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Adenosine A_{2A} receptor gene expression in the normal striatum and after 6-OH-dopamine lesion

A. Kaelin-Lang[1]*, **P. Liniger**[1], **A. Probst**[2], **T. Lauterburg**[1], and **J.-M. Burgunder**[1]

[1] Neuromorphological Laboratory, Department of Neurology, Inselspital, University Hospital, Bern, and
[2] Institute of Pathology, University of Basel, Switzerland

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Summary. Adenosine A_{2A} receptors are present on enkephalinergic medium sized striatal neurons in the rat and have an important function in the modulation of striatal output. In order to establish more accurately whether adenosine transmission is a generalized phenomenon in mammalian striatum we compared the A_{2A} R expression in the mouse, rat, cat and human striatum. Secondly we compared the modulation of enkephalin gene expression and A_{2A} receptor gene expression in rat striatal neurons after 6-OH-dopamine lesion of the substantia nigra. Hybridization histochemistry was performed with a ^{35}S-labelled radioactive oligonucleotide probe. The results showed high expression of A_{2A} adenosine receptor genes only in the medium-sized cells of the striatum in all examined species. In the rat striatum, expression of A_{2A} receptors was not significantly altered after lesion of the dopaminergic pathways with 6-OH-dopamine even though enkephalin gene expression was up-regulated. The absence of a change in A_{2A} receptor gene expression after 6-OH-dopamine treatment speaks against a dependency on dopaminergic innervation. The maintained inhibitory function of A_{2A} R on motor activity in spite of dopamine depletion could be partly responsible for the depression of locomotor activity observed in basal ganglia disorders such as Parkinson's disease.

Keywords: Mouse, cat, human, rat, striatum, adenosine A_{2A} receptors, 6-OH-dopamine.

Introduction

The adenosine A_{2A} receptors (A_{2A}R) play an important role in the control of locomotor activity by the mammalian striatum. The presence of striatal A_{2A}R

* Present address: National Institute for Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, U.S.A.

was demonstrated by binding studies (Jarvis and Williams, 1989) as well as by in situ hybridization histochemistry techniques (Fink et al., 1992). Several studies have shown that the striatal $A_{2A}R$ are colocalized with dopamine D2 receptors on medium-sized cells with striato-pallidal GABA-enkephalinergic projections (Schiffmann et al., 1991a; Fink et al., 1992; Augood and Emson, 1994). Activation of $A_{2A}R$ acts antagonistically to dopamine D2-receptor activation and depresses locomotor activity whereas $A_{2A}R$ blockade enhances locomotor activity (Hauber et al., 1998). The activation of $A_{2A}R$ inhibits GABA release from striatal cells (Kirk and Richardson, 1995) and a postreceptor antagonistic interaction has been demonstrated between $A_{2A}R$ and dopamine D2 receptors (Ferré et al., 1994). This functional antagonism probably underlies the adenosinergic modulation of striatal output (Janusz and Berman, 1992). In order to find out whether adenosine transmission is a generalized phenomenon in mammalian brain we compared $A_{2A}R$ expression in the mouse, rat, cat and human striatum by using in situ hybridization histochemical techniques. Secondly, enkephalin gene expression and $A_{2A}R$ gene expression in the rat striatum were compared after lesion of the rat substantia nigra with 6-OH-dopamine, a widely used animal model of Parkinson's disease.

Materials and methods

Striatum of normal animals

The experiments were carried out in accordance with the NIH guidelines and the regulations of the Swiss cantonal veterinary authority. Adult female Wistar rats (n = 4) maintained on a 12/12 h light/dark cycle were used. Twenty adult mice maintained on a 10/14 h light/dark cycle and one adult cat were used. Rats and mice were killed by decapitation. The deeply anaesthetized, relaxed and intubated cat, used for another study, was killed by phenobarbital overdose. Details on cat anesthesia are described elsewhere (Kaelin-Lang et al., 1999). Brain tissue was rapidly removed, frozen on dry ice or in nitrogen-cooled isopentane and kept at −70°C until further processing.

Human brains

Brains from 3 male subjects (52, 63, 88 years old) were removed 4 to 7 h after death. Tissue samples including the head and the corpus of the caudate nucleus, the lentiform nucleus containing the putamen and globus pallidus were dissected from the fresh brain, frozen and kept at −70°C. No history of neurological disorders, especially Parkinson's disease was known in these patients. The family relatives gave consent for the autopsy.

6-OH-dopamine treatment (Young et al., 1986)

Adult female Wistar rats were anaesthetized with chloral hydrate (10%, 0.0035 × body weight in grams) administered intra-peritoneal (n = 16). After positioning on a small animal holder, a drill hole was made on the right side of the sagittal suture, 4.4 mm caudally and 1.3 mm laterally from bregma, using the atlas of Paxinos and Watson (Paxinos and Watson, 1986). A sharp-tipped cannula was lowered 7.7 mm beneath dura level through the drill hole. The tip of the cannula was placed into the medial forebrain bundle rostral to the substantia nigra. Treated animals (n = 8) were injected with 4 μl of toxin (6,6 mg of 6-hydroxy-dopamine in 1 ml of 0.9% NaCl and 0.1% ascorbic acid) whereas control animals (n = 8) received 4 μl of vehicle over four min. The cannula

was left in place for another 4 min. The brains were removed 2 weeks later as described above.

Hybridization histochemistry

Series of 12 μm adjacent sections of the striatum of all species were cut on a cryostat microtome at −18°C and mounted onto gelatin-coated slides. Coronal sections were cut through the striatum of the injected animals. Hybridization histochemistry was done as described elsewhere (Young et al., 1986; Kaelin-Lang et al., 1998) using an oligonucleotide (48mer: GACCGAGTCCGCTCCCCTGGCAGGGGCTGGCTCTCCATCTGCTTCAGC) recognizing bases 891 to 938 of the A_{2A}R rat gene (Chern et al., 1992). This probe has an homology of 93% (45/48) with the A_{2A} mRNA sequence in the mouse (Ledent et al., 1997). The homology of the whole probe with the human A_{2A} mRNA sequence (bases 1,110 to 1,157) is 90% (43/48) (Furlong et al., 1992). The sequence of the gene coding for the A_{2A}R is not known in the cat. The second oligonucleotide (48mer: ATCTGCATCCTTCTTCATGAAACCGCCATACCTCTTGGCAAGGATCTC) recognizes bases 388 to 435 of the rat enkephalin gene (Young et al., 1986). Labeling was performed using alpha-[^{35}S]deoxyadenosine-triphosphate (Amersham Laboratories) and terminal deoxynucleotidyl-transferase (Boehringer Mannheim).

As controls, oligonucleotides with unrelated sequences and similar G-C content were used on consecutive parallel sections. In order to exclude unspecific binding, incubation was also performed with a 100 times higher concentration of unlabelled probe together with the radioactive probe. The slides were placed for 1 week on X-ray film (Kodak X-omat AR) for visualization. Selected slides were also dipped into Kodak autoradiography emulsion (NTB 3), developed after 6 to 10 weeks and then counterstained with 0,2% toluidine blue. The expression of A_{2A}R mRNA was evaluated using density measurements on film autoradiograms and dark field microscopy (Zeiss Axiophot microscope) with visual counts of the positive cells. Three atlases were used (Snider and Niemer, 1964; Sidman et al., 1971; Paxinos and Watson, 1986).

For the quantification of A_{2A} and enkephalin gene expression, film autoradiograms were digitized using a Cohu CCD camera and analyzed with the "IMAGE" software (NIMH, W. Rasband). The signal density over the caudate/putamen was measured after manual delineation of the region of interest and standardized using sections of brain paste containing known amounts of radioactivity. Four slices cut in the mean part of the striatum were used for each rat. Both ipsilateral and contralateral sides of the striatum were measured in treated and control animals. Differences between the means of the 4 groups (treated rats ipsi- and contralateral, control rats ipsi- and contralateral) were then statistically analyzed with StatView software. ANOVA with Fisher correction was performed. The significance level was set to $p < 0.05$.

Results

High A_{2A}R mRNA expression was found in the striatum, the nucleus accumbens and the olfactory tubercle in all species (Figs. 1, 2, 3). In the striatum, the positive cells were medium-sized neurons (Fig. 2). The large cells were always negative (Fig. 2). The small cell bridges between the nucleus accumbens and the olfactory tubercle in the rat and mouse striatum also revealed a high level of A_{2A}R mRNA. The proportion of A_{2A}R-positive cells in the striatum varied between 18 and 29% of the total cell population. The positive cells were distributed in small clusters of about 10 cells. This clustering was best seen in the mouse and rat striatum. In the human brain, the proportion of A_{2A}R-positive cells varied between 24% and 38% and was slightly higher in the corpus of the caudate nucleus than in other parts of the

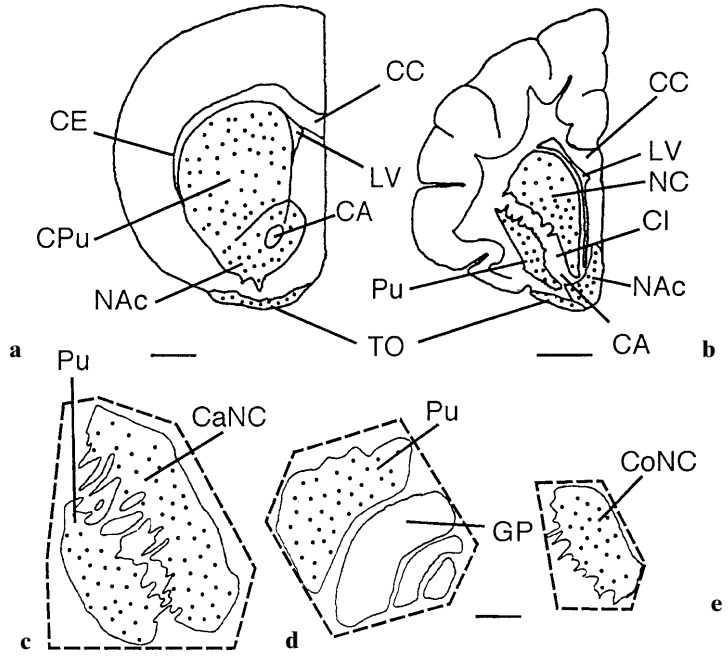

Fig. 1. Localization of $A_{2A}R$ expressing cells (one black circle = 30 neurons in **a**, 90 in **b** and 70 in **c,d** and **e** in the basal ganglia. **a** Rat and mouse striatum, **b** cat striatum; **c** human striatum, head of the nucleus caudatus (CaNC) and putamen (Pu); **d** human putamen (Pu) and globus pallidus pars lateralis and medialis (GP); **e** human striatum, corpus of the caudate nucleus (CoNC). **c,d,e** Dashed lines represent the borders of the removed tissues. *CE* external capsule, *NAc* nucleus accumbens, *TO* olfactory tubercle, *CC* corpus callosum, *CA* anterior white commissure, *CI* internal capsule, *LV* lateral ventricle, (calibration bar in **a**: 1mm, in **b,c,d,e**: 5mm)

striatum. No $A_{2A}R$ gene expression was found in the human globus pallidus. The expression of $A_{2A}R$ gene was completely abolished in control sections of all species in presence of an excess of unlabelled probe confirming the specificity of the oligoprobe used.

The striatum of 6-OH-dopamine treated rats showed an increase in enkephalin mRNA expression on the right injected side (Fig. 3). This increase was highly significant ($p < 0.0001$). The left contralateral side of treated rats as well as the control animals showed no significant change in enkephalin gene expression. This typical pattern of enkephalin gene expression after 6-OH-dopamine treatment confirmed the effectiveness of the 6-OH-dopamine injection. In contrast the analysis of film autoradiography revealed no significant differences in the expression of $A_{2A}R$ between treated and non-treated animals (Fig. 3). In treated animals, no difference was observed between the injected side and the contralateral side. Furthermore, no difference between the injected and the control side of control animals was found.

Discussion

The first binding studies using radioactive labeled CGS21680, a specific ligand, suggested a localization of $A_{2A}R$ mostly in the rat caudate-putamen, nucleus

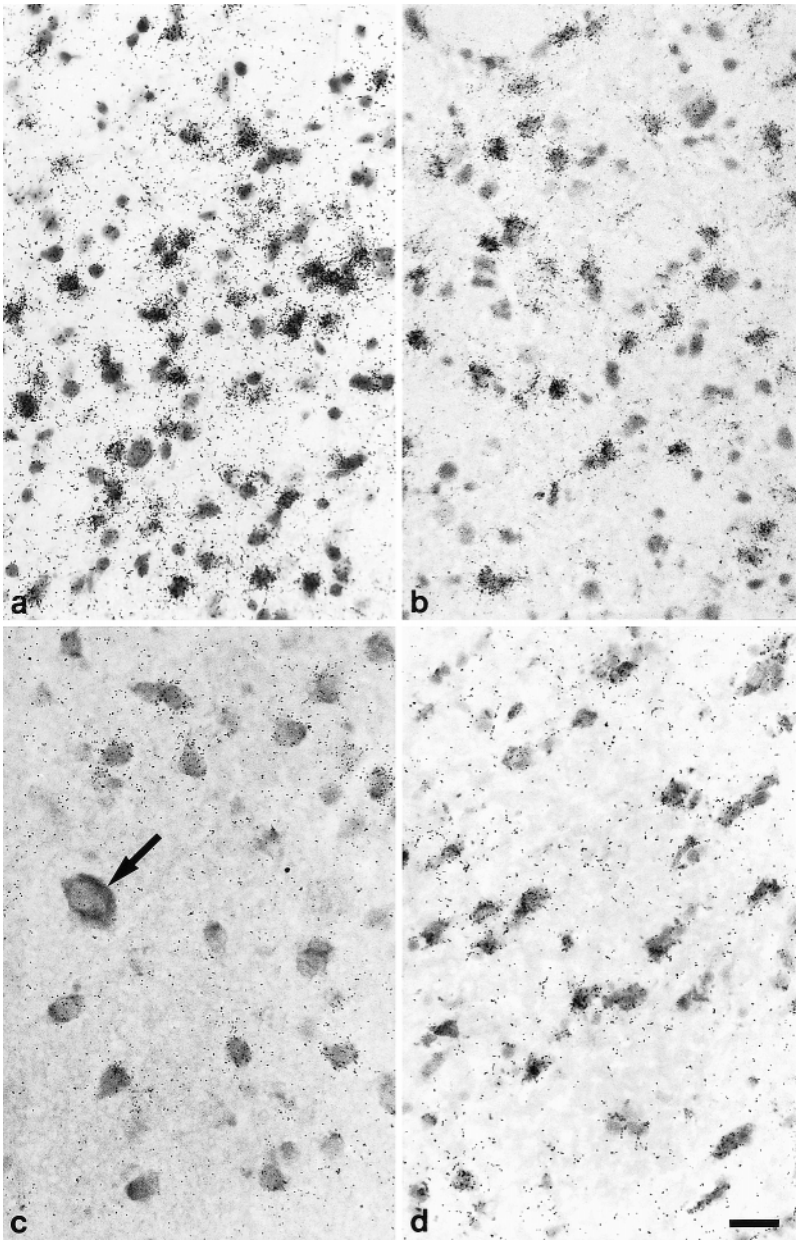

Fig. 2. Expression of $A_{2A}R$ gene in the striatum (bright field microscopy, counterstain with toluidin blue, higher magnification). **a** Mouse; **b** rat; **c** cat; **d** human (head of the caudate nucleus). Arrow: a large cell without $A_{2A}R$ gene expression. (bar: 25 micrometers)

accumbens and olfactory tubercle (Jarvis and Williams, 1989; Wan et al., 1990). Our results confirm previous reports revealing strong $A_{2A}R$ gene expression in medium-sized cells of the caudatum-putamen, the nucleus accumbens, and the olfactory tubercle (Schiffmann et al., 1991b; Fink et al., 1992; Augood and Emson, 1994; Svenningson et al., 1998). In these regions

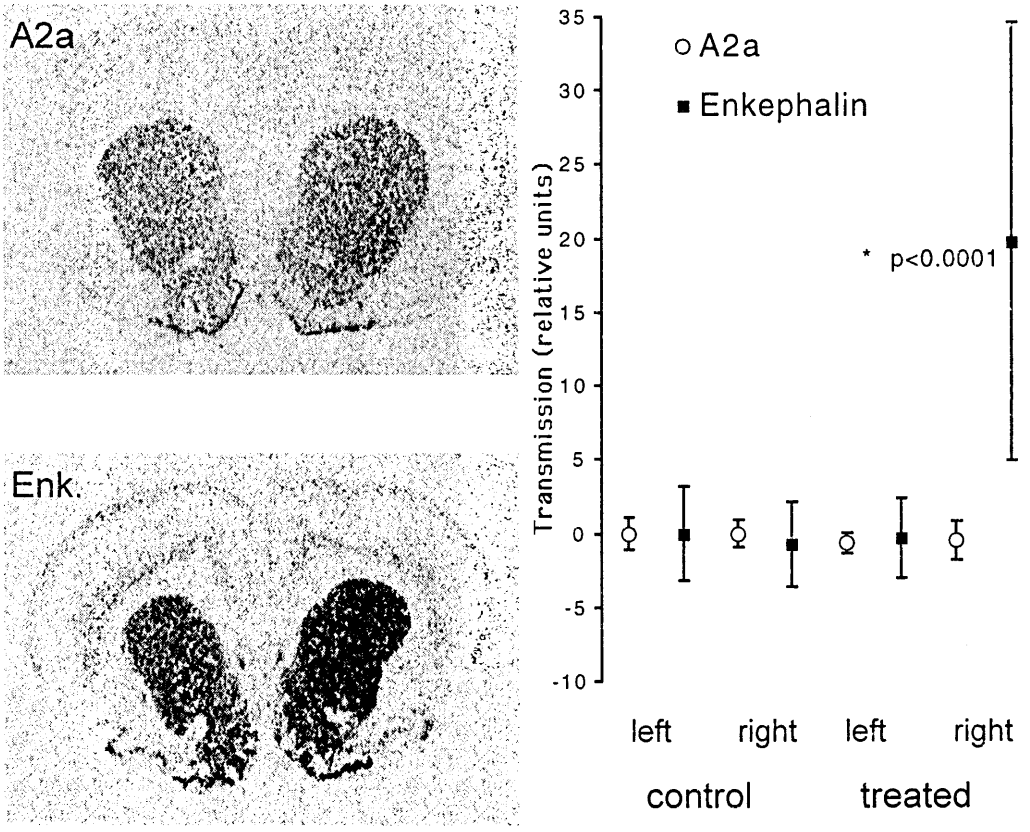

Fig. 3. *Left*: film autoradiography of the striatum of a treated rat. "*A2a*" A_{2A} receptor gene expression, "*Enk*" enkephalin gene-expression. Up-regulation of enkephalin gene expression is seen on the right side, ipsilateral to the injection of 6-OH-dopamine. *Right*: relative transmission values were measured on film autoradiography in the striatum ipsilateral to the 6-OH-dopamine lesioned side (treated right), in the contralateral striatum of the treated animals (treated left) and in both striatum of control animals (control left, control right). No significant differences were seen between the 4 groups for $A_{2A}R$. Enkephalin gene-expression on the right side of treated animals is significantly higher than in the other groups. Data are expressed as mean with SD, the left side of control rats (contralateral to the injection) was used as reference and set to zero

the pattern of $A_{2A}R$ gene expression is similar in the rat, mouse, cat, and human brain. Only the medium-sized striatal cells showed a hybridization signal. This demonstrates the conservation of $A_{2A}R$ gene expression in the medium sized striatal cells among mammalian species including man and indicates an important physiological function of $A_{2A}R$ in the mammalian striatum. In the first in situ hybridization histochemistry studies, $A_{2A}R$ gene expression was found only in the medium sized striatal cells (Schiffmann et al., 1991b; Fink et al., 1992; Augood and Emson, 1994) whereas another report (Dixon et al., 1996) describes a weaker $A_{2A}R$ gene expression in 26% of the large cholinergic striatal cells. Physiological evidences also suggest that $A_{2A}R$ regulate acetylcholine release in striatal cells (Kirk and Richardson, 1995). The differences between our study and the study of Dixon et al. (1996) is

probably due to the different technique and probes used. With the more sensitive technique of RT-PCR (reverse transcription-polymerase chain reaction) $A_{2A}R$ gene expression has been found in tissue extracts from all brain regions (Dixon et al., 1996), speaking for a wide $A_{2A}R$ expression in other non-striatal, neuronal or non neuronal cells. It is therefore possible that $A_{2A}R$ are also expressed in other striatal cells below the detection limits of our technique. However in all previous studies the highest $A_{2A}R$ gene expression was always found in the medium sized striatal cells.

This expression of striatal adenosine $A_{2A}R$ is not altered by 6-OH-dopamine lesion of the nigrostriatal dopaminergic pathway. In contrast, enkephalin gene expression was ipsilateraly up-regulated in the treated animals according to a previous report (Young et al., 1986), although enkephalin and $A_{2A}R$ are expressed in the same cells.

These results suggest that $A_{2A}R$ gene expression in the striatum is not dependent on dopaminergic innervation. In a previous study, no change in A_{2A} binding sites was found in the 6-OH-dopamine model in rats (Morelli et al., 1994) and in guinea pig (Martinez-Mir et al., 1991). In another model, low dose of 6-OH-dopamine injected directly in the striatum (Przedborski et al., 1995) does not change $A_{2A}R$ binding in spite of an increase in dopamine D2 receptors binding sites. Together with our results, this suggests that neither the expression of the A_{2A} gene nor the binding-affinity of the receptor is changed after lesion of nigrostriatal dopaminergic pathways. On the other hand, the $A_{2A}R$ agonist CGS21680 triggers c-fos protein expression in the striatum of 6-OH-dopamine treated animals but not in control animals (Morelli et al., 1995). This suggests a hypersensitivity of striatal neurons to $A_{2A}R$ activation after lesion of the dopaminergic pathway, without changes in $A_{2A}R$ mRNA levels. The loss of the inhibitory function of D2 receptors on adenylate cyclase in GABAergic striatal neurons may explain this hypersensitivity, since activation of D2 receptors inhibit the A_{2A}-induced c-fos expression (Morelli et al., 1995). In other words, it seems that the unchanged $A_{2A}R$ activity in spite of dopamine D2 receptor underfunction causes a relative adenosinergic $A_{2A}R$ hyperfunction in the 6-OH-dopamine-model. $A_{2A}R$ antagonists can modulate this adenosinergic tone, since adenosine antagonists reversed the changes in enkephalin gene expression induced by 6-OH-dopamine lesion of the nigrostriatal pathways (Schiffmann and Vanderhaeghen, 1993).

In contrast to our results, chronic treatment with haloperidol, a typical neuroleptic which antagonizes dopamine D2-receptors, increases the binding of the $A_{2A}R$ agonist CGS21680, suggesting an up-regulation of the $A_{2A}R$ together with the up-regulation of dopamine D2 receptors (Parsons et al., 1995). The differences between the effects of a selective lesion of the nigrostriatal pathways with 6-OH-dopamine and the effects of neuroleptic treatment suggest that $A_{2A}R$ regulation depends on other mechanisms than the nigrostriatal dopaminergic input. It is also possible that neuroleptics modulate $A_{2A}R$ by mechanisms other than D2 antagonism, such as direct influences on $A_{2A}R$ or changes in the global extracellular adenosine concentration (Parsons et al., 1995).

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Authors' address: Dr. A. Kaelin-Lang, National Institutes of Health, National Institute for Neurological Disorders and Stroke, Building 10, Room 5N234, 10 Center Drive, MSC 1430, Bethesda, 20892 MD, USA, e-mail: KaelinA@ninds.nih.gov