DOPAMINE D1, D2 AND MU-OPIOID RECEPTORS ARE CO-EXPRESSED WITH ADENYLYL CYCLASE 5 AND PHOSPHODIESTERASE 7B MRNAS IN STRIATAL RAT CELLS.

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Abstract.

Intracellular cAMP levels are regulated by cAMP synthesis and degradation rate. Nine isoforms of cAMP-synthesizing enzymes called adenylyl-cyclases (ACs) and eleven phosphodiesterases (PDEs) that degrade cyclic nucleotides have been identified. Both types of enzymes exhibit variations not only in their expression pattern distribution throughout the brain, but also in their regulatory characteristics. Different isoforms of ACs and PDEs may be co-expressed in a single cell, thus a gradient of cAMP intracellular levels is formed, which accounts for the diversity of cell responses. Among these isoforms, AC5 and PDE7B are highly expressed in striatum, where the cAMP pathway is implicated in diverse behavioural functions. Striatal AC5 is involved in drug reinforcing actions and motor activity. Less is known about the role of the PDE7B isoenzyme. We performed a double in situ hybridization analysis of the co-expression patterns of AC5 and PDE7B with μ -opioid-receptor, D1- and D2-receptor mRNAs to contribute to a better understanding in the regulation of cAMP levels under dopamine or opiodergic pathway activation in striatum. We found co-expression of AC5 and PDE7B mRNAs in caudate-putamen and nucleus accumbens; we also encountered that more than 50% of MOR, D2- and D1-expressing cells contained AC5 and PDE7B mRNAs. The presence of AC5 and PDE7B mRNAs in D1- and D2-containing cells suggests the participation of these enzymes in striatal functions involving dopaminergic pathways. Co-localization of both isoenzyme mRNAs with MOR expressing cells suggests their involvement in opioid reinforcing effects.

Keywords: addiction, locomotion, AC5, PDE7B, double in situ hybridization.

Abbreviations.

AC adenylyl cyclase
cAMP cyclic adenosine monophosphate
dATP desoxy adenosine triphosphate
DA dopamine
D1 dopamine 1 receptor
D2 dopamine 2 receptor
KO knock-out
MOR μ -opioid receptor
mRNA messenger ribonucleic acid
MSN medium spiny neurons
NTR neurotransmitter receptor
PDE phosphodiesterase
PKA protein kinase A
SN substantia nigra
VTA ventral tegmental area

Introduction.

cAMP signalling cascades are involved in a wide variety of cellular responses. Cyclic nucleotide levels are tightly regulated by cAMP synthesis and degradation rate. Adenylyl cyclases (ACs) are membrane-bound enzymes responsible for the formation of cAMP (Houslay et al. 1997), while phosphodiesterases (PDE) are hydrolyzing enzymes that convert cyclic nucleotides (3', 5'-cyclic nucleotides) into the biological inactive 5'-nucleoside monophosphate. There are ten mammalian AC isoforms (nine transmembrane and one soluble form) (Defer et al. 2000, Hanoune et al. 2001) and 11 PDE families with approximately 50 isoforms (Conti et al. 1999, Houslay et al. 1997). Different isoforms of ACs and PDEs may be co-expressed in a single cell to yield the differential regulatory properties of each isoform. As a result, a gradient of cAMP intracellular levels is originated that accounts for the diversity of cellular responses. Moreover, the specific set of ACs and PDEs isoforms ensures unique responses of different cells to the same stimulus. The analysis of the cellular and sub-cellular expression patterns of specific ACs and PDEs could provide further insight not only in the regulation of cAMP levels but in its functional roles as well.

In brain, cAMP levels have been implicated in genetic transcription processes (Lalli et al. 1994), ionic channel activation, dopaminergic neuron survival (Yamashita et al. 1997), synaptic plasticity (Zhong et al. 1991), neurotransmitter release (Bouron et al. 1999, Chen et al. 1997, Kandel et al. 1982), locomotion, sensory perception, as well as in memory and learning processes (Frey et al. 1993, Tully et al. 2003).

In striatal cells, the increase in cAMP levels leads to activation of protein kinase A (PKA) which plays a role in reward mechanisms of drug consumption, locomotion activation and anxiety (Kim et al. 2008). The striatum contains dopaminergic terminals which receive information from the ventral tegmental area (VTA); 80% of the cells in this structure are medium spiny neurons (MSN) (Coskran et al. 2006, Seeger et al. 2003) containing dopamine 1 (D1) or dopamine 2 (D2) receptors. Striatal cells -integrators of dopaminergic signalling from VTA, substantia nigra (SN), as well as from cortical and thalamic afferent messages- are capable of regulating motor, sensory or cognitive action patterns. Thus, the control of the cAMP/protein kinase A (PKA) pathway is very strict and its impairment could be responsible for the development of psychotic processes, neurological alterations such as Parkinson's and Alzheimer's diseases and anxiety-related disorders.

D1 receptor activation enhances cAMP levels while D2 activation causes a decrease by their coupling to a G_s or G_i protein G to produce positive or negative adenylyl cyclase activity, respectively. Some AC activities and expression have been identified in striatum

(AC1, AC5, AC8, AC9), being AC5 the most abundant in that brain region (Glatt et al. 1993, Matsuoka et al. 1997). AC5 is involved in almost all the known functions regulated by the striatum: its expression is important although not essential for morphine effects after μ -opioid receptor (MOR) activation, which supports its role in drug-preference behaviour (Kim et al. 2006). Besides, AC5 has been proposed as the key AC activity of striatum for the dopamine effects on locomotion induced by D2 receptor agonists, but not by those of D1 (Lee et al. 2002). In ventral striatum (nucleus accumbens), AC5 has been implicated in the anxiety behaviour displayed by animals as a response to stressful stimuli (Kim et al. 2008). Efforts have been made to involve AC5 in behaviours controlled by striatum in studies performed with AC5^{-/-} KO animals, to assay its main role in the locomotion produced by drug addiction. However, it is also possible that the enhancement of other AC activities, that exert a compensatory effect due to the lack of AC5 expression in those animals, has provided false negative results concerning AC5 participation in some striatal processes. Also, since AC5 specific inhibitors are lacking, its participation in different striatal functions is not conclusive.

Reinforcement of drug action and locomotion activation are two functional processes regulated by striatum which are at least in part modulated by the opioidergic system. Ethanol-induced DA release activation in striatum seems to involve μ opioid receptors (MORs), which are highly expressed in this region (Koob 1992). MORs are negatively coupled to AC activity and their activation has been related to the stimulation of the dopaminergic pathway (Nestler 2004). However, it has not yet been defined which AC activities and mRNAs are expressed in cells containing MOR, D1 or D2 receptors, that would contribute to clarify the role of enzymes AC5 and PDE7B in the regulation of cAMP levels in addictive behaviour, locomotion activation by drugs, or cognitive processes.

Given that striatal cells receive diverse afferent signals, they need to integrate and discriminate them to trigger a specific cell response; thus, neurons may be responding to a stimulus and at the same time they must be prepared for a subsequent input. Presence of different sets of synthesizing and degrading ACs and PDEs in striatal cells ensures versatile response patterns to the afferent stimuli. If an incoming signal activates an AC enzyme enhancing cAMP levels, PDEs in striatal cells may contribute to the feedback mechanisms that maintain cAMP content at basal levels. Among the PDEs expressed in striatum (Menniti et al. 2006) (PDE1B, PDE4, PDE7B, PDE10A), PDE7B is an isoenzyme of the PDE7 family that has another member, PDE7A, also expressed in the basal ganglia, although not in the nucleus accumbens, where PDE7B is abundant (Reyes-Irisarri et al. 2005). While PDE7A has been involved in immune cell responses (Yang et al. 2003), PDE7B has been implicated in dopamine signaling: the addition of a DA agonist to striatal

cells enhances PDE7B expression (Sasaki et al. 2004). Among the phosphodiesterases expressed in striatum, PDE7B is the single-substrate degrading PDE with the highest affinity for cAMP; thus, it could be regulating cyclic nucleotide levels at basal conditions (Menniti et al. 2006).

Striatal cells have a differential localization and expression of PDEs. In fact, the participation of these enzymes in motor and cognitive functions might be due to the specific PDE involved. Since the use of PDE7B specific inhibitors has not yet been possible, the identification of PDE7B sub-cellular distribution in striatum by the analysis of the phenotype of its expressing-cells (and its proportion in every cell population) could help to understand regulatory processes involved and to identify the controlling factors associated with PDE activities and expression. It would also contribute to the design of specific pharmacological agents for the treatment of different disorders, minimizing secondary effects of PDE inhibition.

Despite the high striatal expression levels of AC5 and PDE7B, co-localization studies of their mRNAs with MOR and dopamine receptors are lacking. Therefore, the aim of this study is to analyze the cellular phenotype of AC5 and PDE7B expressing-cells in striatum by double *in situ* hybridization histochemistry for both enzyme mRNA expressions, to obtain information on the co-localization of the above mentioned neurotransmitter receptors with these particular enzymes, as well as their potential participation in different basal ganglia functions and the possible regulatory characteristics of the cAMP pathways in which AC5 and PDE7B might be participating.

Results.

Using double *in situ* hybridization histochemistry, we performed co-localization analysis of mRNAs coding for different neurotransmitter receptors (NTRs) viz., MOR (Fig 1A), D1 (Fig 1D), and D2 (Fig 1G), with those of cAMP level-regulating enzymes particularly enriched in striatum, such as phosphodiesterase type 7B (PDE7B) (Fig 1J) (Reyes-Irisarri et al. 2005) and adenylyl cyclase type 5 (AC5) (Fig 1K) (Sanabra and Mengod, unpublished data and (Matsuoka et al. 1997)).

Autoradiographic signals showed specific hybridization patterns for μ -opioid, D1 and D2, receptors and PDE7B in striatum, as it has been described elsewhere (Mansour et al. 1994, Mengod et al. 1989, Mengod et al. 1991, Reyes-Irisarri et al. 2005) (Fig 1A, D, G, J), supporting the selectivity of the probes used.

MOR signal was predominant in the dorsal part of striatum (Fig 1A); several MOR expressing-neurons contained AC5 (Fig 1B, Table 1) or PDE7B mRNA at similar

proportions ($F_{(2,18)} = 0.189$, $p > 0.05$) (Fig 1C, Table 1). The number of cells expressing MOR/AC5 or MOR/PDE7B mRNAs did not vary between antero-posterior striatal levels ($F_{(2,7)} = 0.254$, $p > 0.05$; $F_{(2,8)} = 0.017$, $p > 0.05$, respectively). We observed a similar distribution in nucleus accumbens cells containing simultaneously MOR and AC5 mRNAs or MOR/PDE7B ($F_{(1,4)} = 4.45$, $p > 0.05$) (Table 1).

A larger extent of caudate neurons ($64 \pm 0.6\%$) contained simultaneously MOR and AC5 mRNAs than in n. accumbens ($53 \pm 8\%$) (Table 3) ($F_{(1,13)} = 8.34$, $p < 0.05$); while caudate vs n. accumbens distribution of MOR/PDE7B cells was similar ($F_{(1,5)} = 3.6$, $p > 0.05$) (Table 1).

We counted twice the cells expressing simultaneously AC5 and PDE7B mRNAs. The first time, single digoxigenin labeled cells containing AC5 mRNA were taken as 100%; next we looked for all AC5 positive cells co-expressing PDE7B mRNA. The second time, we counted the radioactively labeled cells containing PDE7B and took this cell population as 100%; subsequently, we found that 65% of PDE7B-expressing cells contained AC5 mRNA (Fig 1L, Table 2).

A similarly high extent of D1 positive neurons expressing AC5 or PDE7B mRNAs in caudate was observed ($F_{(2,21)} = 0.068$, $p > 0.05$) (Fig 1E,F; Table 1), with no difference between rostral and caudal striatum (AC5: $F_{(2,9)} = 1.71$, $p > 0.05$; PDE7B: $F_{(2,9)} = 2.27$, $p > 0.05$). We found a different distribution extent of D1/AC5 cells between dorsal and ventral striatum ($F_{(1,18)} = 7.265$, $p < 0.05$) (Table 1) while the D1/PDE7B population was similar between both areas ($F_{(1,18)} = 0.713$, $p > 0.05$). Although the percentage of D1/AC5 or D1/PDE7B co-expressing cells was similar among the three caudate section levels, nucleus accumbens presented differences (D1/AC5 (72%) vs D1/PDE7B extent (83%)): ($F_{(1,14)} = 23.66$, $p < 0.001$).

Many of the D2 cells expressed AC5 or PDE7B mRNA in a similar extent ($F_{(2,18)} = 0.32$, $p > 0.05$) (Fig 2H, I; Table 1); no difference was found between rostral and caudal striatum (D2/AC5: $F_{(2,9)} = 3.57$, $p > 0.05$; D2/PDE7B: $F_{(2,7)} = 2.59$, $p > 0.05$). In contrast, there were differences between dorsal and ventral distribution of D2/AC5 ($F_{(1,18)} = 19.061$, $p < 0.001$) and D2/PDE7B ($F_{(1,16)} = 23.737$, $p > 0.001$). The percentage of D2/AC5 and D2/PDE7B cells in nucleus accumbens was similar (Table 1).

Regarding AC5 or PDE7B positive cells as 100%, the extent of neurons co-expressing AC5/D1 and PDE7B/D1 was the highest (59, and 58% respectively) and similar to the percentage of AC5/MOR and PDE7B/MOR neurons, supporting the expression of these four mRNAs in a high proportion in striatal neurons. AC5/D2 and PDE7B/D2 cells were present in a lower extent (Table 3).

Discussion.

Regulation of cAMP levels plays a key role for striatum-controlled functions such as movement behaviour and drug reinforcement effects. Opioid or ethanol repeated administration enhances cAMP, CREB and PKA levels and phosphorylated factors as targets for CREB (Koob 1992, Nestler 2004). These increased cAMP levels have been related to behavioural changes induced by drugs, such as aversive state of animals during drug withdrawal (Nestler et al. 1997), as well as drug tolerance, dependence and reward mechanisms.

Locomotion is another behaviour altered by drugs (Barrot et al. 2002, Nestler 2004) and regulated by striatal pathways. This process includes D1 receptor activation by psychostimulant administration, as well as increased cAMP levels in striatum. Thus, enzymes regulating cAMP levels in the striatum are highly important given the variety of drug mechanisms and neurodegenerative processes that depend on dopamine effects for the tuning of cyclic nucleotide content. Among those cAMP synthesizing enzymes expressed in striatum, AC5 is the most abundant. Efforts have been made to relate the activity of this enzyme to striatum-controlled functions, as well as to different behaviours triggered by D1- and D2-receptor activation (Kim et al. 2008, Kim et al. 2006, Lee et al. 2002).

Dopamine released in the striatum acts on medium spiny neurons containing D1 and D2 receptors, to produce an integrated behavioural response of animals to dopamine tone. D2 agonist administration in striatum is able to reduce increased AC activity provoked by D1-stimulation. The importance of AC5 for the balance of these two D1 and D2 regulated pathways in the control of locomotion is evidenced in studies with AC5^{-/-} KO mice, in which the D2-G_{ai}-AC system is dependent on AC5 activity to trigger the behavioural response induced by dopamine agonists; in contrast, for D1 receptors, AC5 seems to be important, but the locomotion displayed by animals also needs of other non AC5 factors (Lee et al. 2002).

Besides the dopamine system, the opioidergic pathway is relevant to drug tolerance and dependence, as well as to reward processes of addiction controlled by striatum (Koob 1992). Dopamine release in striatum affects the opioidergic system directly and induces the characteristic behaviours of drug addiction. AC5 activity has also been involved in the locomotion activation due to morphine administration. In AC5^{-/-} KO mice, morphine and MOR agonists induce a slighter decrease in striatal AC activity after forskolin administration and weaker, though not blocked effects on locomotion and drug preference than wild type animals (Kim et al. 2006). This suggests that AC5 is important but not essential for MOR activation.

In this study, we analyzed whether D1, D2 and MOR receptors are co-expressed with AC5, a cAMP-synthesizing enzyme, and PDE7B, a degrading one. We were also interested in the proportion of striatal receptor-containing cells expressing AC5 and PDE7B mRNAs. By understanding the regulatory characteristics of each enzyme and its predominance in specific striatal cells, our results could provide further insight into cAMP-pathway regulation in striatum as the key to the diverse behavioural functions controlled by this region. It could also help in the determination of the potential role of AC5 and PDE7B activities in drug-related locomotion and reward mechanisms. Furthermore, it could enable the design of specific inhibitors of these enzymes as therapeutic agents to pathologies related to impaired processes controlled by striatum.

Our results showing more than 50% of MOR positive cells co-expressing AC5 mRNA are in agreement with previous studies that suggest AC5 as the main AC activity involved in morphine behavioural changes through MORs (Kim et al. 2006). Other enzymes such as AC1, AC8 or AC9, also expressed in striatum, may account for residual behavioral effects and AC activity of AC5^{-/-} KO animals after morphine administration (Kim et al. 2006). This evidence indicates that each enzyme regulates cAMP levels in a stimulus-specific manner. A high percentage of D1 expressing cells exhibited AC5 mRNA. Given that more caudate-putamen neurons co-expressed D1/AC5 than MOR/AC5 mRNAs (80 vs 64%), it is possible that some D1-mRNA-containing cells express simultaneously AC5 and MOR receptors (McGinty et al. 1998).

We observed co-localization of AC5 with D2 mRNAs; this finding suggests a role for D2 agonists in locomotion, after DA agonist administration through AC5 activation identified in AC5^{-/-} KO animals (Lee et al. 2002). Given the higher percentage of D2/AC5 containing cells than that of MOR/AC5, it is likely that D2/AC5 mRNAs-containing cells are also expressing MORs. This hypothesis could explain the blocked opioid effect on locomotion after morphine administration in AC5^{-/-} KO animals (Lee et al. 2002).

The presence of AC5 in both types of medium spiny neurons may account for the D1 and D2 receptor activation induced by dopamine release participating in the control of locomotion. The higher proportion of D1 and D2 containing cells expressing AC5 may be important for the different cAMP concentrations needed for the diverse behavioural responses displayed by animals after drug administration. Thus, the identification of the phenotype of AC5 expressing cells would be relevant for the design of specific inhibitors with potential use as therapeutic agents in drug addiction.

In nucleus accumbens we found co-localization of AC5 mRNA with D1 and D2 containing cells. In the mesolimbic pathway, dopamine signaling through D1 receptors increases

cAMP levels, when animals are subjected to stressful conditions. Furthermore, activation of the cAMP pathway has been associated with the anxiety behavior displayed by these animals. Since AC5^{-/-} KO mice have an anxiolytic phenotype and higher locomotor and exploring activities; AC5 seems to be the main enzyme implicated in this behaviour. Our results here provide further support for AC5 involvement in reward mechanisms, as well as in mood disorders controlled by nucleus accumbens (Kim et al. 2008) in animals subjected to stress stimuli. Synthesis of other peptides with anxiety-related actions in nucleus accumbens, such as cholecystoquinin (CCK) and dynorphin seems to depend on AC5 activity (Kim et al. 2008). Again, as for dorsal striatum, AC5 may be participating in the balanced response of D1 and D2 containing cells to dopamine and opioid release, as well as, to the feedback regulation of dopaminergic cells in the VTA.

PDE7B is a cAMP degrading enzyme markedly expressed in the striatum with a high degree of specificity for its substrate. Its distribution in medium spiny neurons has been analyzed (Reyes-Irisarri et al. 2005), but the phenotype of the PDE7B containing cells has not been identified. We found here that PDE7B mRNA co-localizes with both D2 and D1 containing neurons in striatum and nucleus accumbens. Since PDE7B mRNA levels are enhanced after D1 agonist administration (Sasaki et al. 2004), it seems that PDE7B activity may return cAMP to basal levels after its increase due to an enhanced AC activity (by D1); this would allow cells to be prepared for the next stimulus. Specific inhibitors of PDE7B may counteract D2 receptor activation, which would in turn potentiate D1 receptor activity, thus participating in the motor control by striatum. PDE7B is known to be absent in dopaminergic cells from the SN and VTA (Reyes-Irisarri et al. 2005) that express D2 with an autorreceptor function. Thus, PDE7B mRNA could be a marker for D2 striatopallidal neurons. Although the former results suggest the non participation of PDE7B in DA release control, it is possible that its activity is involved in DA synthesis and release through the feedback loop of the MSN to SN and VTA, when the dopaminergic tone is increased in striatum.

The low Km of PDE7B for cAMP levels suggests its importance in maintaining cAMP levels in basal conditions, which is relevant for the tonic activation of D2-containing neurons. PDE7B expression in both D1- and D2-expressing striatal neurons suggests its participation in the integrated response of locomotion that depends on the activation of both types of cells.

In this respect, co-localization of PDE7B with D2 containing cells suggests that PDE7B specific inhibitors may exert antipsychotic actions and modulate hyperlocomotion induced by psychostimulants, since inhibiting PDE7B could enhance cAMP levels and counteract D2 receptor activation, as has been proved for other PDEs expressed in striatum, such as

PDE10A (Weber et al. 2009). Also, since PDE7B is constitutively expressed, its activity may account for the tonic activity of the D2-expressing cells in the striatum. However, PDE7B has cAMP as unique substrate in contrast to other phosphodiesterases localized also in striatum, such as PDE10A (this enzyme also degrades cGMP). This characteristic is relevant for the potential counteracting effects of PDE7B inhibitors on D2 activation as therapy for psychosis.

Dopamine has been involved in striatal cell differentiation in rat embryonic cultures through D1 receptor activation (Schmidt et al. 1998); since PDE7B is co-localized with D1 receptors and D1 agonists increase PDE7B expression, it is not unlikely its participation in that cellular process.

Its co-localization with these DA receptors in nucleus accumbens and at the same time with AC5 mRNA, points towards PDE7B as a target for the control of anxiety behaviours involving AC5 activation. Also, since low cAMP levels in nucleus accumbens have been related to the pre-pulse deficiency (PPI) of the startle reaction (Weber et al. 2009), which is considered a behavioural characteristic of schizophrenia appearing in postpartum rats, PDE7B may be a target for the control of this mental illness (Byrnes et al. 2007).

It would be helpful to develop PDE7B inhibitors, since those available that target other PDE activities induce some side effects such as emesis and cardiotoxicity (Barnes et al. 2001).

The presence of PDE7B mRNA in MOR, D1 and D2 expressing cells, together with the observation that all AC5-expressing cells also contain PDE7B mRNA, suggests that all MOR, D1 and D2 positive cells containing AC5 mRNA simultaneously express PDE7B. Thus, PDE7B activity may induce compensating increments of cAMP levels and prepare neurons for a subsequent stimulus after D1 activation. In addition, its constitutive expression (Sasaki et al. 2004) PDE7B may contribute to maintain low cAMP levels in MOR expressing cells, as well as may be in part responsible for the locomotor activation and drug preference induced by morphine.

Its co-localization with MOR in nucleus accumbens indicates that regulation of PDE7B activity may be important for therapy (control) of drug addiction, since increased cAMP levels has been associated with the negative motivational state during drug withdrawal.

The observation of cells co-expressing AC5 and PDE7B enzymes may help to identify their roles as regulating enzymes of cAMP content and their participation in specific striatal functions. Their co-expression in both dorsal striatum and nucleus accumbens suggests that these enzymes may be controlling cAMP levels and in turn CREB phosphorylation among other functions, as well as transcription of opioid peptide genes that are expressed

in both striato-nigral (dynorphin) and striato-pallidal (proenkephalin) cells, which participate in long-term neural and behavioral plasticity in response to drugs. Also, the co-expression of PDE7B and AC5 indicates their possible participation in drug reinforcement mechanisms controlled by VTA neurons, by the activation of the VTA or SN firing rate within the long-loop feedback that includes direct and indirect pathways.

Experimental Procedure.

Tissue preparation

Male Wistar rats averaging 250g in body weight (n=4) were kept in an animal house under controlled light and temperature conditions until they were sacrificed by decapitation. Brains were immediately extracted and frozen on dry ice. Striatal coronal slices (14 μ m thick) were obtained by cutting brains with a microtome-cryostat (Microm HM500 OM, Walldorf, Germany) at -20°C and thaw-mounted onto APTS-coated (3-aminopropyltriethoxysilane; Sigma, St. Louis Mo, USA) slides. Selected sections were located at 1.7, 1, and -0.8 mm coordinates from Bregma (Paxinos G, Watson C. 1998).

Hybridization probes

Oligonucleotides used in this study were complementary to the following base sequences (GenBank Acc. No. inside brackets): AC5, bases 3061-3105, 3172-3216, and 3116-3160 (**NM 022600**); PDE7B, bases 2233-2247, 556-600, 1390-1434, 190-234, and 496-540 (**NM 080894**); μ -opioid receptor, bases 187-231, 1431-1475, 803-850, and 1279-1323 (**NM 013071**); D1 receptor, bases 752-796 (**NM 012546**); D2 receptor bases 347-388 (**NM 012547**). All oligonucleotides were synthesized and HPLC purified by Isogen Bioscience BV (Maarsden, The Netherlands). Evaluation of oligonucleotide sequences with basic local alignment search tool (BLAST) of EMBL and GenBank databases indicated that probes showed no significant similarities with mRNAs others than their corresponding targets in the rat.

Oligonucleotide probes for AC5 and PDE7B were independently labeled with digoxigenin, whereas those for receptors were radiolabeled, except for Figure 1J, in which the PDE7B hybridization signal is radioactive. Oligonucleotides were radioactively labeled at their 3'-end using [α -³³P]dATP (3000 Ci/mmol; New England Nuclear, Boston, MA, USA) for *in situ* hybridization histochemistry experiments; terminal deoxynucleotidyltransferase (Oncogene Research Products, San Diego, CA, USA) was purified using QIA quick Nucleotide

Removal Kit (Qiagen GmbH, Hilden, Germany (Tomiya et al. 1997). Oligonucleotides were non-radioactively labeled with the same enzyme, Dig-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany), in accordance with previously described procedures (Schmitz et al. 1991).

In situ hybridization histochemistry procedures.

Procedures followed for radioactive and non-radioactive labeling of oligonucleotides and for *in situ* hybridization experiments have been published elsewhere (Reyes-Irisarri et al. 2005, Serrats et al. 2003). Briefly, frozen tissue sections were thawed, fixed for 20 min at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS; 1X PBS: 8 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3X PBS at room temperature, twice for 5 min/each in 1X PBS, and incubated for 2 min at 21°C in a pre-digested pronase solution (incubated at 37°C for 4hr and kept frozen in aliquots) (Calbiochem, San Diego, CA, USA) at a final concentration of 24 U/ml in 50 mM Tris-HCl pH 7.5, 5 mM EDTA. Enzymatic activity was stopped by immersion in 2 mg/ml glycine in 1X PBS for 30s. Tissues were finally rinsed in 1X PBS and dehydrated through a graded ethanol series. Radioactively labeled and non-radioactively labeled probes were diluted in a solution containing 50% formamide, 4X SSC (1X SSC: 150 mM NaCl, 15 mM sodium citrate), 1X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl, 20mM phosphate buffer pH 7.0, 250 µg/ml yeast tRNA and 500 µg/ml salmon sperm DNA. Final concentrations of radioactive and Dig-labeled probes in hybridization buffer were in the same range (approximately 1.5 nM). Tissue sections were covered with hybridization solution containing labeled probes, overlaid with Nescofilm coverslips (Bando Chemical Inc., Kobe, Japan) and incubated overnight at 42°C in a humid chamber. Sections were washed four times (45 min/each) in 0.6 M NaCl, 10 mM Tris-HCl pH 7.5 at 60°C, followed by a final 30 min wash in the same buffer at room temperature.

Development of radioactive and non-radioactive hybridization signal.

Hybridized sections were treated as described elsewhere (Landry et al. 2000). Briefly, after washing, slides were immersed for 30 min in a buffer containing 0.1M Tris-HCl pH 7.5, 1M NaCl, 2mM MgCl₂ and 0.5% bovine serum albumin (Sigma) and incubated overnight at 4°C in the same solution with alkaline-phosphatase-conjugated anti-digoxigenin-F(ab) fragments (1:5000; Roche Diagnostics GmbH). Subsequently, slides were washed three times (10 min/each) in the same buffer (without antibody), and twice in an alkaline buffer containing 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 5 mM MgCl₂. Alkaline phosphatase activity was developed by incubating sections with 3.3 mg nitroblue

tetrazolium and 1.65 mg bromochloroindolyl phosphate (Roche Diagnostics GmbH) diluted in 10 ml of alkaline buffer. The enzymatic reaction was blocked by extensive rinsing in the alkaline buffer containing 1 mM EDTA. Sections were then briefly dipped in 70% and 100% ethanol, air-dried, dipped in cholodion and then into Ilford K5 nuclear emulsion (Ilford, Mobberly, Cheshire, UK) diluted 1:1 with distilled water. They were exposed in the dark at 4°C for two weeks for the double-labeled experiment of AC5/PDE7B, and for 4 weeks for the rest of the experiments, and finally developed in Kodak D19 (Kodak, Rochester, NY, USA) for 5 min, and fixed in Ilford Hypam fixer (Ilford). For film autoradiography, hybridized sections were exposed to Biomax-MR (Kodak) films for 3 days at -70°C.

Several routine controls were carried out to determine the specificity of the hybridization signals. As we have previously described (Pompeiano et al. 1992, Reyes-Irisarri et al. 2005), for each of the mRNAs under study, several oligonucleotide probes complementary to different regions of the same mRNA were independently used as hybridization probes in consecutive tissue sections showing identical hybridization patterns. For a given oligonucleotide probe, addition in the hybridization solution of an excess of the same unlabeled oligonucleotide resulted in the complete abolition of the specific hybridization signal. The remaining autoradiographic signal was considered background. If the unlabeled oligonucleotide included in the hybridization solution corresponded to a different oligonucleotide, then the hybridization signal was not affected. The thermal stability of the hybrids was examined by washing at increasing temperatures: a sharp decrease in the hybridization signal was observed at a temperature consistent with the T_m of the hybrids (data not shown).

Analysis of the results.

AC5 or PDE7B mRNA-containing cells were identified as cellular profiles exhibiting a coloured precipitate (alkaline phosphatase reaction product) surrounding or covering the nucleus, clearly distinguishable from the background. MOR, D1 or D2 receptor mRNA hybridization signal was considered positive when accumulation of silver grains over the stained cellular profiles was greater than four times that of the background. Background was defined over those tissue areas negative for hybridization signal. Due to the presence of silver grains at different focal levels, a careful examination of each putatively positive cell was carried out under the microscope to validate positive signals. Cells were counted manually using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a Darklite illuminator (Micro Video Instruments, Avon, MA, USA) that under dark-field provided a dim

but enough light that allowed the visualization of both white silver grains and digoxigenin-coloured cells.

We analyzed 5 fields/side of each dorsal striatum (ten fields/section). Since we had three different locations (at coordinates of 1.7, 1 and -0.8mm) (Paxinos G, Watson C. 1998) sections/location, we examined a total of 60 fields/dorsal striatum/rat. Given that only two locations contained the nucleus accumbens, the total number of fields for this region was 40 fields/rat. Dorsal striatum located at 1.7 and 1.0 mm was counted above an imaginary line drawn at 6 mm of vertical (V) coordinates (at the level of the base of the lateral ventricle) and below the corpus callosum which is easily recognizable. At the location 1.7 mm, the positive cells of the nucleus accumbens were counted below the line mentioned (V 6 mm), lower limit at V 7.8, left lateral limit at 0.5 (or -0.5 for the left section), and right limit at 2.8 mm, being careful to examine only the area nearest to the anterior commissure. For the section at 1.00 mm, nucleus accumbens positive cells were counted inside the limits of an imaginary square figure: left limit L 0.8 mm (or -0.8 mm for the left side of the section), right limit 2.7 mm (or -2.7 mm), upper limit V 6.9 mm and lower limit V 7.8 mm. The section at -0.8 mm contained no nucleus accumbens; thus, dorsal striatum was exclusively examined. We calculated the percentage of NTR-containing cells expressing AC5 or PDE7B (NTR cells were taken as 100%) and the percentage of AC5-expressing cells containing also PDE7B mRNA (AC5 positive cells were considered as 100%) in dorsal and ventral striatum. Also, we made a calculus of the extent of AC5 or PDE7B-expressing cells (each one taken as 100%) that contained NTR mRNA in caudate-putamen and nucleus accumbens.

Preparation of the figures.

Hybridized tissue section images from film autoradiograms were taken with a Wild 420 Leica microscope equipped with a digital camera (DXM1200 F, Nikon) and ACT-1 Nikon Software. Microphotographies of the hybridized tissue slides were performed using a Zeiss axioplan microscope equipped with a digital camera (DXM1200 F, Nikon) and ACT-1 Nikon Software. Bright-field images were captured with transmitted light; dark-field images with a Darklite illuminator (Micro Video Instruments, Avon, MA, USA). Figures were prepared for publication using Adobe Photoshop Software (Adobe Software, San Jose, CA, USA). The contrast and brightness of images were the only variables adjusted digitally. For anatomical reference, sections close to those used were stained with cresyl violet.

Statistical analysis.

Student's *t*-test was performed to compare the percentage of NTR-positive neurons expressing AC5 vs NTR-cells containing PDE7B mRNA, and caudate vs n.accumbens percentage of double-labeled cells; one-way ANOVA analysis was used to identify differences in the number of double-labeled cells between different brain section levels in each experiment (three for caudate and two for n.accumbens). Bonferroni's post-hoc test was used when differences were significant ($p < 0.05$).

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TABLE 1. Expression of AC5 or PDE7B mRNAs in striatal cells containing MOR, D1, or D2 dopamine receptors.

	AC5		PDE7B	
	Caudate-putamen	Nucleus accumbens	Caudate-putamen	Nucleus accumbens
MOR	64 ± 0.6 %	53 ± 8 %	68 ± 2 %	63 ± 0.1 %
D1	80 ± 2 %	72 ± 2 %	81 ± 1 %	83 ± 2 %
D2	65 ± 0.8 %	52 ± 3 %	67 ± 2 %	50 ± 1 %

TABLE 2. Co-expression of AC5 and PDE7B mRNAs in striatal cells.

	AC5	PDE7B
AC5		99.6 ± 0.2 %
PDE7B	65 ± 2 %	

TABLE 3. Expression of MOR, D1, or D2 receptor mRNAs in striatal cells containing AC5 or PDE7B enzyme mRNAs.

ENZYMES	MOR	D1	D2
AC5	50 ± 3 %	59 ± 2 %	42 ± 2 %
PDE7B	56 ± 2 %	58 ± 1 %	41 ± 3 %

Table 1. Expression of AC5 or PDE7B mRNAs in striatal cells containing MOR, D1, or D2 dopamine receptors. Values (in percentage) are the mean ± S.E.M. of cells expressing MOR, D1, or D2 mRNA (taken as 100 %) simultaneously containing AC5 or PDE7B mRNAs in caudate-putamen or nucleus accumbens; values represent the mean of counted cells in an average of 10 fields/each striatum level/rat, n=4. The average number of counted cells expressing each enzyme mRNA was 20 ± 1/field for AC5 and 26 ± 2/ field for PDE7B.

Table 2. Co-expression of AC5 and PDE7B mRNAs in striatal cells. Double labeled cells were counted twice. The first time, single digoxigenin labeled cells containing AC5 mRNA

were taken as 100%, next we obtained the percentage of double labeled cells containing AC5 and PDE7B mRNAs. The second time, radioactively labeled cells expressing PDE7B were taken as 100%. In this manner, the values are either the mean $\pm$ S.E.M. of the percentage of AC5 positive cells (taken as 100%) or the percentage of the PDE7B mRNA containing cells that were double labeled (containing AC5 and PDE7B) in striatum. Values represent the mean of counted cells in an average of 10 fields/each striatum level/rat, n=4. The average number of counted cells expressing AC5 mRNA was 17 and 56 for PDE7B mRNA/field.

Table 3. Expression of MOR, D1, or D2 receptor mRNAs in striatal cells containing AC5 or PDE7B enzyme mRNAs. Values are the mean $\pm$ S.E.M. of the percentage of cells expressing AC5 or PDE7B enzyme mRNAs (taken as 100 %) that simultaneously contained MOR, D1, or D2 mRNA in striatum; values represent the mean of counted cells in an average of 10 fields/each striatum level/rat, n=4.

Fig 1. Panels A, D, G, J, and K show autoradiographic signals of ^{33}P -labelled MOR (A), D1 (D), D2 (G), PDE7B (J), and AC5 (K) oligonucleotides from rat coronal striatal sections obtained by *in situ* hybridization histochemistry. Panels B, E, H are photomicrographs taken with a Darklite illuminator of emulsion dipped sections of the striatum showing simultaneous cellular localization of AC5 mRNA -using digoxigenin labeled oligonucleotides (dark precipitate)- and either ^{33}P -MOR (B), D1 (E), D2 (H), or PDE7B (L) mRNA labels; or cellular localization of PDE7B mRNA labeled with digoxigenin in cells expressing MOR (C), D1 (F), or D2 mRNAs (I) radioactively labeled and detected by double *in situ* hybridization histochemistry. Double-labeled cells expressing two mRNAs are indicated by black and white arrows, while single radioactively-labeled cells are indicated by solid black arrows. Scale bar = 20 μm .