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Certain 1,4-Disubstituted Aromatic Piperidines and Piperazines with Extreme Selectivity for the Dopamine D4 Receptor Interact with a Common Receptor Microdomain

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ABSTRACT

We previously demonstrated that, in the D4 dopamine receptor, the aromatic microdomain that spans the interface of the second and third transmembrane segments influences the high-affinity interactions with the D4-selective ligand L750,667 [3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1*H*-pyrrolo[2,3-*b*]pyridine] and the D2-selective ligands methylspiperone, aripiprazole, and its congener OPC4392 [7-[3-(4-(2,3-dimethylphenyl) piperazinyl) propoxy] 2-(1*H*)-quinolinone] (Schetz et al., 2000). Here we tested a variety of 1,4-disubstituted aromatic piperidines/piperazines (1,4-DAPs) with different subtype selectivities and functional properties against a panel of D4 receptor mutations in the aromatic microdomain to ascertain whether these ligands recognize this common site. Mutant D4 receptors were constructed by substituting the nonconserved amino acid(s) from the corresponding locations in the D2 receptor. The D4-L2.60W, D4-F2.61V, and D4-LM3.28-3.29FV substitutions result in alterations of the relative position of members of the aromatic microdomain. From these results and molecular models of the ligand-receptor complexes, we conclude that 9 of the 11 D4-selective 1,4-DAPs, including L750,667, have a common pattern of ligand-receptor recognition that depends upon favorable interactions with the phenylalanine at position 2.61 (D4-F2.61V, 20–96-fold decrease). Like methylspiperone, aripiprazole, and OPC4392, the two D4-selective 1,4-DAPs that are insensitive to the D4-F2.61V mutation are sensitive to aromatics at position 2.60 (D4-L2.60W, 7–20-fold increase), and they all have longer spacer arms that permit their tethered aromatics to adopt alternative orientations in the binding-site crevice. All 11 of the D4-selective 1,4-DAPs were sensitive to the D4-LM3.28-3.29FV mutation (13–494-fold decrease) but not the moderately D2-selective methylspiperone. The inferences suggest that subtype selectivity involves two different modes of interaction with the microdomain for the D4-selective 1,4-DAPs and a third mode for D2-selective 1,4-DAPs.

Dopamine receptors belong to the class A family of G protein-coupled receptors (GPCRs) that share a rhodopsin-like structure. In humans and mammals, the dopamine receptor family is comprised of five genotypically distinct members, which are subclassified as D1-like (D1 and D5) and D2-like (D2, D3, and D4) receptors on the basis of their similarities in structure, G protein-coupling preferences, and pharmacology. After the discovery that antipsychotic efficacy strongly correlates with the strength of D2 receptor antagonism (Creese et al., 1976), it was realized that not one, but three subtypes of D2-like receptors existed and that many antipsy-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; L750,667, 3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1*H*-pyrrolo[2,3-*b*]pyridine; L745,870, 3-{[4-(4-chlorophenyl) piperazin-1-yl]methyl}-1*H*-pyrrolo[2,3-*b*]pyridine [chlorophenylpiperazinyl methylazaindole (CPPMA)]; FAUC113, 3-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-*a*]pyridine; FAUC213, 2-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-*a*]pyridine; PNU101,387G, (*S*)-(−)-4-[4-[2-(isochroman-1-yl)ethyl]-piperazin-1-yl]benzenesulfonamide (sonepiprazole); TM, transmembrane domain; 1,4-DAP, 1,4-disubstituted aromatic piperidine/piperazine; OPC4392, 7-[3-(4-(2,3-dimethylphenyl) piperazinyl)propoxy] 2-(1*H*)-quinolinone; NGD 94-1, 2-phenyl-4(5)-[4-92-pyrimidinyl)-piperazin-1-yl)-methyl]-imidazole; Ro61-6270, 2-amino-benzoic acid 1-benzyl-piperidin-4-yl ester; PD168,077, *N*-[[4-(2-cyanophenyl)-1-piperazinyl]methyl]-3-methylbenzamide; CP226,269, 5-fluoro-2-[[4-(2-pyridinyl)-1-piperazinyl]methyl]-1*H*-indole; CP293,019, 7-[(4-fluorophenoxy) methyl]-2-(5-fluoro-2-pyrimidinyl) octahydro-97*R*,9a*S*)-2*H*-pyrido[1,2-*a*] pyrazine; RBI257, 1-[4-iodobenzyl]-4-[*N*-(3-isopropoxy-2-pyridinyl)-*N*-methyl]-aminopiperidine; Ro10-4548, RAC-2′-2-hydroxy-3–4-(4-hydroxy-2-methoxyphenyl)-1-piperazinyl-propoxy-acetanilide (CPPMA)]; OPC14597, 7-[4-[4-(2,3-dichlorophenyl)-1-piperazinyl]-butoxy]-3,4-dihydro-2 (1*H*)-quinolinone (aripiprazole); methylspiperone, 8-[4-(4-fluorophenyl)-4-oxobutyl]-(3-methyl-1-phenyl)-1,3,8-triazaspiro[4,5]decan-4-one.

chotic drugs had high affinity for D3 and/or D4 receptor subtypes. Once it became clear that the neuroendocrine and extrapyramidal side effects of antipsychotic drugs were also mediated by blockade of D2 receptors and that D3 and D4 receptor had lower levels of expression and a more restricted distribution, hopes were raised that compounds selective for D3/D4 subtypes would have antipsychotic actions free of D2-mediated side effects.

Interest in developing drugs selective for the D4 subtype was further fueled by two findings. The first was the reported 6-fold increase in the density of striatal (but not limbic) D4 receptors in the postmortem brains of schizophrenics (Seeman et al., 1993). The second was the discovery that clozapine, an efficacious antipsychotic with reduced neuroendocrine and extrapyramidal side effects, has a 3- to 8-fold higher affinity for the D4 versus the D2 and D3 receptor subtypes [Van Tol et al., 1991; Seeman et al., 1997a; Psychoactive Drug Screening Program database (PDSP), 2004 (http://pdsp.cwru.edu/pdsp.asp)]. A variety of D4-selective ligands was developed on the basis of these findings. However, the first of these compounds, L750,667, which was reported to be a highly D4-selective antagonist, failed to show antipsychotic potential in animal models predictive of antipsychotic efficacy in humans (Bristow et al., 1997). In placebo-controlled clinical trials, L745,870, the more bioavailable congener of L750,667, did not alleviate any of the symptoms of schizophrenia (Kramer et al., 1997). Instead, there was a trend toward a worsening of psychotic symptoms. Furthermore, the initial findings concerning increased levels of striatal D4 receptors in postmortem brains of schizophrenics were based on a methodological approach that has been subsequently refuted (Seeman et al., 1997b; Helmeste and Tang, 2000). Moreover, the clozapine-like structural analogs olanzapine and quetiapine both display a clozapine-like atypical antipsychotic clinical profile and have higher affinity for the D2 than the D4 subtype (~3–4- and 14-fold, respectively; PDSP database, 2004). Although these later findings seemed to exclude D4 as a viable antipsychotic drug target, subsequent in vitro studies with L745,870 and other compounds considered initially to be highly D4-selective antagonists provided evidence for their weak partial agonist activity (Gazi et al., 1998, 1999).

It has been demonstrated that L745,870-like derivatives such as FAUC113 also have weak partial agonist activity, which can be completely eliminated by a 2′ substitution, rather than a 3′ substitution, of the diazole moiety of the heterocyclic ring (Lober et al., 2001). The assignment of the 2′-substituted diazole FAUC213 as a "neutral antagonist" using different measures of functional activity (fluorometric imaging plate reader $G\alpha_{qo5}$- based versus mitogenesis-based) has been discussed recently (Stewart et al., 2004). It is remarkable that the D4-selective neutral antagonist FAUC213 was recently shown to have atypical antipsychotic potential in animal models predictive of antipsychotic efficacy in humans (Boeckler et al., 2004), in contrast to the structurally distinct D4-selective neutral antagonist PNU101,387G (sonepiprazole), which has no demonstrable antipsychotic activity in humans (Corrigan et al., 2004). It was previously demonstrated that the D4/D2 pharmacological selectivity profile of L745,870 and its differentially halogenated congener L750,667 become more like the substituted receptor when the corresponding amino acids present in the rat D2 subtype are substituted into a rat D4 receptor background (i.e., D4-F2.61V and D4-LM3.28-3.29FV mutants; Schetz et al., 2000) and when some of these and other reciprocal mutations are made in an N- and C-terminally epitope-tagged human D2 receptor background (Simpson et al., 1999). Such substitutions are tantamount to a repositioning of aromatics in a microdomain that spans TM2/TM3. A subsequent survey of compounds developed to have high selectivity for the D4 receptor revealed that, like L745,870 and L750,677, most are 1,4-disubstituted aromatic piperidines/piperazines (1,4-DAPs) (Oak et al., 2000). In the present study, we show that 9 of 11 highly D4-selective 1,4-DAPs that we tested recognize a common spatial pattern of aromatic residues in the TM2/TM3 microdomain of the binding-site crevice. Docking of all 11 compounds in molecular models of the rat D4 receptor constructed in the structural context of bovine rhodopsin reveals a mode of binding consistent with the idea that most of these 1,4-DAPs have a tethered aromatic oriented to interact with the TM2/TM3 aromatic microdomain. This interaction represents one possible mode of recognition, but ligands that can orient their tethered aromatics so as not to interact with this microdomain in TM2/TM3 take advantage of other modes of molecular recognition in the binding pocket.

Materials and Methods

Reagents. FAUC113 and FAUC213 were synthesized as described previously (Lober et al., 2001). All other drugs were either purchased from Sigma/RBI (Natick, MA) or received as generous gifts from the various sources listed under *Acknowledgments*. Analytical grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and cell culture supplies were purchased from either Sigma-Aldrich or Hyclone Laboratories (Logan, UT). [^{3}H]Methylspiperone (NET856; 84 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

Site-Directed Mutagenesis. Microdomains within TM2 and TM3 of the rat D4 receptor were modified with the corresponding residues from the rat D2L receptor using DpnI-based site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA). Custom mutagenic primers synthesized for the mutagenesis reactions (Bio-Synthesis, Lewisville, TX) were purified by polyacrylamide gel electrophoresis before use. Full-length oligonucleotide sequencing was performed on each mutant receptor to verify the presence of the mutation and absence of unwanted mutations. The location of mutations within the receptor are denoted according to the universal numbering convention for locating the relative position of amino acids in the transmembrane-spanning domains of the biogenic amine family of G protein-coupled receptors developed by Ballesteros and Weinstein (1995). The naming system for each mutant indicates the wild-type receptor background with the abbreviation D2 or D4, followed by the single-lettered code for the wild-type amino acid and its location, and ending with the mutant amino acid. For example, the D4-F2.61V mutant has a D4 background that has been mutated from a phenylalanine at position 2.61 to a valine present at this corresponding position in D2 receptors. The naming convention used here also facilitates the comparison of the present results with the work on other GPCRs.

Transfection. pcDNA3 plasmid constructs containing either the wild-type or a mutant rat dopamine receptor were transiently transfected into COS-7 cells using $CaPO_4$ precipitation (Invitrogen, Carlsbad, CA). Specifically, 20 μg of plasmid DNA was mixed with a final volume of 1 ml of $CaPO_4$/HEPES solution, and the resulting precipitate was added drop-wise to 20 to 30% confluent cells attached to a 150-cm^2 plate in a total volume of 20 ml of Dulbecco's modified Eagle's media supplement with 8% bovine calf serum and antibiotics.

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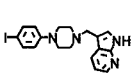
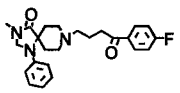
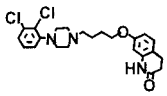
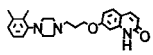
TABLE 1

Affinity of various 1,4-DAPs reported in a previous study by Schetz et al. (2000).

Drug binding affinity (K_i) is expressed as the average ± S.D. with fold changes relative to the wild-type D4 receptor in parentheses.

Receptor	L750,677 	Methylspiperone 	Aripiprazole 	OPC4392 
			nM	
D2-WT	>1500 (>10,000)	0.020 ± 0.004 (0.068)	0.19 ± 0.04 (0.004)	2.6 ± 0.5 (0.042)
D4-WT	0.11 ± 0.02 (1)	0.29 ± 0.030 (1)	47 ± 6.8 (1)	62 ± 12 (1)
D4-L2.60W	0.07 ± 0.02 (0.60)	0.044 ± 0.007 (0.15)	2.3 ± 0.76 (0.05)	3.8 ± 0.71 (0.061)
D4-F2.61V	10.6 ± 2.2 (96)	0.474 ± 0.094 (1.6)	66 ± 17 (1.4)	N.D.
D4-LM3.28-3.29 FV	2.2 ± 0.38 (20)	0.515 ± 0.057 (1.8)	N.D.	N.D.
D4-F2.61V+ LM3.28-3.29FV	15.9 ± 3.3 (145)	0.196 ± 0.075 (0.67)	N.D.	N.D.

N.D., not determined because of limited availability of the test compound.

The media was removed by aspiration the following day and replaced with fresh media. Cells were grown to confluence before they were harvested.

Preparation of Membranes for Binding Assays. COS-7 cells expressing the desired receptor were dislodged by a 5-min incubation in Earle's balanced saline solution lacking Ca^{2+} and Mg^{2+} and supplemented with 5 mM EDTA. After centrifugation, the cell pellet was lysed in lysis buffer (5 mM Tris and 5 mM $MgCl_2$, pH 7.4) at 4°C. The lysate was glass-glass homogenized (eight strokes), and the membranes were centrifuged at 35,000*g* for 30 min. The pellet was resuspended in ice-cold 50 mM Tris, pH 7.4, and centrifuged again. The washed membrane pellet was resuspended by light homogenization (three strokes) in binding buffer (see below) immediately before use.

Radioligand Binding Assays. Membranes containing wild-type or mutant dopamine receptors were assayed for specific [^{3}H]methylspiperone binding activity. The binding buffer consisted of 50 mM Tris, pH 7.4, at 25°C. Nonspecific binding was defined by 5 μM (+)-butaclamol. The reaction was allowed to proceed at 25°C for 1.5 h before rapid filtration through GF/C filters pretreated with 0.3% polyethylenimine. The wash buffer consisted of ice-cold binding buffer (pH 7.4, 0°C). Radioactivity bound to the filters was quantified by scintillation spectroscopy at a counting efficiency of 47%. Membrane protein concentrations were determined using the bicinchoninic acid protein reagent (Pierce, IL) and a bovine serum albumin standard curve. Drug binding affinity values were determined by either saturation isotherms or inhibition curves.

Calculations and Data Analysis. All points for each experiment were sampled in triplicate. The average values of the data from three independent experiments are reported with their associated standard deviation. The equilibrium dissociation constant (K_D) of the primary radioligand was measured by saturation isotherm analysis. The inhibition constant (K_i) values for all compounds were calculated from their IC_{50} values using the Cheng-Prusoff correction: $K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$. This equation assumes a competitive interaction and a pseudo Hill slope = 1. In cases where the best-fit curve did not have a pseudo Hill slope approximating unity, the apparent $K_{0.5}$ values are reported.

All data were analyzed with the statistical and graphing software package Prism 4 (GraphPad Software Inc., San Diego, CA). A 95% confidence interval was used for all curve-fitting procedures and to compare different curve-fitting models. The statistical measures of fit employed were the F-test, the run test, and a correlation coefficient. When analyzing pharmacological differences, any change in affinity that is ≤2.5-fold different from the wild-type background is considered to represent a negligibly small change.

Computational Methods. Three-dimensional molecular models of the seven transmembrane regions of dopamine D2 and D4 receptors were built as described in detail in a recent review (Visiers et al., 2002) using the 2.8 Å crystallographic structure of bovine rhodopsin (Palczewski et al., 2000) as a template for the homology modeling program MODELLER (Sali et al., 1995). The sequence alignment between the transmembrane helices of rhodopsin and the D2 and D4 receptors was taken from the GPCR database (http://www.gpcr.org/7tm/multali/multali.html). The ligands were built using the Builder module of Insight II v. 2000 (Accelyrs Inc., San Diego, CA). The initial structures were energy-optimized with ab initio quantum calculations using Gaussian 95 (Gaussian Inc., Pittsburgh, PA) and the HF 6-31G* basis set. CHARMM-compatible charges for the molecular mechanic calculations were obtained using the CHELPG scheme. A conformational search for the ligands was carried out using the biased Monte Carlo conformational memories method (Guarnieri and Weinstein, 1996). The various conformations were clustered using the XCluster 94 program (MacroModel; Schrödinger, Inc., Portland, OR). Either a representative member of the largest cluster or the conformation that closely resembled the crystallographic conformation of a structurally related ligand was chosen as a candidate for docking studies.

The initial docking of the ligands was done manually with intermolecular interaction energy evaluations using the Docking module of Insight II. Experimentally derived information such as the mutation data for residue D3.32 (Mansour et al., 1992), the orientation of the arginine cage (Ballesteros et al., 1998), and the orientation of the aromatic residues in TM6 (Javitch et al., 1998) were used as guidelines for docking the ligands in the binding sites of the two receptor models. The ligands were anchored by aligning the protonated nitrogen to interact with D3.32. The relative orientation of arm A and B of the 1,4-DAPs was determined by the steric constraints imposed by the cavities on either side of the third helix and guided by the number of favorable interactions that either arm of the ligand could make in a particular orientation. The initial position of the ligands was relaxed by energy minimization of the docked protein-ligand

complex. All simulations were performed with the CHARMM force field (Brooks et al., 1983) and the CHARMM22 parameter set (Mackerell et al., 1998).

Results

Substituting amino acids at positions 2.60, 2.61, and 3.28 of the D4 receptor with the corresponding amino acids of the D2 receptor as well as a combination of the corresponding reciprocal mutations in D2 receptor have been shown to alter the affinity of a few D2- and D4-selective ligands (Simpson et al., 1999; Schetz et al., 2000). These substitutions are tantamount to swapping bulky amino acids (e.g., Phe, Trp, or Met) for small aliphatic amino acids (e.g., Val or Leu). With the exception of the D4-L2.60W mutant, which has a 7-fold increased affinity, all the mutant D4 receptors (D4-F2.61V, D4-LM3.28-3.29FV, and D4-LM3.28-3.29FV+F2.61V) bind [^{3}H]methylspiperone with near wild-type D4 receptor affinity (Table 1). A survey of structurally diverse dopamine receptor ligands in a previous study by Schetz et al. (2000) revealed that only the extremely D4-selective compound L750,667 had a reduced affinity for the D2-like mutation D4-F2.61V, whereas only the D2-selective compounds methylspiperone, aripiprazole, and OPC4392 had increased affinity for a different D2-like mutation: D4-L2.60W. In each case, these observed affinity changes for either the D4-F2.61V or the D4-L2.60W mutant receptors resulted in a more D2-like pharmacological profile. In addition, the reciprocal mutations in a D2 background (rat D2-IFVTL3.27-3.31TLMAM, Schetz et al., 2000; and human D2V2.61F/F3.28L, Simpson et al., 1999) showed an increase in L750,667 and L745,870 affinity, respectively, compared with the wild-type D2 receptor. An observation in the previous study by Schetz et al. (2000) was that all the compounds that were sensitive to mutations at positions 2.60 or 2.61 were 1,4-disubstituted aromatic piperidines/piperazines and, further, that the carbon spacer of the D4-selective compound was considerably shorter (one carbon) than for the D2-selective compounds (four to five carbons). Here we thoroughly investigate the structure-affinity relationships for 10 additional extremely D4-selective 1,4-DAPs for binding to the D4 receptor and mutants affecting the microdomain spanning TM2 and TM3.

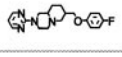
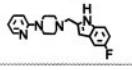
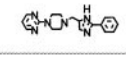
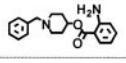
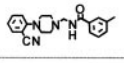
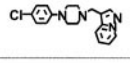
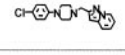
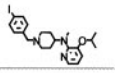
Like L750,667, the D4-selective 1,4-DAPs NGD 94-1, Ro61-6270, PD168,077, CP226,269, CP293,019, RBI257, FAUC113, and FAUC213 bind the D4-F2.61V mutant receptor with significantly reduced affinities (20–86-fold; Table 2). These changes in affinity indicate a more D2 receptor-like pharmacology. In contrast, the binding of the other two D4-selective 1,4-DAPs, PNU101,387G and Ro10-4548, were insensitive to the D4-F2.61V mutation. Instead, the affinity of these drugs was significantly increased by the D4-L2.60W mutation (7–13-fold; Table 3). In the case of PNU101,387G and Ro10-4548, these increases in affinity do not correspond with a more D2-like pharmacology for the D4-L2.60W mutant. All 11 D4-selective 1,4-DAPs bind the D4-LM3.28-3.29FV mutant with reduced affinity, which makes their pharmacology more D2-like. The largest change was measured for CP226,269 (~500-fold), and the smallest changes were for L750,667, FAUC113, and RBI257 (19–24-fold). In contrast, the D4-LM3.28-3.29FV mutation has no effect on the binding of the moderately D2-selective 1,4-DAP methylspiperone. A notable finding among the D4-F2.61-sensitive 1,4-DAPs is that the magnitudes of the affinity changes for L750,667 and FAUC113 are greater for the D4-F2.61V mutation than for the D4-LM3.28-3.29FV mutation, whereas the opposite is true for NGD 94-1, Ro61-6270, PD168,077, CP226,269, CP293,019, RBI257, and FAUC213. The combined mutation of the amino acids located at positions 2.61 and 3.28-3.29 produces a significantly larger, but not additive, reduction in the binding affinity for all the D4-F2.61V-sensitive D4-selective 1,4-DAPs, except for Ro61-6270, RBI257, and CP226,269. The significantly greater magnitudes of the changes make the selectivity profile of some of D4-selective 1,4-DAPs at the combined D4-F2.61V+LM3.28-3.29FV mutant seem even more like the wild-type D2 receptor.

Five of the nine D4-selective 1,4-DAPs that are sensitive to the D4-F2.61V mutation have a one-carbon spacer on arm A that tethers the aromatic moiety to the protonatable amine of their piperizine: L750,667, NGD 94-1, CP226,269, FAUC113, and FAUC213 (Fig. 1). The remaining four, PD168,077, CP293,019, RBI257, and Ro61-6270, have a longer arm A (three to five atoms) extending from their protonatable amines, but in each case there are structural constraints (e.g., a carbon ring as in CP293,019 or an amide as in PD168,077) imposed on the spacer arm near the protonatable

TABLE 2

Affinity of 1,4-Disubstituted Aromatic piperazine/piperidines that are sensitive to the D4-F2.61V mutation

Drug binding affinity (K_i) is expressed as the average ± S.D. with fold changes relative to the wild-type D4 receptor in parentheses.

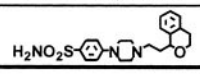
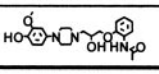
Receptor	CP293,019 	CP226,269 	NGD 94-1 	Ro61-6270 	PD168,077 	FAUC113 	FAUC213 	RBI257 
				nM				
D2-WT	1270 ± 679 (3838)	50 ± 13 (121)	1376 ± 243 (4551)	438 ± 267 (494)	1608 ± 294 (1072)	148 ± 44 (155)	>1000 (>1000)	76 ± 54 (283)
D4-WT	0.33 ± 0.15 (1)	0.41 ± 27 (1)	0.3 ± 0.04 (1)	0.89 ± 0.12 (1)	1.5 ± 0.41 (1)	0.95 ± 0.16 (1)	1.1 ± 0.22 (1)	0.27 ± 0.10 (1)
D4-L2.60W	0.20 ± 0.03 (0.60)	0.19 ± 0.15 (0.46)	0.11 ± 0.03 (0.36)	0.26 ± 0.04 (0.29)	0.40 ± 0.4 (0.27)	0.19 ± 0.10 (0.20)	0.19 ± 0.04 (0.18)	0.12 ± 0.04 (0.46)
D4-F2.61V	9.6 ± 4.7 (29)	16 ± 2.7 (40)	18 ± 8.9 (60)	20 ± 7.4 (22)	30 ± 6.4 (20)	82 ± 54 (86)	26 ± 3.3 (25)	5.6 ± 2.4 (21)
D4-LM3.28-3.29FV	22 ± 11 (66)	202 ± 53 (494)	27 ± 4.1 (89)	52 ± 2.0 (59)	95 ± 12 (64)	19 ± 5.4 (19)	73 ± 22 (70)	6.4 ± 1.8 (24)
D4-F2.61V+ LM3.28-3.29FV	173 ± 78 (522)	217 ± 66 (531)	103 ± 69 (341)	54 ± 7.4 (60)	236 ± 27 (158)	153 ± 72 (160)	524 ± 207 (500)	10 ± 5.6 (38)

amine of their piperazine/piperidine moieties. The common finding for all nine of these 1,4-DAPs is that the vicinal constraints imposed by the shortness, or geometry, of arm A is transferred to their tethered aromatics. The six-membered aromatics tethered to arm B of all the D4-F2.61V-sensitive 1,4-DAPs are either unsubstituted, *para*-halogenated, or mono- or diorthoelectronegative. In contrast, the common structural feature of the five most D4-L2.60W-sensitive 1,4-DAPs is a long arm A (three to five atoms) with an electron-donating oxygen at 3 to 4 carbons from the protonatable amine of the piperidine or piperazine pharmacophore. PNU101,387G has the shortest spacer arm (three atoms) of this series, and it is the only one with a cyclic ether constraining the aromatic at the distal end of arm A–away from the piperazine pharmacophore.

TABLE 3

Affinity of D4-selective compounds that are insensitive to the D4-F2.61V mutation

Drug binding affinity (K_i) is expressed as the average ± S.D. with fold changes relative to the wild-type D4 receptor in parentheses.

Receptor	PNU101,387G 	Ro10-4548 
	nM	
D2-WT	>13,000 (>7500)	>12,000 (>725)
D4-WT	1.8 ± 0.42 (1)	16 ± 3.3 (1)
D4-L2.60W	0.26 ± 0.11 (0.15)	1.3 ± 0.29 (0.080)
D4-F2.61V	5.3 ± 0.57 (3.0)	21 ± 0.78 (1.3)
D4-LM3.28-3.29 FV	67 ± 18 (38)	206 ± 86 (13)
D4-F2.61V+ LM3.28-3.29FV	121 ± 21 (69)	132 ± 66 (8.1)

The differences between the binding-site crevices of D2 and D4 are tantamount to the juxtapositioning of aromatics and small aliphatics at positions 2.61 and 3.28, respectively, thus changing the shape of the hydrophobic face in the crevice. At position 3.29, the change is from a small aliphatic to a rather bulky methionine that has better interactions with aromatics. Scanning cysteine accessibility method analysis of the D2 receptor has shown that the amino acids at positions 2.61, 3.28, and 3.29 are accessible to the binding-site crevice (Javitch et al., 1999), and our molecular model of the D4 receptor indicates the same.

In an effort to better understand the patterns of chemical interactions between 1,4-DAPs and the D2 and D4 receptor binding sites, molecular models were constructed using available experimental data from the literature as well as by defining structural features of both the ligands and their receptors

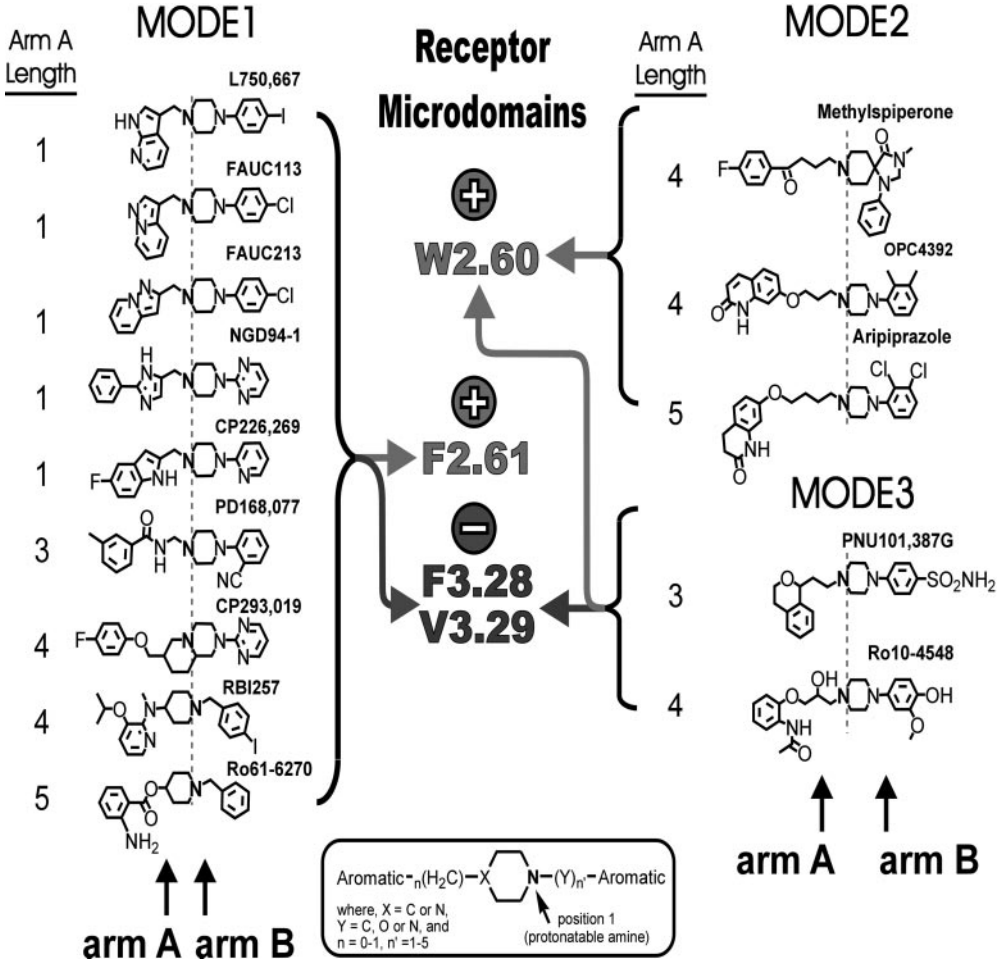


Fig. 1. Schematic summary of the effect of replacing bulky residues with small aliphatic residues at positions 2.60, 2.61, and 3.28-3.29 on the binding of 1,4-DAPs to the D4 receptor. The 2-D hydrogen-suppressed structures of the 1,4-DAPs are aligned (dashed line) with respect to their protonatable amines shown in bold. The effect of specific amino acids at positions 2.60, 2.61, 3.28, and 3.29 on the affinity of specified ligands for the D4 receptor is identified in the central column. Note that F2.61 is wild type, and W2.60, F3.28, and V3.29 are D2-like mutants. The plus sign indicates an increase in affinity, whereas the minus sign indicates a decrease in affinity. The three distinct patterns of sensitivity of 1,4-DAPs for the different mutants defined three distinct modes of binding for the 14 different 1,4-DAPs. Note that ligands that bind in either mode-1 or mode-3 show very high selectivity for the dopamine D4-receptor, whereas those that bind in mode-2 are moderately to highly selective for the D2 dopamine receptor. Arrows designate the spacer arms that tether the aromatics to the central piperidine/piperazine pharmacophore as arms A and B. The numbers next to the chemical structures refer to the length of spacer arm A. This length is calculated as the number of atoms linking the protonatable amine of the piperidine/piperazine pharmacophores to the first aromatic. The pharmacophore of 1,4-DAPs is described in the box with the numbering format and the substituents to the aromatic rings.

(outlined under *Computational Methods*). To characterize the three observed experimental modes of interaction of the 1,4-DAPs with the D4 and D2 receptor, the 1,4-DAPs were classified into three categories, as shown in Fig. 1. All these compounds have a centrally positioned protonated amine (dotted line) that interacts with D3.32 in both D2 and D4 and two aromatic moieties separated by various spacer arm lengths. The model of the D4 receptor is shown in Fig. 2a.

Interaction mode-1 involves compounds that have a short or vicinally constrained arm A extending from the protonatable nitrogen of the pharmacophore and much higher affinity toward the D4 than the D2 receptor. Compounds interacting in this mode are L750,667, CP293,019, CP226,269, NGD 94-1, Ro61-6270, PD168,077, FAUC113, FAUC213, and RBI257. Docking of L750,667 in the wild-type D4 receptor model shown in Fig. 2b indicates that the aryl ring tethered by arm B is involved in favorable aromatic interactions with F2.61. The aryl rings in L750,667 and FAUC113 are involved in displaced pi stacking interactions with the phenyl ring at 2.61, whereas all other ligands belonging to this class interact either in a near-parallel stacking orientation or a T-type orientation (Fig. 2c). It is noteworthy that modeling a valine at position 2.61 demonstrated a loss of the favorable aromatic interaction. At position 3.28, a leucine, as in the wild-type D4 receptor, is preferred because of its small side chain, whereas a phenylalanine interferes sterically with the binding of the

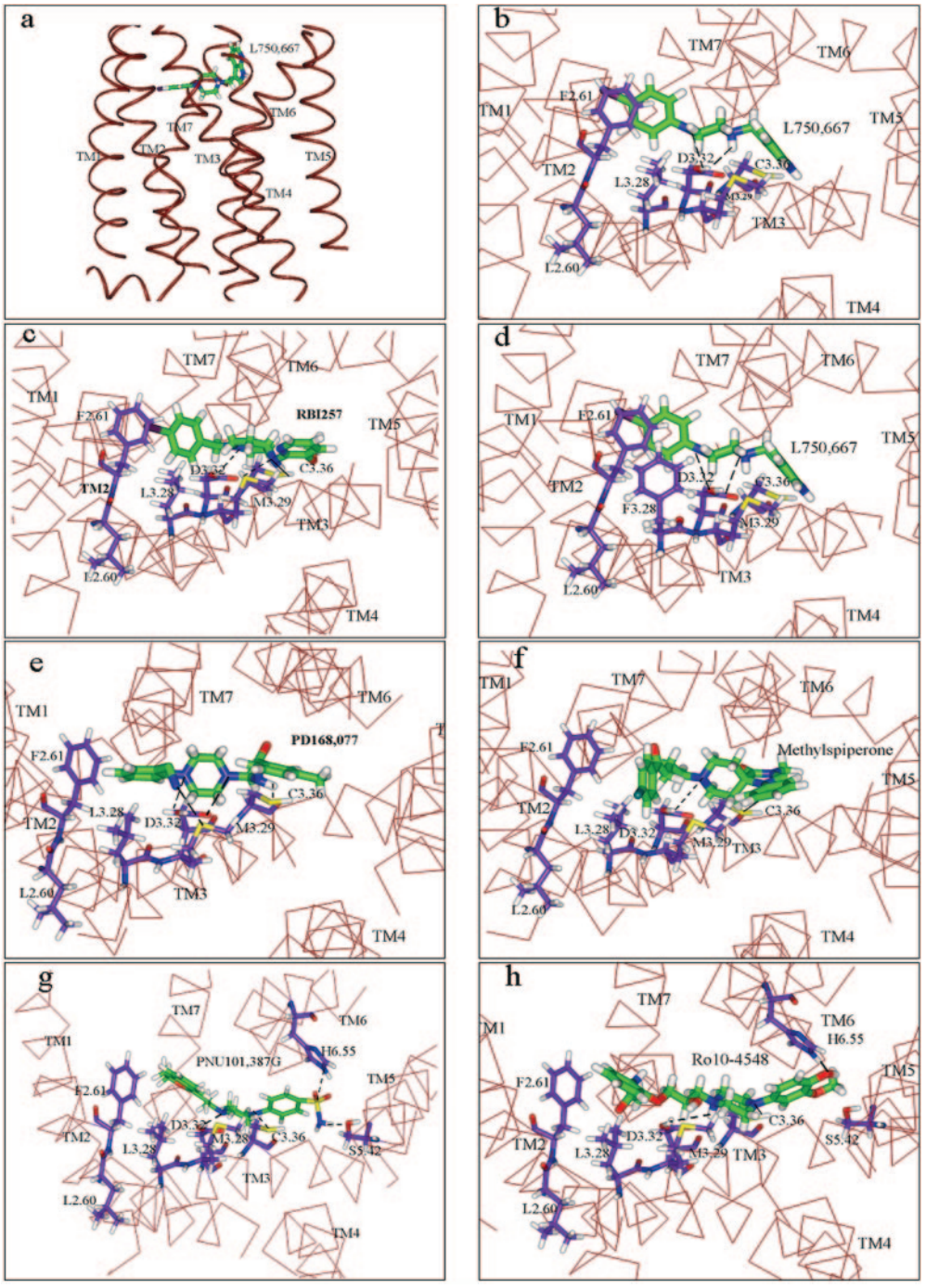


Fig. 2. Representation of the D4 receptor model in a periplasmic view, with docked ligands in the binding site. The TM helices are rendered in red and labeled TM1 to TM7. Residues participating in this study are represented in a stick model and labeled according to their generic number–with carbon in magenta, nitrogen in blue, oxygen in red, and hydrogen in white. The ligands are also represented in a stick model and colored by atom type. Broken lines represent the electrostatic interactions. a, representation of the D4 receptor model perpendicular to the membrane, with L750,667 docked in the binding site; b, L750,667 docked in the binding site; c, RBI257 docked in the binding site (note the tilted T interaction with F2.61); d, F3.28 mutation shown in a ball and stick model (note the steric clash with L750,667); e, PD168,077 docked in the binding site shows the favorable electrostatic interaction with M3.29; f, methylspiperone docked in the binding site has no direct interaction with F2.61; g, PNU101,387G in the binding site has no direct interaction with F2.61 but instead interacts favorably with residues from TM5 and TM6; h, Ro10-4548 in the binding site has no direct interactions with F2.61 and favorable interactions with residues in TM6.

ligand (Fig. 2d). Modeling a valine at position 3.29 instead of a methionine as in the wild-type receptor leads to a loss of a favorable interaction with some mode-1 ligands, specifically with CP226,269 and PD168,077 in the model (Fig. 2e).

Ligands interacting in mode-2 have a long arm A (four to five atoms) and higher affinity for the D2 than the D4 receptor. Methylspiperone, aripiprazole, and OPC4392 belong to this class. Consistent with the experimental data, docking of these ligands in the D4 receptor model indicates that they do not interact directly with F2.61 because of their bent conformation (Fig. 2f). Furthermore, modeling a phenylalanine at position 3.28 does not lead to any steric clashes with the ligand.

PNU101,387G and Ro10-4548, which belong to the mode-3 type of interaction, have a long arm A (three to four atoms) and higher affinity for the D4 than the D2 receptor. Docking of these compounds indicates a similar mode of binding to the D4 receptor, with their arm A oriented toward F2.61 (Fig. 2, g and h). Similar to the mode-2 compounds, these ligands do not have direct aromatic interactions with F2.61. Instead, the charged sulfonamide in PNU101,387G favorably interacts with H6.55 and S5.42, whereas the charged hydroxyl group on the aryl ring of Ro10-4548 favorably interacts with H6.55 in the D4 receptor model. Analysis of the docking of PNU101,387G and Ro10-4548 in the binding site of the D4 receptor indicates that a phenylalanine at position 3.28 would create a moderate steric clash with the ligand.

Discussion

In the absence of crystal structures for D2 and D4 receptors, molecular modeling combined with site-directed mutagenesis and extensive pharmacological analysis provides a powerful tool to address specific ligand-receptor interactions. In this study, we have identified 11 1,4-DAPs that have considerably higher affinity for the D4 than the D2 receptor, and nine of these can be considered to interact with the microdomain comprising residues from TM2 and TM3. Five of these nine compounds have a short arm A (one carbon), and the other four compounds have a long arm A (three to five carbons) that is vicinally constrained. All nine compounds bind to the D4 receptor in a similar mode (mode-1), with their tethered aromatics on arm B pointing toward and interacting with F2.61. Thus, in these nine compounds, the aryl ring is either in a displaced pi-pi stacking or T-type interaction with F2.61. These aromatic interactions between the ligand and receptor play a decisive role in the recognition of the TM2/TM3 aromatic microdomain. Our finding is consistent with the role of aromatic-aromatic interactions in molecular recognition (e.g., see Meyer et al., 2003). Burley and Petsko (1985) have reported aromatic stacking interactions between ring centroids with separations >4.5 Å and <7.0 Å and a dihedral angle between 30 and 90° producing a tilted T or edge-to-face arrangement, which results in favorable aromatic-aromatic interactions contributing up to a 2-kcal/mol change in free energy.

The greater dependence on F2.61 for the binding of the 3′-substituted diazoles, FAUC113 and L750,667 (i.e., the D4-F2.61V mutation leads to a loss of affinity), can be explained by the ability of their arm B aromatics to engage in displaced pi-pi stacking interaction with F2.61, whereas the aryl ring in the other seven ligands belonging to this class is characterized by a greater overlap in pi-pi interactions or a tilted T-type interaction. Furthermore, for mode-1 ligands such as CP293,019 and PD168,077, both of which have an arm A longer than one carbon, the structural constraints in close proximity to the protonatable nitrogen of the pharmacophore are likely to translate into constraints on the possible orientations of both the protonatable nitrogen and its aromatics on arm A, thus stabilizing a specific mode-1 orientation in the binding pocket.

Docking studies reported here showed that a small residue such as leucine is preferred at position 3.28, because it assists the ligands in achieving the favorable interaction with the neighboring D3.32. This was found from modeling studies to be feasible only in the D4 receptor, because in the D2 receptor, a phenylalanine at position 3.28 creates a steric clash with the ligands. The steric hindrance for the docking of ligands in mode-1 (see Fig. 2d) could lead to the loss of affinity observed for the D2 receptor, consistent with the -fold changes reported in Tables 1 and 2. Of the mode-1 binding 1,4-DAPs, only Ro61-6270, RBI257, and CP226,269 did not show a clear synergistic change in affinity for the combined D4-F2.61V+LM3.28-3.29FV mutant. A likely structural explanation in the case of Ro61-6270 and RBI257 is that they can more easily make compensatory adjustments in the binding-site crevice after the mutations, because unlike the other mode-1 compounds, they have a flexible carbon spacer linking their arm B aromatics, thus making it easier for these two ligands to reposition themselves in the modified site.

In this study, mutation of a leucine to a tryptophan at position 2.60 in the D4 receptor leads to an increase in affinity for all the mode-2 and mode-3 ligands and for a few mode-1 ligands. In our model of the wild-type D4 receptor, this leucine is not found to be in direct contact with the ligand (nor does the corresponding residue Thr interact with the chromophore in the crystalline structure of bovine rhodopsin). However, modeling a tryptophan at this position in the D4 receptor suggests unfavorable steric interactions with the residues in the third helix, leading us to consider a possible rearrangement of residues in TM3 in the D2 receptor compared with the D4 receptor. This would enlarge the TM2/TM3 portion of the binding-site crevice such that the increased affinity of the compounds for a D2-like mutation in the D4 receptor is a possible consequence of this local difference in conformation between the two receptors.

In contrast to mode-1 binding 1,4-DAPs, mode-2 and mode-3 binding 1,4-DAPs have either *ortho*- and *meta*-substituted lipophilic substitutions on their arm B aromatics (OPC4392 and aripiprazole), or the qualitative equivalent (methylspiperone), or *para*-substituted or *para*- and *meta*-substituted charged groups (PNU101,387G and Ro10-4548), respectively. None of these substitutions on arm B aromatics can be accommodated in the much narrower hydrophobic TM2/TM3 microdomain portion of the binding-site crevice of the D4 receptor. Instead, mode-2 and mode-3 ligands were modeled to have their arm A aromatics, rather than their arm B aromatics, pointing toward F2.61 (note, however, that the distance precludes a direct interaction with F2.61). This orientation is possible for these ligands, because in each case, arm A is 3 to 4 atoms long, and there is no structural feature that imposes a conformational constraint in close proximity to the protonatable amine of the pharmacophore. Evidence that such a conformation of mode-2 and mode-3 ligands is not

only chemically feasible but also is likely to be favored comes from the crystal structure of spiperone (Koch, 1973), which has its aromatic moiety tethered by a long flexible arm poised almost overhead of its piperidine moiety. Further evidence for such a conformation is the observation that the conformationally rigid PNU101,387G has a distal-cyclized ether tethered to its arm A that would be expected to constrain the aromatic moiety distally and force it to assume a conformation similar to that of spiperone.

Although both mode-2 and mode-3 ligands are believed to adopt a bent conformation in the binding-site crevice, only the mode-3 binding ligands (PNU101,387G and Ro10-4548) are D4-selective, whereas the mode-2 ligands are D2-selective. Part of this difference is understandable given the unfavorable effect that the D4-LM3.28-3.29FV mutant has on the binding of the D4-selective mode-3 ligands, which is caused by the steric clash of F3.28 with the irm A aromatic moiety. This does not occur for the mode-2-binding D2-selective ligand methylspiperone, for which the mutation does not have unfavorable effects. However, the relatively small magnitude of the effect of the D4-LM3.28-3.29FV mutation on mode-3-binding 1,4-DAPs suggests that additional nonconserved receptor microdomains must be important for mode-3 compared with mode-2 binding. It is noteworthy, however, that only the mode-3, D4-selective 1,4-DAPs contain charged substitutions on their arm B aromatics that are oriented so as to enable favorable electrostatic interactions with one or both charged amino acids that are conserved in D2 and D4 receptors: S5.42 and H6.55.

Together, the results offer several novel insights related to the structure-affinity of 1,4-DAPs that are selective for either the D4 or D2 dopamine receptor subtypes. First, there are two distinct but overlapping patterns of microdomain recognition that are exploited by the 1,4-DAPs that are highly selective (>120-fold) for the D4 receptor binding modes 1 and 3. The negative effect of a bulky phenylalanine at 3.28 and a less bulky and electroneutral valine at position 3.29 is shared by both D4-selective modes of binding. Second, the mode-2-binding D2-selective 1,4-DAP methylspiperone has it own pattern of microdomain recognition that partly overlaps with the mode-3-binding D4-selective 1,4-DAPs, i.e., sensitivity to the D4-L2.60W mutant.

A remarkable finding of this study is the apparent lack of correlation between the three identified modes of binding and the known functional properties of the compounds. For example, FAUC213, CP293,019, and Ro61-6270 are antagonists (Hartmann et al., 1996; Sanner et al., 1998; Lober et al., 2001); FAUC113, NGD 94-1, L750,667, and RBI257 are weak partial agonists (Gazi et al., 1998, 1999; Lober et al., 2001); and PD168,077 and CP226,269 are agonists (Glase et al., 1997; Zorn et al., 1997), yet each of these ligands display mode-1 binding. Likewise, within mode-3 binding compounds there is no apparent correlation with their functional properties, because whereas PNU101,387G is an antagonist (Merchant et al., 1996), Ro10-4548 is an agonist (C. Riemer, personal communication). Among mode-2 binding compounds, methylspiperone is an inverse agonist (Wilson et al., 2001), whereas aripiprazole and OPC4392 are (presynaptic) autoreceptor partial agonists and postsynaptic dopamine antagonists (Yasuda et al., 1988; Lawler et al., 1999).

The key structural insights provided by the present ligand-receptor structure-affinity relationship studies have important implications for understanding the properties of the D4 dopamine receptor. Through an iterative process of experimentation and modeling, we have established that, in addition to D3.32, F2.61 is an essential docking site for mode-1-binding 1,4-DAPs. This has important implications for efforts to design D4 receptor subtype-selective ligands, because the majority (9 of 11) of D4-selective 1,4-DAPs, but none of the D2 receptor-selective 1,4-DAPs, display mode-1 binding. In particular, shortening arm A or sterically restricting longer arms vicinal to the protonatable amine of the 1,4-DAP pharmacophore will promote D4 selectivity over D2 receptor-selectivity. It is noteworthy that mode-1 1,4-DAPs exhibit agonist, weak partial agonist, and antagonist functional properties. Therefore, the critical inference is that, at least in the case of mode-1-binding D4-selective 1,4-DAPs, the local orientation of arm B aromatics in the D4-selectivity domain consequently orients their arm A aromatics toward another microdomain between TM5 and TM6. The specific functional phenotype is thus likely to be governed by the chemical nature of the aromatic moiety on arm A and its interaction with the microdomain formed by TM5 and TM6—as recently suggested by Stewart et al. (2004). Visiers et al. (2002) and Ebersole et al. (2003) have already demonstrated that such differential positioning can lead to different functional phenotypes. Our discriminant findings should have clinical relevance as well, because the D4 dopamine receptor has been implicated in the treatment of a broad range of medical conditions, including attention deficit hyperactivity disorder (Avale et al., 2004), substance abuse (Lusher et al., 2001), neurodegeneration (Ishige et al., 2001), and psychosis (Boeckler et al., 2004).

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