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ORIGINAL ARTICLE

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Binding of adenosine receptor ligands to brain of adenosine receptor knock-out mice: evidence that CGS 21680 binds to A_1 receptors in hippocampus

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Abstract The adenosine receptor agonist 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) is generally considered to be a selective adenosine A_{2A} receptor ligand. However, the compound has previously been shown to exhibit binding characteristics that are not compatible with adenosine A_{2A} receptor binding, at least in brain regions other than the striatum. We have examined binding of [^{3}H]CGS 21680 and of antagonist radioligands with high selectivity for adenosine A_1 or A_{2A} receptors to hippocampus and striatum of mice lacking either adenosine A_1 ($A1R^{(-/-)}$) or A_{2A} ($A2AR^{(-/-)}$) receptors. Both receptor autoradiography and membrane binding techniques were used for this purpose and gave similar results. There were no significant changes in the binding of the A_1 receptor antagonist [^{3}H]DPCPX in mice lacking A_{2A} receptors, or in the binding of the A_{2A} receptor antagonists [^{3}H]SCH 58261 and [^{3}H]ZM 241385 in mice lacking A_1 receptors. Furthermore, [^{3}H]CGS 21680 binding in striatum was abolished in the $A2AR^{(-/-)}$, and essentially unaffected in striatum from mice lacking A_1 receptors. In hippocampus, however, binding of [^{3}H]CGS 21680 remained in the $A2AR^{(-/-)}$, whereas binding was virtually abolished in the $A1R^{(-/-)}$. There were no adaptive alterations in A_{2A} receptor expression in this region in $A1R^{(-/-)}$ mice. Thus, most of the [^{3}H]CGS 21680 binding in hippocampus is dependent on the presence of adenosine A_1 receptors, but not on A_{2A} receptors, indicating a novel binding site or novel binding mode.

Keywords Adenosine receptor · Knock-out mice · Striatum · Hippocampus · Receptor autoradiography · CGS 21680

Abbreviations CGS 21680: 2-[*p*-(2-Carboxyethyl) phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine · ZM 241385: 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a] [1,3,5]triazin-5-ylamino]ethyl)phenol · SCH 58261: 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine · DPCPX: 1,3-Dipropyl-8-cyclopentylxanthine · CGS 15943: 9-Chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo-[1,5]quinazolin-5-imine monomethanesulfonate · NECA: 5′-*N*-ethylcarboxamidoadenosine · $A1R^{(-/-)}$: Adenosine A_1 receptor knock-out · $A1R^{(+/-)}$: Adenosine A_1 receptor heterozygote · $A1R^{(+/+)}$: Adenosine A_1 receptor wild-type · $A2AR^{(-/-)}$: Adenosine A_{2A} receptor knock-out · $A2AR^{(+/-)}$: Adenosine A_{2A} receptor heterozygote · $A2AR^{(+/+)}$: Adenosine A_{2A} receptor wild-type · RT-PCR: Reverse transcriptase-polymerase chain reaction

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Introduction

Pharmacological tools are indispensable in the classification of receptors. However, the specificity of these pharmacological tools is often not completely certain. One interesting way of ascertaining the specificity (or lack

thereof) of pharmacological tools is to use mice with a targeted disruption of genes coding for the purported drug target. Here we have used this approach to elucidate some questions that relate to a drug commonly used to study adenosine receptors.

There are four cloned and pharmacologically characterized adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al. 2001). To study the role of A_{2A} receptors, the agonist 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680) has been widely used ever since it was discovered more than 10 years ago (Hutchison et al. 1989; Jarvis et al. 1989). For example, it proved very useful in demonstrating the high density of adenosine A_{2A} receptors in the striatum (Jarvis and Williams 1989; Parkinson and Fredholm 1990). CGS 21680 potently stimulated cAMP formation in striatum but had no significant effect in hippocampus (Lupica et al. 1990; Lopes et al. 1999b). When used at high concentrations, however, it could be shown to inhibit synaptic transmission in hippocampus, suggesting an effect at adenosine A_1 receptors (Lupica et al. 1990). At much lower concentrations, CGS 21680 stimulated the release of acetylcholine from hippocampus (Cunha et al. 1994b, 1995; Jin and Fredholm 1997; Rebola et al. 2002), and increased synaptic transmission modestly, particularly in the presence of an adenosine A_1 receptor ligand (Cunha et al. 1994a). Interestingly, the compound's ability to facilitate hippocampal synaptic transmission in young adult rats was completely dependent on concomitant pharmacological stimulation of adenosine A_1 receptors (Lopes et al. 2002).

Studies on CGS 21680 binding to extrastriatal sites also suggest peculiar properties. Whereas, binding of this agonist to striatum exhibits pharmacology typical for the adenosine A_{2A} receptor, binding to hippocampus shows atypical pharmacology (Johansson et al. 1993; Johansson and Fredholm 1995). Detailed binding studies in the rat show that [^{3}H]CGS 21680 binds to two distinct sites in both striatum and hippocampus (Cunha et al. 1996, 1999). Thus, in striatum more than 80% and in hippocampus less than 20% of the [^{3}H]CGS 21680 binding show typical A_{2A} pharmacology, whereas the remaining sites show high affinity also for adenosine A_1 receptor agonists and antagonists; for example, at these "atypical" binding sites 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) could potently displace [^{3}H]CGS 21680 (Cunha et al. 1996). By contrast, CGS 21680 can only displace a small proportion of [^{3}H]DPCPX bound to hippocampus (Lopes et al. 2002).

These data indicate a possible functional link between adenosine A_1 and A_{2A} receptors in hippocampus. The background for such an interaction is not clear, but the data presented here obtained using mice with targeted deletions of adenosine A_1 ($A1R^{(-/-)}$) and A_{2A} receptors ($A2AR^{(-/-)}$), suggest that a major explanation is that in hippocampus [^{3}H]CGS 21680 interacts not with adenosine A_{2A} but, directly or indirectly, with A_1 receptors.

Materials and methods

Mice We used mice genetically modified to be deficient in different adenosine receptors. Adenosine A_1 receptor deficient mice were on a mixed 129/OlaHsd/C57BL/6 background generated as described (Johansson et al. 2001). Heterozygous animals of the third generation were bred and all experiments were conducted on litter mates genotyped using PCR and/or Southern blot. Adenosine A_{2A} receptor deficient mice, generated as described (Ledent et al. 1997), were on a pure CD1 background. Homozygous animals of the tenth generation were bred to produce offspring with each genotype (controlled using PCR as described; Snell et al. 2000). Animal experimental procedures were approved by Stockholm's Northern animal ethics board (Stockholms norra djurförsöksetiska nämnd), N43/98.

Drugs and chemicals 2-*p*-(2-Carboxyethyl[^{3}H]*N*-phenethylamino-5′-ethylcarboxamidoadenosine ([^{3}H]CGS 21680, specific activity 41–43 Ci/mmol) and 8-cyclopentyl (2,3-[^{3}H]*N*)-1,3-dipropylxanthine ([^{3}H]DPCPX, specific activity 116.0 Ci/mmol) were from DuPont NEN, Stevenage, UK; [^{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) was a kind gift from Dr Ennio Ongini, Schering-Plough, Milan, Italy; (±)N^6-*R*-phenylisopropyladenosine (R-PIA) was from Sigma, Poole, UK; adenosine deaminase (ADA, calf intestine suspension, 200 U/mg, EC 3.5.4.4) from Roche, Germany; 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo-[1,5]quinazolin-5-imine monomethanesulfonate (CGS 15943) was from Research Biochemical International, Natick, MA, USA; 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine (NECA) were from Sigma, Sweden; and 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol ([^{3}H]ZM 241385 specific activity 17 Ci/mmol) was from Tocris, Bristol, UK. All other reagents were of the highest purity available.

Analysis of mRNA with reverse transcriptase-polymerase chain reaction After brief CO_2 anesthesia $A1R^{(+/+)}$, $A1R^{(-/-)}$, and $A2AR^{(-/-)}$ mice were decapitated. The striata and hippocampi were dissected out. Total RNA of each sample was extracted by means of RNeasy RNA extraction kit (Qiagen, Hilden, Germany). RNA was transcribed to cDNA using random hexamers and MuLV reverse transcriptase. Products were thereafter amplified by means of PCR using the adenosine A_{2A} receptor specific primers 5′-CTC CAC CAT GAT GTA CAC CG 3′ and 5′-CAT GGT TTC GGG AGA TGC AG 3′. The cDNA obtained from the $A2AR^{(-/-)}$ served as a negative control. PCR products were analyzed with agarose gel electrophoresis, DNA visualized by ethidium bromide staining and photographed with a video camera.

In another set of experiments, the cDNA was used for semi-quantitative real-time PCR (Heid et al. 1996) in an ABI Prism 7000 Sequence Detector System (Applied

Biosystems). The β-actin and subtype-specific (A_1 and A_{2A}) adenosine receptor primers and probes (MWG-Biotech AG, Ebersberg, Germany) used were synthesized according to sequences previously published (Chunn et al. 2001). The real time PCR reactions were prepared using the TaqMan Universal PCR master mix (Applied Biosystems) and probes labeled at the 5′-end with the reporter dye 6-carboxy-fluorescein (FAM) and at the 3′-end with the quencher dye 6-carboxytetramethyl-rhodamine (TAMRA). The passive reference dye ROX was added to adjust for background variability and for differences in detection sensitivity.

Ligand binding to membranes Groups of between three and five mice of each genotype were killed by decapitation after brief CO_2 anesthesia. The brains were removed and the hippocampi and striata were dissected out at 4°C in sucrose solution (0.32 M) containing 2 mM EGTA, 50 mM Tris–HCl (pH 7.6). Membranes were prepared (Cunha et al. 1996) by homogenization in a Potter-Elvehjem homogenizer followed by centrifugation at 1,000 *g* for 10 min and 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 ADA and incubated for 30 min at 37°C to remove endogenous adenosine. The mixture was then centrifuged at 14,000 *g* for 10 min at 4°C and the pellets resuspended in the incubation solution containing 50 mM Tris–HCl (pH 7.4) and 10 mM $MgCl_2$ (for [^{3}H]CGS 21680 binding experiments) or 2 mM $MgCl_2$ (for [^{3}H]DPCPX binding experiments). The protein concentration was determined using the Bio-Rad protein assay.

In the binding experiments, 280–331 μg of membrane protein was incubated with [^{3}H]CGS 21680 (0.1–20 nM) for 4 h in a final volume of 300 μl in an incubation solution containing 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, and 4 U/ml ADA. Due to the large amount of tissue required, in some of the experiments only 3–5 [^{3}H]CGS 21680 concentration values were tested within the range previously described. Specific binding was determined by subtraction of the non-specific binding, which was measured in the presence of 1 μM CGS 15943. We felt confident in the determination of non-specific binding in these experiments since the results obtained match those previously obtained in rat tissue with high concentrations of different non-selective adenosine receptor ligands (Johansson et al. 1993; Johansson and Fredholm 1995; Cunha et al. 1996, 1999). All binding assays were performed in duplicate. The binding reactions were stopped by vacuum filtration through glass fiber filters (filtermats for receptor binding from Skatron, Molecular Devices, Sunnyvale, CA, USA) using a Skatron 1719 cell harvester and a washing volume of 10 ml at 4°C. The filters were then placed in scintillation vials with 4 ml of scintillation liquid (Ready Safe; Beckman Coultier, Fullerton, CA, USA). Radioactivity was determined after at least 12 h, and the counting efficiency was 55–60%. To ensure a counting error lower than 5% of counts, the samples were counted for 10 min.

The specific binding from saturation experiments was fitted by non-linear regression to a one-site binding graph using the Raphson–Newton method (GraphPad software, San Diego, CA, USA) to determine the binding parameters (dissociation constant, K_d and maximal number of binding sites, B_{max}). Data are mean ± SEM values.

Receptor autoradiography The mice were anesthetized in CO_2 and then decapitated. Brains were immediately dissected out, frozen on dry ice, and thereafter stored at −80°C. Sections of 14 μm thickness were cut in a cryostat +1.42 to +0.62 mm (striatal level), −0.34 to −0.58 mm (globus pallidus level), and −1.70 to −2.18 mm (hippocampus level) from Bregma. Sections were mounted on poly-L-lysine coated slides and stored at −20°C before use.

For detection of adenosine A_1 receptors, slides were incubated with 0.2, 0.5, 1, 2, 5, or 10 nM of the selective receptor antagonist [^{3}H] DPCPX with and without 100 μM guanosine triphosphate (GTP) as described in details elsewhere (Fastbom and Fredholm 1990). For evaluation of non-specific binding, the adenosine analogue R-PIA (20 μM) was added to the incubation solution.

For detection of adenosine A_{2A} receptors, slides were incubated with 0.1, 0.3, 1, 3, or 10 nM of [^{3}H]SCH 58261 (Fredholm et al. 1998) or with 2 nM [^{3}H]ZM 241385 in 50 mM Tris–HCl solution (pH 7.4) containing adenosine deaminase (2 U/ml). For estimation of non-specific binding, the adenosine analogue NECA (50 μM) was added.

[^{3}H]CGS 21680 binding was determined using slides incubated with 0.3, 1, 3, 10, or 30 nM [^{3}H]CGS 21680 as described previously (Johansson and Fredholm 1995). For estimation of non-specific binding, the adenosine receptor agonist 2-chloroadenosine (20 μM) was added to the incubation solution. As previously mentioned, the similarity of the results obtained in autoradiography and membrane binding studies using different non-selective adenosine receptor ligands to define non-specific binding indicates that the ligand chosen here is not affecting the conclusions drawn.

After incubation, dried sections were apposed to ^{3}Hyperfilm (Amersham) together with tritium standards. Optical densities were determined with an MCID M5 system (Imaging Research, St Catharines, ON, Canada) and converted to fmol/mg tissue. Data were analyzed with GraphPad Prism Software (San Diego, CA, USA).

Results

mRNA expression

We first confirmed the lack of adenosine A_{2A} receptor mRNA expression in our A2AR$^{(-/-)}$ mice by reverse transcriptase-polymerase chain reaction (RT-PCR; Fig. 1a). Using semi-quantitative real-time PCR analysis (Fig. 1b), we show that A_{2A} receptors are expressed in hippocampus, in agreement with previous reports (Cunha et al. 1994a; Dixon et al. 1996). However, the expression

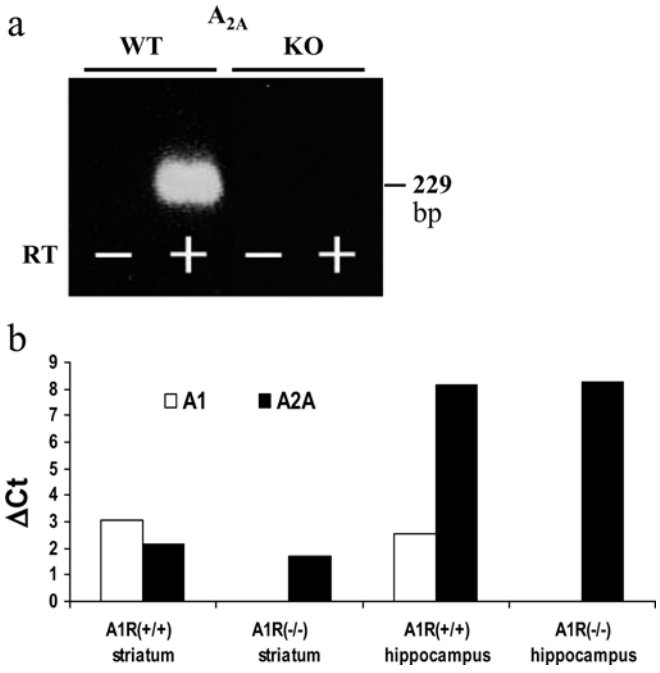

Fig. 1 Adenosine receptor mRNA expression in knock-out mice. **a** Adenosine A_{2A} receptor expression in the striatum in wild-type (*WT*) and adenosine A_{2A} receptor knock-out (*KO*) mice. No A_{2A} receptor message can be detected in the knock-out brain. Representative reverse transcriptase-polymerase chain reaction (*RT-PCR*) data from 1 out of 4 experiments are shown. The experiment was performed in the presence (+) and in the absence (−) of RT. **b** With semi-quantitative real-time PCR the A_1 receptor message appeared to be abundant in both striatum and hippocampus, whereas no signal was detected in the adenosine A_1 receptor knock-out ($A1R^{(-/-)}$). Similar levels of A_{2A} receptor message were detected in hippocampus in both wild-type ($A1R^{(+/+)}$ and $A1R^{(-/-)}$), but at a much lower level than in striatum. The RT-PCR reactions were performed in triplicate. Values are expressed as the difference in the number of cycles needed to reach the detection threshold (Ct = cycle at threshold), using β-actin as reference ($\Delta Ct = Ct_{\text{adenosine receptor}} - Ct_{\beta-\text{actin}}$).

of A_{2A} receptor mRNA in hippocampus was considerably lower than that in striatum in both $A1R^{(+/+)}$ and $A1R^{(-/-)}$ (Fig. 1b). Semi-quantitative real-time PCR analysis of tissue obtained from the striatum of wild-type mice also show that A_{2A} mRNA is somewhat more abundant than A_1 mRNA. In contrast, in the hippocampus A_1 was much more abundant than A_{2A} (the difference between A_1 and A_{2A} mRNA is approximately 50-fold). The A_1 knock-out striatum and hippocampus expressed levels of A_{2A} similar to those found in wild-type tissue. As expected, no adenosine A_1 receptor mRNA signal was found in the $A1R^{(-/-)}$ (Fig. 1b).

Binding of [^{3}H]DPCPX

The first report of the $A1R^{(-/-)}$ mouse showed that [^{3}H]DPCPX binding was essentially eliminated in several brain regions and was reduced to half in heterozygous mice (Johansson et al. 2001). This result was confirmed here as seen in Fig. 2. More importantly, there were no clear-cut effects of knocking out the A_{2A} receptor on [^{3}H]DPCPX binding (see Fig. 2). This is in contrast with a previous report of decreased DPCPX binding in the hippocampus in $A2AR^{(+/-)}$, and increased binding in $A2AR^{(-/-)}$ mice (Snell et al. 2000).

The mice used in the adenosine A_1 and the A_{2A} receptor studies had different genetic backgrounds. Therefore, it is important to note that there were no significant differences in K_d and no major differences in B_{max} in the different mouse strains used here. The ratio of DPCPX binding in presence and absence of GTP, thought to reflect the extracellular adenosine concentration as well as the coupling of the receptor to G proteins (Fastbom and Fredholm 1990), showed no major differences between the A_1 and A_{2A} genotypes used in this study (data not shown). However, the effect could not be estimated in the $A1R^{(-/-)}$ because of the lack of DPCPX binding.

Binding of [^{3}H]SCH 58261 and [^{3}H]ZM 241385

To study the distribution and expression of A_{2A} receptors in the mice, we used both the highly selective antagonist radioligand [^{3}H]SCH 58261 and the somewhat less selective antagonist radioligand [^{3}H]ZM 241385 (Ongini et al. 1999). In accordance with previous autoradiographic studies (Lindström et al. 1996; Fredholm et al. 1998), very little specific binding was observed in any brain region outside the striatum in wild-type mice (Fig. 3). As seen in Fig. 3, the binding to adenosine A_{2A} receptors in caudate putamen and globus pallidus was entirely dependent on the genotype, with no binding being present in $A2AR^{(-/-)}$ mice and half the normal number of binding sites in $A2AR^{(+/-)}$ mice. There were no compensatory changes in [^{3}H]SCH 58261 or [^{3}H]ZM 241385 binding in mice with no adenosine A_1 receptors (Fig. 3).

Binding of [^{3}H]CGS 21680

The agonist radioligand [^{3}H]CGS 21680 also preferentially bound to the striatal regions (Fig. 4), but some weak binding was also seen in the cortex and hippocampus. We confirm the previous finding (Ledent et al. 1997) that binding of [^{3}H]CGS 21680 in the striatum is abolished in the $A2AR^{(-/-)}$ mice. Thus, quantification of the autoradiographic binding experiments showed the expected changes in [^{3}H]CGS 21680 binding in caudate putamen and nucleus accumbens of the $A2AR^{(+/-)}$ and $A2AR^{(-/-)}$ mice (Fig. 5). For example, in the nucleus accumbens the B_{max} was 139 (127–150) fmol/mg (mean and 95% confidence interval [CI] n=3) in the $A2AR^{(+/+)}$ mice, 74 (54–95) fmol/mg (n=5) in the $A2AR^{(+/-)}$ mice, and no binding was detected in the $A2AR^{(-/-)}$ mice (n=4). There were no differences in [^{3}H]CGS 21680 binding in mice with different expression of adenosine A_1 receptors.

We and others have previously demonstrated the existence of specific binding of [^{3}H]CGS 21680 in extrastriatal regions, although the binding density is

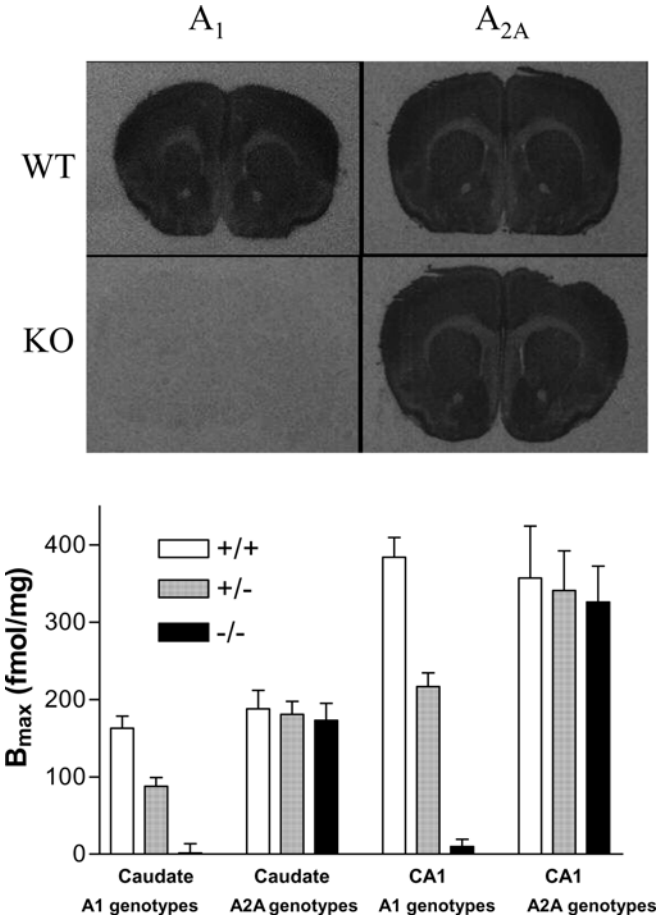

Fig. 2 Binding of [^{3}H]DPCPX to sections of brain from A_1 and A_{2A} receptor knock-out mice and their corresponding wild-types. The *upper four panels* show representative autoradiograms obtained using 10 nM [^{3}H]DPCPX at the level of the striatum in mice. The *lower panel* summarizes the results of binding experiments by plotting the B_{max} values in selected brain regions from experiments using six different concentrations of [^{3}H]DPCPX in 5–6 animals. In the study of $A1R^{(-/-)}$, the B_{max} values were 163 (150–176) fmol/mg and 87 (78–97) fmol/mg gray matter (mean and 95% confidence interval) in the striatum of $A1R^{(+/+)}$ and $A1R^{(+/-)}$ mice. The corresponding K_d values were 0.43 (0.29–0.57) nM and 0.37 (0.20–0.55) nM. No binding was detected in $A1R^{(-/-)}$ mice. The values in CA1 were: B_{max} 384 (360–408) fmol/mg and 218 (204–233) fmol/mg gray matter; K_d 0.49 (0.37–0.63) nM and 0.44 (0.31–0.58) nM. Again, no binding was observed in $A1R^{(-/-)}$ mice. The values in CA3 were: B_{max} 317 (277–358) fmol/mg and 185 (161–209) fmol/mg gray matter; K_d 0.43 (0.19–0.67) nM and 0.32 (0.12–0.52) nM for $A1R^{(+/+)}$ and $A1R^{(+/-)}$ respectively. No binding was observed in $A1R^{(-/-)}$ mice. In the study of $A2AR^{(-/-)}$, the B_{max} values in striatum were: $A2AR^{(+/+)}$ 188 (166–209) fmol/mg; $A2AR^{(+/-)}$ 181 (166–194) fmol/mg; and $A2AR^{(-/-)}$ 173 (155–192) fmol/mg gray matter. The corresponding K_d values were: $A2AR^{(+/+)}$ 0.39 (0.20–0.58) nM; $A2AR^{(+/-)}$ 0.44 (0.29–0.58) nM; $A2AR^{(-/-)}$ 0.38 (0.20–0.56) nM. In CA1 B_{max} values were: $A2AR^{(+/+)}$ 357 (299–416); $A2AR^{(+/-)}$ 341 (298–384); and $A2AR^{(-/-)}$ 325 (286–365). The corresponding K_d values were: $A2AR^{(+/+)}$ 0.47 (0.15–0.79) nM; $A2AR^{(+/-)}$ 0.66 (0.35–0.98) nM; and $A2AR^{(-/-)}$ 0.44 (0.21–0.66) nM. In CA3 B_{max} values were: $A2AR^{(+/+)}$ 332 (279–384); $A2AR^{(+/-)}$ 321 (285–358); and $A2AR^{(-/-)}$ 298 (260–336). The corresponding K_d values were: $A2AR^{(+/+)}$ 0.56 (0.21–0.92) nM; $A2AR^{(+/-)}$ 0.69 (0.39–0.98) nM; and $A2AR^{(-/-)}$ 0.47 (0.22–0.72) nM.

Table 1 Binding of [^{3}H]CGS 21680 to striatal and hippocampal membranes from wild-type ($A1R^{(+/+)}$) and A_1 receptor knock-out mice ($A1R^{(-/-)}$). Results are given as B_{max} (fmol/mg protein; mean ± SEM, n=1). The corresponding K_d values were, in the striatum, 1.99 ±0.86 nM for $A1R^{(+/+)}$s and for $A1R^{(-/-)}K_d$ was kept constant at 1.99 nM; in the hippocampus, 10.98±8.59 nM and 0.95±1.69 nM for $A1R^{(+/+)}$ and $A1R^{(-/-)}$ respectively.

Tissue	$A1R^{(+/+)}$	$A1R^{(-/-)}$
Striatum	171±9	196±11
Hippocampus	59±22	−6±2

considerably lower than that in the basal ganglia (Wan et al. 1990; Johansson et al. 1993; Johansson and Fredholm 1995; Kirk and Richardson 1995; Cunha et al. 1996, 1999). This low abundance binding site is likely to represent binding of [^{3}H]CGS 21680 to adenosine receptor (s) since it is displaced by several adenosine receptor ligands, albeit with a profile that resembles more to A_1 than to A_{2A} receptors (Johansson et al. 1993; Johansson and Fredholm 1995; Kirk and Richardson 1995; Cunha et al. 1996, 1999). Since pharmacological characterization of [^{3}H]CGS 21680 binding with displacement curves failed to provide a clear picture, we hoped that the use of A_1 and A_{2A} receptor knock-out mice would provide a clear answer to whether CGS 21680 binds to A_1 or to A_{2A} receptors in the hippocampus. Indeed, our autoradiographic studies showed that, in contrast to the basal ganglia, [^{3}H]CGS 21680 binding in the CA1 region of the hippocampus was not affected by differences in the A_{2A} receptor genotype. Instead, there were marked differences depending on the adenosine A_1 receptor genotype (Figs. 4, 5). Binding to this hippocampal region was essentially abolished in $A1R^{(-/-)}$ mice and reduced to half in $A1R^{(+/-)}$ mice (Fig. 5). This finding was confirmed by binding experiments using membranes prepared from striatum or hippocampus of $A1R^{(+/+)}$ and $A1R^{(-/-)}$ mice (Table 1). In the striatum, [^{3}H]CGS 21680 bound with high affinity (K_d approximately 2 nM), and binding was not significantly different between the two genotypes. By contrast, in hippocampal membranes from $A1R^{(+/+)}$ mice, K_d was at least 10 times higher and in $A1R^{(-/-)}$ mice no binding at all could be detected.

Discussion

The major finding of the present study is that the hippocampal binding of the purportedly A_{2A} receptor selective agonist [^{3}H]CGS 21680 is lost in animals that do not express adenosine A_1 receptors, despite the fact that there is no indication of adenosine A_{2A} receptor alterations in such animals. Furthermore, in this area [^{3}H]CGS 21680 binding instead remains in animals lacking adenosine A_{2A} receptors. These findings imply that, in some parts of the brain, CGS 21680 binds, and acts, on targets other than adenosine A_{2A} receptors.

It has been repeatedly shown using many different methods that adenosine A_{2A} receptors are highly enriched

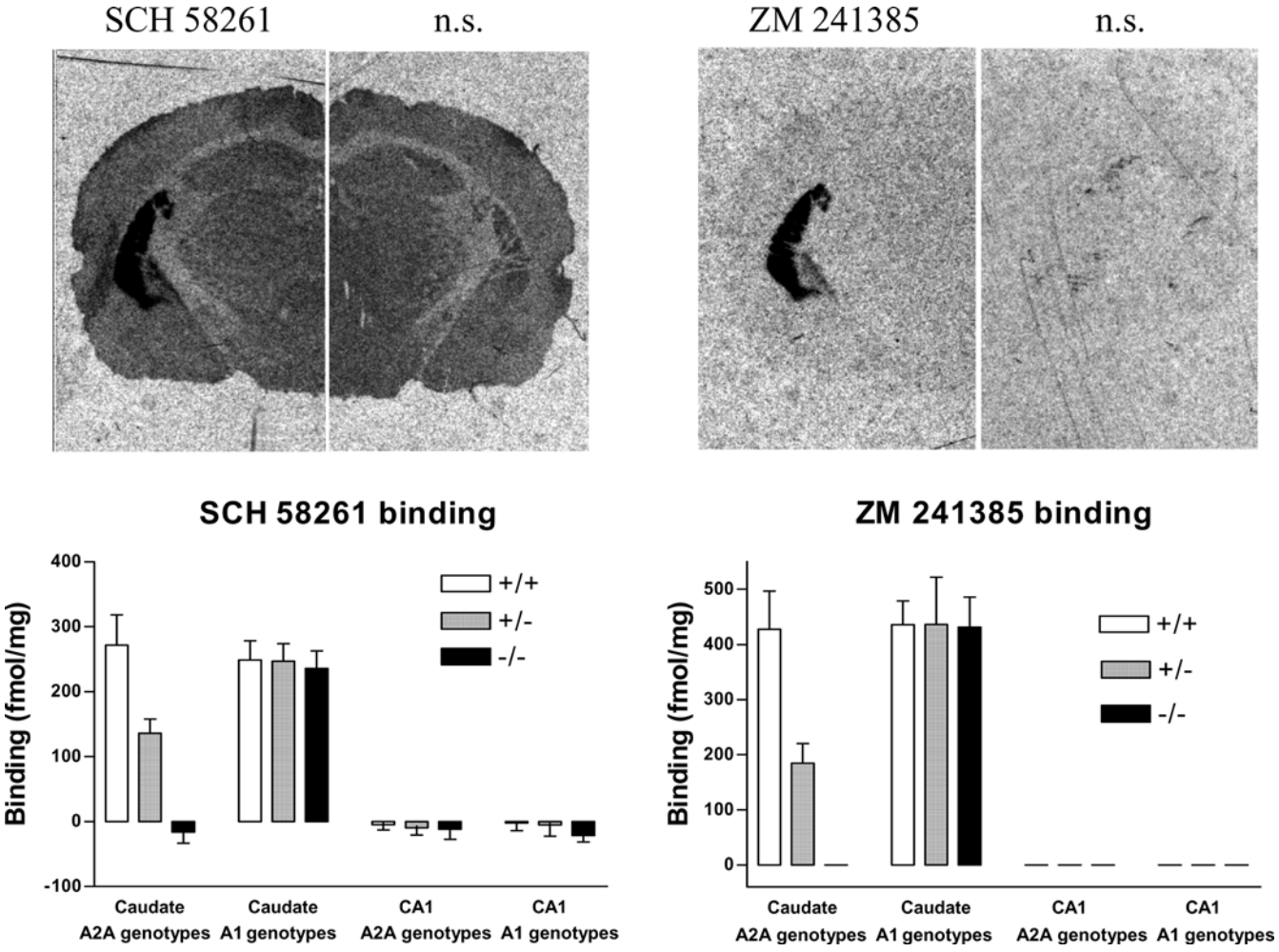

Fig. 3 Binding of adenosine A_{2A} receptor antagonist radioligands to brains from $A1R^{(+/+)}$, $A1R^{(+/-)}$, $A1R^{(-/-)}$, $A2AR^{(+/+)}$, $A2AR^{(+/-)}$, and $A2AR^{(-/-)}$ mice (n=3–6). Autoradiographic experiments were performed as described under Materials and methods. Results are expressed as binding at a concentration of 2 nM ligand (mean ± SEM). In the *upper panels*, autoradiograms with SCH 58261 and ZM 241385 binding in a wild-type animal are shown. The non-specific binding is shown to the right in the autoradiograms. The *lower left panel* summarizes the results of [^{3}H]SCH 58261 binding to sections. There are no significant differences in binding in the caudate between $A1R^{(+/+)}$, $A1R^{(+/-)}$, and $A1R^{(-/-)}$ mice. The $A2AR^{(-/-)}$ mice did not show detectable specific binding of [^{3}H] SCH 58261 in the caudate. The $A2AR^{(+/-)}$ showed an approximately 50% reduction in binding (caudate: 134.5 fmol/mg gray matter, 95% confidence interval (CI) 107.5–161.5) compared with the $A2AR^{(+/+)}$ mice (caudate: 287.9 fmol/mg gray matter, CI 219.4–356.4). However, the binding in CA1 did not significantly differ from zero in any genotype. (The reason for the negative values is that non-specific binding with SCH 58261 is high.) The *lower right panel* shows similar results using [^{3}H]ZM 241385 as the radioligand. In the $A2AR^{(+/-)}$, binding in the caudate was reduced to approximately half compared with wild-type animals. There was no detectable binding in $A2AR^{(-/-)}$ mice (in fact the sections could not be distinguished from the background) and therefore the values are zero. No [^{3}H]ZM 241385 binding was detected in the hippocampus in any genotype. *n.s.* non-specific binding

in caudate putamen, nucleus accumbens and tuberculum olfactorium, and that expression levels elsewhere are low (Jarvis and Williams 1989; Parkinson and Fredholm 1990; Cunha et al. 1994a; Dixon et al. 1996). Here we show that there are no major changes in the density of adenosine A_{2A} receptors, measured with three different radioligands including [^{3}H]CGS 21680, in the areas of high expression (i.e., basal ganglia), in mice that have a targeted deletion of the adenosine A_1 receptor. This implies that the expression of A_{2A} receptors is not critically dependent upon the presence or absence of adenosine A_1 receptors. This is in accordance with the observation that the function of A_{2A} receptors does not seem to be markedly affected by alterations in A_1 receptors: caffeine actions on behavior that are entirely dependent on blockade of A_{2A} receptors in striatum (Ledent et al. 1997; El Yacoubi et al. 2000) are only marginally affected in animals that lack adenosine A_1 receptors (Halldner et al. 2004).

On the other hand purportedly A_{2A} receptor-mediated actions in areas with low levels of A_{2A} receptor expression have been reported to be dependent on adenosine A_1 receptors (O'Kane and Stone 1998; Lopes et al. 1999a, 2002; Cunha and Ribeiro 2000). For example, the effect of CGS 21680 on glutamatergic transmission in hippocampus of young adult rats appears to be strongly dependent on the activity at adenosine A_1 receptors (Lopes et al. 1999a, 2002). Furthermore, CGS 21680 attenuates the neuronal inhibition achieved by an adenosine A_1 receptor

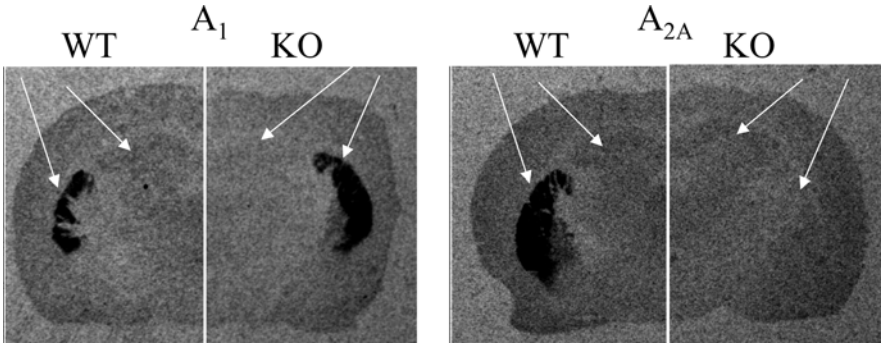

Fig. 4 Typical autoradiograms at a concentration of 30 nM [^{3}H] CGS 21680 with wild-types on the *left* and knock-outs on the *right*. Sections are at a level where the posterior part of the caudate putamen together with globus pallidus can be seen as well as the most anterior part of the hippocampus (*arrows*); the heavier the labeling the darker the image. It is readily seen that the labeling in caudate and in globus pallidus is much stronger than that seen elsewhere, and that this difference is lost in the A2AR$^{(-/-)}$ mice. Note that it is also easier to discern the contours of the hippocampus in the slices that possess adenosine A_1 receptors.

agonist in the hippocampus (Cunha et al. 1994a; O'Kane and Stone 1998). This was taken as evidence for interactions between the two receptor subtypes. The present finding that [^{3}H]CGS 21680 binding in hippocampus is virtually lost in mice lacking A_1 receptors provides an alternative explanation; agents that are presumed to act solely on A_{2A} receptors may in fact also interact with A_1 receptors. The functional antagonistic interaction between A_1 receptor agonists and CGS 21680 that has been repeatedly observed could then be explained if CGS 21680 competes with the binding of A_1 agonists or antagonists. However, CGS 21680 was originally developed as a selective agonist precisely because of its lack of interference, at reasonably low concentrations, with the binding of A_1 agonists or antagonists. Indeed, the previously mentioned study (Lopes et al. 2002) confirmed that CGS 21680 does not interfere significantly with the binding of the A_1 agonist CPA. It was also shown that under conditions favoring CGS 21680 binding (high magnesium) CGS 21680 displaces DPCPX binding only at high concentrations (IC_{50} values approximately 1 μM) in most brain regions, supporting a low affinity to the adenosine A_1 receptor. In contrast, in the same study, 30% of the DPCPX displacement in the CA1 region had an IC_{50}

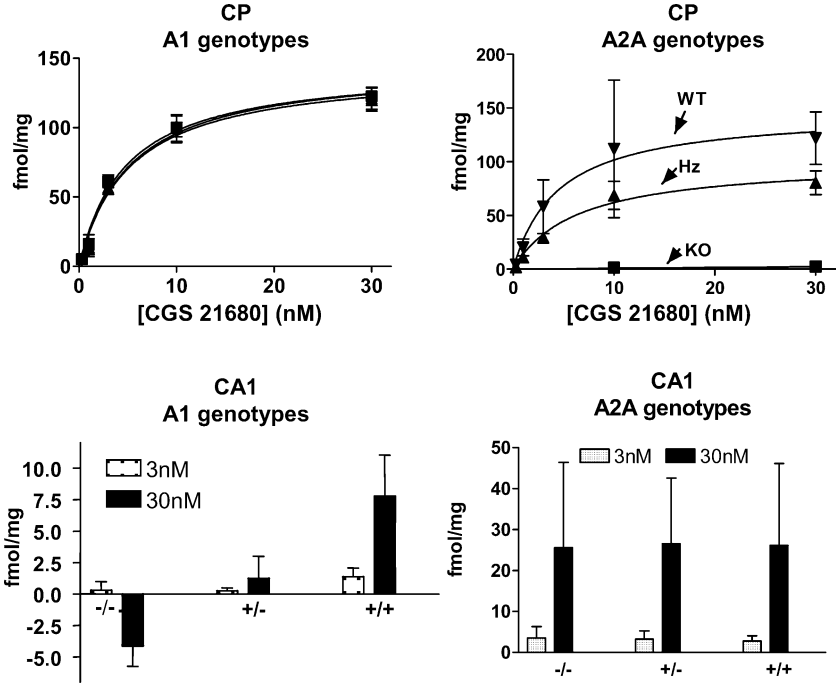

Fig. 5 Binding of [^{3}H]CGS 21680 to brains from A1R$^{(+/+)}$, A1R$^{(+/-)}$, A1R$^{(-/-)}$, A2AR$^{(+/+)}$, A2AR$^{(+/-)}$, and A2AR$^{(-/-)}$ mice (n=5–6). The *two top panels* show the results of saturation binding to caudate putamen (*CP*) in sections. It is readily seen that the binding of [^{3}H]CGS 21680 in this region is dependent on A_{2A} receptor genotype. In the *two lower panels*, it can be seen that this is not the case for the binding of [^{3}H]CGS 21680 to the hippocampus. As binding did not saturate within the concentration range studied (and because non-specific binding became intolerably high at still higher concentrations) results from two concentrations (3 nM and 30 nM) of [^{3}H]CGS 21680 are shown. It is seen that the binding is strongly affected by the presence or absence of adenosine A_1 receptors, but not of adenosine A_{2A} receptors. *Hz* heterozygotes

value of 44 nM. This is interesting in view of the fact that in the same region most of the CGS 21680 binding can be displaced by DPCPX in low nanomolar concentrations (Johansson et al. 1993; Cunha et al. 1996).

Considering that high affinity interactions between [^{3}H]CGS 21680 and the adenosine A_1 receptor are not seen in all locations (for example, no binding could be detected in the A2AR$^{(-/-)}$ striatum), it seems reasonable to assume that such interactions, while dependent on A_1 receptors, also require some other protein. Given that most of the [^{3}H]CGS 21680 binding to hippocampus was unaffected by eliminating A_{2A} receptors by genetic means or by blocking the receptors with antagonists we cannot explain our data based on adenosine A_1/A_{2A} receptor heteromers. Since the binding site we are studying is defined using adenosine-like compounds and since the binding site shows a pharmacology resembling an adenosine receptor, the most parsimonious explanation is that this site represents a particular conformation of the A_1 receptor.

Whereas, the present results show that many of the binding sites for [^{3}H]CGS 21680 are not A_{2A} receptors, our results certainly do not question the presence of effects mediated by A_{2A} receptors outside the striatum. In fact, there is evidence for A_{2A} receptor mRNA in hippocampal CA1 and CA3 pyramidal cells (Lopes et al. 2001) as well as A_{2A} receptor immunoreactivity, binding sites for [^{3}H]SCH 58261 and functional receptors in hippocampal nerve terminals (Rebola et al. 2002, 2003). Also glial cells appear to express some A_{2A} receptors (Kust et al. 1999; Hettinger et al. 2001), and functional adenosine A_{2A} receptors have been found in astrocytes derived from the rat hippocampus (Li 2001). Moreover, there are blood vessels in the hippocampus, and like blood vessels elsewhere (Ngai 2001; Lynge 2000) they express some A_{2A} receptors.

Both the mRNA (Fig. 1b) and the adenosine A_{2A} receptor protein (e.g., Rebola et al. 2003) are detected in the hippocampus, although the receptor protein is expressed at a concentration below the detection level for the binding methods used here. Nevertheless, the results obtained here with adenosine A_1 and A_{2A} receptor knock-outs indicate that the vast majority of the sites with which [^{3}H]CGS 21680 interacts in the hippocampus are not A_{2A} receptors. Consequently, some of the literature implicating A_{2A} receptors in extrastriatal regions of the brain that is predominantly based on studies with CGS 21680 may need to be reconsidered.

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