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Binding of the prototypical adenosine A_{2A} receptor agonist CGS 21680 to the cerebral cortex of adenosine A_1 and A_{2A} receptor knockout mice

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1 2-*p*-(2-carboxyethylphenethylamino-5′-ethylcarboxamidoadenosine) (CGS 21680) is considered the reference compound to study adenosine A_{2A} receptors. However, CGS 21680 binding in the cerebral cortex, where adenosine A_1 receptors are predominant, displays a mixed A_{2A}/A_1 receptor pharmacology. We now use adenosine A_1 and A_{2A} receptor knockout mice to investigate the characteristics of cortical [^{3}H]CGS 21680 binding.

2 [^{3}H]CGS 21680 binding to the cerebral cortex was strongly reduced in adenosine A_1 receptor knockout mice, but only slightly reduced in A_{2A} receptor knockout mice compared with the corresponding wild-type littermates.

3 Another selective A_{2A} receptor ligand, [^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([^{3}H]SCH 58261), displayed a saturable binding to mouse cortical membranes, albeit with a binding density 20 times lower than that of striatal membranes, and this [^{3}H]SCH58261 binding was abolished in both striatal and cortical membranes of A_{2A} receptor knockout mice and unchanged in A_1 receptor knockout mice.

4 The presence of A_{2A} receptors in cortical neurons was further confirmed by Western blot in mouse cortical nerve terminal membranes.

5 It is concluded that, although A_{2A} receptors are present in the cerebral cortex, the purportedly selective A_{2A} receptor agonist [^{3}H]CGS 21680 binds in the cerebral cortex to an entity that requires the presence of adenosine A_1 receptors. Thus, CGS 21680 should be used with care in all preparations where adenosine A_1 receptors out-number A_{2A} receptors.
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Abbreviations: $A_1R^{(-/-)}$, (adenosine A_1 receptor knockout); $A_1R^{(+/-)}$, (adenosine A_1 receptor heterozygot); $A_1R^{(+/+)}$, (adenosine A_1 receptor wild-type); $A_{2A}R^{(-/-)}$, (adenosine A_{2A} receptor knock-out); $A_{2A}R^{(+/-)}$, (adenosine A_{2A} receptor heterozygot); $A_{2A}R^{(+/+)}$, (adenosine A_{2A} receptor wild-type); ADA, (adenosine deaminase); CGS 15943, (9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo-[1,5]quinazolin-5-imine monomethanesulfonate); CGS 21680, (2-[p-(2-carboxyethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine); DPCPX, (1,3-dipropyl-8-cyclopentyl-xanthine); NECA, (5′-N-ethylcarboxamidoadenosine); R-PIA ((±)N^6-R-phenylisopropyladenosine); SCH 58261, (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine); XAC, (8-{4-[(2-aminoethyl) amino]carbonylmethyl-oxyphenyl}xanthine)

Introduction

The purine nucleoside adenosine and its various receptor subtypes play multiple roles in the modulation of the central nervous system (Dunwiddie & Masino, 2001). Adenosine exerts its physiological actions through activation of cell surface receptors (Fredholm *et al*., 2001). Of these, A_1 receptors are widespread throughout the brain, whereas adenosine A_{2A} receptors are far more abundant in the basal ganglia (Fastbom *et al*., 1987; Jarvis & Williams, 1989; Parkinson & Fredholm, 1990). However, there is evidence that adenosine A_{2A} receptors also exist outside the striatum, for example, in the cerebral cortex (reviewed in Cunha, 2001; Fredholm *et al*., 2003). Although these cortical adenosine A_{2A} receptors are not abundant, they may play a fundamental role in the viability of cortical tissue. For instance, the blockade or genetic disruption of A_{2A} receptors confers a profound neuroprotection in situations of ischemia in adult animals (Monopoli *et al*., 1998; Chen *et al*., 1999; Behan & Stone, 2002), whereas gene disruption seems to have the opposite effect in early life (Ådén *et al*., 2003).

A number of potent and rather selective ligands are available for the study of adenosine A_{2A} receptors (Klotz,

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2000). The most widely used adenosine A_{2A} receptor agonist is CGS 21680. It displays both high affinity and selectivity for the rat adenosine A_{2A} receptor (Jarvis *et al*., 1989). However, the binding of CGS 21680 to cortical or hippocampal regions displays several differences from binding to 'typical' striatal-like A_{2A} receptors, especially in terms of pharmacology, which is intermediary between that of A_1 and A_{2A} receptors (Wan *et al*., 1990; James *et al*., 1992; Johansson *et al*., 1993; Kirk & Richardson, 1995; Cunha *et al*., 1996; 1997; 1999; Lindström *et al*., 1996). Given that CGS 21680 remains the drug predominantly used to characterize A_{2A} receptors and to define A_{2A} receptor-mediated effects, it is obviously important to clarify what the 'atypical' binding profile of CGS 21680 binding in the cerebral cortex actually represents.

The recent introduction of adenosine A_1 (Johansson *et al*., 2001) and A_{2A} (Ledent *et al*., 1997; Chen *et al*., 1999) receptor knockout mice has provided a new tool to test the selectivity of adenosine receptor ligands, in particular when ligands are of low affinity or suspected to have poor selectivity between receptor subtypes or when the relative densities of receptor subtypes are considerably different. Thus, instead of carrying out another detailed characterization of CGS 21680 binding, we decided to characterize the impact of the genetic elimination of A_1 or A_{2A} receptors on the binding of CGS 21680 to the cerebral cortex.

Methods

Animals

Adenosine A_1 receptor knockout mice were on a mixed 129/OlaHsd/C57B6 background generated as described (Johansson *et al*., 2001). Heterozygous animals of the third generation were bred and all experiments were conducted on littermates genotyped using PCR. Adenosine A_{2A} receptor knockout mice were either on an outbred CD1 background, generated as described (Ledent *et al*., 1997), or on a pure 129-Steel genetic background, as previously described (Chen *et al*., 1999). Heterozygous mice were bred and littermates with determined genotype (PCR assay) were used. The following symbols are used throughout the text: (+/+) refers to wildtype; (+/−) to heterozygous and (−/−) to homozygous null-mutant mice. All animals were handled according to the EU guidelines (86/609/EEC).

Drugs and solutions

2-*p*-(2-Carboxyethyl-[^{3}H]-*N*-phenethylamino-5′-ethylcarboxamido-adenosine ([^{3}H]CGS 21680, specific activity 41.2 Ci mmol^{-1}) and 8-cyclopentyl(2,3-[^{3}H]N)-1,3-dipropylxanthine ([^{3}H]DPCPX, specific activity 116.0 Ci mmol^{-1}) were from DuPont NEN (Stevenage, U.K.), [^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([^{3}H]SCH 58261, specific activity 77 Ci mmol^{-1}) was prepared by Amersham and was a generous gift of Dr E. Ongini (Shering-Plough, Milan, Italy), (±)N^6-*R*-phenylisopropyladenosine (*R*-PIA) was from Sigma (Poole Dorset, U.K.), adenosine deaminase (ADA, calf intestine suspension, 200 U mg^{-1}, EC 3.5.4.4) was from Roche (Germany) and 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS 15943) and 8-{4-[(2-aminoethyl)amino]-carbonylmethyl-oxyphenyl}xanthine (XAC) were from Research Biochemicals International (Natick, MA, U.S.A.). All drugs were diluted daily into the appropriate media from 10 mM stock solutions made up in dimethylsulfoxide stored at 4°C, except DPCPX, which was dissolved in ethanol. [^{3}H]DPCPX, [^{3}H]SCH 58261, [^{3}H]CGS 21680 and adenosine deaminase were prepared directly into the incubation solution each day. All other reagents were of the highest purity available.

Membrane-binding experiments

Membranes were prepared as previously described (Cunha *et al*., 1996). Briefly, the brains were removed from two to five mice of each genotype and the striata and cortices were dissected out at 4°C in sucrose solution (0.32 M) containing 50 mM Tris-HCl, and 2 mM EGTA, pH 7.6. The tissue was homogenized in a Potter-Elvehjem homogenizer at 4°C. The resulting homogenates were centrifuged at 1000 × *g* for 10 min at 4°C. The supernatants were re-centrifuged at 14 000 × *g* for 12 min at 4°C. The pellets were then resuspended in a solution containing 50 mM Tris-HCl (pH 7.4), 2 mM EGTA, 1 mM EDTA, 2 U ml^{-1} ADA and incubated for 30 min at 37°C to remove endogenous adenosine. After centrifugation at 14 000 × *g* the pellets were resuspended in the incubation solution (50 mM Tris-HCl and 10 mM $MgCl_2$ (for [^{3}H]CGS 21680 and [^{3}H]SCH 58261 experiments) or 2 mM $MgCl_2$ (for [^{3}H]DPCPX experiments), pH 7.4). Aliquots were then frozen at −80°C.

Samples were incubated with 0.1–20 nM [^{3}H]CGS 21680 for 4 h, with 0.3–10 nM [^{3}H]SCH 58261 for 1 h, or with 0.1–10 nM [^{3}H]DPCPX for 2 h at 23–25°C. This was performed with 192–331 μg ([^{3}H]CGS 21680 and [^{3}H]SCH 58261 binding in the cortex) or 22–57 μg ([^{3}H]DPCPX binding and [^{3}H]CGS 21680 or [^{3}H]SCH 58261 binding in the striatum) of membrane protein in a final volume of 300 μl in an incubation solution containing 50 mM Tris-HCl and 10 mM $MgCl_2$ (pH 7.4) for [^{3}H]CGS 21680 and [^{3}H]SCH 58261 experiments and 50 mM Tris-HCl and 2 mM $MgCl_2$ (pH 7.4) for [^{3}H]DPCPX experiments, both containing 4 U ml^{-1} ADA. When tissue from A_1 receptor (−/−) and A_{2A} receptor (−/−) was tested, the tissue from the corresponding wild type (+/+) (i.e., with the same genetic background) was also run in the same assay.

Nonspecific binding was measured in the presence of the A_1/A_{2A} receptor antagonist CGS 15943 (1 μM) for [^{3}H]CGS 21680 experiments, of the A_1/A_{2A} receptor antagonist XAC (1 μM) for [^{3}H]SCH 58261 experiments and of the A_1 receptor agonist R-PIA (100 μM) in the case of [^{3}H]DPCPX experiments. All binding assays were performed in duplicate. The binding reactions were stopped by vacuum filtration. The washing volume was 5 ml for [^{3}H]DPCPX and [^{3}H]SCH 58261 experiments and 10 ml for [^{3}H]CGS 21680 experiments, with the respective incubation buffer maintained at 4°C. The filters were placed in scintillation liquid (Ready Safe, Wallac, Finland) and radioactivity was determined after at least 12 h. The counting efficiency was 55–60%. The protein concentration was determined using the Bio-Rad protein assay based on Bradford dye-binding procedure.

The specific binding from saturation experiments was fitted by nonlinear regression to a one-site binding equation using the Raphson–Newton method, performed with commercial software (GraphPad, San Diego, CA, U.S.A.). Data are the mean ± s.e.m. values or mean (95% confidence interval) of

n experiments. In some cases, the significance was calculated with a paired Student's *t*-test. A value of $P<0.05$ was considered to represent a significant difference.

Autoradiography experiments

Mice were anesthetized with carbon dioxide and thereafter decapitated. The brain was dissected out, directly frozen on dry ice and stored at −80°C. Sections (14 μm) were cut using a cryostat and mounted on Polysine microslides (Menzel-Gläser, Germany). The autoradiographies were performed essentially as described before (Halldner *et al*., 2000). In brief, slides were incubated with 0.2–10 nM of [^{3}H] DPCPX, 0.1–10 nM of [^{3}H]SCH 58261, or 0.3–30 nM [^{3}H]CGS 21680. For evaluation of nonspecific binding, the adenosine analogue R-PIA (20 μM) was added to DPCPX experiments, the adenosine analogue NECA (50 μM) was added to the SCH 58261 experiments and the adenosine receptor agonist 2-chloroadenosine (20 μM) was added to the CGS 21680 experiments. After incubation, dried sections were apposed to ^{3}Hyperfilm (Amersham) together with tritium standards. Optical densities were measured by means of the MCID M5 system (Imaging Research, St. Catherine's, Canada). Results are given as fmol mg^{-1} tissue.

RT–PCR experiments

After brief CO_2 anesthesia, A_1 (+/+), A_1 (−/−), and A_{2A} (−/−) mice were decapitated. The striata and the cortex were dissected out and directly transferred to β-mercaptoethanol-containing RLT buffer (Rneasy, Qiagen). The tissue was homogenized with a syringe and a 30 G needle. Total RNA was extracted by means of Rneasy RNA extraction kit (Qiagen) according to the manufacturer's instructions, and finally dissolved in RNase free water. Total RNA was reverse transcribed (RT) for 55 min at 37°C. Aliquots (1 μl) of cDNA were amplified using the primers 5′-CTC CAC CAT GAT GTA CAC-3′and 5′-CAT GGT TTC GGG AGA TGC AG-3′. PCR conditions consisted of an initial denaturation for 2 min at 94°C followed by 25 cycles of: 94°C, 30 s; 56°C, 60 s; 72°C, 30 s, and then a final 10 min incubation at 72°C. After amplification, the products were electrophoresed through a 0.5 × TBE (Tris-borate/EDTA) 1% agarose gel. Bands were visualized by means of UV light. The A_{2A} (−/−) samples were used as a negative control.

Western blot analysis

The analysis of adenosine A_{2A} receptor immunoreactivity was carried out by Western blot analysis (Rebola *et al*., 2002) in whole membranes of the striatum and in membranes from a Percoll-purified synaptosomal fraction of the cerebral cortex, prepared as previously described (e.g., Cunha *et al*., 1996). After the amount of protein had been determined, each sample was diluted with two volumes of a solution containing 8 M urea, 100 mM dithiothreitol, 2% (w v^{-1}) sodium dodecyl sulfate and 375 mM Tris-HCl (pH 6.8) and incubated for 2 h at 37°C. These diluted samples and the prestained molecular weight markers (Amersham) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% with a 4% concentrating gel) under reducing conditions and electro-transferred to polyvinylidene difluoride membranes (0.45 μm, from Amersham). After blocking for 2 h at room temperature with 5% milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4°C with a goat anti-adenosine A_{2A} receptor antibody (1 : 100 dilution from a 200 μg ml^{-1} stock from Santa Cruz Biotechnology). After four washing periods of 10 min with TBS-T containing 0.5% milk, the membranes were incubated for 90 min at room temperature with the alkaline phosphatase-conjugated anti-goat secondary antibody (1 : 2000 dilution from Calbiochem) in TBS-T containing 1% milk. After five 10-min washes in TBS-T with 0.5% milk, the membranes were incubated with enhanced chemi-fluorescence (ECF) for 5 min and then analyzed with a Storm (Molecular Devices).

Results

Binding of [^{3}H]DPCPX to the cerebral cortex

The pharmacological characterization of adenosine A_1 receptors has mainly been carried out in cerebral cortical preparations. We now confirmed that the selective A_1 receptor antagonist [^{3}H]DPCPX displayed a saturable binding profile in the cerebral cortex of wild-type (+/+) mice (Figure 1a and b). In fact, in the autoradiographic experiments summarized in Figure 1, [^{3}H]DPCPX bound with a K_D of 0.38 nM (95% confidence interval: 0.25 to 0.52 nM) and a B_{max} of 218 (200–236) fmol mg^{-1} gray matter ($n=6$), whereas in membrane-binding studies [^{3}H]DPCPX bound with a K_D of 2.93 nM and a B_{max} of 4196 fmol mg^{-1} protein ($n=1$) to cerebral cortical preparations of wild-type (+/+) mice. As illustrated in Figure 1a, the binding of [^{3}H]DPCPX to cortical tissue was decreased by nearly 50% in adenosine A_1 (+/−) mice, displaying a B_{max} of 109 (100–118) fmol mg^{-1} gray matter and a K_D of 0.35 (0.22–0.49) nM, and the binding disappeared in the A_1 receptor knockout (−/−) mice, as previously reported (Johansson *et al*., 2001). This disappearance of [^{3}H]DPCPX binding to cortical tissue was also found in membrane-binding studies (Figure 1b).

We then compared the binding of [^{3}H]DPCPX to the cerebral cortex of mice with a partial (+/−) or total disruption (−/−) of the adenosine A_{2A} receptor gene with that in control mice (+/+). As illustrated in Figures 1c and d, the binding of [^{3}H]DPCPX to the cerebral cortex was nearly identical in wild-type (+/+), heterozygote (+/−) and A_{2A} receptor knockout (−/−) mice, as assessed both by autoradiography (Figure 1c) and by membrane-binding studies (Figure 1d). For instance, [^{3}H]DPCPX bound with a K_D ranging from 0.25–0.52 nM and B_{max} values ranging from 173 to 188 fmol mg^{-1} gray matter to the striatum of A_{2A} (+/+), A_{2A} (+/−) and A_{2A} (−/−). This indicates that there is no apparent change in the binding properties of cortical adenosine A_1 receptors in A_{2A} receptor knockout animals.

Binding of [^{3}H]CGS 21680 to the basal ganglia

We then wanted to confirm the lack of binding of the prototypical adenosine A_{2A} receptor agonist CGS 21680 to adenosine A_{2A} knockout animals, once again using both autoradiography and membrane binding. For this purpose, we studied the binding of [^{3}H]CGS 21680 to the caudate putamen, which has been the gold standard to characterize adenosine A_{2A} receptor pharmacology. There was a complete lack of

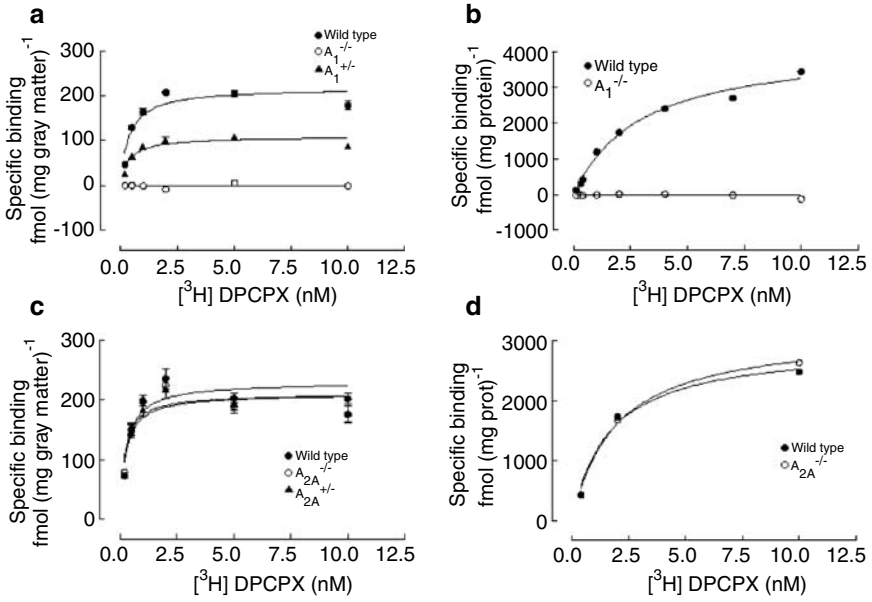

Figure 1 Saturation-binding curves of the selective adenosine A_1 receptor antagonist [^{3}H]DPCPX to the cerebral cortex of adenosine A_1 receptor knockout mice and their respective wild-type littermates (a and b) and to the cerebral cortex of adenosine A_{2A} receptor knockout mice and their respective wild-type littermates (c and d) measured by autoradiography (a and c) or membrane binding (b and d).

[^{3}H]CGS 21680 binding in the caudate putamen of A_{2A} receptor knockout (−/−) mice, while in wild-type (+/+) mice there is an evident saturable curve, as assessed both by autoradiography (not shown) and by membrane-binding studies in striatal membranes (Figure 2a).

We then compared the binding of [^{3}H]CGS 21680 to the basal ganglia in mice with a disruption (−/−) of the adenosine A_1 receptor gene with binding in their controls (+/+). The binding of [^{3}H]CGS 21680 to the striatum was nearly identical in wild-type (+/+) and A_1 knockout (−/−) mice, as assessed both by autoradiography in the caudate putamen (not shown) and by membrane-binding studies in the striatum (Figure 2b). This indicates that there is no apparent change in the binding properties of adenosine A_{2A} receptors in A_1 receptor knockout animals.

Binding of [^{3}H]CGS 21680 to the cerebral cortex

Since [^{3}H]CGS 21680 binding to the cerebral cortex of rats displays a mixed A_1/A_{2A} receptor pharmacology (Wan *et al*., 1990; James *et al*., 1992; Johansson *et al*., 1993; Kirk & Richardson, 1995; Cunha *et al*., 1996; 1997; 1999; Lindström *et al*., 1996), we hoped to clarify the adenosine receptor subtype recognized by CGS 21680 in the cerebral cortex with the use of adenosine A_1 and A_{2A} receptor knockout mice.

To our surprise, autoradiographic quantitation of the [^{3}H]CGS 21680 (3 and 30 nM) binding showed that adenosine A_1 rather than A_{2A} receptors appeared to be involved, since [^{3}H]CGS 21680 binding is abolished in A_1 receptor knockout (−/−) mice (Figure 3a) and is essentially preserved in adenosine A_{2A} receptor knockout (−/−) mice (Figure 3b). However, the strength of this conclusion is partially hampered by the small binding densities of [^{3}H]CGS 21680 binding found in these autoradiographic experiments (see Figure 3).

Therefore, we repeated the same experiments, but now using membrane-binding studies to be able to increase the protein

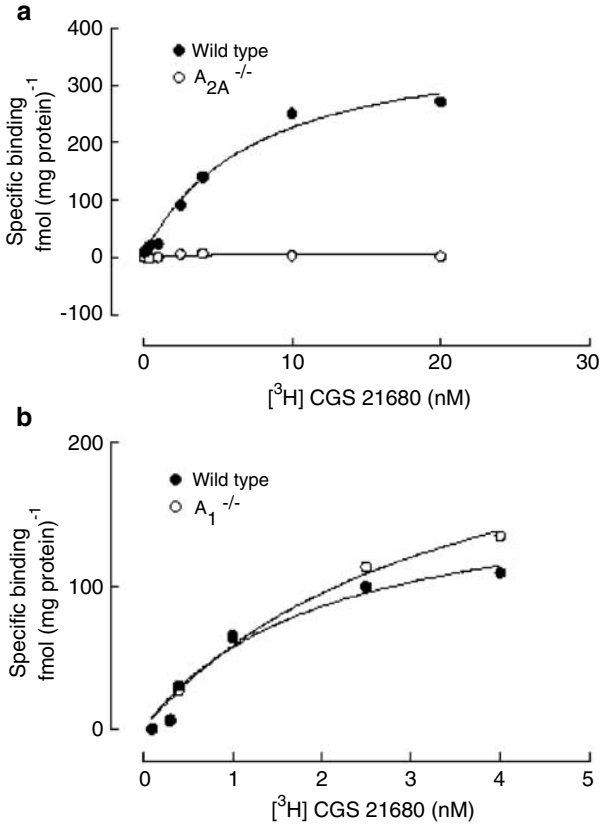

Figure 2 Saturation-binding curves of the adenosine A_{2A} receptor agonist, [^{3}H]CGS 21680, to the basal ganglia of adenosine A_{2A} receptor knockout mice and their wild-type littermates (a) and of adenosine A_1 receptor knockout mice and their wild-type littermates (b) measured by membrane binding. In both panels, the ordinates represent the specific binding of [^{3}H]CGS 21680, obtained upon subtraction of the nonspecific binding determined with 1 μM CGS 15943 from total binding. Nonspecific binding amounted to 20–30% of the total binding.

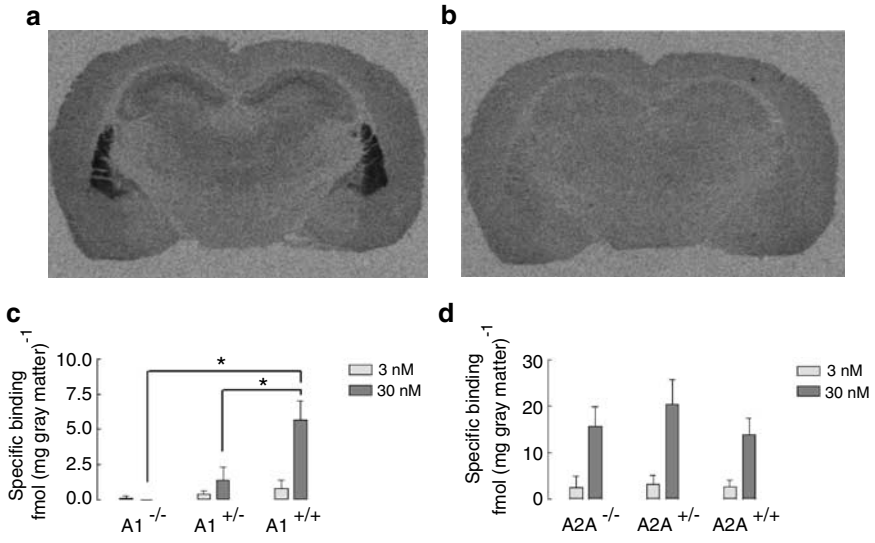

Figure 3 Panels a and b show autoradiograms of [^{3}H]CGS 21680 (30 nM) binding to wild-type mice in the absence (a) or in the presence of 2-chloroadenosine (20 μM) to define nonspecific binding (b). Panels c and d display the average autoradiographic binding data of [^{3}H]CGS 21680 (3 and 30 nM) to the cerebral cortex of adenosine A_1 receptor knockout (−/−), heterozygous (+/−), and their corresponding wildtypes (+/+) (a) and to the cerebral cortex of adenosine A_{2A} receptor knockout (−/−), heterozygous (+/−), and their corresponding wildtypes (+/+) (b). In both panels, the ordinates represent the specific binding of [^{3}H]CGS 21680, obtained upon subtraction of the nonspecific binding determined with 20 μM 2-chloroadenosine from total binding. The data are mean ± s.e.m., $n = 3$–6. Note that the binding of [^{3}H]CGS 21680 to the cerebral cortex is essentially preserved in the adenosine A_{2A} receptor knockout mice and is progressively decreased and abolished in the adenosine A_1 receptor heterozygous and knockout mice.

concentration of cortical membranes in the assay and consequently to amplify the signal, although the binding characteristics of adenosine receptors are expected to be different in autoradiography and membrane-binding experiments (e.g. Fredholm *et al*., 2003). As shown in Figure 4, membrane-binding studies essentially confirmed the major conclusion drawn from autoradiography experiments. Thus, it was observed that the specific binding of [^{3}H]CGS 21680 to cerebral cortical membranes decreased by nearly 80% in adenosine A_1 receptor knockout (−/−) mice (Figure 4a). In contrast, in two experiments, we noted a slight, near 30% decrease (not evaluated statistically) in the binding density of [^{3}H]CGS 21680 in A_{2A} receptor knockout (−/−) mice (Figure 4b) when compared to their wild-type (+/+) littermates.

Binding of [^{3}H]SCH 58261 to the cerebral cortex

One possible explanation is that there are major changes in A_{2A} receptors when the expression of adenosine A_1 receptors in the cerebral cortex is genetically altered. We first confirmed that a low expression (compared to striatum) of A_{2A} receptor mRNA is present in mouse cerebral cortex, as had previously been shown to occur in the rat (Cunha *et al*., 1994), and that the expression remains in A_1 (−/−) (not shown). We next decided to make binding experiments with [^{3}H]SCH 58261, which is described to selectively recognize A_{2A} receptors both in membrane-binding (Zocchi *et al*., 1996) and autoradiography studies (Fredholm *et al*, 1998), and not to interfere with 'atypical' [^{3}H]CGS 21680 binding sites (Lindström *et al*., 1996). In addition, SCH 58261 discriminates between two binding sites of CGS 21680, recognizing the A_{2A} receptor-like binding site and not interfering with the 'atypical' one (Lindström *et al*., 1996).

Using receptor autoradiography, we first characterized [^{3}H]SCH 58261 binding to the caudate putamen of adenosine A_1 and A_{2A} receptor knockout (−/−) mice and their corresponding control littermates. As expected for a selective adenosine A_{2A} receptor ligand, [^{3}H]SCH 58261 binding to the caudate putamen was halved in A_{2A} (+/−) ($B_{max} = 134.5$ fmol mg^{-1} gray matter, 95% confidence interval: 107.5–161.5, compared with a $B_{max} = 287.9$ fmol mg^{-1} gray matter, 95% confidence interval: 219.4–356.4, in wild type mice) and abolished in adenosine A_{2A} receptor knockout (−/−) mice ($n = 5$–6), whereas the averaged K_D values were similar in A_{2A} (+/+) (0.67 nM, 95% confidence interval: 0.07–1.26 nM) and A_{2A} (+/−) (0.48 nM, 95% confidence interval: 0.09–0.87 nM). Furthermore, [^{3}H]SCH 58261 binding in caudate putamen was nearly unchanged in A_1 receptor knockout (−/−) mice, when compared to their corresponding wild-type (+/+) littermates (not shown). However, in these autoradiographic experiments, we observed a very low binding density of [^{3}H]SCH 58261 to the cerebral cortex (lower than 5 fmol mg^{-1} gray matter) that precluded any reliable analysis of the effect of the different adenosine A_1 and A_{2A} receptor genotypes. To overcome this sensitivity problem in the autoradiography experiments, we carried out saturation curves of [^{3}H]SCH 58261 binding in striatal and cortical membranes from adenosine A_{2A} receptor knockout (−/−) mice and their corresponding wild-type littermates.

As illustrated in Figure 5a, in wild-type (+/+) mice, [^{3}H]SCH 58261 bound to striatal membranes with a K_D of 1.02 nM (95% confidence interval: 0.41–1.63 nM) and a B_{max} of 1079 fmol mg^{-1} protein (95% confidence interval: 856–1301 fmol mg^{-1} protein, $n = 4$), whereas the binding density

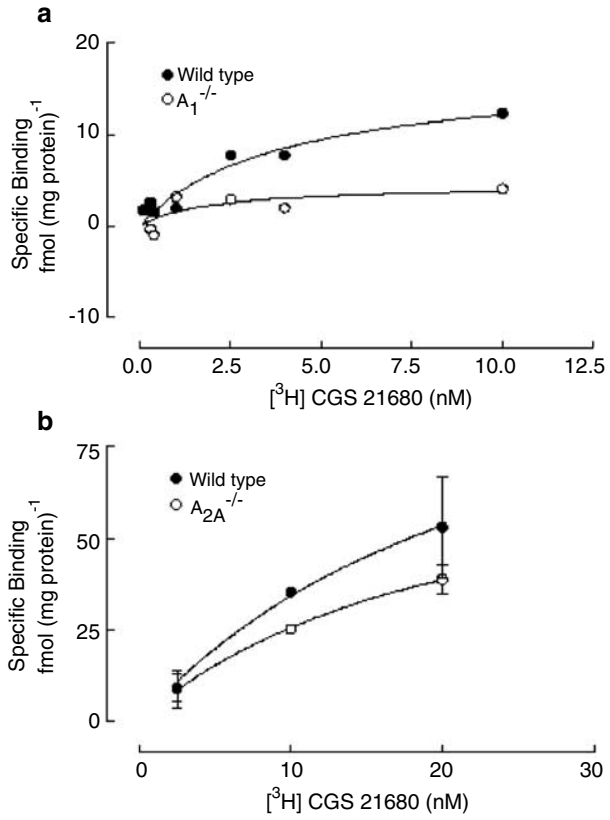

Figure 4 Panel a displays saturation-binding curves of [^{3}H]CGS 21680 binding to membranes from the cerebral cortex of adenosine A_1 receptor knockout mice (open symbols) and corresponding wildtype (filled symbols). The data were obtained from one experiment performed in duplicate. Panel b displays the saturation-binding curve of [^{3}H]CGS 21680 to membranes from the cerebral cortex of adenosine A_{2A} receptor knockout mice (open symbols) and corresponding wild-type (filled symbols) mice. The data are mean ± s.e.m. of two experiments performed in duplicate, which precludes statistical analysis of the results.

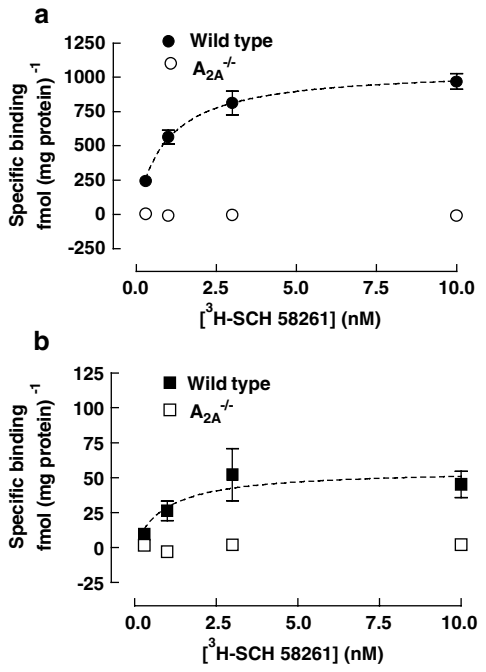

Figure 5 Saturation-binding curves of [^{3}H]SCH 58261 to striatal (a) and cortical (b) membranes of adenosine A_{2A} receptor knockout mice (open symbols) and corresponding wildtype (filled symbols). The ordinates represent the specific binding of [^{3}H]SCH 58261, obtained upon subtraction of the nonspecific binding determined with 1 μM XAC from total binding. Nonspecific binding represented 42–67% of the total binding. The data are mean ± s.e.m. of three to four experiments. Note that the density of binding of [^{3}H]SCH 58261 to striatal membranes is nearly 20-fold greater than that observed in cortical membranes, but for both brain regions [^{3}H]SCH 58261 binding is abolished in A_{2A} receptor knockout mice.

of [^{3}H]SCH 58261 in cortical membranes (Figure 5b) was nearly 20 times lower (B_{max} of 56 fmol mg^{-1}, 95% confidence interval: 10–103 fmol mg^{-1} protein, $n=4$) with a similar affinity (K_D of 1.02 nM, 95% confidence interval: 0.33–1.72 nM, $n=4$). Interestingly, this saturable binding of [^{3}H]SCH 58261 found in the striatum and cerebral cortex was completely abolished in membranes derived from A_{2A} receptor knockout (−/−) mice (Figure 5). This strongly suggests that there are indeed A_{2A} receptors in the cortex as well as in the striatum. The results also confirm that SCH 58261 is a selective ligand for adenosine A_{2A} receptors both in the striatum as well as in the cerebral cortex.

One key issue to validate the conclusion that [^{3}H]CGS 21680 bound to an entity requiring the expression of adenosine A_1 receptors rather than solely to A_{2A} receptors is that the density of A_{2A} receptors in the cerebral cortex is not changed in A_1 receptor knockout (−/−) mice. Since we validated [^{3}H]SCH 58261 as a selective A_{2A} receptor ligand, we compared the binding of [^{3}H]SCH 58261 to cortical membranes of A_1 receptor knockout (−/−) mice and in their corresponding wild type (+/+) littermates. We found no change in the density of [^{3}H]SCH 58261 binding in cortical tissue from the two groups of mice (B_{max} of 45 fmol mg^{-1} protein, 95% confidence interval: 27–62 and 41 fmol mg^{-1} protein, 95% confidence interval: 21–60 fmol mg^{-1} protein in A_1 receptor (+/+) and (−/−) mice, respectively, $n=3$).

Adenosine A_{2A} receptor Western blot analysis in mouse cortical neurons

To further confirm that, even with a low density, adenosine A_{2A} receptors are indeed present in the cortex, we used an immunological approach. We decided to use one of the preparations obtained from brain tissue that displays the lowest level of non-neuronal contaminations, that is, the synaptosomal preparation obtained with the Percoll/sucrose method (Cunha *et al*., 1992). Thus, we investigated by Western blot analysis if the molecular entities recognized by adenosine A_{2A} receptor antibodies in striatal membranes and in membranes from cortical nerve terminals displayed a similar electrophoretic mobility. Figure 6 shows a Western blot, representative of four similar blots carried out in membranes from different animals. It demonstrates the presence of similar bands with a molecular weight of 42 kDa recognized by adenosine A_{2A} receptor antibodies both in the striatum and in cortical nerve terminal membranes. There is a much lower intensity of immunoreactivity in the cortex (note that the amount of protein used was larger in cortical than striatal lanes of the blot shown in Figure 6), and the band is not present in A_{2A} receptor knockout mice (−/−).

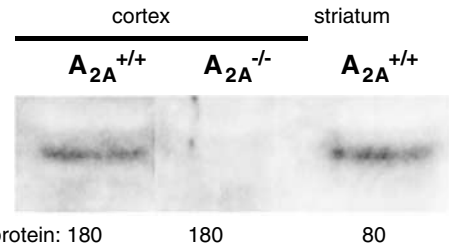

Figure 6 Western blot identifying adenosine A_{2A} receptors in membranes prepared from Percoll-purified nerve terminal membranes prepared from the rat cerebral cortex and from whole membranes of the striatum. The SDS–PAGE gel was loaded with the amount of protein indicated below each lane, and is representative of four similar separations carried out with membranes prepared from different animals.

Discussion

The major conclusion derived from the present study is that, although adenosine A_{2A} receptors are located also in other brain regions than the striatum, the purportedly selective adenosine A_{2A} receptor agonist CGS 21680 binds primarily not to these extrastriatal adenosine A_{2A} receptors, but mainly to a site associated with adenosine A_1 receptors.

The selective adenosine A_{2A} receptor antagonist [^{3}H]SCH 58261 bound to cortical membranes in a manner similar to its binding to striatal membranes, where adenosine A_{2A} receptors were pharmacologically defined (Bruns *et al*., 1986; Jarvis *et al*., 1989; Zocchi *et al*., 1996). Binding of [^{3}H]SCH 58261 was completely abolished in the adenosine A_{2A} receptor knockout mice both in cortical and in striatal membranes, which allows considering SCH 58261 a selective A_{2A} receptor ligand both in the striatum as well as in extrastriatal regions. This confirms previous results showing the existence of adenosine A_{2A} receptor mRNA in cortical tissue both by PCR (Cunha *et al*., 1994; Dixon *et al*., 1996) and by Northern blot analysis (Peterfreund *et al*., 1996), and extend previous observations reporting a specific binding of [^{3}H]SCH 58261 in the cerebral cortex of mice that was attenuated in A_{2A} receptor knockout mice (El Yacoubi *et al*., 2001).

Whereas these results confirm that adenosine A_{2A} receptors are found in cerebral cortex, they do not tell us which cells express them. The cerebral cortex is richly endowed with blood vessels, and, for example, endothelial cells possess adenosine A_{2A} receptors (Feoktistov *et al*., 2002). Furthermore, there is evidence that different glial cells express adenosine A_{2A} receptors (Li *et al*., 2001; Nishizaki *et al*., 2002). The present finding that it was easier to detect [^{3}H]SCH 58261 in homogenates of mouse cortex than in autoradiographic experiments could indicate that the A_{2A} receptors detected in the latter type of experiments are expressed for example, by blood vessels. We do, however, have data that directly support the presence of adenosine A_{2A} receptors in neurons. The finding that adenosine A_{2A} receptor immunoreactivity could be detected in a synaptosomal preparation and that this immunoreactivity is no longer detected in A_{2A} (−/−) mice does provide good evidence for a neuronal localization of at least some adenosine A_{2A} receptors in neurons outside the striatum. This could explain why adenosine A_{2A} receptor antagonists or disruption of the adenosine A_{2A} receptor gene affords neuroprotection (Gao & Phillis, 1994; Jones *et al*., 1998; Monopoli *et al*., 1998; Chen *et al*., 1999; Behan & Stone, 2002, Petroni *et al*., 2002). In particular, the presently observed location of adenosine A_{2A} receptors in nerve terminals of the cerebral cortex provides a molecular support for the proposal that A_{2A} receptors may enhance the release of glutamate in the cerebral cortex (O'Reagan *et al*., 1992; Marchi *et al*., 2002), one of the possible mechanisms to understand neuroprotection afforded by blunting adenosine A_{2A} receptor function (discussed in Fredholm *et al*., 2003).

However, it should be pointed out that the density of these cortical adenosine A_{2A} receptors is considerably lower than the density of striatal adenosine A_{2A} receptors. In fact, the binding density of [^{3}H]SCH 58261 to cortical membranes is about 20 times lower than that of striatal membranes and the immunoreactivity found in cortical nerve terminals is clearly fainter than that found in striatal membranes. This considerably lower abundance of adenosine A_{2A} receptors in the cerebral cortex could have consequences for the ability of endogenous adenosine to activate cortical A_{2A} receptors. It is well known that the efficiency of agonists at A_{2A} receptors is dependent on the level of receptor expression (Svenningsson *et al*., 1999; Arslan *et al*., 2002). There is evidence that the adenosine A_{2A} receptors on striatopallidal GABAergic neurons are tonically activated by endogenous adenosine in the concentration present already under basal conditions (Svenningsson *et al*., 1999; Fredholm *et al*., 2003). By contrast, adenosine A_{2A} receptors in cortex, being much less abundant than in striatum, are likely to be activated only at supraphysiological concentrations, such as those present during hypoxia and ischemia.

The second major conclusion derived from the present study is that CGS 21680, although being a useful adenosine A_{2A} receptor ligand in the striatum, behaves as a nonselective A_{2A} receptor ligand in the cerebral cortex. In fact, in the striatum, the binding of [^{3}H]CGS 21680 is completely abolished in adenosine A_{2A} receptor knockout mice, indicating that in this tissue [^{3}H]CGS 21680 only binds to adenosine A_{2A} receptors. In the cerebral cortex, CGS 21680 also binds to adenosine A_{2A} receptors (see also Cunha *et al*., 1996), as now tentatively concluded by the slight reduction of [^{3}H]CGS 21680 binding (not evaluated statistically) observed in adenosine A_{2A} receptor knockout mice when compared with their respective wild-type littermates (see Figure 4b). However, in the cerebral cortex, CGS 21680 also binds to an entity that is different from adenosine A_{2A} receptors and is essentially dependent on the presence of adenosine A_1 receptors. This is concluded from the marked reduction of [^{3}H]CGS 21680 binding to the cortex of adenosine A_1 receptor knockout mice when compared with their wild-type littermates. This is not likely to be due to a loss of adenosine A_{2A} receptors in A_1 receptor knockout mice, since both the binding of [^{3}H]CGS 21680 to striatal tissue and the binding of [^{3}H]SCH 58261 to cortical membranes were unaffected in A_1 (−/−) mice. Thus, we conclude that [^{3}H]CGS 21680 mostly binds in the cerebral cortex to a molecular entity that requires the presence of adenosine A_1 receptors.

It is important to emphasize that the data so far gathered only allow us to state that [^{3}H]CGS 21680 binds to a molecular entity that requires the A_1 receptor, and does not necessarily imply that [^{3}H]CGS 21680 binds directly to A_1 receptors. In fact, if [^{3}H]CGS 21680 bound to adenosine A_1 receptors, one would expect: (1) that CGS 21680 would bind to

heterologously expressed adenosine A_1 receptors, which was not observed (unpublished observations), and (2) that CGS 21680 would be able to displace the binding of selective adenosine A_1 receptor ligands, and this has not been observed either (Cunha *et al*., 1996; 1997; Lopes *et al*., 2002). Moreover, since adenosine A_1 receptors are also abundant in the striatum (Fastbom *et al*., 1987), one would expect a residual binding of [^{3}H]CGS 21680 in the striatum of adenosine A_{2A} receptor knockout mice and a reduction in striatal [^{3}H]CGS 21680 binding in A_1 receptor knockout mice, neither of which was observed (Figure 2). Thus, one has to conclude that a minor fraction of adenosine A_1 receptors in the cerebral cortex, for some unexplained reason, displays pharmacological properties different from adenosine A_1 receptors in other brain regions, in particular, being able to bind CGS 21680. In fact, several reports have documented the ability of A_1 receptor ligands to displace [^{3}H]CGS 21680 binding to the cerebral cortex (James *et al*., 1992; Johansson *et al*., 1993; Johansson & Fredholm, 1995; Kirk & Richardson, 1995; Cunha *et al*., 1996; Lopes *et al*., 2002). It has been described that cortical A_1 receptors may be found as dimers (Ciruela *et al*., 1995) or dimerize with other receptors (e.g. P_{2Y1}, see Yoshioka *et al*., 2001), and this could confer to them the possibility of binding CGS 21680. It should also be pointed out that adenosine A_1 receptors possess adaptor proteins that control the strength of their coupling to G proteins (Nanoff *et al*., 1995) and it is currently unknown if these adaptor proteins modify the binding profile of adenosine A_1 receptors. Clearly, further studies need to be carried out to explore these possibilities. This effort might not only be of academic interest but may also shed light on physiological processes that have been described as being mediated by 'atypical' A_{2A} receptors in the cortex, namely in terms of behavioral activity (El Yacoubi *et al*., 2000) and in terms of neuroprotection (Latini *et al*., 1999; Tebano *et al*., 2002).

In conclusion, the present paper provides evidence for the presence of adenosine A_{2A} receptors in the cerebral cortex and shows that the prototypical adenosine A_{2A} receptor agonist CGS 21680 is not an adequate tool to characterize these cortical A_{2A} receptors. In fact, although [^{3}H]CGS 21680 also binds to cortical A_{2A} receptors, it mostly binds to an entity that requires the presence of adenosine A_1 receptors. This casts major doubts on the use of CGS 21680 as a selective adenosine A_{2A} receptor agonist in physiological or pharmacological studies, and the effects of CGS 21680 can only be ascribed as being mediated by A_{2A} receptor if data are independent of stimulation or inhibition of adenosine A_1 receptor.

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