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Histidine 6.55 Is a Major Determinant of Ligand-Biased Signaling in Dopamine D_{2L} Receptor

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ABSTRACT

In our previous studies, we demonstrated that the mutation of His393$^{6.55}$ to alanine results in an increased affinity of 1,4-disubstituted phenylpiperazines to the dopamine D_{2L} receptor. This change most likely accounts for the reduced steric hindrance in this part of the binding pocket. In this work, we investigated the role of the steric hindrance imposed by the residue His393$^{6.55}$ for the receptor activation modulated by 1,4-disubstituted aromatic piperidines/piperazines. Site-directed mutagenesis and ligand modifications were used to probe the structural basis of ligand efficacy. The operational model of agonism was used to quantify the ligand bias between the ability of compounds to inhibit cAMP accumulation and stimulate extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. Whereas substantial ligand-biased signaling was observed for the D_{2L} wild-type receptor, an overall increase in agonism was observed for the D_{2L} H393$^{6.55}$A mutant without noteworthy functional selectivity. Targeted chemical modification of the phenylpiperazine moiety at the site of its interaction with the residue His393$^{6.55}$ led to the functionally selective ligand {3-[4-(2,3-dihydro-benzofuran-7-yl)-piperazin-1-yl]-propyl}-pyrazol[1,5-*a*]pyridine-3-carboxamide (FAUC350) that has distinct signaling profiles toward adenylyl cyclase and ERK1/2. FAUC350 behaves as an antagonist in the inhibition of cAMP accumulation and as a partial agonist in the stimulation of ERK1/2 phosphorylation (efficacy = 55%). Overall, the residue His393$^{6.55}$ and proximate molecular substructures of receptor ligands were identified to be crucial for multidimensional ligand efficacy.

Introduction

Because of its implication in various neurological and psychiatric disorders and as a target of antipsychotic drugs, the dopamine D_2 receptor is one of the most studied monoaminergic seven-transmembrane (7TM) receptors. It is well established that, similar to other 7TM receptors, D_2 has the ability to differentially process ligand-based signals to produce a partial activation of cellular signaling pathways in response to some ligands (Burris et al., 2002; Gay et al., 2004; Lane et al., 2007; Urban et al., 2007; Klewe et al., 2008; Masri et al., 2008). Aripiprazole, a 1,4-disubstituted phenylpiperazine, is the first D_2/D_3 dopamine receptor drug that acts as a partial agonist and has been approved for the treatment of psychiatric disorders (Burris et al., 2002). The unique pharmacology of aripiprazole, a drug having both partial agonist and antagonist activity at the D_2 receptor, suggests that functionally selective ligands may provide a new arena for the development of novel therapeutics for psychoses and other disorders (Burris et al., 2002; Mottola et al., 2002; Klewe et al., 2008; Masri et al., 2008). However, to rationally design functionally selective drugs, structure-activity relationships for a biased signaling must be understood (Kenakin and Miller, 2010).

Previous studies on the β_2-adrenergic receptor documented that the activation of a 7TM receptor is a multistep process in which structurally very similar agonists and partial agonists induce distinguishable active states (Ghanouni et al., 2001; Swaminath et al., 2005). The activation of 7TM receptors encompasses the movement of transmembrane helices, in particular of TM6 in a rigid-body fashion, making vertical

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ABBREVIATIONS: 7TM, seven transmembrane; TM, transmembrane domain; CHO, Chinese hamster ovary; 7-OH-DPAT, 7-hydroxy-2-(*N*,*N*-di-*n*-propylamino)tetralin; p$K_{0.5}$, negative decadic logarithm of the concentration of the compound producing 50% inhibition of the specific binding of the radioactive ligand; ERK1/2, extracellular signal-regulated kinase 1/2; ELISA, enzyme-linked immunosorbent assay; FAUC350, {3-[4-(2,3-dihydro-benzofuran-7-yl)-piperazin-1-yl]-propyl}-pyrazol[1,5-*a*]pyridine-3-carboxamide; FAUC335, *N*-[3-[4-(2-methylsulfanylphenyl)piperazin-1-yl]propyl]pyrazolo-[1,5-a]pyridine-3-carboxamide; FAUC321, *N*-[3-[4-(2-methoxyphenyl)piperazin-1-yl]propyl]pyrazolo-[1,5-*a*]pyridine-3-carboxamide; FAUC346, *N*-[4-[4-(2-methoxyphenyl)piperazin-1-yl]butyl]benzo[*b*]thiophene-2-carboxamide; CPD1, *N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)biphenyl-4-carboxamide. PBS, phosphate-buffered saline; SPD, (−)-stepholidine.

"see-saw" movements toward TM3 that result in accumulations around the main ligand-binding pocket at the extracellular side and an opening for G protein binding at the intracellular side (Elling et al., 2006; Schwartz et al., 2006; Nygaard et al., 2009; Bokoch et al., 2010).

We demonstrated that the mutation of His393$^{6.55}$ to alanine results in the increased affinity of 1,4-disubstituted phenylpiperazines at the D_{2L} receptor (Ehrlich et al., 2009). We hypothesized that the additional space created by the H393$^{6.55}$A mutation leads to an increase in conformational freedom of the phenylalanine residues Phe389$^{6.51}$ and Phe390$^{6.52}$. This enables a better accommodation of the phenylpiperazine moiety to the aromatic microdomain of TM6. Molecular dynamics simulation showed that the aromatic substituent of the phenylpiperazine moiety of *N*-[3-[4-(2-methylsulfanylphenyl)piperazin-1-yl]propyl]pyrazolo-[1,5-*a*]-pyridine-3-carboxamide (FAUC335) is in close proximity to the residue His393$^{6.55}$. An interaction between a ligand and His6.55 was also observed in the crystal structure of the highly homologous dopamine D_3 receptor (Chien et al., 2010). The steric hindrance imposed by the bulky phenylpiperazine moiety might prevent the full movement of TM6 after the binding of 1,4-disubstituted phenylpiperazine. This would lead to the reduced efficacy of these ligands, which are typically classified as antagonists or partial agonists at the D_2 receptor (Bettinetti et al., 2002, 2005).

To determine the importance of the residue His393$^{6.55}$ for receptor activation modulated by 1,4-disubstituted phenylpiperazines, we used site-directed mutagenesis and ligand modification to reduce or enhance the steric interactions between the residue 393$^{6.55}$ and the phenylpiperazine moiety. We hypothesized that the reduction of the steric constrains would lead to increased efficacy, whereas the enhancement of the steric constrains would lead to decreased ligand efficacy. The D_{2L} wild-type, D_{2L} H393$^{6.55}$A, and D_{2L} H393$^{6.55}$F receptors have been compared in their ability to bind the ligands, to inhibit the adenylyl cyclase and thus to inhibit cAMP accumulation, and to stimulate the phosphorylation of ERK1/2 after treatment with dopamine-like agonists and partial agonists from the group of 1,4-disubstituted phenylpiperazines, including the tailor-made ligand {3-[4-(2,3-dihydro-benzofuran-7-yl)-piperazin-1-yl]-propyl}-pyrazol[1,5-*a*]pyridine-3-carboxamide (FAUC350). As a molecular indicator of differences in the mechanism of agonist action, the operational model of agonism was used to quantify the ability of an agonist to elicit a response in a given assay, calculate the bias between the signaling pathways, and calculate the bias between the wild-type and mutant receptor (Black and Leff, 1983; Kenakin, 2009; Kenakin and Miller, 2010; Evans et al., 2011).

Materials and Methods

Materials. Dulbecco's modified Eagle's medium/F-12, L-glutamine, fetal bovine serum, penicillin-streptomycin, zeocin, and hygromycin B were purchased from Invitrogen (Carlsbad, CA). Pertussis toxin from *Bordetella pertussis* was purchased from Sigma (St. Louis, MO). [^{3}H]spiperone (97 Ci/mmol) was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Dopamine (3,4-dihydroxyphenethylamine), quinpirole [(−)-quinpirole hydrochloride], spiperone, haloperidol, 7-hydroxy-2-(*N*,*N*-di-*n*-propylamino)tetralin (7-OH-DPAT),

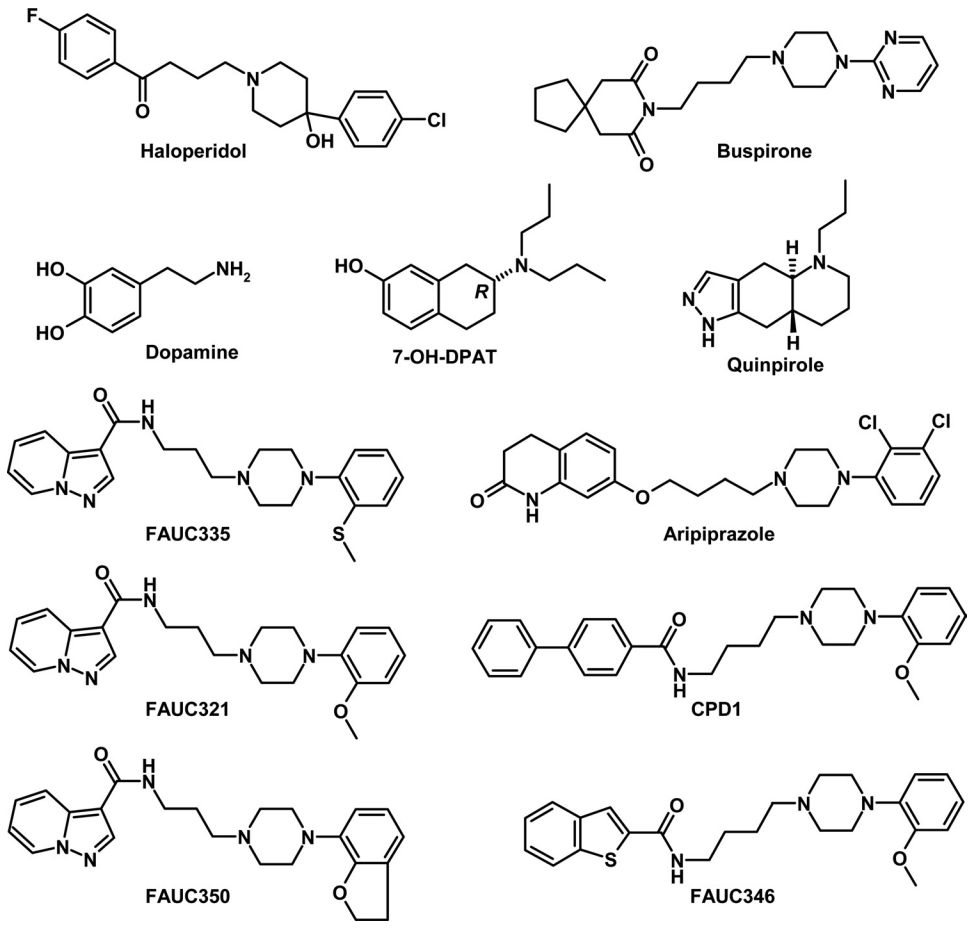


Fig. 1. Chemical structures of the ligands investigated in this study.

and other compounds were purchased from Sigma, unless otherwise stated. The compounds FAUC335 (Ehrlich et al., 2009), *N*-[3-[4-(2-methoxyphenyl)piperazin-1-yl]propyl]pyrazolo-[1,5-*a*]pyridine-3-carboxamide (FAUC321) (Bettinetti et al., 2002), *N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)biphenyl-4-carboxamide (CPD1) (Hackling et al., 2003), *N*-[4-[4-(2-methoxyphenyl)piperazin-1-yl]butyl]benzo[*b*]-thiophene-2-carboxamide (FAUC346) (Bettinetti et al., 2002), and aripiprazole (Oshiro et al., 1998) were synthesized as described previously. See Fig. 1 for structures.

Chemistry. Detailed description of the FAUC350 synthesis and the synthesis scheme are in the Supplemental Data. In general, reagents and dry solvents were obtained from commercial sources unless stated otherwise and were used as received. Reactions were conducted under dry N_2. Evaporations of product solutions were done in vacuo with a rotary evaporator. Column chromatography was performed with 60 μm silica gel. For thin-layer chromatography silica gel, 60-μm plates were used (UV, I_2, or ninhydrin detection). ^{1}H-NMR spectra were recorded at 360 MHz on a Bruker Avance 360 (Bruker, Milan, Italy) in $CDCl_3$ at 300 K; chemical shifts are given in δ relative to tetramethylsilane in ppm (tetramethylsilane = 0). IR spectra were measured on a Jasco (Tokyo, Japan) 410 FT-IR spectrometer. Electron impact mass spectrometry was done by electron impact ionization (70 eV) with a solid inlet on a JEOL (Tokyo, Japan) GCmate II spectrometer. High-resolution mass spectrometry was done at a resolution of M/ΔM = 5000 relative to perfluorokerosene on a JEOL GCmate II spectrometer. Purity was assessed by analytical high-performance liquid chromatography [Agilent 1100 preparative series (Agilent Technologies, Santa Clara, CA), equipped with a multiwavelength detector; column: Zorbax Eclipse XDB-C8 analytical column (Agilent Technologies), 4.6 × 150 mm, 5 μm, flow rate: 0.5 ml/min, detection wavelength: 220 nm]. System 1 (S1): 10 to 75% CH_3OH in H_2O + 0.1% HCO_2H in 18 min; system 2 (S2): 5 to 65% CH_3CN in H_2O + 0.1% HCO_2H in 26 min.

Site-Directed Mutagenesis and Cloning. The cDNA of the human dopamine $D2_{long}$ (D_{2L}) receptor was purchased from the Missouri University of Science and Technology cDNA Resource Center (Rolla, MO). The site-directed mutagenesis was performed as described previously (Ehrlich et al., 2009). The D_{2L} wild-type, D_{2L} H393$^{6.55}$A, and D_{2L} H393$^{6.55}$F receptor cDNAs were subcloned into a pcDNA5/FRT vector (Invitrogen) using NheI/XhoI restriction sites. The entire coding region of the D_{2L} receptor clones was sequenced to ensure that the correct mutation was introduced and to confirm the absence of unwanted mutations.

Cell Lines and Transfection. The Flp-in CHO cell line (Invitrogen) was maintained in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin, and 0.25 μg/ml zeocin and kept in a humid atmosphere at 37°C with 5% CO_2.

The Flp-in CHO cells were transfected with the pOG44 vector encoding Flp recombinase and the pcDNA5/FRT vector encoding specific dopamine receptors at a ratio of 9:1 using *Trans*IT-LT transfection reagent (Mirus Bio Corporation, Madison, WI). Forty-eight hours after transfection, cells were subcultured and the medium was supplemented with 750 μg/ml hygromycin B to obtain colonies stably expressing dopamine receptors. For the maintenance of stably transfected cell lines, the concentration of hygromycin B was reduced to 250 μg/ml to prevent the reversion of transfected Flp-in CHO cells to a nontransfected state.

Cell Harvest and Membrane Preparation. Cells were washed with phosphate-buffered saline (PBS), briefly treated with Tris-EDTA buffer (10 mM Tris, 0.5 mM EDTA, 5 mM KCl, 140 mM NaCl, pH 7.4), and dissociated with a cell scraper. Cells were pelleted at 1000*g* for 6 min at 4°C, resuspended in Tris-EDTA-$MgCl_2$ buffer (50 mM Tris, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, pH 7.4), and subsequently lysed with an Ultra-Turrax (IKA-Werke GmbH & Co. KG, Staufen, Germany). After additional centrifugation at 50,000*g*, the membranes were resuspended in the binding buffer (50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 100 μg/ml bacitracin, 5 μg/ml soybean trypsin inhibitor), and homogenized 10 times with a glass-Teflon homogenizer at 4°C. The homogenized membranes were shock-frozen in liquid nitrogen and stored at −80°C. The protein concentration was determined with the Lowry method with bovine serum albumin used as a standard (Lowry et al., 1951).

Receptor Binding Studies. Competition experiments with human D_{2L} receptors were run with preparations of membranes from CHO cells stably expressing the corresponding receptor and [^{3}H]spiperone at a final concentration of 0.1 to 0.2 nM. The assays were carried out with a protein concentration of 10 μg/ml and K_d values of 0.17 ± 0.06, 0.19 ± 0.04, 0.09 ± 0.01, and 0.09 ± 0.07 nM for the D_{2L} wild-type, D_{2L} H393$^{6.55}$A, D_{2L} H393$^{6.55}$F, and D_{2L} H393$^{6.55}$K receptors, respectively. All assays were performed in 96-well plates at a final volume of 200 μl. After incubation for 1 h at 37°C, we stopped the assay by filtration through Whatman (Clifton, NJ) GF/B filters presoaked with 0.3% polyethylenimine. The filters were rinsed five times with ice-cold Tris-NaCl buffer. After 3 h of drying at 60°C, filters were sealed with melt-on scintillator sheets MeltiLex B/HS (PerkinElmer Life and Analytical Sciences, Waltham, MA), and the filter-bound radioactivity was measured with a MicroBeta TriLux liquid scintillator counter (PerkinElmer Life and Analytical Sciences). Three to six experiments per compound were performed with each concentration in triplicate.

Adenylyl Cyclase Inhibition Assay. Bioluminescence-based cAMP-Glo assay (Promega, Madison, WI) was performed according to the manufacturer's instructions after adjusting the volume of required reagents for use in a white half-area 96-well plate at a final volume of 80 μl. In brief, CHO cells expressing D_{2L} wild-type, D_{2L} H393$^{6.55}$A, or D_{2L} H393$^{6.55}$F receptor were seeded into a white half-area 96-well plate (5000 cells/well) 24 h before the assay. The cells expressed comparable amount of the receptor as determined by saturation experiments (4100 ± 750 fmol/mg for the D_{2L} wild type, 4400 ± 350 fmol/mg for D_{2L} H393$^{6.55}$A, and 3900 ± 440 fmol/mg for D_{2L} H393$^{6.55}$F). Cells were first briefly washed with PBS, pH 7.4 to remove traces of serum and then incubated with various concentrations of substances in the presence of 20 μM forskolin in serum-free medium that contained 500 μM 3-isobutyl-1-methylxanthine and 100 μM 4-(3-butoxy-4-methoxybenzyl)imidazoline, pH 7.4. After 15 min of incubation at 25°C, the cells were lysed with cAMP-Glo lysis buffer. After lysis, the kinase reaction was performed with a reaction buffer containing protein kinase A. At the end of the kinase reaction an equal volume of Kinase-Glo reagent was added. The plates were read with a luminescence protocol on a Victor^{3}V microplate reader (PerkinElmer Life and Analytical Sciences). The experiments were performed three to nine times per compound with each concentration in duplicate. The absolute E_{max} values for quinpirole were 16,000 ± 2400, 13,700 ± 1800, and 12,800 ± 2100 relative luminescence units for the D_{2L} wild-type, D_{2L} H393$^{6.55}$A, and D2L H393$^{6.55}$F, respectively.

PhosphoERK1/2 ELISA Assay. The PathScan phospho-p42/44 mitogen-activated protein kinase (Thr202/Tyr204) sandwich ELISA (Cell Signaling Technology, Danvers, MA) was performed according to the manufacturer's instructions. In brief, 6 × 10^6 CHO cells that expressed D_{2L} wild-type, D_{2L} H393$^{6.55}$A, or D_{2L} H393$^{6.55}$F receptor were seeded in a 100-mm plate. The cells expressed comparable amount of the receptor as determined by saturation experiments (4100 ± 750 fmol/mg for the D_{2L} wild type, 4400 ± 350 fmol/mg for D_{2L} H393$^{6.55}$A, and 3900 ± 440 fmol/mg for D_{2L} H393$^{6.55}$F). The next day, cells were washed once with serum-free media and incubated in the presence of serum-free media for an additional 24 h. For the experiments with the pertussis toxin, 25 ng/ml toxin was added to serum-free media for 24 h. On the day of the experiment, the medium was removed and replaced with serum-free media containing various concentrations of the test substances as indicated and incubated for 5 min at 37°C. A wash with ice-cold PBS and the addition of the lysis buffer stopped the reaction. The plates were kept on ice, and cells were scraped, briefly sonicated (UP50H; Hielscher Ultrasound Technologies, Teltow, Germany), and centrifuged at 15,000*g* for 10 min.

The supernatant was promptly diluted with the sample diluent and incubated overnight at 4°C in the well. After intensive washing steps, the detection of the phosphorylated ERK1/2 followed. The absorbance was read at 450 nm within 2 min after addition of the STOP solution on a Victor^{3}V microplate reader (PerkinElmer Life and Analytical Sciences). The experiment was performed three to four times per compound. The absolute E_{max} values for quinpirole were 1.634 ± 0.155, 1.439 ± 0.117, and 1.569 ± 0.110 relative absorbance units for the D_{2L} wild-type, D_{2L} H393$^{6.55}$A, and D_{2L} H393$^{6.55}$F, respectively.

Data Analysis. The competition curves of the receptor binding experiments and activity assays were analyzed by nonlinear regression using the algorithms in Prism 5.0 (GraphPad Software Inc., San Diego, CA). Competition curves were fitted to the sigmoid curves by nonlinear regression analysis in which the $\log EC_{50}$ value and the Hill coefficient were free parameters. EC_{50} values were transformed to pK_i values according to the equation of Cheng and Prusoff (1973).

Dose-response curves of the activity assays were fitted to the operational model developed by Black and Leff (1983) to obtain an estimate of the transducer constant τ: $E = E_m \times \tau^n[A^n]/((K_A + [A])^n + \tau^n[A]^n)$, where E is the effect, E_m is the maximum possible effect, A is the agonist concentration, n is the transducer slope, and K_A is the dissociation constant of the agonist-receptor complex. The transducer constant τ is a parameter describing the signal transduction efficiency of the system, and the intrinsic efficacy of the agonist was estimated from the fit of the data. Quinpirole was used as the full agonist reference that defines zero and a maximal (100%) response of the system. The curves of all agonists had a Hill coefficient of unity or close to unity. The $\log(\tau/K_A)$ values depicted the "strength" of a given agonist to activate a defined pathway in a defined system. Because agonists are allosteric modulators of 7TM, it is necessary that both efficacy and affinity of the agonist be captured for agonism (Kenakin, 2009; Kenakin and Miller, 2010).

The $\Delta\log(\tau/K_A)$ values were calculated as: $\Delta\log(\tau/K_A)_{agonist/reference} = \log(\tau/K_A)_{agonist} - \log(\tau/K_A)_{reference}$ to compare various agonists within signaling pathway. The $\Delta\Delta\log(\tau/K_A)$ values were calculated as: $\Delta\Delta\log(\tau/K_A)_{H6.55A/wild\ type} = \Delta\log(\tau/K_A)_{H6.55A} - \Delta\log(\tau/K_A)_{wild\ type}$.

The $\Delta\Delta\log(\tau/K_A)$ values provided a scale to compare various agonists between the signaling pathways or between the wild-type D_{2L} and mutant D_{2L} H393$^{6.55}$A receptor. Detailed calculations of all parameters are summarized in Supplemental Tables 3 to 6. The calculations of 95% confidence intervals were made with the program Mathematica 5.0 and based on the algorithm described elsewhere (T. Kenakin, C. Watson, V. Muniz-Medina, A. Christopoulos, S. Novick, manuscript in preparation).

Results

Radioligand Displacement Studies. To determine the influence of mutations H393$^{6.55}$A and H393$^{6.55}$F on the affinity of dopamine receptor agonists, dopamine, quinpirole, and 7-OH-DPAT were chosen. The binding data were fitted in a one-site (monophasic) and a two-site (biphasic) model. A comparison of both models revealed that the two-site (biphasic) model described the binding data for dopamine, quinpirole, and 7-OH-DPAT more accurately. For all other compounds, a one-site (monophasic) model was preferred. For the agonists, changes in the affinities at the high-affinity site will be discussed. At the D_{2L} H393$^{6.55}$A receptor, the affinity of the endogenous ligand dopamine dropped by 28-fold (Table 1). The introduction of the aromatic phenylalanine (H393$^{6.55}$F) or the basic lysine (H393$^{6.55}$K) did not restore the affinity of dopamine (Table 1 and Supplemental Table 1), underscoring the importance of the unique properties of the imidazole side chain of histidine for the binding of dopamine. The D_{2L} H393$^{6.55}$K mutant was expressed at approximately a 10 times lower density (410 ± 120 fmol/mg) compared with wild-type, H393$^{6.55}$A, or H393$^{6.55}$F receptors (4100 ± 750, 4400 ± 350, and 3900 ± 440 fmol/mg, respectively) as determined by [^{3}H]spiperone saturation experiments. The affinities of the synthetic dopamine receptor agonists 7-OH-DPAT and quinpirole were significantly reduced by the D_{2L} H393$^{6.55}$A mutation (12- and 63-fold, respectively). The introduction of an aromatic phenylalanine at position 6.55 (D_{2L} H393$^{6.55}$F) completely restored the affinity of 7-OH-DPAT and quinpirole completely, indicating the significance of the aromatic character of histidine for the binding affinity of these synthetic agonists.

For a range of dopamine antagonists, it was documented that the mutations D_{2L} H393$^{6.55}$L and D_{2L} H393$^{6.55}$C have only moderate compound-specific effects on the affinity of antagonists (Woodward et al., 1994; Javitch et al., 1998). The

TABLE 1

pK_i values for the dopamine receptor antagonists and agonists on D_{2L} wild-type, D_{2L} H393$^{6.55}$A, and D_{2L} H393$^{6.55}$F receptors

The affinities of investigated substances were determined on membrane preparations of stably transfected CHO cells expressing either D_{2L} wild-type, D_{2L} H393$^{6.55}$A, or D_{2L} H393$^{6.55}$F receptors using [^{3}H]spiperone displacement study. Data are derived from normalized curves of three to six experiments done in triplicate. Data were analyzed by nonlinear regression and were best-fit to one-site (monophasic) or two-site (biphasic) competition curves. pK_i values were calculated according to Cheng and Prusoff (1973). The $10^{\Delta pK_{iala/wt}}$ and $10^{\Delta pK_{iphe/wt}}$ values indicate the changes in the affinity. pK_i values are S.E.M. Values in parentheses are Hill slope, but for pK_{high} the values in parentheses are fraction of high-affinity sites.

Compound	pK_i Measured	pK_i for			$\Delta pK_i^{ala/wt}$	$10^{\Delta pK_{iala/wt}}$	$\Delta pK_i^{phe/wt}$	$10^{\Delta pK_{iphe/wt}}$
		D_{2L} Wild-Type	D_{2L} H393$^{6.55}$A	D_{2L} H393$^{6.55}$F				
Haloperidol	$pK_{0.5}$	9.41 ± 0.03 (−0.83)	9.60 ± 0.02 (−0.87)	9.46 ± 0.03 (−0.90)	−0.19	0.65	−0.05	0.89
Buspirone	$pK_{0.5}$	6.64 ± 0.02 (−0.85)	7.00 ± 0.02 (−0.91)	7.70 ± 0.05 (−0.86)	−0.55	0.28	−1.06	0.09
Aripiprazole	$pK_{0.5}$	8.26 ± 0.03 (−0.86)	8.51 ± 0.03 (−0.92)	8.89 ± 0.04 (−0.98)	−0.25	0.56	−0.63	0.23
FAUC335	$pK_{0.5}$	8.85 ± 0.04 (−0.71)	9.35 ± 0.03 (−0.77)	8.72 ± 0.03 (−0.77)	−0.50	0.32	0.13	1.4
FAUC321	$pK_{0.5}$	8.43 ± 0.03 (−0.89)	9.11 ± 0.02 (−0.84)	8.00 ± 0.04 (−0.84)	−0.87	0.13	0.43	2.7
FAUC350	$pK_{0.5}$	7.41 ± 0.03 (−0.92)	8.30 ± 0.04 (−1.00)	7.70 ± 0.04 (−0.75)	−0.89	0.13	−0.29	0.51
CPD1	$pK_{0.5}$	7.43 ± 0.02 (−0.70)	9.00 ± 0.08 (−0.64)	6.89 ± 0.03 (−1.18)	−1.57	0.03	0.54	3.5
FAUC346	$pK_{0.5}$	6.61 ± 0.03 (−1.02)	7.77 ± 0.06 (−0.88)	6.76 ± 0.04 (−0.82)	−1.16	0.07	−0.15	0.71
Dopamine	$pK_{0.5}$	6.35 ± 0.05 (−0.43)	5.24 ± 0.07 (−0.45)	5.40 ± 0.05 (−0.41)	1.11	13	0.95	8.9
	pK_{high}	8.08 ± 0.12 (31%)	6.64 ± 0.15 (36%)	6.82 ± 0.10 (37%)	1.44	28	1.26	18
	pK_{low}	5.70 ± 0.09	4.52 ± 0.14	4.66 ± 0.08	1.18	15	1.04	11
7-OH-DPAT	$pK_{0.5}$	7.16 ± 0.09 (−0.40)	5.92 ± 0.06 (−0.51)	7.05 ± 0.03 (−0.58)	1.24	17	0.11	1.2
	pK_{high}	8.96 ± 0.06 (39%)	7.89 ± 0.20 (24%)	8.77 ± 0.12 (26%)	1.07	12	0.19	1.5
	pK_{low}	6.72 ± 0.04	5.55 ± 0.07	6.62 ± 0.04	1.17	15	0.10	1.3
Quinpirole	$pK_{0.5}$	6.21 ± 0.04 (−0.44)	4.96 ± 0.03 (−0.57)	6.51 ± 0.03 (−0.52)	1.25	18	−0.30	0.50
	pK_{high}	7.89 ± 0.12 (31%)	6.09 ± 0.11 (35%)	7.96 ± 0.13 (31%)	1.80	63	−0.07	0.85
	pK_{low}	5.68 ± 0.08	4.42 ± 0.09	5.96 ± 0.06	1.26	18	−0.28	0.52

same was true for the affinities of the mutants we analyzed. The affinity of the antagonists haloperidol and buspirone increased moderately (up to 3.5-fold) at the D_{2L} $H393^{6.55}A$ mutant. The mutation D_{2L} $H393^{6.55}F$ had no influence on the affinity of haloperidol. The affinity of buspirone increased by 11-fold.

The investigated partial agonists from the group of 1,4-disubstituted phenylpiperazines can be classified by their spacer length and chemical appendage. Whereas FAUC335, FAUC321, and FAUC350 have a propylene spacer (Ehrlich et al., 2009), the homologs CPD1 (Hackling et al., 2003) and FAUC346 (Bettinetti et al., 2002) display butylene spacers between the basic center and the two-atom carboxamide moiety. Aripiprazole combines a butlyene linker with a one-atom heteroaromatic ether group (Oshiro et al., 1998). The affinities of FAUC335, FAUC321, and FAUC350 gained moderately on the affinity at the D_{2L} $H393^{6.55}A$ receptor (between 2.3- and 7.8-fold), and the affinities of CPD1 and FAUC346 increased 14- and 33-fold (Table 1). Aripiprazole demonstrated no significant changes in the affinity at the D_{2L} $H393^{6.55}A$ receptor. At the D_{2L} $H393^{6.55}F$ receptor the affinity of aripiprazole increased 4.3-fold. The affinities of other compounds remained unchanged or mildly decreased (up to 3.5-fold as for CPD1). These findings are in accordance with those of our previous study (Ehrlich et al., 2009), which demonstrated the D_{2L} $H393^{6.55}A$ mutant displays an increased affinity for 1,4-disubstituted phenylpiperazines.

Use of the Operational Model of Functional Selectivity Identified Molecule-Specific Parameters that Lead to a Ligand-Biased Signaling (Functional Selectivity). Dose-response curves of activity assays were fitted to the operational model of agonism (Black and Leff, 1983) to obtain the dissociation constant of the agonist-receptor complex (K_A), and an estimate of the transducer constant τ, that describes the signal transduction efficiency of the system and the intrinsic efficacy of an agonist. Quinpirole was used as the reference full agonist and thus defines zero and a maximal (100%) response of the system. The CHO cells expressed comparable amount of receptors as determined by saturation experiments (4100 ± 750 fmol/mg for the D_{2L} wild type, 4400 ± 350 fmol/mg for D_{2L} $H393^{6.55}A$, and 3900 ± 440 fmol/mg for D_{2L} $H393^{6.55}F$). The log (τ/K_A) values depicted the "strength" of a given agonist to activate a defined pathway in a defined system (Kenakin, 2009; Kenakin and Miller, 2010). To compare various agonists within the signaling pathways, $\Delta\log(\tau/K_A)$ values were calculated. To compare agonists between the signaling pathways or between the wild-type and mutant receptors, $\Delta\Delta\log(\tau/K_A)$ values were calculated. Detailed calculations of all parameters are summarized in Supplemental Tables 4 to 7.

The compounds can be partitioned into three groups based on their ability to inhibit the accumulation of cAMP (Fig. 2A). 7-OH-DPAT and dopamine had comparable $\Delta\log(\tau/K_A)$ values (0.05 and 0.02, respectively) (Fig. 2C). The partial agonists aripiprazole, FAUC335, and FAUC321 had $\Delta\log(\tau/K_A)$ values of −1.52, −1.55, and −0.98, respectively, indicating their moderate ability to inhibit cAMP accumulation. FAUC346 was a very weak partial agonist with the $\Delta\log(\tau/K_A)$ value of −2.50. FAUC350 and CPD1 were antagonists in the investigated pathway with no detectable efficacy. The calculation of the $\Delta\log(\tau/K_A)$ was thus not possible.

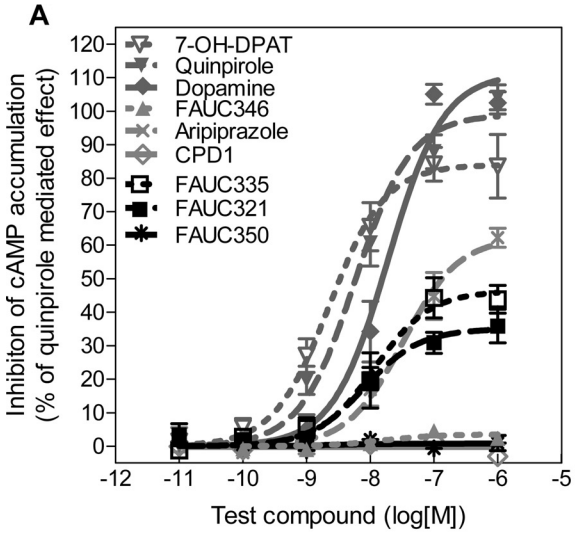
$G_{i/o}$ protein-mediated ERK1/2 phosphorylation reached a maximum approximately 5 min after the stimulation of the D_{2L} receptor (Fig. 2B), and it could be completely blocked by the pertussis toxin (Supplemental Fig. 1). The $\Delta\log(\tau/K_A)$ value of dopamine was 0.21, indicating the activation profile was very similar to the reference agonist quinpirole (Fig. 2C). The ability of 7-OH-DPAT to stimulate the phosphorylation of ERK1/2 was greater than that of dopamine [$\Delta\log(\tau/K_A)$ value 0.76]. All 1,4-disubstituted phenylpiperazines, with the exception of FAUC346, demonstrated improved $\Delta\log(\tau/K_A)$ values, indicating their greater efficiency in modulation of the ERK1/2 phosphorylation. For FAUC350 and CPD1, which were antagonists in the cAMP pathway, the moderate ability to stimulate the ERK1/2 phosphorylation [$\Delta\log(\tau/K_A)$ values −1.00 and −2.57, respectively] was observed.

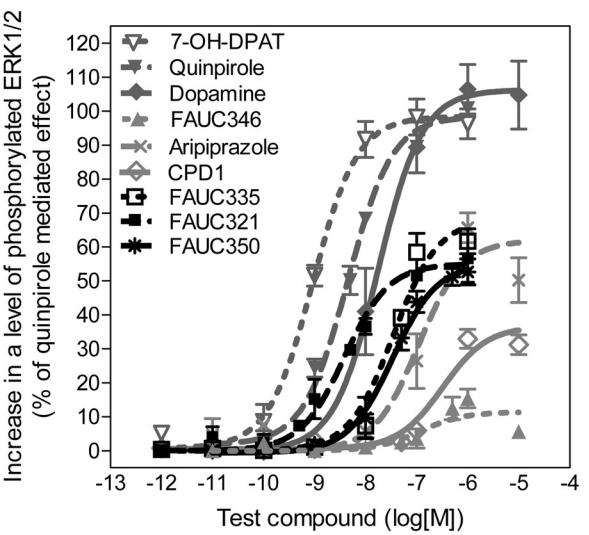
The overall bias [$\Delta\Delta\log(\tau/K_A)$ values] of the compounds for the specific pathway modulated by the activation of the D_{2L} wild-type receptor is depicted in Fig. 2D. Structurally very diverse molecules including dopamine, aripiprazole, and FAUC346 did not show any bias for any of these pathways. CPD1 was moderately active in the ERK1/2 pathway and behaved as an antagonist in cAMP pathway. FAUC321, which differs from FAUC335 only in a methylsulfide substituent instead of a methoxy group in position 2 of the phenylpiperazine ring (Fig. 1), showed a significant preference for the stimulation of ERK1/2 phosphorylation. The fusion of a dihydrofuran ring to the phenylpiperazine unit of FAUC350 abolished the ability of this compound to inhibit the D_{2L} receptor mediated inhibition of cAMP accumulation (no detectable efficacy), but preserved its ability to stimulate ERK1/2 phosphorylation (pEC_{50} 7.46 ± 0.10, efficacy 55 ± 3%) (Supplemental Data). Thus, FAUC350 represents a novel functionally selective D_{2L} ligand with distinct signaling profiles toward adenylyl cyclase and ERK1/2. This example demonstrates that minute changes in the molecular structure suffice to modify ligand-biased signaling.

The Mutation $H393^{6.55}A$ Increased the Efficacy of 1,4-Disubstituted Phenylpiperazines and Abolished the Ability of the Receptor to Recognize Biased Ligands. To determine the influence of $H393^{6.55}A$ mutation on $\Delta\log(\tau/K_A)$ and $\Delta\Delta\log(\tau/K_A)$ values of selected compounds, the ability of the test compounds to inhibit cAMP accumulation and stimulate the ERK1/2 phosphorylation was investigated with CHO cells stably expressing the D_{2L} $H393^{6.55}A$ receptor. The dose-response curves and the calculation of $\Delta\log(\tau/K_A)$ and $\Delta\Delta\log(\tau/K_A)$ values are depicted in Fig. 3. All 1,4-disubstituted phenylpiperazines showed substantial increase in the $\Delta\log(\tau/K_A)$ value up to the value of 1.09 (FAUC321) in the ability to inhibit cAMP accumulation, indicating that the substitution of histidine for alanine increased the ability of 1,4-disubstituted phenylpiperazines to activate the D_{2L} $H393^{6.55}A$ receptor and consequently to inhibit the cAMP accumulation. A similar effect was observed for the ability of compounds to stimulate the ERK1/2 phosphorylation. Comparing overall biases [$\Delta\Delta\log(\tau/K_A)$ values] for the investigated pathways, 7-OH-DPAT, aripiprazole, FAUC335, FAUC321, FAUC350, and FAUC346 showed no significant bias, indicating that the substitution of the $His393^{6.55}$ for alanine largely abolished the ability of compounds to elicit ligand-biased signaling. The only exceptions were dopamine and CDP1. CPD1 behaved as an antagonist in the cAMP pathway at the D_{2L} wild-type receptor and changed the pref-

erence for the signaling pathway by being more efficacious in stimulating the inhibition of cAMP accumulation than the ERK1/2 phosphorylation at the D_{2L} H393$^{6.55}$A receptor.

The Residue His6.55 Is Crucial for Ligand Efficacy and Ligand-Biased Signaling at the D_{2L} Receptor. To quantify ligand-biased signaling and the bias between the





C

	Inhibition of cAMP accumulation	
	Log (τ/K_A)	Δlog (τ/K_A)
Quinpirole	8.68	0.00
7-OH-DPAT	8.73	0.05
Dopamine	8.70	0.02
Aripiprazole	7.16	-1.52
FAUC335	7.13	-1.55
FAUC321	7.70	-0.98
FAUC350	n.a.	n.a.
FAUC346	6.18	-2.50
CPD1	n.a.	n.a.
	Stimulation of ERK1/2 phosphorylation	
Quinpirole	8.41	0.00
7-OH-DPAT	9.17	0.76
Dopamine	8.62	0.21
Aripiprazole	6.67	-1.74
FAUC335	7.29	-1.12
FAUC321	8.19	0.22
FAUC350	7.41	-1.00
FAUC346	5.91	-2.50
CPD1	5.84	-2.57

D

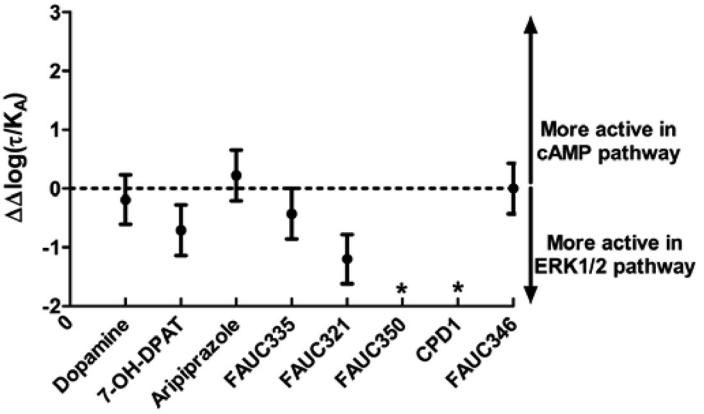
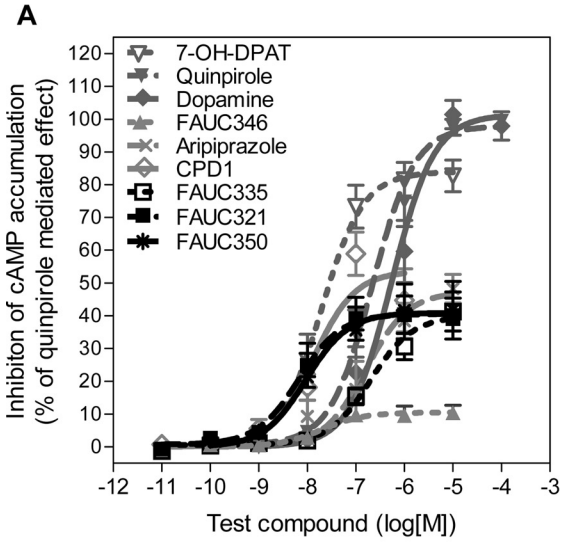
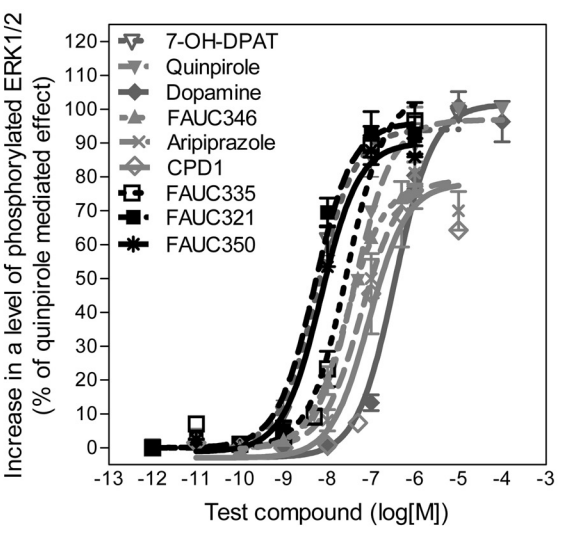


Fig. 2. Use of the operational model of agonism to quantify the ligand bias at the D_{2L} wild-type receptor. The assays were performed on the D_{2L} wild-type receptor expressing CHO cells. A, the inhibition of cAMP accumulation. The cells were incubated with 20 μM forskolin, and the D_{2L} wild-type receptor-mediated inhibition of cAMP accumulation was measured after stimulation with the investigated compounds. Pooled data of three to nine experiments performed in triplicate are shown as normalized curves with error bars representing the S.E.M. The pEC_{50} values and efficacies are summarized in Supplemental Table 2. B, the stimulation of ERK1/2 phosphorylation. Serum-starved cells were stimulated with investigated compounds for 5 min at 37°C. The level of phosphorylated ERK1/2 was detected by ELISA. Pooled data of three to four experiments are shown as normalized curves with error bars representing the S.E.M. The pEC_{50} values and efficacies are summarized in Supplemental Table 3. C, the log(τ/K_A) and Δlog(τ/K_A) values obtained with the operational model of agonism. n.a. indicates not available because of the antagonistic behavior of the compound in the selected signaling pathway. D, the $\Delta\Delta$log(τ/K_A) values as a measure of ligand bias between the pathways. The error bar represents 95% confidence interval. When the range includes zero, the ligands are not biased with the respect to the reference agonist. $*$ indicates the exclusive preference of the compound for the selected signaling pathway. Detailed calculations of Δlog(τ/K_A), $\Delta\Delta$log(τ/K_A) values, and their 95% confidence intervals are summarized in Supplemental Table 4.

D_{2L} wild-type and the D_{2L} H393$^{6.55}$A receptors in the selected pathway, the $\Delta\log(\tau/K_A)$ values from Figs. 2C and 3C were used for the calculation of the $\Delta\Delta\log(\tau/K_A)$ values (describing bias) of compounds for the mutant receptor. The analysis revealed significant bias of compounds for the mutant receptor. All compounds with the exception of dopamine were able to elicit greater response at the D_{2L} H393$^{6.55}$A receptor measured as the inhibition of cAMP accumulation





C

	Inhibition of cAMP accumulation	
	Log (τ/K_A)	Δlog (τ/K_A)
Quinpirole	6.69	0.00
7-OH-DPAT	7.55	0.86
Dopamine	6.75	0.06
Aripiprazole	6.25	-0.44
FAUC335	7.06	0.38
FAUC321	7.78	1.09
FAUC350	7.21	0.52
FAUC346	6.96	0.27
CPD1	7.59	0.90
	Stimulation of ERK1/2 phosphorylation	
Quinpirole	7.36	0.00
7-OH-DPAT	7.71	0.35
Dopamine	6.45	-0.91
Aripiprazole	6.91	-0.45
FAUC335	7.53	0.17
FAUC321	8.26	0.90
FAUC350	8.40	1.04
FAUC346	7.48	0.12
CPD1	6.78	-0.58

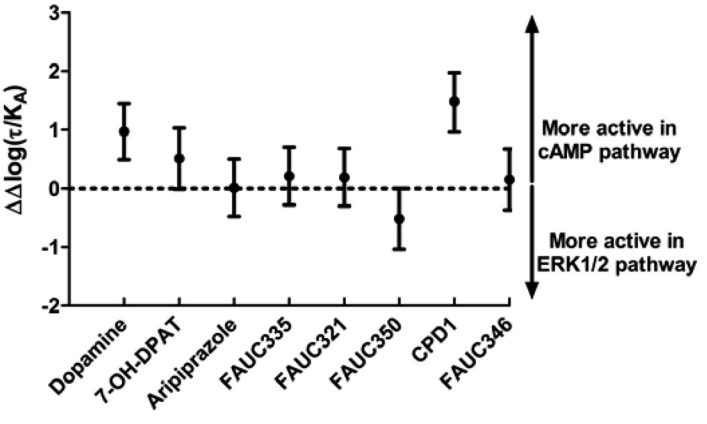


Fig. 3. Use of the operational model of agonism to quantify the ligand bias at the D_{2L} H393$^{6.55}$A receptor. The assays were performed on the D_{2L} H393$^{6.55}$A receptor expressing CHO cells. A, the inhibition of cAMP accumulation. The cells were incubated with 20 μM forskolin, and the D_{2L} H393$^{6.55}$A receptor-mediated inhibition of cAMP accumulation was measured after stimulation with the investigated compounds. Pooled data of three to nine experiments performed in triplicate are shown as normalized curves with error bars representing the S.E.M. The pEC_{50} values and efficacies are summarized in Supplemental Table 2. B, the stimulation of ERK1/2 phosphorylation. Serum-starved cells were stimulated with the investigated compounds for 5 min at 37°C. The level of phosphorylated ERK1/2 was detected by ELISA. Pooled data of three to four experiments are shown as normalized curves with error bars representing the S.E.M. The pEC_{50} values and the efficacies are summarized in Supplemental Table 3. C, the $\log(\tau/K_A)$ and $\Delta\log(\tau/K_A)$ values obtained with the operational model of agonism. D, the $\Delta\Delta\log(\tau/K_A)$ values as a measure of ligand bias between the pathways. The error bars represent 95% confidence interval. When the range includes zero, the ligands are not biased with respect to each other. $*$ indicates exclusive preference of the compound for the selected signaling pathway. Detailed calculations of $\Delta\log(\tau/K_A)$, $\Delta\Delta\log(\tau/K_A)$ values, and their 95% confidence intervals are summarized in Supplemental Table 5.

(Fig. 4A). Dopamine did not discriminate between the D_{2L} H393$^{6.55}$A and D_{2L} wild-type receptors in this assay. The ability of 1,4-disubstituted phenylpiperazines to stimulate the phosphorylation of ERK1/2 was strongly biased for the D_{2L} H393$^{6.55}$A receptor (Fig. 4B). Dopamine and 7-OH-DPAT were more active at the D_{2L} wild-type receptor (Fig. 4B), which is in accordance with their greater binding affinity on this receptor (Table 1). This overall increase in the activity of 1,4-disubstituted phenylpiperazines at the D_{2L} H393$^{6.55}$A receptor implied that the introduction of a smaller alanine at position 393$^{6.55}$ facilitates the movement of TM6 upon ligand binding; this results in the increased efficacy of 1,4-disubstituted phenylpiperazines. Consequently, we anticipated that an introduction of a slightly bulkier residue at the position 393$^{6.55}$ should negatively influence the efficacy of 1,4-disubstituted phenylpiperazines compared with the D_{2L} wild-type receptor. The steric hindrance imposed by the slightly bulkier residue would prevent the full movement of TM6 upon binding of 1,4-disubstituted phenylpiperazines and consequently lead to a reduced efficacy of these compounds.

To prove this hypothesis, the investigated compounds were tested on D_{2L} H393$^{6.55}$F receptor-expressing CHO cells by measuring the inhibition of cAMP accumulation and the stimulation of ERK1/2 phosphorylation. The results obtained from measurements of the inhibition of cAMP accumulation supported our hypothesis. The standard dopamine receptor agonists quinpirole, 7-OH-DPAT, and dopamine preserved their agonist nature also at the D_{2L} H393$^{6.55}$F receptor (Fig. 5A). The ability of 1,4-disubstituted phenylpiperazines to mediate the inhibition of cAMP accumulation was significantly reduced. FAUC350 and FAUC335 were weak partial agonists and aripiprazole and FAUC321 were weak inverse agonists, whereas FAUC346 and CPD1 acted as very strong inverse agonists. The mutation H393$^{6.55}$F considerably reduced the efficacy of 1,4-disubstituted phenylpiperazines.

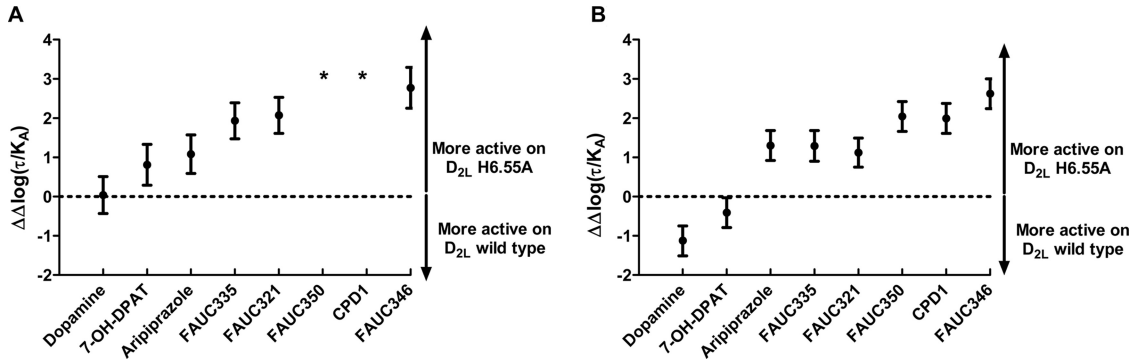


Fig. 4. Use of the operational model of agonism to quantify the ligand bias among the D_{2L} wild-type and D_{2L} H393$^{6.55}$A receptors. The $\Delta\log(\tau/K_A)$ values from Figs. 2C and 3C were used to calculate the $\Delta\Delta\log(\tau/K_A)$ values as a measure of ligand bias between the D_{2L} wild-type and D_{2L} H393$^{6.55}$A receptors. The error bars represent 95% confidence interval. When the range includes zero, the ligands are not biased with respect to each other. * indicates exclusive preference of the compound for the selected signaling pathway. Detailed calculations of $\Delta\log(\tau/K_A)$, $\Delta\Delta\log(\tau/K_A)$ values, and their 95% confidence intervals are summarized in Supplemental Table 6 and 7. A, the $\Delta\Delta\log(\tau/K_A)$ values as a measure of ligand bias between the D_{2L} wild-type and D_{2L} H393$^{6.55}$A receptors in their ability to inhibit cAMP accumulation. B, the $\Delta\Delta\log(\tau/K_A)$ values as a measure of ligand bias between the D_{2L} wild-type and D_{2L} H393$^{6.55}$A receptors in their ability to stimulate ERK1/2 phosphorylation.

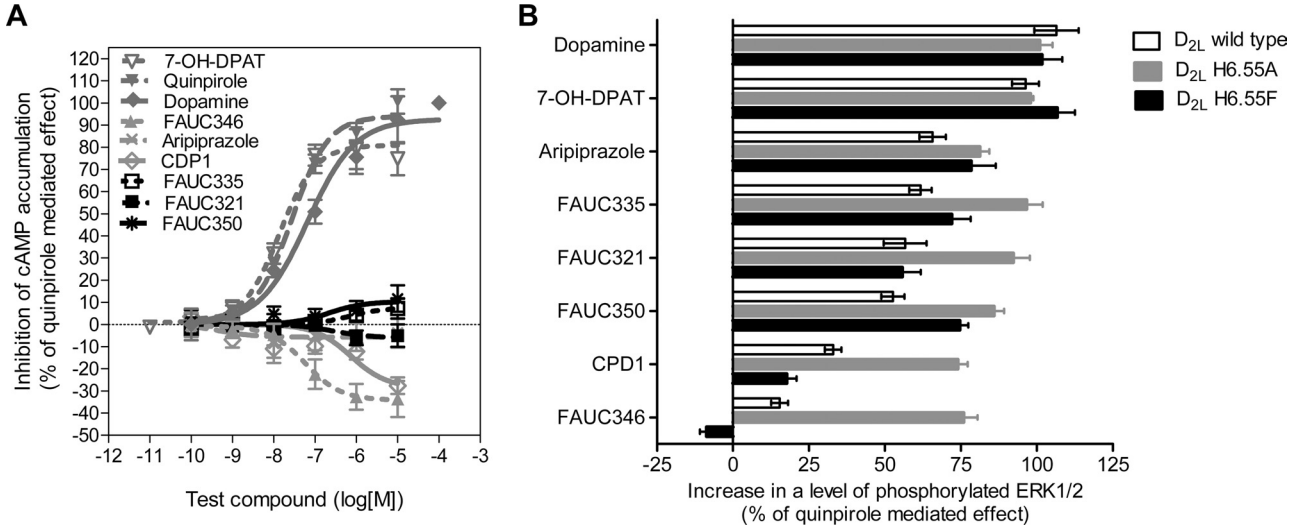


Fig. 5. The ability of investigated compounds to activate the D_{2L} H393$^{6.55}$F receptor. A, the inhibition of cAMP accumulation. The cells were incubated with 20 μM forskolin, and the D_{2L} H393$^{6.55}$F receptor-mediated inhibition of cAMP accumulation was measured after stimulation with the investigated compounds. Pooled data of three to four experiments performed in triplicate are shown as normalized curves with error bars representing the S.E.M. B, the stimulation of ERK1/2 phosphorylation. Serum-starved cells were stimulated with discrete concentration of investigated compounds for 5 min at 37°C. Dopamine, 7-OH-DPAT, and quinpirole were tested at 10 μM, and aripiprazole, FAUC335, FAUC321, FAUC350, FAUC346, and CPD1 were tested at 1 μM. The level of phosphorylated ERK1/2 was detected by ELISA. The mean values with error bars representing the S.E.M. of three to four experiments are shown.

To estimate the ability of the investigated compounds to stimulate D_{2L} $H393^{6.55}F$ receptor-mediated phosphorylation of ERK1/2, we used discrete sample concentrations (between 1 and 10 μM). As expected, the mutation $H393^{6.55}F$ had no influence on the efficacy of dopamine and 7-OH-DPAT (Fig. 5B). We were surprised to find that the mutation $H393^{6.55}F$ had no influence on the efficacy of FAUC335 and FAUC321 and only a minor effect on FAUC350 and CDP1. Only FAUC346 reacted on the mutation $H393^{6.55}F$ with an attenuation of efficacy (from 15% at the D_{2L} wild-type to −8% at the D_{2L} $H393^{6.55}F$ receptor). These observations underscore the role of $His393^{6.55}$ in the D_{2L} receptor as a crucial determinant of multidimensional ligand efficacy.

Discussion

The ability of the D_{2L} receptor to differentially process ligand-biased signals to produce limited activation of downstream signaling pathways in response to some ligands is well documented (Burris et al., 2002; Gay et al., 2004; Lane et al., 2007; Urban et al., 2007; Klewe et al., 2008). Dihydrexidine was the first ligand reported that displayed a functionally selective profile when fully inhibiting cAMP accumulation, but it was not able to inhibit the synthesis and release of dopamine or inhibit the firing of nigral dopaminergic neurons (Mottola et al., 2002). Functional selectivity was also reported for propylnorapomophine, dinapsoline, and (*S*)-(–)-3-(3-hydroxyphenyl)-*N*-propylpiperidine (Gay et al., 2004; Lane et al., 2007). The best example of functional selectivity at the D_2 receptor is aripiprazole, an approved drug for the treatment of psychiatric disorders; it is able to partially activate the G proteins but unable to stimulate β_2-arrestin recruitment (Burris et al., 2002; Urban et al., 2007; Klewe et al., 2008; Masri et al., 2008). Functionally selective ligands may thus provide important tools for the treatment of various disorders.

Despite the great progress in the understanding of 7TM receptor activation, the structure-activity relationships of biased signaling (or functional selectivity) are still inadequately understood. With the combination of medicinal chemistry and the methods of molecular biology, we tried to increase the understanding of the D_{2L} receptor activation with the emphasis on the elucidation of tailor-made ligands and site-directed receptor modifications needed to tune the functional selectivity or ligand bias. The foundation of our present work is built on the observation that a subtle modification of one amino acid in TM6 of the D_{2L} receptor, a region that is critical for ligand binding and receptor activation, can elicit changes in the signaling properties as we reported for the RASSL (Receptor Activated Solely by Synthetic Ligands) D_{2L} $F390^{6.52}W$ (Tschammer et al., 2010). The mutation of $His393^{6.55}$, which is conserved within the D_2-like dopamine receptors, led to an increase in the affinity of 1,4-disubstituted phenylpiperazines to the $H393^{6.55}A$ receptor (Ehrlich et al., 2009). To further explore this increase in affinity at the D_{2L} $H393^{6.55}A$ receptor, the ability of 1,4-disubstituted phenylpiperazines to produce the response the D_{2L} wild-type, D_{2L} $H393^{6.55}A$, and D_{2L} $H393^{6.55}F$ receptors was investigated in whole-cell assays, and the inhibition of adenylyl cyclase and the stimulation of ERK1/2 phosphorylation were measured. Distinct signaling profiles toward adenylyl cyclase and ERK1/2 were previously reported for β_1 and β_2 ligands (Galandrin and Bouvier, 2006). Our data were subjected to the operational model of agonism (Black and Leff, 1983) that was applied to calculate the agonist bias.

Because the agonist bias must be described in terms of both affinity and efficacy (Kenakin, 2009; Kenakin and Miller, 2010; Evans et al., 2011), use of the operational model of agonism enabled us to quantify the bias of a ligand for a selected signaling pathway. The affinity, denoted as K_A, describes the equilibrium dissociation constant of the agonist-receptor complex, and transduction constant, denoted as τ, is a parameter encompassing both the efficacy of the agonist and the sensitivity of a system. The value of $\Delta\log(\tau/K_A)$, relative to a chosen standard for the system quantifies the relative ability of each agonist to produce a response. The difference of the $\Delta\log(\tau/K_A)$ between selected pathways yields $\Delta\Delta\log(\tau/K_A)$, which describes agonist bias or functional selectivity of the ligand.

Use of the operational model of functional selectivity identified molecule-specific parameters essential for fine-tuning of functional selectivity at the D_{2L} receptor. Within the dopamine-simulating recognition element of the ligand, substitution of the methylsulfide group (FAUC335) for the methoxy group (FAUC321) increased the preference for the stimulation of ERK1/2 phosphorylation. The substitution of the methoxy group (FAUC321) for a more sterically demanding dihydrofuran ring (FAUC350) increased the bias of the compound for the ERK1/2 pathway even further and completely abolished the ability of FAUC350 to stimulate the inhibition of cAMP accumulation. This observation underscores the importance of minor structural changes to induce functional selectivity. In fact, FAUC350 displayed distinct signaling profiles toward adenylyl cyclase and ERK1/2. FAUC350 behaved as an antagonist in the inhibition of cAMP accumulation and as a partial agonist in the stimulation of ERK1/2 phosphorylation (efficacy = 55%). According to our molecular dynamics simulation, the aromatic substituent of the phenylpiperazine moiety of FAUC335 is in close proximity to the residue $His393^{6.55}$ of the D_2 receptor (Ehrlich et al., 2009). The latest crystal structure of the highly homologous dopamine D_3 receptor cocrystallized with eticlopride confirmed the interactions between the ligand and His6.55 (Chien et al., 2010).

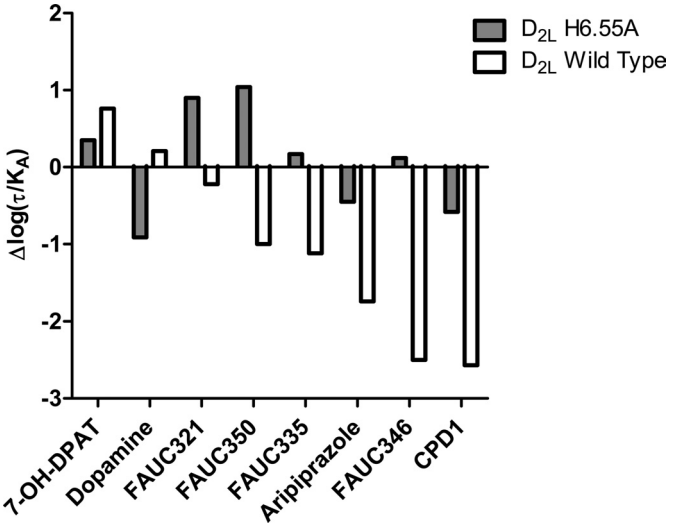


Fig. 6. The D_{2L} $H393^{6.55}A$ mutant tends to be more readily activated by various agonists. The diagram is based on the data obtained for the stimulation of ERK1/2 phosphorylation by D_{2L} wild type and D_{2L} $H393^{6.55}A$ (Figs. 2 and 3 and Supplemental Table 7).

The residue at position 6.55 was postulated to be an important structural determinant for differentiating the pharmacological dual specificities of (−)-stepholidine (SPD) for D_1 and D_2 receptors; SPD operated as an agonist at D_1 and as an antagonist at D_2 (Jin et al., 2002; Fu et al., 2007). In the D_1 receptor, position 6.55 is occupied by Asn292$^{6.55}$. Mutation of the similarly positioned His297$^{6.52}$ in μ-opioid receptor to asparagine enhanced the intrinsic activity of the alkaloid partial agonists, thus identifying a discrete region of the receptor critical for determination of the receptor activation by a specific pharmacophore (Spivak et al., 1997). For the β_2-adrenergic receptor, the inward movement of TM6 permits the interaction of Asn293$^{6.55}$ with agonists and changes the receptor to an active conformation (Wieland et al., 1996; Zuurmond et al., 1999; Bokoch et al., 2010). The increased space in the upper binding pocket would allow an enhanced inward movement of TM6 (Schwartz et al., 2006). The space-generating substitution of His363$^{6.55}$ for alanine in the D_{2L} receptor enabled the increased affinity of 1,4-disubstituted phenylpiperazines and the bias toward agonism. Similar observations have been described for mutations of the ghrelin receptor in the upper part of the binding pocket (Holst et al., 2007). Likewise, subtle changes in the structure of the ligand or the receptor at the site between TM3, TM5, and TM6 can switch the complement fragment 5a anaphylatoxin receptor on or off (Buck and Wells, 2005). As depicted in Fig. 6, which presents our data in terms of decreasing power to induce response [deceasing values of $\Delta\log(\tau/K_A)$] for the wild-type versus the mutant receptor, the precipitous drop-off in agonism goes away and the mutant tends to be more or less homogenously activated by all of the agonists; these types of mutations add a measure of permissivity to agonism.

The substitution of His363$^{6.55}$ for the sterically more demanding phenylalanine caused a moderate drop in the binding affinity and induced the bias of 1,4-disubstituted phenylpiperazines toward antagonism/inverse agonism; this activity was most pronounced in the inhibition of cAMP accumulation. It was proposed that inverse agonists of the β_2-adrenergic receptor may function in part by blocking the motion of TM6 (Bokoch et al., 2010). Similar behavior was predicted for the antagonizing properties of SPD at the D_{2L} receptor (Fu et al., 2007).

The ability of 1,4-disubstituted phenylpiperazines to stimulate D_{2L} H393$^{6.55}$F-mediated ERK1/2 phosphorylation was not impaired, with the exception of FAUC346 that behaved as a weak inverse agonist in the ERK1/2 pathway. Typically, the activation of dopamine D_2 receptors liberates the Gα subunit of the $G_{i/o}$ class of G proteins that leads to the inhibition of the adenylyl cyclase by the Gα_i subunit and thus reduction of cAMP production. The liberated G$\beta\gamma$ subunit stimulates phospholipase C-induced increase in intracellular calcium that ultimately leads to downstream activation (i.e., phosphorylation) of ERK1/2 (Choi et al., 1999; Yan et al., 1999). The Gα_o subunit does not inhibit the adenylate cyclase (Wong et al., 1992) but itself initiates the signaling cascade resulting in ERK1/2 phosphorylation (Antonelli et al., 2000). It was reported that different agonists are able to stabilize different conformations of the receptor with different affinities for the G proteins (G_o versus G_{i2}) (Cordeaux et al., 2001; Lane et al., 2007). These distinct active states with the altered G protein coupling specificity may lead to different functional outcomes, as indirectly observed in our experiments. To determine to which extent 1,4-disubstituted phenylpiperazines and the D_{2L} His363$^{6.55}$ receptor mutants alter G protein coupling will require further studies.

Our observations at the D_{2L} receptor mutants D_{2L} H393$^{6.55}$A and D_{2L} H363$^{6.55}$F contribute additional pieces of information to the understanding of D_{2L} receptor activation and the structure-activity relationships for functional selectivity. In fact, minor ligand or receptor modifications are enough to fine-tune the ligand bias, especially when they are in close proximity to position 6.55 of the receptor. Thus, residue His393$^{6.55}$ and molecular substructures of receptor ligands were identified as crucial determinants of multidimensional ligand efficacy. Our investigations led to the novel functionally selective D_{2L} ligand FAUC350, which behaves as an antagonist in the inhibition of cAMP accumulation and as a partial agonist in the stimulation of ERK1/2 phosphorylation (efficacy = 55%).

Structurally diverse compounds often do not cause any ligand-biased signaling. On the other hand, structurally similar compounds might cause ligand biased signaling as we described for the group of 1,4-disubstituted phenylpiperazines. It is not possible to foresee biased signaling solely from the structural characteristics of a ligand. We propose that once a biased ligand is identified, one can tune the degree of bias with minor structural modifications. Modifying the site of the ligand participating in the interactions with the residue in position 6.55 (His 393$^{6.55}$) of the D_2 receptor, which is involved in receptor activation, will increase the success rate in designing desired biased ligands.

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Authorship Contributions

Participated in research design: Tschammer and Gmeiner.
Conducted experiments: Tschammer and Bollinger.
Contributed new reagents or analytic tools: Bollinger.
Performed data analysis: Tschammer, Kenakin, and Gmeiner.
Wrote or contributed to the writing of the manuscript: Tschammer, Kenakin, and Gmeiner.

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