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# Identification of Transmembrane Regions Critical for Ligand Binding to the Human $D_3$ Dopamine Receptor Using Various $D_3/D_1$ Transmembrane Chimeras

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## ABSTRACT

To investigate the roles of individual transmembrane segments (TM) of the human $D_3$ dopamine receptor in its ligand-receptor interactions, we generated chimeric receptors in which its TMs were replaced, one at a time, partially or entirely, by the corresponding TM of the homologous human $D_1$ receptor. Ligand binding properties of the chimeras, as expressed heterologously in Sf9 cells using recombinant baculoviruses, indicate that the critical binding regions for $D_3$-selective (over $D_1$) ligands reside at narrow regions (6 to 8 residues) near the extracellular surface for TMI, II, IV and VI, while TMV seems to be minimally involved in the ligand selectivity. For TMIII and TMVII, the critical regions seem to be deeper, involving at least the 10 residues near the extracellular surface for TMIII, and the entire TM segment for TMVII. This is based on our current observations that the chimeras with the $D_3$ sequence in the critical regions, although the rest of the TM is of $D_1$ origin (except TMVII), showed the binding properties indistinguishable from those of the wild-type receptor. The chimeras with the $D_1$ sequence in the regions, on the other hand, showed ligand binding characteristics wildly variable depending on substituted TMs: Most marked decreases in ligand affinities were observed with the chimeras of TMIII and VII, and intermediate changes with those of TMIV and VI. Replacements of TMV produced no appreciable effects on the affinities of 14 test ligands (except for one). The chimeras of TMI and II with the $D_1$ sequence in the critical regions showed no appreciable specific binding for several radioactive $D_3$-selective ligands, possibly reflecting their critical roles in assembly and folding of the receptor. These critical regions of the $D_3$ receptor were highly homologous to those of the $D_2$ receptor, except for several nonconservatively substituted residues, which could be exploited to develop ligands selective for the $D_3$ over $D_2$ dopamine receptor or vice versa.

Dopamine receptors are G protein-coupled receptors with seven transmembrane domains, and consist of five distinct subtypes ($D_1$-$D_5$) and their splicing variants, which are classified into the families of $D_1$-like and $D_2$-like receptors (Kebabian and Calne, 1979; Civelli *et al.*, 1993; Gingrich and Caron, 1993; Seeman and Van Tol, 1994). Recently, the $D_2$-like receptors, which include $D_2$, $D_3$ and $D_4$ subtypes, have been the focus of many investigations because of the potential usefulness of their ligands for psychotic disorders and Parkinson's disease (O'Dell *et al.*, 1990; Sokoloff *et al.*, 1990, 1992; Sokoloff and Schwarz, 1995). The $D_3$ dopamine receptor, in particular, has received much attention as a potential target of antipsychotic drugs without extrapyramidal side effects, because of its highly localized distribution in limbic brain regions, in contrast to the $D_2$ dopamine receptors, which are widely distributed in all dopamine projection fields (Sokoloff and Schwarz, 1995). Development of subtype-specific ligands is essential to test this hypothesis, and is critically dependent on the knowledge on ligand binding pockets of individual subtypes.

Ligand binding pockets for the dopamine receptors seem to be primarily contributed by TMs as shown by earlier studies with catecholamine receptors, analogous members of the superfamily of G protein coupled receptors (Frielle *et al.*, 1988; Ostrowski *et al.*, 1992; Strader *et al.*, 1994). Although the sequences of TM segments among the dopamine receptor subtypes are considerably homologous, the ligand binding pockets of $D_2$-like receptors are distinct from those of $D_1$-like receptors (Civelli *et al.*, 1993; Gingrich and Caron, 1993), judging from the existence of numerous ligands with higher affinity for the $D_2$- over $D_1$-like receptors (several orders of magnitude). This provides a unique opportunity to charac-

[1] Our recent mutation of cysteine 114 to serine (i.e., the tenth residue of the TMIII of the human $D_3$ receptor to the corresponding residue of the $D_1$ receptor) led to marked changes in ligand affinities similar to those observed with the chimera TMIII-3. For instance, AJ-76 and UH-232 displayed $K_i$ values 100-fold greater than those obtained with the wild-type receptor. This further supports our proposal of the critical region for TMIII being deeper, involving at least the 10 residues near the extracellular surface.

**ABBREVIATIONS:** TM, transmembrane segment; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine.

terize binding pockets for $D_2$-like dopamine receptors ($D_3$ in particular) via a homolog-scanning mutagenesis (Cunningham *et al.*, 1989). It is likely that $D_3/D_1$ chimeras, for instance, would maintain the receptors without gross disruptions of the wild-type conformation and nonspecific perturbations of the tertiary structures of the receptor (Cunningham *et al.*, 1989). Characterization of such chimeras would enable us to understand contributions of individual TM segments to dopamine ligand binding pockets and would be useful for development of subtype-selective ligands. In this study, we generated a number of human $D_3$ receptor chimeras in which its TM segments, ranging from 22 to 26 amino acid residues, were replaced, one at a time, partially or entirely, with the corresponding segments of human $D_1$ receptor; we report ligand binding properties of the $D_3/D_1$ chimeras, as expressed in Sf9 cells using recombinant baculoviruses carrying the chimeric cDNAs.

## Materials and Methods

**Construction of chimeric receptors.** Initially, we constructed seven $D_3$ chimeras, in which each transmembrane segment of the human $D_3$ dopamine receptor from TMI to TMVII was replaced with the corresponding region of the $D_1$ receptor by using the procedure of gene splicing by overlap extension (Horton *et al.*, 1989). Briefly, a pair of linker primers were synthesized for each chimera. A sense primer contained its 3′ end sequence (about 20 nucleotides) complementary to the proximal part of a target TM of $D_1$ receptor, and its other half (about 20 nucleotides long) complementary to the $D_3$ sequence adjoining its corresponding TM. A similar antisense primer was prepared covering the distal side of the target TM. We used another pair of outside primers that were selected to contain a unique restriction site at the 3′ and 5′ ends of the $D_3$ receptor cDNA, outside of the target TM. After the polymerase chain reaction procedures (Horton *et al.*, 1989), we obtained a $D_3$ cDNA fragment with a $D_1$ TM sequence in the middle and unique restriction sites at the 5′ and 3′ ends. The final polymerase chain reaction fragments were digested with proper restriction enzymes to yield sticky ends and then were cloned into the PCRscript vector containing the $D_3$ dopamine receptor cDNA with the complementary ends. All chimeras were confirmed initially with restriction digestion maps and subsequently with dideoxy sequencing. They are designated as $D_3/D_1$-TMI (31–52), $D_3/D_1$-TMII (66–94), $D_3/D_1$-TMIII (104–126), $D_3/D_1$-TMIV (151–172), $D_3/D_1$-TMV (188–209), $D_3/D_1$-TMVI (327–353) and $D_3/D_1$-TMVII (364–384), with the numbers in the parentheses denoting the range of replaced residues ($D_1$) at the beginning and the end of the $D_3$ TM regions, as noted earlier (Civelli *et al.*, 1993). The numbering refers to the $D_3$ dopamine receptor throughout this report. Two or three additional mutants from each chimera were generated in which a cluster of seven or 14 residues of the amino-terminal (TMX-1 or -2) or seven residues at carboxyl-terminal side (TMX-3) of a given TM was mutated back to the $D_3$ sequence. These chimeras were generated in the same manner as described above, and their correct constructs were confirmed with dideoxy sequencing. Each $D_3$ chimeric insert was transferred to PVL1394 (a shuttle vector for baculovirus), which was used to prepare the recombinant baculovirus, using a Baculo Gold kit (PharMingen, San Diego, CA) following the vender's protocol. The recombinant baculovirus was plaque-purified, and the titer of final purified viral stocks ranged near $1 \times 10^8$ plaque-forming particles/ml. The culture of Sf9 cells ($1 \times 10^6$ cells/ml) was infected with $5 \times 10^6$ viral particles/ml, and harvested 60 to 72 hr after infection. The membranes were prepared as described elsewhere (Pregenzer *et al.*, 1993).

**Binding measurements.** Binding of radioactive ligands was measured in membranes obtained from Sf9 cells expressing recombinant receptors, using filtration techniques as described elsewhere (Pregenzer *et al.*, 1993). Briefly, [$^3$H]spiperone binding was measured in the medium containing 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 20 mM HEPES/Tris, pH 7.4, the radioactive ligand at varying concentrations (0.1 to 30 nM for typical binding profiles), and 5 to 100 $\mu$g of membrane protein depending on the receptor density of a given membranes, in a total volume of 500 $\mu$l at 4° for 60 min. In some experiments, the reaction volume was raised to 2500 $\mu$l to lower the ratio of bound/free ligand. The mixture was filtered over a Whatman GF/B filter (Whatman, Clifton, NJ) under vacuum. The filters were washed three times with 4 ml of an ice-cold 50 mM Tris·HCl buffer, pH 7.4. Nonspecific binding was estimated in the presence of excess unlabeled spiperone (10 $\mu$M). All the stock solutions for ligands were prepared in 0.1% ascorbic acid. Displacement experiments of [$^3$H]spiperone binding by test compounds (competition assay) were carried out in the same assay buffer with the radioactive ligand at 0.5 to 10 nM depending on the dissociation constant of chimeras. The binding data were analyzed using a nonlinear regression method (Sigma Plot), and presented with the mean and standard errors from three or more experiments.

## Results

We examined each chimeric receptor in Sf9 cells with [$^3$H]spiperone binding, a selective antagonist for $D_2$-like receptors ($D_2$, $D_3$ and $D_4$). The ratio of its specific to nonspecific binding at 2 nM was at least 4 or greater with the following chimeras: $D_3/D_1$-TMI-1(39–51), $D_3/D_1$-TMII-3(66–88), $D_3/D_1$-TMIII-2(123–126) and $D_3/D_1$-TMIII-3(104–114), $D_3/D_1$-TMIV-2(166–172), $D_3/D_1$-TMIV-3(151–164), $D_3/D_1$-TMV (188–209), $D_3/D_1$-TMVI (327–353), $D_3/D_1$-TMVI-2(347–353), $D_3/D_1$-TMVI-3(327–346), $D_3/D_1$-TMVII (364–384), $D_3/D_1$-TMVII-1(371–384) and $D_3/D_1$-TMVII-2(381–384). Again, the two numbers in the parenthesis denote the range of residues at the beginning and the end of a $D_3$ TM region that was replaced with the $D_1$ counterparts. Scatchard analysis using the binding equation for a single class of binding sites fit well (linearity) with the binding data from the $D_3$ dopamine receptor as well as those from the above chimeras (data not shown). The dissociation constant for [$^3$H]spiperone ranged from 0.38 to 29 nM, and the maximal binding sites from 3 to 37 pmol/mg protein (Table 1).

For the cells infected with the wild-type baculovirus, the ratio of specific to nonspecific binding of [$^3$H]spiperone was $< 1$. Similar low ratios were observed with the chimeras of $D_3/D_1$-TMI(31–51), $D_3/D_1$-TMI-3(31–41), $D_3/D_1$-TMII(66–94), $D_3/D_1$-TMII-1(75–94), $D_3/D_1$-TMII-2 (89–94), $D_3/D_1$-TMIII(104–126), and $D_3/D_1$-TMIV(151–172). Their ratios did not improve with other commercially available radioactive ligands such as [$^3$H]dopamine, [$^3$H]YM-09151–2 and [$^3$H]raclopride. These chimeras were not examined further in this study.

With the chimeras showing high levels of specific [$^3$H]spiperone binding, competition binding experiments were carried out with various ligands selective for the $D_3$ receptor (representing the $D_2$-like receptors) over the $D_1$ receptor. The ligands represent several well known templates such as butyrophenones (spiperone and haloperidol), ergots (lisuride and cabergoline), aminotetralins (UH-232 and AJ-76), pyrrolidinyl-methyl benzamides (raclopride), and phenothiazines (chlorpromazine). Also included are several agonists such as quinpirole, pramipexole and apomorphine. It should be noted, however, that agonist affinities here represent their low affinity states (uncoupled receptors) because of extremely high density of the cloned receptors (0.3 to 37

TABLE 1
Comparison of the dissociation constant ($K_d$) and the maximal binding sites ($B_{max}$) for [$^3$H]spiperone of the human $D_3$ dopamine receptor and its chimeras containing indicated $D_1$ TM sequences. The binding data fit to a binding model with a single class of binding sites, using Scatchard format. The data represents the mean ± standard error from three measurements.

| [$^3$H] Spiperone | Wild Type | TMI-1 | TM II-3 | TM III-2 | TM III-3 | TM IV-2 | TM IV-3 | TM V | TM VI | TM VI-2 | TM VI-3 | TM VII | TM VII-1 | TM VII-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $K_d$ (nM) | 0.42 ± 0.1 | 0.38 ± 0.03 | 0.46 ± 0.1 | 0.5 ± 0.1 | 6.4 ± 0.6 | 6.3 ± 0.6 | 0.58 ± 0.1 | 1.6 ± 0.1 | 0.72 ± 0.1 | 1.1 ± 0.1 | 0.6 ± 0.07 | 25.3 ± 3.0 | 15.6 ± 1 | 0.9 ± 0.1 |
| $B_{max}$ (pmol/mg of protein) | 25.9 ± 0.6 | 21.8 ± 0.7 | 3.5 ± 0.1 | 10 ± 0.3 | 6.2 ± 0.3 | 5.2 ± 0.3 | 3.0 ± 0.1 | 20.3 ± 0.5 | 26 ± 1 | 0.30 ± 0.05 | 3.4 ± 0.1 | 8.1 ± 0.5 | 4.3 ± 0.3 | 37 ± 3.5 |

pmol/mg protein). Table 2 lists the $K_i$ values for the test ligands in the wild-type receptor ($D_3$) and the chimeras, as obtained using Cheng-Prusoff equation (Cheng and Prusoff, 1973). We also found that all the chimeras we examined here interacted poorly with SCH23390, a $D_1$-specific ligand ($K_d$ of 0.37 nM for $D_1$), with the $K_i$ values of above 1000 nM as measured by its ability to inhibit [$^3$H]spiperone binding.

**TMI mutant.** The TMI sequence (Y31-C51) of the $D_3$ receptor shows that twelve residues are divergent from the $D_1$ receptor. The chimera TMI-1 (39–50) contains the five divergent $D_1$ residues near the carboxyl-terminal side, but retains the $D_3$ sequence at the amino-terminal side (near the extracellular surface). In the chimera, the dissociation constant ($K_d$) for [$^3$H]spiperone was 0.38 ± 0.03 nM, similar to that for the wild-type receptor (0.42 ± 0.1 nM), and the $K_i$ values for the other ligands were indistinguishable from those for the wild-type receptor (Fig. 1). This indicates a minimal contribution of the five divergent residues near the carboxyl-terminal side of TMI (near the intracellular surface) to ligand selectivity. The chimeras with the $D_1$ sequence at the amino-terminal side [TMI(31–52) or TMI-3 (31–41)], on the other hand, displayed no noticeable level of specific binding of [$^3$H]spiperone, [$^3$H]dopamine, [$^3$H]raclopride, and [$^3$H]YM-09151–2. This could imply that the amino-terminal side of TMI (near the extracellular surface) is important not only in ligand binding but also in assembly and folding of the receptor.

**TMII mutant.** Twelve divergent residues are found in the TMII sequence (Y66-G94) of the $D_3$ and $D_1$ receptors. The only TMII chimera we were able to examine here was the TMII-3(66–88) with the $D_1$ sequence at the amino-terminal side (including eight divergent residues) and the $D_3$ sequence at the carboxyl-terminal side (89–94) near the extracellular surface. With this chimera, we obtained the a $K_d$ value for [$^3$H]spiperone of 0.54 ± 0.04 nM, which is similar to that for the wild-type receptor (0.42 ± 0.1 nM) (Table 1). Also, all the test ligands displayed $K_i$ values nearly identical to those obtained with the wild-type receptor (Table 2; Fig. 1). This indicates that the eight divergent residues at the amino-terminal side of TMII, near the intracellular surface, may not be critical for ligand binding. The chimeras with the $D_1$ sequence at the carboxyl-terminal side (near extracellular surface), on the other hand, showed no appreciable level of specific binding, as is the case for TMI. Point mutations in the region would be useful for further characterization of its functional roles.

**TMIII mutants.** The TMIII sequence (D104-I126) of the $D_3$ receptor shows nine residues divergent from the $D_1$ receptor. The chimera TMIII-3(104–114) includes the $D_1$ segment, with eight divergent residues that span the amino-terminal side of the TMIII near the extracellular surface. This mutation differentially affected the affinity of the test ligands (Table 2; Fig. 2), ranging from 15-fold to more than 5000-fold affinity changes. Those with moderate affinity changes (a ≤ 40-fold increase in their $K_i$ values) are spiperone, butaclamol, haloperidol, the ergots (lisuride and cabergoline). Those with noticeable affinity changes (100–500-fold increases in $K_i$ value, as noted in parentheses) are YM-09151–2 (97), chlorpromazine (155), AJ-76 (321), apomorphine (454), and UH-232 (575). Those with marked affinity changes include quinpirole (775), pramipexole (1366), and raclopride (5500).

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TABLE 2

Comparison of $K_i$ values for 14 $D_3$-selective ligands (over $D_1$) in the human $D_3$ dopamine receptors and its $D_1$ chimeras. Binding of [$^3$H]spiperone was measured in the presence of test drugs at various concentrations in the Sf9 cell membranes expressing the wild-type receptor or its chimeras with an indicated TM from $D_1$ dopamine receptor. The half-maximal inhibitory concentrations ($IC_{50}$) were computed and converted to $K_i$ values, using the Cheng-Prusoff equation. The data represent the mean ± standard error from three dose-response profiles.

| | Wt ($D_3$) | TMI-1 | TM II-3 | TM III-2 | TM III-3 | TM IV-2 | TM IV-3 | TM V | TM VI | TM VI-2 | TM VI-3 | TM VII | TM VII-1 | TM VII-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spiperone | 0.42 ± 0.1 | 0.38 ± 0.03 | 0.46 ± 0.03 | 0.5 ± 0.1 | 6.4 ± 0.6 | 6.3 ± 0.6 | 0.58 ± 0.1 | 1.6 ± 0.1 | 0.7 ± 0.1 | 1.1 ± 0.1 | 0.60 ± 0.07 | 25.3 ± 3.0 | 15.6 ± 1 | 0.9 ± 0.1 |
| Butaclamol | 3 ± 0.8 | 2.7 ± 0.1 | 3.6 ± 0.3 | 2.1 ± 0.1 | 89 ± 9 | 51 ± 1 | 3.2 ± 0.1 | 3 ± 0.1 | 3.8 ± 1.5 | 5.5 ± 0.4 | 9.4 ± 0.8 | 134 ± 36 | 30 ± 3 | 6.3 ± 0.5 |
| Chlorpromazine | 1 ± 0.2 | 0.4 ± 0.02 | 1.4 ± 0.2 | 1.2 ± 0.1 | 155 ± 17 | 30 ± 1.6 | 4.3 ± 0.2 | 1.2 ± 0.2 | 5.5 ± 0.5 | 5.8 ± 0.3 | 2.9 ± 0.2 | 40 ± 3 | 19 ± 2.4 | 3.1 ± 0.4 |
| YM 9151-02 | 0.3 ± 0.1 | 0.16 ± 0.04 | 0.6 ± 0.1 | 0.2 ± 0.05 | 29 ± 3 | 1.0 ± 0.1 | 0.6 ± 0.1 | 0.5 ± 0.1 | 0.3 ± 0.1 | 0.28 ± 0.03 | 0.28 ± 0.1 | 29 ± 3 | 47 ± 2 | 1.2 ± 0.2 |
| UH-232 | 4.8 ± 0.5 | 2.8 ± 0.6 | 5 ± 0.3 | 3.9 ± 0.2 | 2,761 ± 426 | 17 ± 1.2 | 5.1 ± 0.3 | 4.1 ± 0.9 | 7 ± 1 | 8.3 ± 0.3 | 10.9 ± 1.1 | 267 ± 72 | 50 ± 3 | 13 ± 1.6 |
| AJ-76 | 31.1 ± 4.6 | 39.1 ± 2.7 | 30 ± 2 | 29 ± 1.6 | 9,994 ± 1,818 | 139 ± 7 | 31.6 ± 2.2 | 26 ± 1.3 | 27 ± 8.7 | 32 ± 2.5 | 67.7 ± 4.6 | 9,893 ± 1,103 | 51 ± 4 | 149 ± 6 |
| Haloperidal | 4.8 ± 0.3 | 1.3 ± 0.2 | 6.5 ± 0.9 | 4.6 ± 0.3 | 115 ± 6 | 279 ± 17 | 29 ± 2 | 20 ± 1.2 | 6.6 ± 0.4 | 14.7 ± 1.1 | 7.8 ± 1.0 | 686 ± 61 | 21 ± 7 | 25 ± 1 |
| Raclopride | 3.7 ± 1.5 | 2.5 ± 0.2 | 4.1 ± 0.3 | 2.6 ± 0.1 | 20,356 ± 2,506 | 142 ± 10 | 4.9 ± 0.6 | 905 ± 106 | 26 ± 1.4 | 20.2 ± 1.2 | 7.7 ± 0.4 | 15,880 ± 951 | 2,622 ± 221 | 10 ± 0.5 |
| (−)3-PPP | 84 ± 9 | 132 ± 10 | 64.5 ± 5.7 | 96 ± 4 | 20,100 ± 4,001 | 1,471 ± 241 | 61 ± 3 | 129 ± 21 | 141 ± 18 | 407 ± 18 | 72.5 ± 12.5 | >50,000 | 8,434 ± 617 | 419 ± 37 |
| Quinpirole | 14 ± 1 | 26 ± 1.8 | 17 ± 1.1 | 9.6 ± 0.3 | 10,856 ± 1,741 | 1,190 ± 111 | 9 ± 2 | 38 ± 3.2 | 3,297 ± 214 | 4,525 ± 471 | 14 ± 1.3 | 43,000 ± 6,870 | 11,195 ± 1,274 | 634 ± 41 |
| Apomorphine | 23 ± 2 | 16.8 ± 3.1 | 12 ± 1.3 | 25.6 ± 0.8 | 10,442 ± 2,116 | 53 ± 2 | 10 ± 1 | 25 ± 12 | 9 ± 1 | 31 ± 3 | 13.3 ± 1.3 | 5,496 ± 766 | 1,003 ± 194 | 646 ± 23 |
| Pramipexole | 5.4 ± 0.6 | 3.7 ± 0.8 | 3 ± 0.2 | 4.1 ± 0.3 | 7,379 ± 213 | 402 ± 2 | 3 ± 0.2 | 5.9 ± 0.3 | 215 ± 83 | 502 ± 29 | 2.3 ± 0.2 | 29,028 ± 7,016 | 1,192 ± 192 | 112 ± 5 |
| Lisuride | 1.1 ± 0.1 | 0.8 ± 0.15 | 1.9 ± 0.2 | 0.8 ± 0.1 | 26.6 ± 1.5 | 0.9 ± 0.3 | 1.4 ± 0.2 | 1.3 ± 0.1 | 0.9 ± 0.1 | 0.8 ± 0.1 | 2.1 ± 0.3 | 3.2 ± 0.6 | 1.1 ± 0.1 | 3.7 ± 0.1 |
| Cabergoline | 1.3 ± 0.1 | 1.8 ± 0.3 | 4.1 ± 0.4 | 1.3 ± 0.1 | 50.4 ± 7.7 | 2 ± 0.1 | 1.8 ± 0.2 | 1.4 ± 0.1 | 1.3 ± 0.2 | 2.3 ± 0.5 | 2.5 ± 0.5 | 3.7 ± 0.3 | 1.5 ± 0.2 | 6 ± 0.4 |

Wt, wild-type receptor.

The TMIII-2 chimera contains the $D_1$ TM III sequence at the carboxyl-terminal side, including the two remaining divergent residues, and the $D_3$ sequence at the amino-terminal side near the extracellular surface. This alteration had no appreciable effect on the affinity of all the tested compounds; their $K_i$ values were nearly identical to those observed with the wild-type receptor (Table 2; Fig. 2).

**TM IV mutants.** Comparison of the TMIV sequence of the $D_3$ (A151-F172) and $D_1$ receptor reveals 18 divergent residues. The chimera TMIV-3 (151–165), containing the $D_1$ sequence at the amino-terminal side (near the intracellular surface) with 11 divergent residues, showed ligand $K_i$ values $\cong$ or $<$ 6-fold changes compared with those for the wild-type receptor (Table 2; Fig. 2). This indicates marginal contributions to ligand binding by the amino-terminal region (A151 to S165), which includes the 11 divergent residues.

The chimera TMIV-2 (166–172) contains the $D_1$ sequence at the carboxyl-terminal side (near the extracellular surface) with seven divergent residues. This mutation differentially affected the affinity of the test ligands (Fig. 2). The compounds with minimal affinity changes ($\leq$ 4-fold) were the aminotetralins (UH-232 and AJ-76), YM-09151–2, apomorphine, (−)3-PPP, and the ergots (lisuride and cabergoline). Those with noticeable increases in their $K_i$ (15- to 100-fold) are spiperone (15), butaclamol (17), chlorpromazine (30), and raclopride (38), haloperidol (58), pramipexole (74) and quinpirole (85). Apparently, the seven divergent residues at the carboxyl-terminal side (near the extracellular surface) of TM IV seem to play considerable roles in ligand binding for the $D_3$ receptor.

**TM V mutant.** Despite 13 divergent residues between the TMV of $D_1$ and $D_3$ receptors (F188-A209), the chimera TMV(188–209), containing the $D_1$ TM V segment in its entirety, had no appreciable effect on the affinity of most test ligands, except for raclopride, which showed a 240-fold increase in its $K_i$ (Fig. 3). Because of this lack of general effects on ligand affinity by TMV, here we did not study TMV further.

**TM VI mutant.** The TM VI sequence (A327-T353) of the $D_3$ receptor shows 19 residues divergent from the $D_1$ receptor. The chimera TMVI(327–353), containing the $D_1$ TM VI segment in its entirety, altered affinities of only few ligands. Quinpirole and pramipexole showed a 235- and 45-fold decrease in their affinity to the chimera, respectively, whereas the other ligands showed no noticeable affinity changes compared with those observed with the wild-type receptor (Table 2; Fig. 3). The chimera TMVI-2 (347–353) contains the seven divergent $D_1$ residues at the carboxyl-terminal side (near the extracellular surface) and displayed ligand affinity changes nearly identical to those observed with the chimera TMVI, with the $K_i$ values of quinpirole and pramipexole 323 and 93 times greater, respectively, than the wild-type receptor, but no appreciable affinity changes with the rest of test ligands (Table 2; Fig. 3). On the other hand, the chimera TMVI-3 (327–346), which contains the $D_3$ sequence at the carboxyl-terminal side and the $D_1$ sequence at the amino-terminal side (near the intracellular surface) including 12 divergent residues, displayed ligand-binding characteristics nearly identical to those of the wild-type receptor, including its affinities for quinpirole and pramipexole (Table 2). Thus, the key molecular determinant for quinpirole and pramipexole seems to

reside at the carboxyl-terminal side (near the extracellular surface) of TMVI.

**TM VII mutants.** Twelve divergent residues are found in the TM VII sequence (L364-T384) when comparing the $D_3$ and $D_1$ receptors. Three TM VII chimeras were examined in this study: TMVII(364–384), TMVII-1(371–384), and TMVII-2(381–384). The chimera TMVII(364–384), containing the $D_1$ sequence in its entirety, bound most test ligands with noticeably low affinity, although this affinity varied from ligand to ligand (Fig. 2). Only the ergots (lisuride and cabergoline) showed no appreciable change in their affinities, displaying only 2- to 3-fold increases in their $K_i$ values compared with those obtained with the wild-type receptor. The ligands with moderate affinity decreases (40- to 150-fold) include butaclamol (45), chlorpromazine (40), UH-232 (56), spiperone (60), YM-09151–2 (97), and haloperidol (144). The ligands of markedly low affinity (over 200-fold) included apomorphine (239), AJ-76 (318), quinpirole (3071), raclopride (4291), and pramipexole (5375). Moreover, (−)3-PPP even at 50 $\mu$M showed no detectable inhibition of [$^3$H]spiperone binding (in Fig. 2, we indicated a 600-fold change in affinity for comparison's sake).

In the chimera TMVII-1(371–384), where the six divergent residues at the amino-terminal side (near the extracellular surface) were reverted to the $D_3$ residues, the binding affinities of several ligands improved compared with those observed with the chimera TMVII (Table 2). The most pronounced improvement was noted with AJ-76, a 200-fold decrease in its $K_i$, nearly approaching its wild-type value, followed by haloperidol and pramipexole with about 20 to 30-fold affinity increases. Only marginal improvements (ranging from 2- to 5-fold changes) were seen with the rest of the test compounds. In the chimera TMVII-2(381–384), where the $D_3$ sequence extended almost all the way to the carboxyl-terminal side except for two divergent residues (381 and 384), most ligands displayed binding affinity comparable with those observed with the wild-type receptor, including spiperone, butaclamol, chlorpromazine, YM 09151–2, UH-232, raclopride and (−)3-PPP (Fig. 2). Interestingly, however, the affinities of quinpirole, apomorphine, and pramipexole were still 45-, 28-, and 21-fold lower, respectively, compared with their wild-type values. This suggests that the remaining two divergent residues, V381 and T384, at the extreme carboxyl-terminal side of TMVII, may contribute to interactions of these ligands with the receptor.

## Discussion

We found no systematic studies on $D_3/D_1$ chimeras in the literature, but few analogous ones such as the $D_1$ chimera with the $D_2$ carboxyl-terminal region, TMVII and TMVI (MacKenzie *et al.*, 1993; Kozell *et al.*, 1994). This chimera

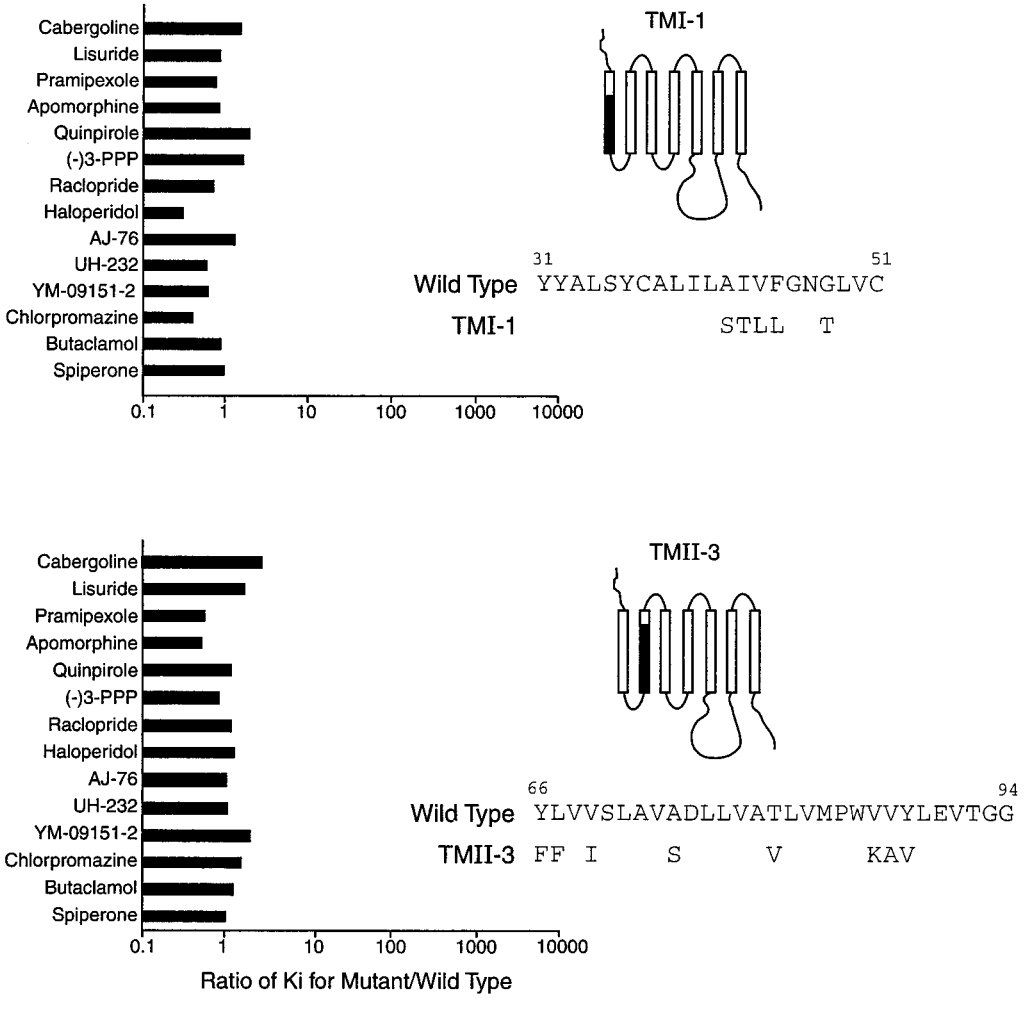


**Fig. 1.** Comparison of relative affinity changes of test ligands in $D_3/D_1$ chimeras involving TMI and II. Relative affinity of a given ligand was represented by the ratio of its $K_i$ value in the chimeras to that in the wild-type receptor. The plots include two chimeras, TMI-1 and TMII-3, showing their binding affinities indistinguishable from those of the wild-type receptor. The diagram depicts $D_3$ dopamine receptors with seven transmembrane segments (rectangular box) and the filled region represent the $D_1$ sequence in the chimeras. For each chimera, the divergent residues in the chimera were indicated in the single-letter codes.

retained binding affinity to [$^3$H]SCH23390, a selective ligand for the $D_1$ receptor, and at the same time improved affinity toward quinpirole, a selective agonist for $D_2$-like receptors. This suggests the importance of the region of TM VI and TM VII for quinpirole binding but provided little information about contributions by individual TM segments to ligand binding pockets. In this study, with a number of ligands selective for the $D_3$ over the $D_1$ dopamine receptor, we characterized the binding properties of various $D_3$ chimeric receptors in which its individual TM segments were replaced one at a time, partially or entirely, with the corresponding $D_1$ TM sequence. Our results showed that relative changes in ligand affinity were widely variable from chimera to chimera, depending on substituted TM segments. The chimeras with the $D_1$ TMIII (TM III-3 in particular) or TMVII segment showed marked affinity changes for a broad spectrum of ligands, with a maximal affinity decrease of nearly 5000-fold (e.g., raclopride and pramipexole). The replacement of TMIV with the corresponding $D_1$ sequence near the extracellular surface (TM IV-2) produced rather moderate affinity alterations for most ligands, with a maximal affinity decrease up to 80-fold for pramipexole and quinpirole. Similar replacements of TMV or -VI sequence, despite 13 and 19 divergent residues, respectively, selectively affected the affinities of few ligands, raclopride for the TMV chimera (a 244-fold increase in its $K_i$ value), and quinpirole and pramipexole for the TMVI chimera (236- and 40-fold increases in their $K_i$ values, respectively), with no noticeable effects on the affinity of the other test ligands.

In fact, all the chimeras examined here produced, more or less, differential effects on affinities of test ligands of various chemical templates, as if only a particular microbinding region or regions were perturbed in a given chimera. For instance, in the TM VII chimera, we observed a 5000-fold increase in the $K_i$ value for raclopride (benzamide), but no appreciable change in those for lisuride and cabergoline (ergots). These disparities among test ligands support the view that the observed affinity changes arise from alterations in

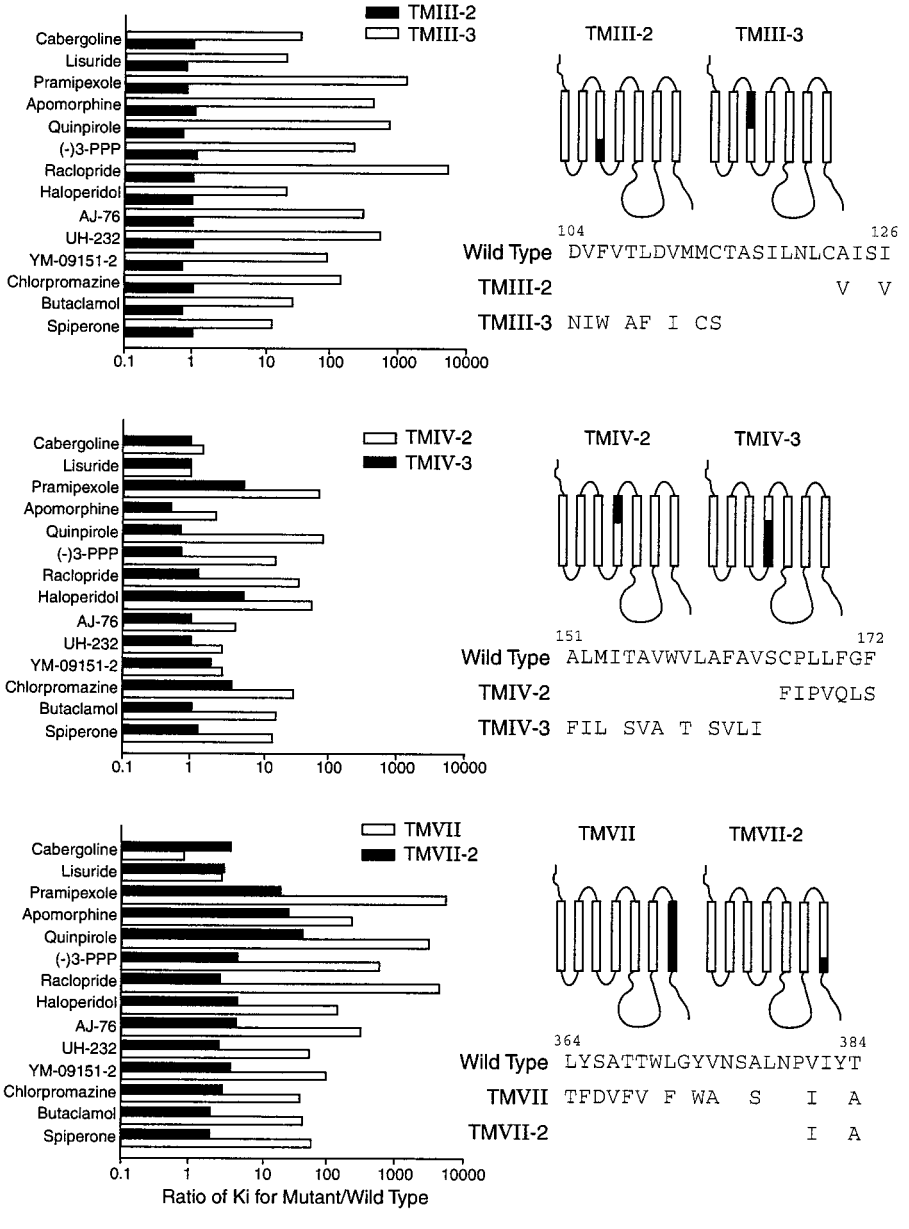


**Fig. 2.** Comparison of relative affinity changes of test ligands in $D_3/D_1$ chimeras involving TMIII, IV and VII. Relative affinity of a given ligand was represented by the ratio of its $K_i$ value in the chimeras to that in the wild-type receptor. For each chimera, the divergent residues in the chimera were indicated in the single-letter code. The plots include the chimeras with three TM segments affecting affinities for a broad spectrum of ligands, and intend to contrast differential effects of two distinct regions in a TM segment. The bar for (−)3-PPP in TMVII at the bottom represents a lower limit for its $K_i$ change, because its $K_i$ value is >50,000 nM, an upper limit in our assay.

critical binding domains for particular ligands, rather than from such nonspecific changes as global perturbations of receptor conformations. This apparent lack of nonspecific receptor alterations among the $D_3/D_1$ chimeras could be attributed not only to the high sequence homology between the $D_1$ and $D_3$ TM segments but also to substitutions of only small and discrete TM regions, one at a time, in these chimeras. Despite these efforts, however, several chimeras, particularly those involving regions toward the extracellular side of TM I and II, could not be examined here because of their low levels of specific binding for available $D_3$-selective radioactive ligands. This perhaps could be interpreted to mean that TMI and II may play critical roles in assembly and/or folding of the receptors, and will necessitate even smaller substitutions, including point mutations, to assess their roles in receptor-ligand interactions. Besides TMI and II, the chimeras with the whole segment of $D_1$ TMIII or IV also failed to show binding activity for $D_3$ ligands (despite high homologies to the $D_3$ counterparts), but these TMs differ from the TMI and II in that substitutions with smaller $D_1$ TM fragments, including the critical regions retained ligand binding activity, albeit of lower affinity compared with the wild-type receptor. It seems that overall free energy changes from the two smaller substitutions in TMIII (TMII-2 and TMIII-3), for instance, could not predict the energy change encountered with the substitution of the whole TM segment. This unpredictable (probably much greater) free energy change could arise from structural interdependency of the residues within a helical structure as well as their cooperative interface interactions with the adjacent transmembrane helices. Otherwise, free energy changes from the smaller substitutions would be additive, as known for mutations of structurally independent regions. Also there are many conceivable scenarios leading to the chimeras' failure to show ligand binding activity, such as their failed expression, their rapid degradation, a failure of their translocation to surface membranes, or their severely disturbed binding pockets. Discrimination of these various conformational possibilities for the chimeras with $D_1$ TMI, II, III, and IV could be attempted in the future with various techniques in cell biology, including the uses of site-specific antibodies to the $D_3$ receptor.

Here we evaluated how an overall similarity (or dissimilarity) of the substituted TM sequence for a given chimera is related to its relative changes in ligand affinity. As computed the similarity in the primary sequences of substituted TMs, using the Dayhoff Table (Schwarz and Dayhoff, 1978), we found that the overall similarity of the substituted region to the original $D_3$ TM sequences shows no relationship with the degree of ligand affinity changes. For instance, the order of similarity (%) among the key chimeras follows TM III-3 (91.3%) > TMV (72.9%) > TM IV-2(72.7%) > TMVII (61.9%) > TM VI (59.3%), and the order hardly reflects their relative

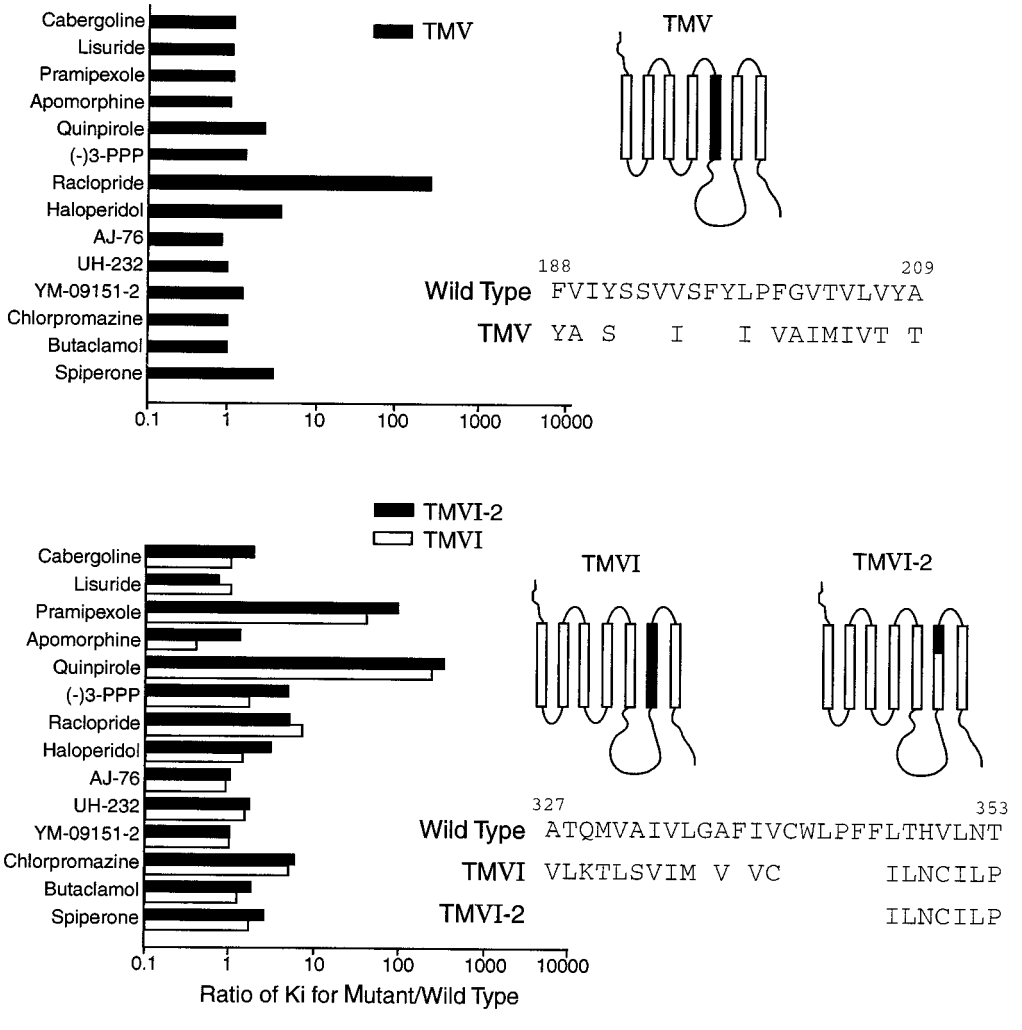


**Fig. 3.** Comparison of relative affinity changes of test ligands in $D_3/D_1$ chimeras involving TMV, TMVI and TMVI-2. The plots include chimeras affecting affinities of selective ligands, raclopride for TMV and quinpirole and pramipexole for TMVI. For the TMVI chimeras, the plot underscores the fact that the seven divergent $D_1$ residues at the carboxyl-terminal side (near extracellular surface) influence the affinity of quinpirole and pramipexole. The TMVI-3 with the 12 divergent residues at the amino-terminal side (near intracellular surface) showed ligand binding properties indistinguishable form the wild-type $D_3$ receptor, including quinpirole and pramipexole (Table 2). Other details for the plots were as described in the legend of Fig. 1.

impact on ligand affinity (see the text, Table 2, and Figs. 1, 2, and 3). This again points out that alterations of critical binding regions in the chimeras, rather than overall dissimilarities in their sequences, are responsible for observed ligand affinity changes. Again, this negligible general structural factor is largely attributable to substitution of a small, discrete region for a given chimera with a homologous segment and could be recognized by the use of diverse ligands selective for the $D_3$ over $D_1$ receptor. From these considerations, we propose that individual TM segments disproportionately contribute to receptor ligand interactions of the $D_3$ dopamine receptor, with the order of decreasing impact, TM VII, TMIII > TM IV >TM VI > TMV.

Furthermore, the current study enabled us to focus on the molecular determinants of individual TM segments for the $D_3$ receptor ligand interactions. Our observations indicate that the key molecular determinants of most TM segments confined to narrow regions near the extracellular surface. For instance, the TMIV-3 chimera that contains the seven residues of the $D_3$ origin at the carboxyl-terminal side (near the extracellular surface), bound all the ligands with affinity comparable with that of the wild-type receptor, despite its $D_1$ sequence at the amino-terminal side (A151-F164), which includes 11 divergent residues. On the other hand, the TMIV-2 chimera with the $D_1$ sequence at the carboxyl-terminal side and the $D_3$ sequence at the amino-terminal side, (its orientation of $D_1$ sequence opposite to the TMIV-3 chimera), showed considerable changes in ligand affinity (Fig. 2). This difference in the behaviors of the TMIV-2 and TMIV-3 chimera could not be predicted from their overall sequence similarity to the wild-type receptor (63.7% for TMIV-3 and 72.7% for TMIV-2), and underscores the view that the key molecular determinants of the TMIV for ligand binding are confined to the residues near the extracellular surface. Similarly, the TMVI-3 with the $D_3$ sequence near the extracellular surface bound all the ligands with affinities comparable with that of the wild-type receptor despite its 12 divergent $D_1$ residues near the intracellular surface. On the other hand, the TMVI-2 with the seven divergent $D_1$ residues near the extracellular surface showed selective affinity changes for quinpirole and pramipexole that were very similar to those of the TMVI, which contains the entire $D_1$ TMVI sequence. The same view can be applied to the TMI, TMII, and TMVI. That is, no appreciable affinity changes for the test ligands were observed with the TMI-1, II-3, and VI-3, with the $D_3$-sequence (6 to 8 residues deep) near the extracellular surface despite their $D_1$ sequences in the rest of the TM segments. With the TMV, we did not pursue further because there were no noticeable affinity changes for test ligands other than raclopride. For the TMIII segment, the chimera with the $D_1$ sequence near the extracellular surface (TMIII-3, 104–114) produced marked changes in ligand affinities compared with the wild-type receptor.[1] This is also consistent with the view that the key molecular determinants for ligand binding confined to the regions near the extracellular surface, but the question of how far this critical region extends could not be

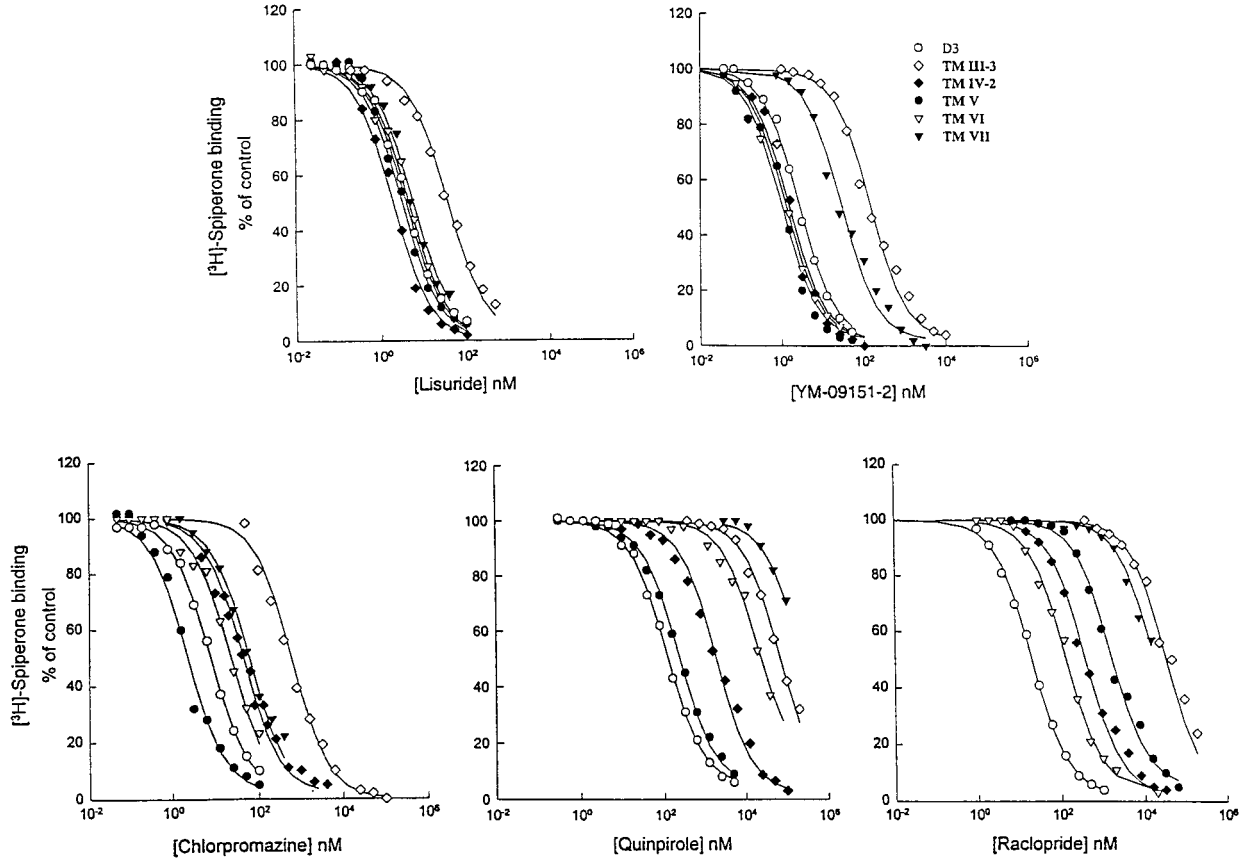


**Fig. 4.** Dose-dependent binding profiles for five representative ligands displaying differential sensitivity to $D_3/D_1$ chimeras. [$^3$H]Spiperone binding was measured in the presence of indicated ligands at various concentrations. The concentration of [$^3$H]spiperone was varied, but was kept at less than its $K_d$ value for individual chimeras, so that their $IC_{50}$ values in the plots are reasonably close to their $K_i$ values.

answered with our current study because of the high sequence homology of the rest of the TMIII sequence (115TASILNLC122) between the $D_1$ and $D_3$ receptor. For example, the TMIII-2 chimera contains only two divergent $D_1$ residues, A123V and I126V, at the distal carboxyl-terminal side (near the intracellular surface), and showed no changes in ligand binding properties.

For the TMVII segment, the critical binding region was probed with the three TMVII chimeras that have the $D_3$ sequence extending progressively from the extracellular to intracellular surface (e.g., no $D_3$ sequence for the TMVII chimera); the seven $D_3$ residues near extracellular surface for TMVII-1; and the entire $D_3$ sequence except for V381I and T384A at the extreme carboxyl-terminal side for the TMVII-2. Test ligand affinities steadily improved as the $D_3$ sequence extended from the amino-terminal (extracellular surface) to the carboxyl-terminal side (intracellular surface), although the improvements were not uniform among the ligands (Table 2). Interestingly, the TMVII-2 chimera with only two divergent residues (V381I and T384A) showed 20 to 45-fold lower affinities to quinpirole, apomorphine, and pramipexole. Structurally, the two residues seem to reside at the nearly same face of the TMVII helix (3.6 amino acid residues per helical turn), and are adjacent to Pro380, a strictly conserved residue among dopamine receptors. It has been shown previously that the corresponding proline in $D_2$ is exposed to extracellular water-accessible binding-site crevice, as probed with the cysteine scanning mutagenesis, using a charged, hydrophilic sulfhydryl-specific agent (Fu *et al.*, 1996). Pro380 probably contributes to ligand binding pockets, and its immediate surroundings could be altered by the mutations of the two adjacent residues, leading to the selective alterations of several agonist affinities. The fact that only the agonist affinities were altered by the mutations could be interpreted to mean that the region including Pro380 is somehow linked to receptor-G protein coupling. It seems that the contributions of the TMVII to ligand binding arise not only from the residues near the extracellular surface but also from those near the intracellular surface on the carboxyl-terminal side, even if not directly.

We list below the $K_i$ values of test ligands as measured by competition assay with [$^3$H]SCH23390 in human $D_1$ receptors expressed in Sf9 cells, under experimental conditions identical to the current study; for spiperone, 634 ± 98 nM; butaclamol, 19.8 ± 1.3 nM; chlorpromazine, 79 ± 4 nM; YM 09151–2, 3,072 ± 692 nM; UH-232, 312 ± 42 nM; AJ-76, 63,272 ± 3,841 nM; haloperidol, 55 ± 3 nM; raclopride, ≫64,000 nM; (−)3-PPP, 8,088 ± 498 nM; quinpirole, >64,000; pramipexole, >64,000; lisuride, 148 ± 24 nM, and cabergoline, 2,315 ± 110 nM. The affinity to $D_1$ is quite variable among the test ligands; from the compounds of no appreciable affinity represented by raclopride (≫64,000 nM) to those of relatively high affinity represented by butaclamol (19.8 nM). It is of some interest to evaluate how the $D_1$ affinity of individual test ligands is reflected in their affinity to the $D_3/D_1$ chimeras where one $D_1$ TM is placed in the $D_3$ receptor environments. One would expect a tendency that the ligands with lower affinities to $D_1$ would display more markedly altered affinities to the chimeras, and this seems to be true for most compounds (e.g., raclopride and pramipexole). Also, one tends to expect that ligand affinity changes in $D_3/D_1$ chimeras would be limited within the range between its $D_3$ and $D_1$ values. This expectation, however, is based on the assumptions that (a) a given ligand shares the same set of binding regions between the $D_3$ and $D_1$ receptors, which could be distributed in several TMs, and (b) that the substituted $D_1$ region contributes to the ligand binding in the same way as its $D_3$ counterpart, except for the impact from divergent residues. This seems to be untrue for some compounds. For instance, the $K_i$ values of butaclamol for certain $D_3/D_1$ chimeras far exceed its $D_1$ $K_i$ value. This could be interpreted to mean that butaclamol employs a set of binding regions in $D_3$ different from those in $D_1$, although the two receptors are homologous. Future study on the roles of individual $D_1$ TMs in ligand binding is needed, and one may construct $D_1$ chimeric receptors where one $D_3$ TM is placed in the $D_1$ receptor environments. Studies with such chimeras would reveal whether ligand binding regions for a given ligand reside at the same set of TMs in $D_1$ as observed with the $D_3$. With such information on hand, one could dissect molecular basis of ligand selectivity for $D_3/D_1$ receptor.

Structural studies with bacteriorhodopsin (Henderson *et al.*, 1990) and β-adrenergic receptors (Frielle *et al.*, 1988; Strader *et al.*, 1994) indicate that TMIII and VII are most centrally located in the ligand binding pocket, which is flanked by the TM I, II, IV, V and VI. This model is compatible with our results with the $D_3$ receptor in that replacements of TMIII and TMVII with the corresponding $D_1$ sequence markedly affected the affinity for a broad spectrum of ligands while replacements of the flanking TMs had moderate (for TMIV) or no effects on ligand affinity except for few ligands (TMV or VI). Furthermore, asymmetrical involvements of TMs in the binding pocket are implied from our results; that is, the critical binding regions on the $D_3$ receptor are confined to the six to eight residues near the extracellular surface for TMI, II, IV and VI, but extend considerably deeper to the intracellular surface for the TMVII, more or less a funnel-shaped binding pocket with its narrow apex provided by the TMVII and probably TMIII segments.

As discussed above, individual ligands showed widely variable binding affinities toward a given chimera. This probably reflects the supposition that the binding pockets for individual ligands consist of multiple regions, some of which are common and overlapping among the ligands but others of which are highly individualized. Here, we attempted to sort out the test ligands for their sensitivity to the various $D_3/D_1$ chimeras. Fig. 4 shows the competition binding profiles (with

TABLE 3
Alignment of the amino acid sequences of TM segments from the human $D_2$ and $D_3$ dopamine receptors. The divergent residues are underlined; those nonconservatively altered are L34, S35, Y36, C37, A38 (TMI), T92 (TMII), F172 (TMIV), and T353 (TMVII).

| | |
|---|---|
| TMI | D2–YYATLLTLLIAVIVFGNVLVCMA |
| | D3–YYALSYCALILAIVFGNGLVCMA |
| TMII | D2–YLIVSLAVADLLVATLVMPWVVYLEVVG |
| | D3–YLVVSLAVADLLVATLVMPWVVYLEVTG |
| TMIII | D2–DIFVTLDVMMCTASILNLCAISI |
| | D3–DVFVTLDVMMCTASILNLCAISI |
| TMIV | D2–TVMISIVWVLSFTISCPLLFGL |
| | D3–ALMITAVWVLAFAVSCPLLFGF |
| TMV | D2–FVVYSSIVSFYVPFIVTLLVYI |
| | D3–FVIYSSVVSFYLPFGVTVLVYA |
| TMVI | D2–ATQMLAIVLGVFIICWLPFFITHILNI |
| | D3–ATQMVAIVLGAFIVCWLPFFLTHVLNI |
| TMVII | D2–LYSAFTWLGYVNSAVNPIIYT |
| | D3–LYSATTWLGYVNSALNPVIYT |

[$^3$H]spiperone) for the five representative ligands showing differential sensitivities to the various chimeras we examined here. Raclopride is the most sensitive ligand, displaying markedly low affinity to the chimeras containing the $D_1$ TMIII, IV, V, VI, or VII. Quinpirole (also pramipexole) was sensitive to the above chimeras except for the TMV chimera. Chlorpromazine, representing the group of butaclamol and haloperidol, was sensitive to only the TMIII, IV, and VII chimera. YM-09151–2, representing the group of spiperone, UH-232, AJ-76, (−)3-PPP, and apomorphine, was sensitive to only the TM III and VII chimeras. Finally, the ergots (lisuride and cabergoline) showed no sensitivity to the chimeras except for the TMIII chimera, in which they showed about 30-fold lower affinity. This insensitivity of ergots is remarkable considering their high selectivity for the $D_3$ over $D_1$ receptors [e.g., the $K_i$ value of cabergoline was 1800-fold less for the $D_3$ than for the $D_1$ receptor, as measured with competition experiments using [$^3$H]SCH23390 and [$^3$H]spiperone under identical conditions (data not shown)]. This implies that the subtype selectivity of the ergots may arise from their selective interactions with the TMIII and the residues in the extracellular loops.

This grouping tends to assign compounds with the same chemical template into the same class, such as the ergots (lisuride and cabergoline) and aminotetralins (UH-232 and AJ-76, as one might expect, but also collects chemically divergent ligands into a group, such as quinpirole and pramipexole. The latter situation may arise from similarity in their steric configurations and would be useful for further classifications of ligands. It will be also of future interest to see how single point mutations would affect ligand affinity within such groups.

Because most of the test ligands we examined here bound the $D_3$ and $D_2$ receptors with similar high affinity, one would predict a high sequence identity of the critical binding regions between the $D_3$ and $D_2$ receptors. Indeed, as shown in Table 3, the sequence of the $D_{2long}$ receptor is nearly identical in the hot binding regions except for eight residues, L34, S35, Y36, C37, A38 (TMI), T92 (TMII), F172 (TMIV), and T353 (TMVII), which are nonconservatively substituted on the $D_2$ receptor. These structural differences could be exploited to develop ligands selective for the $D_3$ over $D_2$ dopamine receptor or vice versa.





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