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Receptor conformations involved in dopamine D_{2L} receptor functional selectivity induced by selected transmembrane-5 serine mutations

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Mutation-induced changes in functional selectivity

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List of Abbreviations

AC	adenylate cyclase
AA	arachidonic acid release
DA	dopamine
DHX	dihydroxidine; (trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo- [a]phenanthridine)
DNS	dinapsoline; 8,9-dihydroxy-2,3,7,11b-tetrahydro-1H-naph[1,2,3- <i>de</i>]- isoquinoline
DNX	dinoxyline; 8,9-dihydroxy-1,2,3,11b-tetrahydrochromeno[4,3,2,- <i>de</i>]- isoquinoline
GIRK	G protein inward-rectifying potassium channel
GPCR	G protein-coupled receptor
HB	hydrogen bond
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	Isobutylmethylxanthine
$K_{0.5}$	apparent affinity constant ($n_H < 1.0$); equals K_I when $n_H = 1$.
MAPK	mitogen-activated protein kinase
$*n_H$	Hill coefficient

ABSTRACT

Although functional selectivity is now widely accepted, the molecular basis is poorly understood. We have studied how aspects of transmembrane region 5 of the dopamine D_{2L} receptor interacts with three rationally selected rigid ligands (dihydropyridine, dinapsoline, and dinoxaline) and the reference compounds dopamine and quinpirole. As was expected from homology modeling, mutation of three TM5 serine residues to alanine (S5.42A, S5.43A, S5.46A) had little effect on antagonist affinity. All three mutations decreased the affinity of the agonist ligands to different degrees, with S5.46A being somewhat less affected. Four functions [adenylate cyclase (AC), Erk1/2 phosphorylation (MAPK), arachidonic acid release (AA), GTP γ S binding (GTP γ S)] were assessed. The intrinsic activity (IA) of quinpirole was unaffected by any of the mutations, whereas S5.42A and S5.46A mutations abolished the activity of dopamine and the three rigid ligands, although dihydropyridine retained IA at MAPK function only with S5.42A. Remarkably, S5.43A did not markedly affect IA for AC and MAPK for any of the ligands, but eliminated AA activity for dinapsoline and dihydropyridine, but not dinoxaline. These data suggest that this mutation did not disrupt the overall conformation or signaling ability of the mutant receptors, but differentially affected ligand activation. Computational studies indicate that there are multiple receptor conformations stabilized by these D₂ agonists. This has led to models showing the stabilized conformations and interhelical and receptor-ligand contacts corresponding to the different activation pathways stabilized by various agonists. These data provide a basis for understanding D_{2L} functional selectivity and rationally discovering functionally selective D₂ drugs.

INTRODUCTION

Functional selectivity is the phenomenon by which the binding of a ligand to a receptor results in markedly different levels of activation (or lack thereof) of one or several of the signaling pathways linked to the targeted receptor. Although this signaling bias runs counter to classical concepts of drug-receptor mechanisms, it has become firmly established and has been demonstrated for dozens of receptors (Neve, 2009; Urban *et al.*, 2007). As well as being heuristically interesting, it is also generally appreciated that functionally selective ligands with the “correct” bias may yield improved therapeutic indices versus drugs that are “typical” agonists or antagonists (Mailman, 2007). It is generally thought that the functionally selected properties of a ligand are a result of the differential stabilization and/or induction of active states of the target receptor that are associated with specific signaling pathways (Urban *et al.*, 2007; Kenakin, 1995; Kenakin, 2007). There is, however, little data that have explored how this may occur.

One of the earliest demonstrations of functional selectivity was with the dopamine D₂ receptor (Lawler *et al.*, 1994; Lawler *et al.*, 1999). In particular, studies with a series of relatively rigid, conformationally-restrained D₂ ligands showed extreme bias in signaling, in some cases as extreme as full agonist and pure antagonist (Kilts *et al.*, 2002; Mottola *et al.*, 2002; Mottola *et al.*, 1991; Gay *et al.*, 2004). Thus, in Chinese hamster ovary (CHO) cells expressing hD_{2L} receptors, Gay *et al.* (2004) reported that dinapsoline (DNS) was a full agonist at several functions [e.g., fully inhibiting cAMP accumulation (AC) and fully stimulating p44/p42 MAP kinase phosphorylation (MAPK)], yet was a partial agonist at stimulating G protein-coupled inward rectifying potassium channels (GIRK) and at D_{2L}-mediated release of [³H]-arachidonic acid (AA). Conversely,

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dinoxylene (DNX) was a full agonist at all four effector pathways (Gay *et al.*, 2004). These data are noteworthy because there are only subtle differences in the chemical structures of DNS and DNX (see Figure 1).

Subtle structural difference in a ligand may lead to functional selectivity (Gay *et al.*, 2004; Ryman-Rasmussen *et al.*, 2005). Thus, we hypothesized that modification of the D_{2L} receptor in regions where such ligands could be informative about how functionally selective signaling occurs. The current experiments address this hypothesis by studying the role of specific binding interactions that occur between the D_{2L} receptor and three rigid analogs, dihydroxidine (DHX) (Lovenberg *et al.*, 1989), DNS (Ghosh *et al.*, 1996), and DNX (Grubbs *et al.*, 2004). These ligands were originally designed as novel D₁ agonists in which the accessory phenyl ring was expected to confer both high D₁ affinity and high D₁:D₂ selectivity (Nichols, 1983; Charifson *et al.*, 1989; Mottola *et al.*, 1996). Unexpectedly, all three compounds also had significant D₂ affinity, leading to the discovery of D₂ functionally selective signaling for DHX and DNS (Kilts *et al.*, 2002; Mottola *et al.*, 2002; Gay *et al.*, 2004).

In the current study, computational approaches were used to hypothesize specific binding interactions within each ligand-receptor complex. The affinity and functional properties of the probe and reference ligands was then assessed using four distinct functional endpoints. The resulting data are consistent with the hypothesis that subtle changes in ligand-specific interactions induced by mutation of selected amino acids of the receptor may stabilize and/or induce certain receptor conformations that lead to functional selectivity (i.e., differential activation of one or more signaling pathways).

MATERIALS AND METHODS

Materials

Dinapsoline [(+)-8,9-dihydroxy-2,3,7,11b-tetrahydro-1H-naphth[1,2,3-*de*]isoquinoline], dinoxyline [(+)-8,9-dihydroxy-1,2,3,11b-tetrahydrochromeno[4,3,2,-*de*]isoquinoline], and dihydrexidine [trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[*a*]phenanthridine] were synthesized following published procedures (Brewster *et al.*, 1990; Ghosh *et al.*, 1996; Grubbs *et al.*, 2004). Dopamine, quinpirole, isobutylmethylxanthine, EDTA, dithiothreitol, sucrose, pepstatin A, leupeptin, PMSF, fetal bovine serum and other standard chemical reagents were purchased from Sigma/RBI (St. Louis MO & Natick, MA). [³H]-N-methylspiperone, [³H]-arachidonic acid [or [5, 6, 8, 9, 11, 12, 14, 15-³H(N)]-AA], and [³⁵S]-GTPγS were purchased from Amersham Biosciences Inc. (Piscataway, NJ). [¹²⁵I] for cAMP assays was purchased from NEN/Perkin Elmer (Boston, MA), and processed as described by Brown *et al.* (2009). cAMP primary antibody was obtained from Dr. Gary Brooker (George Washington University, Washington DC) and secondary antibody, rabbit anti-goat IgG, was purchased from Advanced Magnetics (Cambridge, MA). HEPES buffer was purchased from Research Organics (Cleveland, OH). Ham's F-12, Opti-Mem, penicillin, streptomycin, primers and lipofectamine were purchased from Invitrogen (Carlsbad, CA). Hygromycin B was purchased from Roche Applied Science (Indianapolis, IN). Primary antibody to phospho-p44/p42 MAPK and secondary antibody, anti-rabbit HRP-conjugated, were purchased from Cell Signaling Technology Inc. (Beverly, MA).

Molecular biology and cell culture.

CHO hD_{2L} wild type and mutant cells were maintained in Ham's F-12 media supplemented with 10% fetal bovine serum, 100X penicillin-streptomycin, and 100 µg/mL hygromycin. Mutant cell lines were constructed using the pcDNA5/FRT plasmid obtained from Invitrogen. Following subcloning of the hD_{2L} receptor into the plasmid, point mutations were introduced using standard PCR techniques. Stable transfections of point mutants into CHO K1 cells were conducted using modifications of a previously published protocol (Milligan, 1999). Throughout this paper, the identification of the mutated amino acid residue will be made using universal notation proposed by Ballesteros and Weinstein (1995) in which the first number denotes the TM helix in which the residue is located, and the second number pair is the location of the residue under question relative to the most conserved residue for that helix. The residues we studied (listed in both universal notation and absolute position) were S5.42A (S193A), S5.43A (S194A), and S5.46A (S197A).

Radioreceptor assays

Membranes for radioreceptor assays were prepared by rinsing cells with phosphate-buffered saline, and then lysing with a solution containing 2 mM HEPES, 2 mM EDTA, 1 mM dithiothreitol, 1 µg/mL pepstatin A, 0.5 µg/mL leupeptin, and 0.05 µg/mL PMSF. Cell fragments were scraped, homogenized, and centrifuged at 30,000 g for 30 min. Following centrifugation, cell pellets were resuspended, homogenized and placed into storage buffer (50 mM HEPES, 0.32 M sucrose, 1 µg/mL pepstatin A, 0.5 µg/mL leupeptin, and 0.05 µg/mL PMSF) and stored at -80 °C. Saturation binding assays were conducted using protocols described previously (Gay *et al.*, 2004) but with varying

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concentrations of [³H]-N-methyl-spiperone to determine the B_{max} and K_D for each membrane preparation (e.g. hD_{2L} WT; hD_{2L} S5.42A, etc.). Domperidone (10 μM) was used to define non-specific binding. Competition binding assays also utilized [³H]-N-methylspiperone.

Because these experiments incorporated detailed functional analysis (*vide infra*) including GTPγS activation, we did not make use of a non-hydrolyzable GTP analog like Gpp(NH)p to estimate the proportion of receptors that were G protein-coupled. In retrospect, this might have been useful and should be considered in future testing of the hypotheses outlined in the Discussion.

GTPγS assay

Measurement of [³⁵S]-GTPγS binding was determined as described previously (Shapiro *et al.*, 2003), with non-specific binding defined by 10 μM cold GTPγS. Assay tubes contained 150-200 pM [³⁵S]-GTPγS, binding buffer (50 mM HEPES, 100 mM NaCl, 4 mM MgCl₂, 1 mM EDTA, 0.1% BSA, 0.1% ascorbic acid, pH 7.4 with NaOH), 10 μM GDP, and varying concentrations of agonists and/or antagonist. Membranes (approximately 100 μg protein/mL) were incubated with test compounds for 15 min at 30 °C before addition of [³⁵S]-GTPγS. After an additional 30 min incubation, the assay was terminated by filtration (Packard Filtermate 190 harvester) with ice cold wash buffer (50 mM HEPES, 4 mM mgCl₂, pH 7.4 with KOH), and radioactivity quantified by liquid scintillation spectrometry (Packard TopCount NXT).

cAMP accumulation assay

Measurement of dopamine receptor agonist inhibition of forskolin-stimulated cAMP accumulation was performed in whole cell preparations as described previously

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(Gay *et al.*, 2004). In brief, CHO cells were seeded in 24-well plates at a density of 2.5×10^6 cells/well, and grown for 48 hr in Ham's F-12 media supplemented with 10% fetal bovine serum and 100X penicillin-streptomycin. Cells were preincubated for 5 min prior in fresh media (serum-free media containing 25 mM HEPES, 500 μ M isobutylmethylxanthine, and 0.1 % ascorbic acid) at 37 °C. Assay medium then was aspirated, and fresh assay media containing forskolin and/or various concentrations of the test compounds was added. The plates were incubated for 15 min at 37 °C, cells rinsed with fresh assay medium, aspirated, and the reaction halted using 0.1 N HCl. The cAMP was quantified using a modified radioimmunoassay previously described (Harper and Brooker, 1975).

MAP kinase assay

Measurement of dopamine receptor agonist stimulation of p44/p42 MAPK was performed in whole cell preparations by modifying a previously published protocol (Versteeg *et al.*, 2000). CHO cells were seeded in 96-well plates at a density of 5×10^6 cells/well and grown for 48 hr in Ham's F-12 media supplemented with 10% fetal bovine serum at 37 C. Cells were serum starved for 6 hr prior to stimulation. Appropriate drug dilutions of the test compounds were added to each well at a volume of 100 μ L for 10 min. The reaction then was terminated, and the cells fixed by aspirating the wells and adding 100 μ L of 4% formaldehyde PBS solution for 20 min. Cells were washed three times with 100 μ L wash buffer (0.1% Triton X-100/PBS solution), followed by a 20 min incubation with 0.6% H₂O₂ Triton/PBS solution to quench endogenous peroxidases. After washing the cells three times again with wash buffer, and after a 1 h incubation with 10% BSA in Triton/PBS solution (to block nonspecific antibody binding), cells were incubated

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overnight (about 12 hr) with a 1:250 dilution of PhosphoPlus® p44/42 primary antibody in the Triton/PBS solution (100 µL) containing 5% BSA at 4 °C. Cells were washed three times with wash buffer for five min, and incubated with 100 µL HRP-conjugated goat anti-rabbit secondary antibody (1:100 dilution) with 5% BSA at room temperature for 1 h. Again, cells were washed three times with wash buffer for five min, and then twice with PBS. Cells were then incubated with 50 µL of an *o*-phenylenediamine (OPD) solution (0.4 mg/mL OPD, 17.8 mg/mL Na₂HPO₄·7H₂O, 7.3 mg/mL citric acid and 0.015% H₂O₂) for 15 min at room temperature in the dark. The reaction was terminated by the addition of 25 µL of 1 M H₂SO₄ that causes a light-to-dark orange color change (A₄₉₀ - A₆₅₀) that is proportional to phosphorylation.

Arachidonic acid assay

Measurement of dopamine receptor agonist potentiation of ATP-stimulated [³H]-arachidonic acid (AA) release was measured in whole cell preparations using modifications of a previously published method (Berg *et al.*, 1998). CHO cells were seeded in 24-well plates at a density of 5 x 10⁵ cells/well and grown for 24 hr in Ham's F-12 media supplemented with 10% fetal bovine serum at 37 C. Cells are serum starved with 500 µL of serum free Ham's F-12 containing 0.5 µCi/mL [³H]AA for 5 hr at 37 C. Ten-µL aliquots were removed to compare with the original tritiated loading media to determine the time course and total cellular uptake of [³H]-AA. Cells were washed three-times for 5 min each with Hank's balanced salt solution (HBSS) containing 0.5% fatty acid-free BSA and antagonists for respective wells (500 µL/well/wash). Cells then were incubated with agonists for 15 min with or without ATP dissolved in HBSS/BSA (ATP being added last and in timed increments of 5 sec between wells).

Data and Statistical Analysis

Data were analyzed using Prism Version 4.0/5.0 (GraphPad Software, San Diego CA). Saturation analysis was conducted using a one-site binding model (Prism). Competition data used non-linear regression and a sigmoidal equation to determine IC₅₀ and E_{max} values. The IC₅₀s were corrected for radioligand concentration, and are reported as corrected affinity values (K_{0.5}) adjusted by the Cheng-Prusoff equation for bimolecular interactions (Cheng and Prusoff, 1973) regardless of n_H. Intrinsic activity and potency estimates were made from sigmoidal fits of dose response data using Prism. Statistical analyses were conducted using SigmaStat 2.03 or InStat 3, using algorithms specified with each experiment.

Computational strategy

Homology model of human D_{2L} receptor

The human D_{2L} was modeled using three closely related GPCR crystal structures as templates: the β₂-adrenergic receptor (PDB ID: 2RH1); dopamine D₃ receptor (PDB ID: 3PBL); and bovine rhodopsin (PDB ID: 1GZM). Due to the distant homology and different transmembrane packing compared to the biogenic amine receptor crystals, the adenosine receptor was not used as a template for modeling the D_{2L}. The disulfide-bonded cystines between ECL2 and TM3 were identified from sequence alignment with other class A GPCRs. The homology model was predicted using the software MODELLER (Fiser and Sali, 2003), followed by optimization of polar residues and energy minimization.

Ligand docking

The agonist structures were modeled using the SCHRODINGER Maestro™ interface (Schrodinger, LLC, Portland, Oregon, 2006). The ligand structures were optimized and partial charges were calculated (QM, basis set 6-31G**) using the software Jaguar (Schrodinger, LLC, Portland, Oregon, 2006). Multiple conformations of the ligands were generated using Monte Carlo sampling (Macromodel, Maestro Schrodinger, LLC, Portland, Oregon, 2006), and these conformations were docked into the D_{2L} receptor model using Glide standard precision module of Maestro. During docking, the vdW radii of both ligands and the receptor were scaled by 0.5 to increase diversity of the docked poses. The resulting ligand poses were clustered and the top ranking pose from each cluster was chosen based on binding energy. The final bound pose for each agonist was selected from these top ranking poses by visual inspection.

Ligand induced receptor conformational changes (LITiCon)

LITiCon method has been discussed in detail earlier (Bhattacharya *et al.*, 2008b; Bhattacharya *et al.*, 2010). Here we detail the steps of the method as applied to hD2L. We identified that TM helices 3, 5 and 6 are in direct contact with all the agonists and hence would undergo conformational changes due to ligand binding. We performed simultaneous systematic spanning of the rotational orientation of TM helices 3, 5 and 6 in 10° increment with respect to the initial state. While TM3, TM6 were rotated between ±50°, TM5 was rotated from -50° to +20°. This process generated 968 receptor conformations. For each conformation, the following steps were performed:

- Optimization of all side-chain conformations using SCWRL 3.0 (Canutescu and Dunbrack, Jr., 2003).

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- Conjugate gradient minimization of the potential energy of the ligand in the field of the rest of protein fixed until convergence of 0.3 kcal/mol-Å RMS deviation in force per atom is achieved.
- Calculation of the ligand binding energy defined as the difference of the potential energy of the ligand with protein fixed, and the potential energy of the free ligand calculated in water using Generalized Born solvation method (Zamanakos, 2001).
- Inter-helical and ligand-receptor hydrogen bonds using HBPLUS 3.0 (McDonald and Thornton, 1994). This generates a three dimensional binding energy landscape in the rotational degrees of freedom of TM3, 5, 6.
- Identified all the local minima in this landscape and sorted them by total number of interhelical hydrogen bonds (HB) and ligand-receptor HB and then by binding energy. The final ligand stabilized receptor structural model was selected based on low binding energy and high number of HBs.

RESULTS

Ligand rationale

For these studies, we compared the actions of the endogenous ligand dopamine and the prototypical D₂ agonist quinpirole with those of dihydrexidine (DHX), dinapsoline (DNS), and dinoxyline (DNX). The D₂ functional selectivity of dihydrexidine *in vitro* is consistent with both its D₂ functional selectivity *in situ* (Mottola *et al.*, 2002; Kilts *et al.*, 2002), and with its lack of amphetamine-like behavioral actions (Darney, Jr. *et al.*, 1991) despite being a full D₁ and D₂ agonist at the canonical signaling pathway. Of particular importance for the present study is the fact that dihydrexidine has a fused ring structure with no rotatable bonds. This relatively rigid structure decreases the degrees of

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freedom in interpreting data of the type this study was designed to generate. The second important ligand used was dinapsoline, another rigid compound that also has been shown to have functionally selective properties with the D₂ wild-type receptor (Gay *et al.*, 2004). The third related ligand was dinoxylone, a compound that differs from dinapsoline only in an ether-methylene bridge substitution, a feature hypothesized to influence receptor interactions in subtle, but meaningful, ways (*vide infra*). The structures of the eutomers of these compounds are shown in Figure 1. In all cases, the D₂ affinity of the distomer is at least 100-fold less than the eutomer at the wild-type D₂ receptor, and because of limited or unavailability of pure eutomer, racemic mixtures were used for this research. The reported K_{0.5} values are based on the concentration of the racemate, and were not corrected for the effects of the distomer. The synthetic ligands had no functional effect in any of these assays performed in wild-type CHO K1 cells not containing the D_{2L} receptor. The D₂ antagonist domperidone (10 μM) was tested against WT and all receptor mutants, and as expected, no functional activation was seen at any endpoint tested. Domperidone completely blocked quinpirole activation of all effector endpoints with both WT and mutant receptors.

Effect of S5.42A, S5.43A, and S5.46A mutants on receptor expression and antagonist radioligand binding

Based on the large body of data for aminergic GPCRs and on the D₂ receptor that elucidated many aspects of the role of TM5 serines in interaction with ligands (Strader *et al.*, 1989; Cox *et al.*, 1992; Mansour *et al.*, 1992; Woodward *et al.*, 1996; Wiens *et al.*, 1998; Shi and Javitch, 2002), we chose to explore the effects of the TM5 serine residues S5.42, S5.43, and S5.46 on the functional efficacy of the above mentioned ligands for

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various signaling pathways. The hD_{2L} WT, S5.42A, S5.43A, and S5.46A receptors were expressed stably in CHO K1 cells, and characterized using saturation radioreceptor assays using [³H]-N-methylspiperone. As is shown in Table 1 and Supplementary Figure 1, these mutations had only slight effects on the K_D, ranging from 0.50 nM for S5.46A to 1.4 nM for S5.43A (WT 0.58 nM). The expression level also was similar for the WT, S5.42A, and S5.46A (2.3-4.0 pmol/mg protein), whereas the S5.43A expressed at a somewhat higher density (20.7 pmol/mg protein).

Effect of S5.42A, S5.43A, and S5.46A on affinity of agonist probe ligands

The affinity for each probe ligand was determined using competition radioreceptor assays versus [³H]-N-methylspiperone in membranes from both WT and mutant receptors. An apparent affinity constant, K_{0.5} (Table 1), was determined from experimental IC₅₀ values corrected for radioligand K_D and concentration using the bimolecular Cheng-Prusoff relationship (Cheng and Prusoff, 1973). Competition binding studies also were conducted with several structurally distinct antagonists to rule out gross structural changes induced by the receptor (data not shown). Although all of the antagonists had slightly decreased affinity (as found for N-methylspiperone), the rank order of their affinity was unchanged, consistent with the hypothesis that these mutations caused no major changes to the overall receptor structure.

Representative data for each agonist probe ligand at each receptor are shown in Figure 2 and summarized in Table 1. It should be noted that although the differences between drugs was not always statistically significant when comparing the means (Table 1), the rank order was identical in each of the experimental replicates. The differential effects that these mutations had on the rigid probe ligands are striking. For example, the

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S5.42A and S5.46A mutations caused a much greater loss of affinity to DNX than to the structurally similar DNS. These differential changes can be seen by comparing the rank orders of affinity.

WT: DNX > DNS > quinpirole = dopamine = DHX;

S5.42A: DNS > quinpirole > DNX > DHX >> dopamine;

S5.43A: DNS = DNX > DHX >> quinpirole = dopamine;

***S5.46A*: DNS > DNX > DHX > quinpirole = dopamine. Effect of mutations on**

Ligand-induced GTP γ S binding

An estimate of G protein turnover was conducted by assessing the ligand-induced binding of the non-hydrolyzable GTP analog [³⁵S]-GTP γ S (see Figure 3 and Table 2). As has been reported previously (Gay *et al.*, 2004), DHX and DNS were partial agonist at stimulating GTP γ S binding, a property shared by DNX. The most remarkable finding in this experiment related to S5.43A. As noted above, this mutation caused a decrease in affinity of all of the ligands (Table 1, Figure 2), yet the potency of the synthetic ligands in this assay was either unaffected or actually increased (significant for DNX). In addition, S5.43A actually increased the intrinsic activity of DNS. Conversely, S5.42A and S5.46A tended to cause a loss of both potency and intrinsic activity for all of the rigid ligands. Quinpirole was relatively unaffected with no change evident in intrinsic activity and a significant decrease in potency at only S5.46A.

Functional biochemical differences caused by S5.42A

Measurement was conducted on the first of three D₂-linked pathways, agonist inhibition of forskolin-stimulated cAMP accumulation in a whole cell assay system, for WT and S5.42A receptors (Figure 4, Table 3, and Supplementary Figure 2). In the WT

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receptor, all dopamine agonists (DNS, DNX, DHX, quinpirole, and dopamine) robustly inhibited forskolin-stimulated cAMP accumulation. Conversely, inhibition of cAMP accumulation with the S5.42A mutant was abolished with all ligands tested except quinpirole. These data suggest that S5.42A is critical for ligand-receptor mediated conformational changes associated with inhibitory actions of the D_{2L} receptor at cAMP accumulation (AC).

Measurement of a second pathway, agonist stimulation and subsequent phosphorylation of the p44/p42 MAP kinase, then was conducted in a whole cell assay system for WT and S5.42A receptors (Figure 4, Table 3, and Supplementary Figure 3). In the WT receptor system, all dopamine agonists (DNS, DNX, DHX, quinpirole, and dopamine) fully activated MAPK. S5.42A only minimally affected the actions of quinpirole (EC₅₀ = 44 nM) compared to WT (EC₅₀ = 23 nM). Conversely, the S5.42A mutation had minimal effects on the potency DHX but decreased intrinsic activity somewhat, yet caused a complete loss of function at all of the other endpoints with all three rigid probe ligands.

Measurement of a third pathway, agonist stimulated [³H]-arachidonic acid release, was conducted in a whole cell assay system for WT and S5.42A (Figure 4, Table 3, and Supplementary Figure 4). The potency of quinpirole was decreased by the S5.42A mutation (EC₅₀ = 223 nM vs. 63 nM in WT), but intrinsic activity was unaffected. Conversely, all of the other ligands were inactive with the S5.42A receptor (see Table 3). The MAPK and AA functional data provide strong evidence that functional effects caused by the S5.42A mutation are ligand dependent, and do not represent a general dysfunction of the receptor.

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Functional biochemical differences caused by S5.43A mutant

The hypothesis that S5.43 is critical for forming intrahelical H-bonds that stabilize the TM5 alpha helix was tested using functional profiling as detailed above (see Figure 4 and Table 3). We hypothesized that mutation of S5.43A would have minimal effect on intrinsic activity at all endpoints measured with our test ligands. In support of that hypothesis, S5.43A had no effect on AC inhibition versus WT, although changes in rank order of potency were observed [WT: DNX > quinpirole > dopamine > DHX > DNS; S5.43A: quinpirole > dopamine > DNX > DNS > DHX (see Table 3 for EC50s)].

The MAPK data are shown in Figure 4, Table 3 and Supplementary Figure 3. All of the test ligands (DNS, DNX, DHX, quinpirole, and dopamine) fully activated MAPK in both WT and the S5.43A mutant, although changes in rank order of potency were observed [WT: dopamine = quinpirole = DNX > DNS > DHX vs. S5.43A: quinpirole > dopamine > DNS > DHX > DNX (see Table 3 for EC50s)]. The S5.43A mutation had dramatic effects on agonist stimulated [³H]-arachidonic acid release (see Figures 4, Table 3 and Supplementary Figure 4). With the mutant receptor, quinpirole and DNX were minimally affected, but activity of the other ligands was lost. Of note, the structurally similar ligands DNS and DNX were affected oppositely with S5.43A. These data indicate that the effects of the S5.43A mutation were seen primarily on one function (AA), but not on two others (AC, MAPK), at which the full agonist activity at the WT receptor was maintained.

Functional biochemical differences caused by S5.46A

S5.46 is the TM5 serine residue located deepest in the binding pocket of hD_{2L}. The S5.46A mutation did not affect the inhibition of AC by quinpirole, whereas none

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of the other ligands retained activity. In a similar fashion, at both MAPK and AA release, the activity of DNS, DNX, DHX, and dopamine were absent with the S5.46A mutant. Quinpirole maintained its intrinsic activity, but had modest loss of potency (5-8 fold) at both functions.

DISCUSSION

The rigid ligands used here were developed as D₁ agonists, but were previously shown also to be functionally selective D₂ ligands both in heterologous systems (Gay *et al.*, 2004; Kilts *et al.*, 2002) and *in situ* (Kilts *et al.*, 2002; Mottola *et al.*, 2002), and have atypical behavioral properties expected of canonical D₁:D₂ agonists (Smith *et al.*, 1997; Darney, Jr. *et al.*, 1991; Gulwadi *et al.*, 2001). Thus, these ligands provide useful probes for our studies, and resulting hypotheses may explain novel actions of these compounds *in vivo*.

Table 4 provides a non-quantitative summary of this study that will permit more facile integration of these complex data. The S5.46A mutation caused a total loss of intrinsic activity for dopamine, dihydrexidine, dinapsoline, and dinoxyline, yet left the quinpirole's intrinsic activity unaffected, suggesting that S5.46A did not disrupt the signaling ability. S5.43A mutation did not markedly affect the intrinsic activity of quinpirole at any function, nor did it affect the activity of DNX at any function, yet completely eliminated intrinsic activity for dopamine, dihydrexidine, and dinapsoline at AA release, but not other functions. Finally, S5.42A did not affect the intrinsic activity of quinpirole, but with one exception (dihydrexidine at MAPK) eliminated the intrinsic activity of dopamine and the rigid ligands. Functional selectivity also can be expressed as large and differential changes in potency (Gay *et al.*, 2004) as shown in Table 4. A recent

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report showed that H6.55 mutations of the D_{2L} can abolish the bias of some functionally selective ligands (Tschammer *et al.*, 2011), but the current study demonstrates that bias in a single function also can be introduced by a point mutation (Figure 4).

Strengths and weaknesses of this experimental system

It is now realized that GPCR functional selectivity may not only involve differential activation of G protein heterotrimers, but also many other interactors (Urban *et al.*, 2007). Thus, even in the same cell, the same receptor may be involved in different “signalsomes,” and a measured endpoint may be dependent on activity of two or more signalsomes. Although it may seem desirable to have a totally defined system in which to study different ligand-induced/stabilized, this is not only technically difficult, but no available system recapitulates the characteristics seen consistently *in situ* and in single native cells.

Although we assume that mutation-induced functional selectivity is a consequence largely of alterations the ligand-receptor interactions, it is possible that ligand-independent, mutation-induced alterations in the signalsome itself are involved. Two lines of evidence suggest this is not a major factor here. The reference ligand quinpirole has markedly different predicted docking poses to the D_{2L} receptor. In our assays, quinpirole-induced functional activity was similar in mutant and WT receptors, suggesting that functionally-selective signaling of rigid ligands is not due simply to disruption of D₂ signaling. Moreover, in the current studies, the effects on intrinsic activity were often dramatic (e.g., 100% or 0% intrinsic activity), but if differential changes were occurring via two alternate routes, partial agonism might have been expected.

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This raises an ancillary point. The S5.43A line had a much higher receptor expression than the WT, S5.42A, or S5.46A lines (Table 1), suggesting that the failure to find partial agonism is a receptor reserve artifact. Yet partial agonism at GTP γ S binding, uncorrelated with receptor expression, was seen for all three rigid ligands. Indeed, the high expressing S5.43A increased the potency of DNX in stimulating GTP γ S binding, yet the same mutation markedly decreased DNX potency at adenylyl cyclase. Thus, while receptor reserve may have influenced these results, the effect was probably minor. In the case of S5.43A, the mutation caused a major disruption only of a single function, and only for some ligands. Together, this suggests that the functional effects reflect alterations in ligand-receptor interactions, and not gross disruption of receptor function.

Computational Analysis

As noted above, none of the mutations affected quinpirole activation, possibly because the full agonists (quinpirole, DA, DNS, DNX and DHX) stabilize an ensemble of receptor conformations that show differential coupling affinities to downstream proteins (Vaidehi and Kenakin, 2010). To test this hypothesis, we computed the binding energy (BE) landscapes of the agonists and D_{2L} using the computational method LITiCon that has been validated for prediction of activation of rhodopsin (Bhattacharya *et al.*, 2008b) and β 2-adrenergic receptor (Bhattacharya *et al.*, 2008a; Bhattacharya and Vaidehi, 2010). As seen in Figure 5C, the BE landscapes of dopamine shows two distinct minima (agonist states 1 & 2). The BE landscape of the other agonists are qualitatively similar (supplementary Figure S5). Both minima are characterized by small movements of TM3, and a counterclockwise rotation of TM5 (from the extracellular side) that brings S5.43 closer to the binding pocket. The difference between the two agonist-stabilized receptor

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conformations comes from TM6 movement. In receptor state 1, movement of TM6 brings H6.55 closer to TM5, whereas in state 2, TM6 movement brings H6.55 closer to TM7.

The agonist preferred receptor conformations are stabilized by both interhelical and receptor-ligand contacts. S5.42 makes a hydrogen bond (HB) with the catechol hydroxyl groups of DA, DNS and DNX (m-OH of DA, p-OH of DNS, DNX, Figure 5B & 5E) in both agonist states 1 and 2. This HB is missing in the DHX bound structure (distance: 5.8Å), and therefore S5.42 contributes towards the stability of the receptor active states of DA, DNS, DNX, but to a lesser extent for DHX. This is in agreement with the functional selectivity profile of the S5.42A mutant, which completely abolishes activation of all pathways for all the agonists except DHX and quinpirole, which still maintains partial activity towards the MAPK pathway. In conjunction with the predicted DHX bind site, this suggests that S5.42 does not form a HB with DHX in the MAPK specific D_{2L} conformation. The lack of HB with S5.42 could be compensated by stronger HB of DHX with the other two serines or tighter aromatic contact between the agonist and the phenylalanines on TM5 and TM6. This is not, however, clear from the models. Thus S5.42A has less drastic effect on DHX mediated MAPK activation.

In contrast to S5.42, the effect of S5.43 is more pathway-specific. In the activation assays, the S5.43A mutant only affected the AA pathway while the other two pathways were unaffected for all agonists. For the AA pathway, S5.43A abolished the intrinsic activity of DA, DNS and DHX, while retaining partial activity for DNX and quinpirole. In our model, the receptor/agonist state 1 is stabilized by a HB between S5.43 and H6.55 (Figure 5B). In contrast, the receptor/agonist state 2 is stabilized by the HB between H6.55 and Y7.35 (Figure 5A). Thus our model, in conjunction with the results

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from the S5.43A mutant, indicates that agonist state 1 may represent the receptor conformation that mediates AA activation, whereas slightly different agonist state 2 corresponds to the cAMP and MAPK activation, although this hypothesis will require further experimentation to test. In the DNX and quinpirole bound structures, the side chain of H6.55 is proximal to the ether oxygen of DNX and imidazole nitrogen of quinpirole respectively. Thus in the S5.43A mutant, DNX and quinpirole can stabilize agonist state 2 by forming HB with H6.55, whereas the other ligands (DA, DNS, DHX) that lack the polar motifs of DNX and quinpirole cannot form this HB, and hence cannot stabilize state 1. These ligands require S5.43 to stabilize agonist state 1, and thus lose AA activity in the S5.43A mutant. This explains the insensitivity of S5.43A mutation towards DNX and quinpirole-mediated AA activation.

S5.46A eliminates activation of the three pathways for all agonists except quinpirole. In our agonist-stabilized model of the D₂ receptor, S5.46 forms a HB with T3.37 that stabilizes both agonist states 1 and 2 (Figure 5). Thus mutating S5.46 to Ala destabilizes both the active states and abolishes activity of all the three pathways. Among the agonists studied here, quinpirole shows a different activation profile compared to the rest of the agonists. In the experimental activation assays, none of the serine mutations affected quinpirole mediated D₂ activation. Unlike the catechol agonists, the nitrogen atoms on the imidazole of quinpirole are distant from the serine residues on TM5 precluding the strong HB with the serines in the agonist-stabilized states. This explains the insensitivity of the serine mutations towards quinpirole-mediated D₂ activation. To understand the mechanism of quinpirole activation, comparison of the interactions in the ligand binding pocket of both in the inactive and agonist-stabilized conformations

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showed that the n-propyl group of quinpirole is buried deep into the binding pocket allowing direct contact with W6.48. The steric interaction could induce a change of rotamer of the W6.48 side chain, which in turn creates a steric clash with the side chain of F5.47 (Figure 5F). In order to relieve the clash between W6.48 and F5.47, TM5 rotates counterclockwise leading to the activation of the receptor. Thus W6.48 and F5.47 form an aromatic switch that stabilizes the active states of quinpirole-bound hD_{2L}. Our model also suggests that H6.55 is important for quinpirole mediated AA activation, since the HB between quinpirole and H6.55 contributes to the stability of agonist state 1.

Differences in the agonist stabilized receptor conformations could lead to functional selectivity in the D_{2L} receptor. Selective conformational stabilization by structurally distinct agonists was reported in β_2 -adrenergic receptor (Ghanouni *et al.*, 2001; Swaminath *et al.*, 2005). Here we find that the three serine residues on TM5 interact differently with the agonist to stabilize distinct D_{2L} conformations for all agonists except quinpirole. The S5.46-T3.37 HB stabilizes the active state conformations of TM5 making S5.46 critical for all three activation pathways. In contrast S5.43 stabilizes only one of the active states (AA pathway) by forming HB with H6.55. S5.42A shows differential effect on the different agonists (less effect on DHX-MAPK activation) depending on the strength of the HB with various agonists. Besides the serines, the residue H6.55 is predicted to be important for the functionality of the D₂ receptor, and data to this effect was recently reported (Tschammer *et al.*, 2011). In β -adrenergic receptors, the residue in the analogous position of H6.55 (N6.55) has been shown to be important for agonist activity. In the β_2 -adrenergic receptor, the N6.55L mutation reduced the activity of norepinephrine (Wieland *et al.*, 1996). In the agonist bound crystal

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structures of the β_1 -adrenoreceptor (Warne *et al.*, 2011), N6.55 forms a HB with S5.43 similar to the predicted agonist-bound structures of the D₂. In this work, H6.55 is predicted to stabilize the D₂ conformation responsible for AA activity by forming HB with S5.43 and polar groups of quinpirole and DNX. Unlike the serine mutations that only affect the agonists with catechol motif, the H6.55A mutation is predicted to affect the activation of all agonists studied here including quinpirole. The model of D₂ activation that emerges from this work is summarized in Figure 6. Although the computational model presented here explains the differences between AA vs. cAMP/MAPK pathways, we attribute changes in functional selectivity to ligand-receptor interactions, but neglects effects on receptor-protein interactors. Thus, functional selectivity can be engendered by mutations that perturb arrestin binding (Lan *et al.*, 2009b; Lan *et al.*, 2009a). Nonetheless, our data demonstrate that ligand-receptor interactions can be a determinant of signaling bias, and the one clearly most relevant to drug discovery.

Conclusions

Antagonists were essentially unaffected by these TM5 serine mutations because their binding to the D₂ receptor, unlike agonists, is less dependent on interactions with these residues. The effects on the binding of these agonist ligands also were largely predictable by existing molecular models, whereas these mutations caused patterns of effects on the functional activity of the probe ligands not directly predictable from binding. One hypothesis is that ligands can cause receptor populations to shift between groups of discrete active states that are linked to specific signaling pathways. Alternatively, each ligand may induce a discrete range of conformational changes that

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affect, in a graded fashion, whether specific signaling pathways are activated. At present, the former hypothesis is one that has been largely favored, and is the one that would explain the constitutive activity often seen in cellular systems. In any event, the ability to form and test hypotheses about how such changes occur can be heuristically interesting, but also may lead to structure-based discovery of novel functionally selective ligands.

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AUTHORSHIP CONTRIBUTIONS

Participated in Research Design: Fowler. Mailman, Bhattacharya, Vaidehi

Conducted Experiments: Fowler. Urban, Bhattacharya

Contributed New Reagents or Analytical Tools: Fowler, Bhattacharya, Vaidehi

Performed data Analysis: Fowler. Mailman, Bhattacharya, Vaidehi

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REFERENCES

- Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci* **25**:366-428.
- Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P and Clarke WP (1998) Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* **54**:94-104.
- Bhattacharya S, Hall SE, Li H and Vaidehi N (2008a) Ligand-stabilized conformational states of human beta(2) adrenergic receptor: insight into G-protein-coupled receptor activation. *Biophys J* **94**:2027-2042.
- Bhattacharya S, Hall SE and Vaidehi N (2008b) Agonist-induced conformational changes in bovine rhodopsin: insight into activation of G-protein-coupled receptors. *J Mol Biol* **382**:539-555.
- Bhattacharya S, Subramanian G, Hall S, Lin J, Laoui A and Vaidehi N (2010) Allosteric antagonist binding sites in class B GPCRs: corticotropin receptor 1. *J Comput Aided Mol Des* **24**:659-674.
- Bhattacharya S and Vaidehi N (2010) Computational mapping of the conformational transitions in agonist selective pathways of a G-protein coupled receptor. *J Am Chem Soc* **132**:5205-5214.
- Brewster WK, Nichols DE, Riggs RM, Mottola DM, Lovenberg TW, Lewis MH and Mailman RB (1990) trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[a]phenanthridine: a highly potent selective dopamine D1 full agonist. *J Med Chem* **33**:1756-1764.
- Brown JT, Kant A and Mailman RB (2009) Rapid, semi-automated, and inexpensive radioimmunoassay of cAMP: Application in GPCR-mediated adenylate cyclase assays. *J Neurosci Methods* **177**:261-266.
- Canutescu AA and Dunbrack RL, Jr. (2003) Cyclic coordinate descent: A robotics algorithm for protein loop closure. *Protein Sci* **12**:963-972.
- Charifson PS, Bowen JP, Wyrick SD, Hoffman AJ, Cory M, McPhail AT and Mailman RB (1989) Conformational analysis and molecular modeling of 1-phenyl-, 4-phenyl-, and 1-benzyl-1,2,3,4-tetrahydroisoquinolines as D₁ dopamine receptor ligands. *J Med Chem* **32**:2050-2058.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.

- Cox BA, Henningsen RA, Spanoyannis A, Neve RL and Neve KA (1992) Contributions of conserved serine residues to the interactions of ligands with dopamine D₂ receptors. *J Neurochem* **59**:627-635.
- Darney KJ, Jr., Lewis MH, Brewster WK, Nichols DE and Mailman RB (1991) Behavioral effects in the rat of dihydrexidine, a high-potency, full-efficacy D₁ dopamine receptor agonist. *Neuropsychopharmacology* **5**:187-195.
- Fiser A and Sali A (2003) Modeller: generation and refinement of homology-based protein structure models. *Methods Enzymol* **374**:461-491.
- Gay EA, Urban JD, Nichols DE, Oxford GS and Mailman RB (2004) Functional selectivity of D₂ receptor ligands in a Chinese hamster ovary hD₂L cell line: evidence for induction of ligand-specific receptor states. *Mol Pharmacol* **66**:97-105.
- Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR and Kobilka BK (2001) Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor. *J Biol Chem* **276**:24433-24436.
- Ghosh D, Snyder SE, Watts VJ, Mailman RB and Nichols DE (1996) 9-Dihydroxy-2,3,7,11b-tetrahydro-1H-naph[1,2,3-de]isoquinoline: a potent full dopamine D₁ agonist containing a rigid-beta-phenyldopamine pharmacophore. *J Med Chem* **39**:549-555.
- Grubbs RA, Lewis MM, Owens-Vance C, Gay EA, Jassen AK, Mailman RB and Nichols DE (2004) 8,9-dihydroxy-1,2,3,11b-tetrahydrochromeno[4,3,2,-de]isoquinoline (dinoxyline), a high affinity and potent agonist at all dopamine receptor isoforms. *Bioorg Med Chem* **12**:1403-1412.
- Gulwadi AG, Korpinen CD, Mailman RB, Nichols DE, Sit SY and Taber MT (2001) Dinapsoline: characterization of a D₁ dopamine receptor agonist in a rat model of Parkinson's disease. *J Pharmacol Exp Ther* **296**:338-344.
- Harper JF and Brooker G (1975) Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'0 acetylation by acetic anhydride in aqueous solution. *J Cyclic Nucleotide Res* **1**:207-218.
- Kenakin T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16**:232-238.
- Kenakin TP (2007) Functional Selectivity through Protean and Biased Agonism: Who Steers the Ship? *Mol Pharmacol*.
- Kilts JD, Connery HS, Arrington EG, Lewis MM, Lawler CP, Oxford GS, O'Malley KL, Todd RD, Blake BL, Nichols DE and Mailman RB (2002) Functional selectivity of dopamine receptor agonists. II. Actions of dihydrexidine in D₂L receptor-transfected MN9D cells and pituitary lactotrophs. *J Pharmacol Exp Ther* **301**:1179-1189.

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- Lan H, Liu Y, Bell MI, Gurevich VV and Neve KA (2009a) A dopamine D2 receptor mutant capable of G protein-mediated signaling but deficient in arrestin binding. *Mol Pharmacol* **75**:113-123.
- Lan H, Teeter MM, Gurevich VV and Neve KA (2009b) An intracellular loop 2 amino acid residue determines differential binding of arrestin to the dopamine D2 and D3 receptors. *Mol Pharmacol* **75**:19-26.
- Lawler CP, Prioleau C, Lewis MM, Mak C, Jiang D, Schetz JA, Gonzalez AM, Sibley DR and Mailman RB (1999) Interactions of the novel antipsychotic aripiprazole (OPC-14597) with dopamine and serotonin receptor subtypes. *Neuropsychopharmacology* **20**:612-627.
- Lawler CP, Watts VJ, Booth RG, Southerland SB, and Mailman RB. (1994) Discrete functional selectivity of drugs: OPC-14597, a selective antagonist for post-synaptic dopamine D2 receptors. *Society for Neuroscience Abstracts* **20**:525.
- Lovenberg TW, Brewster WK, Mottola DM, Lee RC, Riggs RM, Nichols DE, Lewis MH and Mailman RB (1989) Dihydropyridine, a novel selective high potency full dopamine D-1 receptor agonist. *Eur J Pharmacol* **166**:111-113.
- Mailman RB (2007) GPCR functional selectivity has therapeutic impact. *Trends Pharmacol Sci* **28**:390-396.
- Mansour A, Meng F, Meador-Woodruff JH, Taylor LP, Civelli O and Akil H (1992) Site-directed mutagenesis of the human dopamine D₂ receptor. *Eur J Pharmacol* **227**:205-214.
- McDonald IK and Thornton JM (1994) Satisfying hydrogen bonding potential in proteins. *J Mol Biol* **238**:777-793.
- Milligan G. (1999) Signal Transduction: A Practical Approach.
- Mottola DM, Cook LL, Jones SR, Booth RG, Nichols DE and Mailman RB (1991) Dihydropyridine, a selective dopamine receptor agonist that may discriminate postsynaptic D₂ receptors. *Soc Neurosci Abstr* **17**:818.
- Mottola DM, Kilts JD, Lewis MM, Connery HS, Walker QD, Jones SR, Booth RG, Hyslop DK, Piercey M, Wightman RM, Lawler CP, Nichols DE and Mailman RB (2002) Functional selectivity of dopamine receptor agonists. I. Selective activation of postsynaptic dopamine D2 receptors linked to adenylate cyclase. *J Pharmacol Exp Ther* **301**:1166-1178.
- Mottola DM, Laiter S, Watts VJ, Tropsha A, Wyrick SD, Nichols DE and Mailman RB (1996) Conformational analysis of D1 dopamine receptor agonists: pharmacophore assessment and receptor mapping. *J Med Chem* **39**:285-296.

MOLPHARM#75457

- Neve KA (2009) *Functional Selectivity of G Protein-Coupled Receptor Ligands*. Humana, New York.
- Nichols DE (1983) The development of novel dopamine agonists, in *Dopamine Receptors* (Kaiser C and Keibadian JW eds) pp 201-218, American Chemical Society, Washington, D.C.
- Ryman-Rasmussen JP, Nichols DE and Mailman RB (2005) Differential activation of adenylate cyclase and receptor internalization by novel dopamine D1 receptor agonists. *Mol Pharmacol* **68**:1039-1048.
- Shapiro DA, Renock S, Arrington E, Sibley DR, Chiodo LA, Roth BL and Mailman RB (2003) Aripiprazole, a novel atypical antipsychotic drug with a unique and robust pharmacology. *Neuropsychopharmacology* **28**:1400-1411.
- Shi L and Javitch JA (2002) The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu Rev Pharmacol Toxicol* **42**:437-467.
- Smith HP, Nichols DE, Mailman RB and Lawler CP (1997) Locomotor inhibition, yawning and vacuous chewing induced by a novel dopamine D2 post-synaptic receptor agonist. *Eur J Pharmacol* **323**:27-36.
- Strader CD, Candelore MR, Hill WS, Sigal IS and Dixon RA (1989) Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. *J Biol Chem* **264**:13572-13578.
- Swaminath G, Deupi X, Lee TW, Zhu W, Thian FS, Kobilka TS and Kobilka B (2005) Probing the beta2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. *J Biol Chem* **280**:22165-22171.
- Tschammer N, Bollinger S, Kenakin T and Gmeiner P (2011) Histidine 6.55 is a major determinant of ligand-biased signaling in dopamine D2L receptor. *Mol Pharmacol* **79**:575-585.
- Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M and Mailman RB (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**:1-13.
- Vaidehi N and Kenakin T (2010) The role of conformational ensembles of seven transmembrane receptors in functional selectivity. *Curr Opin Pharmacol* **10**:775-781.

MOLPHARM#75457

- Versteeg HH, Nijhuis E, van den Brink GR, Evertzen M, Pynaert GN, van Deventer SJ, Coffey PJ and Peppelenbosch MP (2000) A new phosphospecific cell-based ELISA for p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase B and cAMP-response-element-binding protein. *Biochem J* **350 Pt 3**:717-722.
- Warne T, Moukhametzianov R, Baker JG, Nehme R, Edwards PC, Leslie AG, Schertler GF and Tate CG (2011) The structural basis for agonist and partial agonist action on a beta(1)-adrenergic receptor. *Nature* **469**:241-244.
- Wieland K, Zuurmond HM, Krasel C, Ijzerman AP and Lohse MJ (1996) Involvement of Asn-293 in stereospecific agonist recognition and in activation of the beta 2-adrenergic receptor. *Proc Natl Acad Sci USA* **93**:9276-9281.
- Wiens BL, Nelson CS and Neve KA (1998) Contribution of serine residues to constitutive and agonist-induced signaling via the D2S dopamine receptor: evidence for multiple, agonist-specific active conformations. *Mol Pharmacol* **54**:435-444.
- Woodward R, Coley C, Daniell S, Naylor LH and Strange PG (1996) Investigation of the role of conserved serine residues in the long form of the rat D2 dopamine receptor using site-directed mutagenesis. *J Neurochem* **66**:394-402.
- Zamanakos, G. (2001) A fast and accurate analytic method for the computation of solvent effects in molecular simulations. Thesis/Dissertation. California Institute of Technology. Pasadena, CA.

FOOTNOTES

J. Corey Fowler and Supriyo Bhattacharya contributed equally to this work.

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Conflict-of-interest

The intellectual property for some of the compounds used in this study were assigned by Dr. Mailman to the University of North Carolina and outlicensed to Biovalve Technologies Inc. that was acquired by Valeritas. None of the other authors report a conflict-of-interest.

LEGENDS FOR FIGURES

Figure 1. Structures of probe ligands used in this study.

Figure 2. Competition binding of test compounds with hD_{2L} WT and mutant receptors. Membranes were incubated with [³H]-N-methylspiperone as described in the Methods. Analysis was conducted using non-linear regression and a sigmoidal equation to determine IC₅₀s, reported as corrected affinity values (K_{0.5}). Assays were conducted in triplicate and data represent 3-4 independent experiments. The legend in each panel is listed in order of decreasing affinity.

Figure 3. GTPγS turnover experiments of test compounds with hD_{2L} WT and mutant receptors. Membrane fragments were incubated for 15 min with varying concentrations of test compounds until reaching equilibrium. 0.2 nM [³⁵S]-GTPγS was then added for 30 min and stimulation was measured. Quinpirole and dopamine had identical intrinsic activity in WT cells, and the actions of quinpirole were not affected by the mutations. Hence all of the data is referenced to quinpirole activity in WT cells. Analysis was conducted using non-linear regression and a sigmoidal equation to determine IC₅₀s reported below in Table 2. Assays were conducted in triplicate and data represent 3-4 independent experiments. The legend in each panel is listed in order of decreasing potency.

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Figure 4. Ligand effects on intrinsic activity of second messenger pathways of hD_{2L} with mutations at S5.42A, S5.43A, and S5.46A. *Top row:* Ligand-mediated inhibition of forskolin-stimulated cAMP accumulation (AC). [For the sake of comparison, in this panel greater receptor-mediated inhibition is presented as a taller histogram bar when it actually represents greater inhibition]; *Middle row:* Ligand-mediated activation of p44/p42 MAP Kinase (MAPK); *Bottom row:* Ligand-mediated potentiation of [³H]-arachidonic acid release. Note that agonists inhibit AC, but stimulate MAPK and AA release. Data are representative of E_{max} values for 3-5 independent experiments conducted in triplicate. * p < 0.05 (One way ANOVA, post hoc Dunnett's). ** p < 0.05 (Kruskal-Wallis one way ANOVA, post hoc Dunn's).

Figure 5. Stabilization of multiple receptor conformations (agonist states 1 & 2) by the D₂ agonists. A) binding site of DA in agonist state 2; B) binding site of DA in agonist state 1; C) BE landscape of DA showing the different agonist stabilized states; D) binding site of DHX in agonist state 1; E) binding site of DNX in agonist state 1; F) binding site of quinpirole in agonist state 1. The red dotted lines represent HB contacts. In the quinpirole bound receptor, the moieties involved in stabilizing the active states are shaded.

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Figure 6. Model of functional selectivity in hD_{2L}. The schematics show the receptor conformations corresponding to the different activation pathways stabilized by various agonists and the interhelical and receptor-ligand contacts looking from the extracellular side: **A)** cAMP/MAPK activation by ligands DA, DNS, DNX (agonist state 2 in Figure 5); **B)** AA activation by DA, DHX, DNS (agonist state 1 in Figure 5); **C)** AA activation by DNX; **D)** mechanism of activation by quinpirole for all three pathways. The orientation of TM6 where H6.55 faces TM5 corresponds to the AA pathway, whereas H6.55 facing TM7 represents cAMP/MAPK activation.

TABLES

Table 1. Competition binding data for [³H]-N-methylspiperone sites at TM5 serine mutants.

<i>Mutant</i>	WT	S5.42A	S5.43A	S5.46A
<i>K_{0.5} (nM)</i>				
DNS	144 ± 17	660 ± 190	750 ± 370	330 ± 160
DNX	83 ± 5	2,700 ± 990*	770 ± 90	580 ± 340
DHX	490 ± 91	7,400 ± 1,000*	2,600 ± 50	1,600 ± 40
quinpirole	365 ± 6	1,040 ± 100	27,000 ± 10,000*	2,900 ± 820
dopamine	450 ± 170	80,000 ± 3,000*	31,000 ± 6,000*	3,500 ± 1,500

Values represent mean ± SEM. The expression of these receptors was: WT: K_D =0.58 and B_{max}=4.8 pmol/mg; S5.42A: K_D=0.59 and B_{max}= 4.0 pmol/mg; S5.43A: K_D =1.4 nM and B_{max}=20.74 pmol/mg; S5.46A: K_D =0.50 nM and B_{max}=2.34 pmol/mg.

* Significantly different from WT (P<0.05; One-Way ANOVA and Tukey-Kramer Multiple Comparisons Test).

Table 2. Potency of probe ligands in affecting GTP γ S binding.

<i>Mutant</i>	WT	S5.42A	S5.43A	S5.46A
	<i>EC50 (nM)</i>			
DNS	290 \pm 70	--*	135 \pm 25	--*
DNX	550 \pm 30	--*	69 \pm 17*	660 \pm 250
DHX	370 \pm 180	1,300 \pm 410	92 \pm 28	6,600 \pm 6,200
Quinpirole	740 \pm 120	1,700 \pm 860	203 \pm 5	7,200 \pm 1900*
Dopamine	1,700 \pm 1,040	10,700 \pm 1,500*	4,300 \pm 1600	--*

Values represent EC50 \pm S.E.M. for 3-4 independent experiments conducted in triplicate.

* Significantly different from WT (P<0.05; One-Way ANOVA and Tukey-Kramer Multiple Comparisons Test).

-- Potency cannot be measured as no functional activity was detected.

Table 3. Effect of serine mutations on functional potencies of probe ligands.

<i>Ligand</i>	<i>EC50 (nM)</i>			
Dopamine	WT	S5.42	S5.43A	S5.46
AC	46 ± 22	--*	19 ± 3*	--*
MAPK	28 ± 7	--*	114 ± 40*	--*
AA-release	49 ± 26	--*	--*	--*
Quinpirole	WT	S5.42	S5.43A	S5.46
AC	16 ± 6	7.8 ± 1.4	3 ± 1	54 ± 11
MAPK	26 ± 5	44 ± 12	26 ± 11	193 ± 28
AA-release	63 ± 12	223 ± 67*	35 ± 27	292 ± 16*
DNX	WT	S5.42	S5.43A	S5.46
AC	5.6 ± 0.3	--*	36 ± 17	--*
MAPK	27 ± 17	--*	2,100 ± 1,300	--*
AA-release	101 ± 12	--*	40 ± 18*	--*
DNS	WT	S5.42	S5.43A	S5.46
AC	193 ± 63	--*	119 ± 48	--*
MAPK	72 ± 68	--*	155 ± 9	--*
AA-release	340 ± 180	--*	--*	--*
DHX	WT	S5.42	S5.43A	S5.46
AC	93 ± 10	--*	125 ± 28	--*
MAPK	31 ± 105	69 ± 6.1	400 ± 130	--*
AA-release	395 ± 119	--*	--*	--*

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

* Significantly different from WT (P<0.05; One-Way ANOVA and Tukey-Kramer Multiple Comparisons Test).

Table 4. Qualitative summary of effects of mutations on probe ligands relative to WT.

Assay	S5.42A					S5.43A					S5.46A				
	DHX	DNS	DNX	QP	DA	DHX	DNS	DNX	QP	DA	DHX	DNS	DNX	QP	DA
Binding	↓↓	↓	↓↓	↔↓	↓↓↓	↓	↓	↓	↓↓↓	↓↓↓	↔	↔↓	↓	↓	↓
GTPγS (EC ₅₀)	↔↓	ID	ID	↔	↓	↔↑	↔↑	↑	↔↑	↔↓	↓↓	ID	↔	↓↓	-
AC (EC ₅₀)	ID	ID	ID	↑	ID	↔	↔	↓	↓	↔	ID	ID	ID	↔↑	-
MAPK (EC ₅₀)	↔↑	ID	ID	↔	ID	↔	↔	↓↓↓	↔	↔↓	ID	ID	ID	↓	-
AA (EC ₅₀)	ID	ID	ID	↔↓	ID	ID	ID	↔↓	↔↓	ID	ID	ID	ID	↓	-
GTPγS (E _{max})	↓↓	↓↓↓	↓↓	↔	↓↓	↔	↑↑	↔	↔	↔	↓↓	↓↓↓	↓↓↓	↔	↓↓↓
AC (E _{max})	↓↓↓	↓↓↓	↓↓↓	↔	↓↓↓	↔	↔	↔	↔	↔	↓↓↓	↓↓↓	↓↓↓	↔	↓↓↓
MAPK (E _{max})	↓↓	↓↓↓	↓↓↓	↓	↓↓↓	↔	↔	↔	↔	↔↑	↓↓↓	↓↓↓	↓↓↓	↔	↓↓↓
AA (E _{max})	↓↓↓	↓↓↓	↓↓↓	↓↓	↓↓↓	↓↓↓	↓↓↓	↓	↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↔↓	↓↓↓

↑ Positive effect (i.e. decreased K_{0.5}, decreased EC₅₀, or increased E_{max})

↔ Minimal or no effect

↓ Detrimental effect (i.e. increased K_{0.5}, increased EC₅₀, or decreased E_{max})

↓↔ Trend downwards

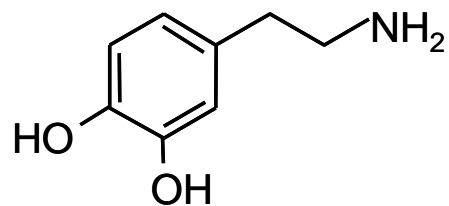
↔↑ Trend upwards

ID Indeterminable

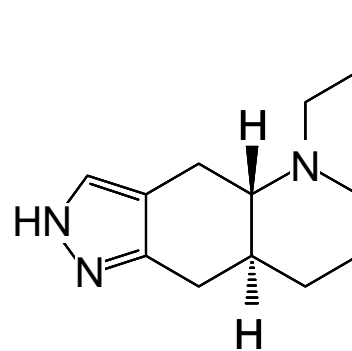
Note ↓ = 5-9 fold; ↓↓ = 10-50 fold; ↓↓↓ = > 50 fold

QP = quinpirole; DA = dopamine

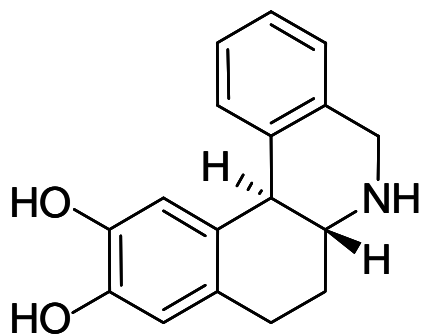
Figure 1.



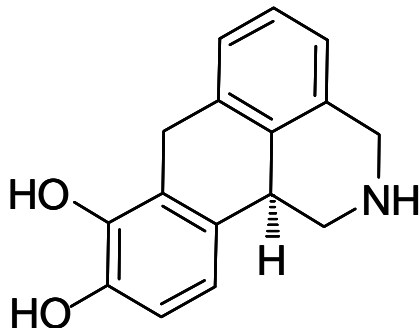
dopamine



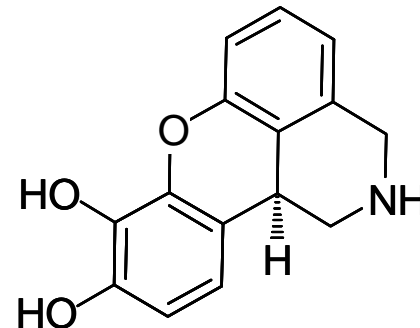
quinpirole



dihydrexidine



dinapsoline



dinoxylone

Figure 2

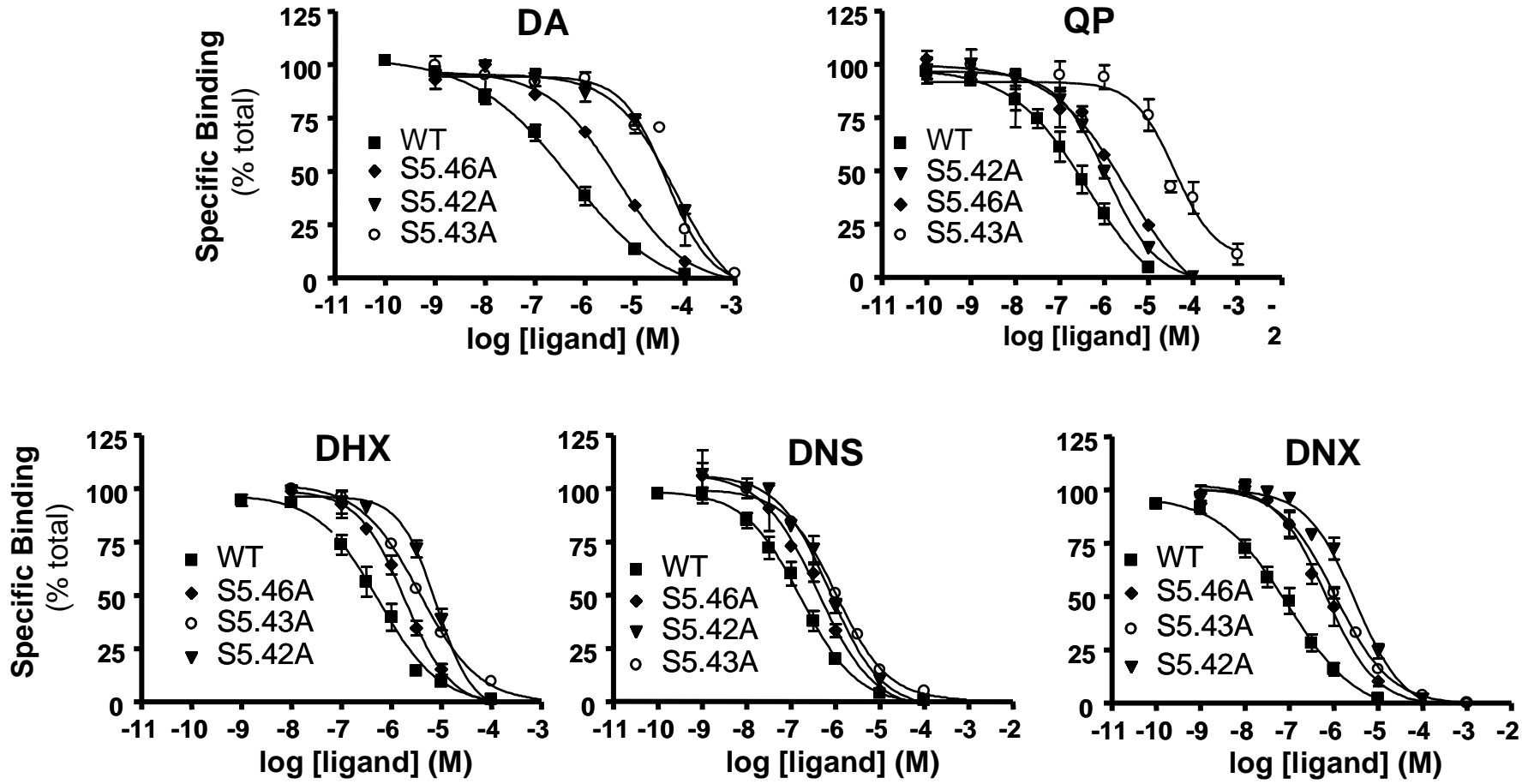


Figure 3

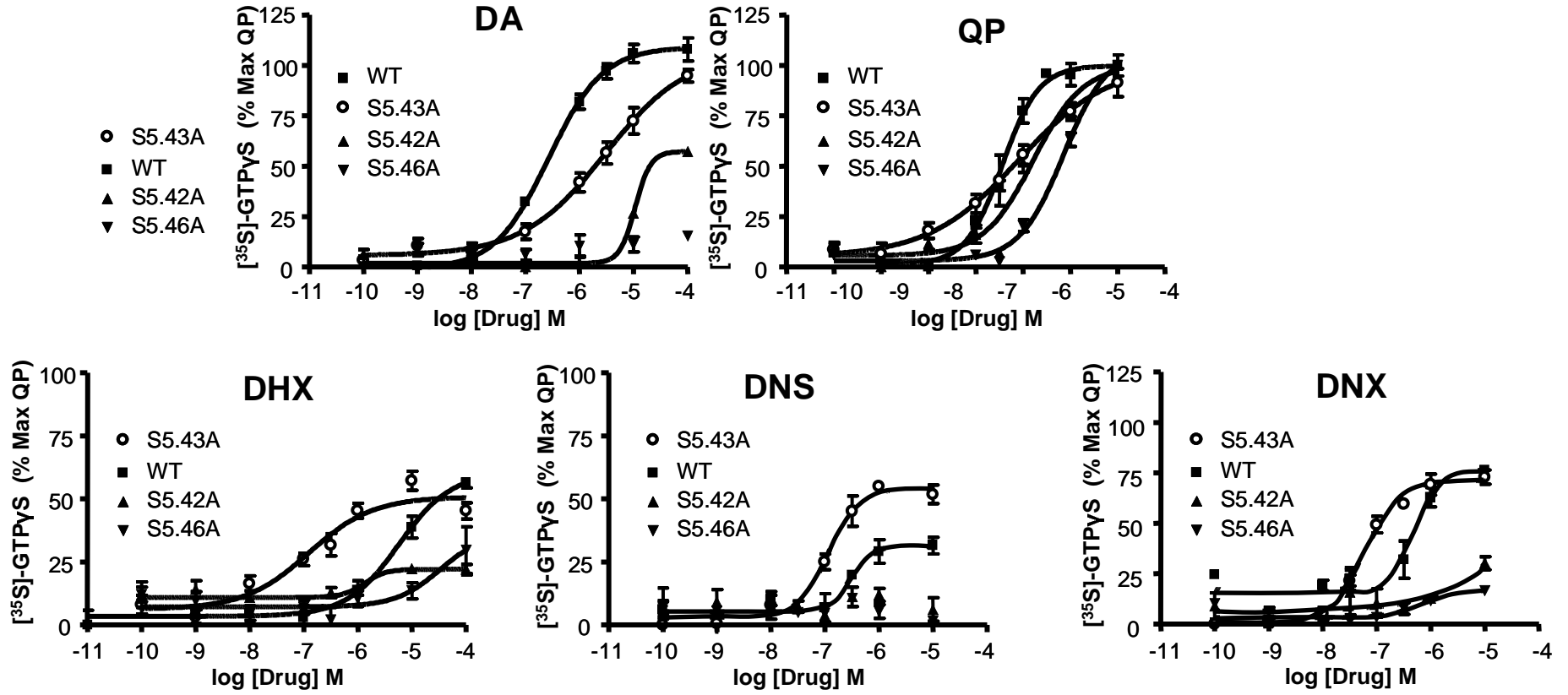


Figure 4

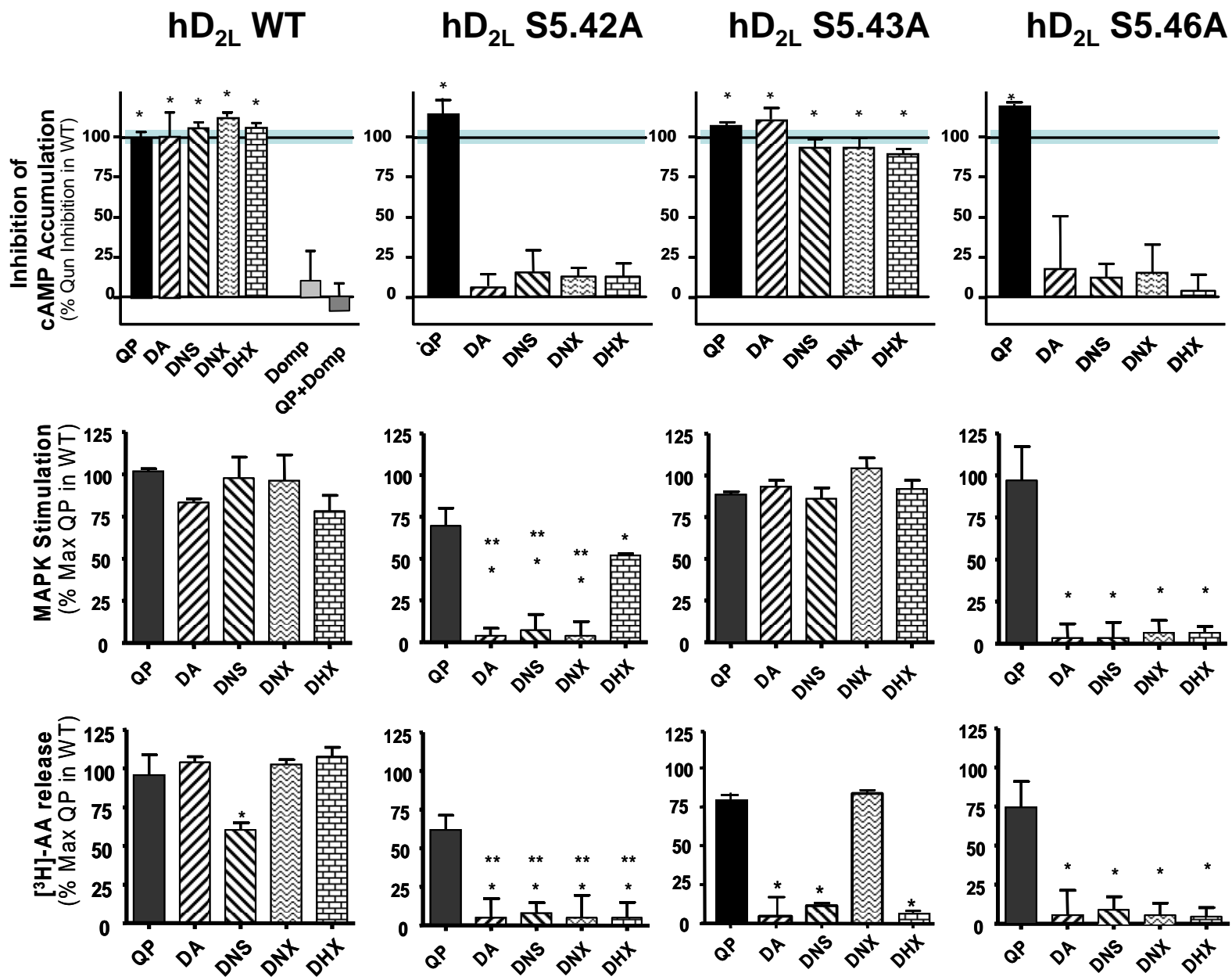


Figure 5

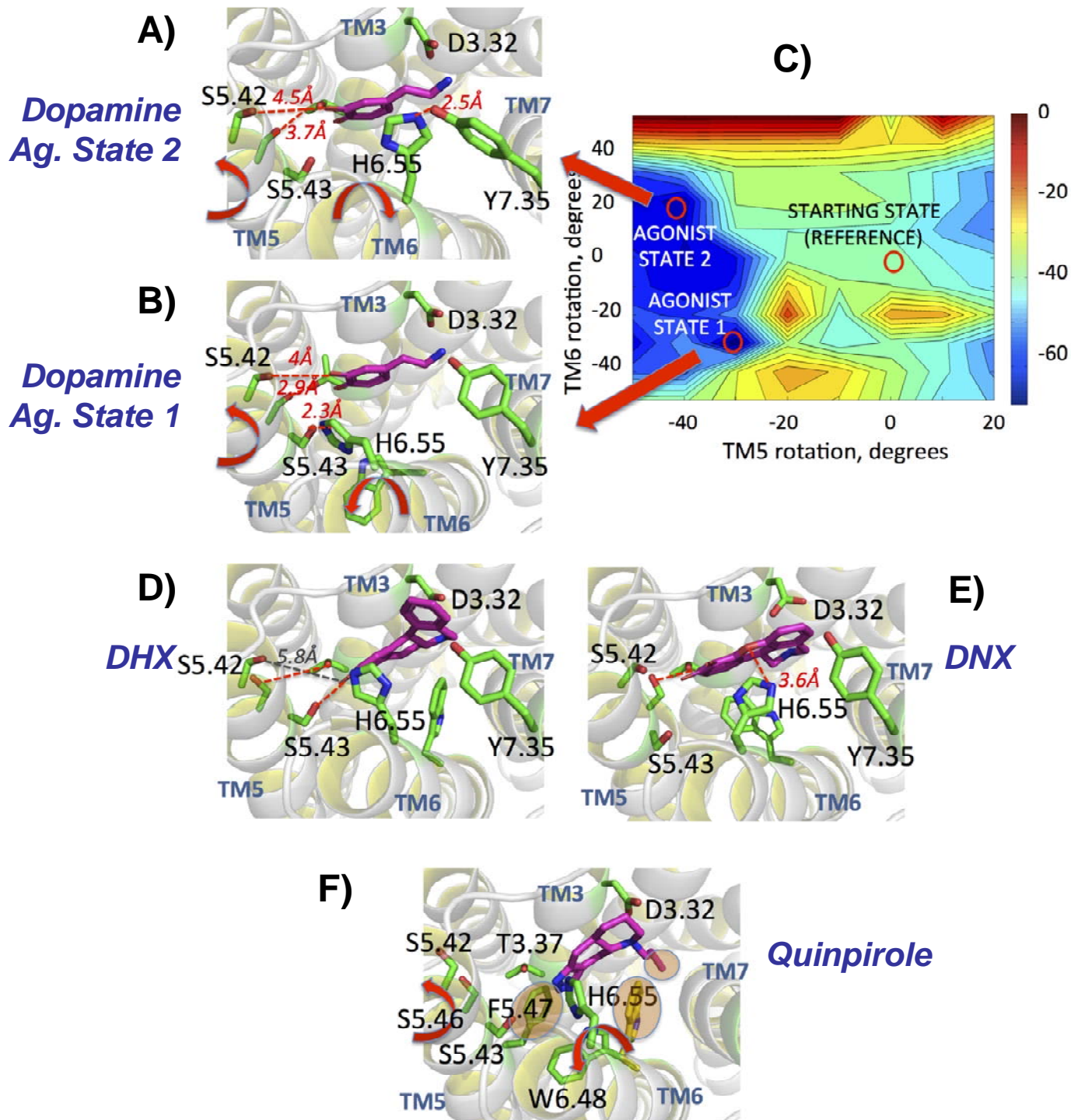


Figure 6

