Molecular Pharmacology Fast Forward. Published on September 24, 2004 as doi:10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

"MOL 1321"

Certain 1,4-disubstituted aromatic piperidines and piperazines with extreme selectivity for the dopamine D4 receptor interact with a common receptor microdomain.

Sandhya Kortagere, Peter Gmeiner, Harel Weinstein, John A. Schetz

Department of Physiology & Biophysics, Weill Medical College of Cornell University (WCMC), Box 75, 1300 York Ave., New York, NY 10021. (S.K and H.W)

Department of Medicinal Chemistry, Emil Fischer Center, Friedrich-Alexander University, Schuhstraße 19, D-91052 Erlangen, Germany. (P.G)

Department of Pharmacology & Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107-2699. (J.A.S)

Department of Pharmacology, School of Pharmacy, University of Mississippi, P.O. Box 1848, University, MS 38677-1848. (J.A.S)

"MOL 1321"

Running title: Similar receptor determinants for 1,4-DAPs.

Correspondence should be addressed to: John A. Schetz, Ph.D., Department of Pharmacology & Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107-2699. Phone: 817-735-2056, FAX: 817-735-2091, e-mail: jschetz@hsc.unt.edu

Number of:

Text pages: 37

Tables: 3

Figures: 2

References: 43

Words in Abstract: 260

Words in Introduction: 804

Words in Discussion: 1645

Abbreviations.

L750,667 is 3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine.

L745,870 is 3-{[4-(4-chlorophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine and is also known as CPPMA, which stands for chlorophenylpiperazinyl methylazaindole.

NGD 94-1 is 2-phenyl-4(5)-[4-92-pyrimidinyl)-piperazin-1-yl)-methyl]-imidazole.

PD168,077 is N-[[4-(2-Cyanophenyl)-1-piperazinyl]methyl]-3-methylbenzamide.

FAUC213 is 2-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-a]pyridine.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

FAUC113 is 3-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-a]pyridine.

PNU101,387G is

(S)-(-)-4-[4-[2-(isochroman-1-yl)ethyl]-piperazin-1-yl]benzenesulfonamide.

RBI257 is 1-[4-iodobenzyl]-4-[N-(3-isopropoxy-2-pyridinyl)-N-methyl]-aminopiperidine.

CP293,019 is 7-[(4-Fluorophenoxy)methyl]-2-(5-fluoro-2-pyrimidinyl) octahydro -97R,9aS)-2H-pyrido[1,2-a] pyrazine.

CP226,269 is 5-fluoro-2-[[4-(2-pyridinyl)-1-piperazinyl]methyl]-1H-indole.

OPC4392 is 7-[3-(4-(2,3-dimethylphenyl) piperazinyl) propoxy] 2-(1H)-quinolinone.

OPC14597 is 7-[4-[4-(2,3-dichlorophenyl)-1-piperazinyl]-butoxy]-3,4-dihydro-2 (1H)-quinolinone also called aripiprazole.

Methylspiperone is 8-[4-(4-Fluorophenyl)-4-oxobutyl]-(3-methyl-1-phenyl)-1,3,8-triazaspiro[4,5]decan-4-one hydrochloride.

Ro61-6270 is 2-Amino-benzoic acid 1-benzyl-piperidin-4-yl ester.

Ro10-4548 is RAC-2'-,2-hydroxy-3-4-(4-hydroxy-2-methoxyphenyl)-1-piperazinyl-propoxy-acetanilide.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

We previously demonstrated that in the D4 dopamine receptor the aromatic microdomain that spans the interface of the second and third transmembrane (TM) segments influences the high affinity interactions with the D4-selective ligand L750,667 and the D2-selective ligands methylspiperone, aripiprazole and its congener OPC4392 (Schetz *et al.*, 2000). Here we tested a variety of 1,4-disubstituted aromatic piperidines/piperazines (1,4-DAP) with different subtype-selectivities and functional properties against a panel of D4 receptor mutations in the aromatic microdomain to ascertain whether these ligands are recognizing this common site. Mutant D4 receptors were constructed by substituting the non-conserved 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 nine of the eleven 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 eleven of the D4-selective 1,4-DAPs were sensitive to the D4-LM3.28-3.29FV mutation (13 to 494-fold decrease), but not the moderately D2-selective methylspiperone. The inferences suggest

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

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.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

Dopamine receptors belong to the class-A family of G protein-coupled receptors (GPCR) 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, D4) receptors on the basis of their similarities in structure, G protein coupling preferences, and pharmacology. Subsequent to 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 antipsychotic drugs had high affinity for D3 and/or D4 receptor subtypes as well. 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 post-mortem 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 3-8-fold higher affinity for the D4 versus the D2 and D3 receptor subtypes (Van Tol *et al.*, 1991; Seeman *et al.*, 1997b, PDSP database, 2004, http://pdsp.cwru.edu/pdsp.asp). A variety of D4-selective ligands were developed on the basis of these findings. However, the first of such compounds, L750,667, which was reported to be a highly D4-selective antagonist, failed to show

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

antipsychotic potential in animal models predictive of antipsychotic efficacy in humans (Bristow *et al.*, 1997). In placebo-controlled clinical trials, the more bioavailable congener of L750,667, namely L745,870, did not alleviate any of the symptoms of schizophrenia (Kramer *et al.*, 1997). Instead there was a trend towards a worsening of psychotic symptoms. Further, the initial findings concerning increased levels of striatal D4 receptors in post-mortem brains of schizophrenics were based on a methodological approach that has been refuted subsequently (Helmeste and Tang, 2000, Seeman *et al.*, 1997a). Moreover, the clozapine-like structural analogues, olanzapine and quetiapine, both display a clozapine-like atypical antipsychotic clinical profile and both have higher affinity for the D2 than the D4 subtype (~3-4-fold and 14-fold, respectively, PDSP database, 2004). While 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, Gazi *et al.*, 1999).

Recently, it was 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 ($G\alpha_{qo5}$-based FLIPR *vs.* mitogenesis-based) has been discussed recently (Stewart *et al.*, 2004). Remarkably, 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 D2-like 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-terminally 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 report we show that nine of eleven 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 eleven 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.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

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 (Saint Louis, MO) or received as generous gifts from the various sources listed in the acknowledgements. Analytical grade chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and cell culture supplies were purchased from either Sigma Chemical Company or Hyclone Laboratories. [^{3}H]methylspiperone (NET856, 84 Ci/mmol) was purchased from Dupont NEN.

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, CA). Custom mutagenic primers synthesized for the mutagenesis reactions (Biosynthesis Inc., Lewisville, TX) were PAGE-purified prior to use. Full-length oligonucleotide sequencing was performed on each mutant receptor to verify the presence of the mutation and the 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, CA). Specifically, 20 µg of plasmid DNA were mixed with a final volume of 1 ml $CaPO_4$/HEPES solution and the resulting precipitate was added drop wise to 20-30% confluent cells attached to a 150 cm^2 plate in a total volume of 20 ml of DMEM media supplement with 8% bovine calf serum and antibiotics. The following day, the media was removed by aspiration 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 5 min incubation in Earle's balanced saline solution (EBSS) 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, 5 mM $MgCl_2$, pH 7.4 at 4ºC). The lysate was glass-glass homogenized (8 strokes) and the membranes were centrifuged at 35,000xg for 30 min. The pellet was re-suspended in cold 50 mM Tris, pH 7.4 at 25ºC and centrifuged again. The washed membrane pellet was re-suspended by light homogenization (3 strokes) in binding buffer (see below) immediately before use.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

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 25ºC. Non-specific binding was defined by 5 µM (+)-butaclamol. The reaction was allowed to proceed at 25ºC for 1.5 hours before rapid filtration through GF/C filters pretreated with 0.3% polyethyleneimine. The wash buffer consisted of ice-cold binding buffer (pH=7.4, 0ºC). Radioactivity bound to the filters was quantified by scintillation spectroscopy at a counting efficiency of 47%. Membrane protein concentrations were determined using the bicinchonic acid (BCA) 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 or 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: $K_i = IC_{50}/(1+[ligand]/K_D)$. 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. A 95% confidence interval was used for all curve-fitting procedures and for comparing

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 receptor were built as described in detail in a recent review (Visiers *et al.*, 2002) using the 2.8Å 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 INSIGHTII (Accelyrs Inc, 2000). The initial structures were energy optimized with *ab-initio* quantum calculations using GAUSSIAN (Gaussian Inc; Pittsburg, PA, 1995) and the HF 6-31G* basis set. CHARMM compatible charges for the molecular mechanics calculations were obtained using the CHELPG scheme. Conformational search for the ligands were carried out using the biased Monte-Carlo conformational memories method (Guarnieri *et al.*, 1996). The various conformations were clustered using the XCLUSTER program (MacroModel, Schrödinger, Portland, OR, 1994). Either a representative member of the largest cluster or that conformation which closely resembled the crystallographic conformation of a functionally related ligand was chosen as a candidate for docking studies.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

The initial docking of the ligands was done manually with intermolecular interaction energy evaluations using the DOCKING module of INSIGHTII. Experimentally derived information such as the mutation data for residue D3.32 (Mansour *et al.,* 1992), the orientation of the arginine cage (Ballesteros, 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 the arm A and the arm B of the 1,4-DAPs were determined by the steric constraints imposed by the cavities on either side of the third helix, and were also guided by the number of favorable interactions 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 forcefield (Brooks *et al.,* 1983) and the charmm22 parameter set (Mackerell *et al.,* 1998).

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Substituting amino acids at position 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 [3H]methylspiperone with near *wild type* D4 receptor affinity (Table I). A survey of structurally diverse dopamine receptors 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, while only the D2-selective compounds methylspiperone, aripiprazole, 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. Also, 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 to 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 position 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

(1 carbon) than for the D2-selective compounds (4-5 carbons). Here we thoroughly investigate the structure-affinity relationships (SAR) for an additional ten extremely D4-selective 1,4-DAPs for binding to the D4 receptor and mutants affecting the microdomain between 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- to 86-fold, Table II). These changes in affinity are indicative of 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- to 13-fold, Table III). 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 eleven D4-selective 1,4-DAPs bind the D4-LM3.28-3.29FV mutant with reduced affinity, which makes their pharmacology more D2 receptor-like. The largest change was measured for CP226,269 (~500-fold) and the smallest changes were for L750,667, FAUC113 and RBI257 (19- to 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 amongst the D4-F2.61-senstive 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, while 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 appear 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 piperidine: 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 (3-5 atoms) extending from their protonatable amines, but in each case there are structural constraints (e.g., carbon ring as in CP293,019 or an amide as in PD168,077) imposed on the spacer arm near the protonatable 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 di-orthoelectronegative. In contrast, the common structural feature of the five most D4-L2.60W-sensitive 1,4-DAPs is a long arm A (3-5 –atoms) with an electron donating oxygen at 3-4 carbons from the protonatable amine of the piperidine or piperazine pharmacophore. PNU101,387G has the shortest spacer arm (3 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.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

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 (SCAM) 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 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 (outlined in the computational methods section). 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 a vicinally constrained arm A extending from the protonatable nitrogen of the pharmacophore and much higher affinity towards 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 D4 receptor *wild type* 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 pi-stacking interactions with the phenyl ring at 2.61, while all other ligands belonging to this class interact either in a near parallel stacking orientation or a T-type orientation (Fig. 2c). Notably, 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 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 (4-5 atoms) and higher affinity for D2 than for 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 due to 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 (3-4 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 towards F2.61 (Fig. 2g and Fig. 2h). 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, while

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 eleven 1,4-DAPs that have considerably higher affinity for the D4 than the D2 receptor, and nine of them can be considered to interact with the microdomain comprising residues from TM2 and TM3. Five of these nine compounds have a short arm A (1 carbon) and the other four compounds have a long arm A (3-5) that is vicinally constrained. All of them bind to the D4 receptor in a similar mode (mode-1), with their tethered aromatics on arm B pointing towards 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 the 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 *et al.* (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 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 a displaced pi-pi stacking interactions with F2.61, while the aryl ring in the other seven ligands belonging to this

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

class makes a near pi-pi stack to a tilted T-type interaction. Further, for mode-1 ligands, such as CP293,019 and PD168,077 whose arm A is 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 like leucine is preferred at position 3.28, as 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 D2 a phenylalanine at position 3.28 creates steric clashes with the ligand. 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 I and II. 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 following 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, the 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 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 to the D4 receptor. This would enlarge the TM2/3 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 the 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, rather than their arm B aromatics, pointing towards 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 the arm A is 3-4 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 is also likely to be favored, comes from the crystal structure of spiperone (Koch *et al.*, 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, while the mode-2 ligands are D2-selective. Part of this difference is understandable from the unfavorable effect that the D4-LM3.28-3.29FV mutant has on the binding of the D4-selective mode-3 ligands, which is due to the steric clash of F3.28 with the 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 to mode-2 binding. Notably, however, 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.

Taken 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, which 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 in 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 (Lober *et al.*, 2001, Sanner *et al.*, 1998; Hartmann *et al.*, 1996), FAUC113, NGD 94-1, L750,667, RBI257 are weak partial agonists (Lober *et al.*, 2001; Gazi *et al*., 1998; Gazi *et al.*, 1999), 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. Similarly, there is no apparent correlation within mode-3 binding compounds and their functional properties, as PNU101,387G is an antagonist (Merchant *et al.*, 1996), whereas Ro10-4548 is an agonist (Dr. Claus Riemer, *personal communication*). Amongst mode-2 binding compounds, methylspiperone is an inverse agonist (Wilson *et al.*, 2001), whereas aripiprazole and OPC4392 are (presynaptic) autoreceptor partial agonists and post-synaptic dopamine antagonists (Yasuda *et al.*, 1988; Lawler *et al*., 1999).

The key structural insights provided by the present ligand-receptor SAR 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, as the majority (9 out 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. Notably, mode-1 1,4-DAPs exhibit agonist, weak partial agonist and antagonist functional properties. The critical inference is, therefore, that at least in the case of mode-1 binding D4-selective 1,4-DAPs, the local orientation of the arm B aromatics in the D4-selectivity domain consequently orients their arm A aromatics towards 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-TM6 - as recently suggested by Stewart *et al.*, 2004. That such differential positioning can lead to different functional phenotypes has already been demonstrated for other GPCRs (Ebersole *et al.*, 2003; Visiers *et al.*, 2002). 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).

Acknowledgements.

The authors wish to thank Dr. David R. Sibley for providing the rat dopamine receptor clones and Mr. Miao Huang for his technical assistance in the later stages of this work. The authors wish to thank the following people and their organizations for supplying samples of compounds that were not commercially available: Dr. Claus Riemer (Hoffman La Roche) for Ro10-4548 and Ro61-6270, Donnie Owens (Pfizer) for CP293,019 and CP226,269, Dr. Edwin Steinbrecher (Pharmacia) for PNU101,387G, and Dr. Andrew Thurkauf (Neurogen) for NGD 94-1.

References

Avale ME, Falzone TL, Gelman DM, Low MJ, Grandy DK, Rubinstein M (2004) The dopamine D4 receptor is essential for hyperactivity and impaired behavioral inhibition in a mouse model of attention deficit/hyperactivity disorder. *Mol Psychiatry*. (in press)

Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci* **25**: 366-428.

Ballesteros J, Kitanovic S, Guarnieri F, Davies P, Fromme BJ, Konvicka K, Chi L, Millar RP, Davidson JS, Weinstein H, *et al*. (1998) Functional microdomains in G-protein-coupled receptors: the conserved arginine cage motif in the gonadotropin-releasing hormone receptor. *J Biol Chem* **273**: 10445-10453.

Boeckler F, Russig H, Zhang W, Löber S, Schetz J, Hübner H, Ferger B, Gmeiner P, Feldon J (2004) FAUC 213, a highly selective dopamine D(4) receptor full antagonist, exhibits atypical antipsychotic properties in behavioural and neurochemical models of schizophrenia. *Psychopharmacology*. **175(1)**: 7-17.

Bristow LJ, Collinson N, Cook GP, Curtis N, Freedman SB, Kulagowski JJ, Leeson PD, Patel S, Ragan CI, Ridgill M, Saywell KL, Tricklebank MD (1997) L-745,870, a subtype selective dopamine D4 receptor antagonist, does not exhibit a neuroleptic-like profile in

rodent behavioral tests. *J Pharmacol Exp Ther*. **283**:1256-1263.

Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S and Karplus M (1983) CHARMM: a program for macromolecular energy, miniminzation and dynamics calculations. *J Comput Chem* **4**: 187-217.

Burley S and Petsko G (1985) Aromatic-aromatic interaction: A mechanism of protein structure stabilization. *Science* **229**: 23-28.

Corrigan MH, Gallen CC, Bonura ML, Merchant KM (2004) Sonepiprazole Study Group. Effectiveness of the selective D4 antagonist sonepiprazole in schizophrenia: a placebo-controlled trial. *Biol Psychiatry*. **55**:445-451.

Creese I, Burt DR, and Snyder SH (1976) Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. *Science* **192**:481-483.

Ebersole BJ, Visiers I, Weinstein H, Sealfon SC (2003) Molecular basis of partial agonism: orientation of indoleamine ligands in the binding pocket of the human serotonin 5-HT2A receptor determines relative efficacy. *Mol Pharmacol* **63(1):**36-43.

Gazi L, Bobirnac I, Danzeisen M, Schupbach E, Bruinvels AT, Geisse S, Sommer B, Hoyer D, Tricklebank M, Schoeffter P (1998) The agonist activities of the putative

antipsychotic agents, L745,870 and U-101958 in HEK293 cells expressing the human dopamine D4.4 receptor. *Br J Pharmacol* **124**:889-896.

Gazi L, Sommer B, Nozulak J, Schoeffter P (1999) NGD 94-1 as an agonist at human recombinant dopamine D4.4 receptors expressed in HEK293 cells. *Eur J Pharmacol* **372**:R9-10.

Glase SA, Akunne HC, Georgic LM, Heffner TG, MacKenzie RG, Manley PJ, Pugsley TA, Wise LD (1997) Substituted [(4-phenylpiperazinyl)-methyl]benzamides: selective dopamine D4 agonists. *J Med Chem* **40**:1771-1772.

Guarnieri F, Weinstein H (1996) Conformational Memories and the Exploration of biologically relevant peptide conformations; An illustration for the Gonadotropin-Releasing Harmone. *J Am Chem Soc* **118**: 5580-5589

Hartmann DS, Smeyne R, Zenner MT, Goepfert C, Schlaeger EI, Jenck F, Civelli O, Godel T, Riemer C (1996) R0 61-6270, a new highly selective dopamine D4 receptor antagonist induces c-fos expression in mouse cortex (abstract 202.1). *Soc Neurosci Abstr* **22**:498.

Helmeste DM, Tang SW (2000) Dopamine D4 receptors. *Jpn J Pharmacol*. **82**:1-14.

Ishige K, Chen Q, Sagara Y, Schubert D (2001) The activation of dopamine D4 receptors inhibits oxidative stress-induced nerve cell death. *J Neurosci* **21**:6069-6076.

Javitch JA, Ballesteros JA, Weinstein H, Chen J (1998) A cluster of aromatic residues in the sixth membrane-spanning segment of the dopamine D2 receptor is accessible in the binding-site crevice. *Biochemistry* **37**: 998-1006.

Javitch JA, Ballesteros JA, Chen J, Chiappa V, Simpson MM (1999) Electrostatic and aromatic microdomains within the binding-site crevice of the D2 receptor: contributions of the second membrane-spanning segment. *Biochemistry* **38(25):**7961-7968.

Koch MHJ (1973) The crystal and molecular structure of 8-[3-(*p*-fluorobenzoyl)propyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one, $C_{23}H_{26}N_3O_2F$, *Acta Cryst* **B29**: 379-382.

Kramer MS, Last B, Getson A, Reines SA(1997) The effects of a selective D4 dopamine receptor antagonist (L-745,870) in acutely psychotic inpatients with schizophrenia. D4 Dopamine Antagonist Group. *Arch Gen Psychiatry.* **54**:567-572.

Lawler CP, Prioleau C, Lewis MM, Mak C, Jiang D, Schetz JA, Gonzalez AM, Sibley DR, Mailman RB (1999) Interactions of the novel antipsychotic aripiprazole (OPC-14597) with dopamine and serotonin receptor subtypes. *Neuropyschopharmacology* **20**:617-627.

Lober S, Hubner H, Utz W, Gmeiner P (2001) Rationally based efficacy tuning of selective dopamine D4 receptor ligands leading to the complete antagonist 2-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-a]pyridine (FAUC 213). *J Med Chem* **44**:2691-2694.

Lusher JM, Chandler C, Ball D (2001) Dopamine D4 receptor gene (DRD4) is associated with Novelty Seeking (NS) and substance abuse: the saga continues... *Mol Psychiatry* **6**:497-499.

Mackerell AD Jr, Bashford D, Bellot M, Dunbrack RL, Evanseck JD, Field MJ, Fischer S, Gao J, Guo H, Ha S, *et al*. (1998) All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J Phys Chem B* **102**: 3586-3616.

Mansour A, Meng F, Meador-Woodruff JH, Talyor LP, Civelli O and Akil H (1992) Site-directed mutagensis of the human dopamine D2 receptor. *Euro J Pharm* **227**:205-214.

Merchant KM, Gill GS, Harris DW, Huff RM, Eaton MJ, Lookingland K, Lutzke BS, Mccall RB, Piercey MF, Schreur PJ, Sethy VH, Smith MW, Svensson KA, Tang AH, Vonvoigtlander PF, Tenbrink RE (1996) Pharmacological characterization of U-101387, a dopamine D4 receptor selective antagonist. *J Pharmacol Exp Ther* **279**:1392-1403.

Meyer EA, Castellano RK, Diederich F (2003) Interactions with Aromatic Rings in

Chemical and Biological Recognition *Angew Chem Int Ed Engl*. **42(11):**1210-1250.

Oak JN, Oldenhof J, Van Tol HH (2000) The dopamine D(4) receptor: one decade of research. *Eur J Pharmacol* **405(1-3):**303-327.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289(5480):**739-745.

Sali A, Potterton L, Yuan F, van Vlijmen H, Karplus M (1995) Evaluation of comparative protein modeling by MODELLER. *Proteins Struct Funct Genet* **23**: 318-326.

Sanner MA, Chappie TA, Dunaiskis AR, Fliri AF, Desai KA, Zorn SH, Jackson ER, Johnson CG, Morrone JM, Seymour PA, Majchrzak MJ, Faraci WS, Collins JL, Duignan DB, Prete Di CC, Lee JS, Trozzi A (1998) Synthesis, SAR and pharmacology of CP-293,019: a potent, selective dopamine D4 receptor antagonist. *Bioorg Med Chem Lett* **8**:725-730.

Schetz JA, Benjamin PS and Sibley DR (2000) Nonconserved residues in the second transmembrane spanning domain of the D(4) dopamine receptor are molecular determinants of D(4) selective pharmacology. *Mol. Pharmacol*. **57**:144-152.

Seeman P, Guan HC, Van Tol HH, Niznik HB (1993) Low density of dopamine D4 receptors in Parkinson's, schizophrenia, and control brain striata. *Synapse* **14(4):**247-253.

Seeman P, Guan HC, Nobrega J, Jiwa D, Markstein R, Balk JH, Picetti R, Borrelli E, Van Tol HH (1997a) Dopamine D2-like sites in schizophrenia, but not in Alzheimer's, Huntington's, or control brains, for [3H]benzquinoline. *Synapse* **25(2)** 137-146.

Seeman P, Corbett R, Van Tol HH (1997b) Atypical neuroleptics have low affinity for dopamine D2 receptors or are selective for D4 receptors. *Neuropsychopharmacology* **16(2):**93-110; discussion 111-135.

Simpson MM, Ballesteros JA, Chiappa V, Chen J, Suehiro M, Hartman DS, Godel T, Snyder LA, Sakmar TP, Javitch JA (1999) Dopamine D4/D2 receptor selectivity is determined by a divergent microdomain contained within the second, third, and seventh membrane-spanning segments. *Mol Pharmacol* **56**:1116-1126.

Stewart AO, Cowart MD, Moreland RB, Latshaw SP, Matulenko MA, Bhatia PA, Wang X, Daanen JF, Nelson SL, Terranova MA, Namovic MT, Donnelly-Roberts DL, Miller LN, Nakane M, Sullivan JP, Brioni JD (2004) Dopamine D4 ligands and models of receptor activation: 2-(4-pyridin-2-ylpiperazin-1-ylmethyl)-1H-benzimidazole and related heteroarylmethylarylpiperazines exhibit a substituent effect responsible for additional efficacy tuning. *J Med Chem.* **47**:2348-55.

Van Tol HH, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O (1991) Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature* **350**:610-614.

Visiers I, Ballesteros JA, Weinstein H (2002) Three-dimensional representations of G protein-coupled receptor structures and mechanisms. *Meth Enzymol* **343**:329-371

Wilson J, Lin H, Fu D, Javitch JA, Strange PG (2001) Mechanisms of inverse agonism of antipsychotic drugs at the D(2) dopamine receptor: use of a mutant D(2) dopamine receptor that adopts the activated conformation., *J. Neurochem* **77**:493-504.

Yasuda Y, Kikuchi T, Suzuki S, Tsutsui M, Yamada K, Hiyama T(1988) 7-[3-(4-[2,3-Dimethylphenyl]piperazinyl)propoxy]-2(1H)-quinolinone (OPC-4392), a presynaptic dopamine autoreceptor agonist and postsynaptic D2 receptor antagonist. *Life Sci* **42**:1941-1954.

Zorn SH, Jackson E. Johnson CG, Lewis JM, Fliri A (1997) CP-226,269 is a selective dopamine D4 receptor agonists (abstract 271.11) *Soc. Neurosci Abstr* **23**:685.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This work was supported by in part by grants R01 MH063162-01 (to J.A.S.) and K05 DA00060 (to H.W.)

Reprints requests to:

John A. Schetz, Ph.D., Department of Pharmacology & Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107-2699.

Phone: 817-735-2056, FAX: 817-735-2091, e-mail:jschetz@hsc.unt.edu

Sandhya Kortagere[1], Peter Gmeiner[2], Harel Weinstein[1], John A. Schetz[3,4]

[1] Department of Physiology & Biophysics, Weill Medical College of CORNELL University (WCMC), Box 75, 1300 York Ave., New York, NY 10021.

[2] Department of Medicinal Chemistry, Emil Fischer Center, Friedrich-Alexander University, Schuhstraße 19, D-91052 Erlangen, Germany.

[3] Department of Pharmacology & Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107-2699.

[4] Department of Pharmacology, School of Pharmacy, University of Mississippi, P.O. Box 1848, University, MS 38677-1848.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

"MOL 1321"

Figure Legend

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 text. 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 while 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 fourteen 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, while 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 arm 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.

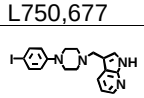
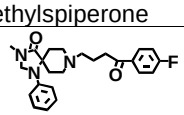
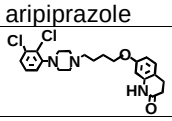
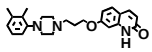
Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

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 labeled according to their generic number, with carbon in magenta, nitrogen in blue, oxygen in red and hydrogen in white and are represented in a stick model. 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) Mutation F3.28 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 interactions 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 has favorable interactions with residues in TM6.

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

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

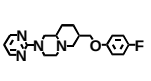
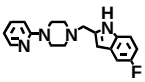
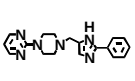
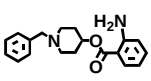
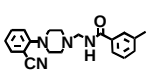
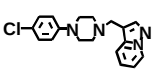
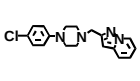
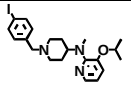
Receptor	Drug binding affinity (Ki, nM) expressed as an average ± standard deviation and fold changes in parenthesis			
	L750,677	methylspiperone	aripiprazole	OPC4392
				
D2-WT	>1500 (>10,000)	0.020±0.004 (0.068)	0.19±0.04 (0.004)	2.6±0.5 (0.042)
D4-WT	0.11±0.02 (1)	0.29±0.030 (1)	47±6.8 (1)	62±12 (1)
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)	ND[a]
D4-LM3.28-3.29 FV	2.2±0.38 (20)	0.515±0.057 (1.8)	ND[a]	ND[a]
D4-F2.61V+ LM3.28-3.29FV	15.9±3.3 (145)	0.196±0.075 (0.67)	ND[a]	ND[a]

[a] ND means Not Determined due to limited availability of the test compound

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

"MOL 1321"

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

Receptor	Drug binding affinity (Ki, nM) expressed as an average ± standard deviation and fold changes in parenthesis							
	CP293,019	CP226,269	NGD 94-1	Ro61-6270	PD168,077	FAUC113	FAUC213	RBI257
								
D2-WT	1270±679 (3838)	50±13 (121)	1376±243 (4551)	438±267 (494)	1608±294 (1072)	148±44 (155)	>1,000 (>1,000)	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)

Molecular Pharmacology Fast Forward. Published on September 24, 2004 as DOI: 10.1124/mol.104.001321
This article has not been copyedited and formatted. The final version may differ from this version.

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

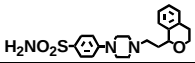
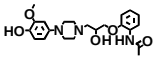
Receptor	Drug binding affinity (Ki, nM) expressed as an average ± standard deviation and fold changes in parenthesis	
	PNU101,387G	Ro10-4548
		
D2-WT	>13,000 (>7500)	>12,000 (>725)
D4-WT	1.8±0.42 (1)	16±3.3 (1)
D4-L2.60W	0.26±0.11 (0.15)	1.3±0.29 (0.080)
D4-F2.61V	5.3±0.57 (3.0)	21±0.78 (1.3)
D4-LM3.28-3.29 FV	67±18 (38)	206±86 (13)
D4-F2.61V+ LM3.28-3.29FV	121±21 (69)	132±66 (8.1)

Figure 1

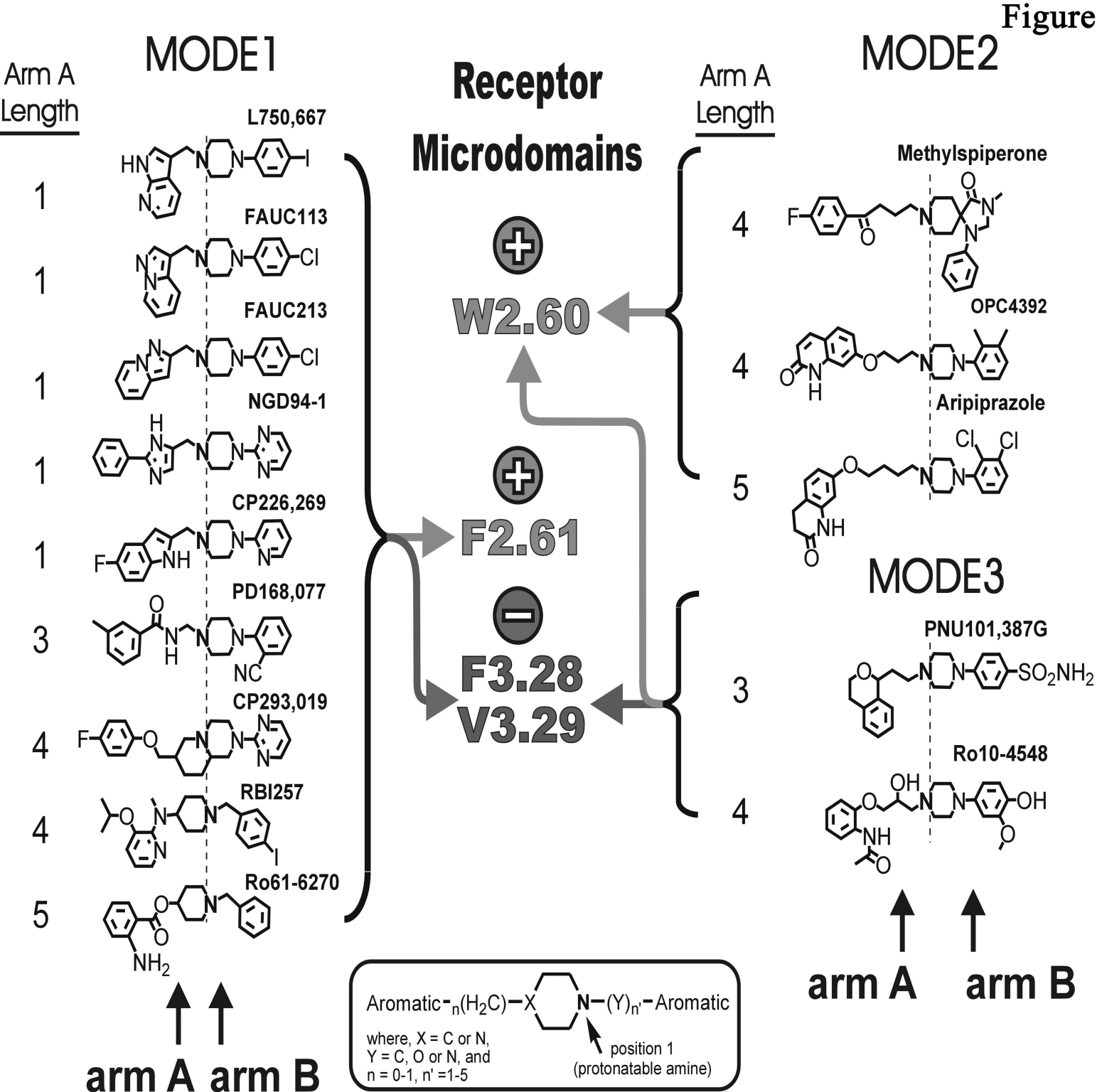
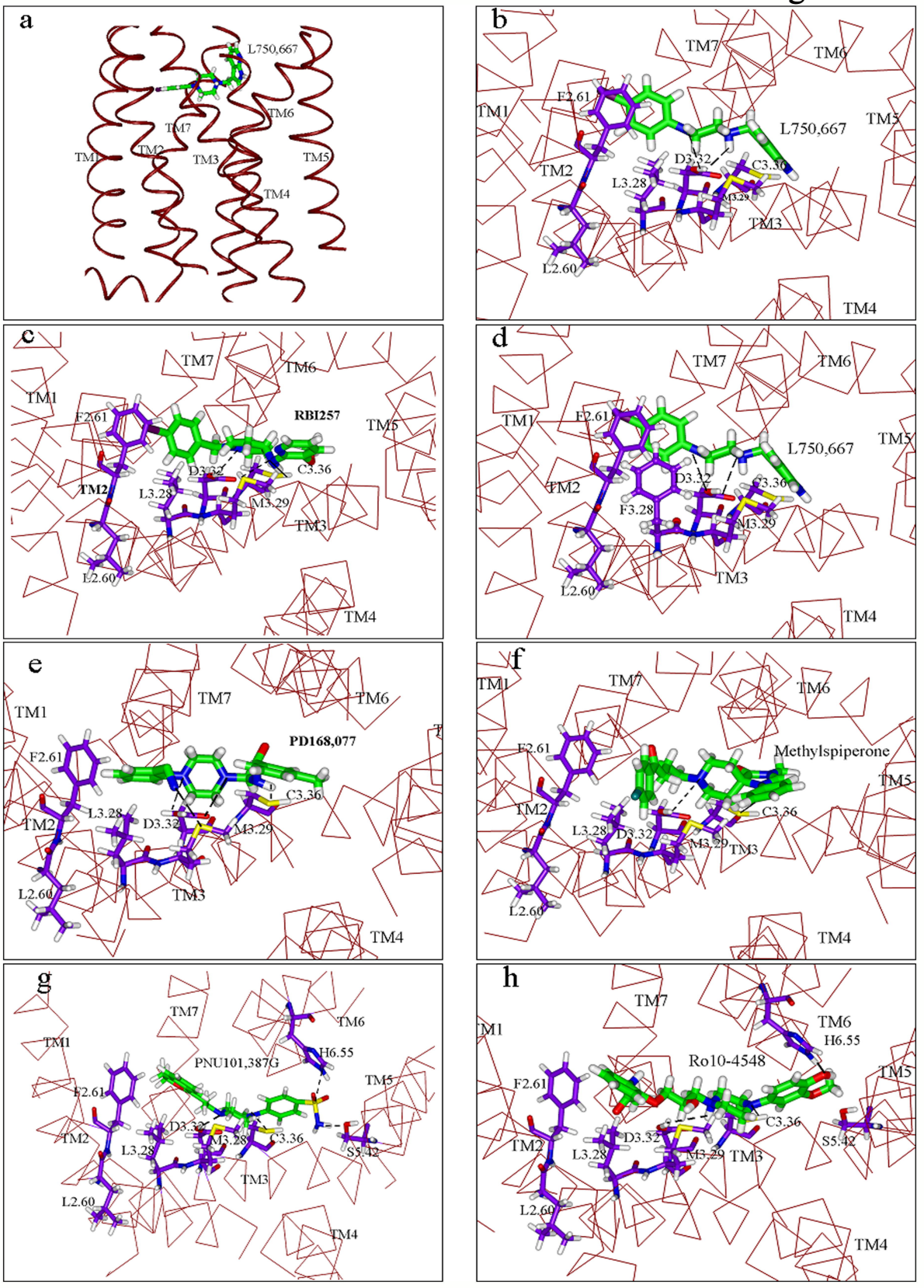


Figure 2


a
L750,667
TM1
TM2
TM3
TM4
TM5
TM6
TM7
b
TM7
TM6
TM1
F2.61
L750,667
TM5
TM2
D3.32
C3.36
L3.28
M3.29
TM3
L2.60
TM4
c
TM7
TM6
TM1
F2.61
RBI257
TM5
D3.32
C3.36
TM2
L3.28
M3.29
TM3
L2.60
TM4
d
TM7
TM6
TM1
F2.61
L750,667
TM5
TM2
D3.32
C3.36
F3.28
M3.29
TM3
L2.60
TM4
e
TM7
TM6
TM1
PD168,077
F2.61
C3.36
L3.28
TM2
D3.32
M3.29
L2.60
TM3
TM4
f
TM1
TM7
TM6
F2.61
Methylspiperone
TM5
TM2
C3.36
L3.28
D3.32
M3.29
TM3
L2.60
TM4
g
TM7
TM6
TM1
PNU101,387G
H6.55
TM5
F2.61
D3.32
TM2
M3.28
C3.36
S5.42
L3.28
TM3
L2.60
TM4
h
TM7
TM6
M1
H6.55
Ro10-4548
TM5
F2.61
C3.36
TM2
D3.32
S5.42
L3.28
M3.29
TM3
L2.60
TM4