Published in final edited form as:

J Neurochem. 2009 July ; 110(1): 45–57. doi:10.1111/j.1471-4159.2009.06103.x.

Three amino acids in the D₂ dopamine receptor regulate selective ligand function and affinity

David F. Cummings*, Spencer S. Ericksen[†], and John A. Schetz*

*Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, Texas, USA

[†]Department of Physiology & Biophysics, Weill Medical College of Cornell University (WCMC), New York, New York, USA

Abstract

The D₂ dopamine receptor is an important therapeutic target for the treatment of psychotic, agitated, and abnormal behavioral states. To better understand the specific interactions of subtype-selective ligands with dopamine receptor subtypes, seven ligands with high selectivity (>120-fold) for the D₄ subtype of dopamine receptor were tested on wild-type and mutant D₂ receptors. Five of the selective ligands were observed to have 21-fold to 293-fold increases in D₂ receptor affinity when three non-conserved amino acids in TM2 and TM3 were mutated to the corresponding D₄ amino acids. The two ligands with the greatest improvement in affinity for the D₂ mutant receptor [i.e., 3-{{4-(4-iodophenyl) piperazin-1-yl}methyl}-1H-pyrrolo[2,3-b]pyridine (L-750,667) and 1-[4-iodobenzyl]-4-[N-(3-isopropoxy-2-pyridinyl)-N-methyl]-aminopiperidine (RBI-257)] were investigated in functional assays. Consistent with their higher affinity for the mutant than for the wild-type receptor, concentrations of L-750,667 or RBI-257 that produced large reductions in the potency of quinpirole's functional response in the mutant did not significantly reduce quinpirole's functional response in the wild-type D₂ receptor. In contrast to RBI-257 which is an antagonist at all receptors, L-750,667 is a partial agonist at the wild-type D₂ but an antagonist at both the mutant D₂ and wild-type D₄ receptors. Our study demonstrates for the first time that the TM2/3 microdomain of the D₂ dopamine receptor not only regulates the selective affinity of ligands, but in selected cases can also regulate their function. Utilizing a new docking technique that incorporates receptor backbone flexibility, the three non-conserved amino acids that encompass the TM2/3 microdomain were found to account in large part for the differences in intermolecular steric contacts between the ligands and receptors. Consistent with the experimental data, this model illustrates the interactions between a variety of subtype-selective ligands and the wild-type D₂, mutant D₂, or wild-type D₄ receptors.

Keywords

attention deficit hyperactivity disorder; dopamine receptor; erectile dysfunction; G-protein-coupled receptor; schizophrenia; structure–activity relationship

Modulation of dopaminergic pathways is a principal aim of numerous pharmacological interventions because dopamine receptors play an integral role in movement, emotional cognition, memory, and attention. Parkinson's disease and schizophrenia are but two examples of severely debilitating disorders mitigated by dopaminergic treatments (Ustun *et al.* 1999) that prolong life and improve its quality. Still there is much to be learned concerning the molecular basis of selective ligand actions on dopamine receptors and analogous heterotrimeric G-protein-coupled receptor systems.

Dopamine receptors are biogenic amine heterotrimeric G protein-coupled receptors sharing structural homology with rhodopsin. Five genes coding for five subtypes of the dopamine receptor (D₁–D₅) are known to exist in mammals. With the exception of the D₂ receptor partial agonist, aripiprazole, the most commonly prescribed neuroleptics are thought to attenuate psychosis by antagonizing the D₂ receptor subtype (Lawler *et al.* 1999; Shapiro *et al.* 2003; Schetz and Sibley 2007). Although the D₄ receptor was previously considered a drug target for the treatment of psychosis (Sanyal and Van Tol 1997), the failure of three clinical trials suggests that this is not the case (Kramer *et al.* 1997; Truffinet *et al.* 1999; Corrigan *et al.* 2004). However, the D₄ receptor has remained a potentially important clinical target because of its roles in regulating hyperactivity and CNS-mediated penile tumescence (Schetz and Sibley 2007; Schetz 2009).

Previous work on the D₄ receptor revealed that the non-conserved amino acids at positions 2.60, 2.61, 3.28, and 3.29 provided key structural determinants for drug selectivity between D₂ and D₄ receptor subtypes (Schetz *et al.* 2000). This conclusion was reached by analyzing reciprocal mutations, i.e., mutations where one or more amino acids in the D₄ sequence were substituted with the corresponding amino acid of the D₂ sequence. A more comprehensive study focusing on a large number of D₄-selective ligands from the 1,4-disubstituted aromatic piperazine/piperidine (1,4-DAP) structural class revealed that a majority of 1,4-DAPs have a pattern of molecular recognition (mode-1) dominated by amino acids occupying positions 2.61, 3.28, and 3.29 (Kortagere *et al.* 2004). Related studies in a D₂ receptor background have corroborated the importance of amino acids at position 2.61, 3.28, and 3.29 in the D₂ receptor as binding determinants for several D₄-selective ligands (Simpson *et al.* 1999); however, the functional properties of D₂ mutant receptors with improved affinity for specific D₄-selective drugs have never been investigated.

We report here for the first time the functional properties of a combined TM2/TM3 reciprocal mutant D₂ receptor (D₂-V2.61F + FV3.28-3.29LM) and demonstrate that it not only has increased affinity and improved functional sensitivity for D₄-selective ligands, but that such ligands [L-750,667 and 1-[4-iodobenzyl]-4-[N-(3-isopropoxy-2-pyridinyl)-N-methyl]-aminopiperidine (RBI-257)] exert the same functional effects on the D₂ mutant as they do on the wild-type D₄ receptor. These findings suggest that a D₂ receptor microdomain which controls a ligand's selectivity can also control a ligand's function.

Materials and methods

Reagents

Cell culture media was purchased from Hyclone (Logan, UT, USA). [^3H]Methylspiperone, 8-[4-(4-fluorophenyl)-4-oxobutyl]-(3-methyl-1-phenyl)-1,3,8-triazaspiro[4,5]decan-4-one hydrochloride ([^3H] MSP; NET-856, 70–80 Ci/mmol) was purchased from Perkin Elmer (St Louis, MO, USA). Other drugs were purchased from Sigma (St Louis, MO, USA) or Tocris (Ellisville, MO, USA).

Site-directed mutagenesis and transfections

Mutagenesis was accomplished using a QwikChangeTM kit (Stratagene, CA, USA) and confirmed by full-length sequencing. Mutant receptors were named by slight modification of the receptor nomenclature system as described previously (Ballesteros and Weinstein 1995; Ericksen *et al.* 2009): each amino acid in a transmembrane segment (TMS) is described by coordinates based on its position relative to the most conserved amino acid of that TMS (position 50). For example, D₂-V2.61F denotes a valine to phenylalanine mutation at position 61 of TMS 2 in the D₂ receptor. Plasmid DNA was transfected into cells by calcium phosphate precipitation as described previously (Ericksen *et al.* 2009). Stable receptor expression was maintained under 100 $\mu\text{g}/\text{mL}$ G418 drug selection pressure.

Membrane preparation and radioligand binding assays

Cell membranes were prepared as described previously (Ericksen *et al.* 2009) by suspension in lysis buffer (5 mM Tris, 5 mM MgCl_2 , pH 7.4, 4°C) for 5–10 min followed by homogenization with eight strokes of a Dounce homogenizer and centrifugation at 28 000 g for 45 min. The resulting pellet was resuspended in cold binding buffer and recentrifuged. The final membrane pellet was resuspended in cold binding buffer, homogenized with four strokes, and stored on ice until use. Radioligand binding buffer consisted of 50 mM Tris adjusted to pH 7.4 at 25°C with 1 N KOH or 1 N HCl (Ericksen *et al.* 2009). For saturation isotherms, cell membranes were equilibrated (90 min) with increasing concentrations of [^3H]MSP in the presence or absence of 5 μM (+)-butaclamol, a drug used to define non-specific binding. For competition binding experiments, a fixed concentration of 0.5 nM [^3H]MSP was equilibrated (90 min) with increasing concentrations of non-radiolabeled competing ligand. Equilibrated samples were rapidly filtered through Whatman GF/C filters (Brandel, Gaithersburg, MD, USA) pre-treated with 0.3% poly(ethyleneimine) (10 min) and washed three times with 3 mL of ice-cold binding buffer (50 mM Tris, pH 7.4, 0°C). Radioactivity was quantified in a scintillation counter. Membrane protein concentration was determined by bicinchoninic acid assay (Pierce, IL, USA).

cAMP functional assays

Intracellular cAMP concentrations were determined using a cAMP AlphascreenTM detection kit (Perkin Elmer, Waltham, MA, USA) as described previously (Ericksen *et al.* 2009). Briefly, human embryonic kidney (HEK293) cells were seeded at 50 000 cells/well in a poly-L-lysine-coated 96-well microtiter plate. Stimulation buffer (Dulbecco's modified Eagle's medium, 20 mM HEPES, 100 μM sodium metabisulfite, 30 μM Ro 20-1724), cell

lysis buffer (0.3% Tween 20, 20 mM HEPES, 1 $\mu\text{g}/\mu\text{L}$ bovine serum albumin), and bead buffer (20 mM HEPES, 30 μM Ro 20-1724, 1 $\mu\text{g}/\mu\text{L}$ bovine serum albumin, 1 $\times$ Hank's Balanced Salt Solution) were prepared the next morning and adjusted to pH 7.4 with 1 N cell culture tested NaOH (Sigma-Aldrich, St Louis, MO, USA). Drug dilutions prepared in stimulation buffer containing 6 μM forskolin were equilibrated (37°C, 5% CO_2) for 30 min. Cells were challenged with the equilibrated dilutions for 20 min (37°C, 5% CO_2) followed by 5-min centrifugation at 1500 g . Cells were lysed by 90 min of shaking in 100 μL of cell lysis buffer. Prior to quantification, half units of acceptor and donor beads pre-incubated with biotinylated cAMP (3.76 nM) were equilibrated (1 h) with 30 μL of each sample in an opaque 96-well Costar plate (Corning, NY, USA) protected from light. Samples were read on a Perkin-Elmer Alpha Fusion™ plate reader in alpha mode using a 2-sec window (680 nm excitation at 0.6 s/sample and 520–620 emission 1.4 s/sample).

Calculations and data analysis

All data points for each experiment were sampled in triplicate and each experiment was repeated three times, unless noted otherwise. All data were analyzed using Prism version 4.0 (Graphpad Software Inc., San Diego, CA, USA). Geometric means and standard deviations are reported in the tables and SEM are plotted in the graphs. For saturation isotherm analysis, specifically bound [^3H]MSP was calculated by subtracting non-specifically bound [^3H]MSP (defined as binding in the presence of 5 μM (+)-butaclamol) from the total bound [^3H]MSP at each concentration of [^3H]MSP. The equilibrium dissociation constant (K_D) for [^3H]MSP and the receptor density (B_{max}) were determined by best-fitting the specific binding data versus the concentration for free radioligand to a single-site square hyperbola curve. Inhibition constants (K_i) for unlabeled ligands were calculated using the Cheng-Prusoff equation: $K_i = \text{IC}_{50}/(1 + [\text{radioligand}]/K_D)$. For cases where drug solubility was limited, IC_{50} values were generated by extrapolating concentration–response curves to zero. All cAMP values were converted to the amount of cAMP generated per milligram of sample protein (cAMP/mg) then normalized relative to the amount of cAMP generated by 6 μM forskolin in the absence of agonist. Efficacy was determined by subtracting the best-fit values for the lowest horizontal asymptote from the highest horizontal asymptote of the fitted sigmoidal semi-log dose–response curves. Statistical analyses of the curve-fitting procedures included the run test, F -test, and Pearson's correlation coefficient. Values were analyzed by one-way ANOVA and Dunnett's *post hoc* analysis with significance established at the 95% confidence level ($p < 0.05$).

Construction of receptor homology models

D₂ and D₄ receptor structures were constructed with Modeller 9v1 (San Francisco, CA, USA) (Šali and Blundell 1993) using both the bovine rhodopsin (Li *et al.* 2004) (1GZM) and β_2 adrenergic (Cherezov *et al.* 2007) (2RH1) structures as templates. For each dopamine receptor model, 1000 structures were generated and ranked using Modeller's objective function. The models were then aligned and clustered using the GRONingen Machine for Chemical Simulations (GROMACS, v3.3; Uppsala, Sweden) (Van Der Spoel *et al.* 2005) *gcluster* utility (cutoff = 0.20 Å). The most representative (central) structure from the first (wild-type D₂ receptor) and second (wild-type D₄ receptor) most populated clusters were used for minimization, normal mode analysis (NMA), and docking. These structures were

minimized *in vacuo* using the L-BGFS (low-memory Broyden–Fletcher–Goldfarb–Shanno, quasi-Newtonian algorithm for energy minimization) method in two stages, each applied to convergence ($F_{\max} < 10$ kJ/mol/nm) using GROMACS v3.3 (Van Der Spoel *et al.* 2005) with the molecular systems parameterized according to the Optimized Potentials for Liquid Simulations (OPLS) all atom forcefield (Jorgensen *et al.* 1996). Electrostatic interactions were treated by the particle-mesh Ewald method. In the first stage all backbone protein atoms were restrained with half-harmonic force restraints ($k = 1000$ kJ/mol/nm). In the second minimization stage, only C_{α} atoms were restrained.

Building ensembles of receptor conformers

To obtain receptor conformers based on perturbations along low-frequency normal modes, each minimized receptor model was reduced to a C_{α} -only elastic network model and submitted to NMA deformation and refinement web server for NMA and automated decoy generation using default parameters with the exception of the distance weighting parameter. For the distance weighting parameter a non-default value of 3.0 Å was applied as recommended for C_{α} -only models and the approximate average C_{α} root mean square deviation (Å) of the decoy value was set to 1 Å to focus on smaller backbone motions near the initial conformation. (<http://lorentz.immstr.pasteur.fr/decoys/submission.php>) (Lindahl *et al.* 2006). Based on the motion of the first five, non-trivial (non-zero eigenvalue), normal modes, 1000 decoy structures were generated from random linear combinations of movements along the five mode eigenvectors. These five modes embody TMS movements that appeared to influence binding crevice geometry. Vibrational modes beyond the lowest five frequencies were excluded because they involved motion in the non-binding cleft regions or relatively low amplitude motions in the TM core region. For the resulting 1000 decoys generated for each receptor we rebuilt all-atom structures on the C_{α} frames. Backbone and side chain atoms were built onto the fixed C_{α} template with Modeller 9v1, followed by minimization, a short 15 ps MD run, and minimization with C_{α} atoms fixed for each procedure. The structure was then minimized to convergence with positional restraints on the C_{α} carbons in GROMACS, as described above. For use in docking, the 1000 rebuilt and minimized decoys for each receptor were clustered, as described above, into a more manageable subset of 24–25 representative conformers.

Docking ligands into receptor conformers

The ligands were constructed in Discovery Studio (Accelrys, Inc., San Diego, CA, USA) and geometry optimized in Gaussian03 with *ab initio* quantum mechanical calculations using the Hartree-Fock 6-31G** basis set (Frisch *et al.* 2004). The partial charges were set according to the automated Gasteiger partial charge assignment of AutoDockTools 1.4.5 (The Scripps Research Institute, La Jolla, CA, USA). Each ligand was docked 25 times to each receptor conformation (24–25 conformers) using the default Lamarckian genetic algorithm search routine in AutoDock 4.0 (Morris *et al.* 1998) with non-default parameters for the genetic algorithm population size (increased to 500) and maximum number of energy evaluations (ramped up to 2.5×10^6). Selective side chain flexibility was allowed within the docking routine: the rotation of dihedrals in the side chains of 2.61, 3.28, 3.29, 3.32, and 6.51 were explored because of their apparently critical positions in the ligand binding site and influences on cleft shape. After docking with each receptor conformation, poses were

clustered using a Root Mean Square Deviation (RMSD) tolerance of 3.0 Å and then ranked by mean energy. Poses with reinforced ionic interaction between the ligand's protonatable amine and the conserved aspartate at position 3.32 (D3.32) were visually screened for mode-1 (Kortagere *et al.* 2004) orientations in the binding cleft (see Results for a description). Complexes with poses meeting these criteria were included in the subset used to obtain the contact-likelihood matrices (CLMs) from the intermolecular contacts extracted with Ligplot v4.4.2 (London, UK) (Wallace *et al.* 1995). PyMOL v0.99 (DeLano Scientific LLC, San Carlos, CA, USA) was used for rendering figures.

Results

To characterize the subtype-selective binding domain in a D₂ receptor background, we tested an extensive panel of D₄-selective 1,4-DAPs that were previously examined in the D₄ receptor background (Kortagere *et al.* 2004). When mutant D₂-FV3.28-3.29LM and D₂-V2.61F + FV3.28-3.29LM receptors were transiently expressed in COS-7 cells, the levels of expression and the affinities for the moderately D₂-selective 1,4,-DAP [³H]MSP were comparable with the wild-type D₂ receptor (1.1-fold to 1.3-fold and 1.7-fold to 2.3-fold, respectively, Floresca *et al.* 2005). The affinities of the mutant D₂ receptors for the agonist (–)-quinpirole, which lacks a 1,4-DAP structural motif, were similar to those of the wild-type D₂ receptor (1.2-fold to 3.8-fold, Fig. 1 and Table 1). The small to moderately improved affinities (4.8-fold to 22-fold) observed for the mutant D₂-FV3.28-3.29LM receptor for five of the 1,4-DAPs [3-{[4-(4-chlorophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine] (L-745,870, also known as CPPMA, which stands for chlorophenylpiperazinyl methylazaindole), L-750,667, 2-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-a]pyridine (FAUC213), 2-amino-benzoic acid 1-benzyl-piperidin-4-yl ester (Ro 61-6270), RBI-257] improved further (21-fold to 292-fold) for the mutant D₂-V2.61F + FV3.28-3.29LM receptor (Table 1). The two remaining 1,4-DAPs 2-phenyl-4(5)-[4-(2-pyrimidinyl)-piperazin-1-yl]-methyl-imidazole (NGD 94-1) and *N*-[[4-(2-cyanophenyl)-1-piperazinyl] methyl]-3-methylbenzamide (PD168,077)] had higher affinities for the mutant D₂-FV3.28-3.29LM receptor (13-fold and 34-fold, respectively), than for the mutant D₂-V2.61F + FV3.28-3.29LM receptor (4.8-fold to 6.4-fold, respectively) whose affinities were closer to the wild-type D₂ receptor (Table 1). RBI-257 and L-750,667, the compounds with the largest improvements in affinity for the D₂-V2.61F + FV3.28-3.29LM receptor relative to the wild-type D₂, were selected for further characterization in functional assays.

Although identification of binding site residues that could impart 1,4-DAP recognition and D₂/D₄ preference was important, we were more interested in characterizing changes in subtype-specific function related to the subtype-selective binding domain of the D₂ receptor. COS-7 cells, while appropriate for binding experiments, were unable to inhibit cAMP formation through activation of D₂-like dopamine receptors (data not shown). Therefore the HEK293 line, which lacks endogenous dopamine receptors but contains G proteins capable of mediating the cAMP responses of transfected D₂ or D₄ dopamine receptors (Gazi *et al.* 1998; Chemel *et al.* 2006) was selected for functional assessments. Wild-type D₂ and mutant D₂-V2.61F + FV3.28-3.29LM receptors were stably expressed in HEK293 cells and characterized by receptor binding studies. The cell surface density of dopamine receptors

expressed in several stable HEK293 clones was determined by [³H]MSP saturation isotherm analysis. The corresponding [³H]MSP affinity values (K_D) were 0.78 ± 0.39 nM for the wild-type D₂ receptor and 1.6 ± 0.76 nM for the D₂-V2.61F + FV3.28-3.29LM receptor. Note that while absolute affinity values for [³H]MSP were quite different for wild-type D₂ and mutant D₂-V2.61F + FV3.28-3.29LM receptors expressed in COS-7 and HEK293 cells, the relative difference between wild-type and mutant D₂ receptors within a cell type were essentially the same (2.3-fold vs. 2.1-fold, respectively). To limit any ambiguity caused by unequal receptor expression, clones expressing wild-type or mutant receptors were matched by receptor density. HEK293 cell lines expressing 4.6 ± 1.6 pmole/mg protein of wild-type D₂-WT receptor or 4.6 ± 2.5 pmole/mg protein of mutant D₂-V2.61F + FV3.28-3.29LM receptor were analyzed for their ability to inhibit cAMP formation.

Because RBI-257 had strikingly different affinity for the wild-type and mutant D₂ receptors, preliminary tests to determine the most appropriate concentration range for investigating the antagonism of (–)-quinpirole function at the wild-type D₂ and mutant D₂-V2.61F + FV3.28-3.29LM receptors were performed (data not shown). Based on these results, the following concentrations of RBI-257 were selected: 0.2 and 2 μ M for the D₂-V2.61F + FV3.28-3.29LM receptor and 2 and 20 μ M for the D₂-WT receptor. Similar tests performed for L-750,667 resulted in the selection of 0.5 μ M L-750,667 as an appropriate single concentration for comparing functional antagonism of (–)-quinpirole at the wild-type D₂ and mutant D₂-V2.61F + FV3.28-3.29LM receptors. Dose–response curves demonstrated that the mutant receptor had 4.3-fold lower potency and 18% less efficacy for the agonist (–)-quinpirole than did the wild-type D₂ receptor (Table 2). Further, the right shift in (–)-quinpirole potency induced by RBI-257 occurred at a much lower RBI-257 concentration in the mutant D₂-V2.61F + FV3.28-3.29LM than in the wild-type D₂ receptor: a >5000-fold potency shift was observed in the presence of 2 μ M RBI-257 for the mutant D₂-V2.61F + FV3.28-3.29LM receptor with no significant shift for the wild-type D₂ receptor (Fig. 2a and b and Table 2). However, a 10-fold higher concentration of RBI-257 was able to significantly shift the potency (120-fold) of (–)-quinpirole for the wild-type receptor (Table 2 and Fig. 2a). A similar pattern of shifted (–)-quinpirole potency was observed for L-750,667 (Table 2 and Fig. 2d). Neither RBI-257 nor L-750,667 had statistically significant effects on the efficacy of (–)-quinpirole (–20% effect, Table 2 and Fig. 2). No attempt was made to quantify the functional antagonism of these ligands as pK_b values (Schild analysis) because D₄ selective ligands and (–)-quinpirole do not compete for the same binding site (Table 1) which is an assumption that must be met to yield meaningful values (Kenakin 1997).

Assays of cAMP accumulation designed to differentiate the functional properties of D₄-selective 1,4-DAPs revealed that RBI-257 was an antagonist at the wild-type D₂, wild-type D₄, and the mutant D₂-V2.61F + FV3.28-3.29LM receptors (Fig. 3b and d). In contrast, L-750,667, which acted as an antagonist at the wild-type D₄ and mutant D₂-V2.61F + FV3.28-3.29LM receptors, exhibited partial agonism at the wild-type D₂ receptor (Fig. 3b–d). Neither (–)-quinpirole nor L-750,667 was able to reduce forskolin-stimulated cAMP accumulation in untransfected HEK293 cells (Fig. 3a), i.e., those lacking dopamine receptors. Further confirmation of the partial agonist properties of L-750,667 at the wild-type D₂, but not the mutant D₂-V2.61F + FV3.28-3.29LM receptor, was established by

evaluating the effect of increasing concentrations of L-750,667 at a fixed 60 nM concentration of (–)-quinpirole (Fig. 4). At higher doses in the mutant D₂-V2.61F + FV3.28-3.29LM receptor, L-750,667 completely reversed the (–)-quinpirole-stimulated functional response confirming functional antagonism of the mutant. In contrast, the highest concentrations of L-750,667 were only able to partly decrease (–)-quinpirole-stimulated wild-type D₂ receptor inhibition of cAMP formation. This reduced maximal functional response confirmed that L-750,667 was a partial agonist of the wild-type D₂ receptor. The EC₅₀ for antagonism of the (–)-quinpirole functional response by L-750,667 was 3.2-fold higher for the mutant than the wild-type receptor (0.57 ± 0.27 and 1.9 ± 2.3 μ M respectively, Fig. 4).

To examine the role that residues conferring D₄/D₂ selectivity have on the positioning of 1,4-DAPs in the binding cleft, several 1,4-DAPs (L-750,667, RBI-257, NGD 94-1, and PD168,077) were docked into wild-type D₂, mutant D₂-V2.61F + FV3.28-3.29LM, and wild-type D₄ homology models. The resulting complexes (600–625) were screened using two geometric criteria: (i) an H-bond reinforced ionic interface formed by the protonatable amine group of the ligand interacting with the conserved aspartate at position 3.32 of biogenic amine receptors (for review, see Floresca and Schetz 2004) and (ii) pose orientations where the short arm of the 1,4-DAP (Arm-B, Kortagere *et al.* 2004) was directed, in accordance with prior experimental evidence from our lab, at the region of TMS 2 and 3 rather than TMS 5 and 6 (Kortagere *et al.* 2004). These criteria focused our investigations to the ligand poses that were most suitable for residue contact analysis.

When ligand poses that met our criteria were examined, consistent patterns of intermolecular contacts emerged that reconciled some of the observed selectivity features attributed to positions 2.61, 3.28, and 3.29. The poses observed for L-750,667 in both the mutant D₂-V2.61F + FV3.28-3.29LM and wild-type D₄ receptors directed the *p*-iodophenyl ring of Arm-B toward F2.61, a resident of the cleft formed by TMS 2, 3, and 7 (Fig. 5a top and middle panels). A similar orientation was observed for L-745,870, the *p*-chloro analog of L-750,667 in the D₂-V2.61F receptor (Ericksen *et al.* 2009). However, despite similarities between the ligand orientation and contact residues in wild-type D₄ and mutant D₂-V2.61F + FV3.28-3.29LM receptors in the wild-type D₂ receptor, L-750,667 was rotated counter-clockwise in the binding pocket (from an extracellular perspective) which allowed the *p*-iodophenyl ring to make significant contact with the phenylalanine of 3.28 within the TM2/3 cleft (Fig. 5a). These changes in the preferred binding configuration for L-750,667 and RBI-257 (5B, right panel; wire model not shown) suggest that positions 2.61, 3.28, and 3.29 provide recognition features integral to positioning 1,4-DAPs with *p*-halogenated Arm-B moieties in the binding pocket.

To quantitatively compare the observed intermolecular contact distributions for 1,4-DAPs docked to the receptor models, tendencies within a ligand–receptor pairing for ligand–residue interactions were plotted as CLMs (Fig. 5b). Each CLM was constructed from the sum of specific ligand–residue contacts (< 3.9 Å) normalized against the total amount of residue contacts (< 3.9 Å) achieved within the entire subset of ligand–receptor poses that passed the screening criteria (see details above). For L-750,667 and RBI-257, the distribution of CLM index values was better correlated between the wild-type D₄ and mutant

D₂-V2.61F + FV3.28-3.29LM receptors (correlation = 0.867), than the wild-type D₂ and mutant D₂-V2.61F + FV3.28-3.29LM receptors (correlation = 0.723). This showed quantitatively that the distribution of contacts achieved for poses of L-750,667 in the mutant D₂-V2.61F + FV3.28-3.29LM receptor were more like the poses observed at wild-type D₄ receptor and less like the poses observed at the wild-type D₂ receptor. A similar trend (0.863 vs. 0.806) in the CLM was observed for RBI-257. While this trend was consistent for *p*-halogenated 1,4-DAPs (L-750,667 and RBI-257) the results for *o*-electronegative 1,4-DAPs (NGD 94-1 and PD168,077) exhibited complex patterning of the CLM in which mutant D₂-V2.61F + FV3.28-3.29LM receptor poses were dissimilar to poses in both wild-type D₂ and wild-type D₄ receptors. These findings for NGD 94-1 and PD168,077 suggested that 1,4-DAPs with an ortho-electronegative Arm-B group did not adopt D₄-like binding modes in the mutant D₂-V2.61F + FV3.28-3.29LM receptor and that mutations of the TM2/3 microdomain were not sufficient for conferring ortho-electronegative 1,4-DAP selectivity to the mutant receptor. Our observation was supported by the revertant affinities of NGD 94-1 and PD168,077 for the mutant D₂-V2.61F + FV3.28-3.29LM receptor, meaning that this mutant's affinity profile looked more like the wild-type D₂ receptor than did the mutant D₂-FV3.28-3.29LM (Table 1).

Despite specific differences in ligand interaction between receptor subtypes, Fig. 5b revealed a general qualitative similarity within the CLMs. For 1,4-DAPs, the contact residues with the strongest ligand-residue interactions were typically the following positions (in order of general prominence in contact index value): V/F2.61, V/M3.29, F6.51, D3.32, F/L3.28, V3.33, T7.39, V5.39, L4.61, Y/V7.35, H6.55, F/Y5.38, S7.36, S5.42, Y7.43, C3.36, W7.40, V2.57, C3.25, and L/S2.64. Interestingly, 2.61, 3.28, and 3.29 represented three of the top five contact sites in terms of CLM index values and comprised three of the six contact positions that varied in residue identity between the wild-type D₂ and wild-type D₄ receptors.

Discussion

The three amino acid TM2/3 microdomain that forms the extracellular portion of the binding site crevice has been suggested, on the basis of radioligand binding studies, to be a site of interaction for ligands with high selectivity for the D₄ subtype of dopamine receptor (Simpson *et al.* 1999; Schetz *et al.* 2000; Kortagere *et al.* 2004). Recognition that the receptor background (i.e., D₂ vs. D₄), ligand structures, and binding reaction conditions (Floresca *et al.* 2005; Ericksen *et al.* 2009) greatly influenced the experimental outcome of previous TM2/3 microdomain studies (Simpson *et al.* 1999; Schetz *et al.* 2000; Kortagere *et al.* 2004) lead us to ask whether the difference in the observed binding affinities for D₄ selective ligands were the result of localized changes in the TM2/3 microdomain or global changes in the receptor complex. It was reasoned that rigorous assessment of the overall state of the mutant receptor should include a measure of its functional properties relative to the wild-type receptor, as has only been carried out here. Assessment of the functional properties of a combined TM2/3 reciprocal D₂ mutant receptor (D₂-V2.61F + FV3.28-3.29LM) revealed improved sensitivity of the mutant receptor for the D₄-selective ligands L-750,667 and RBI-257. Further, these ligands produced the same functional outcome at both the D₂ mutant and wild-type D₄ receptors. In contrast, non-selective

ligands, such as MSP and (–)-quinpirole, were insensitive to these TM2/3 microdomain alterations. These findings demonstrated that the three amino acids exerted localized effects while regulating the affinity and function of D₄-selective ligands. A molecular docking technique that permits flexibility in the receptor backbone was used to enhance the accommodation of 1,4-DAPs into representations of the receptor structure. Consistent with the empirical results, the docking studies suggested that the three amino acid TM2/3 microdomain accounts not only for a large portion of the differences in receptor phenotypes, but also for a large portion of the differences in steric contacts between D₄-selective ligands, L-750,667 and RBI-257, and wild-type or mutant dopamine receptors.

Similar to D₂ and D₄ dopamine receptors, positions 2.61, 3.28, and 3.29 are implicated in various biogenic amine receptor–ligand interaction domains. For example, in muscarinic receptors positions 2.61, 3.28, and 3.29 are significant mediators of muscarinic ligand selectivity and function (Drubbisch *et al.* 1992; Matsui *et al.* 1995; Lu and Hulme 1999). However, the pattern of recognition between ligands with selectivity for D₁ versus D₂ receptor subtypes appears to be more complex. In the macaque D₁ dopamine receptor, reciprocal mutations at position 3.28 with residues of the rat D₂L receptor revealed moderate to large increases in affinity for the D₂-like selective ligands spiperone, domperidone, and YM-09151-02 (224-fold, 45-fold, and 24-fold, respectively), but less than 4-fold changes in affinity for D₂-like selective ligands haloperidol, piquindone, raclopride, sulpiride, and tropapride, as well as the D₁-like selective ligand SCH23390 (Lan *et al.* 2006). However, the reciprocal mutant D₂ receptor, D₂-F3.28W, did not replicate the substantial changes in affinity observed for the mutant D₁ receptor, i.e., less than a 2-fold change for any of the listed ligands.

Positions 2.61, 3.28, and 3.29 play an important role in rhodopsin folding and conformational stability. For instance, a T2.61I mutation causes congenital night blindness (al-Jandal *et al.* 1999), while a G3.29D mutation results in retinitis pigmentosa (Millan *et al.* 1995). The increased reactivity of both the T2.61I and E3.28A mutants to hydroxylamine in the dark state combined with decreased meta II state decays suggests that these mutant receptors exhibit pathological instability of the rhodopsin resting-state (Sakmar *et al.* 1991; Han *et al.* 1996; Ramon *et al.* 2003). The poorly expressed mutants E3.28V, E3.28I, E3.28M, E3.28W, and T2.61K did not reconstitute to form pigments in the presence of 11-*cis*-retinal (Han *et al.* 1996; Ramon *et al.* 2003). These findings demonstrated that positions 2.61, 3.28, and 3.29 were located in conformationally sensitive regions of rhodopsin. In the case of 2.61 and 3.28, the proximity of the retinal Schiff base allows for potential proton exchange with an appropriate amino acid residue. This releases 11-*cis*-retinal from the binding pocket in a manner analogous to the dissociation of a ligand from a biogenic amine receptor. As a whole, these experiments demonstrate the importance of these three positions for understanding ligand interactions with certain biogenic amine receptors.

While a great deal had been revealed in previous studies concerning the discriminant structural features of 1,4-DAP ligands and the receptor residues governing the strength of ligand interactions with specific D₂ or D₄ receptor microdomains, virtually nothing was known in this context about receptor activation states (Floresca and Schetz 2004). Thus, we measured the functional properties of a combined TM2/3 reciprocal mutation D₂ receptor

and report here for the first time that the D₂-V2.61F + FV3.28-3.29LM mutant not only has increased affinity for the D₄-selective ligands L-750,667 and RBI-257, but also requires a much lower concentration of these antagonists to produce a rightward shift of the agonist functional response. We observed that both of these ligands exert the same functional effects on the D₂-V2.61F + FV3.28-3.29LM mutant as they do on the wild-type D₄ receptor. In the case of L-750,667, this amounts to conversion of the D₂-V2.61F + FV3.28-3.29LM mutant from a D₂ receptor background that was activated by this ligand to one like the wild-type D₄ receptor that was not. The switch in the functional effect of L-750,667 for the D₂-V2.61F + FV3.28-3.29LM mutant was not because of a concentration effect, because L-750,667 had higher affinity and potency for this mutant than it did for the wild-type D₂ receptor. Further, the switch in functional effect for the mutant persisted at dose ranges well beyond that needed to produce an observable reversal of (–)-quinpirole's functional effect for the mutant or the wild-type D₂ receptor. In contrast to L-750,667, the functional profile of RBI-257 was not changed in the mutant receptor; however, since RBI-257 is an antagonist at both the D₂ and D₄ receptor subtypes, no change in functional properties was expected for the mutant receptor.

To gain a molecular perspective on the interactions of 1,4-DAPs with the dopamine receptor subtypes, we docked several 1,4-DAPs into our initial wild-type D₄, wild-type D₂, and mutant D₂-V2.61F + FV3.28-3.29LM receptor homology models. However, we initially failed to observe any 1,4-DAP poses satisfying our experimental interaction criteria. This failure was attributed to the limitations of our initial docking strategy. In general, ligand docking strategies have been plagued by two major limitations that hinder their applicability and accuracy (Perola *et al.* 2004; Sousa *et al.* 2006). One problem is the poor performance of scoring functions which rank the likelihood of ligand–receptor complex geometries by attempting to estimate the relative pose energies. The other major problem (one we appeared to encounter here) is the static receptor approximation as currently implemented in grid-based docking approaches. Lately, some progress has been made in addressing this latter issue by expanding the ligand docking search over multiple receptor conformations based on NMA (Cavasotto *et al.* 2005; Lindahl and Delarue 2005). In a strategy similar to that used by Cavasotto *et al.* we explored relatively small backbone conformational changes by docking not into merely one receptor conformer, but by docking into an ensemble of receptor conformers generated by movements along a combination of the lowest frequency normal mode vectors. By incorporating receptor backbone flexibility into our docking study, we were able to identify 1,4-DAP binding poses that produced the expected ionic interaction with D3.32, and furthermore, were able to utilize this well-established interaction as a selection criterion (Floresca and Schetz 2004) rather than only relying on the scoring function.

The poses that resulted from the enhanced strategy, in agreement with the current data and related studies of D₂ and D₄ dopamine receptors (Simpson *et al.* 1999; Schetz *et al.* 2000; Kortagere *et al.* 2004), suggested that positions 2.61, 3.28, and 3.29 are primary determinants of 1,4-DAP recognition in the D₂ dopamine receptor. These three positions accounted for a large proportion of the intermolecular contacts made by docked 1,4-DAPs. For L-750,667 and RBI-257, positions 2.61, 3.28, and 3.29 appeared to be responsible for mimicry of wild-type D₄ binding in the D₂-V2.61F + FV3.28-3.29LM receptor. For Ro

61-6270, L-745,870, and 2-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-a]pyridine (FAUC213), a similar pattern holds: the increase of affinity with the addition of a phenylalanine mutation at 2.61 suggested a similar process of interaction for all five of these ligands at the mutant D₂-V2.61F + FV3.29-3.29LM receptor (Table 1). However, this trend did not hold true for NGD 94-1 and PD168,077. These ligands lost affinity when the valine at 2.61 was mutated in the mutant D₂-FV3.28-3.29LM receptor to create the mutant D₂-V2.61F + FV3.29-3.29LM receptor. Careful examination of ligand molecular structure revealed that ligands possessing para-halogenated or unhalogenated Arm-B aryl rings exhibited a trend of increased affinity when more subtype selective amino acids were mutated. This trend did not hold true for ligands with ortho-electronegative groups (ring nitrogens or nitrile groups).

From close inspection of the CLMs (Fig. 5b), it is interesting to note that the differences in ligand positioning (Fig. 5a) could potentially explain functional differences for certain 1,4-DAPs. For example, contact between the D₂ receptor and the pyrrolopyridine ring of L-750,667 yielded the index values observed for residues F5.47, W6.48, F6.51, and F6.52 (comprising 19.6% of total intermolecular contacts). These contact positions are thought to influence the rotamer state of W6.48 and thus play a role in triggering early steps in the cascade of events leading to receptor activation (Lin and Sakmar 1996; Ebersole and Sealton 2002). The CLM index values for these four residues decreased for the D₂-V2.61F + FV3.28-3.29LM mutant (12.2%) and wild-type D₄ (12.4%) in agreement with the finding that L-750,667 lost partial agonism in the mutant D₂ receptor (Fig. 3c). While it is speculative to suggest a role between docking contacts and receptor efficacy, it remains quite likely that ligand positioning plays a key role in receptor activation. More informed conclusions may be drawn from assessing the dynamic nature of ligand–receptor interactions to show how the ‘trigger’ positions are specifically affected by a given ligand’s occupancy. In summary, substitution of amino acids in the TM2/3 microdomain of the D₂ receptor for the corresponding amino acids in the D₄ receptor yielded a mutant D₂-V2.61F + FV3.28-3.29LM receptor with pharmacology and function that reflected that of the wild-type D₄ receptor.

Acknowledgments

This work was supported by in part by grants R01 MH063162 (JAS) and T32 DA007274 (SSE). The authors wish to thank Dr Christina Z. Floresca and Shiuwei Chen for their assistance with early phases of this work, and the following people and their organizations for supplying samples of compounds that were not commercially available: Dr Claus Riemer (Hoffman La Roche) for Ro 61-6270 and Dr Andrew Thurkauf (Neurogen) for NGD 94-1.

Abbreviations used

1,4-DAPS	1,4-disubstituted aromatic piperidines and piperazines
CLM	contact likelihood matrix
FAUC213	2-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-a]pyridine
GROMACS	GRoningen Machine for Chemical Simulations

HEK293	human embryonic kidney cells
L-745,870	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
L-750,667	3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine
MSP	methylspiperone,8-[4-(4-fluorophenyl)-4-oxobutyl]-(3-methyl-1-phenyl)-1,3,8-triazaspiro[4,5]decan-4-one hydrochloride
NGD 94-1	2-phenyl-4(5)-[4-(2-pyrimidinyl)-piperazin-1-yl]-methyl]-imidazole
NMA	normal mode analysis
PD168,077	<i>N</i> -[[4-(2-cyanophenyl)-1-piperazinyl]methyl]-3-methylbenzamide
RBI-257	1-[4-iodobenzyl]-4-[<i>N</i> -(3-isopropoxy-2-pyridinyl)- <i>N</i> -methyl]-aminopiperidine
Ro 61-6270	2-aminobenzoic acid 1-benzyl-piperidin-4-yl ester
TMS	transmembrane segment

References

- Ballesteros JA, Weinstein H. Integrated methods for modeling G-protein-coupled receptors. *Methods Neurosci.* 1995; 25:366–428.
- Cavasotto CN, Kovacs JA, Abagyan RA. Representing receptor flexibility in ligand docking through relevant normal modes. *J. Am. Chem. Soc.* 2005; 127:9632–9640. [PubMed: 15984891]
- Chemel BR, Roth BL, Armbruster B, Watts VJ, Nichols DE. WAY-100635 is a potent dopamine D4 receptor agonist. *Psychopharmacology (Berl).* 2006; 188:244–251. [PubMed: 16915381]
- Cherezov V, Rosenbaum DM, Hanson MA, et al. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science.* 2007; 318:1258–1265. [PubMed: 17962520]
- Corrigan MH, Gallen CC, Bonura ML, Merchant KM. Sonepiprazole Study Group. Effectiveness of the selective D4 antagonist sonepiprazole in schizophrenia: a placebo-controlled trial. *Biol. Psychiatry.* 2004; 55:445–451. [PubMed: 15023570]
- Drubbisch V, Lameh J, Philip M, Sharma YK, Sadee W. Mapping the ligand binding pocket of the human muscarinic cholinergic receptor Hm1: contribution of tyrosine-82. *Pharm. Res.* 1992; 9:1644–1647. [PubMed: 1488411]
- Ebersole BJ, Sealfon SC. Strategies for mapping the binding site of the serotonin 5-HT2A receptor. *Methods Enzymol.* 2002; 343:123–136. [PubMed: 11665561]
- Ericksen SS, Cummings DF, Weinstein H, Schetz JA. Ligand selectivity of D2 dopamine receptors is modulated by changes in local dynamics produced by sodium binding. *J. Pharmacol. Exp. Ther.* 2009; 328:40–54. [PubMed: 18849360]

- Floresca CZ, Schetz JA. Dopamine receptor microdomains involved in molecular recognition and the regulation of drug affinity and function. *J. Recept. Signal Transduct. Res.* 2004; 24:207–239. [PubMed: 15521362]
- Floresca CZ, Chen S, Kortagere S, Schetz JA. Reciprocal mutations in TM2/TM3 in a D2 dopamine receptor background confirms the importance of this microdomain as a selective determinant of para-halogenated 1,4-disubstituted aromatic piperazines. *Arch. Pharm. (Weinheim)*. 2005; 338:268–275. [PubMed: 15952239]
- Frisch, MJ.; Trucks, GW.; Schlegel, HB., et al. Gaussian 03 (Revision C.02). Wallingford, CT: Gaussian, Inc.; 2004.
- Gazi L, Bobirnac I, Danzeisen M, Schubach E, Bruinvels AT, Geisse S, Sommer B, Hoyer D, Tricklebank M, Schoeffter P. The agonist activities of the putative antipsychotic agents, L-745,870 and U-101958 in HEK293 cells expressing the human dopamine D4.4 receptor. *Br. J. Pharmacol.* 1998; 124:889–896. [PubMed: 9692773]
- Han M, Lin SW, Smith SO, Sakmar TP. The effects of amino acid replacements of glycine 121 on transmembrane helix 3 of rhodopsin. *J. Biol. Chem.* 1996; 271:32330–32336. [PubMed: 8943295]
- al-Jandal N, Farrar GJ, Kiang AS, Humphries MM, Bannon N, Findlay JB, Humphries P, Kenna PF. A novel mutation within the rhodopsin gene (Thr-94-Ile) causing autosomal dominant congenital stationary night blindness. *Hum. Mutat.* 1999; 13:75–81. [PubMed: 9888392]
- Jorgensen WL, Maxwell DS, Tirado-Rives J. Development and testing of the OPLS all-atom force field on conformational energetics and properties of organic liquids. *J. Am. Chem. Soc.* 1996; 118:11225–11236.
- Kenakin, TP. Pharmacologic Analysis of Drug-Receptor Interaction. 3rd. Philadelphia, PA: Lippincott-Raven Press; 1997.
- Kortagere S, Gmeiner P, Weinstein H, Schetz JA. Certain 1,4-disubstituted aromatic piperidines and piperazines with extreme selectivity for the dopamine D4 receptor interact with a~common receptor microdomain. *Mol. Pharmacol.* 2004; 66:1491–1499. [PubMed: 15448188]
- Kramer MS, Last B, Getson A, Reines SA. 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.* 1997; 54:567–572. [PubMed: 9193198]
- Lan H, Durand CJ, Teeter MM, Neve KA. Structural determinants of pharmacological specificity between D(1) and D(2) dopamine receptors. *Mol. Pharmacol.* 2006; 69:185–194. [PubMed: 16236817]
- Lawler CP, Prioleau C, Lewis MM, Mak C, Jiang D, Schetz JA, Gonzalez AM, Sibley DR, Mailman RB. Interactions of the novel antipsychotic aripiprazole (OPC-14597) with dopamine and serotonin receptor subtypes. *Neuropsychopharmacology.* 1999; 20:612–627. [PubMed: 10327430]
- Li J, Edwards PC, Burghammer M, Villa C, Schertler GF. Structure of bovine rhodopsin in a trigonal crystal form. *J. Mol. Biol.* 2004; 343:1409–1438. [PubMed: 15491621]
- Lin SW, Sakmar TP. Specific tryptophan UV-absorbance changes are probes of the transition of rhodopsin to its active state. *Biochemistry.* 1996; 35:11149–11159. [PubMed: 8780519]
- Lindahl E, Delarue M. Refinement of docked protein-ligand and protein-DNA structures using low frequency normal mode amplitude optimization. *Nucleic Acids Res.* 2005; 33:4496–4506. [PubMed: 16087736]
- Lindahl E, Azuara C, Koehl P, Delarue M. NOMAD-Ref: visualization, deformation and refinement of macromolecular structures based on all-atom normal mode analysis. *Nucleic Acids Res.* 2006; 34(Web Server issue):W52–W56. [PubMed: 16845062]
- Lu ZL, Hulme EC. The functional topography of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. *J. Biol. Chem.* 1999; 274:7309–7315. [PubMed: 10066794]
- Matsui H, Lazareno S, Birdsall NJ. Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. *Mol. Pharmacol.* 1995; 47:88–98. [PubMed: 7838137]
- Millan JM, Fuchs S, Paricio N, Wedemann H, Gal A, Najera C, Prieto F. Gly114Asp mutation of rhodopsin in autosomal dominant retinitis pigmentosa. *Mol. Cell. Probes.* 1995; 9(1):67–69. [PubMed: 7760863]

- Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* 1998; 19:1639–1662.
- Perola E, Walters WP, Charifson PS. A detailed comparison of current docking and scoring methods on systems of pharmaceutical relevance. *Proteins.* 2004; 56:235–249. [PubMed: 15211508]
- Ramon E, del Valle LJ, Garriga P. Unusual thermal and conformational properties of the rhodopsin congenital night blindness mutant Thr-94 → Ile. *J. Biol. Chem.* 2003; 278:6427–6432. [PubMed: 12466267]
- Sakmar TP, Franke RR, Khorana HG. The role of the retinylidene Schiff base counterion in rhodopsin in determining wavelength absorbance and Schiff base pKa. *Proc. Natl Acad. Sci. USA.* 1991; 88:3079–3083. [PubMed: 2014228]
- Šali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* 1993; 234:779–815. [PubMed: 8254673]
- Sanyal S, Van Tol HH. Review the role of dopamine D4 receptors in schizophrenia and antipsychotic action. *J. Psychiatr. Res.* 1997; 31:219–232. [PubMed: 9278187]
- Schetz JA. Dopamine Receptors: Introduction. 2009 IUPHAR database (IUPHAR-DB), <http://www.iuphar-b.org/GPCR/Introduction-DisplayForward?chapterID=1282>.
- Schetz, JA.; Sibley, D. Dopaminergic neurotransmission. In: Sibley, D.; Hanin, I.; Kuhar, MJ.; Skolnick, P., editors. *Handbook of Contemporary Neuropharmacology*. Hoboken, NJ: John Wiley & Sons, Inc; 2007. p. 221-256.
- Schetz JA, Benjamin PS, Sibley DR. Nonconserved residues in the second transmembrane-spanning domain of the D(4) dopamine receptor are molecular determinants of D(4)-selective pharmacology. *Mol. Pharmacol.* 2000; 57:144–152. [PubMed: 10617689]
- Shapiro DA, Renock S, Arrington E, Chiodo LA, Liu LX, Sibley DR, Roth BL, Mailman R. Aripiprazole, a novel atypical antipsychotic drug with a unique and robust pharmacology. *Neuropsychopharmacology.* 2003; 28:1400–1411. [PubMed: 12784105]
- Simpson MM, Ballesteros JA, Chiappa V, Chen J, Suehiro M, Hartman DS, Godel T, Snyder LA, Sakmar TP, Javitch JA. Dopamine D4/D2 receptor selectivity is determined by a divergent aromatic microdomain contained within the second, third, and seventh membrane-spanning segments. *Mol. Pharmacol.* 1999; 56:1116–1126. [PubMed: 10570038]
- Sousa SF, Fernandes PA, Ramos MJ. Protein-ligand docking: current status and future challenges. *Proteins.* 2006; 65:15–26. [PubMed: 16862531]
- Truffinet P, Tamminga CA, Fabre LF, Meltzer HY, Riviere ME, Papillon-Downey C. Placebo-controlled study of the D4/5-HT2A antagonist fananserin in the treatment of schizophrenia. *Am. J. Psychiatry.* 1999; 156:419–425. [PubMed: 10080558]
- Ustun TB, Rehm J, Chatterji S, Saxena S, Trotter R, Room R, Bickenbach J. Multiple-informant ranking of the disabling effects of different health conditions in 14 countries. WHO/NIH Joint Project CAR Study Group. *Lancet.* 1999; 354:111–115. [PubMed: 10408486]
- Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ. GROMACS: fast, flexible, and free. *J. Comput. Chem.* 2005; 26:1701–1718. [PubMed: 16211538]
- Wallace AC, Laskowski RA, Thornton JM. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng.* 1995; 8:127–134. [PubMed: 7630882]

Fig. 1.

D₄-selective ligands L-750,667 and RBI-257 have significantly enhanced affinity for the mutant D₂-FV3.28-3.29LM and D₂-V2.61F + FV3.28-3.29LM receptors relative to the wild-type D₂ receptor. (–)-Quinpirole did not display significantly enhanced affinity at these mutants. Graphs are the averaged values of three radiolabeled competition binding experiments between [³H]methylspiperone and (a) L-750,667, (b) RBI-257, or (c) (–)-quinpirole; at the wild-type D₂ (●), mutant D₂-FV3.28-3.29LM (■) or D₂-V2.61F + FV3.28-3.29LM (▼) receptors. Data are graphed as the geometric mean ± SEM.

Corresponding affinity values and fold changes relative to the wild-type D₂ receptor are listed in Table 1.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

Much lower concentrations of RBI-257 are needed to antagonize the (–)-quinpirole-induced functional activation of the mutant D₂-V2.61F + FV3.28-3.29LM in comparison with the wild-type D₂ receptor. In the presence of 2 μM RBI-257 a greater than 5000-fold decrease in (–)-quinpirole potency was observed for the mutant but not the wild-type receptor. (a) Functional activation of the wild-type D₂ receptor by (–)-quinpirole alone (○) or co-incubated with 2 μM (▲) or 20 μM (●) of RBI-257. (b) Functional activation of the mutant D₂-V2.61F + FV3.28-3.29LM receptor by (–)-quinpirole alone (□) or co-incubated with 0.2 μM (■) or 2 μM (▲) of RBI-257. (c) (–)-Quinpirole in the absence (○) or presence (●) of 500 nM L-750,667 at the wild-type D₂ receptor. (d) (–)-Quinpirole in the absence (□) or presence (■) of 500 nM L-750,667 at the mutant D₂-V2.61F + FV3.28-3.29LM receptor. Graphs represent the geometric mean ± SEM of three experiments. The corresponding potency and efficacy values are listed in Table 2.

Fig. 3.

L-750,667 exhibits partial agonism at only the wild-type D₂ receptor. While L-750,667 was identified as a partial agonist of the wild-type D₂ receptor, this ligand behaved as an antagonist at both the mutant D₂-V2.61F + FV3.28-3.29LM and the wild-type D₄ receptors. Overall, the functional profile observed for the mutant D₂ receptor was similar to the wild-type D₄ receptor but not the wild-type D₂ receptor. No significant response was observed for (a) untransfected HEK293 cells. HEK 293 cells stably expressing either (b) wild-type D₂ receptors, (c) mutant D₂-V2.61F + FV3.28-3.29LM receptors or (d) wild-type D₄ receptors

when stimulated by (–)-quinpirole had respective $65 \pm 1.4\%$, $53 \pm 7.4\%$, and $49 \pm 5.1\%$ decreases in the concentration of forskolin-stimulated cAMP. Only at the wild-type D₂ receptor, did L-750,667 significantly decrease the concentration of forskolin-induced cAMP ($42 \pm 9.2\%$, $*p < 0.05$). Values are the geometric mean $\pm$ -SEM of three experiments. Statistically significant decreases in cAMP relative to the forskolin control were determined at 95% confidence by one-way ANOVA with Dunnett's *post hoc* analysis and indicated by asterisks (*).

Fig. 4.

The dose-dependent partial reversal of (–)-quinpirole agonism by L-750,667 confirms this ligand as a partial agonist of the wild-type D₂ receptor. The full agonist effect of 60 nM (–)-quinpirole was reversed in a dose-dependent manner by L-750,667 in wild-type D₂ (●) and mutant D₂-V2.61F + FV3.28-3.29LM (■) receptors. In contrast to the complete reversal (EC₅₀ = 0.57 μM) of (–)-quinpirole agonism at the D₂-V2.61F + FV3.28-3.29LM receptor, the partial reversal (EC₅₀ = 1.9 μM) of (–)-quinpirole agonism at the wild-type D₂ receptor confirmed L-750,667 as an unequivocal wild-type D₂ receptor partial agonist. Data are graphed as the geometric mean ± SEM.

Fig. 5.

The TM2/3 microdomain accounts for a large portion of the intermolecular contacts between 1,4-DAPs and the D₂ or D₄ receptor backgrounds. (a) Representative poses of L-750,667 were bound to (Top panel: wild-type D₄; Middle panel, mutant D₂-V2.61F + FV3.28-3.29LM, or Bottom panel) wild-type D₂ receptors. Blue ribbons represent the receptor backbones, yellow sticks represent the side chains of primary contact residues for each pose, and orange sticks represent L-750,667. In achieving a similar set of intermolecular contacts, the orientation of L-750,667 bound to the mutant D₂ receptor was very similar to the ligand orientation in the wild-type D₄ receptor. In contrast to the orientation in the wild-type D₄ receptor, the counter-clockwise rotated orientation of

L-750,667 (from an extracellular perspective) in the wild-type D2 receptor was achieved by the promotion of contact between the para-halogenated Arm-B aryl moiety and the TM2/3 interface. Thus, the binding orientation of L-750,667 was found to be sensitive to the identity of positions 2.61, 3.28, and 3.29. (b) Normalized ligand–receptor CLMs. The contact likelihood index, a measure of the probability the ligand will interact with specific binding cleft residues, is taken from the summation of intermolecular contacts ($< 3.9 \text{ \AA}$) counted for the subset of docking poses that meet the experimentally suggested interaction criteria as defined in Results. Matrix elements are colored according to the normalized likelihood of contact index value and scaled from blue (low) to red (high). No residue type is specified for positions that vary between the wild-type D₂ and D₄ backgrounds. Correlations for contact index value distributions between receptor constructs and each ligand are given at the bottom of the columns. The two para-halogenated (L-750,667 and RBI257) and two ortho-electronegative (NGD 94-1 and PD168,077) 1,4-DAPs used to construct this matrix show that Arm-B positions 2.61, 3.28, and 3.29 appear to provide key determinants for the D₂/D₄ selectivity of L-750,667 and RBI257, based on their influence on the ligand–receptor contact distributions. The role for these positions is less clear for NGD 94-1 and PD168,077 based on this contact model which falls in line with experimental observations of these ortho-electronegative 1,4-DAP.

Table 1

Affinities of D₄-selective ligands for wild-type D₂, mutant D₂-FV3.28-3.29LM, or mutant D₂-V2.61F + LM3.28-3.29FV receptors expressed in COS-7 cells

Ligand	Structure	D ₂ -WT	D ₂ -FV3.28-3.29LM	D ₂ -V2.61F + FV3.28-3.29LM	D ₄ -WT
L-745,870 (CPPMA)		656 ± 227 (1)	117 ± 95 [*] (↓5.6)	11 ± 4.3 [*] (↓60)	0.32 ± 0.14 (↓2050)
L-750,667 ^a		1400 ± 950 (1)	150 ± 87 (↓9.3)	14 ± 4.2 [*] (↓100)	0.11 ± 0.02 ^b (↓13400)
RBI-257		85 ± 12 (1)	3.8 ± 0.73 [*] (↓22)	0.29 ± 0.07 [*] (↓293)	0.27 ± 0.10 ^c (↓315)
FAUC213 ^c		1300 ± 640 (1)	190 ± 100 (↓6.8)	32 ± 16 [*] (↓41)	1.1 ± 0.22 ^c (↓1030)
Ro 61-6270		655 ± 274 (1)	136 ± 43 [*] (↓4.8)	31 ± 2.5 [*] (↓21)	0.89 ± 0.12 ^c (↓736)
NGD 94-1		817 ± 284 (1)	64 ± 34 [*] (↓13)	127 ± 17 [*] (↓6.4)	0.3 ± 0.04 ^c (↓2720)
PD168,077		1380 ± 64 (1)	41 ± 4.4 [*] (↓34)	287 ± 30 (↓4.8)	1.5 ± 0.41 ^c (↓540)
(-)-Quinpirole		812 ± 617 (1)	2461 ± 2230 (↑3.0)	3075 ± 3009 (↑3.8)	ND

^aFloresca *et al.* 2005;

^bSchetz *et al.* 2000;

^cKortagere *et al.* 2004.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Affinity values (K_i or K_D) are the geometric mean $\pm$ SD of three experiments. Affinity values for the wild-type D4 receptor are listed for comparison. Fold change relative to the wild-type D2 receptor is shown within parentheses below the corresponding affinity values. Decreases in K_i values (corresponding to increased affinity) are denoted with $\downarrow$ arrows and increases in K_i values (corresponding to decreased affinity) are denoted with $\uparrow$ arrows.

Significant changes in mutant receptor affinity relative to the wild-type D2 receptor were evaluated by Dunnett's Multiple Comparison Test ($*p < 0.05$).

ND means not determined.

Table 2

Potency and percent efficacy values for (–)-quinpirole alone and with competing ligand (L-750,667 or RBI-257) at the wild-type D₂ and mutant D₂-V2.61F + FV3.28-3.29LM receptors

Ligands	D ₂ -WT		D ₂ -V2.61F + FV3.28-3.29LM	
	EC ₅₀ (nM)	% Efficacy	EC ₅₀ (nM)	% Efficacy
(–)-Quinpirole	0.46 ± 0.06 (1)	73 ± 2.0 (1)	2.0 ± 0.94 (1)	60 ± 6.5 (1)
(–)-Quinpirole + 0.2 μM RBI-257	N.D.	N.D.	21 ± 5.4 (↑11)	60 ± 8.0 (1)
(–)-Quinpirole + 2.0 μM RBI-257	0.70 ± 0.20 (↑1.5)	78 ± 5.2 (↑1.1)	>10,000* (↑>5000)	N.D.
(–)-Quinpirole + 20 μM RBI-257	55 ± 26* (↑120)	64 ± 9.9 (↓1.2)	N.D.	N.D.
(–)-Quinpirole + 0.5 μM L-750,667	0.37 ± 0.14 (↓1.2)	64 ± 8.7 (↓1.2)	98 ± 50* (↑49)	68 ± 7.8 (↑1.1)

ND, not determined.

Potency (EC₅₀) and percent efficacy are expressed as the geometric mean ± SD of three experiments. Fold changes relative to (–)-quinpirole response in the absence of competing ligand are in parentheses with decreases denoted by ↓ arrows (higher relative potency or lower relative efficacy) and increases denoted with ↑ arrows (lower relative potency or higher relative efficacy).

Significance relative to the wild-type D₂ receptor was evaluated by one-way ANOVA with Dunnett's *post hoc* analysis **p* < 0.05.