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Characterization of the rat A_{2A} adenosine receptor gene: a 4.8-kb promoter-proximal DNA fragment confers selective expression in the central nervous system

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Abstract

We isolated and characterized a 4.8-kb 5′ flanking region of the rat A_{2A} adenosine receptor (A_{2A}-R) gene in the present study. Promoter activity was observed with this DNA fragment in PC12 cells and C6 cells which contain endogenous A_{2A}-Rs. A fusion fragment consisting of the 4.8-kb promoter-proximal DNA fragment of the A_{2A}-R gene, and the coding region of lacZ was utilized to produce mice harbouring the fusion gene. In three independent founder lines, proteins and transcripts of the transgene were found in many areas of the central nervous system (CNS), but not in three peripheral tissues examined. Double immunohistochemical analyses revealed that the transgene was coexpressed with endogenous A_{2A}-R and proper neuronal markers in the brain. Specifically, the transgene in the striatum was found in the enkephalin-containing GABAergic neurons and in the cholinergic neurons as was found for the endogenous A_{2A}-R. However, a selectively enriched striatal expression of the transgene was not found as was observed for the endogenous A_{2A}-R. Collectively, the 4.8-kb promoter-proximal DNA fragment of the rat A_{2A}-R gene contains important element(s) to direct its expression in the CNS where functional A_{2A}-R are found, but were not sufficient to confer the highly concentrated expression of the striatal A_{2A}-R. Furthermore, expressions of A_{2A}-R and the transgene were found in both neurons and astrocytes, suggesting that adenosine might mediate its function through A_{2A}-R in both cell types.

Introduction

Adenosine is an important neuromodulator which regulates many physiological functions (such as modulation of transmitter release, vasodilatation and renal autoregulation) via four distinct adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3; Marshall, 2000; Klinger *et al*., 2002; Welch, 2002). We previously cloned the cDNA and the gene of the rat A_{2A} adenosine receptor (A_{2A}-R), which contains seven transmembrane domains and belongs to the G protein-coupled receptor family (Chern *et al*., 1992; Chu *et al*., 1996; Lee *et al*., 1999). Stimulation of the A_{2A}-R results in activation of adenylyl cyclase (AC) and protein kinase C (Lai *et al*., 1997; Lai *et al*., 1999). In the central nervous system (CNS), expression of A_{2A}-R is transiently regulated in various areas (e.g. the striatum, cortex and hippocampus) in the developing rat brain (Weaver, 1993), suggesting that adenosine may play an important role in brain development by interacting, at least partly, with transiently expressed A_{2A}-R. Soon after neurogenesis, the transcript of the rat A_{2A}-R gene is heavily expressed by striatal neurons (Weaver, 1993) and colocalizes with the D2 dopamine receptor (D2-R) in GABAergic striatopallidal neurons (Ferre *et al*., 1993). In addition to the intense expression in the striatum, A_{2A}-R was expressed at low levels in many regions (e.g. the cortex and hippocampus) of the brain and has been implicated in the regulation of specific functions (e.g. neuronal protection and modulation of synaptic transmission; Jones *et al*., 1998; Chen *et al*., 1999; Lee *et al*., 1999; Cunha & Ribeiro, 2000). Many peripheral tissues, including heart, lung and platelets, also express the A_{2A}-R (Ledent *et al*., 1997; Lee *et al*., 1999). Collectively, tight control of A_{2A}-R gene expression is very important for neuronal development, basal ganglia activity and many other peripheral functions.

We previously characterized the rat A_{2A}-R gene (Chu *et al*., 1996) and demonstrated that this gene is composed of at least two exons and encodes two clusters of alternative transcripts initiated from two independent promoters. The major difference in the transcripts from these two alternative promoters is the length of their 5′-untranslated regions (5′UTR), which suppress A_{2A}-R gene expression at the translational level (Lee *et al*., 1999). Moreover, we reported recently that nuclear factor 1 (NF1) is an important transcriptional modulator of rat A_{2A}-R gene (Lee *et al*., 2003). In the present study, we further characterize the A_{2A}-R gene using a combination of cell cultures and transgenic mouse approaches. We present data to demonstrate that the 4.8-kb promoter-proximal DNA fragment (consisting of a 4.3-kb 5′-flanking region and the 0.5-kb 5′UTR) of the rat A_{2A}-R gene contains

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important *cis* element(s) to direct the expression of transgene in many brain areas where functional A_{2A}-R is expressed. Most importantly, these results reveal, for the first time, the existence of A_{2A}-R in astrocytes. This finding is of particular interest because functions of A_{2A}-R in the CNS might be mediated by not only neurons but also by astrocytes. The cell type(s) targeted by A_{2A}-R-related drugs in the CNS therefore might need further examination.

Materials and methods

Polymerase chain reaction (PCR) and DNA walking of the rat A_{2A}-R gene

The 5′-flanking region of the rat A_{2A}-R gene was obtained by a combination of nested PCR and DNA walking techniques. DNA walking was carried out following the manufacturer's protocol (PromoterFinder™; Clontech, Palo Alto, CA, USA). The A_{2A}-R-specific primer used for the first PCR reactions is as follows: 5′-ATCCACACGGCCCATCACACGAGCA-3′ (corresponding to the rat A_{2A}-R gene from bases +89 to +65, with +1 as the translational start site; Chu et al., 1996). The PCR reaction was first conducted at high temperature (94 °C, 25 s; 72 °C, 10 min) for seven cycles, and then at low temperature for another 32 cycles (94 °C, 25 s; 67 °C, 10 min). Products of the first PCR reaction were used as templates for the second PCR reaction. The A_{2A}-R-specific primers used for the second PCR reaction are as follows: 5′-AGCTCCCCGAAGACGTTCTCACAGA-3′ (corresponding to rat A_{2A}-R from bases −141 to −165), or 5′-GGTGGGAGCAGCC-ACACTTGGGCAG-3′, corresponding to rat A_{2A}-R from bases −1 to −25). The second PCR reaction was initially conducted at high temperature (94 °C, 25 s; 72 °C, 10 min) for five cycles, and then at low temperature for another 22 cycles (94 °C, 25 s; 67 °C, 10 min). DNA fragments amplified from the Sca I-library were inserted into the Sma I site of the pGL2-basic plasmid (Promega, Madison, WI, USA), which contains firefly luciferase (F-LUC) as a reporter gene, and are designated pGL2-(−4785/−141) or pGL2-(−4785/−1). The nucleotide sequence (GenBank accession no. AF107208; Fig. 1A) of this 4.8-kb DNA fragment was verified using constructs created from at least two independent PCRs.

Cell culture

PC12 cells were originally obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Gibco) plus 10% horse serum (Gibco) in an incubation chamber gassed with 10% CO_2−90% air at 37 °C. Rat C6 glioma cells were kindly provided by Dr K. King (Academia Sinica, Taiwan), and were grown in DMEM supplemented with 10% fetal bovine serum in 10% CO_2−90% air.

Transfection and reporter assay

Cells (7.5×10^5 per 35-mm well) were transfected using lipofectamine™ 2000 (Invitrogen, Taipei, Taiwan) following the manufacturer's protocol. Briefly, 3 μL of the lipofectamine™ 2000 reagent was diluted into 0.5 mL of serum-free medium, then added to 70 pmol of the desired F-LUC reporter gene constructs mixed with 10 pmol of the pRL-TK construct containing *Renilla* luciferase (R-LUC) as the

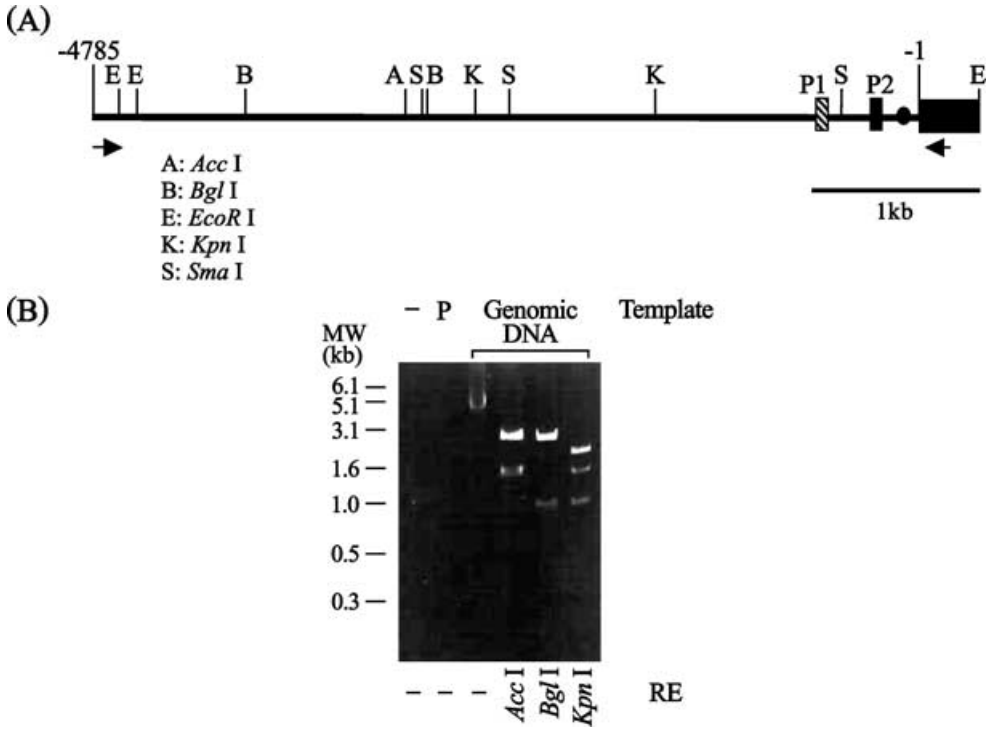

FIG. 1. Isolation and characterization of the 5′-flanking region of the A_{2A}-R gene which exhibits promoter activity in PC12 cells. (A) Mapping of the 4.8-kb promoter-proximal DNA fragment of the A_{2A}-R gene based on information from cDNA and genomic clones. The approximate location of the coding region is indicated by a closed box with +1 as the translational start site (Chu *et al.*, 1996). The 4.8-kb promoter-proximal DNA fragment (−4785/−1) is located immediately upstream of the translational start site. Abbreviations for restriction enzymes sites are: A, *Acc* I; B, *Bgl* I; E, *EcoR* I; K, *Kpn* I; S, *Sma I*. (B) A DNA fragment amplified from rat genomic DNA using two A_{2A}-R-specific primers (5′-GCTAGACCTTCCCCTCCCGTGG-GTT-3′ and 5′-ATCCACACGGCCCATCACA-CGAGCA-3′, corresponding to rat A_{2A}-R from bases −4776 to −4752 and +89 to +65, respectively). Digestion of the resultant DNA fragment by *Acc* I, *Bgl* I, or *Kpn* I produced correct DNA fragments as predicted (3 and 1.8 kb; 2.8, 1.05 and 0.95 kb; 2.2, 1.6 and 1 kb, respectively). Arrows in A indicate the positions of the primers used in the PCR reaction. Two negative control reactions ('−' and 'P') using no template or pGL2-(−4785/−1) as the template, respectively, produced no product as predicted.

reporter gene (Promega). The pRL-TK was used as an internal control. Forty-eight hours after transfection, the cells were lysed and the luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocol. The light produced was measured with a TD-20/20 Luminometer (Promega). Results were obtained using at least two different preparations of plasmids.

Preparation of A_{2A}-R promoter-n-lacZ transgenic mice

To produce the A_{2A}-R promoter-n-lacZ fusion reporter gene [(−4785/−1)-n-lacZ] construct, the DNA fragment of the rat A_{2A}-R gene (−4785/−1) was subcloned into the *Sph* I site of a pBluescript II-KS-n-gal vector (a generous gift from Dr Tang, IBMS, Academia Sinica). The A_{2A}-R promoter-n-lacZ reporter fragment was excised from the vector sequences by digestion with *Xho* I plus *Hind* III, and then injected into FVB mouse oocytes (Hogan *et al*., 1994) at the Transgenic Core Facility at the Institute of Molecular Biology, Academia Sinica and the Level Transgenic Center (Taipei, Taiwan). Transgenic founder lines were identified by the PCR genotyping technique of genomic DNA extracted from tail tissues (Hogan *et al*., 1994) using primers located in the transgene (5′-AGGGTAAGAA-TGGTTACTTGCCACTTGCGGCCA-3′ and 5′-CGACCTTACGCT-TCTTCTTTGG-3′). Transgenic mice were further identified using the Southern blotting technique. Mouse genomic DNA (8 μg) from tails was digested with *Apa* I and subjected to Southern blot analysis by hybridization with a ^{32}P-labelled DNA fragment of the rat A_{2A}-R gene (−2283 to −1387, with +1 as the translational start site) as a probe. Existence of the transgene (881 bp) was visualized by autoradiography. Due to the high sequence homology between the rat and mouse A_{2A}-R genes, a larger faint band (≈ 1.2 kb) resulting from the mouse A_{2A}-R gene could also be detected by this DNA probe. The copy number of the integrated transgene was estimated based on a comparison of the intensity of the transgene band with that of the signal of the endogenous mouse A_{2A}-R gene obtained from mouse genomic DNA on the same Southern blot. The copy numbers of the integrated transgene for the transgenic lines #C and #B4 were 4 and 10, respectively (data not shown). The identified founder lines were mated to FVB mice to establish transgenic lines. The F0–F4 generations of the A_{2A}-R promoter-n-lacZ heterozygous littermates and wild-type littermates were analysed for β-galactosidase expression. Animals were housed at the Institute of Biomedical Sciences Animal Care Facility under a 12-h light–dark cycle. Animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee of IBMS, Academia Sinica.

Brain tissue preparation

Animals were deeply anaesthetized with sodium pentobarbital (50 mg/kg) and intracardialy perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains and spinal cords were carefully removed, postfixed with 4% paraformaldehyde in 0.1 M PB for 2–5 h, and then immersed in 30% glycerol in 0.1 M PB. Tissues were cut at 20 μm on a freezing microtome (CM3050, Leica Microsystems Nussloch GmbH, Nussloch, Germany).

Adenylyl cyclase assay

AC activity was assayed as previously described (Chern *et al*., 1993). Briefly, tissues were sonicated using a W-380 sonicator (Ultrasonics, Farmingdale, NY, USA) at a setting of 20% power output for a total of 90 s. The homogenate was centrifuged at 50 000 ***g*** for 30 min to collect the P1 membrane fractions. The AC activity assay was performed at 37 °C for 10 min in a 400-μL reaction mixture containing (in mM) ATP, 1; NaCl, 100; Hepes, 50; $MgCl_2$, 6; and EGTA, 0.2; with GTP, 1 μM; rolipram, 100 μM; and membrane protein, 10 μg. Reactions were stopped by addition of 0.6 mL of 10% TCA. The cAMP formed was isolated by Dowex chromatography (Sigma Chem., St Louis, MO, USA) and assayed by radioimmunoassay. The enzyme activity was linear for up to 30 min with membrane proteins up to 40 μg.

β-Galactosidase detection

To determine the expression of β-galactosidase in the transgenic animals, tissues were first homogenized using a douncer in a reporter lysis buffer (Promega), and then sonicated using a sonicator (Ultrasonic Inc., Farmingdale, NY, USA) at a setting of 30% output power for a total of 2 min on ice. After low-speed centrifugation (1000 ***g***, 10 min), the supernatant was collected to determine the activity of β-galactosidase as described elsewhere (Nielsen *et al*., 1983) using o-nitrophenyl-β-D-galactopyranoside as the substrate. The product was detected by measuring its absorbance at 420 nm using a spectrophotometer. The β-galactosidase activity was linear with time for OD_{420} up to 1. To ensure that the relative β-galactosidase activity was determined in the linear range of the assay, 10–100 μg of protein was assayed in triplicate reactions.

For β-galactosidase histochemistry, tissue sections were rinsed twice with PBS and stained with X-gal for 2–40 h in staining buffer (X-gal, 1 mg/mL; $MgCl_2$, 1.3 mM; potassium ferricyanide, 3 mM; potassium ferrocyanide, 3 mM; sodium deoxycholate, 0.01%; and NP-40, 0.02% in 0.1 M sodium phosphate buffer, pH 7.3). Sections were then rinsed with PBS and counterstained with eosin.

In situ *hybridization*

The regional distribution of the A_{2A}-R transcripts was determined using the *in situ* hybridization technique as previously described (Lin *et al*., 1998). The DNA fragment containing −221 to +1 bp of the A_{2A}-R gene was subcloned into a pGEM-7 vector (Promega). RNA probes were transcribed by T7 or SP6 RNA polymerase in the presence of ^{33}P-UTP (2000–4000 Ci/mmole; Amersham) using an *in vitro* transcription kit following the manufacturer's protocol (Promega). Hybridization was performed in a solution containing 10^7 cpm/mL of probes at 55 °C overnight, followed by standard washing procedures. Brain sections were exposed to BioMax-MR (Kodak) and autoradiographs were analysed with Adobe PhotoShop 4.0 (Adobe).

Preparation and characterization of the anti-A_{2A}-R antibody

An oligopeptide corresponding to the hypervariable C terminal region (amino acids 394–410) of the rat A_{2A}-R was conjugated to bovine serum albumin (BSA) using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), and injected into male New Zealand white rabbits to raise the polyclonal anti-A_{2A}-R antibody (Harlow & Lane, 1988). The resultant antibody was purified using an affinity column of the antigen peptide conjugated to SulfoLinked beads through the cysteine residue (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's protocol, and was designated A_{2A}-R_C. To characterize the specificity of A_{2A}-R_C, the antigen peptide was also conjugated to an irrelavent protein, ovalbumin, using MBS as described above. For Western blot analyses, we used a 1 : 1000 dilution for the anti A_{2A}-R_C antiserum following the standard protocol as described elsewhere (Chern *et al*., 1993).

Immunohistochemistry and quantification

Single antigen immunostaining was carried out by the avidin–biotin–peroxidase complex (ABC) method as previously described (Liu *et al*., 1998). In general, we used a 1 : 1000 dilution for the anti A_{2A}-R_C antiserum. Controls for the specificity of A_{2A}-R_C were performed by

incubating sections with the A_{2A}-R_C preadsorbed with the peptide antigen conjugated to an irrelevant protein (ovalbumin). A_{2A}-R immunostaining was completely abolished by the preadsorption treatment. Adsorption with the same amount of ovalbumin alone did not affect the immunostaining signal.

Double immunofluorescence staining was carried out as previously described (Liu *et al*., 1998). Sections were incubated at 4 °C for 36–48 h in a mixture of two different primary antibodies of different species, and were then incubated in a mixture of secondary antibodies containing 0.1% normal goat serum with either 1 : 500 fluorescein isothicyanate (FITC)-conjugated goat antirabbit IgG (Cappel™ Res., Durham, NC, USA) and 1 : 500 rhodamine-conjugated goat antimouse IgG (Cappel™ Res.) for 2 h, or 1 : 500 Texas Red-conjugated goat antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and 1 : 500 DTAF-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratory) for 2 h, followed by standard washing procedures. The pattern of double immunostaining was studied with the aid of a laser confocal microscope (Bio-Rad, MRC-1000; Hercules, CA, USA). Elimination of the primary antibody resulted in a loss of immunofluorescence staining. The monoclonal antiβ-galactosidase antibody (1 : 10 000; Boehringer Mannheim Biochemica, Mannheim, Germany), the polyclonal antiβ-galactosidase antibody (1 : 10 000; Chemicon International Inc., Temecula, CA, USA), the monoclonal antimicrotubule-associated protein-2 (MAP2) antibody (1 : 2000; Boehringer Mannheim Biochemicals), the monoclonal anti-Tuj1 antibody (1 : 8000; Promega), the monoclonal glial fibrillary acidic protein (GFAP) antibody (1 : 1000; Sigma, St Louis, MO, USA), the polyclonal anti-GFAP antibody (1 : 2000; Sigma), and the monoclonal anticholine acetyltransferase (ChAT) antibody (1 : 400; Chemicon) were purchased from the indicated company and used at the indicated dilution. The polyclonal antienkephalin antibody was a generous gift from Professor Elde (University of Minnesota, USA). For some of the coexpression analyses, expression of the transgene was visualized by β-galactosidase histochemical staining with X-gal for 0.5–40 h as described above, followed by the single antigen immunohistochemistry method using 0.05% 3′,3′-diaminobenzidine (DAB) as the substrate.

X-gal- and A_{2A}-R-stained coronal brain sections were quantified by counting the numbers of transgene-positive cells and that of double-labelled cells in defined areas of photomicrographs taken using a microscope in phase contrast. Double-labelled cells were normalized to the number of total transgene-positive cells, and are presented as the percentage of coexpressing cells. For each quantification, ≈500–1000 transgene-positive cells were counted. Data points represent the mean ± SEM of data collected from three brains of the indicated mouse line.

PCR and Southern blot analysis of the lacZ transgene

Total RNA was purified from the indicated mouse tissue using the Tri Reagent Kit (Molecular Research Center, Cincinnati, OH, USA). Production of cDNA was carried out using Superscript II reverse transcriptase (Gibco BRL, Grand Island, NY, USA) following the manufacturer's protocol. DNA amplification was performed in a solution containing Tris-HCl, 10 mM; KCl, 50 mM; $MgCl_2$, 1.5 mM; gelatin, 0.001% (w/v); with 0.6 mM of the desired primers, 0.2 mM each of deoxynucleotide triphosphate and cDNA template, and 2 units of *Pfu* thermostable DNA polymerase (Stratagene, La Jolla, CA, USA) per 50 μL of reaction solution. To detect the expression of the transgene, a 500-bp DNA fragment was amplified using two lacZ-specific primers (5′-GGCTGCTCCAAAGAAGAAG-3′ and 5′-TACTGTCGTCGTCCCCTCAAAC-3′) for 30 cycles (94 °C, 1 min; 57 °C, 1 min; 72 °C, 2 min). To detect the expression of the endogenous gene, a 514-bp DNA fragment was amplified using two primers specific for the transcript of the mouse A_{2A}-R gene (5′-GCCATCGACAGATACATCG-3′ and 5′-AGGTACATGAGCCAAGGAGG-3′) for 30 cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1.5 min). The resultant PCR products were separated on agarose gels (2%) and transferred to nylon membranes (GeneScreen plus, DuPont, MA, USA). Specificity of the PCR product was verified by hybridization with a ^{32}P-labelled primer (5′-CGTTGCACCACAGATGAAAC-3′ and 5′-GAAGCAGATGGAGAGCCAAC-3′for the lacZ and the mouse A_{2A}-R gene, respectively)

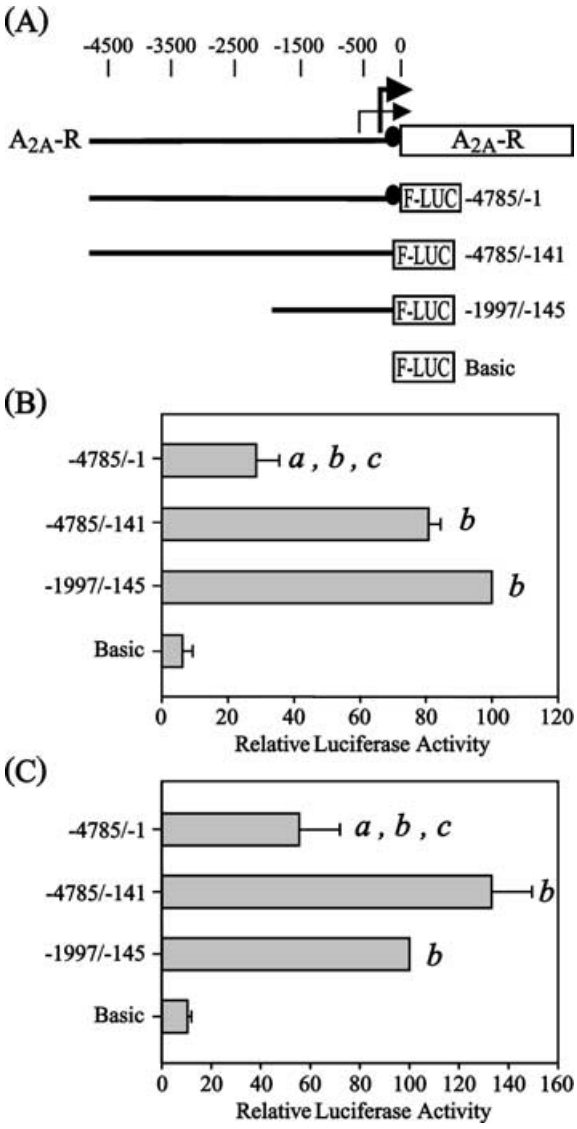

FIG. 2. The 5′-flanking region of the A_{2A}-R gene exhibits promoter activity in PC12 cells and C6 cells. (A) Schematic structures of the A_{2A}-R promoter constructs are illustrated. The indicated DNA fragment containing the 5′-flanking region and the indicated 5′UTR of the rat A_{2A}-R gene were subcloned into pGL2-basic, which contains F-LUC as the reporter gene. The indicated construct was transfected in parallel with pGL2-basic, which lacks promoter activity, into (B) PC12 cells or (C) C6 cells. Efficiency of transfection was monitored by cotransfecting pRL-TK as an internal control as described in Materials and methods. Values represent the mean ± SEM of at least three determinations and are expressed as percentages of the promoter activity of pGL2-(−1997/−145). The arrows mark the two transcriptional start sites of the rat A_{2A}-R gene (Chu *et al*., 1996). F-LUC, coding region of luciferase. Statistical significance: $^{a}P < 0.05$ compared to pGL2-(−1997/−145); $^{b}P < 0.05$ compared to pGL2-basic; $^{c}P < 0.05$ compared to pGL2-(−4785/−141). Specific comparisons were performed by one-way ANOVA followed by the Scheffé *F*-test.

at 42 °C for 16 h. Membranes were washed in 2 × standard saline citrate (SSC; 0.3 M NaCl and 0.03 Na citrate, pH 7) twice for 5 min at RT, in 2 × SSC containing 0.4% sodium dodecyl sulphate (SDS) twice for 20 min at 42 °C, and then in 0.1 × SSC twice for 20 min at RT. The membrane was air-dried and subjected to autoradiography.

Results

To characterize the transcriptional regulation of the rat A_{2A}-R gene *in vivo*, we obtained a 4.8-kb promoter-proximal DNA fragment of the A_{2A}-R gene using a combination of PCR and a DNA walking technique. To confirm that the 5′-flanking region of the rat A_{2A}-R gene obtained as described above was authentic, a 5′-flanking DNA fragment was amplified from rat genomic DNA using two A_{2A}-R-specific primers (5′-GCTAGACCTTCCCCTCCCGTGGGTT-3′ and 5′-ATCCACACGGCCCATCACACGAGCA-3′, corresponding to the rat A_{2A}-R gene from bases −4776 to −4752 and +89 to +65, respectively). As shown in Fig. 1B, digesting the resultant DNA fragment with *Acc* I, *Bgl* I, or *Kpn* I produced correct DNA fragments as predicted (3 and 1.8 kb; 2.8, 1.05 and 0.95 kb; and 2.2, 1.6 and 1 kb, respectively).

The promoter activity of this 5′-flanking region of the A_{2A}-R gene was analysed in PC12 cells and C6 cells, which express high and low amounts of A_{2A}-R, respectively (Lee *et al*., 1999; Lee *et al*., 2003). Two DNA fragments of the rat A_{2A}-R gene were inserted into the *Sma I* site of the pGL2-basic plasmid, which contains F-LUC as the reporter gene, and is designated pGL2-(−4785/−141) or pGL2-(−4785/−1). The promoter activities were determined by luciferase activity after transient transfection (Fig. 2). Transfection of pGL2-(−4785/−141) and pGL2-(−1997/−145) resulted in significant promoter activity as characterized elsewhere (Chu *et al*., 1996; Lee *et al*., 2003). As predicted, inclusion of the uAUG-5 codon (Lee *et al*., 1999) markedly reduced the activity of F-LUC with pGL2-(−4785/−1) in both cell types tested. The negative control, pGL2-basic with no. 5′-flanking sequence, produced very low luciferase activity.

A transgenic approach was then used to determine whether the 4.8-kb promoter-proximal DNA fragment of the A_{2A}-R gene plays an important role in the expression of the A_{2A}-R in the brain. A fusion gene consisting of a 4.8-kb DNA fragment of the A_{2A}-R gene and the coding region of lacZ was created and utilized to produce mice harbouring the indicated fusion gene (Fig. 3A). A nuclear localization sequence was fused to the N terminus of the transgene. Expression of the transgene therefore appears in the nucleus. To monitor the expression of endogenous A_{2A}-R, an oligopeptide corresponding to the hypervariable C terminal region (amino acids 394–410) of the rat A_{2A}-R was used to prepare the anti-A_{2A}-R antiserum, designated $A_{2A}R_C$, in rabbits. No sequence homology was found between amino acids 394–410 of the A_{2A}-R and the corresponding regions of the other adenosine receptor isoforms. By the Western blot analysis, an immunoreactive band of ≈48 kDa was observed in the plasma membrane fractions collected from the rat striatum, while addition of excess antigen peptide caused the disappearance of the A_{2A}-R-immunoreactive band (Fig. 3B).

In total, 35 independent transgenic founders were identified by the PCR typing analysis of tail genomic DNA. Expression of the β-galactosidase transgene driven by the A_{2A}-R promoter fragment in various tissues was analysed using either histochemical staining with X-gal or enzymatic assays. Expression of the transgene was widespread in various brain regions with variable levels of intensity. No blue β-galactosidase-positive signal was observed in the wild-type nontransgenic FVB mice (Fig. 3D). In three independent transgenic lines (#C, #D and #B4), expression of the transgene was found in the cortex, hippocampus, striatum and cerebellum (Figs 3 and 4, Table 1; also see Supplemental Materials Fig. S1). Except for a low expression level of β-galactosidase in the heart of line #C, no expression of the transgene was detected in other peripheral tissues examined. The #D founder line is infertile, and therefore was not used for detailed characterization except for the initial identification of transgene expression. Consistent observations described below were made using two other independent founder lines (#C and #B4). To verify the expression pattern of the transgene, we also determined the transcript level of lacZ using the RT-PCR technique. As shown in Fig. 5, lacZ transcript was detected in five areas of the CNS but not in three peripheral tissues tested. In line with the β-galactosidase activity assay (Table 1), low levels of the LacZ transcript were found only in the heart of line #C (Fig. 5B) but not in line #B4 (Fig. 5A). As expected, no LacZ transcript was detected in the CNS of the wildtype FVB (Fig. 5C). In contrast, expression of endogenous A_{2A}-R transcripts detected using the RT-PCR technique could be observed in all tissues examined with different levels of expression (Fig. 6). The expression profiles of endogenous A_{2A}-R in wildtype FVB and two transgenic founder lines (#B4 and #C) were very similar. In addition, A_{2A}-R immunoreactivities in the brains of wildtype FVB mice, two transgenic mouse lines (#B4 and #C) and SD rats were very similar (Supplemental Materials, Fig. S2). Collectively, insertion of the transgene did not influence the expression of endogenous A_{2A}-R in the transgenic founder lines.

Other laboratories (Rosin *et al*., 1998) and our own (Lee *et al*., 1999) have shown that the expression level of A_{2A}-R in the striatum is markedly higher than that in the hippocampus or in the cortex. However, we did not observe a significantly higher transgene expression in the striatum than in other areas of the brain (Fig. 3C). Nevertheless, the transgene was coexpressed with the endogenous A_{2A}-R in the striatum as demonstrated using double-immunohistochemical staining (Fig. 3E). In addition, identical to the endogenous A_{2A}-R in the striatum (Ferre *et al*., 1993; Kirk & Richardson, 1994), the transgene was found in GABAergic enkephalin-containing neurons and cholinergic neurons (Fig. 3F and G, respectively). It should be mentioned that detection of the transgene by the histochemical staining with X-gal or by immunostaining using the antiβ-galactosidase antibodies revealed identical expression profile of transgene in the brain (data not shown). Because immunostaining of endogenous A_{2A}-R using DAB as the substrate is more sensitive than the signals obtained from the immunofluorescence method, we determined the coexpression of the endogenous A_{2A}-R and the transgene mostly using a combination of the DAB-immunostaining of endogenous A_{2A}-R

FIG. 4. Expression of the A_{2A}-R/β-galactosidase transgene in the hippocampus and the cortex is consistent with that of the endogenous A_{2A}-R. (A) *In situ* hybridization of the endogenous A_{2A}-R in the rat cortex and hippocampus. Coronal brain slices (25 μm) from SD rats were hybridized with an antisense ^{33}P-labelled cRNA probe encoding a common region (−221 to +1 bp of the A_{2A}-R gene; the translational start site was designated +1; Chu *et al*., 1996) to detect the total A_{2A}-R transcripts. (B) Brain slices hybridized with a sense ^{33}P-labelled cRNA probe (−221 to +1 bp of the A_{2A}-R gene) was used as a negative control as shown in. (C) Photomicrograph illustrating A_{2A}-R immunoreactivity in the hippocampus and cortex. (D) No signal was detected in the cortex and hippocampus in the presence of an excess amount (1 mg/mL) of the corresponding antigen peptide conjugated to an irrelevant protein (ovalbumin). Addition of the same amount of ovalbumin alone did not affect the immunostaining signal. (E) Expression of the transgene in the transgenic line #B4 was examined by histochemical staining with X-gal. Co-expression of the transgene (blue, β-galactosidase histochemistry) and the endogenous A_{2A}-R (brown, immunocytochemical staining) in the cortex (F) and in the hippocampus (G) are demonstrated in the transgenic line #B4 as indicated. Scale bar, 10 μm (F and G).

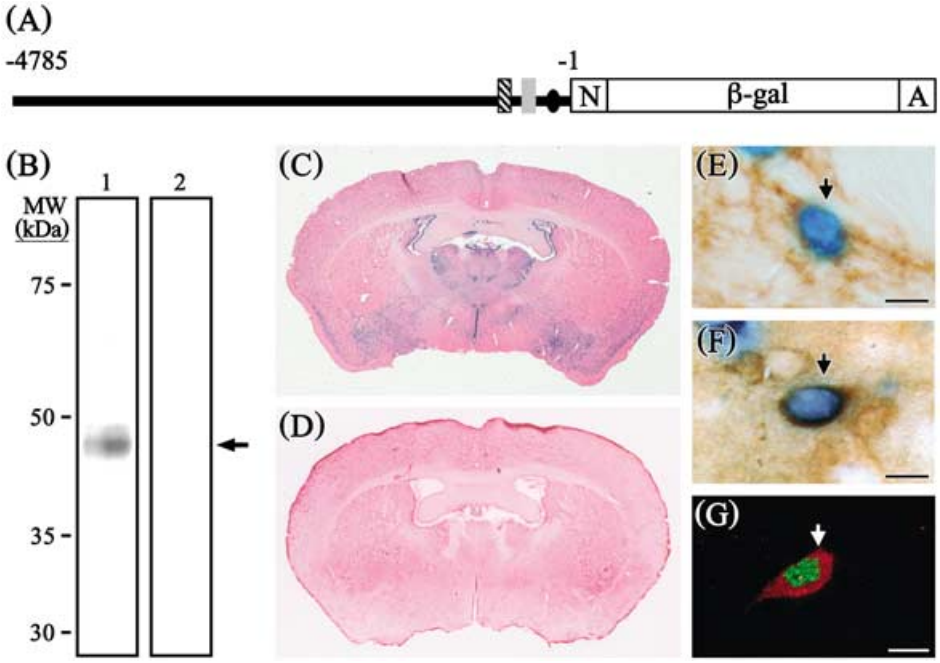

FIG. 3. Expression of the A_{2A}-R/β-galactosidase transgene in the striatum. (A) Schematic representation of the A_{2A}-R/β-galactosidase transgene. The DNA fragment containing 4.3 kb of the 5′-flanking region and the 5′UTR of the rat A_{2A}-R gene was fused to a nuclear β-galactosidase expression cassette (N-β-gal). N, nuclear localization sequence; β-gal, coding region of β-galactosidase; A, SV40 late polyadenylation signal. Sequences of the A_{2A}-R gene are numbered +1 from the translational start site. Shaded boxes mark the position of the two independent promoters (P1 and P2; Chu *et al*., 1996). The closed circle represents the inhibitory upstream AUG codon (Lee *et al*., 1999). (B) Immunoblots of the rat striatum membrane fraction (50 μg per lane) using the anti-A_{2A}-R antibody (1 : 1000 dilution) in the absence (lane 1) or presence (lane 2) of an excess amount of the corresponding antigen peptide (500 μg/mL). The arrow marks the predicted size of the endogenous A_{2A}-R protein. (C) Expression of the transgene in a coronal brain section of transgenic line #B4 was examined by histochemical staining with X-gal. β-Galactosidase-positive regions in the brain appear blue. Wide and general expression of the transgene was also observed in two other transgenic lines (#C and #D). (D) A coronal brain section from the wildtype FVB as the negative control. (E) Co-expression of the transgene (blue, β-galactosidase histochemistry) and the endogenous A_{2A}-R (brown, immunocytochemical staining) in the striatum of the transgenic line #B4. (F) Co-expression of the transgene (blue, β-galactosidase histochemistry) and enkephalin (brown, immunocytochemical staining) in the striatum of the transgenic line #B4. (G) Confocal image illustrating coexpression of the transgene (green) and a marker (ChAT, red) of the cholinergic neurons in the striatum of the transgenic line #B4. Due to the presence of a nuclear localization sequence in the transgene, expression of the transgene appears in the nucleus. When β-galactosidase histochemistry was used to detect the transgene, the blue colour indicated the location of the product of the transgene; it can be readily observed in the nucleus and areas around the nucleus due to the spread of the transgene product. The apparent size of the nucleus with the transgene therefore might appear larger than its actual size. Scale bars, 10 μm (E–G).

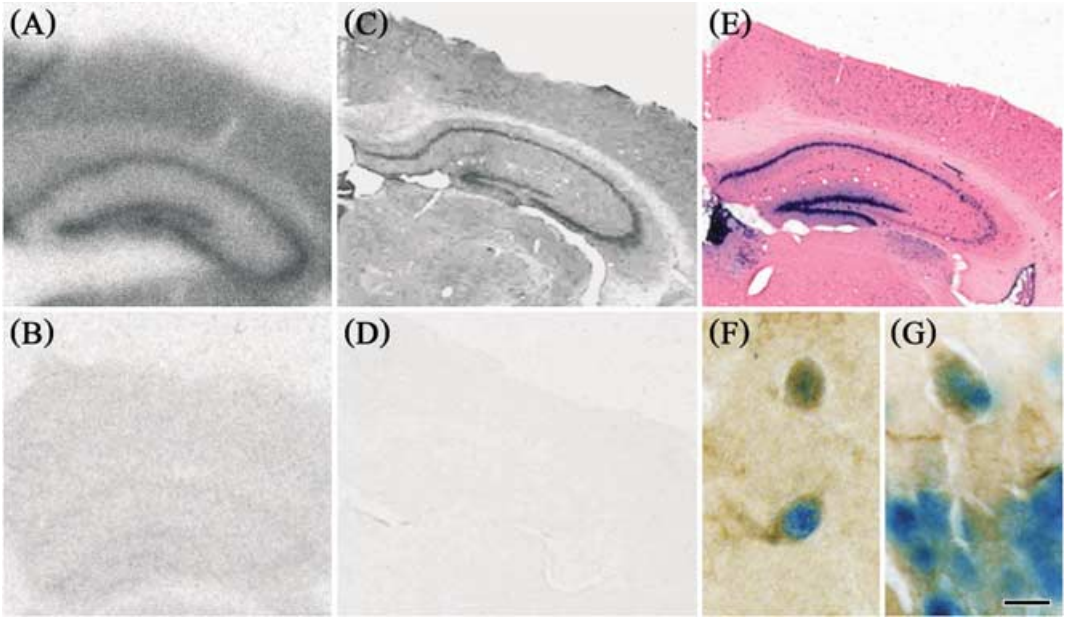

TABLE 1. Summary of the expression of A_{2A}-R/β-galactosidase transgene and endogenous A_{2A}-R

Tissue	β-Galactosidase (independent founder line)			Endogenous A2A-R		
	#C	#D[¶]	#B4	Rat mRNA[*]	Rat immunoreaction	Mouse immunoreaction
Cerebral cortex	+	+	+	+	+	+
Striatum	+	+	+	+++	+++	+++
Hippocampus	+[†]	+	+	+	+	+
Thalamus/hypothalamus	+++[‡]	+	+	ND	+	+
Cerebellum	+++	+	+[§]	+	+	+
Spinal Cord	+	ND	+	ND	ND	+
Heart	+	+	−	++	ND	ND
Lung	−	−	−	++	ND	ND
Liver	−	−	−	++	ND	ND
Kidney	−	−	−	++	ND	ND

+++, intense signal; ++, moderate signal; +, detectable signal; ND, not determined. The activity of β-galactosidase was determined either by enzymatic assay or by histochemical staining with the X-gal substrate as detailed in Materials and methods. Although some variation existed in the expression levels as indicated in the table, the transgene was consistently expressed in five areas of the CNS in three independent founders (#B4, #C, and #D). In contrast, except for the heart, the transgene was not detected in three peripheral tissues (lung, liver, and kidney) where moderate levels of endogenous A_{2A}-R were found. [*]The endogenous A_{2A}-R transcript measured by a real-time PCR technique (Lee *et al*., 1999). [†]The number of transgene-expressing pyramidal cells in the hippocampus of line #C was lower than those in the other two founders (lines #B and #D). [‡]Transgene expression in the thalamus/hypothalamus areas of line #C was higher than those in lines #B4 and #D. Nevertheless, the transgene remained highly coexpressed with endogenous A_{2A}-R (Supplementary Materials, Fig. S3). [§]Transgene expression in the cerebellum of line #B4 was lower than those in the lines #C and #D. [¶]The #D founder line is infertile, and therefore was only characterized by histochemical staining with the X-gal substrate. Coronal brain sections, stained for the transgene (β-galactosidase) or endogenous A_{2A}-R, of wildtype FVB mice and the transgenic founders are also presented in the section 'Supplementary Materials' (Figs S1 and S2).

and the histochemical staining of β-galactosidase using X-gal in the following experiments.

Consistent with previous findings that stimulation of the A_{2A}-R modulates important functions in the cortex and hippocampus (Chen *et al*., 1999; Lopes *et al*., 1999; Okada *et al*., 1999), expression of A_{2A}-R transcripts in the cortex and hippocampus were clearly demonstrated using *in situ* hybridization (Fig. 4A and B). Marked intensity of the endogenous A_{2A}-R transcripts was present in the pyramidal layers of CA1, CA2, CA3 and the dentate gyrus. The A_{2A}-R-immunoreactive signals were also detected in the cortex and hippocampus (Fig. 4C). The addition of excess antigen peptide resulted in disappearance of the A_{2A}-R-immunoreactive signals (Fig. 4D). This finding is consistent with those of previous studies in which low levels of A_{2A}-R were detected in many areas of the brain (Rosin *et al*., 1998; Lee *et al*., 1999). Moreover, treating plasma membranes collected from the cortex and the hippocampus with an A_{2A}-R-selective agonist, 2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS21680), evoked an increase in AC activity as shown in Table 2. In line with previous findings from other laboratories, our data also suggest that functional A_{2A}-Rs exist in the cortex and the hippocampus. Most importantly, expression patterns of the A_{2A}-R/lacZ transgene in the representative transgenic founder lines correlated well with those of the endogenous A_{2A}-R (Fig. 4A, C and E). Double-immunohistochemical staining further demonstrated that the A_{2A}-R/transgene was largely coexpressed with the endogenous A_{2A}-R in the cortex and hippocampus (Fig. 4F and G, respectively). In the cortex, percentages of the transgene-positive cells which also expressed endogenous A_{2A}-R were $92.3 \pm 3.8\%$ and $74.0 \pm 7.0\%$ for the transgenic lines #B4 and #C, respectively. In the hippocampus, the percentages of cells coexpressing the transgene and A_{2A}-R were $95.7 \pm 1.8\%$ and $75.0 \pm 6.9\%$ for lines #B4 and #C, respectively. These data support the high fidelity of transgene expression. Co-expression of the endogenous A_{2A}-R and the transgene was also observed in the spinal cord (data not shown). Moreover, the endogenous A_{2A}-R (Figs 7B and C, and 8B and C) and the transgene (Figs 7D and E, and 8D and E) were found in both neurons and astrocytes in the cortex (Fig. 7), the hippocampus (Fig. 8) and other areas (e.g. hypothalamus, striatum; data not shown). Adenosine therefore might exert its action through the A_{2A}-R in both neurons and astrocytes in the adult brain.

Discussion

Because A_{2A}-R has been implicated in functions of a wide variety of tissues including the CNS, the heart, the kidney and other peripheral tissues (Weaver, 1993; Rosin *et al*., 1998; Lasley & Smart, 2001; Klinger *et al*., 2002; Welch, 2002), detailed characterization of its gene structure and regulation might impart important knowledge towards understanding its action. We had previously cloned an ≈1.8-kb 5′-flanking region of the rat A_{2A}-R gene and demonstrated that this gene contains at least two TATA-less independent promoters, two exons and one ≈7.2-kb intron (Chu *et al*., 1996). This gene structure is similar to that of the human A_{2A}-R gene, which has been localized to chromosome 22q11.2 and is composed of two exons separated by a 6.4-kb intron (Le *et al*., 1996). In the present study, we have further isolated and characterized a 4.8-kb promoter-proximal DNA fragment (including a 4.3-kb 5′ flanking region and the 0.5-kb 5′UTR) of the rat A_{2A}-R gene (Fig. 1). Comparison of the rat and human A_{2A}-R genomic sequences (accession numbers AF107208 and ap000355.gb_pr5, respectively) reveals 65% identity in the 4.3-kb 5′-flanking region, supporting the interspecies importance of this 5′-flanking region in regulation of the A_{2A}-R. Promoter analysis demonstrated that this DNA fragment drove the expression of a luciferase reporter gene in PC12 and C6 cells expressing the endogenous A_{2A}-Rs (Fig. 2). Using a transgenic approach, we found that this 4.8-kb promoter-proximal DNA fragment of the A_{2A}-R gene directed its expression in cells expressing endogenous A_{2A}-R in the CNS, but not in several peripheral tissues examined (except for the heart). This observation is of particular interest because the A_{2A}-R was found not only in the CNS but also in many peripheral tissues with expression levels markedly higher than those found in the brain regions (except for the striatum). As shown earlier (Lee *et al*., 1999), the amounts of A_{2A}-R transcripts in the kidney and the lung are at least three times higher than those in the

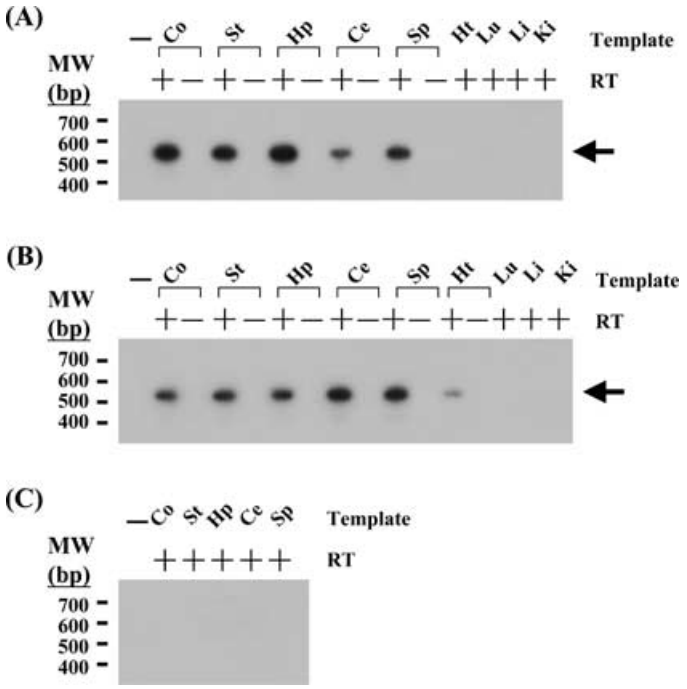

FIG. 5. Expression of transgene was detected in five areas of the CNS. Total RNA was harvested from (A) the transgenic line #B4, (B) the transgenic line #C and (C) the wild-type FVB mice. The transgene fragments were amplified from the indicated cDNA prepared in the presence (+) or absence (−) of reverse transcriptase (RT). PCR products were analysed using ethidium bromide–agarose (2%) electrophoresis and transblotted for Southern blot analysis to verify the specificity. Arrows mark the amplified transgene DNA fragments. Molecular weight markers (MW) are indicated at the left. −, no template. Note that no transcript was detected in the wildtype FVB (C). Co, cortex; St, striatum; Hp, hippocampus; Ce, cerebellum; Sp, spinal cord; Ht, heart; Lu, lung; Li, liver; Ki, kidney.

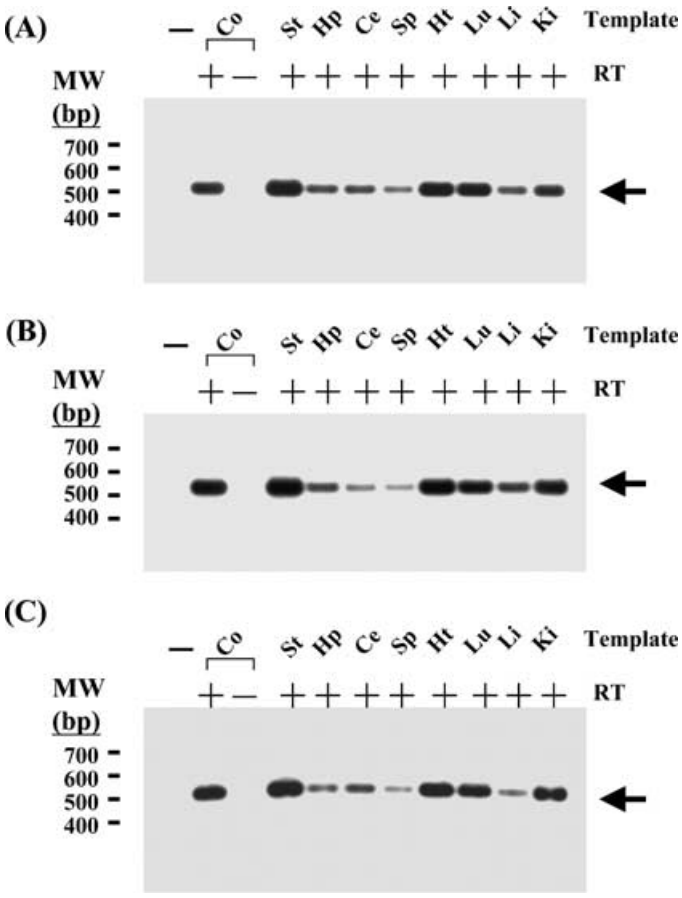

FIG. 6. Insertion of the transgene did not interfere with the expression of endogenous A_{2A}-R. Total RNA was harvested from (A) the transgenic line #B4, (B) the transgenic line #C and (C) the wild-type FVB mice. The transgene fragments were amplified from the indicated cDNA prepared in the presence (+) or absence (−) of reverse transcriptase (RT). PCR products were analysed using ethidium bromide–agarose (1%) electrophoresis and transblotted for Southern blot analysis to verify the specificity. Arrows mark the amplified A_{2A}-R DNA fragments. Molecular weight markers (MW) are indicated at the left. −, no template. Co, cortex; St, striatum; Hp, hippocampus; Ce, cerebellum; Sp, spinal cord; Ht, heart; Lu, lung; Li, liver; Ki, kidney.

cortex and hippocampus using a quantitative PCR technique. In the presence study, we found that protein and transcript of the transgene directed by the 4.8-kb A_{2A}-R promoter could be readily detected in the CNS using various techniques, but not in three peripheral tissues which contain endogenous A_{2A}-R (Figs 3–8; Table 1). This 4.8-kb DNA fragment therefore appears to contain *cis* element(s) necessary for the expression of A_{2A}-R in the CNS, but not in several major peripheral tissues (i.e. lung, kidney and liver).

As summarized in Table 1 (see also Supplementary Materials, Figs. S1 and S2), endogenous A_{2A}-R and the transgene were located in the CNS with similar patterns. Quantitative analysis of the double immunohistochemical-stained brain sections further showed that the transgene was mostly coexpressed with endogenous A_{2A}-R in the cortex and hippocampus of the transgenic lines #B4 (>90%) and #C (>70%). Thus, except for the striatum, this 4.8-kb 5′ flanking region conferred a relatively faithful expression profile of the transgene in brain areas where A_{2A}-R exists. Note that the expression level of the transgene in each founder could be affected by the position where the transgene was inserted, which therefore might have caused differences in expression levels of the transgene in different brain areas. For example, transgene expression in the thalamus–hypothalamus and cerebellum areas of line #C was higher than those of lines #B4 and #D (Table 1; Supplementary Material, Fig. S1). It appeared that the transgene of line #C might have been affected by an enhancer for these areas around the transgene insertion site. Importantly, although the expression level of transgene in this area was higher than expected, the transgene remained highly coexpressed with endogenous A_{2A}-R (Supplementary Material, Fig. S3). There were also minor discrepancies in the transgene expression patterns between founders. For instance, the number of transgene-expressing pyramidal cells in the hippocampus of line #C was lower than those in the other two founders (lines #B and #D). Again, the insertion position of the transgene might have caused such variation. Nevertheless, the overall CNS-selective expression of the transgene was consistent in these three independent founders, supporting our hypothesis that this 4.8-kb promoter-proximal DNA fragment contains important *cis* element(s) to direct expression of the transgene in many brain areas where functional A_{2A}-R is expressed.

Multiple promoter involvement for a single gene has many precedent examples. We previously reported that the 5′UTRs of A_{2A}-R transcripts derived from both promoters of the rat A_{2A}-R gene suppress gene expression at the translational level via an upstream open reading frame (uORF). The negative regulation by 5′UTR of the rat A_{2A}-R gene was also confirmed in the present study (Fig. 2). Because 5′UTR of the A_{2A}-R gene exhibits strong interspecies homology, it will be of great interest to determine whether the post-transcriptional regulation (e.g. translation efficiency, transcript stability) by 5′UTR can be a general mechanism which modulates expression of the A_{2A}-R gene *in vivo* under certain physiological or pathological conditions. In the

TABLE 2. Expression of functional A_{2A}-Rs in various areas of the brain

	Adenylyl cyclase (pmol/mg/min)	
Tissue	CGS21680 (10 μM)	Forskolin (100 μM)
Striatum	41.3 ± 7.2	78299.1 ± 10320.7
Hippocampus	45.4 ± 10.2	31495.1 ± 8804.9
Cortex	100.1 ± 9.5	17572.8 ± 2083.6

Membrane fractions were prepared from the indicated tissues. Adenylyl cyclase activities were measured in the presence of CGS21680 (10 μM) or forskolin (100 μM) as indicated, and were subtracted from those in the absence of stimuli. The basal, nonstimulated adenylyl cyclase activities of the striatum, the hippocampus and the cortex are 35.2 ± 2.9, 219.7 ± 25.7 and 428.7 ± 11.6 pmol/mg/min, respectively. Each assay was carried out in triplicate. Values are the mean ± SEM of 9–15 determinants from 3–5 independent experiments.

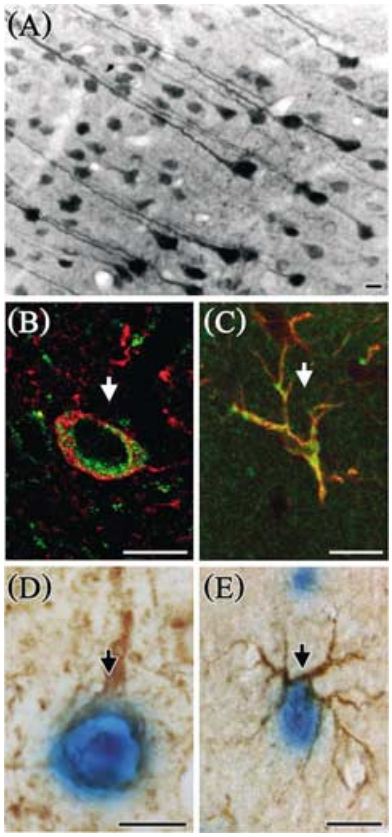

FIG. 7. The endogenous A_{2A}-R and the transgene are expressed in both neurons and astrocytes in the cortex. (A) Photomicrograph illustrating A_{2A}-R immunoreactivity in the FVB mouse cortex. (B) The confocal image illustrates coexpression of the A_{2A}-R (green) and a marker (Tuj1, red) of neurons. (C) The confocal image illustrates coexpression of the endogenous A_{2A}-R (green) and a marker (GFAP, red) of astrocytes. (D and E) Co-expression of the transgene (blue; β-galactosidase histochemistry) and a neuron marker (MAP2, brown; immunocytochemical staining; D) or the astrocyte marker (GFAP, brown, immunocytochemical staining; E) are illustrated in the cortex of transgenic line #B4. Similar results were also observed in transgenic line #C. Scale bars, 10 μm.

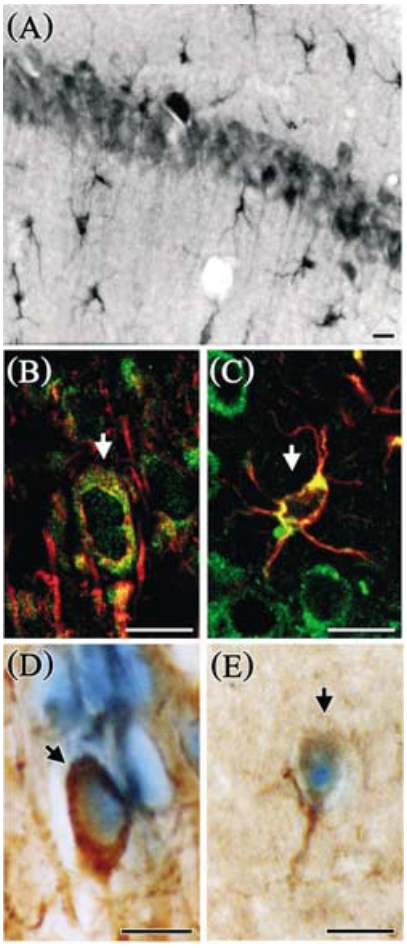

FIG. 8. The endogenous A_{2A}-R and the transgene are expressed in both neurons and astrocytes in the hippocampus. (A) Photomicrograph illustrating A_{2A}-R immunoreactivity in the FVB mouse hippocampus. (B) A confocal image illustrating coexpression of the endogenous A_{2A}-R (green) and a marker (Tuj1, red) of neurons. (C) A confocal image illustrating coexpression of A_{2A}-R (green) and a marker (GFAP, red) of astrocytes. Colocalization of A_{2A}-R and GFAP appears yellow. (D and E) Co-expression of the transgene (blue, β-galactosidase histochemistry) and a neuron marker (MAP2, brown; immunocytochemical staining; D) or the astrocyte marker (GFAP, brown, immunocytochemical staining; E) are illustrated in the hippocampus of transgenic line #B4. Similar results were also observed in transgenic line #C. Scale bars, 10 μm.

current study, the data presented herein suggest that important *cis* element(s) might exist in the 4.8-kb promoter-proximal DNA fragment of the rat A_{2A}-R gene which regulates the expression of the rat A_{2A}-R gene in the CNS at the transcriptional level. As for many other G protein-coupled receptors (e.g. the A_1 adenosine receptor; Ren & Stiles, 1994; Rivkees *et al*., 1999), regulation at the transcriptional and translational levels might both be crucial for proper expression and regulation of the A_{2A}-R gene. With the currently available evidence, we cannot exclude the possibility that additional promoter(s) of the A_{2A}-R gene might exist which control its expression in the peripheral tissues. It is also possible that, although using the same promoter(s), important DNA element(s) are missing in the 4.8-kb DNA fragment which confers its peripheral expression. Another type of DNA element(s) which maybe absent in this promoter-proximal DNA fragment is the component(s) that grants the striatum-enriched expression of A_{2A}-R. As we reported earlier, the levels of striatal A_{2A}-R transcripts are ≈100-fold higher than other areas of the brain (Lee *et al*., 1999). Unlike the endogenous A_{2A}-R, levels of striatal transgene expression were similar to those of other brain areas in three transgenic founder lines (Table 1). The faithful coexistence (Figs 3 and 4) eliminates the possibility that such an expression profile of transgene was due to certain aspects of the construct used. In contrast, however, these data suggest that the 4.8-kb DNA fragment may not contain the necessary

DNA component(s), such as enhancers controlling the expression level (striatum) and tissue specificity (periphery), to reach complete concordance between transgene and endogenous A_{2A}-R expressions. Such DNA components remain to be identified, and might be positioned further upstream of the 4.8-kb promoter-proximal DNA fragment or in the 7.2-kb intron of the A_{2A}-R gene as reported for other genes (Burke *et al*., 1999; Liu *et al*., 2000). Although less likely, we cannot completely rule out the possibility that the lack of enriched striatal expression of the transgene might be due to the absence of rat-specific transcription factors of the rat A_{2A}-R gene in mice. Although 5′UTR has been implicated in tissue-specific expression of other genes (Lo & Lau, 1999), 5′UTR of the rat A_{2A}-R gene might not contribute to the lack of proper expression in the striatum and peripheral tissues because the entire 5′UTR was included in the transgenic construct (Fig. 3). Because the 3′UTR of the A_{2A}-R gene among different species is also highly homologous, we have attempted to determine the effect of 3′UTR on the tissue expression *in vivo* using a transgenic approach by inserting the entire 3′UTR of the rat A_{2A}-R gene downstream of theA_{2A}-R promoter/n-lacZ transgene for the production of transgenic mice. The expression pattern of the transgene in these mice was very similar to the results found in the absence of 3′UTR (data not shown). 3′UTR of the rat A_{2A}-R gene thus might not directly participate in the brain-selective expression of the transgene under physiological conditions.

Mounting evidence suggests that the A_{2A}-R is involved in many important functions of the CNS. In the striatum where the A_{2A}-R is highly enriched, the A_{2A}-R was shown to form complexes with D2 dopamine receptors (D2-Rs) and to antagonize the action of D2-R *in vivo* (Aoyama *et al*., 2000; Hillion *et al*., 2002). Moreover, the A_{2A}-R is involved in NMDA receptor-evoked elevation of cAMP (Nash & Brotchie, 2000). In areas where only low levels of the A_{2A}-R are found, the A_{2A}-R also plays significant regulatory roles. In the hippocampus, activation of the A_{2A}-R protects against kainate-induced excitotoxicity (Jones *et al*., 1998), facilitates synaptic transmission (Cunha & Ribeiro, 2000), modulates the dynamics of neuronal plasticity (Fujii *et al*., 2000) and increases glial glutamate efflux (Li *et al*., 2001). In the spinal cord, A_{2A}-R stimulation modulates the non-NMDA receptor-mediated excitatory postsynaptic currents and has been implicated in preclinical models of pain (Patel *et al*., 2001). Deficiency of the A_{2A}-R results in alternations in various neuronal responses, including a functional hypodopaminergic state (Dassesse *et al*., 2001), an attenuation of brain injury induced by transient focal ischemia in mice (Chen *et al*., 1999) and a partial reduction in the catalepsy caused by manipulating the dopaminergic and cholinergic systems (El Yacoubi *et al*., 2001b). Due to these modulating actions of A_{2A}-R, it has been suggested that antagonists of the A_{2A}-R possess beneficial effects on Parkinson's disease (Grondin *et al*., 1999) and depression (El Yacoubi *et al*., 2001a). As an important neuromodulator, expression of the A_{2A}-R has been shown to be located in neurons of the striatum (Schiffmann & Vanderhaeghen, 1993). One important finding of the present study is to clearly demonstrate, for the first time, that A_{2A}-R also exists in astrocytes in various areas of the brain. The action of adenosine mediated by stimulation of the A_{2A}-R therefore might occur not only in neurons but also in astrocytes. Data presented herein provide a foundation for understanding the molecular basis of A_{2A}-R expression and important insights into the cell types involved in the action of adenosine (and A_{2A}-R-related drugs) in the CNS.

Supplementary material

The following material can be accessed at http://www.blackwellpublishing.com/products/journals/suppmat/EJN/EJN2907/EJN2907sm.htm

FIG. S1. Expression of the transgene in the brains of three independent founders. The transgene was detected by staining coronal brain sections of the wildtype FVB (A, B, C), #B4 (D, E, F), #C (G, H, I), and #D (J, K, L) with X-gal.? β-Galactosidase-positive regions in the brain appear blue. Coronal brain sections from the wildtype FVB mouse serve as the negative control.

FIG. S2. Expression of A_{2A}-R in the brains of the SD rat, FVB mice, and two transgenic founders. Photomicrographs illustrating A_{2A}-R immunoreactivity in coronal brain sections of the SD rat (A, B, C), FVB mouse (D, E, F), #B4 (G, H, I), and #C (J, K, L). No signal was detected in the presence of an excess amount (1 mg/mL) of the corresponding antigen peptide as described and shown in Fig. 4.

FIG. S3. Coexpression of the transgene (blue, β-galactosidase histochemistry) and endogenous A_{2A}-R (brown, immunocytochemical staining) in the thalamus/hypothalamus of the transgenic line #C. The blue colour indicates the location of the product of the transgene; it can be readily observed in the nucleus and areas around the nucleus due to the spread of the transgene product. The scale bar indicates 20 μm.

Acknowledgements

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Abbreviations

5′UTR, 5′ untranslated region.; A_{2A}-R, A_{2A} adenosine receptor; ABC, avidin–biotin–peroxidase complex; AC, adenylyl cyclase; BSA, bovine serum albumin; CGS21680, 2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine hydrochloride; ChAT, choline acetyltransferase; CNS, central nervous system; D2-R, D2 dopamine receptor; DAB, 3′,3′-diaminobenzidine; F-LUC, firefly luciferase; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein-2; MBS, m-maleimidobenzoyl-N-hydroxysuccinimide ester; PB, phosphate buffer; PCR, polymerase chain reaction; R-LUC, *Renilla* luciferase; RT, room temperature; SDS, sodium dodecyl sulphate; SSC, standard saline citrate.

References

Aoyama, S., Kase, H. & Borre, E. (2000) Rescue of locomotor impairment in dopamine D2 receptor-deficient mice by an adenosine A2A receptor antagonist. *J. Neurosci.*, **20**, 5848–5852.

Burke, Z., Wells, T., Carter, D., Klein, D. & Baler, R. (1999) Genetic targeting: the serotonin N-acetyltransferase promoter imparts circadian expression selectively in the pineal gland and retina of transgenic rats. *J. Neurochem.*, **73**, 1343–1349.

Chen, J.-F., Huang, Z., Ma, J., Zhu, J., Moratalla, R., Standaert, D., Moskowitz, M.A., Fink, J.S. & Schwarzschild, M.A. (1999) A_{2A} adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J. Neurosci.*, **19**, 9192–9200.

Chern, Y., King, K., Lai, H.-L. & Lai, H.-T. (1992) Molecular cloning of a novel adenosine receptor gene from rat brain. *Biochem. Biophys. Res. Comm.*, **185**, 304–309.

Chern, Y., Lai, H.-L., Fong, J.-C. & Liang, Y. (1993) Multiple mechanisms for desensitization of A2a adenosine receptor-mediated cAMP elevation in rat pheochromocytoma PC12 cells. *Mol. Pharmacol.*, **44**, 950–958.

Chu, Y.-Y., Tu, K.-H., Lee, Y.-C., Kuo, Z.-J., Lai, H.-L. & Chern, Y. (1996) Characterization of the rat A2a adenosine receptor gene. *DNA Cell Biol.*, **15**, 329–337.

Cunha, R.A. & Ribeiro, J.A. (2000) Adenosine A_{2A} receptor facilitation of synaptic transmission in the CA1 area of the rat hippocampus requires protein kinase C but not protein kinase A activation. *Neurosci. Lett.*, **289**, 127–130.

Dassesse, D., Massie, A., Ferrari, R., Ledent, C., Parmentier, M., Arckens, L., Zoli, M. & Schiffmann, S.N. (2001) Functional striatal hypodopaminergic activity in mice lacking adenosine A_{2A} receptors. *J. Neurochem.*, **78**, 183–198.

El Yacoubi, M., Ledent, C., Parmentier, M., Bertorelli, R., Ongini, E., Costentin, J. & Vaugeois, J.M. (2001a) Adenosine A_{2A} receptor antagonists are potential antidepressants: evidence based on pharmacology and A2A receptor knockout mice. *Br. J. Pharmacol.*, **134**, 68–77.

El Yacoubi, M., Ledent, C., Parmentier, M., Costentin, J. & Vaugeois, J.M. (2001b) Adenosine A_{2A} receptor knockout mice are partially protected against drug-induced catalepsy. *Neuroreport*, **12**, 983–986.

Ferre, S., O'Connor, W.T., Fuxe, K. & Ungerstedt, U. (1993) The striopallidal neuron: a main locus for adenosine–dopamine interactions in the brain. *J. Neurosci.*, **13**, 5402–4506.

Fujii, S., Kato, H., Ito, K., Itoh, S., Yamazaki, Y., Sasaki, H. & Kuroda, Y. (2000) Effects of A1 and A2 adenosine receptor antagonists on the induction and reversal of long-term potentiation in guinea pig hippocampal slices of CA1 neurons. *Cell Mol. Neurobio.*, **20**, 331–350.

Grondin, R., Bedard, P.J., Hadj-Tahar, A., Gregoire, L., Mori, A. & Kase, H. (1999) Antiparkinsonian effect of a new selective adenosine A_{2A} receptor antagonist in MPTP-treated monkeys. *Neurology*, **52**, 1673–1677.

Harlow, E. & Lane, D. (1988) *Antibodies: a Laboratory Manual*, 1st edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 59–137.

Hillion, J., Canaals, M., Torvinen, M., Casado, V., Scott, R., Terasmaa, A., Hansson, A., Watson, S., Olah, M.E., Mallol, J., Franco, R., Ferre, S. & Fuxe, K. (2002) Coaggregation, cointernalization, and codesensitization of adenosine A_{2A} receptors and dopamine D2 receptors. *J. Biol. Chem.*, **277**, 18091–18097.

Hogan, B., Beddington, R., Constantini, F. & Lacy, E. (1994) Isolating high-molecular-weight DNA from mouse tails. In Hogan, B., Beddington, R., Costantini, F. & Lacy, E. (Eds), *Manipulating the Mouse Embryo: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 296–299.

Jones, P.A., Smith, R.A. & Stone, T.W. (1998) Protection against kainate-induced excitotoxicity by adenosine A_{2A} receptor agonists and antagonists. *Neuroscience*, **85**, 229–237.

Kirk, I.P. & Richardson, P.J. (1994) Adenosine A2a receptor-mediated modulation of striatal [^{3}H]GABA and [^{3}H]acetylcholine release. *J. Neurochem.*, **62**, 960–966.

Klinger, M., Freissmuth, M. & Nanoff, C. (2002) Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell Signal*, **14**, 99–108.

Lai, H.-L., Lin, T.-H., Kao, Y.-Y., Lin, W.-J., Hwang, M.-J. & Chern, Y. (1999) The N terminus domain of type VI adenylyl cyclase mediates its inhibition by protein kinase C. *Mol. Pharmacol.*, **56**, 644–650.

Lai, H.L., Yang, T.-H., Messing, R.O., Ching, Y.-H., Lin, S.-C. & Chern, Y. (1997) Protein kinase C inhibits adenylyl cyclase type VI activity during desensitization of the A2a-adenosine receptor-mediated cAMP response. *J. Biol. Chem.*, **272**, 4970–4977.

Lasley, R.D. & Smart, E.J. (2001) Cardiac myocyte adenosine receptors and caveolae. *Trends Cardiovasc. Med.*, **11**, 259–263.

Le, F., Townsend-Nicholson, A., Baker, E., Sutherland, G.R. & Schofield, P.R. (1996) Characterization and chromosomal localization of the human A2a adenosine receptor gene: ADORA2A. *Biochem. Biophys. Res. Commun.*, **223**, 461–467.

Ledent, C., Vaugeois, J.M., Schiffmann, S.N., Pedrazzini, T., Yacoubi, M.E., Vanderhaeghen, J.J., Costentin, J., Heath, J.K., Vassart, G. & Parmentier, M. (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature*, **388**, 674–678.

Lee, Y.-C., Chang, C.-W., Su, C.-W., Lin, T.-N., Sun, S.H., Lai, H.-L. & Chern, Y. (1999) The 5′ untranslated regions of the rat A2A adenosine receptor gene function as negative translational regulators. *J. Neurochem.*, **73**, 1790–1798.

Lee, Y.-C., Lai, H.-L., Sun, C.-N., Chien, C.-L. & Chern, Y. (2003) Identification of nuclear factor 1 (NF1) as a transcriptional modulator of rat A_{2A} adenosine receptor. *Mol. Brain Res.*, **111**, 61–73.

Li, X.X., Nomura, T., Aihara, H. & Nishizaki, T. (2001) Adenosine enhances glial glutamate efflux via A2a adenosine receptors. *Life Sci.*, **68**, 1343–1350.

Lin, T.-N., Wang, P.Y., Chi, S.-I. & Kuo, J.-S. (1998) Differential regulation of ciliary neurotrophic factor (CNTF) and CNTF receptor alpha (CNTFR alpha) expression following focal cerebral ischemia. *Mol. Brain Res.*, **55**, 71–80.

Liu, Y., Li, H., Tanaka, K., Tsumaki, N. & Yamada, Y. (2000) Identification of an enhancer sequence within the first intron required for cartilage-specific transcription of the alpha2 (XI) collagen gene. *J. Biol. Chem.*, **275**, 12712–12718.

Liu, F.-C., Wu, G.-C., Hsieh, S.-T., Lai, H.-L., Wang, H.-F., Wang, T.-W. & Chern, Y. (1998) Expression of type VI adenylyl cyclase in the central nervous system: implication for a potential regulator of multiple signals in different neurotransmitter systems. *FEBS. Lett.*, **436**, 92–98.

Lo, N.W. & Lau, J.T. (1999) Transcription of the beta-galactoside alpha2,6-sialyltransferase gene (SIAT1) in B-lymphocytes: cell type-specific expression correlates with presence of the divergent 5′-untranslated sequence. *Glycobiology*, **9**, 907–914.

Lopes, L.V., Cunha, R.A. & Ribeiro, J.A. (1999) Cross talk between A (1) and A (2A) adenosine receptors in the hippocampus and cortex of young adult and old rats. *J. Neurophysiol.*, **82**, 3196–3203.

Marshall, J.M. (2000) Adenosine and muscle vasodilatation in acute systemic hypoxia. *Acta Physiol. Scand.*, **168**, 561–573.

Nash, J.E. & Brotchie, J.M. (2000) A common signaling pathway for striatal NMDA and adenosine A2a receptors: implications for the treatment of Parkinson's disease. *J. Neurosci.*, **20**, 7782–7789.

Nielsen, D.A., Chou, J., Mackrell, A.J., Casadaban, M.J. & Steiner, D.F. (1983) Expression of a preproinsulin-beta-galactosidase gene fusion in mammalian cells. *Proc. Natl Acad. Sci. USA*, **80**, 5198–5202.

Okada, M., Kawata, Y., Murakami, T., Wada, K., Mizuno, K., Kondo, T. & Kaneko, S. (1999) Differential effects of adenosine receptor subtypes on release and reuptake of hippocampal serotonin. *Eur. J. Neurosci.*, **11**, 1–9.

Patel, M.K., Pinnock, R.D. & Lee, K. (2001) Adenosine exerts multiple effects in dorsal horn neurones of the adult rat spinal cord. *Brain Res.*, **920**, 19–26.

Ren, H. & Stiles, G.L. (1994) Posttranscriptional mRNA processing as a mechanism for regulation of human A1 adenosine receptor expression. *Proc. Natl Acad. Sci. USA*, **91**, 4864–4866.

Rivkees, S.A., Chen, M., Kulkarni, J., Browne, J. & Zhao, Z. (1999) Characterization of the murine A1 adenosine receptor promoter, potent regulation by GATA-4 and Nkx2.5. *J. Biol. Chem.*, **274**, 14204–14209.

Rosin, D.L., Robeva, A., Woodard, R.L., Guyenet, P.G. & Linden, J. (1998) Immunohistochemical localization of adenosine A2A receptors in the rat central nervous system. *J. Comp. Neurol.*, **401**, 163–186.

Schiffmann, S.N. & Vanderhaeghen, J.J. (1993) Adenosine A2 receptors regulate the gene expression of striatopallidal and striatonigral neurons. *J. Neurosci.*, **13**, 1080–1087.

Weaver, D.R. (1993) A2a adenosine receptor gene expression in developing rat brain. *Mol. Brain Res.*, **20**, 313–327.

Welch, W.J. (2002) Adenosine A1 receptor antagonists in the kidney: effects in fluid-retaining disorders. *Curr. Opin. Pharmacol.*, **2**, 165–170.