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Structural Determinants of Pharmacological Specificity Between D_1 and D_2 Dopamine Receptors

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ABSTRACT

To test the hypothesis that pharmacological differentiation between D_1 and D_2 dopamine receptors results from interactions of selective ligands with nonconserved residues lining the binding pocket, we mutated amino acid residues in the D_2 receptor to the corresponding aligned residues in the D_1 receptor and vice versa and expressed the receptors in human embryonic kidney 293 cells. Determinations of the affinity of the 14 mutant D_2 receptors and 11 mutant D_1 receptors for D_1- and D_2-selective antagonists, and rhodopsin-based homology models of the two receptors, identified two residues whose direct interactions with certain ligands probably contribute to ligand selectivity. The D_1 receptor mutant $W99^{3.28}F$ showed dramatically increased affinity for several D_2-selective antagonists, particularly spiperone (225-fold), whereas the D_2 receptor mutant $Y417^{7.43}W$ had greatly decreased affinity for benzamide ligands such as raclopride (200-fold) and sulpiride (125-fold). The binding of the D_1-selective ligand *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390) was unaffected, indicating that SCH23390 makes little contact with these ancillary pocket residues. Mutation of $A/V^{5.39}$ caused modest but consistent and reciprocal changes in affinity of the receptors for D_1 and D_2-selective ligands, perhaps reflecting altered packing of the interface of helices 5 and 6. We also obtained some evidence that residues in the second extracellular loop contribute to ligand binding. We conclude that additional determinants of D_1/D_2 receptor-selective binding are located either in that loop or in the transmembrane helices but, like residue 5.39, indirectly influence the interactions of selective ligands with conserved residues by altering the shape of the primary and ancillary binding pockets.

Dopamine modulates diverse biological functions, including movement, endocrine function, and memory formation, through activation of five distinct dopamine receptor subtypes that belong to the G protein-coupled receptor (GPCR) superfamily and are grouped into two subfamilies, D_1-like dopamine receptors and D_2-like dopamine receptors, based on their structure, pharmacology, and transduction pathways. The D_1 and D_2 receptors are the most abundant dopamine receptor subtypes and are most similar to the traditional pharmacologically defined D_1 and D_2 receptors (Kebabian and Calne, 1979). The D_1 receptor has a long carboxyl terminus and a short third intracellular loop, couples to the adenylate cyclase stimulatory G proteins $G\alpha_{s/olf}$, and stimulates cyclic AMP accumulation. In contrast, the D_2 receptor has a short carboxyl terminus and a long third intracellular loop, couples to the pertussis toxin-sensitive G proteins $G\alpha_{i/o}$, inhibits cyclic AMP accumulation, and also modulates a variety of $G\beta\gamma$-regulated effectors such as calcium and potassium ion channels, mitogen-activated protein kinases, and phospholipases (Neve et al., 2004).

D_1 and D_2 receptor-selective agonists and antagonists are current or potential therapeutic drugs for treatment of schizophrenia, Parkinson's disease, and other neuropsychiatric disorders (Sidhu et al., 2003). Although there are numerous drugs that are highly selective for the D_2 receptor over the D_1 receptor, the chemical diversity of D_1 receptor-selective drugs is lower, and there is little information on the structural features of the two receptors that contribute to D_1/D_2 pharmacological selectivity. The primary binding pocket in catecholamine receptors includes residues in transmembrane helix (TM) 3, TM5, and TM6; in particular, Asp3.32 and a cluster of three Ser residues in TM5 interact with the protonated nitrogen and catechol hydroxyls, respec-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; TM, transmembrane helix; EL2, second extracellular loop; SCH23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; YM09151-2, *cis*-*N*-[1-benzyl-2-methyl-pyrrolidin-3-yl]-5-chloro-2-methoxy-4-methylaminobenzamide; CPPMA, (3-[4-(4-chlorophenyl)piperazin-1-yl]methyl-1*H*-pyrrolo[2,3-*b*] pyridine (chlorophenylpiperazinyl methyl-azaindole).

tively, in catecholamine ligands (Strader et al., 1989). In D_2 receptor homology models, we have identified an ancillary binding pocket composed of a cluster of aromatic and nonpolar residues between transmembrane helices 2, 3, and 7 on the extracellular side of the primary binding pocket (Teeter et al., 1994; Neve et al., 2003). We have speculated that these ancillary pocket residues stabilize the binding of drugs with aromatic or nonpolar groups oriented toward the ancillary pocket and that ancillary pocket residues contribute to pharmacological selectivity. For example, several ancillary pocket residues are part of an aromatic microdomain that is important for selectivity between dopamine D_2 and D_4 receptors (Simpson et al., 1999).

We now describe the pharmacological characterization of D_1 and D_2 receptor mutants in which one or more residues were mutated to the corresponding residue(s) in the other receptor subtype. The mutation effects on ligand affinity were rationalized by ligand docking in rhodopsin-based homology models of the D_1 and D_2 receptors. Mutations of residues at three positions in the receptor transmembrane helices, including two ancillary pocket residues, changed receptor affinity for some ligands in a manner consistent with the hypothesis that the residues contribute to pharmacological specificity: position 3.28 (Trp99 in the D_1 receptor), position 7.43 (Tyr417 in the D_2 receptor), and position 5.39 (Ala195 and Val190 in the D_1 and D_2 receptors, respectively). Receptor modeling and ligand docking studies suggest that Trp99 and Tyr417 interact directly with some ligands but that position 5.39 contributes to pharmacological selectivity indirectly by determining the distance between other binding site residues. Our data also provide some indication that residues in the second extracellular loop (EL2) contribute to D_1/D_2 selectivity.

Materials and Methods

Materials. [^{3}H]Spiperone (107 Ci/mmol) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and [^{3}H]SCH23390 (86 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Serum was purchased from Hyclone Laboratories (Logan, UT). (+)-Butaclamol, SCH23390, *S*-(−)-raclopride, domperidone, haloperidol, spiperone, *S*-(−)-sulpiride, and most other drugs and reagents, including culture medium, were purchased from Sigma-Aldrich (St. Louis, MO). Piquindone, tropapride, and YM09151-2 were obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program (Bethesda, MD).

Numbering of Residues. Residues are numbered according to their positions in the rat D_{2L} (long alternatively spliced form of the D_2) receptor sequence (Monsma et al., 1989) or in the rhesus macaque D_1 receptor sequence (Machida et al., 1992). To simplify the identification of corresponding residues in D_1 and D_2 receptors, we also used an index system in which each residue has a number that denotes the TM in which it lies and its location relative to the most conserved residue in that helix (Ballesteros and Weinstein, 1995). The most conserved residue within each helix is assigned the number 50; e.g., the most highly conserved residue in TM3 of the D_2 receptor, Arg132, has the index number 3.50 and is designated Arg132$^{3.50}$. One residue toward the N terminus from Arg132 is Asp131$^{3.49}$, and one residue toward the C terminus is Tyr133$^{3.51}$. The position and index numbers of TM residues mutated in this study are provided in Table 1.

Production of Cell Lines. Mutants of the rat D_{2L} (long alternatively spliced form of the D_2) receptor and the rhesus macaque D_1 receptor were constructed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Double mutants were obtained through one or two cycles of mutagenesis, whereas triple/quadruple mutants were achieved through two or three cycles. Wild-type and mutant receptors in pcDNA3.1 were transfected into human embryonic kidney 293 cells with Lipofectamine (Invitrogen, Carlsbad, CA), and clonal cell lines stably expressing the receptors were isolated after selection with G418 (800 μg/ml). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 5% iron-supplemented calf bovine serum, 5% fetal bovine serum, and 600 μg/ml G418 at 37°C and 10% CO_2.

Radioligand Binding Assays. Cells were lysed in ice-cold hypotonic buffer (1 mM Na^+ HEPES, pH 7.4, and 2 mM EDTA) for 15 min, scraped from the plate, and centrifuged at 17,000*g* for 20 min. The resulting crude membrane fraction was resuspended with a Brinkmann Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 6 for 8 to 10 s in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 0.9% NaCl). Membrane proteins (40–100 μg) were incubated in duplicate for 45 min at 37°C, in the case of D_2 receptor, in a total reaction volume of 1 ml with [^{3}H]spiperone at concentrations ranging from 0.01 to 0.4 nM for saturation binding or ~0.1 nM with the appropriate concentration of the competing drug for competition binding. For characterization of wild-type and mutant D_1 receptors, incubations were carried out in 0.5 ml final volume containing [^{3}H]SCH23390 at concentrations ranging from 0.1 to 3.0 nM for saturation binding or ~1.0 nM with the appropriate concentration of the competing drug for competition binding. (+)-Butaclamol (2 μM) was used to define nonspecific binding. Data for saturation and competition binding were analyzed by nonlinear regression using the computer program Prism (GraphPad Software, San Diego, CA) to determine K_d and IC_{50} values. Apparent affinity (K_i) values were calculated from the IC_{50} values by the method of Cheng and Prusoff (1973). In all assays, the free concentration of radioligand was calculated as the concentration added minus the concentration specifically bound.

Receptor Homology Modeling and Ligand Docking. Based on known homology of rhodopsin and dopamine receptors, the sequences of the dopamine D_1 and D_2 receptors were aligned with rhodopsin. The alignments agreed with those found in the G protein-coupled receptor database (http://www.gpcr.org). Modeling procedures were similar to those previously used to model the sodium site in the D_2 receptor (Neve et al., 2001). In brief, amino acids for the respective receptor were substituted for the side chains of rhodopsin in the crystal structure (1l9h; Okada et al., 2002), and geometry around Pro substitutions was adjusted using a Pro template (Teeter et al., 1994). Improvement of poor contacts by rotamer change and repacking of helices (primarily TM5 and TM6) was accomplished

TABLE 1

Numbering of residues mutated in this study

Numbering is provided for both receptors at positions that were mutated in either receptor. For TM residues, the index number is that of Ballesteros and Weinstein (1995), and for EL2 residues, the index denotes the position of the residue relative to the conserved Cys in that loop.

D_2 Residue	D_1 Residue	Index Number
Phe110[a]	Trp99	3.28
Ile183	Asp187	+1
Ile184	Ser188	+2
Ala185	Ser189	+3
Asn186	Leu190	+4
Phe189	Tyr194	5.38
Val190	Ala195	5.39
His394	Asn292	6.55
Leu408	Thr312	7.34
Tyr409[a]	Phe313	7.35
Ser410	Asp314	7.36
Thr413[a]	Val317	7.39
Tyr417[a]	Trp321	7.43

[a] Residues predicted to be in the ancillary binding pocket according to our D_2 receptor homology model (Neve et al., 2003).

manually using the program CHAIN (Sack, 1988). Only transmembrane helices were modeled for this study because binding and specificity sites are substantially in these regions. No energy minimization was used, but close contacts were eliminated manually. This modeling procedure has accurately predicted Na^+ binding residues, as confirmed by mutagenesis (Teeter et al., 1994; Neve et al., 2001). Our modeling approach relies heavily on the experimentally determined X-ray structure of rhodopsin (Teeter et al., 1994; Neve et al., 2003).

Ligands were docked into the binding site using previously identified polar groups on the protein as attachment points (Strader et al., 1989): the conserved negatively-charged residue $Asp^{3.32}$, which binds to the positively charged nitrogen in the aminergic ligands, and the Ser residues 5.42 and 5.46, which interact with polar atoms on the ligand (the O, N, -OH groups, or halogens Cl or F). Ligand conformations were either from crystal structures of the ligands or from ab initio calculations. The crystal structures of piquindone (Olson et al., 1981), spiperone (Liang et al., 1998), and haloperidol (Reed and Schaefer, 1973) were described previously. For tropapride, eight conformations were generated from the degrees of freedom and subjected to ab initio quantum mechanical calculations using the basis sets 3-21G* and 6-31G* in the program Spartan (Wavefunction, Inc., Irvine, CA), producing minimized conformations of approximately equal energy. One conformation matched three of the available crystal structures and fit well in our D_2 receptor model (Teeter et al., 2001). The structure of SCH23390 was based on energy minimization and analysis of conformationally constrained analogs (Pettersson et al., 1990).

Once ligands were docked, interactions in the ancillary pocket could be assessed, as described below. Aromatic and aliphatic groups that could bind in the hydrophobic ancillary pocket have varying degrees of rigidity relative to the docked portion of the structure, ranging from spiperone as most rigid to haloperidol as least rigid.

Our model derived from rhodopsin is expected to be the inactive state structure of a GPCR because the rhodopsin crystal structure is in the ground state (i.e., bound to 11-*cis*-retinal). Although the D_2 receptor residue $Ser194^{5.43}$ has also been identified from mutagenesis as important for the binding of agonists (Cox et al., 1992), it cannot readily interact directly with the ligand in our ground-state model of the dopamine receptors and may be used for the activated state of the receptor.

Results and Discussion

Mutations Based on Solvent Accessibility and Nonconservation. Seven mutant D_2 receptors were constructed based on the criteria of Simpson et al. (1999) for identifying amino acid residues that potentially contribute to receptor subtype selectivity: the residues must be exposed in the binding pocket, and residue side chain properties should not be conserved between D_1 and D_2 receptors. One residue, $Phe110^{3.28}$, is in TM3 and is predicted to be in the ancillary binding pocket (Teeter et al., 1994; Neve et al., 2003), and a second residue is in TM6 ($His394^{6.55}$). Five residues are in TM7, with three of them ($Tyr409^{7.35}$, $Thr413^{7.39}$, and $Tyr417^{7.43}$) predicted to be in the ancillary binding pocket (Table 1). Each residue was mutated to the corresponding residue in the D_1 receptor. Mutant receptors were stably expressed in human embryonic kidney 293 cells, and drug affinity was determined by saturation analysis of the binding of the D_2-like receptor radioligand [^{3}H]spiperone and competition analysis of the binding of seven additional D_2-selective antagonists and the D_1-selective antagonist SCH23390 (Table 2; Fig. 1).

D_2-Y417W had substantially decreased affinity for most D_2-selective antagonists, consistent with data from other receptors implicating residue 7.43 in ligand binding (Matsui et al., 1995; Cavalli et al., 1996; Roth et al., 1997; Mialet et al., 2000). Substituted benzamides (sulpiride, raclopride, tropapride, and YM-09151-02) were particularly sensitive to this mutation, with their binding reduced 60- to 200-fold. Each of the other mutations caused a modest reduction in affinity for one or more D_2-selective antagonists. None of the mutants had markedly increased affinity for SCH23390, in contrast to what would be expected if they contributed to the D_1 receptor selectivity of this ligand. The lack of effect of mutation of the residues in TM7 on the binding of SCH23390 is inconsistent with our prior analysis of chimeric D_1/D_2 receptors, which identified this region as being particularly important for the selective binding of SCH23390 and several other benzazepine ligands (Kozell et al., 1994), but our model supports the conclusion that SCH23390 does not contact these ancillary pocket residues (see below).

The sensitivity of ligands to the D_2-Y417W mutation can be explained by the larger size of the Trp residue and its different orientation in the ancillary pocket (Fig. 2). For the benzamides, the orientation of the benzyl and ethyl substituents on the five-membered pyridyl ring with the charged nitrogen is key (Fig. 1). These all extend toward the cytoplasmic side of the ancillary pocket where they contact residue D_2-417. When Tyr417 is mutated to the bulkier Trp, the affinity of these ligands is decreased.

We created D_1 receptor mutants that were reciprocals of four of the D_2 receptor mutants (Table 1). Mutations that contribute to subtype selectivity would be expected to decrease the affinity of SCH23390 and increase the affinity of D_2 receptor ligands. Consistent with this expectation, each mutation caused a modest but statistically significant reduction in affinity for [^{3}H]SCH23390, as determined by saturation analysis (Table 2). In contrast, three of the mutations caused little gain of affinity for D_2-selective ligands; D_1-$N292^{6.55}H$, D_1-$V317^{7.39}T$, and D_1-$W321^{7.43}Y$, reciprocals of

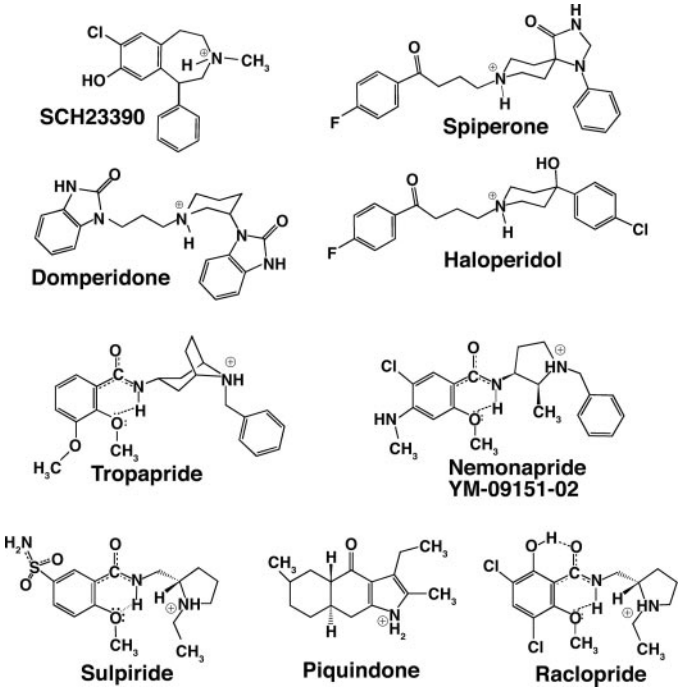

Fig. 1. Structures of ligands used in this study.

TABLE 2

Pharmacological characterization of single-residue mutants of the D_1 and D_2 receptors

Affinity values (expressed as mean pK_d or pK_i ± S.E.) are shown for each ligand and at the indicated wild-type or mutant receptor. The number below the affinity value in each cell is the ratio of the K_d or K_i of the wild-type receptor to that of the mutant (mean ± S.E.); a number greater than 1 indicates that the mutation enhanced the affinity of the receptor for a given ligand, whereas a number less than 1 indicates a loss of affinity. Affinity values for spiperone at wild-type and mutant D_2 receptors and for SCH23390 at wild-type and mutant D_1 receptors are the pK_d determined by saturation analysis of radioligand binding. The pK values for wild-type receptors (D_2-WT and D_1-WT) are the means of all experiments for a given drug, whereas the -fold change for a particular mutant and the statistical significance of the difference in pK values were calculated from only the experiments in which that mutant and the wild-type receptor were tested together. The number of experiments used to determine the -fold change is in parentheses. An asterisk denotes a pK value that was significantly different from wild type ($P < 0.05$). Mutation-induced affinity changes that are greater than 10-fold are indicated by bold font.

Receptor	Drug Affinity								
	SCH23390	Spiperone	Domperidone	Haloperidol	Piquindone	Raclopride	Sulpiride	Tropapride	YM-09151-02
D_2-WT	5.73 ± 0.26 1 (7)	10.31 ± 0.07 1 (18)	9.23 ± 0.08 1 (6)	8.85 ± 0.13 1 (6)	8.25 ± 0.10 1 (7)	8.57 ± 0.10 1 (6)	8.45 ± 0.07 1 (9)	10.46 ± 0.10 1 (9)	10.23 ± 0.16 1 (6)
D_2-F110W	6.26 ± 0.04 1.5 ± 0.1 (4)*	10.04 ± 0.05 0.6 ± 0.1 (4)*	8.59 ± 0.04 0.3 ± 0.02 (3)*	8.88 ± 0.08 0.7 ± 0.1 (3)	8.25 ± 0.01 1.6 ± 0.4 (4)	8.35 ± 0.13 0.9 ± 0.1 (3)	8.12 ± 0.02 0.7 ± 0.03 (4)*	9.77 ± 0.05 0.4 ± 0.1 (5)*	9.66 ± 0.19 0.5 ± 0.04 (3)
D_2-H394N	6.58 ± 0.05 1.7 ± 0.1 (3)*	10.01 ± 0.10 0.2 ± 0.03 (5)*	8.94 ± 0.03 0.3 ± 0.01 (3)*	8.20 ± 0.17 0.4 ± 0.1 (3)	7.59 ± 0.03 0.1 ± 0.01(3)*	8.04 ± 0.12 0.2 ± 0.02 (3)*	**7.05 ± 0.11** **0.04 ± 0.01 (5)***	10.55 ± 0.09 0.7 ± 0.1 (4)	10.28 ± 0.13 0.6 ± 0.1 (3)
D_2-L408T	6.26 ± 0.06 0.8 ± 0.1 (3)	10.65 ± 0.05 0.9 ± 0.1 (5)	9.34 ± 0.05 0.9 ± 0.04 (3)	8.73 ± 0.21 1.3 ± 0.2 (3)	8.26 ± 0.06 0.6 ± 0.1 (3)*	8.53 ± 0.13 0.6 ± 0.1 (3)	8.44 ± 0.05 0.8 ± 0.1 (5)	10.62 ± 0.08 0.8 ± 0.1 (4)	10.52 ± 0.18 1.0 ± 0.3 (3)
D_2-Y409F	6.36 ± 0.07 1.0 ± 0.2 (3)	10.92 ± 0.08 1.7 ± 0.2 (5)	9.44 ± 0.05 1.1 ± 0.1 (3)	8.52 ± 0.08 0.8 ± 0.1 (3)	7.76 ± 0.05 0.2 ± 0.01 (3)*	8.01 ± 0.12 0.2 ± 0.02 (3)*	8.57 ± 0.08 1.0 ± 0.2 (5)	10.81 ± 0.12 1.3 ± 0.3 (4)	11.00 ± 0.00 3.0 ± 0.4 (3)*
D_2-S410D	6.30 ± 0.04 0.9 ± 0.1 (3)	10.22 ± 0.08 0.3 ± 0.1 (4)*	9.05 ± 0.03 0.4 ± 0.02 (3)*	8.38 ± 0.16 0.6 ± 0.1 (3)	8.26 ± 0.03 0.6 ± 0.03 (3)*	8.64 ± 0.11 0.8 ± 0.04 (3)	8.54 ± 0.13 1.0 ± 0.3 (4)	10.62 ± 0.08 0.8 ± 0.1 (4)	10.54 ± 0.09 1.0 ± 0.03 (3)
D_2-T413V	5.47 ± 0.28 1.1 ± 0.3 (3)	10.01 ± 0.03 0.5 ± 0.03 (4)*	8.51 ± 0.03 0.3 ± 0.02 (3)*	8.50 ± 0.07 0.3 ± 0.1 (3)*	8.25 ± 0.06 1.6 ± 0.4 (4)	8.28 ± 0.13 0.8 ± 0.1 (3)	7.92 ± 0.08 0.4 ± 0.1 (4)*	9.38 ± 0.10 0.2 ± 0.04 (5)*	9.51 ± 0.09 0.4 ± 0.1 (3)
D_2-Y417W	5.24 ± 0.64 1.5 ± 0.4 (4)	**9.29 ± 0.04** **0.1 ± 0.01 (4)***	8.54 ± 0.03 0.3 ± 0.02 (3)*	8.64 ± 0.13 0.4 ± 0.1 (3)	7.27 ± 0.02 0.2 ± 0.04 (4)*	**6.10 ± 0.13** **0.005 ± 0.000 (3)***	**6.21 ± 0.02** **0.008 ± 0.001 (4)***	**8.32 ± 0.12** **0.016 ± 0.004 (4)***	**8.00 ± 0.10** **0.014 ± 0.004 (3)***
D_1-WT	9.26 ± 0.04 1 (5)	6.36 ± 0.03 1 (4)	5.83 ± 0.02 1 (3)	7.17 ± 0.01 1 (3)	5.70 ± 0.01 1 (3)	3.82 ± 0.15 1 (3)	4.77 ± 0.08 1 (3)	6.08 ± 0.02 1 (3)	6.09 ± 0.02 1 (3)
D_1-W99F	9.12 ± 0.02 0.7 ± 0.1 (5)*	8.71 ± 0.02 **225 ± 14 (4)***	**7.48 ± 0.03** **45 ± 4.6 (3)***	7.60 ± 0.01 2.7 ± 0.03 (3)*	5.44 ± 0.04 0.5 ± 0.04 (3)*	3.83 ± 0.23 1.0 ± 0.3 (3)	4.21 ± 0.12 0.3 ± 0.03 (3)*	6.57 ± 0.03 3.1 ± 0.1 (3)*	**7.47 ± 0.03** **24 ± 1.2 (3)***
D_1-N292H	8.58 ± 0.03 0.2 ± 0.01 (5)*	6.13 ± 0.12 0.7 ± 0.2 (4)	5.64 ± 0.02 0.7 ± 0.1 (3)*	6.75 ± 0.04 0.4 ± 0.04 (3)*	5.22 ± 0.01 0.3 ± 0.01 (3)*	3.13 ± 0.11 0.2 ± 0.01 (3)*	4.41 ± 0.07 0.4 ± 0.04 (3)*	5.56 ± 0.01 0.3 ± 0.01 (3)*	5.86 ± 0.05 0.6 ± 0.03 (3)*
D_1-V317T	8.93 ± 0.10 0.5 ± 0.1 (5)*	5.88 ± 0.03 0.3 ± 0.02 (4)*	5.57 ± 0.05 0.6 ± 0.04 (3)*	6.91 ± 0.02 0.6 ± 0.03 (3)*	5.76 ± 0.04 1.2 ± 0.1 (3)	3.81 ± 0.12 1.0 ± 0.04 (3)	5.11 ± 0.13 2.2 ± 0.5 (3)	**4.78 ± 0.19** **0.05 ± 0.02 (3)***	5.69 ± 0.03 0.4 ± 0.01 (3)*
D_1-W321Y	8.81 ± 0.03 0.4 ± 0.04 (5)*	6.09 ± 0.02 0.5 ± 0.04 (4)*	5.81 ± 0.03 0.9 ± 0.04 (3)	6.62 ± 0.04 0.3 ± 0.02 (3)*	6.30 ± 0.03 3.9 ± 0.1 (3)*	3.56 ± 0.14 0.6 ± 0.1 (3)	4.73 ± 0.05 0.9 ± 0.1 (3)	5.49 ± 0.09 0.3 ± 0.04 (3)*	6.02 ± 0.01 0.9 ± 0.1 (3)

D_2 receptor mutations that generally decreased the affinity of D_2 ligands, had unchanged or slightly decreased affinity for D_2 ligands except for an almost 4-fold increase in the affinity of D_1-W321Y for piquindone. The mutant D_1-W99F, however, had a 225-fold increase in apparent affinity for spiperone, with smaller increases of 45-, 24-, 3.1-, and 2.7-fold for domperidone, YM-0915-02, tropapride, and haloperidol, respectively. This was surprising because the reciprocal mutation F110$^{3.28}$W had little effect on the affinity of the D_2 receptor for ligands. Figure 3 depicts the dramatic leftward shift in the spiperone competition binding curve (toward D_2 wild type) observed for D_1-W99F.

The increased affinity for D_2 antagonists that results from the D_1-W99F mutation could be due to both the altered size of this residue at the ancillary pocket opening and the orientation of the aromatic group on the ring with the protonated nitrogen of the ligand. The 225-fold increase in spiperone binding affinity probably comes from the smaller Phe side chain that opens the ancillary pocket in the D_1 receptor. When residue 3.28 is Trp, the pocket is effectively closed (Fig. 4A). The mutant Phe residue also has a favorable stacking interaction with the nonpolar N1-phenyl ring that is relatively rigidly held in spiperone (Fig. 4B).

Haloperidol matches spiperone in structure, except for the more flexible chlorophenyl substituent *para-* to the nitrogen in the pyrrole ring (Fig. 1). That the flexible chlorophenyl substituent can rotate away from Trp99 in native D_1 as well as its less optimal stacking with Phe99 in the mutant receptor (Fig. 4C) make the improvement in affinity of D_1-W99F for haloperidol relatively smaller than the considerable binding improvement for spiperone. It is interesting that differences between the interactions of haloperidol and spiperone with this residue (Trp99) can account for the entire difference in D_1/D_2 selectivity for spiperone (0.05 nM K_d and 400 nM K_i at D_2 and D_1, respectively, in the experiments in which the wild-type receptors were analyzed together with the mutants D_1-W99F and D_2-F110W; almost 8000-fold selective) and haloperidol (0.8 nM and 68 nM K_i at D_2 and D_1, respectively; 85-fold selective); both ligands are approximately 35-fold selective for the D_2 receptor over D_1-W99F (spiperone and haloperidol K_i for mutant receptor of 1.9 and 25 nM, respectively).

Whereas spiperone has a relatively rigidly held phenyl ring, the corresponding substituent on domperidone and YM-09151-02 is free to rotate on the central ring. In domperidone, the substituent is *meta-* to the nitrogen in the central piperidine ring rather than *para-* as in spiperone, and in YM-09151-02, the substituent is bound to the charged nitrogen of the pyrrole ring (Fig. 1). The similarity in the substituent for domperidone and spiperone despite large differences in the effect of the W99F mutation suggests that the orientation of the substituent may be the more important factor in the relative effect of Trp99 on receptor affinity for the two ligands. This argument also applies to YM-09151-02, where the orientation of the phenyl substituent is less favorable for stacking with the mutant Phe99. For D_2-selective ligands whose binding affinity is only slightly elevated or unaffected by the W99F mutation, geometry and flexibility both come into play. The ethyl or benzyl substituents of sulpiride, raclopride, and tropapride are relatively flexible and point toward residue 7.43 rather than residue 3.28 (see above and Fig. 2) so that the removal of Trp99$^{3.28}$ enhances their binding weakly or not at all.

Residue 3.28 also contributes modestly to ligand selectivity between D_2 and D_4 receptors, because D_2-F110L has slightly decreased affinity for [^{3}H]spiperone and 5-fold enhanced affinity for the D_4-selective ligand CPPMA (Simpson et al., 1999), and mutation of residues Leu$^{3.28}$ and Met$^{3.29}$ in the D_4 receptor to the corresponding D_2 receptor residues decreases the affinity of many D_4-selective ligands (Kortagere et al., 2004). Thus, the aromaticity and shape of the side chain at this position affects the receptor subtype selectivity of ligands to an extent that depends on the geometry, flexibility, and stacking potential of ligand substituents that are oriented toward outer TM3 and the opening of the ancillary binding pocket.

Although mutations of the two ancillary pocket residues 3.28 and 7.43 have effects that suggest a contribution to the

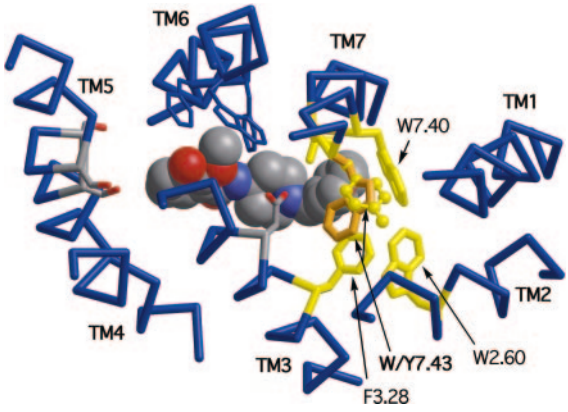

Fig. 2. Tropapride binding in the D_2 receptor is decreased by the point mutation of Y417W. The view is from the intracellular side of the primary and ancillary binding pockets. In yellow are important residues in the ancillary binding pocket: 2.60, 3.28, 7.40, and 7.43. Residues are numbered according to the index of Ballesteros and Weinstein (1995). The differing orientations relative to the ligand of Trp and Tyr at position 7.43 are shown. The D_2 residue Tyr417 (yellow, ball and stick) extends across the top of the pocket, whereas the D_1 residue Trp (gold) extends into the pocket overlapping the benzyl group of tropapride and decreases this ligand's binding to the mutated receptor. Primary binding pocket residues Asp114$^{3.32}$ and Ser193$^{5.42}$ and Ser197$^{5.46}$ are also depicted, with oxygen in red and carbon in gray. TM6 aromatic residues in deep blue (Trp358$^{6.48}$, Phe361$^{6.51}$, and Phe362$^{6.52}$) line the binding pocket. The backbone is drawn from Cα to Cα of the respective helices. Tropapride, shown as space filling, has colors as above plus nitrogen in blue.

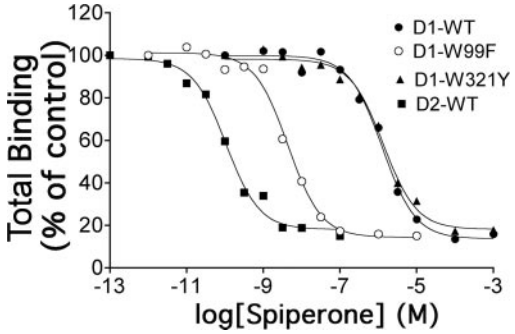

Fig. 3. The mutation W99F greatly enhanced the apparent affinity of the D_1 receptor for the D_2 antagonist spiperone. Data are shown from one of three or more independent experiments in which inhibition of the binding of radioligand to the indicated receptor (wild-type D_1 and D_2, and the D_1 receptor mutants W99$^{3.28}$F and W321$^{7.43}$Y) was determined. Data are plotted as a percentage of the total binding in the absence of spiperone versus the logarithm of the concentration of spiperone. The radioligand was [^{3}H]SCH23390 for the D_1 wild-type and mutant receptors and [^{3}H]spiperone for the D_2 receptor.

D_2 receptor selectivity of ligands, none of the mutations substantially changed receptor affinity for the D_1-selective ligand SCH23390 (Table 2). D_2-selective antagonists such as spiperone are longer than SCH23390 (Fig. 1). Although the distance from polar halide or -OH to the protonated nitrogen is comparable, the D_2-selective ligands have relatively rigid groups that extend beyond the protonated nitrogen and are parallel to the rest of the molecule, reaching into the ancillary pocket, which leads from the primary binding pocket perpendicular to the helix axes. SCH23390, however, contains a phenyl ring perpendicular to the rest of the ligand extending from the ring containing the protonated nitrogen (Pettersson et al., 1990). SCH23390 docked in the D_2 receptor model has few interactions in the ancillary pocket because its 1-phenyl substituent extends toward the extracellular surface of the receptor, parallel to the helix axes.

Lack of Reciprocal Effects. The absence of a D_1/D_2-reciprocal effect for the mutations at 3.28 and 7.43 is puzzling. Why did mutation of residue 3.28 enhance binding of some D_2-selective ligands to the D_1 receptor without decreasing their binding to the D_2 receptor, and why did mutation of residue 7.43 decrease binding of some D_2-selective ligands to the D_2 receptor without enhancing their binding to the D_1 receptor? Our D_2 receptor model, depicting tight packing of hydrophobic residues in the ancillary pocket (Neve et al., 2003), suggested the hypothesis that the absence of a D_1/D_2-reciprocal effect for mutations at positions 3.28 and 7.43 reflected the context in which the point mutation was made. For example, perhaps changing between Phe and Trp at position 3.28 affects the binding of spiperone only in a receptor (e.g., the D_1 receptor) that also has the bulkier Trp at position 7.43.

To test this hypothesis, we combined the two mutations in the double-mutant receptors D_1-W99F/W321Y and D_2-F110W/Y417W. We also created the triple mutants D_1-W99F/V317T/W321Y and D_2-F110W/T413V/Y417W, because Thr413$^{7.39}$ is located within the ancillary binding pocket together with residues 3.28 and 7.43 in our D_2 receptor model (Neve et al., 2003), and its mutation from Thr to Val modestly reduced D_2 receptor affinity for many D_2-selective ligands (Table 2). We predicted that combining the mutations would have additive or synergistic effects on ligand affinity. In contrast to our prediction, the D_1 double and triple mutants had lower affinity for spiperone (i.e., were less D_2-like) than the single mutant W99F (Table 3). Furthermore, the extra mutations caused no further increase in affinity for YM-09151-02 or piquindone over that observed for D_1-W99F or D_1-W321Y, respectively (Tables 2 and 3). On the other hand, the 10-fold decrease in affinity of D_1-W99F/W321Y and D_1-W99F/V317T/W321Y for SCH23390 was greater than the decrease resulting from single mutations of any of the residues, and the D_2-F110W/Y417W double mutant had de-

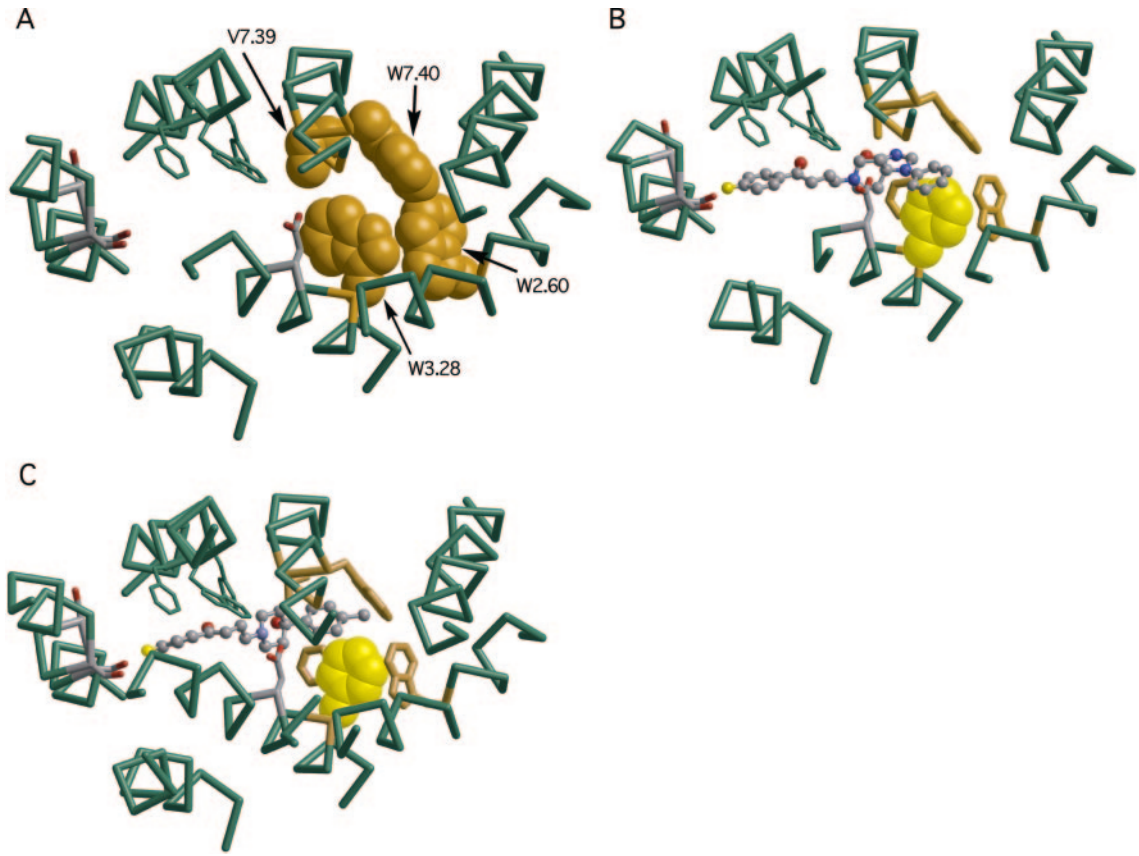

Fig. 4. W99F mutation opens the ancillary pocket for binding of D_2 receptor-selective ligands. View of the D_1 receptor from the intracellular side shows the ancillary pocket residues (gold, space filling). Side chains of conserved aromatic residues on TM6 involved in binding are shown in green. The key contact residues in the binding site (Asp110$^{3.32}$ and Ser residues on TM5) have oxygen colored red and carbon gray. A, ancillary pocket of D_1 receptor is constricted by Trp99$^{3.28}$. B and C, depiction of spiperone (B) or haloperidol (C) docked into the D_1 receptor with both the D_2 residue Phe (yellow, space filling) and wild-type D_1 residue Trp99 (gold) shown at position 3.28. Trp99 is partially obscured behind Phe$^{3.28}$. The phenyl ring of spiperone overlaps with Trp99 but is well stacked with Phe. In contrast, the chlorophenyl substituent in haloperidol is able to move away from Trp99 and is edge-to-edge with Phe at that position.

creased affinity for [^{3}H]spiperone and tropapride that was roughly equivalent to the additive effects of the two single mutants. Adding the third mutation to the D_2 receptor (D_2-F110W/T413V/Y417W) had little or no additional effect. Overall, these results provided only slight support for our hypothesis that residues at positions 3.28, 7.43, and 7.39 have additive or synergistic effects on the affinity of subtype-selective ligands.

Do D_1 and D_2 Receptors Have the Same Binding Pockets? An alternative hypothesis for the lack of reciprocal effects of the mutations on the binding of D_2-selective ligands is that the specificity/binding sites may not be identical for D_1 and D_2 receptors. Whereas the central hydrogen bonding and electrostatic interactions in the binding site (Ser residues on TM5 and Asp on TM3; see Fig. 2) are conserved between the two receptors, the selectivity (ancillary) pockets may be quite different. As noted above, D_2-selective ligands have relatively rigid groups, extending beyond the protonated nitrogen, that are parallel to the rest of the molecule and to the membrane plane and that reach into the ancillary binding pocket, whereas SCH23390 contains a phenyl ring perpendicular to the rest of the molecule and the membrane plane and parallel to the helix axes. Furthermore, as discussed below, the position of TM5 relative to TM6 seems to differ in the D_1 and D_2 receptors. In the D_1 receptor, this would move the fluorine-substituted ring on spiperone that binds to the Ser residues closer to TM6 and move the rigid N1-phenyl ring closer to the mutated residue in the ancillary pocket (W99F$^{3.28}$). According to our model, on the other hand, reducing the size of the side chain at position 7.43 from Trp to Tyr in D_1-W321Y does not enhance the binding of substituted benzamides because these D_2-selective ligands, with the exception of piquindone, are prevented from reaching 7.43 by the bulky Trp$^{3.28}$ (Figs. 2 and 4A). Although one would predict that opening up the ancillary pocket by removing Trp99 in the double mutant D_1-W99F/W321Y would cause benzamide ligands to bind with more D_2-like affinity, we speculate that replacing the two Trp residues with smaller aromatic residues may destabilize helix packing and the ancillary pocket.

Why is piquindone, with modestly enhanced binding to D_1-W321Y (Table 2), an exception to this rule? Structure-activity relations for this Na^+-dependent ligand and its derivatives (Teeter and DuRand, 1996) support its binding in a small cleft, adjacent to the ancillary pocket, that stretches in an intracellular direction from Asp114$^{3.32}$ in TM3 toward Na^+-binding pocket residues, including Asp80$^{2.50}$ (Neve et al., 2001). In the D_1 receptor, this puts piquindone in Van der

TABLE 3

Pharmacological characterization of multiple-residue mutants of the D_1 and D_2 receptors

Affinity values (expressed as mean pK_d or pK_i ± S.E.) are shown for each ligand at the indicated wild-type or mutant receptor. The number below the affinity value is the ratio of the K_d or K_i of the wild-type receptor to that of the mutant (mean ± S.E.); a number greater than 1 indicates that the mutation enhanced the affinity of the receptor for the ligand, whereas a number less than 1 indicates a loss of affinity. Single-residue mutations to determine the basis for the effects of the 5.38/5.39 double mutant are also included in this table. Affinity values for spiperone at wild-type and mutant D_2 receptors and for SCH23390 at wild-type and mutant D_1 receptors are the pK_d determined by saturation analysis of radioligand binding. The pK values for wild-type receptors (D_2-WT and D_1-WT) are the means of all experiments for a given drug, whereas the -fold change for a particular mutant and the statistical significance of the difference in pK values were calculated from only the experiments in which that mutant and the wild-type receptor were tested together. The number of experiments used to determine the -fold change is in parentheses. An asterisk denotes a pK value that was significantly different from wild type ($P < 0.05$). Mutation-induced affinity changes that were greater than 10-fold are indicated by bold font.

Receptor	Drug Affinity				
	SCH23390	Spiperone	Piquindone	Tropapride	YM-09151-02
D_1-WT	9.18 ± 0.03	6.33 ± 0.04	5.57 ± 0.07	5.60 ± 0.1	6.07 ± 0.05
	1 (21)	1 (9)	1 (6)	1 (6)	1 (3)
D_1-W99F/W321Y	**8.23 ± 0.04**	**7.59 ± 0.05**	6.13 ± 0.03	6.22 ± 0.09	**7.39 ± 0.05**
	0.1 ± 0.01 (3)*	**22 ± 1 (3)***	2.5 ± 0.2 (3)*	8.8 ± 1.9 (3)*	**20.7 ± 0.3 (3)***
D_1-W99F/V317T/W321Y	**8.21 ± 0.06**	7.07 ± 0.02	6.02 ± 0.03	6.09 ± 0.10	**7.48 ± 0.03**
	0.1 ± 0.02 (5)*	5.5 ± 0.2 (3)*	2.0 ± 0.1 (3)*	6.5 ± 1.4 (3)*	**25.7 ± 2.4 (3)***
D_1-Y194F/A195V	**7.90 ± 0.03**	7.00 ± 0.02	**6.84 ± 0.00**	6.27 ± 0.06	6.93 ± 0.09
	0.07 ± 0.01 (4)*	3.7 ± 0.4 (3)*	**12.5 ± 0.6 (3)***	9.4 ± 1.5 (3)*	7.2 ± 0.6 (3)*
D_1-Y194F	8.91 ± 0.08	N.D.	5.25 ± 0.12	5.59 ± 0.00	N.D.
	0.5 ± 0.1 (4)*		0.7 ± 0.2 (3)	0.6 ± 0.1 (3)	
D_1-A195V	**8.17 ± 0.05**	N.D.	6.07 ± 0.07	6.46 ± 0.02	N.D.
	0.09 ± 0.03 (4)*		4.6 ± 0.5 (3)*	4.4 ± 1.1 (3)*	
D_1-E2L0.3	9.28 ± 0.10	6.15 ± 0.10	5.52 ± 0.03	5.08 ± 0.08	5.77 ± 0.04
DSS/IIA	1.3 ± 0.3 (4)	0.7 ± 0.2 (3)	0.6 ± 0.03 (3)*	0.5 ± 0.1 (3)	0.5 ± 0.1 (3)*
D_1-E2L0.4	**7.77 ± 0.03**	5.87 ± 0.08	5.41 ± 0.06	4.97 ± 0.19	5.78 ± 0.14
DSSL/IIAN	**0.05 ± 0.004 (6)***	0.3 ± 0.1 (3)*	0.5 ± 0.1 (3)*	0.6 ± 0.2 (3)	0.5 ± 0.1 (3)
D_2-WT	6.06 ± 0.02	10.04 ± 0.03	N.D.	9.95 ± 0.05	N.D.
	1 (6)	1 (12)		1 (6)	
D_2-F110W/Y417W	6.35 ± 0.03	**8.52 ± 0.10**	N.D.	**7.61 ± 0.49**	N.D.
	1.9 ± 0.3 (3)*	**0.04 ± 0.01 (3)***		**0.004 ± 0.001 (3)***	
D_2-F110W/T413V/Y417W	6.15 ± 0.07	**8.89 ± 0.09**	N.D.	**7.53 ± 0.04**	N.D.
	1.2 ± 0.2 (3)	**0.09 ± 0.02 (3)***		**0.003 ± 0.0005 (3)***	
D_2-F189Y/V190V	6.46 ± 0.06	9.56 ± 0.06	N.D.	9.71 ± 0.09	N.D.
	2.7 ± 0.3 (3)*	0.3 ± 0.04 (7)*		0.7 ± 0.1 (3)	
D_2-F189Y	5.91 ± 0.03	9.66 ± 0.04	N.D.	9.78 ± 0.05	N.D.
	0.8 ± 0.1 (3)	0.4 ± 0.1 (7)*		0.8 ± 0.1 (3)	
D_2-V190A	6.31 ± 0.06	9.47 ± 0.08	N.D.	9.54 ± 0.07	N.D.
	1.9 ± 0.2 (3)*	0.3 ± 0.04 (7)*		0.5 ± 0.1 (3)*	
D_2-E2L0.3	5.52 ± 0.07	9.89 ± 0.03	N.D.	**8.32 ± 0.03**	N.D.
IIA/DSS	0.3 ± 0.03 (3)*	0.7 ± 0.1 (5)*		**0.02 ± 0.005 (3)***	
D_2-E2L0.4	5.46 ± 0.02	9.33 ± 0.07	N.D.	**7.51 ± 0.46**	N.D.
IIAN/DSSL	0.2 ± 0.02 (3)*	0.2 ± 0.04 (5)*		**0.004 ± 0.001 (3)***	

N.D., not determined.

Waals contact with Trp321 at the intracellular end of the ancillary pocket, and mutation to Tyr opens up this pocket. Thus, binding of piquindone is enhanced not by the W99F mutation at the mouth of the ancillary pocket but rather by the W321Y mutation. Finally, the lack of a gain of affinity for D_2-selective ligands with the mutant D_1-W321Y could also be explained by assuming that Tyr417 in the D_2 receptor does *not* interact directly with benzamide ligands and that the loss of affinity for these ligands is an indirect consequence of a mutation-induced perturbation of helix packing.

Mutations Based on Proximity to Primary Binding Residues. Residues that are one helix turn away from key ligand-contacting residues are frequently important for pharmacological specificity (Shi and Javitch, 2002), with a good example being residue 3.28, which is one turn away from the TM3 $Asp^{3.32}$ residue that is the primary contact residue for biogenic amine ligands (Shi and Javitch, 2002). We therefore made the double mutant D_1-Y194F/A195V. $Tyr194^{5.38}$ and $Ala195^{5.39}$ are approximately one helix turn away from two serine residues that are important for agonist binding to dopamine receptors (Cox et al., 1992; Neve et al., 2003) and are part of a stretch of 11 contiguous residues in TM5 that, in the D_2 receptor, are exposed to the water-accessible binding pocket as indicated by their high or moderate reactivity with water-soluble cysteine-modifying reagents (Javitch et al., 1995). The Ala/Val substitution at position 5.39 is quite conservative. The Tyr/Phe substitution at position 5.38 is less conservative but seemed unlikely to be a major determinant of D_1/D_2 subtype selectivity because the D_4 receptor has the Tyr residue that is shared by all of the D_1-like receptors at this position instead of the Phe shared by the other D_2-like receptors. Nevertheless, the D_1-Y194F/A195V double mutant showed strong evidence for the presence of selectivity determinants at this locus, with 4- to 12-fold enhanced affinities for the four D_2-selective antagonists tested and 14-fold decreased affinity for [^{3}H]SCH23390 (Table 3).

To explore this region further, we tested the two single mutants D_1-Y194F and D_1-A195V, as well as the reciprocal mutants D_2-F189Y/V190A, D_2-F189Y, and D_2-V190A. We observed that the affinity of the mutant D_1-A195V for [^{3}H]SCH23390 was decreased 11-fold, whereas the affinity of D_1-Y194F for [^{3}H]SCH23390 was decreased only 2-fold (Table 3); competition analysis further showed that the mutant D_1-A195V had increased affinity for the D_2-selective antagonists tropapride and piquindone. Thus, most of the effect of the double mutation on the binding of these subtype-selective ligands could be explained by mutation of Ala195. The reciprocal D_2 mutants had changes in affinity that were smaller than those observed for the D_1 mutants but in the direction consistent with the hypothesis that the residue at position 5.39 contributes to D_1/D_2 selectivity (Table 3).

It is interesting that changing the residue at position 5.39 in the α_{1b}-adrenoceptor from Ala to Val, its corresponding residue in the α_{1a}-adrenoceptor, confers on the receptor a more α_{1a}-like pharmacological profile (Perez et al., 1998). This effect was additive with the effect of a mutation from Leu to Met at position 6.55, although for the dopamine receptors we found only a modest effect of mutating D_2-$His394^{6.55}$ or D_1-$Asn292^{6.55}$ (Table 2).

How does the relatively conservative Ala/Val substitution, in the amino-terminal part of TM5, reciprocally affect the binding of ligands that differentiate between D_1 and D_2 receptors? In our D_1 and D_2 receptor models, residue 5.39 packs against residue 6.59 on the extracellular side of TM6 (Fig. 5). In the D_2 receptor, these residues are relatively large (Val packs against Ile) compared with the D_1 receptor, where Ala contacts Pro. Thus, the helices at the extracellular TM5/TM6 interface of the D_1 receptor are closer than the corresponding residues are in the D_2 receptor. In the model, the Cβ-Cβ distance between residues 5.39 and 6.59 is less than 4 Å for D_1 and more than 5 Å for D_2.

Residues in TM5 and TM6 make important contributions to the ligand binding site. In particular, the TM5 Ser residues at one end of the ligand binding pocket probably interact directly with ligand and create a polar environment for ligands. Hydrophobic Trp and Phe residues in TM6 cradle the ligand binding site on one side. The shorter distance in the D_1 receptor at positions 5.39 and 6.59 results in the Ser residues being closer to TM6 in the D_1 receptor (9 Å from $Ser^{5.42}$ Cβ to $Phe^{6.52}$ Cβ) than in the D_2 receptor (10 Å). This brings SCH23390 closer to the aromatic residues on TM6. When Ala in the D_1 receptor is mutated to the Val (as in D_2), the tight packing with Pro causes an increase in the TM5-TM6 distance and movement of a ligand away from the aromatic residues in TM6, enhancing the binding of D_2 ligands, which have a wider profile in the binding pocket, and decreasing affinity for SCH23390.

An interesting aspect of the effect of position 5.39 on D_1/D_2 selectivity is that docking ligands in our receptor models provided no indication of a direct interaction with this residue, one to two helix turns above the primary binding pocket residues in TM5 and TM6. Instead of interacting directly with ligands, position 5.39 seems to affect ligand binding by altering the relative positions of other primary binding pocket residues that are conserved between D_1 and D_2 receptors.

Mutations in the Second Extracellular Loop. The EL2 of rhodopsin-family GPCRs has been suggested to play a role

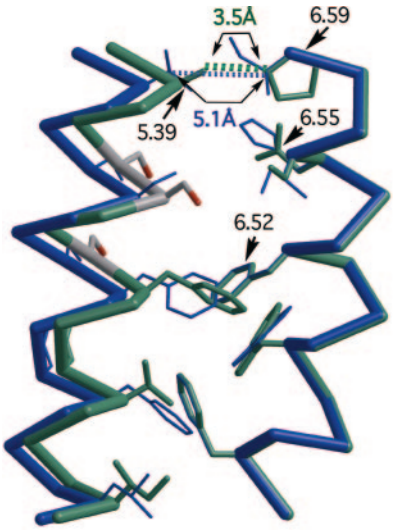

Fig. 5. Difference between TM5 to TM6 helix contacts at the Val/Ala mutation site (residue 5.39) contribute to D_1/D_2 receptor binding differences. Residues 5.39 and 6.59 toward the extracellular face of the membrane (top of figure) pack more closely for D_1 (green) than for D_2 (blue). In the D_1 receptor, the Cβ-Cβ distance for residues 5.39 and 6.59 (Ala and Pro) is relatively close (3.5 Å, large green dots). In the D_2 receptor, the Cβ-Cβ distance for residues 5.39 and 6.59 is longer (~5 Å, fine blue dots) because the Val to Ile contact residues are larger. The positions of two TM6 residues discussed in the text are also indicated.

in pharmacological specificity (Shi and Javitch, 2002). This is consistent with the structure of EL2 in rhodopsin, where the ligand is covalently attached to the receptor and does not dissociate; EL2 is inserted into the binding pocket in such a way that several residues, surrounding a Cys residue that forms a highly conserved disulfide bond with a Cys residue in TM3, contact retinal (Palczewski et al., 2000). The pharmacological profiles of subtypes of α-adrenoceptors (Zhao et al., 1996), 5-hydroxytryptamine receptors (Wurch and Pauwels, 2000), and adenosine receptors (Olah et al., 1994; Kim et al., 1996) are also influenced by residues in EL2. For example, switching three consecutive residues that follow the conserved cysteine in EL2 between α_{1a}- and α_{1b}-adrenoceptors is sufficient to switch the subtype selectivity of some antagonists (Zhao et al., 1996), and much of the difference between the affinity of canine and human 5-HT_{1D} receptors for ketanserin can be attributed to the presence of a Gln or Leu residue immediately after the conserved Cys in EL2 (Wurch and Pauwels, 2000). Shi and Javitch (2004) identified five residues in EL2 of the D_2 receptor that line the binding site crevice, as determined by the substituted cysteine accessibility method, including two residues (Ile184 and Asn186, +2 and +4 relative to the conserved Cys182) that are protected from cysteine-modifying reagents by antagonist binding. Ile184 is shared by D_2 and D_3 receptors, with a conservative Leu substitution in the D_4 receptor. Asn186 is also conserved in D_2 and D_3 receptors, but the D_4 receptor has an Asp residue at that position. To test the hypothesis that these residues contribute to D_1/D_2 receptor pharmacological selectivity, we mutated three (EL2.3) or four (EL2.4) consecutive residues immediately after the conserved Cys in EL2 in the D_1 and D_2 receptors to the corresponding residues in the other subtype. D_2-EL2.3 and D_2-EL2.4 both had substantially decreased affinity for the D_2 receptor antagonist tropapride and modestly decreased affinity for [^{3}H]spiperone, but both mutants also had modestly decreased affinity for SCH23390 (Table 3). Likewise, D_1-EL2.4 had substantially decreased affinity for [^{3}H]SCH23390 but unchanged or slightly decreased affinity for the D_2 receptor-selective antagonists (Table 3). The loss of affinity observed for some ligands provides some support for the hypothesis that this region of EL2 in dopamine receptors contributes to forming the ligand binding pocket, but the lack of any gain-of-function (i.e., increased affinity, which is the most rigorous criterion for identifying receptor determinants of pharmacological selectivity) weakens the hypothesis that EL2 contributes to D_1/D_2 receptor selectivity. These results, however, should be interpreted with caution. Residues at positions −1 and −5 relative to the conserved Cys were also identified as lining the binding site crevice of the D_2 receptor but were not tested in these studies because the presence of a ligand did not protect them from cysteine-modifying reagents (Shi and Javitch, 2004). Furthermore, the EL2 is considerably longer in the D_1 receptor than in the D_2 receptor and possibly arrayed very differently in the two receptors in a way that cannot be mimicked by simply exchanging three or four residues. We have not modeled the loops because of their considerable difference and our philosophy to be initially conservative in modeling large differences from rhodopsin.

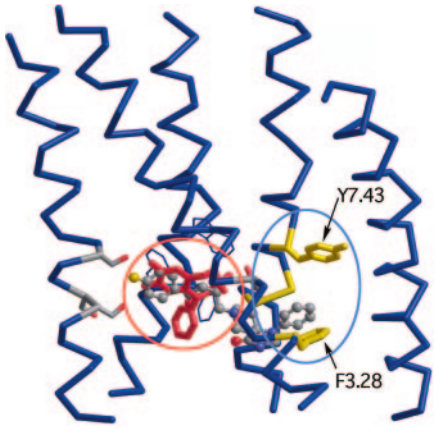

Fig. 6. Regions that contribute to selective high-affinity binding to D_1 and D_2 receptors are nonoverlapping. D_2 receptor model is depicted with the extracellular face of the membrane at the bottom. For the D_2 receptor, with spiperone docked in the binding pocket, ancillary pocket residues (yellow with blue circle) seem most important. For the D_1 receptor, regions of the primary binding pocket in contact with the benzazepine rings of SCH23390 (red circle) seem to contribute most to specificity.

Dopamine Receptor Ligand Specificity Regions. The specificity regions identified in this study seem to be quite distinct for the D_1 and D_2 receptors (Fig. 6). The D_2 receptor contains a specificity pocket consisting of aromatic groups that can increase ligand binding affinity. Protein aromatic groups are well suited to packing with ligand aromatic groups because their rotation can accommodate different geometries in the ligand. In addition, rotation of the protein aromatic group can permit a more closely packed pocket in the absence of the ligand than can other side chains. According to our models, this pocket is not accessible in the D_1 receptor unless it is opened up by mutation of Trp99$^{3.28}$.

In the case of the D_1 receptor, it is the packing of TM5 and TM6 and the size of the primary binding pocket in the vicinity of the aromatic rings of SCH23390 that contribute to D_1 receptor-selective binding. Although we have not modeled the loops for the receptor because of the lack of structural information, the 1-phenyl of SCH23390 is oriented toward and possibly interacts with EL2 so that residues there could influence specificity. Our exploration of residues near the conserved Cys in EL2 has not yet identified such residues.

Summary and Conclusions. To identify structural determinants of D_1/D_2 receptor pharmacological specificity, we mutated residues based on several criteria. Some residues were selected because they are accessible in the binding site crevice and differ nonconservatively between D_1 and D_2 receptors while being shared within the D_1-like and D_2-like subclasses. Others were selected based on their proximity to primary binding pocket residues or to test the hypothesis that a region of EL2 immediately C-terminal to a conserved Cys residue contributes to pharmacological specificity for these receptors.

We identified two residues ot contribute to the selectivity of certain D_2 receptor-selective ligands by making direct contact with ligand substituents: residues 3.28 and 7.43. In the D_1 receptor, the mutation W99F$^{3.28}$ enhanced the affinity of ligands that are sufficiently long and inflexible to interact negatively with the bulkier Trp residue, particularly if the ligand geometry permitted a stacking interaction with the Phe residue; this Trp residue accounted for all of the difference in selectivity between the structurally related compounds spiperone and haloperidol. In the D_2 receptor, our model suggests that the mutation Y417$^{7.43}$W greatly de-

creased the affinity of ligands such as benzamides because of the larger size and differing orientation of Trp. In addition, a Val/Ala switch at position 5.39 had reciprocal effects on the binding of D_1- and D_2-selective antagonists, consistent with a role for this residue in pharmacological selectivity. Our D_1 and D_2 receptor models suggest that changes at this position alter the size of the binding pocket by modulating the distance between the extracellular ends of TM5 and TM6. Finally, we obtained modest support for the hypothesis that residues following the conserved Cys in EL2 contribute to pharmacological specificity.

Overall, we have observed that structural determinants of D_1/D_2 receptor-selective binding vary among different classes of dopamine ligands and even within a group of structurally similar ligands. We have mutated most residues that are believed to be exposed to the binding site crevice, that differ between D_1 and D_2 receptors, and that are conserved within the D_1-like and D_2-like subclasses, and we conclude that residues contributing to pharmacological specificity are not in the same location on the two receptors (Fig. 6). Furthermore, the residues that have been identified as contributing to pharmacological specificity can account for only a fraction of the difference between D_1 and D_2 receptors. We hypothesize that additional significant determinants of D_1/D_2 receptor-selective binding either are in EL2 or, like Ala/Val$^{5.39}$, affect the overall shape of the primary and ancillary binding pockets rather than interacting directly with ligands.

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