Ring Substituents on Substituted Benzamide Ligands Indirectly Mediate Interactions with Position 7.39 of Transmembrane Helix 7 of the D4 Dopamine Receptor^[S]

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ABSTRACT

In an effort to delineate how specific molecular interactions of dopamine receptor ligand classes vary between D2-like dopamine receptor subtypes, a conserved threonine in transmembrane (TM) helix 7 (Thr7.39), implicated as a key ligand interaction site with biogenic amine G protein-coupled receptors, was substituted with alanine in D2 and D4 receptors. Interrogation of different ligand chemotypes for sensitivity to this substitution revealed enhanced affinity in the D4, but not the D2 receptor, specifically for substituted benzamides (SBAs) having polar 4- (*para*) and/or 5- (*meta*) benzamide ring substituents. D4-T7.39A was fully functional, and the mutation did not alter the sodium-mediated positive and negative allostery observed with SBAs and agonists, respectively. With the exception of the non-SBA ligand (+)-butaclamol, which, in contrast to certain SBAs, had decreased affinity for the D4-T7.39A mutant, the

interactions of numerous other ligands were unaffected by this mutation. SBAs were docked into D4 models in the same mode as observed for eticlopride in the D3 crystal structure. In this mode, interactions with TM5 and TM6 residues constrain the SBA ring position that produces distal steric crowding between pyrrolidinyldiethylamine moieties and D4-Thr7.39. Ligand-residue interaction energy profiles suggest this crowding is mitigated by substitution with a smaller alanine. The profiles indicate sites that contribute to the SBA binding interaction and site-specific energy changes imparted by the D4-T7.39A mutation. Substantial interaction energy changes are observed at only a few positions, some of which are not conserved among the dopamine receptor subtypes and thus seem to account for this D4 subtype-specific structure-activity relationship.

Introduction

Dopamine receptors are biogenic amine G protein-coupled receptors (GPCRs) with each subtype having a distinct central nervous system distribution and neurophysiological involve-

ment. Each of the five subtypes is a potential target for therapeutic intervention in a variety of dysfunctional or pathological states, including erectile dysfunction (Brioni et al., 2004; De-poortère et al., 2009), migraines (Charbit et al., 2010), attention deficit/hyperactivity disorders, addiction (Swift, 2010), eating disorders (Bello and Hajnal, 2010), Parkinson's disease (Stocchi, 2009), and schizophrenic psychosis (Seeman, 2010). Most small molecules that interact with dopamine receptors are not specific toward an individual subtype and often interact with other members of the GPCR superfamily (Hopkins et al., 2006). For example, it is still debated as to whether the benefits of an atypical antipsychotic dibenzodiazepine such as clozapine [8-chloro-11-(4-methylpiperazin-1-yl)-5*H*-dibenzo[*b,e*][1,4]diazepine] arise from dopamine receptor interactions, serotonin receptor interactions, or both (Meltzer and Huang, 2008). Although a number of highly D4-selective ligands have been described, poor ligand selectivities for GPCR-targeted drugs may obscure the mechanisms of therapeutic action and contribute to adverse side effects.

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Because the crystal structure for the D3 subtype of dopamine receptor has been made available (Chien et al., 2010), more reliable molecular models of the dopamine receptor subtypes can be constructed. Upon inspection of the D2-like receptors (D2, D3, and D4), D2 shares identity with 17 of the 18 (94%) contact residues reported for eticlopride [3-chloro-5-ethyl-*N*-[(2*S*)-1-ethylpyrrolidin-2-yl] methyl]-6-hydroxy-2-methoxybenzamide within the D3 structure, whereas the D4 receptor shares only 13 of the 18 (72%) observed contact residue identities. As expected from these observations, the affinities of numerous substituted benzamides (SBAs) have been known for years to be similar between D2 and D3 receptor subtypes (Malmberg et al., 1993; Tang et al., 1994; Lawler et al., 1999; Abbas et al., 2009). At the D4 subtype, however, the affinities for SBAs are rarely similar to the two other D2-like receptors (Scatton et al., 2001; Burstein et al., 2005).

In this article, we examine the specific role of position Thr7.39 in dopamine receptor D4/D2 subtype-selective recognition of various therapeutically relevant ligand chemotypes, including a large panel of SBAs. Residue position 7.39 has been implicated in ligand recognition for several biogenic amine GPCRs and is conserved among D2-like subtypes. In the D3 subtype, aminotetralin agonist selectivities are affected by mutations at position 7.39 (T369V), despite little change in affinities for the agonist dopamine or the SBA antagonist raclopride (Lundstrom et al., 1998). In α_2 adrenergic receptor, an F7.39N mutation promoted stronger binding for aryloxyalkylamine ligands such as alprenolol, pinolol, and propranolol (Suryanarayana et al., 1991). In β_2 adrenergic receptor, N7.39V produced large (100-fold) reductions in affinity for the aryloxyalkylamine ligand class, an effect attributed to the loss of an intermolecular H-bond with the aryloxy (ether) oxygen (Suryanarayana et al., 1991; Suryanarayana and Kobilka, 1993). However, N7.39Q/T actually promoted more than 10-fold stronger affinity for the antagonist yohimbine and N7.39Q/T/A mutants produced functional responses to *p*-clonidine, which acts as an antagonist in the wild type. Furthermore, position 7.39 varies across serotonin receptor subtypes and plays a key role in aryloxyalkylamine selectivity in 5HT_{1A} (Guan et al., 1992), 5HT_{1B} (Oksenberg et al., 1992; Parker et al., 1993), 5-HT_{1D α /B}, 5-HT_{1E}, and 5-HT_{1F} receptors (Adham et al., 1994).

Using members of the SBA class of ligands as probes, we have uncovered a D4-specific structure-activity relationship (SAR) between benzamide ring substituents and sensitivity to T7.39A substitution. Specifically this substitution leads to enhanced binding affinity for SBAs whose benzamide ring has a polar H-bond accepting *meta* (5-) substituent and/or an H-bond donating/accepting *para* (4-) substituent. This SAR is absent in the D2 receptor and thus suggests differences in SBA recognition features in D2 and D4 receptor subtypes that could potentially be exploited to enhance dopamine receptor subtype targeting by this ligand class.

Materials and Methods

Reagents. Hyclone bovine calf serum and powdered DMEM were purchased from Thermo Fisher Scientific (Waltham, MA) and used to make DMEM complete, a growth medium consisting of DMEM supplemented with 10% bovine calf serum, 50 U/ml penicillin G, 50 μ g/ml streptomycin sulfate, and 100 μ M sodium pyruvate. The 10 $\times$ Hanks' balanced salt solution purchased from Invitrogen (Carlsbad, CA) was diluted as necessary for functional assays. [³H]methylspiperone (NET-856; 80–85 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Tris buffer reagents were purchased from United States Biologicals (Swampscott, MA). With the exceptions of *N*-[2-[2-hydroxy-3-[4-(4-hydroxy-3-methoxyphenyl)piperazin-1-yl]propoxy]phenyl]acetamide (Ro10-4548), 4-[4-[2-[(1*S*)-3,4-dihydro-1*H*-isochromen-1-yl]ethyl]piperazin-1-yl]benzenesulfonamide (PNU101,387G), and 5-fluoro-2-[[4-(2-pyridinyl)-1-piperazinyl]methyl]-1*H*-indole (CP226,269), which were kind gifts from Roche Diagnostics (Indianapolis, IN), Eli Lilly & Co. (Indianapolis, IN), and Pfizer (New York, NY), respectively, all ligands were purchased from Sigma (St. Louis, MO) or Tocris Bioscience (Ellisville, MO).

Membrane Preparation. Wild-type and mutant rat D4 dopamine receptors were stably expressed in HEK293 cells by using the same methodology as described previously (Kortagere et al., 2004; Ericksen et al., 2009). In brief, plasmid DNA containing the receptor and a resistance gene for G418 were transfected by CaPO₄ precipitation into a low confluence of HEK293 cells. Monoclonal colonies were isolated by challenging the transfection plates with DMEM containing 2 mg/ml G418 for 2 weeks. Stable receptor expression of expanded clones was confirmed by saturation isotherm analysis 3 to 4 weeks later. The HEK293 cells that stably expressed mutant receptor were maintained (37°C, 5% CO₂) in DMEM containing 100 μ g/ml G418 and used to prepare cell membranes for radioligand binding assays as described previously (Ericksen et al., 2009). In brief, HEK293 cells expressing dopamine receptors were detached from 175-cm² culture flasks using 5 mM EDTA lifting buffer (Dulbecco's phosphate-buffered saline without Ca²⁺ and Mg²⁺ supplemented with 5 mM EDTA). These cells were pelleted by centrifugation at 700g before resuspension in lysis buffer (5 mM Tris and 5 mM MgCl₂, pH 7.4 at 4°C). After 5 to 10 min the cell lysate was homogenized (Dounce homogenizer, eight strokes) and centrifuged at 28,000g for 30 min. The pellet was resuspended in binding buffer (50 mM Tris, pH 7.4 at 4°C) and recentrifuged at 28,000g for 30 min. This purified membrane pellet was rehomogenized (Dounce homogenizer, four strokes) in binding buffer (50 mM Tris, pH 7.4 at 4°C) and stored on ice for use the same day. Binding buffers were pH-adjusted by using 1 N KOH and 1 N HCl.

Radioligand Binding Studies. As with our previous studies using HEK293 cells (Ericksen et al., 2009), membranes expressing the D4-WT, D2-WT, D4-T7.39A, or D2-T7.39A receptors were challenged with [³H]methylspiperone alone and in competition with other D4 receptor ligands to characterize affinity shifts for mutants relative to the wild-type background. In brief, 0.5 nM [³H]*N*-methylspiperone, purified HEK293 cell membranes containing dopamine receptors, and various concentrations of dopaminergic ligands in binding buffer (50 mM Tris, pH 7.4 at 25°C) in a total volume of 1 ml were allowed to equilibrate at room temperature for 90 min. Receptors were then isolated by rapid filtration through GF/C filters pretreated for 10 min with 0.3% polyethyleneimine (Sigma) and three

ABBREVIATIONS: GPCR, G protein-coupled receptor; ABT-724, 2-[[4-(pyridin-2-yl)piperazin-1-yl]methyl]-1*H*-benzimidazole; CP226,269, 5-fluoro-2-[[4-(2-pyridinyl)-1-piperazinyl]methyl]-1*H*-indole; DAP, diaryl-piperidine/piperazine; DMEM, Dulbecco's modified Eagle's medium; EL2, extracellular loop 2; 5-HT, 5-hydroxytryptamine; HEK, human embryonic kidney; IFD, induced fit docking; MIA, methylisobutylamiloride; NGD 94-1, 2-[4-[[2-(phenyl-1*H*-imidazol-5-yl)methyl]piperazin-1-yl]pyrimidine; PNU101,387G, 4-[4-[2-[(1*S*)-3,4-dihydro-1*H*-isochromen-1-yl]ethyl]piperazin-1-yl]benzenesulfonamide; RMSD, root mean square deviation; Ro10-4548, *N*-[2-[2-hydroxy-3-[4-(4-hydroxy-3-methoxyphenyl)piperazin-1-yl]propoxy]phenyl]acetamide; Ro20-1724, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone; SAR, structure-activity relationship; SBA, substituted benzamide; TM, transmembrane; vdW, van der Waals; WT, wild type.

rapid washes with 3.5 ml of ice-cold binding buffer (50 mM Tris, pH 7.4 at 0°C). Dried filters were cut into individual scintillation vials, filled with 3.5 ml of scintillation fluid, mixed, and counted in the scintillation counter. Nonspecific interactions of the radioligand were defined by the competition of [³H]N-methylspiperone with 5 μ M (+)-butaclamol [3-(1,1-dimethylethyl)-2,3,4,4a,8,9,13b,14-octahydro-1H-benzo[6,7]cyclohepta[1,2,3-de]pyrido[2,1-a]isoquinolin-3-ol]. The amount of membrane protein was determined by bicinchoninic acid assay (Thermo Fisher Scientific) and adjusted to within the range of 0.2 to 0.4 mg/ml for each assay.

Assessing Dopamine Receptor Function by cAMP Signaling. HEK293 cells stably expressing wild-type and mutant dopamine receptors were assessed for their ability to inhibit a forskolin [(3R,4aR,5S,6S,6aS,10S,10aR,10bS)-3-ethenyl-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1-oxo-5,6,6a,8,9,10-hexahydro-2H-benzof[chromen-5-yl]acetate]-stimulated intracellular cAMP signal in the presence of various agonists. Activation of the D4 dopamine receptor then depressed this cAMP signal in our cells. Antagonists were tested for their ability to prevent a full agonist response induced by (–)-quinpirole [(4aR,8aR)-5-propyl-1,4,4a,6,7,8,8a,9-octahydropyrazolo[3,4-g]quinoline] and therefore preserve the accumulation of intracellular cAMP. Intracellular cAMP concentration was determined by using a cAMP Alphascreen detection kit (PerkinElmer Life and Analytical Sciences) and a PerkinElmer Life and Analytical Sciences Fusion plate analyzer as described previously (Ericksen et al., 2009). In brief, HEK293 cells seeded at a density of 50,000 cells per well (200 μ l of DMEM per well) were allowed to attach overnight to sterile 96-well, poly-L-lysine-coated microtiter plates (poly-L-lysine; Sigma). After incubation for 16 to 18 h, the growth medium was removed, and the cells were challenged for 25 min at 37°C (ambient CO₂) with temperature-equilibrated drug dilutions containing 6 μ M forskolin dissolved in stimulation buffer [1× Hanks' basic salt solution, 50 mM HEPES, 100 μ M sodium metabisulfite, and 30 μ M 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone (Ro20-1724), pH 7.4 at 37°C]. In experiments using antagonists, antagonists and agonist were added simultaneously. Microtiter plates were centrifuged at 1500g for 5 min after the drug incubation time had elapsed. The removal of the supernatant from each well was quickly followed by the addition of lysis buffer (100 μ l of 0.3% Tween 20, 20 mM HEPES, 1 μ g/ μ l bovine serum albumin, and 30 μ M Ro20-1724, pH 7.4 at 25°C) and lytic freezing at –80°C overnight. Lysates were thawed the next morning at 37°C on the benchtop (ambient CO₂). The quantification of intracellular cAMP was determined by combining in an opaque 96-well Costar plate (Corning Life Sciences, Lowell, MA) 10 μ l of cell lysate with 10 μ l of 0.5 U (9.35 μ g/ml) acceptor beads previously dark-adapted in bead buffer for 2 h (20 mM HEPES, 30 μ M Ro20-1724, 1 μ g/ μ l bovine serum albumin, and 1× Hanks' basic salt solution, pH 7.4 at 25°C). While protected from light, the Costar plates containing the lysate and acceptor bead mixture were centrifuged at 5g for 2 min. Thirty minutes postcentrifugation, 10 μ l of 0.5 U (12.5 μ g/ml) donor beads equilibrated in darkness with 5 units of biotinylated cAMP (3.76 nM) in bead buffer for 2.5 h were added to the dark-equilibrated lysate/acceptor bead mixture. These plates were carefully centrifuged for another 2 min at 5g while covered with aluminum foil. The donor bead/biotinylated cAMP complexes were allowed a minimum of 2 h to compete with cAMP for acceptor bead occupancy before quantification in the PerkinElmer Life and Analytical Sciences Fusion plate analyzer.

Calculations and Data Analysis. All data were analyzed and graphed by using Prism version 4.0 (GraphPad Software Inc., San Diego, CA). Each data table reports the geometric mean and S.D. for experiments repeated three times with two or more sample replicates per experiment except where noted. Error margins depicted in the graphs are S.E.M. For radioligand competition assays, data from two or more sample replicates were averaged for each individual experiment, the nonspecific binding as defined by 5 μ M (+)-butaclamol was subtracted, and the resulting specific binding was normalized as the amount of radioligand specifically bound in the presence of competing drug divided by the amount specifically bound in the absence of drug. These data were then graphed to generate the individual IC₅₀. The average IC₅₀ value for a set of three radiolabeled

competition experiments and the average equilibrium dissociation constant (K_D) of [³H]methylspiperone for each receptor were used to find the inhibition constant (K_i) defined in the Cheng-Prusoff equation as $K_i = IC_{50}/(1 + [\text{radioligand}]/K_D)$ (Cheng and Prusoff, 1973). K_i values were analyzed for significance by one-way analysis of variance with a Dunnett's post hoc analysis. In cases where the pseudo-Hill slope was not equal to 1.0, $K_{0.5}$ values were substituted for K_i values. For cAMP assessments, the average cAMP accumulated per milligram of membrane protein was determined by comparing the average raw cpm of three replicates that contained cells, 6 μ M forskolin, and experimental drugs, to the average cpm values generated by a cAMP standard curve. The magnitude of the cAMP change was obtained by normalizing the average cAMP level (femtomole per milligram of protein) of each sample to the average cAMP level generated by unopposed 6 μ M forskolin exposure. The resultant value was plotted as a percentage of the maximal cAMP level that the cells generated in this assay. Basal levels of cAMP accumulation are the levels of intracellular cAMP in the absence of forskolin stimulation. To represent basal levels as nonzero values in the normalization of data, 0% is defined as the cAMP signal for buffer controls containing no cells. Efficacy was determined by subtracting the lowest horizontal asymptote from the highest horizontal asymptote as defined by graphing the sigmoidal semilog concentration-response curve. Half-maximal potency (EC₅₀) and efficacy values generated from three cAMP functional experiments were analyzed by one-way analysis of variance with a Dunnett's post hoc analysis, and significance was established at the 95% confidence level ($p \leq 0.05$).

Induced Fit Docking and Pose Analysis. Wild-type rat D4 dopamine receptor homology models were constructed with Modeler 9v1 (<http://salilab.org/modeller/9v1/release.html>) using the template structure of the D3 dopamine receptor (Protein Data Bank accession no. 3PBL) (Chien et al., 2010). The β_2 adrenergic receptor (Protein Data Bank accession no. 2RH1) (Cherezov et al., 2007) served as a supplemental template to prevent overfitting and either fill in missing coordinates or provide alternative coordinates for any potentially erroneously fit coordinates in the D3 structure. After generating 1000 models, the top-ranking structure according to Modeler 9v1's default molecular probability density function for scoring (based on satisfaction of template-dependent geometric constraints) was used as the initial conformation for docking. A set of SBAs, (S)-amisulpride, 4-amino-5-bromo-N-[2-(diethylamino)ethyl]-2-methoxybenzamide (bromopride), (S)-eticlopride, (S)-nafadotride [N-((2S)-1-butylpyrrolidin-2-yl)methyl]-4-cyano-1-methoxy-2-naphthamide], (S)-raclopride, (S)-remoxipride [(S)-3-bromo-N-[(1-ethylpyrrolidin-2-yl)methyl]-2,6-dimethoxy-benzamide], (S)-sulpiride [N-((2S)-1-ethylpyrrolidin-2-yl)methyl]-2-methoxy-5-sulfamoylbenzamide], and tiapride [N-(2-diethylaminoethyl)-2-methoxy-5-methylsulfonylbenzamide], were constructed with Maestro 9.0 (Schrödinger, Inc., New York, NY). Ground-state geometries and electronic configurations were computed with Gaussian03 (Gaussian, Inc., Wallingford, CT) using ab initio Hartree-Fock quantum mechanical calculations with the 6-31G** basis set. However, in the case of bromopride and remoxipride, the 3-21G** basis set was required to accommodate their bromine orbitals. Using Maestro 9.0, the receptor models (wild-type and T7.39A mutant) were prepared for docking by automated assignment of bond orders and formal charges, addition of hydrogen atoms for suitable protonation states (termini were capped with neutral functionalities), determination of χ "flips" for residues with frequently ambiguous coordinates (Asn, Gln, and His) to enhance internal H-bonding, and all-atom energy minimization before docking. Both receptor and ligand structures were automatically parameterized for minimization and induced fit docking (IFD) according to the OPLS 2001 all-atom force field. A maximum of 500 ligand poses was obtained for each receptor construct by using Schrödinger's IFD protocol to search a cubic region, with edge lengths of 18 Å, centered in the binding cleft between the C α atoms of Asp3.32 and Phe6.52. During the initial Glide procedure within IFD, the side chain of residue Leu180^(Cys + 2) located two residues C-terminal from disul-

fide Cys178 on extracellular loop 2 (EL2)] was temporarily mutated to alanine to expand and smooth the binding cavity to facilitate broader initial exploration of configuration space. The original side chain was then restored in later phases of the procedure. Residues within 6 Å of the docked ligand pose were included in the final optimization procedure to produce unique ligand-receptor configurations.

Because a general SBA binding mode is likely to be shared among the D2-like dopamine receptor subtypes, we screened favorable output poses for similarity to the observed eticlopride pose in the D3 crystal structure complex. Although computationally expensive, we performed an unbiased docking of all 10 substituted benzamides, including eticlopride. Results obtained from these unbiased searches revealed some very similar poses to that of bound eticlopride cocrystallized in the D3 receptor. This gave us some confidence in our assumption of an eticlopride-like pose for the SBA series studied here in the context of the D4 receptor. An eticlopride-like pose was frequently identified in each case (RMSD <1.0 Å), except in the case of amisulpride [4-amino-*N*-[(1-ethylpyrrolidin-2-yl)methyl]-5-ethylsulfonyl-2-methoxy-benzamide], where despite additional sampling (docking runs), we obtained poses only approximately similar (RMSD 3.8–4.1 Å) to the eticlopride-like poses. The resulting docked complexes from IFD were superimposed on chain A of the D3 crystal structure complex. Then, using the program VMD 1.9 (University of Illinois, Urbana-Champaign, IL), we calculated RMSD between our ligand pose and the eticlopride coordinates in D3 based on shared nonhydrogen atoms in the SBA pharmacophore. For the lowest RMSD docking pose in each SBA-receptor complex, van der Waals (vdW), electrostatic, and total intermolecular mechanics energy components were computed between the SBA and each residue in the D4 receptor model by using the NAMD2 energy evaluation feature as implemented within VMD 1.9 and the CHARMM22 force field supplemented with ligand parameters obtained from the SwissParam server (<http://swissparam.ch/>). PyMOL 0.99rc6 (Delano Scientific, LLC; <http://www.delanoscientific.com/>) was used for rendering figures, and Microsoft (Redmond, WA) Excel 2007 was used to produce the colored contact energy matrices.

Results

Receptor Expression and Functional Viability. Cloned wild-type and T7.39A mutant D2 and D4 dopamine receptors were each stably expressed in HEK293 cells. The membrane densities of expressed receptors were determined by saturation isotherm analysis with the radiolabeled antagonist [³H]methylspiperone. Individual cell lines expressing high receptor densities ($B_{\max} = 1.9$ –8.6 pmol/mg membrane protein) of one of the four wild-type or T7.39A mutant receptors (Table 1) were selected for further studies. This included investigation of each receptor's binding properties for [³H]methylspiperone and 22 additional ligands: selective and

nonselective agonists and antagonists and two allosteric modulators (Schetz and Sibley, 2001) (Fig. 1). As expected, the D2 receptor bound [³H]methylspiperone with higher affinity compared with the D4 receptor (Table 1). Both T7.39A mutant receptors showed approximately 2-fold (1.9–2.5) higher affinity for [³H]methylspiperone than their corresponding wild-type receptors. Comparable affinities to their respective wild-type receptors and high expression levels suggested native folds for both constructs.

The functions of D4 constructs were also tested to examine the effect of the T7.39A substitution on receptor activation by agonists of different structures. The D4-T7.39A mutant was activated by two structurally distinct agonists, demonstrating functional viability despite the mutation (Table 2; Fig. 2). The D4-selective agonist 2-[4-[(2-phenyl-1*H*-imidazol-5-yl)methyl]piperazin-1-yl]pyrimidine (NGD 94-1) had similar potencies and efficacies at the D4-WT and D4-T7.39A mutant receptors, with slightly enhanced potency (1.9-fold) and efficacy (116% of WT) at the mutant receptor, whereas dopamine was 5.4-fold less potent at the D4-T7.39A mutant than the D4-WT receptor with 26% decreased efficacy. By analogy with other biogenic amine GPCRs (Suryanarayana and Kobilka, 1993; Wacker et al., 2010; Warne et al., 2011), the reductions in dopamine's potency and efficacy were not unexpected because favorable interactions of a ligand's protonatable amine with side chains at position 7.39 resulting in a narrowing in the binding crevice between TM5-TM7 correlate with agonist activity (Warne et al., 2011). We have shown previously that dopamine interacts with TM5 serines in the D4 receptor (Cummings et al., 2010), and here we demonstrate that the loss of the interaction of dopamine with Thr7.39 hampers dopamine's ability to activate the D4-T7.39A mutant receptor. The functional data presented here indicate that the T7.39A mutant remains functional and the substitution results in localized changes in the mutant receptor, implying a near-native receptor fold.

Benzazepine Selectivity. Next, we probed the influence of conserved threonine at position 7.39 in both D2 and D4 subtypes on the binding for some dibenzodiazepine (benzazepine) antipsychotic ligands with ranging wild-type D2/D4 selectivities (Table 3). The benzazepines were examined because an adjacent position, Val7.35 (Tyr408 in D2), had been shown earlier to have a role in clozapine selectivity (Simpson et al., 1999). For the compounds tested against the D2 subtype, no change in affinity was observed for the T7.39A substitution. The same substitution in the D4 subtype produced no significant change for olanzapine [2-methyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*][1,5]benzodiazepine] and quetiapine [2-(2-(4-dibenzo[*b,f*][1,4]thiazepine-11-yl-1-piperazinyl)ethoxy)ethanol] and small reductions in affinity (4.1- to 5.4-fold) for loxapine [2-chloro-11-(4-methylpiperazin-1-yl)dibenzo[*b,f*][1,4]oxazepine] and clozapine. The largest effect was a moderate 11-fold decrease in affinity observed for (+)-butaclamol, which deviates structurally from the other four compounds tested in that it is a pentacyclic benzocycloheptane, whereas the other compounds share the tricyclic benzazepine pharmacophore.

Endogenous and 1,4-Diaryl-Piperidine/Piperazine Agonist Recognition. In addition to the benzazepines, we tested other ligand types with respect to 7.39 interactions in the D4 receptor (Tables 4 and 5). The endogenous agonist dopamine has only slightly decreased affinity for the D4-

TABLE 1
Affinities of [³H]*N*-methylspiperone for wild-type and mutant D2 and D4 receptors

Binding affinities (K_D) and receptor densities ($B_{\max}$) are expressed as the mean $\pm$ S.D. of three separate experiments. Fold change relative to the appropriate wild-type receptor is indicated in parentheses with down arrows ($\downarrow$) indicating a decrease in K_D value (higher relative affinity) or decrease in $B_{\max}$ value (decreased relative receptor density) and up arrows ($\uparrow$) indicating an increase in K_D value (lower relative affinity) or increase in $B_{\max}$ value (increased relative receptor density).

Receptor	[³ H] <i>N</i> -Methylspiperone	
	$B_{\max} \pm$ S.D.	$K_D \pm$ S.D.
	fmol/mg protein	pM
Wild-type D2	8647 $\pm$ 2430 (1)	86 $\pm$ 24 (1)
D2-T7.39A	4211 $\pm$ 688 (2.1 $\downarrow$)	46 $\pm$ 6 (1.9 $\downarrow$)
Wild-type D4	1900 $\pm$ 381 (1)	206 $\pm$ 44 (1)
D4-T7.39A	6913 $\pm$ 2854 (3.6 $\uparrow$)	84 $\pm$ 12 (2.5 $\downarrow$)

Ligand Name	Chemical Structure	Ligand Name	Chemical Structure
Dopamine		Sulpiride
NGD94-1		Amisulpride
ABT-724		Tiapride
MIA		Bromopride
CP226,269		Metoclopramide
Aripiprazole		Eticlopride
Butaclamol		Nafadotride
Clozapine		Remoxipride
Olanzapine		Raclopride
Quetiapine		Nemonapride
Loxapine		Methylspiperone

Fig. 1. Chemical structures of the organic ligands used in this study.

TABLE 2
Potencies and efficacies for dopamine and NGD 94-1 at wild-type and mutant D4 receptors
Data for the potencies (EC₅₀, nM) and relative efficacies of dopamine and NGD 94-1 are expressed as the mean ± S.D. of three separate experiments. Increased (↑) or decreased (↓) values relative to the wild-type D4 receptor are expressed as fold changes within parentheses.

Receptor	NGD 94-1		Dopamine	
	Potency	Relative Efficacy	Potency	Relative Efficacy
	nM	%	nM	%
Wild-type D4	0.57 ± 0.11 (1)	44 ± 14 (1)	4.1 ± 1.9 (1)	74 ± 14 (1)
D4-T7.39A	0.30 ± 0.04 (1.9 ↓)	51 ± 7.9 (1.2 ↑)	22 ± 13.6 (5.4 ↑)	55 ± 3.4 (1.3 ↓)

T7.39A receptor compared with the wild type. Weakened affinity was expected considering that the homologous residue in adrenergic receptors, Asn7.39 (in the β_{1/2} adrenergic receptors), seems to play a role in anchoring the amine portion of some ligands (Suryanarayana and Kobilka, 1993; Wacker et al., 2010; Warne et al., 2011). Selected members of

Fig. 2. Functional analysis of the D4-T7.39A mutant receptor indicates a near-native fold. The D4-T7.39A mutant has near wild-type functional properties when stimulated by the endogenous agonist dopamine and the D4-selective partial agonist NGD 94-1. The functional properties of D4 wild-type and T7.39A mutant receptors stably expressed in HEK293 cells were assessed by measuring forskolin-stimulated changes in intracellular cAMP accumulation. A, compared with the wild-type D4 receptor, the potency and efficacy of dopamine is modestly reduced in the T7.39A mutant. This is expected from localized changes caused by the mutation (see *Results* for details). B, NGD 94-1 has similar potency and efficacy for the D4 wild-type and T7.39A receptors. Overall, these changes are consistent with a near-native fold for the D4-T7.39A mutant receptor.

the 1,4-diaryl-piperidine/piperazine (DAP) class of ligands with partial efficacy, 7-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butoxy]-3,4-dihydroquinolin-2(1*H*)-one (aripiprazole), CP226,269, NGD 94-1, and 2-[(4-pyridin-2-yl)piperazin-1-yl methyl]-1*H*-benzimidazole (ABT-724) (Table 2) (Lawler et al., 1999; Newman-Tancredi et al., 2008; Cummings et al., 2010), show little change in affinity after the T7.39A substitution. The allosteric modulators zinc and 3-amino-6-chloro-*N*-(diaminomethylidene)-5-[methyl(2-methylpropyl)amino]pyrazine-2-carboxamide (methylisobutylamiloride, MIA) also produce no significant change in affinity at the D4-T7.39A mutant, further suggesting that this mutation does not grossly affect the protein fold or substantially shift the distribution of conformational states.

Sodium Allostery. In previous work, we identified mutations in the cleft-facing residues of TM2 and TM3 that intensified the allosteric response of the D2 receptor to physiological concentrations (140 mM) of sodium involving 1,4-DAP ligand affinity enhancements (Erickson et al., 2009). Here, we examined the effect of T7.39A substitution in the D4 subtype on sodium allostery (Tables 4 and 5). For dopamine,

aripiprazole, and CP226,269 weakened affinity is observed in the presence of a high concentration of sodium, as expected for agonists. However, the extent to which sodium negatively modulates ligand affinities is similar for D4-WT (ranging from 3.1- to 9.5-fold) compared with D4-T7.39A (ranging from 5.0- to 12-fold) receptors (compare Tables 4 and 5). Thus the mutation imparts little effect on sodium allostery for these agonists. For the SBAs tested at D4-WT and D4-T7.39A, sodium induced no effect or only weak allosteric enhancements in affinity (< 3-fold). This lack of an effect on affinity at the wild-type D4 receptor is consistent with reports that increased SBA binding in the presence of high (but physiological) sodium concentrations results from increases in apparent receptor density (number of binding sites) (Schetz et al., 1999), rather than stronger affinity as in the case of D2 receptors. This different response of SBAs to D2 and D4 receptors reflects the difference in sodium's allosteric mechanism for these two receptor subtypes: a positive heterotropic cooperativity in D2 versus a noncompetitive positive allosteric modulation in D4.

Substituted Benzamides. Next, we probed position 7.39 to elucidate its role in D2 and D4 subtype recognition of SBA ligands, a structural class of dopamine receptor antagonists (Table 6). The SBA affinities for the D2 subtype vary widely, with K_i values in the test set spanning five orders of magnitude. However, the affinity with which these compounds bind the D2 receptor subtype remained unaffected by the T7.39A substitution. In contrast, this substitution in D4 produced varied and often pronounced enhancements of SBA affinity. We measured significantly stronger affinities (up to 21-fold) for sulpiride, amisulpride, bromopride, metoclopramide, and tiapride (Table 6). It is noteworthy that these five SBAs, which showed the strongest enhancements, had polar H-bond accepting *meta* (5-) substituents such as sulfonamide, sulfone, or a halide and/or H-bond donor/acceptor amine *para* (4-) substituents on the benzamide ring moiety. This enhanced affinity was somewhat surprising in that the T7.39A substitution removes a cleft-accessible hydroxyl group and should be expected to weaken, rather than strengthen, the binding affinity for ligands with additional polar ring substituents. Nemonapride [*cis*-5-chloro-2-methoxy-4-(methylamino)-*N*-[2-methyl-1-(phenylmethyl)-3-pyrrolidinyl]benzamide], although having polar 5- and 4-substituents, was insensitive

TABLE 3

Binding affinities for benzazepines at D2 and D4 subtypes and their corresponding T7.39A mutants

The D4-T7.39A mutant receptor has a moderately lower-affinity interaction for the benzazepine-like (+)-butaclamol and small decreases for several different benzazepines. These compounds, however, show little sensitivity to the T7.39A substitution at the D2 subtype. Affinities (K_i) are expressed as the mean $\pm$ S.D. (nM) of three or more separate experiments. Increased ($\uparrow$) or decreased ($\downarrow$) K_i values relative to the wild-type receptor are listed in parentheses as fold changes.

Receptor	(+)-Butaclamol	Clozapine	Olanzapine	Quetiapine	Loxapine
Wild-type D2	0.14 $\pm$ 0.08 (1)	N.D.	5.4 $\pm$ 1.17 (1)	78.5 $\pm$ 14.2 (1)	N.D.
D2-T7.39A	0.32 $\pm$ 0.11 (2.3 $\uparrow$)	N.D.	7.8 $\pm$ 1.93 (1.4 $\uparrow$)	105.6 $\pm$ 20.1 (1.3 $\uparrow$)	N.D.
Wild-type D4	34 $\pm$ 10 (1)	1.1 $\pm$ 0.74 (1)	4.35 $\pm$ 1.43 (1)	588 $\pm$ 222 (1)	1.9 $\pm$ 0.34 (1)
D4-T7.39A	355 $\pm$ 66 (11 $\uparrow$)	5.6 $\pm$ 2.8 (5.4 $\uparrow$)	10.6 $\pm$ 2.31 (2.4 $\uparrow$)	1419 $\pm$ 784 (2.4 $\uparrow$)	7.7 $\pm$ 1.1 (4.1 $\uparrow$)

N.D., not determined.

TABLE 4

Affinities for selective and nonselective D4 receptor ligands at wild-type and mutant D4 receptors

Affinities (K_i) are expressed as the mean $\pm$ S.D. (nM) of three or more separate experiments. Increased ($\uparrow$) or decreased ($\downarrow$) K_i values relative to the wild-type D4 receptor are listed in parentheses as fold changes.

Receptor	NGD 94-1	Dopamine	ABT-724	Aripiprazole	CP226,269	MIA	Zinc
Wild-type D4	0.25 $\pm$ 0.064 (1)	12 $\pm$ 6.0 (1)	8.0 $\pm$ 0.49 (1)	77 $\pm$ 32 (1)	0.080 $\pm$ 0.072 (1)	105 $\pm$ 42 (1)	5,083 $\pm$ 3,586 (1)
D4-T7.39A	0.080 $\pm$ 0.026 (3.2 $\downarrow$)	35 $\pm$ 33 (2.9 $\uparrow$)	6.3 $\pm$ 1.1 (1.3 $\downarrow$)	49 $\pm$ 9.9 (1.6 $\downarrow$)	0.26 $\pm$ 0.0084 (3.2 $\uparrow$)	34 $\pm$ 7.1 (3.1 $\downarrow$)	4,984 $\pm$ 3,829 (1.0 $\downarrow$)

TABLE 5
Affinities for selective and non-selective D4 receptor ligands in the presence of 140 mM NaCl at wild-type and mutant D4 receptors
Affinities (K_i) are expressed as the mean $\pm$ S.D. (nM) of three or more separate experiments. Increased ($\uparrow$) or decreased ($\downarrow$) K_i values relative to the wild-type D4 receptor are listed in parentheses as fold changes.

Receptor	Sulpiride + NaCl	Tiapiride + NaCl	Eticlopride + NaCl	Nafadotride + NaCl	Remoxipride + NaCl	Dopamine + NaCl	Aripiprazole + NaCl	CP226,269 + NaCl
Wild-type D4	674 $\pm$ 140 (1)	811 $\pm$ 117 (1)	29 $\pm$ 1.7 (1)	142 $\pm$ 48 (1)	2900 $\pm$ 293 (1)	85 $\pm$ 50 (1)	241 $\pm$ 176 (1)	0.76 $\pm$ 0.96 (1)
D4-T7.39A	41 $\pm$ 18 (16 $\downarrow$)	78 $\pm$ 19 (10 $\downarrow$)	6.4 $\pm$ 1.3 (4.6 $\downarrow$)	171 $\pm$ 20 (1.2 $\uparrow$)	1544 $\pm$ 154 (1.9 $\downarrow$)	421 $\pm$ 236 (5.0 $\uparrow$)	247 $\pm$ 132 (1.0 $\uparrow$)	2.4 $\pm$ 1.6 (3.1 $\uparrow$)

TABLE 6
Substituted benzamides with a sulfonamide, sulfone, and amine substitution have enhanced affinity for the D4-T7.39A, but not the D2-T7.39A, mutant receptors
Affinities (K_i) are expressed as the mean $\pm$ S.D. (nM) of three or more separate experiments. Increased ($\uparrow$) or decreased ($\downarrow$) K_i values relative to the wild-type receptor are listed in parentheses as fold changes.

Receptor	Sulpiride	Amisulpride	Bromopride	Metoclopramide	Tiapiride	Eticlopride	Nafadotride	Remoxipride	Raclopride	Nemonapride
Wild-type D4	1,069 $\pm$ 179 (1)	2653 $\pm$ 155 (1)	990 $\pm$ 78 (1)	537 $\pm$ 286 (1)	1415 $\pm$ 38 (1)	48 $\pm$ 0.90 (1)	510 $\pm$ 135 (1)	2054 $\pm$ 82 (1)	2368 $\pm$ 575 (1)	0.24 $\pm$ 0.082 (1)
D4-T7.39A	50 $\pm$ 4.6 (21 $\downarrow$)	135 $\pm$ 35 (20 $\downarrow$)	65 $\pm$ 12 (15 $\downarrow$)	51 $\pm$ 23 (11 $\downarrow$)	126 $\pm$ 25 (11 $\downarrow$)	7.5 $\pm$ 0.53 (6.4 $\downarrow$)	478 $\pm$ 73 (1.1 $\downarrow$)	1732 $\pm$ 293 (1.2 $\downarrow$)	2615 $\pm$ 680 (1.2 $\downarrow$)	0.45 $\pm$ 0.026 (1.9 $\uparrow$)
Wild-type D2	471 $\pm$ 43.2 (1)	84 $\pm$ 6.3 (1)	315 $\pm$ 45.1 (1)	N.D.	4279 $\pm$ 557 (1)	0.56 $\pm$ 0.06 (1)	N.D.	N.D.	N.D.	N.D.
D2-T7.39A	244 $\pm$ 33.3 (1.9 $\downarrow$)	81 $\pm$ 6.5 (1.03 $\downarrow$)	212 $\pm$ 21.9 (1.5 $\downarrow$)	N.D.	3770 $\pm$ 426 (1.1 $\downarrow$)	0.83 $\pm$ 0.17 (1.5 $\uparrow$)	N.D.	N.D.	N.D.	N.D.

N.D., not determined.

Fig. 3. SBA structures ordered horizontally by the degree of observed binding affinity enhancement upon D4-T7.39A substitution. Polar substituents on the benzamide ring, located both *meta* (5-) and *para* (4-) to the amide, seem to play a critical role in the observed SAR. It is noteworthy that in the pose that we believe is most likely for these ligands the benzamide ring substituents do not directly interact with the 7.39 position. Rather, in this mode, they orient the ring such that the distal pyrrolidino or diethylamine may form hydrophobic contacts with residues on TM2, TM3, or TM7.

to the D4-T7.39A mutation. This particular ligand, however, diverges structurally from the other SBAs tested (Fig. 3) because of the unique position of the amine nitrogen within

the pyrrolidine ring and the benzyl substituent extending from this nitrogen. Nemonapride was thus not considered further with respect to the observed SAR. With the exception

Fig. 4. Representative SBA poses after docking into D4 receptor models. Representative poses shown here were selected by criterion of lowest RMSD with the known binding position of eticlopride pharmacophore atoms. A, T7.39A-sensitive SBAs with polar 4- and 5-benzamide ring substituents docked into D4-WT. B, T7.39A-insensitive SBAs with nonpolar benzamide ring substituents docked into D4-WT. C, T7.39A-sensitive SBAs docked into D4-T7.39A. D, T7.39A-insensitive SBAs docked into D4-T7.39A. Selected contact residues are shown as sticks and numbered by the Ballesteros-Weinstein index. TM1 to TM7 are colored by spectrum from blue to red.

Fig. 5. (I and II) Complete ligand-residue interaction energy maps for docked SBAs. Positive (unfavorable) energies are shown in shades of red; negative (favorable) interaction energies are shown in shades of blue. A, the electrostatic component of the computed interaction energy for the SBAs at the WT and T7.39A mutant. B, the van der Waals component. C, the total interaction energy (the sum of electrostatic and van der Waals components). D, the residue-specific total interaction energy differences for each ligand with respect to the WT and T7.39A mutant constructs. Here, red and blue indicate more favorable interactions with WT and T7.39A, respectively.

E

D

C

B

A

II

Fig. 5. (I and II) Continued.

of eticlopride, which had an intermediate increase in affinity, SBAs lacking polar groups at these positions, including nafadotride, remoxipride, and raclopride, were insensitive to the T7.39A substitution (Table 6; Fig. 3).

In contrast to the D4 subtype, the SBAs tested were uniformly insensitive to the T7.39A substitution in D2 with respect to affinity changes. This suggests that interactions between position 7.39 and the polar ring substituents are not likely to be mediated through direct contact because the loss of threonine's side-chain hydroxyl group would be anticipated to reduce affinity with directly interacting polar groups. This was affirmed by the binding mode observed for eticlopride in the D3 crystal structure (Supplemental Fig. 1) where only the hydrophobic pyrrolidinyl group and/or alkyl substituents of this ring made contacts near Thr7.39 and the benzamide ring *p*- and *m*-substituents were directed toward TM5 and TM6.

The position of conserved residue Thr7.39 in our dopamine receptor structural models is adjacent to TM2 and TM3. It has been shown previously that residue positions in these TMs are critical determinants for D2/D4 selectivity in the 1,4-DAP class of ligands (Kortagere et al., 2004; Ericksen et al., 2009). In a previous study, moderate enhancements in the affinity for the SBAs raclopride and nafadotride were observed in D4 constructs with multiple D2 residues swapped into the TM3 cleft-facing positions, but not for TM2 cleft-facing substitutions (Schetz and Sibley, 2000). From previously published contact measurements of the crystalline structure of the D3-eticlopride complex (Chien et al., 2010), eticlopride makes relatively few contacts with positions Val2.61 (Phe2.61 in D4) and Phe3.28 (Leu3.28 in D4). It is likely that SBAs do not make extensive contacts with residues in TM2 and occupy a binding mode that is then distinct from those we have proposed previously for the 1,4-DAPs (Kortagere et al., 2004; Cummings et al., 2009) where one of the aryl substituents is directed prominently into the TM2/TM3 interface (Kortagere et al., 2004; Cummings et al., 2009; Ericksen et al., 2009).

To gain a molecular perspective on the role of position 7.39 on SBA selectivity for the D4 receptor, we docked a set of eight SBAs with ranging sensitivity toward the T7.39A mutation into D4-WT and D4-T7.39A receptor models. Among the resulting poses, we obtain a cluster where the benzamide ring is oriented into the orthosteric pocket between TM3, TM5, and TM6, and their pyrrolidinyl/diethyl amine end is oriented to form the expected H-bond reinforced ionic interactions with Asp3.32 (Floresca and Schetz, 2004) and hydrophobic interactions with the γ -methyl of Thr7.39 (Fig. 4; Supplemental Fig. 2). This cluster of poses matches the mode of binding observed for eticlopride in the D3 crystal structure (Chien et al., 2010) (Supplemental Fig. 1). The orthosteric pocket of the cleft is the region of occupancy expected for the catechol ring of catecholamine agonists in amine receptors and is observed for the ring structures of antagonists (or partial inverse agonists) cyanopindolol [(*S*)-4-[3-(*tert*-butylamino)-2-hydroxypropoxy]-1*H*-indole-2-carbonitrile] and carazolol [1-(9*H*-carbazol-4-yloxy)-3-(propan-2-ylamino)propan-2-ol] in β 1 and β 2 adrenergic receptor crystal structures, respectively (Cherezov et al., 2007; Warne et al., 2008). To determine contact residues in D2-like receptors, we selected a representative pose for each SBA as the one being most similar in position to that of eticlopride in the D3 crystal structure. After aligning our D4 mod-

els to the D3 coordinates of the D3-eticlopride crystal structure complex, the representative pose was taken as that with the lowest RMSD with respect to the pharmacophore atoms (amine, amide group, and benzamide ring carbons) shared with eticlopride in the D3 structure. In every case except for amisulpride, a low RMSD pose (< 0.75 Å) was identified that clearly matched the eticlopride pose in the D3 structure. Because we failed to find a suitable match for amisulpride, a favorable (low energy), but slightly different, mode of binding was used for amisulpride. This pose maintains the expected overall orientation and H-bond reinforced ionic interaction with D3.32; however, it is noticeably deeper in the pocket than eticlopride in the D3 structure, diverging 3.8 and 4.1 Å RMSD from eticlopride in D3 in D4-WT and D4-T7.39A, respectively (Supplemental Fig. 3). The shift in binding position is likely caused by a steric conflict arising from the large 5-ethylsulfonyl group on the benzamide ring.

Residue-ligand interaction energy maps were then calculated for the representative pose for each SBA ligand (Fig. 5), separating the interactions into electrostatic (Fig. 5, I and II, A) and vdW (Fig. 5, I and II, B) components, and residue interaction energy totals (Fig. 5, I and II, C). Inspection of the contact maps confirms that the contact distributions are not only similar to those reported for eticlopride in the eticlopride-D3 crystalline complex, but also consistent among the different SBAs and between the wild-type and mutant D4 constructs. A banded pattern of interaction is evident for the extracellular portions of TM2, TM3, TM5, TM6, and TM7, reflecting ligand contacts with the regular intervals of cleft-facing segments of the helical structures. Only very weak interaction energy contributions are made from residues in TM1 and TM4. It is noteworthy that the net contribution made by vdW components to the interaction energy total (E_{total}) are significant (23.4%) despite the fact that electrostatic interactions (76.6%) are longer range and fall off much more slowly (with distance squared) than vdW interactions (with distance to the sixth power). Moreover, if the strong H-bond reinforced ionic interaction with Asp3.32 is neglected, the vdW interactions then comprise approximately 60% of the total interaction energy. Because of the observed mixtures of favorable (positive) and unfavorable (negative) electrostatic interactions that negate each other's contribution to the total energy, a net electrostatic contribution to the total interaction energy predominates only at TM3 and TM7. The vdW contributions are more significant at TM2 and TM6 because of the aromatic cluster of receptor residues. Both types of interactions are significant with the subtype-variable EL2 segment (C-terminal to the disulfide cysteine) and the connecting TM5. Residues with significant vdW interactions are fewer but generally interact favorably with the exception of positions 7.39 and 7.43 (Fig. 5, I and II, B). As expected, the vdW repulsion from Thr7.39 in D4-WT seems to be mitigated by the T7.39A substitution in the most sensitive SBAs. However, this does not explain the observed SAR because the T7.39A mutation also diminishes the site's favorable electrostatic interactions with the sensitive ligands, offsetting the energetic reward for reducing the apparent vdW clashes. From the interaction energy maps (Fig. 5, I and II, A–C), we computed the changes in residue-ligand interaction energies (difference maps) for each SBA between D4-WT and D4-T7.39A receptors (Fig. 5, I and II, D). From the difference maps, it is confirmed that the interactions specifically with 7.39 are, in terms of net interaction ener-

gies, made less favorable by the mutation (Fig. 5, I and II, D). It is noteworthy that in the case of the mutation-sensitive SBAs, the mutation produces more favorable interactions with residues one intracellular helical turn (below) from 7.39 (7.41–7.44) and less favorable electrostatic interactions with residues one extracellular helical turn (above) from 7.39. In contrast, the insensitive SBAs show an opposite trend in the mutant where some steric clashes are exacerbated with contact residues one helical turn below 7.39, whereas electrostatic interactions are enhanced one helical turn above 7.39 (Fig. 5II, D).

Although an account of the TM7 interactions with the ligand does not provide a simple basis for the observed SAR, favorable benzamide ring interactions at TM5 and TM6 compensate for weakened favorable electrostatic interactions at TM7 and favor the mutation-sensitive SBAs in the T7.39A construct as indicated by the total interaction energies summed over all residues in Fig. 5, I and II, D. Note that the compensatory changes in TM5 and TM6 total interaction energy components in the difference map vary substantially with the ligands, reflecting the variation in ring substitutions among the SBAs tested. These results suggest that the SAR with respect to the T7.39A effect on SBAs arises as a consequence of the complexity of interactions between the ring substituents and TM5 and TM6. Encouragingly, the mutation-induced shifts in net energy changes calculated for each pose approximately fit with the observed rank order of affinity shifts for the SBAs, supporting our assumption that most of these ligands bind in a very similar orientation to that of eticlopride in the D3 crystal structure.

Discussion

In our analysis of the role of conserved residue Thr7.39 with regard to D2/D4 dopamine receptor ligand selectivity for several different ligand chemotypes, we found the benzazepines, 1,4-DAPs, and dopamine exhibit weak sensitivity to the alanine substitution, showing small or no reductions in affinity (less than 5-fold). However, particular SBAs were sensitive to the T7.39A substitution, exhibiting improved affinity. Limited to the D4 subtype, this effect seems to depend on the benzamide ring's 4- and 5-substituents. In SBAs with the strongest affinity increases (11- to 21-fold), a polar ring substituent, such as sulfone or sulfonamide, occupied the 5-position as in the cases of amisulpride, sulpiride, and tiapride or a primary amine occupied the 4-position as in the case of bromopride and metoclopramide, or both as in the case of amisulpride.

After docking a set of SBAs with varied experimental response to the T7.39A substitution in D4, we selected a pose for each SBA in each construct that best matched eticlopride in the D3 crystal structure (Chien et al., 2010), except in the case of amisulpride where an alternative mode was observed. For the selected poses, we measured a consistent pattern of interactions between SBAs and cleft residues. In this mode, the benzamide ring is directed into the primary orthosteric cleft lined by residues of TM3, TM5, TM6, and EL2. An amide substituent bridges the ring to a tertiary amine group that makes an H-bond reinforced salt bridge (reinforced ionic bond) with D3.32. The amine group's ethyl groups or pyrrolidyl ring interacts with residues of TM2, TM3, and TM7 (Figs. 4 and 5, I and II, A-C), primarily with Met3.29,

Thr7.39, Val3.33, Tyr7.43, Glu2.65, Leu3.28, and Phe2.61, in decreasing order of total absolute interaction energy (Table 7). Absolute values assess the overall strength of interaction, whether favorable or unfavorable.

Here, we consider interaction energies as approximations to residue contributions to the ligand binding free energy because we apply one fixed representative pose to our analysis rather than a representative ensemble reflecting a more physical distribution of states from an equilibrated bulk system at physiological temperature corresponding to experimental conditions. Entropic contributions and the important solvation, ion, and membrane effects were also neglected. For further studies, we plan to apply free-energy methods with molecular dynamics simulations to look more precisely at the molecular determinants of subtype selectivity. However, the energy profiles computed here are useful in that they indicate key interaction residues for the SBAs as well as those with interactions that change in response to the T7.39A mutation.

To explain the D4-specific SAR, we first examined subtype differences in the local structural environment of Thr7.39. This environment includes the TM2/3 microdomain region established to confer D2/4 selectivities for numerous 1,4-DAPs (Kortagere et al., 2004). D4 residues in this region that differ from the D2/3 subtypes include Phe2.61, Leu3.28, Met3.29, and Val7.35, which provide contact surfaces for the SBA moiety pyrrolidine ring moiety or alkyl substituents of the tertiary amines. The D4 receptor's Val7.35 seemed a likely candidate for conferring the SAR because a tyrosine occupies 7.35 in the D2/3 subtypes. However, T7.39A substitution had little effect on SBA affinities in D2, suggesting that any steric conflicts arising from the bulky tyrosine ring are not mitigated by T7.39A substitution. In addition, the enhanced affinity does not depend on the hydrophobic substituents of the tertiary amine groups of the

TABLE 7

SBA contact residues ranked by mutation-induced interaction energy changes (ΔE_{total}) with corresponding dopamine receptor subtype residue identities

Bold type indicates a significant difference in side chain polarity. Italic type indicates minor residue variation between D2 and D4 subtypes.

D2	D3	D4	Index	D4 Residue	$\Delta E_{\text{total}}^*$	E_{total}^{**}
<i>kcal / mol</i>						
ASP	ASP	ASP	3.32	112	40.0	1367.5
TYR	TYR	TYR	7.43	358	31.9	47.8
ASN	ASN	ARG	6.58	337	27.8	43.7
ALA	SER	GLU	183 ^{(C + 3)***}	183	24.0	40.7
THR	THR	THR	7.39	354	23.5	68.7
VAL	VAL	VAL	5.39	188	20.4	33.3
PHE	PHE	PHE	6.52	331	19.0	29.4
TRP	TRP	TRP	6.48	327	17.3	36.1
GLY	GLY	GLY	7.42	357	17.3	59.8
PHE	PHE	PHE	6.51	330	14.6	80.9
VAL	VAL	<i>MET</i>	3.29	109	14.2	79.8
HIS	HIS	HIS	6.55	334	12.2	116.2
SER	SER	SER	5.42	191	10.8	73.2
SER	SER	SER	5.43	192	9.3	44.3
<i>ILE</i>	<i>ILE</i>	<i>LEU</i>	182 ^{(C + 2)***}	182	8.1	39.8
CYS	CYS	CYS	180 ^{(C + 0)***}	180	7.6	54.0
VAL	VAL	<i>PHE</i>	2.61	88	6.6	16.3
<i>PHE</i>	<i>THR</i>	<i>VAL</i>	7.38	353	6.3	21.1
VAL	VAL	VAL	3.33	113	6.2	57.7
GLU	GLU	GLU	2.65	92	6.1	35.4

* ΔE_{total} absolute values for a given residue, summed over all selected SBA docked poses.

** Sum of E_{total} absolute values for a given residue, summed over all selected SBA poses.

*** Residues in EL2 are designated by position with respect to the disulfide bridge cysteine 180^(C + 0).

SBA. The effect, although achieved through the T7.39A mutation, instead depends on the SBA ring substituents that interact with D4-specific residues in TM5/6 on the opposite side of the binding cleft.

A comparison of aligned subtype binding site sequences shows D4 diverges substantially from the D2/3 subtypes. The contact positions that exhibit greatest mutation-induced shift in interaction energy (ΔE_{total}) are Asp3.32, Tyr7.43, Arg6.58, Glu183^(C + 3), Thr7.39, and Val5.39 (the top 20 are listed in order in Table 7 and reordered by E_{total} in Table 8). Among positions having both strong E_{total} and ΔE_{total} , Arg6.58 (Asn6.58 in the D2/3 subtypes) and Glu183^(C + 3) (Ala/Ser in D2/3) differ most between D2 and D4 subtypes in chemical properties (black highlights in Fig. 5E and bold type in Tables 7 and 8). Based on our interaction energy analysis, positions that potentially confer subtype-selectivity of the SAR involving the SBAs also include Met3.29[Val], Val7.35[Tyr], Leu182^(C + 2)[Ile], Val7.38[Phe], and Phe2.61[Val] (D2 subtype residues in brackets).

Our ligand-receptor interaction maps (Fig. 5, I and II, A–C) show abundant SBA interactions with conserved residues on TM5 at Val5.39, Ser5.42, and Ser5.43 and TM6 at Trp6.48, Phe6.51, Phe6.52, and His6.55. Isolating the interactions for only the 4- and 5-substituents (Supplemental Tables 1 and 2) reveals contacts with TM5, TM6, and EL2. Trends in interaction energy arising merely from these substituents generally follow those of the total interaction energies for the entire ligand (Tables 7 and 8), further suggesting that these substituents play a key role in the SAR. In previous studies with the D2/D4 subtypes involving the SBA ligands, remoxipride, sulpiride, raclopride, and epidipride, variable responses were observed for mutations at each of the three conserved serines in TM5 of dopamine receptors (for review see Floresca and Schetz, 2004). D2-S5.42A showed a 9.5-fold increase in affinity for remoxipride, whereas D2-C3.36S+S5.42C exhibited an 11.4-fold

increase in affinity for sulpiride relative to the D2-C3.36S mutant. D2-S5.43A showed 4.6- and 3.6-fold weakened affinities for sulpiride and epidipride, respectively. The D2-S5.46A substitution produced 6.8- and 4.2-fold losses in affinity for raclopride in two different reports, and D2-C3.36S+S5.46C caused a 14-fold weakened affinity for sulpiride relative to the D2-C3.36S mutant. In the D4 subtype, we recently reported 5.1- and 4.7-fold decreases in sulpiride affinity at D4-S5.42A and D4-S5.46A, respectively (Cummings et al., 2010). In regard to TM6 mutations, a D2-H6.55L mutant was reported to have moderately reduced affinity (~8-fold) for sulpiride and sultopride, which are closely related analogs, each having either a 5-position sulfone or sulfonamide on the substituted benzamide ring (Woodward et al., 1994). Likewise, a D2-C3.36S+H6.55C mutant has a moderately decreased (7.7-fold) affinity for sulpiride relative to the D2-C3.36S mutant to which it was compared. Although a D2-F6.52A mutant was unable to bind a number of radioligands, including [³H]raclopride, only a small reduction (3.4-fold) in sulpiride affinity was observed for the D2-C3.36S+F6.52C mutant relative to a D2-C3.36S mutant.

To explain the affinity enhancements in D4 arising from T7.39A substitution, we propose that H-bonds between the side chains of the conserved TM5 serines and the SBAs' polar 4- and 5-ring substituents impose restraints on the ligand position that, in turn, produce a distal steric conflict between pyrrolidinyl/diethylamine moieties and Thr7.39 in the mutation-sensitive SBAs. Contacts with the conserved serines are more abundant for the T7.39A-sensitive ligands in the wild-type receptor (Fig. 5, I and II, A and B), which might suggest some crowding in this region. The T7.39A substitution increases accessible volume for the SBAs' hydrophobic pyrrolidinyl/diethylamine moieties to occupy. The T7.39A-insensitive SBAs are less constrained because of the absence or apolarity of equivalent ring substituents, which probably participate in nonspecific interactions with the TM5/TM6 contacts and are not as spatially constrained by geometrically dependent H-bonding. The lack of specific constraints on the benzamide ring position affords more "wobble room," which manifests as insensitivity toward steric changes at position 7.39.

It is noteworthy that, although contact residues in TM5 and TM6 are conserved across the D2-like subtypes and cannot account for the D4 subtype-selective phenomenon, many residues in the local environment of the contact residues do indeed differ. TM5 positions Asp5.37[Ala] and Tyr5.38[Phe] have charged and polar side chains in D4 subtype. Adjacent positions in TM6 include Thr6.57[Leu] and Arg6.58[Asn] in the D4 subtype. Furthermore, positions on the C-terminal segment after the disulfide bridge at Cys^(Cys + 0) of EL2 leading into TM5 hold charged residues Glu183^(Cys + 3)[Asn], Asp184^(Cys + 4)[Phe], and Arg185^(Cys + 5)[Ala]. This region in D4 forming the extracellular lid of the orthosteric pocket is significantly more polar and charged and perhaps accounts for subtype variability in the role of polar 4- and 5-substituents on the SBAs. Moreover, the subtype residue differences in this region probably influence water accessibility to TM5 and TM6 cleft residues with polar side chains (His6.55, Ser5.42, Ser5.43, and Ser5.46) and local cleft water structure and H-bonding networks that play into the D4 subtype-selective effect for SBAs with polar substituents.

In conclusion, we have examined the role of conserved TM7 position 7.39 in D4 dopamine receptor recognition of some

TABLE 8

SBA contact residues ranked by total interaction energies (E_{total}) with corresponding dopamine receptor subtype residue identities

Bold type indicates a significant difference in side chain polarity. Italic type indicates minor residue variation between D2 and D4 subtypes.

D2	D3	D4	Index	D4 Residue	$\Delta E_{\text{total}}^*$	E_{total}^{**}
kcal/mol						
ASP	ASP	ASP	3.32	112	40.0	1367.5
HIS	HIS	HIS	6.55	334	12.2	116.2
PHE	PHE	PHE	6.51	330	14.6	80.9
VAL	VAL	<i>MET</i>	3.29	109	14.2	79.8
SER	SER	SER	5.42	191	10.8	73.2
THR	THR	THR	7.39	354	23.5	68.7
GLY	GLY	GLY	7.42	357	17.3	59.8
VAL	VAL	VAL	3.33	113	6.2	57.7
CYS	CYS	CYS	180 ^(C + 0) ***	180	7.6	54.0
TYR	TYR	TYR	7.43	358	31.9	47.8
SER	SER	SER	5.43	192	9.3	44.3
ASN	ASN	ARG	6.58	337	27.8	43.7
TYR	TYR	VAL	7.35	350	4.8	41.9
ALA	SER	GLU	183 ^(C + 3) ***	183	24.0	40.7
<i>ILE</i>	<i>ILE</i>	<i>LEU</i>	182 ^(C + 2) ***	182	8.1	39.8
SER	SER	SER	7.36	351	5.6	37.2
TRP	TRP	TRP	6.48	327	17.3	36.1
ILE	SER	CYS	181 ^(C + 1) ***	181	6.0	35.5
GLU	GLU	GLU	2.65	92	6.1	35.4
VAL	VAL	VAL	5.39	188	20.4	33.3

* ΔE_{total} absolute values for a given residue, summed over all selected SBA docked poses.

** E_{total} absolute values for a given residue, summed over all selected SBA poses.

*** Residues in EL2 are designated by position with respect to the disulfide bridge cysteine 180^(C + 0).

therapeutically relevant ligand chemotypes. In the SBA class of ligands, we have uncovered a D4-specific SAR between substitution pattern on the benzamide ring and sensitivity to T7.39A substitution, leading to enhanced binding affinity for specific SBAs. SBA contact patterns in our models, which are supported by our experiments and the available D3-eticlopride structure, imply a subtype-dependent mechanism whereby the pattern of substitutions around the benzamide ring leads to a different configuration of interactions with conserved contact residues in TM5 and TM6. This in turn affects hydrophobic pyrrolidinyl/diethylamine group interactions in the secondary cleft region between TM2, TM3, and TM7, accounting for sensitivity to the alanine substitution at Thr7.39. Experimental analysis of a conserved contact residue, Thr7.39, in the context of structural maps of SBA-receptor interactions has provided a basis for understanding and perhaps modulating dopamine receptor subtype selectivity for this particular class of ligands.

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Authorship Contributions

Participated in research design: Ericksen and Schetz.

Conducted experiments: Ericksen, Cummings, Teer, Amdani, and Schetz.

Contributed new reagents or analytic tools: Ericksen and Schetz.

Performed data analysis: Ericksen, Cummings and Schetz.

Wrote or contributed to the writing of the manuscript: Ericksen, Cummings and Schetz.

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