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CHARACTERIZATION OF A NOVEL EXON WITHIN THE D3 RECEPTOR GENE GIVING RISE TO AN mRNA ISOFORM EXPRESSED IN RAT BRAIN

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A novel D3 receptor cDNA containing an insertion of 84 bp has been isolated from a series of alternatively spliced D3 receptor cDNAs by polymerase chain reaction on RNA isolated from rat basal forebrain. Sequence analysis of both subcloned cDNAs and genomic DNA clones, has identified a 84 bp insertion resulting in a 28 amino acid insertion in the first extracellular loop. A comparison of the cDNA sequence of the novel D3 cDNA and the isolated genomic sequence indicates that the novel insertion corresponds to a new exon and arises via an alternative splicing event. The mRNA for the novel insertion could be detected by PCR in several areas of the brain co-localized with the wild type form of the D3 receptor. Moreover, genomic Southern blot analyses suggest that the D3-receptor exon giving rise to this novel D3 mRNA variant might exist in other species.

The diverse physiological effects of dopamine in mammalian brain are mediated by a variety of receptors, which have been classified on the basis of pharmacological, biochemical, functional and molecular differences (1). A cDNA was recently cloned coding for the dopamine D3 receptor (2), which is predominantly expressed in mesolimbic structures that are involved in the control of motor, affective, cognitive and neuroendocrine functions (3). Unlike the D1 and D5 dopamine receptor genes (4, 5), which are intronless, the D3 receptor gene contains at least six exons (6), indicating that multiple transcripts might originate via alternative splicing mechanisms giving rise to different receptor isoforms. Indeed, shorter mRNA variants of the original D3 sequence have been reported that potentially code for truncated receptors of 100 and 109 amino acids (6, 7). These alternatively-spliced mRNAs contain specific exonic-deletions that result in frameshifts and premature termination. A third isoform has recently been isolated from mouse olfactory tubercle, which alternatively splices out 63 bases within the third intracellular loop, producing a D3 receptor with identical binding characteristics to the full length D3 receptor (8). A fourth isoform with an internal 54 bp deletion resulting in a 428 amino acids produces a protein with no binding activity (6).

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Here, in addition to the previously reported alternatively spliced mRNAs, we report the existence of a longer form of the D3 receptor mRNA ($rD3^{in}$), containing an insertion of 84 bp, arising from an extra exon in the D3 gene. We have characterized the respective intron-exon sequence and show that it is differentially expressed in brain structures when compared to the previously cloned or wild-type D3 receptor. Transient expression of the $rD3^{in}$ gene in COS cells did not produce any reproducible specific binding of dopaminergic ligands.

MATERIALS AND METHODS

a) Cloning of novel D3 receptor isoforms

Basal forebrain tissue from 5 male Wistar rats were dissected and total RNA extracted in guanidinium isothiocyanate and purified through a CsTFA gradient (Pharmacia). Oligodeoxynucleotide primers (Table 1) were synthesized on an Applied Biosystems 380A DNA synthesizer. First strand cDNA was generated from 12 µg of total RNA using 5 units of AMV reverse transcriptase (Promega) with 1 µM primer PCR-2. A first round of polymerase chain reaction (PCR) with 1 µg of cDNA was carried out with PCR-1 and PCR-2 (20 cycles), followed by a second round of PCR (30 cycles) with primers PCR-3 and PCR-4 in a thermocycler (Techne). PCR reactions (100 µl) were carried out with 1 µM primers in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ and 40 units/ml Taq polymerase (Serva). PCR cycle times/temperatures were 2 min/94°C, 3 min/58°C and 3 min/72°C. A band of approximately 1.4 kb was isolated from an agarose gel and subcloned into pCDL-SRα296 (9). Single colonies were picked and rat D3 gene insertions were sequenced by the Sanger method. 1 µg of rat genomic DNA (Clontech) was amplified by PCR using primers RDI-1 and RDI-3 or RDI-2 and RDI-4 using the standard conditions for 30 cycles. A fragment of approximately 280 bp was isolated, subcloned and sequenced.

b) Analysis of expression of novel D3 isoforms

Total RNA was prepared from various regions of brain tissue from male Wistar rats (BRL). For single stranded cDNA synthesis, 3 µg total RNA was treated with AMV reverse transcriptase using PCR-6 as primer in a 20 µl reaction followed by PCR using primers PCR-5 and PCR-6 (35). Gel-electrophoresis, ^{32}P-end labeling of oligonucleotides and Southern blot hybridizations were performed according to standard protocols (10). A double stranded cDNA probe specific for the 84 bp insert was produced by using two oligonucleotides, IN-S and IN-AS, which overlap in the 3'-region (table 1). The two oligos were re-annealed and filled-out using Klenow fragment of DNA polymerase with α-[^{32}P]dXTPs (10). The probe was hybridized to a ZOOBLOT (Clontech) containing Eco RI digested genomic DNA from different species. Genomic Southern blot hybridizations were performed overnight at 37°C in 5xSSPE/0.1%SDS, 20 µg/ml tRNA, 20 µg/ml denatured DNA. Low stringency washes were performed in 2xSSPE/0.05% SDS at RT for one hour, followed by a high stringency wash in 2xSSPE/0.1% SDS at 65°C for 30 min.

c) Transient transfections and binding assays

cDNAs for the D3 receptor isoforms were transiently transfected into COS-M6 cells (11) for receptor binding studies. The cells were harvested after 72h and membranes prepared and tested for [^{125}I]iodospiperone binding as described (2).

RESULTS

PCR primers were designed to amplify the full coding sequence for the rat D3 receptor (table 1) based on the published sequence (2). Primers PCR-1 and PCR-2 were used in a first round of amplification (20 cycles), and primers PCR-3 and PCR-4 were used to reamplify cDNA products (30 cycles) from the first reaction generating a band of approximately 1.4 kbp. Upon subcloning

Table I
Sequence of oligonucleotides designed for PCR or hybridization probes

oligo	sequence (5'->3')	reference
PCR-1	ACATTTTGGAGTCGCGTTCCTCTGTGTG	bases 50-77 (ref.2)
PCR-2	GTGCGGTCTCTTCTCCTCCTTCAGCAGG	bases 1415-1442* (ref.2)
PCR-3	CCATGGCACCTCTGAGCCAGATAAGC	bases 80-105 (ref.2)
PCR-4	CCTTCAGCAGGACAGGATCTTGAGG	bases 1401-1425* (ref.2)
PCR-5	TCCTACTGTGCTCTCATCCTAGCC	bases 184-207 (ref.2)
PCR-6	AGGACAGTCACCCCGAAGGGAACGTA	bases 673-698* (ref.2)
P1	ATACGCCAGGCTGTCTGGGATTG	bases 5'-29'* (fig 1b)
P2	GACTCCACCTGTCACCTCCAAGTACACCAC	bases 333-366* (ref.2)
IN-S	CAATCAATCCCAGACAGCCTGGCGTATCCTCTAT GCCTGCCAGCCCTCTGGT	bases 5'-52' (fig 1b)
IN-AS	CTTCCTGGCTCCCTGTGGCGGGTGCCATCAGAAC CAGAGGGCTGGCAG	bases 37'-84'* (fig 1b)
RDI-1	CTAGTGGCCACGTTGGTGAT	bases 310-329 (ref.2)
RDI-2	ACAGCCTGGCGTATCCTCTA	bases 14'-33' (fig 1b)
RDI-3	GGCGGGTGCCATCAGAACCA	bases 49'-68'* (fig 1b)
RDI-4	GCTGATGGCACAGAGGTTCA	bases 437-456* (ref.2)

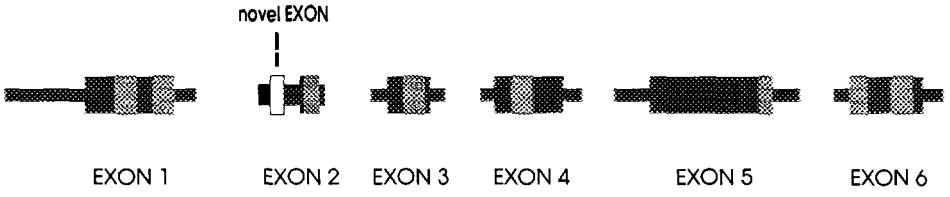
* = complement.

the amplified band isolated, 12 clones were isolated which contained cDNA insertions of approximately 1225 bp (1 clone), 1300 bp (2 clones), 1350 bp (8 clones) and 1440 bp (1 clone). These differences were mapped to the region including transmembrane domains II to V. Upon sequencing the four different cDNA inserts, we found: a 126 bp deletion of the third transmembrane domain leading to production of a putative protein of 404 amino acids; a 54 bp deletion of the second extracellular loop leading to the production of a putative receptor containing 428 aa; the original 1358 bp clone as reported by Sokoloff et al. (2) and a clone containing an insertion ($rD3^{in}$) of 84 bp between the first and second exons, potentially coding for an extra 27 amino acids positioned between the putative first and second transmembrane domains but interrupted by a TGA codon (fig 1A).

In order to test the hypothesis that the additional cDNA corresponded to a previously unidentified exon in the D3 gene, we amplified PCR fragments from rat genomic DNA. Primer sets targeting the regions flanking the borders of the putative exon were used (fig 1b), such that PCR products would be amplified, depending on the length of the intron(s). We found that while one set of primers (RDI-2 and RDI-4) gave a short band of about 400 bp, the other set (RDI-1 and RDI-3) gave no band, probably due to the length of the size of the intron. This 400 bp band was subcloned and sequenced and found to contain a short intron of 108 bp (fig 1). This sequencing of the genomic fragment confirmed that the inserted 84 bp sequence is most likely coded by an extra exon, as well as confirming the existence of an in-frame TGA codon that was found in the original cDNA.

In order to assess the expression of the $rD3^{in}$ exon in rat brain, primers within the first and fifth transmembrane domains and flanking the insertion, PCR-5 and PCR-6, were used in a reverse transcriptase/PCR with RNA prepared from different brain regions. From total RNA, we could

A



B

EXON 1

292 CTGGCTGTGGCCGACCTGCTAGTGGCCACGTTGGTGATGCCGTGGGTGGTGTACTTGGAG
L A V A D L L V A T L V M P W V V Y L E

...intron A...

new EXON

1' CAATCAATCCCAGACAGCCTGGCGTATCCTCTATGCCTGCCAGCCCTCTGGTTCTGATGG
Q S I P D S L A Y P L C L P A L W F * W

new intron A'

61' CACCCGCCACAGGGAGCCAGGAAGgtgtgttggcatctctgatgaacagctcaggggagg
H P P Q G A R K

121' caagagagttgattgggttattgttctttccctggagaaaacactaacctccttcttatc

EXON 2

181' tctaatgcccagGTGACAGGTGGAGTCTGGAATTTCAGCCGCATTTGCTGTGACGTTTTT
V T G G V W N F S R I C C D V F

Figure 1. A) Genomic structure of the rat D3 receptor; large bars represent the coding sequence, hatched boxes correspond to transmembrane domains, white box indicates location of novel exon and intron A', **B) DNA and amino acid sequences of the novel D3 exon and intron A';** numbering of EXON 1 follows ref.2, novel D3 exon (1'-84') and novel intron A' (85'-192').

generate specific cDNA fragments of 600 bp and 518 bp after 35 cycles of PCR amplification. However the 600 bp band was detected at a much lower intensity than the fragment of 518 bp, as visualized by ethidium bromide-stained gel (not shown). Specificity of the cDNA fragments was confirmed by Southern blot analysis using hybridization to oligonucleotide probes (P1and P2), showing that only the upper band hybridized to the P1 oligonucleotide specific for the $rD3^{in}$ sequence.

Hybridization of probe P1 to the novel $rD3^{in}$ exon sequence shows that the insert form of the D3 has a more restricted regional distribution when compared to the expression of the wild-type D3 receptor mRNA (fig 2). The expression of the wild-type D3 mRNA, as detected by probe P2 which excludes the $rD3^{in}$ exon, is high in the hypothalamus, pons-medulla and mesencephalon, moderate in olfactory tubercle, striatum and septum, and barely detectable in olfactory bulb,

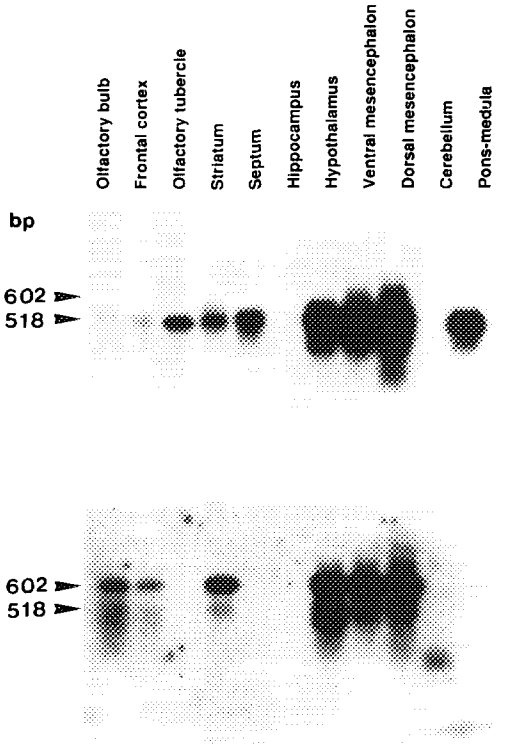


Figure 2. Regional distribution of D3 receptor mRNA isoforms in the rat brain; Southern blot analysis of the PCR fragments were detected by ^{32}P-end labeled probes P2, specific for the wild type D3 (upper) and IN-AS, specific to the novel exon (lower).

cortex, hippocampus and cerebellum. The expression of rD3^{in} is, in general, about 5 to 10 fold less abundant than the wild-type D3 mRNA (data not shown). Expression is highest in hypothalamus and mesencephalon, lower in striatum, olfactory bulb and cortex and, in contrast to the wild type D3 form, barely detectable in pons-medulla, septum, and olfactory tubercle. There is a distinct increase in the expression of the rD3^{in} variant with respect to the D3 in the olfactory bulb and striatum.

To verify whether this novel D3 exon is phylogenetically conserved, we probed for the rD3^{in} variant exon sequence on a genomic Southern blot. Under the high stringency wash conditions, the rD3^{in}-specific probe showed specific hybridization signals with rat and mouse genomic DNA (fig 3A), while under low stringency conditions, faint hybridization signals appeared for human, monkey, dog and cow genomic DNA (fig 3B).

DISCUSSION

Alternative splicing is one mechanism by which genes can give rise to functionally and anatomically distinct protein products. The genes for the D2, D3 and D4 dopamine family of G-protein coupled receptors have all been shown to potentially alter their coded receptors by either

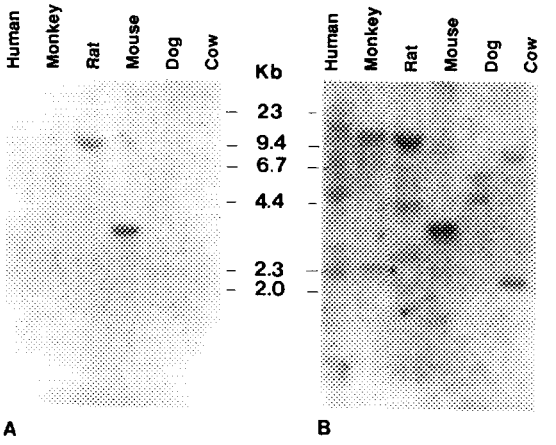


Figure 3. Genomic Southern blot analysis of D3 novel exon in different species. Zooblot was hybridized to a dsDNA probe (IN-S/IN-AS) synthesized specifically to target the novel exon sequence. High stringency conditions show a specific hybridization signal in rat and mouse genomic DNA (A), while under lower stringency conditions, faint hybridization signals are detected in other species as well (B).

alternative mRNA splicing (D2 and D3) or by polymorphic variations in the genome (D4). In addition to the previously described D3 mRNA splice variations (6, 7, 8), alternative splicing of the D2 mRNA provides for an additional 29 amino acids in the third intracellular loop (12) and polymorphic variations in the gene encoding the human D4 receptor encodes a number of a repeated 16 amino acid sequences within the third intracytoplasmic loop (13). The potential to generate heterogeneity in neurotransmitter receptors by alternative splicing has been previously reported for GABAa (14, 15), glutamate (16), substance P (17), luteinizing hormone (18) and follitropin receptors (19).

We have isolated several alternatively spliced D3 receptor cDNAs, which potentially code for a series of novel D3 receptor isoforms. Two correspond to D3 mRNA with deleted exons; a 54 bp deletion and identical to that reported by Giros et al. (4). We also identified an alternatively spliced mRNA, which contains an 84 bp insertion, $rD3^{in}$, adding 27 amino acids and a TGA stop codon into the first extracellular loop. Analysis of the sequence of the three clones show that none have used the expected splice acceptor sites predicted by the genomic D3 DNA sequence for the wild-type D3 cDNA. Following transient transfection of all the D3 isoforms into COS-M6 cells, significant [^{125}I]iodospiperone binding was only observed for the wild-type D3 receptor. A low level of binding (< 1 fmol/mg protein) was found for $rD3^{in}$, but was not reproducible suggesting that receptor protein is produced inefficiently, if at all, from this sequence.

The expression of this novel D3 receptor mRNA was further characterized in adult rat brain. The mRNA for this form of the D3 receptor has been detected in most mesolimbic brain areas, co-expressed with the normal form of D3 receptor (3). But the levels of expression of the novel D3 mRNA vary with respect to the wild type form of the D3 mRNA in different regions of the mesolimbic system, suggesting that it may contribute to gene expression and may have a physiological role.

In addition, we have subcloned by PCR the region of the rat genome which encodes the extra exon coding the 84 bp insertion and shown that it is separated from the second exon by a short 108 bp intron. The existence of the extra exon was confirmed by genomic southern blotting. DNA sequence analysis of both the $rD3^{in}$ cDNA and the genomic DNA show a TGA-stop codon in the middle of the putative 28 amino acids. Although this may lead to the expression of a truncated protein, it is also possible that the TGA codon in the novel D3 exon sequence codes for a seleno-cysteine (20) and by the use of a special selenocysteinyl-tRNA recognizing the UGA codon is able to produce a functional D3 receptor. Curiously, all mRNAs for these D3 isoforms were isolated from samples of adult rat basal forebrain and the physiological significance of such a high rate of aberrant, non-productive mRNA splicing remains to be explained.

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