to interfere with this transducing pathway on the effect of A_1 R and A_{2A} R receptor agonists on GABA transport. In addition, we also tested the blocker of phospholipase C (PLC) to evaluate a putative transducing pathway classically associated with $G_{q/11}$.

The inhibitory action of A_1 R agonist on GAT-1 (Fig. 8A) and GAT-3 (Fig. 8C) still occurred in the presence of PLC blocker U73122 (3 μ M; Smith et al., 1990), but it was totally abolished by the blockade of PKA by Rp-cAMPs (100 μ M; Wang et al., 1991). The activation of AC with a supramaximal concentration of forskolin (10 μ M; Awad et al., 1983) increased GABA transport and occluded the inhibitory effect of the A_1 R agonist (CPA) during GABA transport (Fig. 8A,C). The facilitatory effect of the A_{2A} R agonist CGS 21680 was not affected by the PLC inhibitor U73122 but was totally impaired by the PKA blocker Rp-cAMPs. The AC activator forskolin mimicked the action of the A_{2A} R agonist, and its facilitatory effect was not additive with that of CGS 21680, indicating a common mechanism (Fig. 8B,D).

Figure 5. A_1 R– A_{2A} R heteromer in astrocytes is coupled to both $G_{i/o}$ and G_s . In **a**, [35 S]GTP- γ assays were performed as described in Materials and Methods to test $G_{i/o}$ activity (left), G_s activity (middle), or $G_{q/11}$ activity (right) using membranes from astrocytes treated for 10 min with medium, the A_{2A} R antagonist SCH 58261 (50 nM), or the A_1 R antagonist DPCPX (50 nM) before the activation with A_1 R agonist CGS 21680 (30 nM) or A_1 R agonist CPA (30 nM) or ACh (10 μ M) as positive control. In **b** and **c**, astrocytes were treated with medium, PTX (5 μ g/ml), or ChTx (5 μ g/ml) before stimulation with CPA (30 nM) or CGS 21680 (30 nM), and GAT-1- and GAT-3-mediated [3H]GABA uptake was measured as indicated in Materials and Methods. Toxins were preincubated with the astrocytes for 4 h and then removed before uptake assays. **d**, CellKey label-free assays were performed in CHO cells stable expressing A_1 R (left), A_{2A} R (middle), or both (right), treated with medium, PTX (10 ng/ml), or ChTx (100 ng/ml), and stimulated or not with CGS 21680 (10 nM) or CPA (10 nM). Results are as mean $\pm$ SEM from four to eight independent experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test; * $p < 0.001$ compared with control (100%, white bar), ** $p < 0.001$ compared with cells treated only with the agonist. NS, $p > 0.05$.

Discussion

The work now reported clearly shows that GABA uptake by astrocytes is under modulation by extracellular adenosine, which, by interacting with a functional unit constituted by A_1 R– A_{2A} R heteromers coupled to two distinct G-proteins, $G_{i/o}$ and G_s , can either boost or depress the amount of inhibitory neurotransmitter available to neurons.

Using an adenosine analog, CADO, with high structural similarity to adenosine but with the advantage of not being taken up by the cells nor metabolized by ecto-enzymes, we observed that submicromolar concentrations of this agonist inhibit GABA uptake, whereas at low micromolar concentrations, there is an enhancement of GABA transport. Considering that the affinity of adenosine for the A_1 R is slightly higher than for the A_{2A} R (Fredholm et al., 2001), it is likely that the inhibition was mediated by A_1 R and facilitated by A_{2A} R. Accordingly, the A_1 R-selective agonist CPA inhibited GABA uptake into astrocytes, whereas the

Figure 7. Heterotetramers formed by A_1 R– A_1 R and A_{2A} R– A_{2A} R homodimers. Heterotetramers constituted by two A_1 R and two A_{2A} R protomers were demonstrated by BRET with BilFC assays (see Materials and Methods). In **a**, a schematic representation of the technique is given. One receptor fused to the N-terminal fragment (nRluc8) and another receptor fused to the C-terminal fragment (cRluc8) of the Rluc8 act as BRET donor after Rluc8 reconstitution by a close receptor–receptor interaction and one receptor fused to an YFP Venus N-terminal fragment (nVenus) and another receptor fused to the YFP Venus C-terminal fragment (cVenus) act as BRET acceptor after YFP Venus reconstitution by a close receptor–receptor interaction. In **b**, BRET saturation curve was obtained in HEK-293 cells cotransfected with 1.5 μ g of the two cDNA corresponding to A_1 R–nRluc8 and A_{2A} R–cRluc8 and with increasing amounts of the two cDNAs corresponding to A_1 R–nVenus and A_{2A} R–cVenus (equal amounts of the two cDNAs). mBU (net BRET $\times$ 1000; see Materials and Methods) are represented in front of the ratio between the fluorescence of the acceptor and the luciferase activity of the donor (YFP/Rluc8). In **c**, results from cells expressing equivalent amounts of A_1 R–nRluc8, A_{2A} R–cRluc8, and A_1 R–nVenus or A_{2A} R–cVenus, expressing A_1 R–nVenus, A_{2A} R–cVenus, and A_1 R–nRluc8 or A_{2A} R–cRluc8, expressing A_1 R–nRluc8, A_{2A} R–cRluc8, and YFP Venus, or expressing A_1 R–nVenus, A_{2A} R–cVenus, and Rluc8 are given as negative controls and compared with a positive control. Data are means $\pm$ SD of three different experiments grouped as a function of the amount of BRET acceptor.

A_{2A} R-selective agonist CGS 21680 facilitated it. Unexpectedly, the blockade of either receptor with selective antagonists prevented the effects mediated by either agonist, a strong indication that A_1 R and A_{2A} R are interacting at the molecular level in pri-

Figure 8. A_1 R– A_{2A} R heteromer signaling. Astrocytes were treated for 15 min with 1 U/ml ADA (see Materials and Methods) before the addition of medium, the PLC inhibitor U73122 (3 μ M), the PKA inhibitor Rp-cAMPs (100 μ M), or the AC enhancer forskolin (10 μ M). After 20 min, the A_1 R agonist CPA (30 nM) (**a**, **c**) or the A_{2A} R agonist CGS 21680 (30 nM) (**b**, **d**) were added, and the GAT-1-mediated (**a**, **b**) or GAT-3-mediated (**c**, **d**) mediated [3 H]GABA uptake was measured as indicated in Materials and Methods. Results are mean $\pm$ SEM from 4–10 independent experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test; * p < 0.001 versus control (100%, white columns), $^{**}p$ < 0.001 versus cells treated with the agonist alone. **e**, Schematic representation of A_1 R– A_{2A} R heteromer function. At low levels, adenosine binds preferentially to the A_1 R protomer of the heteromer, which will activate $G_{i/o}$ protein, and through a mechanism that involves AC and PKA activity, leads to a decrease (–) in GABA uptake mediated by GAT-1 and GAT-3. At higher concentrations, adenosine activates the A_{2A} R protomer of the heteromer inhibiting A_1 R and, through G_s protein, couples to the AC/cAMP/PKA pathway, leading to an enhancement (+) of GABA uptake.

mary cortical astrocytes. Abundant evidence of A_1 R– A_{2A} R functional crosstalk has been described, namely, A_{2A} R activation attenuates A_1 R-mediated responses in the hippocampus (Cunha et al., 1994; Lopes et al., 1999), at the neuromuscular junction (Correia-de-Sá and Ribeiro, 1994), and in transfected cells (Ciruela et al., 2006); however, no attempt has been made to unequivocally and directly identify A_1 R– A_{2A} R heteromerization in neural cells. Thus, we looked for energy transfer in primary cultures of astrocytes transfected with cDNAs for A_{2A} R–RLuc and A_1 R–YFP. In these assays, a positive, specific, and satura-

ble BRET signal for the energy transference between A_{2A}R–RLuc and A₁R–YFP was detected in living primary astrocytes. Moreover, the heteromer in native astrocytes was detected by looking for a fingerprint that consists of changes in ligand binding characteristics of A₁R when the A_{2A}R is activated. These results complemented and strengthened the evidence for A₁R–A_{2A}R heteromerization in nontransfected astrocytes.

Biotinylation assays showed that exposure to either A₁R or A_{2A}R agonists led to similar decreases in surface expression of A₁R, and to similar increases in the A₁R levels in intracellular fractions. It then appears that binding of a single ligand to the heteromer is sufficient to promote internalization of the two receptors. The previous blockade of A₁R or A_{2A}R prevents the heteromer internalization mediated by both A₁R and A_{2A}R agonists, suggesting that internalization of heteromer in response to agonists is a consequence of heteromer activity. Confocal imaging of transfected HEK-293T cells confirmed that exposure to an A₁R agonist led to internalization of not only A₁R but also A_{2A}R and, conversely, exposure to an A_{2A}R agonist led to internalization of not only A_{2A}R but also A₁R. Thus, in this aspect, A₁–A_{2A}R heteromers behaved as the β 2-adrenoceptor– δ -opioid (Jordan et al., 2001) and μ -opioid–tachykinin NK1 (Pfeiffer et al., 2003) receptor heteromers but different from other heteromers whose protomers do not co-internalize (Smith and Milligan, 2010). Agonist exposure did not affect BRET, suggesting that agonist binding does not induce pronounced allosteric modifications in the receptors. It therefore appears that the A₁R–A_{2A}R heteromer in astrocytes mostly works as an integral entity, leaving and probably also reaching the cell surface as a heteromeric structure. Furthermore, because antagonists did not modify the receptor levels at the membrane or the BRET signal, the loss of effect of one agonist on previous blockade of the other receptor cannot be attributed to heteromer disruption or formation. Cross-antagonism, which is considered a heteromer fingerprint (Ferre et al., 2009), is likely attributable to conformational changes induced by the antagonist and leads to a nonfunctional state of the signaling receptor by uncoupling it from G-protein-mediated signaling.

Heteromers may couple to G-proteins differently from those to which each individual receptor partner usually couple. This is indeed the case of the dopamine D₁–D₂ receptor heteromer, which couples to G_q (Rashid et al., 2007) in a clear shift from the canonical D₁ coupling to G_s and D₂ coupling to G_{i/o}. To identify the transducing system operated by the A₁R–A_{2A}R heteromer in astrocytes, we first used an approach similar to that used by Rashid et al. (2007), i.e., [³⁵S]GTP- γ binding followed by differential immunoprecipitation. Data obtained allowed to conclude that the A₁R–A_{2A}R heteromer in astrocytes seems to be coupled to both G_{i/o}- and G_s-proteins. Interestingly, the cross-antagonism was also evident in these assays. Thus, the A₁R agonist, but not the A_{2A}R agonist, increased G_{i/o} activity, but the enhancement was also prevented when the A_{2A}R antagonist was present. Reciprocally, G_s activation, which was restricted to the A_{2A}R agonist, was prevented when the A₁R was blocked with the antagonist. Similar conclusions could be drawn from GABA uptake assays because toxin-induced prevention of coupling of receptors to either G_s or G_{i/o} led to reciprocal impaired function of A₁R or A_{2A}R agonists to modulate GABA transport. The cross-inhibition of heteromer function by the toxins was observed in both cultured astrocytes and heterologous cells coexpressing A₁R and A_{2A}R but not in cells expressing only one receptor subtype. Altogether, these data provide strong evidence for a heteromeric functional entity regulating GABA uptake by astrocytes. This functional unit consists of an A₁R–A_{2A}R heteromer/G_{i/o}–G_s com-

plex, which signals through G_s when the A_{2A}R protomer is activated and through G_i when the A₁R protomer is activated. Most importantly, the blockade of a single partner in the complex led to adjustments in the whole unit. BRET with a double luminescence/fluorescence molecular complementation approach showed that A₁R–A_{2A}R heteromers appear as heteromers of homomers with a minimal structure consisting of an A₁R–A₁R–A_{2A}R–A_{2A}R complex. The heterotetramer makes it possible to accommodate the two different G-proteins; indeed, two G-protein-coupled receptor molecules cannot bind to more than a single G-protein (Han et al., 2009). The transducing system operated by the heteromer seems to involve the AC, consistent with the G-protein data and the data obtained in the presence of forskolin or the inhibitor of the PKA (Rp-cAMPs), both of which occluded the effects of the A₁R- or A_{2A}R-selective agonists during GABA uptake.

A 10-fold rise in concentration of the nonselective ligand CADO was enough to gate A_{2A}R activation and engage a completely opposite modulation of GABA uptake. Assuming a near 10-fold higher potency of CADO compared with adenosine (Ribeiro and Sebastião, 1987), the shift from inhibition to enhancement of GABA uptake might occur at low micromolar concentrations of extracellular adenosine. These concentrations are easily attained at tripartite synapse, in which astrocytes and neurons release considerable amounts of ATP, which are degraded into ADP, AMP, and adenosine by ecto-5'-nucleotidases. The higher the release of ATP, as at high neuronal firing rates in reciprocal neuron-to-astrocyte communication at the tripartite synapse (Fields and Burnstock, 2006), the higher the expected concentration of extracellular adenosine. It is therefore likely that sustained neuronal firing promotes activation of the A_{2A}R protomer of the A₁R–A_{2A}R heteromer, leading to facilitation of GABA uptake. Activation of GABA uptake by astrocytes will lead to a decrease in ambient GABA and a subsequent depression of tonic GABAergic inhibition, resulting in enhanced excitatory tonus. Conversely, at submicromolar adenosine concentrations, there is a preferential activation of the A₁R protomer of the A₁R–A_{2A}R heteromer, and GABA uptake by astrocytes would be inhibited and tonic inhibition by GABA would be enhanced. Thus, through an adenosine action on A₁R–A_{2A}R heteromers, astrocytes might behave as dual amplifiers, facilitating excitation at intense astrocytic to neuronal signaling and increasing inhibition at low neuronal firing rates. This switch in neural activity may require a highly efficient control to avoid sudden state transitions, and this seems to be the main advantage of heteromerization of A₁R and A_{2A}R in astrocytes. Indeed, overstimulation of just one of the receptor protomers leads to internalization of the whole functional unit, therefore allowing a double brake in the system and avoiding an abrupt inhibitory signaling and a sudden switch from excitation to inhibition as a consequence of desensitization of only the excitatory protomer.

To conclude, we herein provide strong and complementary evidence that, during GABA uptake, adenosine has a biphasic effect on GABA uptake, which is mediated by A₁R–A_{2A}R heteromers coupled to both G_{i/o}- and G_s-proteins. Extracellular adenosine acting on these A₁R–A_{2A}R functional units operates in a concerted way to balance a PKA-dependent action on GABA uptake as indicated in the scheme of Figure 8E. The neural output would be inhibitory at low firing rates and facilitatory at high firing rates. We therefore disclose an as-yet unknown way through which adenosine by acting on adenosine receptors in astrocytes may significantly contribute to neurotransmission in a dual manner, which depends on the concentration of the nucle-

oside that is in turn dependent on neuronal firing activity. This adds a novel conceptual way to understand the fundamental role of astrocytes at tripartite synapses.

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