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Dopamine D4 Receptor Repeat: Analysis of Different Native and Mutant Forms of the Human and Rat Genes

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SUMMARY

Recent molecular characterization of the human D4 gene has revealed the existence of various polymorphic forms of this receptor. These variations are found in the putative third cytoplasmic loop region and encode a variable number of repeats of 16 amino acids in length. In the present study we have compared the pharmacological binding profiles of seven different polymorphic variants of the human D4 receptor, the rat D4 receptor, and two different human D4 receptor mutants that were deleted in the repeat sequence. For this purpose we cloned the rat D4 receptor gene and compared its gene structure and its pharmacological binding profile with those of the D4.4 and D4.7 genes. The rat and human D4 genes display a high degree of sequence similarity, especially in the coding regions. An Alu repeat sequence was identified in the first intron of the human D4 gene but is not present in the rat D4 gene. Furthermore, using the polymerase chain reaction we cloned 3-, 5-, 6-, and 9-fold repeat sequences. These cloned repeat sequences were used for the reconstruction of full length cDNAs encoding D4.3, D4.5, D4.6, and D4.9, respectively. These novel forms of the human D4 receptor, as well as the previously cloned D4.2, D4.4, and D4.7 forms, were transiently expressed in COS-7 cells. All of the different forms of the human and rat D4 receptors and repeat deletion mutants displayed similar binding profiles for all ligands tested, although small differences were observed. The affinity for dopamine could be decreased by guanosine-5′-(β,γ-imido)triphosphate with the different forms of the D4 receptor, including the two receptor mutants that were deleted in the repeat sequence. These data suggest that the polymorphic repeat sequence has little influence on D4 binding profiles and might not be essential for G protein interaction.

Various novel dopamine receptors that were previously unrecognized in brain tissues have been cloned in the past few years (1–4). This late recognition was most likely due to their low abundance and their similar pharmacology, compared with the classical dopamine D1 and D2 receptors. After their discovery it has been of major interest to characterize the different receptor forms pharmacologically and functionally, because the dopamine system has been targeted for treatment of psychomotor disorders such as Parkinsons's disease, schizophrenia, Alzheimer's disease, and Huntington's disease (5, 6).

The dopamine D4 receptor structurally resembles the D2 and D3 receptors and displays a pharmacological profile similar to that of the D2 receptor (1). However, the atypical neuroleptic agent clozapine displays an approximately 10-fold higher affinity for the cloned D4 receptor, compared with the cloned D2 and D3 receptors (1). This higher affinity of clozapine for D4 receptors is in good agreement with the free plasma water and/or spinal fluid levels of clozapine in effective schizophrenia treatment and suggests that the dopamine D4 receptor is involved in mediating the antipsychotic effects of clozapine (7). Clozapine is of particular value because it has fewer extrapyramidal side effects than classical neuroleptic agents, does not cause tardive dyskinesia, and has been used succesfully in schizophrenic patients who were otherwise resistant to treatment (8, 9). The observation that D4 receptor levels are increased in the postmortem brain of schizophrenic patients also suggests a role for this dopamine receptor as a clinical target to control psychosis (10).

The dopamine D4 receptor displays a mRNA distribution profile distinct from that of the D2 and D3 receptors. Although it has been localized in classical D2 receptor-containing areas such as the striatum, it has relatively higher mRNA levels in limbic regions such as the frontal cortex, hippocampus, and amygdala (1, 11, 12). In the periphery, the D4 receptor has been identified in rat heart and retina (13, 14), as well as in lympho-

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ABBREVIATIONS: bp, base pair(s); Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PHNO, 4-propyl-9-hydroxy-2,3,4a,5,6,10b-hexahydro-4*H*-naphth[1,2b][1,4]oxazine; CHO, Chinese hamster ovary.

cytes localized in the lamina propriae of the fundus of the stomach (15), which suggests that this receptor might be the peripheral DA2 receptor.

Further molecular characterization of the human dopamine receptor gene resulted in the identification of a polymorphic region within the putative third cytoplasmic domain (16, 17). This polymorphic region consists of a variable number of tandem repeats of 48 bp in length (16 amino acids). We have thus far identified 19 different repeat units that are found at variable positions within the repeat region and thereby encode structurally different proteins (17). The rat dopamine D4 receptor displays little sequence identity with the human D4 receptor within the third cytoplasmic loop region, and the rat D4 gene does not have a well conserved repeat sequence (13, 18).

The structural differences between the rat D4 receptor and various forms of the human D4 receptor and their pharmacological profiles are of importance for determination of structure-activity relationships and for rational drug design. For this purpose we have cloned and sequenced the entire rat D4 receptor gene, including all of its introns, and compared it structurally and pharmacologically with the human D4.4 and D4.7 genes. Furthermore, cloning and reconstruction of cDNAs encoding 3-, 5-, 6-, and 9-fold repeats allowed us to extend these comparative studies with the D4.2, D4.3, D4.4, D4.5, D4.6, D4.7, and D4.9 receptors. By deleting the repeat sequence in the human D4 receptor, we could determine indirectly whether this sequence confers sensitivity to guanine nucleotides to dopamine binding.

Experimental Procedures

Materials. COS-7 cells were obtained from the American Type Culture Collection, and the expression vector pCD-PS was a generous gift from Dr. T. Storman (National Institutes of Health, Bethesda, MD). Fetal calf serum and Dulbecco's modified Eagle's medium were obtained from GIBCO/BRL Ltd. (Grand Island, NY) and Central Media Preparation Service (University of Toronto), respectively. The cloning vector pBluescript SK− and the *Escherichia coli* strain XL-I were purchased from Stratagene (La Jolla, CA). The rat genomic library in the bacteriophage EMBL3 and its host *E. coli* strain Y1090 were purchased from Clontech (Palo Alto, CA). The restriction endonucleases *Tth*III1, *Eco*47III, and *Eco*NI were obtained from New England Biolabs (Beverly, MA), whereas all other restriction enonucleases used were obtained from GIBCO/BRL. DNA polymerase (Klenow fragment) and T4 DNA ligase were purchased from GIBCO/BRL, calf intestinal alkaline phosphatase from Boehringer Mannheim (Laval, Quebec, Canada), materials required for the PCR from Perkin Elmer (Norwalk, CT), materials required for sequence analysis from United States Biochemicals (Cleveland, OH), and materials required for large-scale plasmid preparation from QIAGEN (Chatsworth, CA). Oligonucleotide primers required for sequence analysis and PCR were synthesized at the Biotechnology Service Center (Banting and Best Institute, University of Toronto). [^{3}H]Spiperone (120 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Drugs for ligand binding studies were obtained as follows: Sigma Chemical Co. (St. Louis, MO), dopamine and Gpp(NH)p; Astra Arcus AB (Södertälje, Sweden), raclopride; Janssen Pharmaceutica (Beerse, Belgium), haloperidol; Sandoz Pharma Ltd. (Basel, Switzerland), clozapine; Yamanouchi Pharmaceutical Co. (Tokyo, Japan), YM-09151–2 [(n)emonapride]; Merck Sharp & Dohme Research Laboratories (Rahway, NJ), (+)-PHNO; and Research Biochemicals Inc. (Natick, MA), LY171555 [(−)-quinpirole] and (+)-butaclamol.

Molecular cloning techniques. Standard molecular techniques needed for the isolation and cloning of DNA fragments, such as plasmid preparation, restriction endonuclease digestion, DNA ligation and modification, and gel electrophoresis, were essentially done according to the instructions of the manufacturers of the enzymes used and/or standard protocols as described by Sambrook *et al.* (19). Nucleotide sequence analysis was done by the dideoxy chain termination method, using 7-deaza-dGTP and Sequenase version 2.0 (United States Biochemicals).

Cloning of the rat D4 gene. Five hundred thousand independent clones from a rat genomic library were plated and transferred in duplicate to nylon filters (Colony Plaque Screen; NEN-DuPont, Boston, MA). The filters were prehybridized for 6 hr at 42° in 40% formamide, 0.2% polyvinylpyrrolidone (M_r 40,000), 0.2% Ficoll (M_r 400,000), 0.05 M Tris·HCl, pH 7.5, 1 M NaCl, 0.1% tetrasodium pyrophosphate, 1% sodium dodecyl sulfate, 100 μg/ml denatured salmon sperm DNA. The filters were then hybridized under similar conditions for 16 hr in the presence of 10^6 dpm/ml ^{32}P-labeled, 700-bp, *Eco*RI-*Xho*I fragment (random-primed labeling kit; Boehringer Mannheim) of the human D4 cDNA clone $D_2$10S (1) and were washed for 1 hr in 2× standard saline citrate (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 1% sodium dodecyl sulfate, at 55°. Four different hybridizing clones were identified and plaque purified. Restriction endonuclease sites and Southern blot analysis indicated that one of the clones contained a 3.8-kilobase *Bam*HI fragment that encoded the entire rat D4 gene. This *Bam*HI fragment was subcloned into the vector pBluescript, and both strands were completely analyzed by sequence analysis.

PCR amplification and cloning of the human D4 repeat sequence. Human genomic DNA isolated from human blood samples using proteinase K and phenol/chloroform extraction was used for the amplification of the D4 repeat sequence by PCR. Amplification by PCR from 100 ng of human genomic DNA was done in 25-μl volumes as described by us previously (17). The primers 5′-GCGACTACG-TGGTCTACTCG-3′ and 5′-GGTCTGCGGTGGAGTCTG-3′ were used for amplification of the repeat sequence. The amplification protocol consisted of 20 cycles of 94° (20 sec), 54° (20 sec), and 72° (40 sec), followed by a final 4-min incubation at 72°, using a Perkin-Elmer 9600 thermocycler. Amplified DNA was analyzed by agarose (2%) gel electrophoresis. Genomic DNA from individuals that showed amplified fragments corresponding in size to 3-, 5-, 6-, and 9-fold repeats was amplified again. This amplified material was treated with DNA polymerase Klenow fragment and phosphorylated using T4 polynucleotide kinase. With the exception of the 9-fold repeat sequence fragment, all fragments were then size-separated on 2% low-melting point agarose gels (SeaPlaque; FMC), isolated from the gels, and cloned into the *Sma*I site of pBluescript. The amplified 9-fold repeat fragment was digested with *Pst*I before agarose gel electrophoresis and, after its isolation, was subcloned into pBluescript linearized with *Pst*I and *Sma*I. The different clones were characterized by sequence analysis.

Construction of D4 expression vectors. The entire human D4.4 and D4.7 genes were cloned as an *Apa*I-*Pst*I fragment into the corresponding restriction endonuclease sites in pBluescript, excised as a *Kpn*I-*Xba*I fragment from pBluescript, and subcloned into the *Kpn*I-*Spe*I restriction endonuclease sites of the expression vector pCD-PS. The rat D4 gene in pBluescript was excised as a *Sma*I-*Xba*I fragment and then cloned into *Sma*I-*Spe*I sites of pCD-PS. The cDNAs of D4.2, D4.4, and D4.7 were cloned into pCD-PS as described previously (16). Full length clones of cDNAs with 3-, 5-, 6-, and 9-fold repeats in the expression vector pCD-PS were reconstructed essentially by replacing the internal *Eco*47III-*Tth*III1 fragment encoding the repeat sequence of the D4.7 cDNA with the *Eco*47III-*Tth*III1 fragments of the cloned PCR fragments for 3-, 5-, 6-, and 9-fold repeats (see Fig. 2).

The deletion mutant D4Δ*Eco*47III was created by excision of the *Eco*47III-*Tth*III1 fragment containing the polymorphic repeat sequence and subsequent religation of the D4 receptor cDNA after fill-in of the *Tth*III1 5′ overhang sequence. The deletion mutant D4Δ*Eco*NI was made by excision of the *Eco*NI- *Tth*III1 fragment containing the repeat

sequence and subsequent religation of the remaining D4 cDNA (see Fig. 2).

Expression studies. The different dopamine D4 receptor clones were expressed transiently in COS-7 cells. Before transfection, COS-7 cells growing on 150-mm tissue culture plates (Nunc, Denmark) were harvested by aspiration of the medium, incubation for 1–2 min in PBS, pH 7.2, containing 2 mg/ml trypsin, and collection of the cells in PBS. The cells were then centrifugated for 10 min at 700 × *g* and resuspended in PBS. The cells (5×10^7 cells/ml of PBS) were transfected at 4° with the different D4 expression vectors (10 μg of DNA/5×10^7 cells) by electroporation using a BTX 600 Electro Cell Manipulator (0.65 kV/cm, 2.9 msec). The transfected cells were seeded at 5×10^6 cells/100-mm plate and grown for 3 days in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. On the third day after transfection the medium was aspirated from the plates and the cells were harvested in PBS by being scraped from the plates with a cell scraper. The cells were then centrifuged for 10 min at 1500 × *g* and the cell pellets were stored at −80°.

Ligand binding studies. The transfected cells were homogenized (Polytron, setting 5, 5 sec) at 4° in binding buffer (50 mM Tris·HCl, pH 7.4, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, unless specified differently). The homogenates were centrifuged for 15 min at 39,000 × *g* and the pellet was resuspended in binding buffer at a concentration of approximately 1 mg/ml. For saturation binding analysis 250 μl of homogenate were incubated in duplicate with increasing concentrations (10–3000 pM) of [^{3}H]spiperone (120 μCi/mmol). Competition binding analysis was done by coincubation of 200–250 pM [^{3}H]spiperone and increasing concentrations (10–3000 pM) of the competing ligand, in either the presence or the absence of 200 μM Gpp(NH)p. Nonspecific binding was determined by coincubation of [^{3}H]spiperone with 30 μM dopamine. The samples were incubated in a final volume of 1 ml for 2 hr at room temperature and then filtered using a cell harvester (Skatron Instruments, Lier, Norway), as described previously (1). [^{3}H]Spiperone retained on the filters was detected by liquid scintillation counting. The ligand binding data were analyzed by the nonlinear least-square curve-fitting program LIGAND.

Results and Discussion

Comparative analysis of the rat and human D4 genes. Sequence analysis of the 3.8-kilobase genomic *Bam*HI fragment containing the putative rat D4 receptor gene indicated that we had isolated a genomic clone for this receptor that was identical to the rat D4 gene reported by O'Malley *et al.* (13). Comparative analysis of the complete nucleotide sequence of the human D4 receptor gene and the rat D4 receptor gene (EuGene release 3.2; sequence analysis program LAWRENCE to find homology domains between nucleotide sequences followed by sequence analysis program MONTE to test the significance of nucleotide sequence similarities) showed significant homologies between the coding regions of the human gene and the putative coding region of the rat gene (data not shown) (GenBank database: human D4 gene, accession numbers L12397, L12398, and X58497; rat D4 gene, accession number U03551). Based on these homologies and the previously identified splice junction sites of the human D4 receptor, we propose a coding sequence for the rat D4 receptor that differs from the sequence proposed by O'Malley *et al.* (13) by the inclusion of two extra amino acids (valine and glutamine) at the intron 1/exon 2 border (Fig. 1a). The sequence of the first intron of the rat sequence has not been previously characterized and is found to be 364 bp smaller than the human sequence. Apparent is the large sequence drift between the rat and human sequences in the noncoding regions, compared with the coding regions. However, two regions in intron 1 (rat nucleotides 395–442/human nucleotides 402–443 and rat nucleotides 810–992/human nucleotides 874–1073; the first nucleotide of intron 1 is denoted as 1) show significant sequence similarity (EuGene release 3.2, LAWRENCE followed by MONTE) between the rat and human genes; the first region comprises a CA repeat. Whether these sequences are of functional importance is not clear. Immediately downstream from these homologous regions in intron 1 is an apparent gap in the nucleotide sequence in the rat intron, compared with the human sequence. This region in the human gene displays a strong sequence similarity with human specific Alu repeat sequences and is not present in the rat gene (20, 21). Nucleotides 1223–1501 of intron 1 display 78% sequence similarity with the entire Alu consensus sequence (20) and are immediately followed by a sequence (nucleotides 1502–1672) that displays 78% sequence similarity with the second half of the Alu consensus sequence (Fig. 1b). Comparison of the first complete Alu sequence with the five identified subfamilies of Alu elements, i.e., older, galago, diverged, major, and conserved, gives sequence similarities of 77%, 70%, 77%, 77%, and 48%, respectively. These Alu sequences are found throughout the genome of primates and, based on sequence similarities, might have been derived from the 7SL RNA gene. To date there is no known function for this family of short interspersed repeated DNA elements (22).

When translated, the coding region of the rat gene displays only 75% similarity with the human receptor, in contrast to the high amino acid similarity between human and rat D2 and D3 receptors (>90%), suggesting that this entire region of the genome has been subject to relatively large evolutionary changes. This relatively large sequence difference in the coding region is predominantly located within the putative third cytoplasmic loop, which displays approximately 57% sequence similarity. This region contains a polymorphic repeat sequence in the human gene that is not present in the rat gene (13) (Fig. 2a). Although the third cytoplasmic loop region of G protein-coupled receptors has been shown to be involved in signal transduction (23–25), the implications of the sequence differences are as yet unknown. In this respect it is interesting to note the presence of 21 serine and threonine residues in this region of the rat receptor, compared with seven such residues in the human receptor. Twelve of these residues are located in the region that aligns with the repeat sequence in the human receptor. Because several of these amino acid residues are potential substrates for protein kinases, there might be differences in control of receptor desensitization between the two species. Moreover, the D2 short and long forms, which differ by 29 amino acids in a region of the third cytoplasmic loop in a position similar to the location of the polymorphic repeat sequence of the D4 receptor, demonstrate differences in characteristics of signal transduction (26, 27).

Identification of novel repeat sequences of the D4 gene. By making use of the PCR we have been able to amplify the polymorphic human D4 repeat sequence from the genomic DNA of individuals identified as having a D4 receptor allele corresponding in size to a 3-, 5-, 6-, or 9-fold repeat sequence. These amplified sequences were further studied by sequence analysis (see Fig. 2b). The sequences of the 3-fold repeat ($\alpha\gamma\zeta$) and the 5-fold repeat ($\alpha\beta\eta\epsilon\zeta$) are identical to previously reported sequences (17). However, the 6-fold repeat sequence ($\alpha\beta\theta\epsilon\beta\zeta$) is a new variant of the previously reported 6-fold repeat sequences. The isolated 9-fold repeat sequence

a.

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                          intron 1  /  exon 2

                                    Val Gln Gly Gly Ala Trp
D4 human          tcgccgcgcag GTC CAG GGT GGC GCG TGG
D4 rat            ttcgtggccag GTC CAG GGT GGC GTG TGG
                                    Val Gln Gly Gly Val Trp
```

b.

```
                1
Alu             GGCCGG--GCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGCG
D4 human        ....A.AA..-..-..AT..................G............AA....A..A.
              1223

                GATCACCTGAGGTCAGGAGTTCGAGACCAGCCTGGCCAACATGGTGAAACC---CC-G-T
                ...-.---.---.------..-............GA.....----.--..AGG..T.G.

                CTCTACTAAAAATACAAAAAT-T-AGCCGGGCGTGGTGGC-GCGTGCCTGTAATCCCAGC
                ......A......TTT.....A.C.......G........T...-..-...TG.......

                TACTCGGGAG-GCTGAGGCAGG-AGAATCGCTTGAACCCGGGAGGCGGAGGTTGCAGTGA
                ..T.....G.A.........-..C..........A....T.....................

                GCCGAGATCGCGCCACTGCACTCCAGCCTGGGCGACA-GAGCGAGACTCCGTCTCAAAAA
                .TT......T.A.....................A...A......A...............

                 291
                AAAA
                .C..
                1501


               121
Alu             AAA-AATACAA-AAATTAGCCGGGCGTGGTGGCGCGTGCCTGTAATCCCAGCTACTCGGG
D4 human        ...G..-.-..C....C.......G...........C.G..................T..
              1502

                AGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGG-CGGAGGTTGCAGTGAGCCGA-GAT
                ..A......TG....G.........G.T.A...ATT.C-...C.A.........-.T...

                                                                        291
                CGCGCCACTGCACTCCAGCCTG-GGCGACAGAGCGAGACTCCGT--CTCAAAAAAAAA
                G.A.....A...........-.C..T....C........-..-.AA.......C...G
                                                                       1672
```

Fig. 1. a, Nucleotide sequence of the 3′ splice junction site of intron 1 of the human D4 receptor (16), compared with the homologous region of the rat gene. *Lowercase letters*, intron sequence; *uppercase letters*, coding sequence. The putative amino acid sequences of the depicted region for the human and rat genes are indicated *above* and *below the nucleotide sequence*, respectively. b, Alignment of identified Alu sequences in intron 1 of the human D4 gene with the Alu consensus sequence (20). Sequence similarity of the identified regions with the Alu consensus sequence is 78%. The first sequence alignment (nucleotides 1223–1501 of intron 1) maintains this maximum sequence similarity with the entire Alu consensus sequence (nucleotides 1–291), whereas the second sequence alignment shows such a sequence similarity only with the next 170 nucleotides immediately adjacent to the first Alu sequence (nucleotides 1502–1672 of intron 1). ·, Nucleotides of the D4 Alu sequence identical to the Alu consensus sequence; –, gaps created to achieve maximum sequence homology.

($\alpha\beta\eta\pi\beta\epsilon\beta\epsilon\zeta$) is entirely novel, because no such variant has previously been reported. None of the characterized repeat sequences contained a 48-bp repeat unit different from the 19 units reported previously. However, the novel 6- and 9-fold repeats have increased the number of different observed haplotypes to 27. Both of these haplotypes code for a novel putative protein sequence, resulting in the identification of 20 putative different D4 receptor variants to date. All of the isolated variants have as first and last repeat units the α and ζ units, respectively. Only one exception to this has been reported previously, in which a rare haplotype with seven repeat units was found to end in a ξ sequence. However, the ζ and ξ units code for identical amino acid sequences. The high conservation of the α and ζ sequence and the fact that no individuals with less than the $\alpha\zeta$ repeat have been observed suggest a functional importance of this region. Because the third cytoplasmic loop

a.

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                           α
        TM5                                                          ┌──────────┐ ┌──
D4.2 human  LLLYWA TFRGLQRWEVARRAKLHGRAPRRPSGPGPP-S-PTPPAPRLPQDPCGPDCAPPAPGL
D4   rat    ...... .....R...A..HT...S............V.D..QG-.-.FS.-.-.---..S.S.

                ζ
────────────────────────────┐                                     TM6
---P-----P--D----PCGSN-CAPPDAVRAAALPPQTPPQTRRRRRRAKITGRERKAMR VLP
RTS.TVSSR.ES.LSQS..-.PG.LL...--.L.Q..A-.SS-..K.G............ ...
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b.

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Eco47III                        EcoNI                          TthIII1
 ↓                                ↓                               ↓
LQRWEVARRAKLHGRAPRRPSGPGPPSPTP    α   -  -  -  -  -  -  -  ζ  PDAVR D4.2
..............................    α   -  -  γ  -  -  -  -  ζ  ..... D4.3
..............................    α   β  -  δ  -  -  -  -  ζ  ..... D4.4
..............................    α   β  η  -  -  -  -  ε  ζ  ..... D4.5
..............................    α   β  -  θ  -  ε  β  -  ζ  ..... D4.6
..............................    α   β  η  -  -  ε  β  ε  ζ  ..... D4.7
..............................    α   β  η  π  β  ε  β  ε  ζ  ..... D4.9
..............................PPAPRL-  -  -  -  -  -  -  -  -  --P.. D4ΔEcoNI
..----------------------------    -   -  -  -  -  -  -  -  -  --P.. D4ΔEco47III1
```

Fig. 2. a, Alignment of the amino acid sequence of the putative third cytoplasmic loop region of the human and rat D4 receptors. The last and first amino acids of the proposed transmembrane regions (*TM*) 5 and 6, respectively, are shown and are separated from the loop sequence by a space. ·, Amino acids of the rat receptor that are identical to those of the human receptor; –, gaps in the sequences to achieve maximum sequence homology. The α and ζ repeat sequences in the human D4 receptor are *overlined*. b, Amino acid sequence alignment of the polymorphic region of the cloned human D4 receptor variants (D4.2, D4.3, D4.4, D4.5, D4.6, D4.7, and D4.9) and the third cytoplasmic loop deletion mutants (D4Δ*Eco*NI and D4Δ*Eco*47III). The positions of the *Eco*47III1, *Eco*NI, and *Tth*III1 restriction sites used in the construction of the deletion mutants are indicated. The complete amino acid sequences of the repeat units (α, β, γ, δ, η, θ, ϵ, and ζ) are identical to those published previously (17). ·, Amino acids in the alignment identical to those in the top sequence (D4.2); –, sequence gaps.

region has been shown to play an important role in signal transduction, it is possible that the repeat sequence might participate in or modulate such a function (26, 27). On the other hand, regions that have been implicated to be essential in G protein coupling in other receptors are located at the carboxyl terminus of the third cytplasmic loop (23–25).

Pharmacological analysis of the rat and human D4 genes. Although there are no differences in pharmacological profiles between the expressed human D4 cDNAs and genes, we compared the rat D4 genes with the human D4 genes (Table 1), because the genomic constructs display comparable levels of expression of the D4 receptor when transiently expressed in COS-7 cells (B_{max} of approximately 100–200 fmol/mg of protein), whereas with the human D4 cDNA constructs higher levels of expression are achieved (500–1000 fmol/mg of protein). The human D4 genes display a characteristic pharmacological profile for the D2 receptor antagonists spiperone, emonapride, haloperidol, clozapine, and butaclamol, as described previously (1, 16). The relatively high affinity of clozapine, compared with the D2 receptor, is the most distinguishing feature. The rat D4 receptor displays a similar pharmacological profile for these antagonists, although haloperidol and butaclamol seem to have somewhat diminished affinities, compared with the human receptor. Nevertheless, the rat D4 receptor displays high affinity for clozapine, as does the human homologue. Although in the first report by O'Malley *et al.* (13) the affinities of the rat D4 receptor for butaclamol and clozapine were determined to be 109 pM and 1.2 nM, respectively, our data do not support these values. Our analyses indicate the affinities of butaclamol and clozapine to be 352 nM and 17 nM, respectively, which are in agreement with the affinities observed for the human receptor.

Dopamine displays similar affinities for the human and rat D4 receptors, whereas the D2 receptor agonists quinpirole and PHNO seem to have slightly lower affinities for the rat D4 receptor (Table 1). However, the three different receptor agonists display comparable rank orders of potency. Although the affinities in Table 1 are derived from single-site analysis with the nonlinear curve-fitting program LIGAND, most curves display a significantly better fit ($p < 0.05$) in a two-site analysis, which is indicative of the presence of high and low affinity states of the receptor (Fig. 3). Because it has been shown

TABLE 1

Affinity dissociation constants for the expressed human and rat D4 genes

Expression and binding analyses of the genes encoding the human dopamine D4.4 and D4.7 receptors (D4.4G and D4.7G) and the rat dopamine D4 receptor (rat D4G) were performed. The affinity dissociation constants for the antagonists are the means of two to six independent measurements. The average percentage coefficient of variation of the individual affinity dissociation constants is 18%. The average range for the affinity dissociation constants is the mean ± 29%. The affinity dissociation constants for the agonists are the mean of two or three independent measurements. The average percentage coefficient of variation of the individual affinity dissociation constants is 20%. The average range for the affinity dissociation constants is the mean ± 29%.

Ligand	NaCl	k_i		
		Human D4.4G	Human D4.7G	Rat D4G
		nM		
Antagonist				
Spiperone[a]		0.071	0.062	0.143
Emonapride		0.153	0.247	0.347
Haloperidol		1.25	0.83	5.93
Clozapine		11.0	21.4	16.6
Butaclamol		79	67	352
Agonist				
Dopamine	–[b]	7	7	3
Dopamine + Gpp(NH)p[c]	–	19	24	30
Quinpirole	+	71	58	278
Quinpirole	–	7	7	45
PHNO	+	23	21	97

[a] [^{3}H]Spiperone, k_d.
[b] + and –, presence and absence of 120 mM sodium chloride in the binding buffer.
[c] 200 μM Gpp(NH)p.

previously that omission of sodium chloride increases the percentage of receptor sites in the high affinity state (28), we have done our competition binding assays with dopamine and quinpirole in the presence and absence of sodium chloride. Omission of sodium chloride in the binding assay results in 5–10-fold improved affinities for these agonists and also results in more consistent detection of high affinity sites. Moreover, the observed affinities for dopamine in the absence of sodium chloride are in close agreement with the EC_{50} values for dopamine-mediated inhibition of forskolin-stimulated cAMP levels in CHO cells expressing the D4 receptor.[1] Inclusion of 200 μM levels of the nonhydrolyzable GTP analogue Gpp(NH)p in the dopamine displacement binding assay indeed results in a rightward shift of the competition curves (Fig. 3), indicating functional coupling (28–30) of the human and rat D4 receptors in COS-7 cells.

Pharmacological analysis of the different polymorphic D4 receptor forms. By reconstruction and expression of full length cDNAs coding for the various forms of the D4 receptor, the influence of the polymorphic repeat sequence on the binding characteristics of several ligands was investigated. Saturation binding analysis with [^{3}H]spiperone demonstrated that the seven forms tested display similar affinities (K_d ~ 90 pM). The levels of expression were approximately 500–1000 fmol/mg of protein. Consecutive pharmacological analysis of the D2 antagonists haloperidol, clozapine, and raclopride by competition with [^{3}H]spiperone binding also demonstrated no major differences in affinity among the different D4 variants (haloperidol, K_i ~ 1 nM; clozapine, K_i ~ 13 nM; raclopride, K_i ~ 2000 nM) (see Table 2). However, as reported previously (1, 16), clozapine has an approximately 10-fold higher affinity for the D4 receptor, compared with the D2 receptor, whereas raclopride has an approximately 1000-fold lower affinity for the D4 receptor, compared with the D2 receptor. These data confirm previously reported pharmacological profiles for the D4.2, D4.4, and D4.7 receptors and extend those observations to other D4 variants having 3-, 5-, 6-, and 9-fold repeat units.

The different D4 variants also seem to have similar affinities for dopamine (k_i ~ 15 nM) (Table 2). Analysis of the dopamine competition curves indicated that 18 of 20 displayed a significantly better fit ($p < 0.05$) for a two-site model than a single-site model. Because untransfected COS-7 cells have no endogenous [^{3}H]spiperone binding, these two sites probably represent two different affinity states of the D4 receptor (average K_{high} ~ 6 nM; average K_{low} ~ 250 nM). Inclusion of 200 μM Gpp(NH)p in the competition binding assay resulted in decreased affinity for dopamine (k_i ~ 25 nM) in all dopamine competition assays [all dopamine competition assays in the presence and absence of Gpp(NH)p were done in parallel]. Only 10 of the 20 dopamine plus Gpp(NH)p competition curves had a significantly better fit for a two-site model (K_{high} ~ 13 nM; K_{low} ~ 400 nM). The observation that when the low affinity site contributes only a limited number of sites (<25%) the K_{low} tends to be of a higher value (>100 nM) suggests that the k_i is probably a more accurate estimate of the affinity for the low affinity site. A similar observation was made for the dopamine competition curves in the absence of Gpp(NH)p, that is, when the number of low affinity sites comprises a small portion of the total amount of sites, their affinity seems to be relatively low. However, when low and high affinity sites are present in more equivalent amounts, the K_{low} seems to be in better agreement with the k_i derived from the dopamine competition curves in the presence of Gpp(NH)p. This suggests that the pooled averages of K_{low} of 250–400 nM are probably overestimated, and a K_{low} of 25–100 nM might be more accurate.

The observation that the high affinity state of the receptor can be converted to the low affinity state by the nonhydrolyzable GTP analogue Gpp(NH)p suggests that this receptor is functionally coupled to a G protein-regulated signal transduction system in COS-7 cells (28–30). It has been shown that D4 receptors can inhibit the activation of adenylyl cyclase in CHO cells (31). Whether this signal transduction system is also used in COS-7 cells remains to be determined.

Measurement of basal dopamine levels in rat brain by microdialysis indicates that extracellular dopamine concentrations might be as high as 50 nM (32, 33). Our data showing the relatively high affinity of dopamine for the D4 receptor in the high affinity state (1–10 nM) suggest that the D4 receptor might be able to respond effectively to small changes in dopamine levels. However, if one speculates that the dopamine concentrations are severalfold higher in the synaptic cleft, then the receptor would be of more importance for tonic control by dopamine, especially if one considers that several dopamine competition assays indicate high affinity states for the D4 receptor of <1 nM (see Fig. 3).

It has been shown previously that human D4.2 and D4.4 receptors differ from the D4.7 receptor in sodium chloride sensitivity of clozapine binding. Here we have measured in parallel, in the presence and absence of sodium chloride in the binding buffer, the affinities of both [^{3}H]spiperone and clozapine for seven different D4 variants (Fig. 4). The data pre-

[1] V. Asghari, S. Sanyal, S. Buchwaldt, A. Paterson, V. Jovanovic, and H. H. M. Van Tol. Modulation of intracellular cAMP levels by different human dopamine D4 receptor variants. Manuscript in preparation.

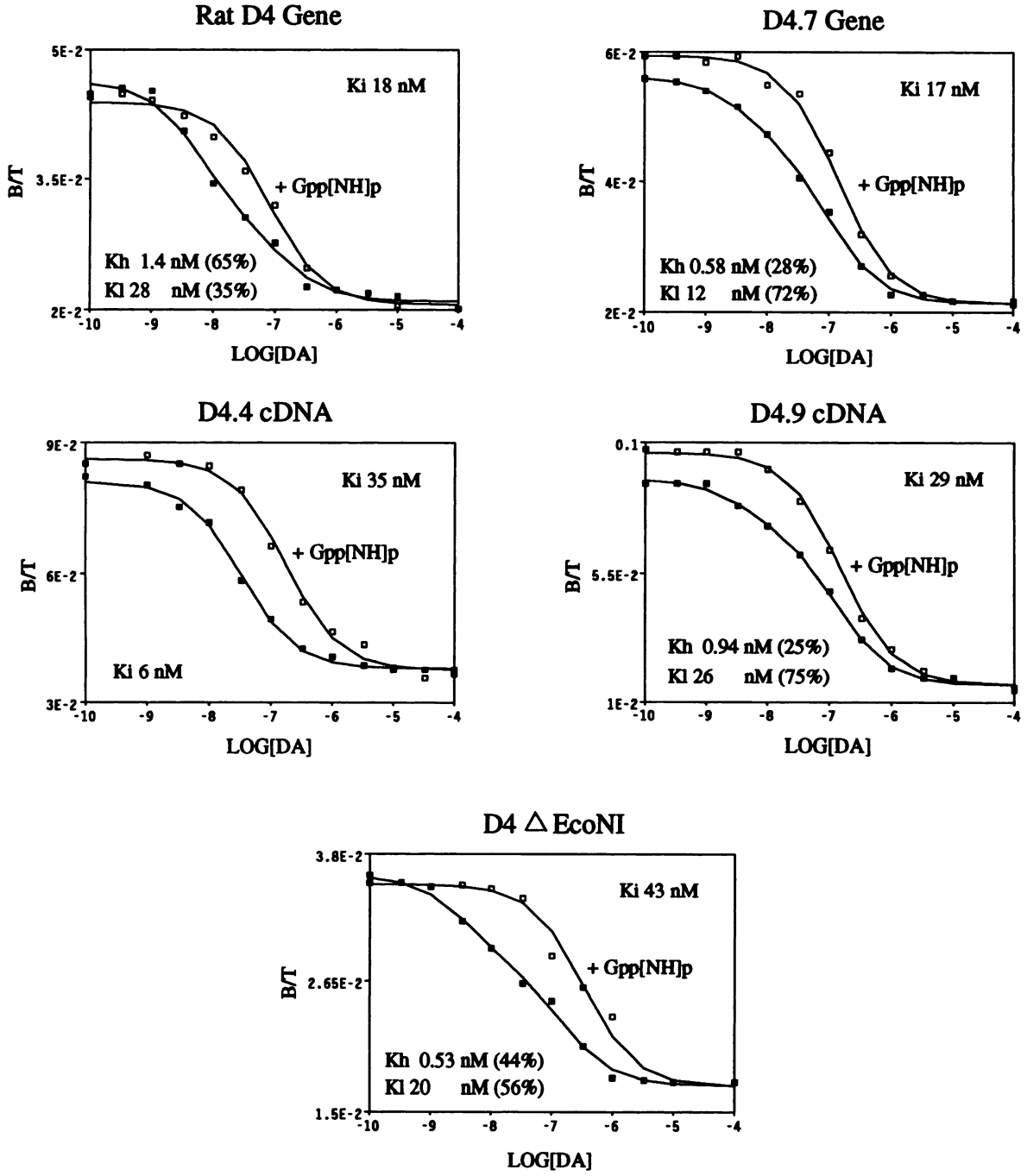

Fig. 3. Dopamine competition curves for the rat D4 gene, human D4.7 gene, human D4.4 cDNA, D4.9 cDNA, and D4Δ*Eco*NI deletion mutant expressed in COS-7 cells. Competition binding analysis was done with [^{3}H]spiperone (250 pM) and various concentrations of dopamine, in the presence (□) and absence (■) of 200 μM Gpp(NH)p. The concentrations of dopamine (*DA*) are given as the logarithm of the concentration (in μM) (*x*-axis), and the amount of specific binding is given as the ratio of bound versus total [^{3}H]spiperone in the binding assay (*y*-axis). As determined by the LIGAND program, most of the dopamine binding in the absence of Gpp(NH)p, in contrast to that in the presence of Gpp(NH)p, displayed significantly better fits for a two-affinity state model ($p < 0.05$). The affinity dissociation constants and percentages of sites contributing to the different affinity states of the dopamine competion curves in the absence of Gpp(NH)p are given in the *bottom left corners*, and the affinity dissociation constants for the dopamine competition curves in the presence of Gpp(NH)p are indicated in the *top right corners*.

sented in Fig. 4 suggest that the difference in sodium chloride sensitivity of clozapine binding is dependent on the number of repeat units in the D4 variant. Two-way analysis of variance (number of repeat units × sodium chloride) indicated significant differences between groups [$F(3,52) = 32.48$, $p < 0.0001$]. The affinity of clozapine is increased by the exclusion of sodium chloride in the binding buffer [$F(1,52) = 84.71$, $p < 0.0001$] and affected by the number of repeat units [$F(1,52) = 8.34$, $p < 0.006$]. Furthermore, these analyses revealed an interaction between the two factors (number of repeat units and sodium chloride) [$F(1,52) = 4.4$, $p < 0.04$], suggesting that the relationship between the number of repeats and the affinity for clozapine is different between the groups including or excluding sodium chloride. These data are essentially in agreement with

TABLE 2

Affinity dissociation constants for mutant and normal D4 receptor variants

Expression and binding analyses of cDNAs encoding two mutants with deletions in the third cytoplasmic loop (D4Δ*Eco*47 and D4Δ*Eco*NI) and seven natural occuring variants (D4.2, D4.3, D4.4, D4.5, D4.6, D4.7, and D4.9) of the human D4 receptor were performed. The affinity dissociation constants are the means of two independent duplicate measurements. The average percentage coefficient of variation of the affinity dissociation constants is <10%. The average range for the affinity dissociation constants of the antagonists (spiperone, haloperidol, clozapine, and raclopride) is the mean ± 9%. The average percentage coefficient of variation of the individual affinity dissociation constants for dopamine is 13%. The average range for the affinity dissociation constants is the mean ± 14%. Binding of dopamine was done in the absence of 120 mM sodium chloride in the binding buffer. The affinity dissociation constants are given for the single-site model; however, 18 of 20 dopamine curves displayed a better fit ($p < 0.05$) in a two-site model (K_{high} = 6.2 ± 1.1 nM; K_{low} = 254 ± 65 nM; mean ± standard error). In contrast, only 10 of 20 dopamine plus Gpp(NH)p curves gave a better fit for a two-site model (K_{high} = 12.9 ± 1.7 nM; K_{low} = 400 ± 103 nM).

D4 type	k_i					
	Spiperone[a]	Haloperidol	Clozapine	Raclopride	Dopamine	Dopamine + Gpp(NH)p[b]
	nM					
D4.2	0.063	0.85	10.6	1320	10	17
D4.3	0.084	0.91	14.5	2460	14	21
D4.4	0.110	1.35	10.1	2020	10	27
D4.5	0.088	1.02	16.9	2070	17	26
D4.6	0.108	0.94	10.9	2135	19	30
D4.7	0.104	1.04	11.8	1955	16	21
D4.9	0.119	1.28	16.2	2570	21	37
D4Δ*Eco*47III1	0.102	0.93	17.9	2045	29	45
D4Δ*Eco*NI	0.068	0.64	8.5	1470	8	32

[a] [^{3}H]Spiperone, k_d.
[b] 200 μM Gpp(NH)p.

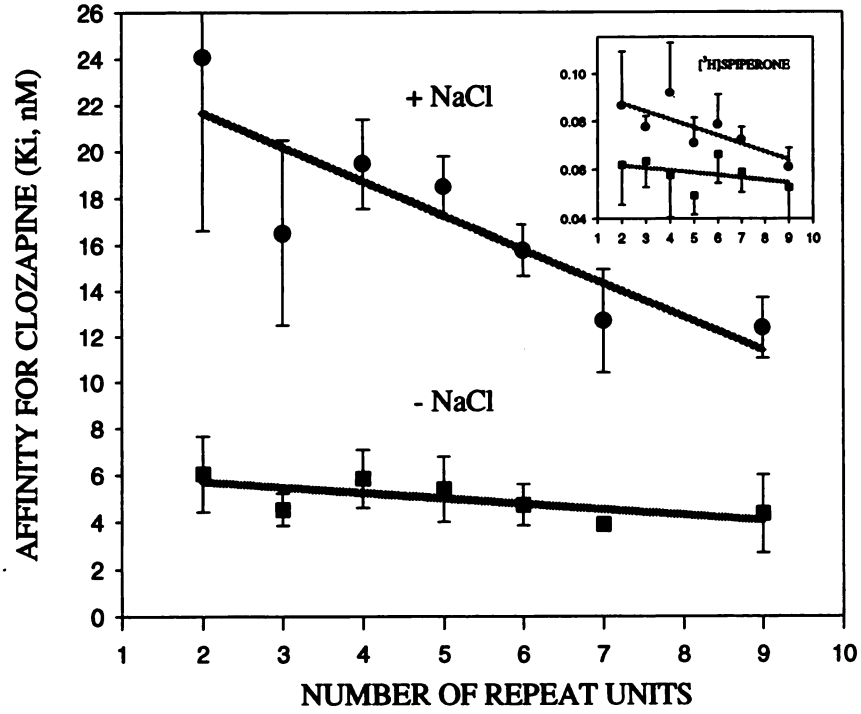

Fig. 4. Affinity of clozapine for seven different D4 variants (*y*-axis, K_i, mean ± standard error), which vary in the number of repeat units (*x*-axis, 2–7 and 9 repeat units), in the presence (●) or the absence (■) of sodium chloride in the binding buffer. Binding assays in the presence and the absence of sodium chloride in the binding buffer (see Experimental Procedures) were done in parallel for each D4 variant. The average affinities for clozapine were calculated from four independent experiments performed in duplicate. The affinity for [^{3}H]spiperone (*inset*) was measured by saturation binding analysis, and the affinity for clozapine was determined by competition with [^{3}H]spiperone binding. *Gray line*, regression for the affinity of the ligand and the number of repeat units in the different D4 variants.

our previous published observations on the differences in sodium chloride sensitivity of clozapine binding between D4 variants (16). Despite the observed trend in differences in affinity for clozapine with the number of repeat units in the third cytoplasmic loop, these differences are rather small and it has to be questioned whether such differences are of physiological or clinical relevance. The observation that there are no major differences in affinity for clozapine between different polymorphic forms of the D4 receptor suggests that variations in responsiveness to clozapine treatment in schizophrenic patients (9) are probably unrelated to the type of D4 receptor variant.

Pharmacological analysis of D4 repeat deletion mutants. Two deletion mutants were constructed for the third cytoplasmic loop region where the repeat sequence is located. In the *Eco*NI-*Tth*III1 mutant (D4Δ*Eco*NI) the repeat sequence is deleted except for the first 5 amino acids from the α repeat unit, whereas in the *Eco*47III-*Tth*III mutant (D4Δ*Eco*47III1) the first 27 upstream amino acids have also been deleted (Fig. 2b). Both mutants displayed a pharmacological profile for the tested antagonists similar to that of the nonmutated D4 receptor variants. Furthermore, the D4Δ*Eco*NI deletion mutant displayed a sensitivity to sodium chloride similar to that seen for the other D4 variants (data not shown). This further indicates that the polymorphic repeat region in the third cytoplasmic loop of the D4 receptor has little influence on binding profiles.

Displacement of [^{3}H]spiperone binding by dopamine was also not affected by these deletions. Both mutants displayed a k_i of approximately 18 nM. The dopamine competition curves for the D4 *Eco*NI-*Tth*III1 deletion mutant displayed a better fit for two affinity sites, with an average K_{high} of 1.4 nM. Inclusion of 200 μM Gpp(NH)p in the dopamine competition assays resulted in decreased affinity for dopamine and conversion of the competition curves to a single affinity site. The guanine nucleotide effect was most dramatic for the *Eco*NI-*Tth*III1 deletion mutant (see Fig. 3). Because the *Eco*47III-*Tth*III1 deletion mutant comprises a larger region of the third cytoplasmic loop, it is possible that this deletion already interferes with G protein coupling. The observation that the repeat deletion mutants are still sensitive to guanine nucleotides suggests that the repeat sequence is not essential for G protein coupling. However, the possibility cannot be excluded that there are differences in the efficacy of signal transduction between these mutants and the different polymorphic variants.

Conclusion

The dopamine D4 receptor has received much attention as a potential novel target for antipsychotic drug development, especially because this receptor has, like the muscarinic and 5-hydroxytryptamine type 2 receptors, a relatively high affinity for the atypical antipsychotic agent clozapine (7). The observation that the D4 receptor densities are increased approximately 6-fold in postmortem caudate putamen tissue of schizophrenic patients, compared with unaffected individuals (10), further supports this receptor being a promising target for schizophrenia treatment. However, the large number of polymorphic variants of the human D4 receptor (17) and the absence of such a polymorphic region in the rat D4 receptor make it desirable to investigate whether this region can affect the pharmacological binding profile with respect to the initial pharmacological screening of novel D4-specific ligands.

The cloning and expression of the rat D4 receptor and its pharmacological comparison with the human D4 receptor do not reveal major species differences; however, quinpirole, PHNO, and butaclamol may have a slightly lower affinity for the rat D4 receptor than the human receptor. Dopamine binding to both the rat and human receptors expressed in COS-7 cells is sensitive to guanine nucleotides, suggesting a G protein interaction for both of these receptors.

Our continued screening for new variants of the human D4 receptor has resulted in the identification of two novel polymorphisms, encoding 6- and 9-fold 16-amino acid repeat sequences in the third cytoplasmic loop. Expression and pharmacological characterization of seven different variants show that these have similar pharmacological profiles, suggesting that the polymorphic region has little effect on ligand binding, which is further supported by the fact that the repeat sequence deletion mutants are pharmacologically almost indistinguishable from the native D4 variants. Although detailed analysis of clozapine binding and sensitivity to sodium chloride indicated that the affinity of the D4 receptor for this antipsychotic agent is variable among D4 subtypes, the observed differences are <2-fold. Furthermore, the rat D4 receptor, which has relatively low sequence identity to the human receptor in the third cytoplasmic loop, does not display any major pharmacological differences, compared with the human variants.

The observation that there are no major pharmacological differences among all of the tested D4 variants and mutants does not preclude the possibility that these receptors differ in efficacy or specificity of signal transduction. However, the report that none of the polymorphic variants shows an association with clozapine responsiveness seems to be in agreement with the almost identical binding profiles for the different receptor variants (34). To date, linkage and association studies between the different D4 receptor variants and schizophrenia have been negative (35, 36). This fact does not support the D4 receptor as a primary cause of this disorder, but it does not exclude it as a clinical target for treatment. In this respect, the increased D4 receptor levels and effectiveness of clozapine treatment in schizophrenic patients makes the D4 receptor an attractive alternative for the development of novel antipsychotic agents. Furthermore, the presented evidence that there seem to be no major pharmacological differences between the D4 variants encourages the design of specific D4 ligands that, based on pharmacological profiles, can act uniformly on patients with the different D4 variants.

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