

Differential expression of the mouse D₂ dopamine receptor isoforms

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We have identified and characterized the cDNAs corresponding to the mouse D₂ dopamine receptors. We show that in the mouse the D₂ dopamine receptor is found in two forms, generated by alternative splicing of the same gene. mRNA distribution analysis of areas expressing the D₂ receptors shows that the larger form is the most abundant, except in the brain stem where the shorter form is predominant. Membranes of mammalian cells transiently transfected with both forms of D₂ receptor bind [³H]apiprone with a high affinity.

Mouse D₂ dopamine receptor; mRNA distribution; Expression

1. INTRODUCTION

Dopamine receptors can be divided into two classes D₁ and D₂ [1,2]. D₁ receptors activate the adenylyl cyclase pathway while D₂ receptors act as inhibitors. The dopamine D₂ receptor is highly represented in the central nervous system, especially in areas like the striatum and substantia nigra as well as in the pituitary gland [3,4]. Abnormal regulation of the receptor's levels seems to be associated with disorders such as Parkinson's disease [5] and schizophrenia in humans [6]. In the anterior pituitary gland dopamine acts through a D₂ type receptor and causes inhibition of prolactin production and release [7]. Activation of the D₂ receptor lowers the intracellular level of cAMP by a mechanism which appears to involve coupling of the receptor to an inhibitory G protein (G_i) [8]. The gene for a D₂ dopamine receptor has been cloned from rat brain; it encodes a predicted protein of 415 amino acids (aa) [9] which belongs to the family of G protein-coupled membrane receptors [10]. Recently a longer cDNA isoform encoding a predicted protein of 444 aa has been cloned from rat, human and bovine tissues [11–16].

We have isolated a cDNA from a mouse pituitary library encoding the 444 aa form of the D₂ receptor, here referred to as D₂A. This cDNA is highly homologous at the nucleotide and amino acid level to both forms of the rat D₂ receptor. Analysis of mouse genomic DNA suggests that the two forms originate by

alternative splicing. We have analyzed the distribution of the two forms of D₂ dopamine receptors in the mouse brain. The results show that the shorter form, here referred to as D₂B, is the least abundant in most CNS areas, with the exception of the brain stem where it is predominant.

2. MATERIALS AND METHODS

A mouse pituitary cDNA library was constructed in λzapII (stratagene) starting from 3 µg polyA⁺ RNA from female mice pituitaries. The cDNAs were synthesized with a cDNA cloning kit from Pharmacia, containing *Eco*RI–*Nor*I adaptors at each site, inserted into the *Eco*RI site of λzapII arms. The library (1·10⁶ pfu) was transferred on Nitrocellulose filters (Schleicher and Schuell) and screened with two degenerate ³²P-labelled oligonucleotides (Spec. act. 10⁶/µg) specific to conserved sequences present in the

CACCC
VI (5'-TT ATCTGCTGGCTGCCCTTCTTC 3')
TGTGG

and VII

T G T T TAG
(5'-TGGCT GGCTA G CAA 3')
A T C C CTC

transmembrane domains of receptors binding cationic amines [17]. The hybridization conditions were 5×SSC, 5×Denhardt's, 0.1% sodium pyrophosphate, 1% SDS and salmon sperm DNA (100 µg ml⁻¹) at 42°C. Filters were washed in 2×SSC and 0.1% SDS at 42°C. 3 overlapping clones were rescued from the λzap vector and sequenced on both strands by the Sanger dideoxy chain termination method using Sequenase (US Biochemical Corporation) [18]. To obtain the cDNA corresponding to the shorter form of the mouse D₂ Dopamine receptor, D₂B, cDNA was synthesized from 1 µg of total RNA from mouse brain stem in a 20 µl reaction containing 50 mM Tris-HCl, pH 8.3, 150 mM KCl, 10 mM MgCl₂, 15 mM DTT, 0.5 mM dNTP, 0.5 µg of amplimer A (5'-GGCCTTCTGCCACAGCTT-3') complementary to the D₂A sequence from nucleotide 1404 to 1423 of the coding sequence and 10 U AMV reverse transcriptase. After incubating at 42°C for 1 h, reactions were inactivated by heating at 65°C for 10 min and 4 µl were added to a PCR reaction

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The PCR reactions were made in 50 μ l containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM dNTP, 0.5 μ g amplifier A and 0.5 μ g amplifier B (5'-GTCACCTGCTC-GTCTAT-3') complementary to the D₂A sequence from nucleotide 609 to 628 of the coding sequence. Reactions were heated at 94°C for 2 min, then 2.5 U of Taq polymerase were added and the temperature was cycled with the following profile: 94°C, 0.5 min; 55°C, 1 min, 72°C, 2 min. After 40 cycles the PCR reaction was digested with *Hinc* II that liberates a 498 bp long fragment. A *Hinc* II digestion was also performed on the D₂A cDNA. We exchanged the 498 bp fragment specific to the D₂B form with the 585 bp long fragment of the D₂A form. The cDNA obtained was sequenced to ensure the absence of PCR artefacts.

Quantitative S₁ nuclease analysis was performed on 10 μ g of total RNA, prepared by the LiCl-urea method [19]. The hybridization conditions were 50% formamide, 40 mM Pipes (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl at 37°C for 12 h. Digestions were made in 0.2 M NaCl 0.05 M sodium acetate (pH 4.6), 4.5 mM ZnSO₄, with 300 U S₁ (Appligene, Strasbourg, France) at 25°C for 2 h. The digestions were located on a 8% polyacrylamide/urea gel.

For eukaryotic expression, cDNA fragments containing the entire coding region for the mouse D₂A and D₂B receptor were inserted in both orientation in the pSG5 vector [20] to create pSVD₂A* (sense orientation) or pSVD₂A (antisense orientation) and pSVD₂B* or pSVD₂B. Cos7 cells were transfected with either pSVD₂A* or pSVD₂B* (10 μ g DNA/plate) by the calcium phosphate method [21], washed after 12 h and harvested after 36 h. Membranes were prepared as already described [22]. Binding assays were carried out with 40 μ g protein/sample [22] using [³H]spiperone and bromocriptine at the concentration indicated in Fig. 4. Data were analysed by non-linear least squares regression [23].

3. RESULTS

3.1. Cloning of the D₂ dopamine receptor cDNAs

Two sets of oligonucleotides were generated to screen a mouse pituitary cDNA library. The sequence of the oligonucleotides was based on a comparison of the

AGCGGTGCCC		-1
GAGGGGCGCGGCTGGCTGATCGCGGGGAGCTGGAAGCCTCGAGCAGCGCGGCTTCTCTGGCGCGCGGCGCTATGGCTTGAAGAGCGTGCCACCGATGGCGCCCACTGCCCC		
ATG GAT CCA CTG ACC CTG TCC TGG TAC GAT CAT GAT CTG GAG AGG CAG ACC TGG AGC CGG CCC TTC ACC GGG TCC GAA GCG AAG CCA GAC		90
Met Asp Pro Leu Asn Leu Ser Trp Tyr Asp Asp Asp Leu Glu Arg Gln Asn Trp Ser Arg Pro Phe Asn Gly Ser Glu Gly Lys Ala Asp		30
AGG CCC CAC TAC AAC TAC TAT GGC ATG CTG CTC ACC CTC CTC ATC TTT ATC ATC GTC TTT GGC AAT GTG CTG GTG TGC ATG GCT GTA TCA		180
Arg Pro His Tyr Leu Thr Tyr Tyr Ala Met Leu Leu Thr Leu Leu Ile Phe Ile Ile Val Phe Gly Asn Val Leu Val Cys Met Ala Val Ser		60
CGA GAG AAG GCT TTG CAG ACC ACC ACC AAC CTC GCT GAG GTC AGC CTC GCT GCT GGC GAT CTT CTG GTG GGC ACA CTG GTT ATG CCC TGG		270
Arg Glu Lys Ala Leu Gln Thr Thr Thr Asn Tyr Leu Ile Val Ser Leu Ala Val Ala Asp Leu Leu Val Ala Thr Leu Val Met Pro Trp		90
GTC CTC TAT CTG GAG GTG GTG GGT GAG TGG AAA TTC ACC AGG ATT CAC TGT GAC ATC TTT GTC ACT CTG GAT GTC ATG ATG TGC ACA GCA		360
Val Val Tyr Leu Leu Val Val Gly Glu Trp Phe Ser Arg Tyr Thr Ala Val Ala Met Pro Met Leu Tyr Asn Thr Arg Tyr Ser Ser Lys Arg		120
AGC ATC TTG AAC CTG TGT GCC ATC AGC ATC GAC AGG TAC ACA GCT GTG GGC ATG CCT ATG TTG TAT AAC ACA CGC TAC AGC TCC AAG CGC		450
Ser Ile Leu Asn Leu Cys Ala Ile Ser Ile Asp Arg Tyr Thr Ala Val Ala Met Pro Met Leu Tyr Asn Thr Arg Tyr Ser Ser Lys Arg		150
CGA GGT ACT GTC ATG ATC GCC ATT GTC TGG GTC CTG TCC TTC ACC ATC TCT TGC CCA CTG CTC TTT GGA CTC AAC AAC ACA GAC CAG AAT		540
Arg Val Thr Val Met Ile Val Trp Val Thr Leu Ser Phe Thr Ile Ser Cys Pro Leu Leu Phe Gly Leu Asn Asn Ala Asp Gln Ser		180
GAG TGT ATC ATT GCC AAC CCT GCC TTC GTG GTC TAC TCC TCC ATC GTC TCG TTC TAC GTG CCC TTC ATC GTC ACC CTG CTG GTC TAT ATC		630
Glu Cys Ile Ile Ala Asn Pro Ala Phe Val Val Tyr Ser Ser Ile Val Ser Phe Tyr Val Pro Phe Ile Val Thr Leu Leu Val Tyr Ile		210
AAA ATC TAC ATC GTT CTC CGC AAG CGT CGG AAG CGG GTC AAC ACC AAG CGT AGC AGC CGA GCT TTC AGA GCC AAC CTG AAG ACA CCA CTC		720
Lys Ile Tyr Ile Val Leu Arg Lys Arg Arg Lys Arg Val Asn Thr Lys Arg Ser Ser Arg Ala Asn Leu Lys Thr Pro Leu		240
AAG GGC AAC TGT ACC CAC GCT GAG GAC ATG AAA CTC TGC ACC GTT ATC ATG AAG TCT AAT GGG AGT TTC CCA GTG AAC AGG CGG AGA ATG		810
Lys Gly Asn Cys Thr His Pro Glu Asp Met Lys Leu Cys Thr Val Ile Met Lys Ser Asn Gly Ser Phe Pro Val Asn Arg Arg Arg Met		270
GAT GCT GCC CGC CGA GCT CAG GAG CTG GAA ATG GAG ATG CTG TCA AGC ACC AGC CCC CCA GAG AGG ACC CGG TAT AGC CCC ATC CCT CCC		900
Asp Ala Ala Arg Arg Ala Gln Glu Leu Met Glu Met Lys Ser Thr Ser Pro Pro Glu Arg Thr Arg Tyr Ser Pro Ile Pro Pro		300
AGT CAC CAC CAG CTC ACT CTC CCC GAT CCA TCC CAC CAC GGT CTA CAT ACC AAC CCT GAC AGT CCT GCC AAA CCA GAA AAG AAT GGG CAT		990
Ser His His Gln Leu Thr Leu Pro Asp Pro Ser His His Gly Leu His Ser Asn Pro Asp Ser Pro Ala Lys Pro Glu Lys Asn Gly His		330
GCC AAG ATT GTC AAT CCC AGG ATT GCC AAG TTC TTT GAG ATC CAG ACC ATG CCC AAT GGC AAA ACC CAG ACC TCC CTT AAG ACN ATG AGC		1080
Ala Lys Ile Val Asn Pro Arg Ile Ala Lys Phe Phe Glu Ile Gln Thr Met Pro Asn Gly Lys Thr Arg Thr Ser Leu Lys Thr Met Ser		360
CGC AGG AAG CTC TCC CAG CAG AAG GAG AAG AAA GCC ACT CAG ATG CTT GCC ATT GTT CTT GGT GTG TTC ATC ATC TGC TGG CTG CCC TTC		1170
Arg Arg Lys Leu Ser Gln Gln Lys Lys Ala Thr Gln Met Leu Ala Ile Val Leu Gly Val Phe Ile Ile Cys Trp Leu Pro Phe		390
TTC ATC ACC CAC ATC CTG AAT ATA CAC TGT GAC TGC AAC ATC CCA CCA GTC CTC TAC AGC GGC TTC ACA TGG CTG GGC TAT GTC AAC AGT		1260
Phe Ile Thr His Ile Leu Asn Ile His Cys Asp Cys Asn Ile Pro Pro Val Leu Tyr Ser Ala Phe Thr Trp Leu Gly Tyr Val Asn Ser		420
GCC GTG AAC CCC ATC ATC TAT ACC ACC TTC AAC ATT GAG TTC CGC AAG GCC TTC ATG AAG ATC CTG CAC TGC TGA GTGCGCCCTTGCTGCAC		1354
Ala Val Asn Pro Ile Ile Tyr Thr Thr Phe Asn Ile Glu Phe Arg Lys Ala Phe Met Lys Ile Leu His Cys		444
AGCAGCTGCTGGCGCTCCCTGCTAGGCAGGCCAGCTCATCCCTGCAAGCTGTGGGAGAAAGGCCACAGATGGAATCGGCTTCTCTTGACCTGACGGCTCTGCAGTGTAGCT		1473
TGGCTGGGTGCCCTCTCTGCCACACACCTTATCCTGTCAGGGTAGGGCAGGGAGACTGGTATGTATACAGCTCTGGGGTTGGATCCATGGCTCAGAGCAGCTCACAGATGCCCC		1592
TTTCAGATGACAGTCTGTCTCTTGGCACCAAGAGGAGCAGCCTTCTCTGACCTTCTCTCAGGCACGGAAGCTAGCTCAGTAGCGGAGCACACCTTGATTGTGGCTTGCCCTGG		1711
CCCTTGCTTGCTTATGTGGATCAGGTGGTAGAAGAGAAGGACAGTCTTACTTTACAGGACCATAGGAAAGCAGGGAACATGCCAAGGCTCCAGGTGACGTATGCTCGGGAGA		1830
CACACATAAACACAGGTAGCTCCACGGACCCAGAGAACTGAGGCTGAAATCTGTTTCCACCCCACTCTAGTGTGAATCCCTACTTCCATAGCAGTGGGTATTGCTATGTTCT		1949
CCACTGTTATAGAAATCCATGGGGTTTCTGACCTTCGGGGGAAATAACTCTAATCTCAAGGGCCCCAAGAGAGACTGTAAAGAGAAAAATAGCTGATTTCCTCTACCTTCCATC		2068
CACCTCCGCCACTTCTGACATACATTGGACATAGCCATTCGCCACAGCAGATGCTGGACAGCTGGGAAGTTGAGCCTTGGACAGTGTGGAGCTGAAGTTGGAGGTGGTAACCTGGG		2187
GCCTTGGGCGGGGGGTGTGATATTTCCCTCTTCCAAGTCTCTCTCTGCGCAGTGCCTCTGCTTASAGGAGGCTGTGGATGGGGCTGCTGGGCTGCTGATACCATGGGTCTGGC		2306
CCTAGTGAGGGTGGGGAAGCTGCAGCTTGGAGGGGTCTGGGCTCCAACTCTGTAACATCACCATACATGCACCAAAACCAATAAACCTTGACAGAGTCATCCACGGAAGA		2420

Fig. 1. Sequence analysis of the mouse Dopamine D₂A receptor cDNA. Nucleotide and predicted amino acid sequences are shown and numbered in the right hand margin beginning with the first methionine of the open reading frame. Asterisks (*) indicate potential N-linked glycosylation sites. Putative protein kinase A sites are indicated (□). Triangles (Δ) indicate putative protein kinase C sites, casein kinase II sites are designated by an arrow. The additional 29 aa specific to this isoform are underlined with dashed lines. The poly-A adenylation site is underlined as well as a small open reading frame in the 5' untranslated region in frame with the first ATG. The putative 7 transmembrane domains are boxed.

amino acid sequences of G protein-coupled receptors binding cationic amines (for review see ref. 17). A cDNA was isolated of 2549 bp with an open reading frame coding for a 444 aa long protein (Fig. 1). The sequence was recognized as encoding a D₂ receptor because of its high homology to the D₂ receptor isolated from rat brain cDNA [9], except for an insertion of 87 base pairs in the region corresponding to the putative third intracytoplasmic domain. A similar form of the D₂ receptor has been characterized in other species [11–16].

Southern genomic blot analysis using specific oligonucleotides showed the presence of a single band suggesting that the two cDNAs are products of the same gene (Fig. 2), and they are likely to be generated by alternative splicing as it has been shown in rat and humans [12,13,15].

3.2. mRNA distribution analysis

In order to gain insights into the function of the two D₂ dopamine receptor isoforms, we investigated their relative distribution in areas in which the D₂ receptor is expressed. Therefore we performed a quantitative S₁ nuclease mapping on mRNAs from pituitary and different areas of the brain of adult mice (Fig 3). A 75-mer oligonucleotide was synthesized spanning the region of the putative third intracytoplasmic domain, where the insertion is located. Experiments were performed in probe excess, to ensure a correct quantification of the data. This analysis shows that in pituitary and striatum D₂A is at least 3-fold more abundant than D₂B. Interestingly this ratio is completely inverted in the brain stem where D₂B is predominant (see Fig 3; D₂A and D₂B). The differential distribution of the D₂ mRNAs in the brainstem obtained by S₁ nuclease

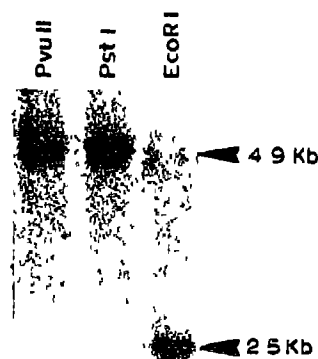


Fig 2 Southern blot analysis of mouse genomic DNA. 10 μ g of mouse genomic DNA were digested by either *Pst*I, *Pvu*II or *Eco*RI as indicated on each lane. After transfer the filter was hybridized with ³²P-labelled oligonucleotides complementary to either D₂A (from nucleotide 724–784) or D₂A and D₂B (from nucleotide 843–903). The approximate size of the single bands detected by hybridization with either probes, is as indicated.

analysis, reveals a difference in the ratio D₂A:D₂B which has not been detected before by Northern or PCR analysis. Northern analysis is not suitable for a direct quantification of the relative presence of the two forms of D₂ receptors, because of the lack of sequences specific to the D₂B isoform. PCR analysis [13] also failed to reveal such a difference, probably due to a lack of quantification in the PCR reactions, in which it is likely that the longer form is better amplified, as it always appears predominant.

3.3. Functional expression

To determine whether D₂A and D₂B cDNAs encoded functional receptors we introduced the sense or antisense expression vectors by transient transfection in the Cos7 cells. Membranes from transfected and non-transfected cells were tested for their ability to bind [³H]spiperone [22], a D₂ specific ligand. Binding of [³H]spiperone to these membranes was reversible saturable and with high affinity for both types of receptors (data not shown). Fig. 4 demonstrates the ability of a dopaminergic ligand, bromocriptine, to compete with high affinity for specific [³H]spiperone binding to transfected Cos7 cell membranes (D₂A IC₅₀ = 5 · 10⁻⁸ M; D₂B IC₅₀ = 8 · 10⁻⁸ M). Untransfected Cos 7 cells as well as those transfected with the antisense constructs were unable to bind [³H]spiperone, ruling out the possibility that an endogenous dopamine receptor could be responsible for the observed binding.



Fig 3 Quantitative S₁ nuclease analysis of mouse brain and pituitary mRNA. 10 μ g of total mRNA were hybridized to a synthetic 75-mer oligonucleotide, ³²P-end labelled by T4 polynucleotide kinase, complementary to the D₂ receptors mRNA from nucleotide 792 to 860 to which a 6 nucleotides non-specific tail was added (spec. act. 10⁸/μg). This probe was homologous for 51 nucleotides to both forms (415–444) of D₂ receptors while 18 nucleotides were specific to the D₂A (444) form. The protected fragments were 51 nucleotides long (D₂415) and 69 nucleotides long (D₂444) as expected, the 75 nucleotides full-length probe is indicated. The marker used is a ³²P-labelled pBR 322 digested by *Msp*I, the size of the fragments shown in the picture are in order 90, 76 and 67 base pairs. The tissue from where the mRNA was extracted are indicated above each lane. The relative amount of mRNA corresponding to either form of D₂ receptor was estimated by densitometric scanning of the autoradiogram.

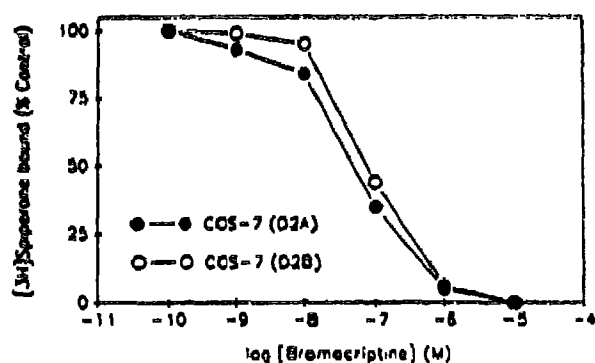


Fig. 4. Binding assay. Competition analysis of the dopaminergic ligand bromocriptine for [3 H]piperone (0.5 nM) binding in cos 7 cells. Bromocriptine concentrations are as indicated in the figure.

4. DISCUSSION

The D_2 dopamine receptor has been shown to generate the activation of different signal transduction pathways upon binding to its ligand [24]. These effects are mediated through its interactions with at least two G proteins [25]. In this paper we report the isolation of two forms of the D_2 dopamine receptors from mouse, D_2A and D_2B . These two forms generated by alternative splicing differ by an insertion of 29 aa between position 241 and 271 in the putative third intracytoplasmic domain of the D_2B receptor. This domain has been shown to play an important role in the coupling to G proteins in the case of the β_2 - and α_2 -adrenergic receptors [26]. The finding of two isoforms for the dopamine D_2 receptor, that differ only in this domain, raises the possibility of a differential coupling of these two receptors to G proteins. The two dopamine D_2 receptor amino acid sequences are highly conserved, including the 29 residue insertion, 100% from mouse to rat (97% homology at the nucleotide level, in the translated region) and 95.7% from rodents to human (90.45% at the nucleotide level, in the translated region), suggesting functional constraints on these sequences.

In order to gain insight into their function, we analyzed the RNA distribution of the D_2 receptor's isoforms. The D_2A form is the most abundant throughout all the areas tested with the exception of the brainstem, where the D_2B form is predominant. This finding points out the interesting possibility that the D_2B form could represent the presynaptic D_2 auto-receptor, able to regulate dopamine synthesis and release, since the brainstem contains the nuclei (substantia nigra) from where the dopaminergic fibers arise. We could not detect the presence of D_2 receptors in the adult mouse liver, like others did in rat [13]. We do not know whether this is due to the limit of detection of our probe, or whether the other authors used a fetal liver, as it was not specified in their paper. Concerning their bind-

ing affinity, classical pharmacological studies did not permit to discriminate between them as both receptors seem to have the same affinity for the ligand tested, when transfected in cos7 cells, as well as in other cell lines [12,13,14].

It has to be taken into account that the cell lines used could be deficient or limiting in one of the specific G proteins that bind to these receptors [27]. Alternatively, the high concentration of DNA transfected into these cells, necessary to observe binding, could result in high levels of receptors that would outnumber the available G proteins; thus the binding observed would correspond mostly to receptors that are not coupled to G proteins. Alternatively the difference between these two receptors could reside in the activation of several second messenger pathways in a differential manner. For instance, it has been shown that the α_2 -adrenergic receptors can both activate phospholipase C and inhibit adenylate cyclase [28].

Further studies will be aimed to detect a functional difference between the two forms of the dopamine D_2 receptors: measuring the effect of their expression on the adenylyl cyclase as well as on other signal transduction pathways in different cell lines.

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