Histidine 6.55 is a major determinant of ligand biased signaling in dopamine D_2L receptor

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Non-standard abbreviations:
7TM receptor, seven transmembrane receptor; TM, transmembrane domain; CHO, Chinese hamster ovarian cells; EDTA, ethylenediaminetetraacetic acid; 7-OH-DPAT, 7-hydroxy-2-(N,N-di-n-propylamino)tetralin; IBMX, 3-isobutyl-1-methylxanthine; Ro 20-1724, [4-(3-
butoxy-4-methoxybenzyl)imidazoline; $pK_{0.5}$, negative decadic logarithm of the concentration of the compound producing 50% inhibition of the specific binding of the radioactive ligand; EC$_{50}$, half maximal effective concentration; IC$_{50}$, half maximal inhibitory concentration; PKA, protein kinase A; ERK1/2, extracellular regulated kinase 1/2.
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ABSTRACT

In our previous studies, we demonstrated that the mutation of His393 to alanine results in an increased affinity of 1,4-disubstituted phenylpiperazines to the dopamine D2L 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 for the receptor activation modulated by 1,4-DAPs. Site-directed mutagenesis and ligand modifications were used to probe the structural basis of ligand efficacy. The operational model of agonism was employed to quantify the ligand bias between the ability of compounds to inhibit cAMP accumulation and to stimulate ERK1/2 phosphorylation. Whereas substantial ligand biased signaling was observed for the D2L wild type receptor, an overall increase in agonism was observed for the D2L His393Ala mutant without noteworthy functional selectivity. Targeted chemical modification of the phenylpiperazine moiety at the site of its interaction with the residue His393 led to the functionally selective ligand 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 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 D₂ receptor is one of the most studied monoaminergic seven transmembrane (7TM) receptors. It is well established that, similar to other 7TM receptors, D₂ 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; Klewe et al., 2008; Lane et al., 2007; Masri et al., 2008; Urban et al., 2007). Aripiprazole, a 1,4-disubstituted phenylpiperazine, is the first D₂/D₃ 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 D₂ 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; Klewe et al., 2008; Masri et al., 2008; Mottola et al., 2002). 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 β₂-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 “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 (Bokoch et al., 2010; Elling et al., 2006; Nygaard et al., 2009; Schwartz et al., 2006).

We demonstrated that the mutation of His393⁶.₅₅ to alanine results in the increased affinity of 1,4-disubstituted phenylpiperazines at the D₂L receptor (Ehrlich et al., 2009).
hypothesized that the additional space created by the His393<sup>6.55</sup>Ala mutation leads to an increase in conformational freedom of the phenylalanine residues Phe389<sup>6.51</sup> and Phe390<sup>6.52</sup>. 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 FAUC335 is in close proximity to the residue His393<sup>6.55</sup>. An interaction between a ligand and His6.55 was observed also in the crystal structure of the highly homologous dopamine D<sub>3</sub> 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<sub>2</sub> receptor (Bettinetti et al., 2005; Bettinetti et al., 2002).

To determine the importance of the residue His393<sup>6.55</sup> for receptor activation modulated by 1,4-disubstituted phenylpiperazines, we employed site-directed mutagenesis and ligand modification to reduce or enhance the steric interactions between the residue 393<sup>6.55</sup> 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<sub>2L</sub> wild type, D<sub>2L</sub> His393<sup>6.55</sup>Ala and D<sub>2L</sub> His393<sup>6.55</sup>Phe 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 FAUC350. As a molecular indicator of differences in 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, to calculate the bias between the signaling pathways, and to calculate the bias between the wild type and mutant receptor (Black and Leff, 1983; Evans et al., 2010; Kenakin and Miller, 2010; Kenakin, 2009).
MATERIAL AND METHODS

Materials. Dulbecco’s modified Eagle’s medium/F-12, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, zeocin and hygromycin-B were purchased from Invitrogen. Pertussis toxin from Bordetella pertussis was purchased from Sigma. [3H]spiperone (97 Ci/mmol) was purchased from Amersham. Dopamine (3,4-dihydroxyphenethylamine), quinpirole, (-)-quinpirole hydrochloride, spiperone, haloperidol, 7-OH-DPAT (R-(+)-7-hydroxy-2-(N,N-di-n-propylamino)tetratin hydrobromide) and other compounds were purchased from Sigma, unless otherwise stated. The compounds FAUC335 (Ehrlich et al., 2009), FAUC321 (Bettinetti et al., 2002), CPD1 (Hackling et al., 2003), FAUC346 (Bettinetti et al., 2002) and aripiprazole (Oshiro et al., 1998) were synthesized as previously described.

Chemistry. Detailed description of the FAUC350 synthesis and the synthesis scheme (Scheme 1) 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 N2. Evaporations of product solutions were done in vacuo with a rotary evaporator. Column chromatography was performed with 60 µm silica gel. For TLC silica gel, 60 µm plates were used (UV, I2 or ninhydrin detection). 1H-NMR spectra were recorded at 360 MHz on a Bruker Avance 360 in CDCl3 at 300 K; chemical shifts are given in δ relative to TMS in parts per million (ppm) relative to TMS = 0. IR spectra were measured on a Jasco 410 FT-IR spectrometer. EIMS was done by EI ionization (70 eV) with a solid inlet on a Jeol GCmate II spectrometer. HRMS was done at a resolution of M/ΔM = 5000 relative to PFK on a Jeol GCmate II spectrometer. Purity was assessed by analytical HPLC (Agilent 1100 preparative series, equipped with a multi-wavelength detector; column: Zorbax Eclipse XDB-C8 analytical column, 4.6 × 150 mm, 5 µm, flow rate: 0.5 ml/min, detection wavelength: 220 nm). System 1 (S1): 10–75% CH3OH in H2O + 0.1% HCO2H in 18 min, System 2 (S2): 5-65% CH3CN in H2O + 0.1% HCO2H in 26 min.
Site-Directed Mutagenesis and Cloning. The cDNA of the human dopamine D2L (D2L) receptor was purchased from the Missouri S&T cDNA Resource Center. The site-directed mutagenesis was performed as previously described (Ehrlich et al., 2009). The D2L wild type, D2L His393tyrAla and D2L His393tyrPhe receptor cDNAs were subcloned into a pcDNA5/FRT vector (Invitrogen) using NheI/XhoI restriction sites. The entire coding region of the D2L 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 DMEM/F-12 supplemented with 10% FBS, 2 mM L-glutamine, 1% Pen-Strep and 0.25 μg/mL zeocin and kept in a humid atmosphere at 37°C with 5% CO2.

The Flp-in™ CHO cells were transfected with the pOG44 vector encoding Flp recombinase and the pcDNA5/FRT vector encoding specific dopamine receptor at a ratio of 9:1 using TransIT®-LT transfection reagent (Mirus Bio Corporation). 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 non-transfected 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-MgCl2 buffer (50 mM Tris, 5 mM EDTA, 1.5 mM CaCl2, 5 mM MgCl2, 5 mM KCl, 120 mM NaCl, pH 7.4) and subsequently lysed with an Ultraturrax. After additional centrifugation at 50,000 g, the membranes were resuspended in the binding buffer (50 mM Tris, 1 mM EDTA, 5 mM MgCl2, 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.

**Receptor Binding Studies.** Competition experiments with human D2L receptors were run with preparations of membranes from CHO cells stably expressing the corresponding receptor and [3H]spiperone at a final concentration of 0.1-0.2 nM. The assays were carried out with a protein concentration of 10 µg/mL and Kd values of 0.17 ± 0.06 nM, 0.19 ± 0.04, 0.09 ± 0.01 nM, and 0.09 ± 0.07 nM for the D2L wild type, D2L His3936.55Ala, D2L His3936.55Phe and D2L His3936.55Lys receptors, respectively. All assays were performed in 96-well plates at a final volume of 200 µL. After incubation for 1 hr at 37°C, we stopped the assay by filtration through Whatman GF/B filters presoaked with 0.3% polyethylenimine. The filters were rinsed 5 times with ice cold Tris-NaCl buffer. After 3 hr of drying at 60°C, filters were sealed with melt-on scintillator sheets MeltiLex B/HS (Perkin-Elmer), and the filter-bound radioactivity was measured with a MicroBeta TriLux liquid scintillator counter (Perkin-Elmer). Three to six experiments per compound were performed with each concentration in triplicate.

**Adenylyl cyclase inhibition assay.** Bioluminescence based cAMP-Glo™ assay (Promega) was performed according to the manufacturers instructions after adjusting the volume of required reagents for use in a white half-area 96-well plate at the final volume 80 µL. Briefly, CHO cells expressing D2L wild type, D2L His3936.55Ala or D2L His3936.55Phe receptor were seeded into a white half-area 96-well plate (5000 cells/well) 24 hr prior to the assay. The cells expressed comparable amount of the receptor as determined by saturation experiments (4100 ± 750 fmol/mg for the D2L wild type, 4400 ± 350 fmol/mg for D2L His3936.55Ala and 3900 ± 440 fmol/mg of protein for D2L His3936.55Phe). Cells were first
briefly washed with phosphate buffered saline (PBS, pH 7.4) to remove traces of serum and were incubated with various concentrations of substances in the presence of 20 µM forskolin in serum-free medium that contained 500 µM IBMX and 100 µM Ro 20-1724, 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 PKA. 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 microplate reader Victor3V (Perkin-Elmer). The experiments were performed three to nine times per compound with each concentration in duplicate. The absolute \( E_{\text{max}} \) values for quinpirole were 16 000 ± 2400 RLU (relative luminescence units), 13 700 ± 1800 RLU and 12 800 ± 2100 RLU for the D2L wild type, D2L His3936.55Ala and D2L His3936.55Phe, respectively.

**PhosphoERK1/2 ELISA Assay.** The PathScan phospho-p42/44 MAPK (Thr202/Tyr204) Sandwich ELISA (Cell Signaling) was performed according to manufactures instructions. Briefly, 6x10^6 CHO cells that expressed D2L wild type, D2L His3936.55Ala or D2L His3936.55Phe 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 D2L wild type, 4400 ± 350 fmol/mg for D2L His3936.55Ala and 3900 ± 440 fmol/mg of protein for D2L His3936.55Phe). The next day, cells were washed once with the serum free media and incubated in the presence of serum free media for additional 24 hr. For the experiments with the pertussis toxin, 25 ng/mL of the toxin was added to the serum free media for 24 hr. On the day of the experiment, the medium was removed and replaced with the 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, cells scraped, briefly sonicated (UP50H, Hielscher Ultrasound Technologies) 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 Victor^3V (Perkin Elmer) microplate reader. The experiment was performed three to four times per compound. The absolute Emax values for quinpirole were 1.634 ± 0.155 RAU (relative absorbance units), 1.439 ± 0.117 RAU and 1.569 ± 0.110 RAU for the D_2L wild type, D_2L His393^6.55Ala and D_2L His393^6.55Phe, 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, San Diego, CA). Competition curves were fitted to the sigmoid curves by non-linear regression analysis in which the logEC_{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:

\[
E = E_m \times \frac{\tau^n[A]^n}{(K_A + [A])^n + \tau^n[A]^n}
\]  

with E denoting the effect, E_m the maximum possible effect, A the agonist concentration, and K_A 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 is 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 (τ/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 and Miller, 2010; Kenakin, 2009). The \( \Delta \log(\tau/K_A) \) values were calculated as:

\[
\Delta \log(\tau/K_A)_{\text{agonist/reference}} = \log(\tau/K_A)_{\text{agonist}} - \log(\tau/K_A)_{\text{reference}}
\]  

(2)

to compare various agonists within signaling pathway. The \( \Delta \Delta \log(\tau/K_A) \) values were calculated as:

\[
\Delta \Delta \log(\tau/K_A)_{\text{H6.55A/wild type}} = \Delta \log(\tau/K_A)_{\text{H6.55A}} - \Delta \log(\tau/K_A)_{\text{wild type}}
\]  

(3)

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} His393^{6.55}Ala receptor. Detailed calculations of all parameters are summarized in the Supplemental Tables 3-6. The calculations of 95% confidence intervals were made with the program Mathematica 5.0 and were based on the algorithm developed by Watson et al. (Quantifying Functional Selectivity (Biased Agonism), in preparation).
RESULTS

Radioligand Displacement Studies.

To determine the influence of mutations His393<sup>6.55</sup>Ala and His393<sup>6.55</sup>Phe 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<sub>2L</sub> His393<sup>6.55</sup>Ala receptor, the affinity of the endogenous ligand dopamine dropped by 28-fold (Table 1). The introduction of the aromatic phenylalanine (His393<sup>6.55</sup>Phe) or the basic lysine (His393<sup>6.55</sup>Lys) did not restore the affinity of dopamine (Table 1, Supplemental Table 1), underscoring the importance of the unique properties of the imidazole side chain of histidine for the binding of dopamine. The D<sub>2L</sub> His393<sup>6.55</sup>Lys mutant was expressed at about a 10-times lower density (410 ± 120 fmol/mg) compared to wild type, His393<sup>6.55</sup>Ala or His393<sup>6.55</sup>Phe receptors (4100 ± 750 fmol/mg, 4400 ± 350 fmol/mg and 3900 ± 440 fmol/mg, respectively) as determined by [3H]spiperone saturation experiments. The affinities of the synthetic dopamine receptor agonists 7-OH-DPAT and quinpirole were significantly reduced by the D<sub>2L</sub> His393<sup>6.55</sup>Ala mutation (12– and 63– fold, respectively). The introduction of an aromatic phenylalanine at position 6.55 (D<sub>2L</sub> His393<sup>6.55</sup>Phe) 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<sub>2L</sub> His393<sup>6.55</sup>Leu and D<sub>2L</sub> His393<sup>6.55</sup>Cys have only moderate compound-specific effects on the affinity of antagonists (Javitch et al., 1998; Woodward et al., 1994). The 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$_{2L}$ His$^{393}_{6.55}$Ala mutant. The mutation D$_{2L}$ His$^{393}_{6.55}$Phe 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 their 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 butylene 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$_{2L}$ His$^{393}_{6.55}$Ala 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$_{2L}$ His$^{393}_{6.55}$Ala receptor. At the D$_{2L}$ His$^{393}_{6.55}$Phe 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$_{2L}$ His$^{393}_{6.55}$Ala 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 $\tau$, 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 ($4100 \pm 750$ fmol/mg for the D$_{2L}$ wild type, $4400 \pm 350$ fmol/mg for D$_{2L}$ His393$^{6.55}$Ala and $3900 \pm 440$ fmol/mg of protein for D$_{2L}$ His393$^{6.55}$Phe). The log ($\tau/K_A$) values depicted the “strength” of a given agonist to activate a defined pathway in a defined system (Kenakin and Miller, 2010; Kenakin, 2009). 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 receptor, $\Delta \Delta \log(\tau/K_A)$ values were calculated. Detailed calculations of all parameters are summarized in the Supplemental Tables 4-7.

The compounds can be partitioned into three groups based on their ability to inhibit the accumulation of cAMP (Figure 2A). 7-OH-DPAT and dopamine had comparable $\Delta \log(\tau/K_A)$ values (0.05 and 0.02, respectively) (Figure 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 about 5 min after the stimulation of the D$_{2L}$ receptor (Figure 2B) and it could be completely blocked by the
pertussis toxin (Supplemental Figure 1). The $\Delta \log(\tau/K_A)$ value of dopamine was 0.21, indicating the activation profile very similar to the reference agonist quinpirole (Figure 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 Figure 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 (Figure 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 His393$^{6.55}$Ala increased the efficacy of 1,4-disubstituted phenylpiperazines and abolished the ability of the receptor to recognize biased ligands.
To determine the influence of His393\textsuperscript{6.55}Ala 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 to stimulate the ERK1/2 phosphorylation was investigated with CHO cells stably expressing the D\textsubscript{2L} His393\textsuperscript{6.55}Ala 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 Figure 3A-D. 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\textsubscript{2L} His393\textsuperscript{6.55}Ala 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\textsuperscript{6.55} for alanine largely abolished the ability of compounds to elicit ligand-biased signaling. The only exceptions were dopamine and CDP1. CDP1 behaved as an antagonist in the cAMP pathway at the D\textsubscript{2L} wild type receptor and changed the preference for the signaling pathway by being more efficacious in stimulating the inhibition of cAMP accumulation than the ERK1/2 phosphorylation at the D\textsubscript{2L} His393\textsuperscript{6.55}Ala receptor.

The residue His6.55 is crucial for ligand efficacy and ligand biased signaling at the D\textsubscript{2L} receptor.

To quantify ligand biased signaling and the bias between the D\textsubscript{2L} wild type and the D\textsubscript{2L} His393\textsuperscript{6.55}Ala receptor in the selected pathway, the $\Delta \log(\tau/K_a)$ values from Figure 2C and Figure 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 D2L His393^6.55^Ala receptor measured as the inhibition of cAMP accumulation (Figure 4A). Dopamine did not discriminate between the D2L His393^6.55^Ala and D2L wild type receptor in this assay. The ability of 1,4-disubstituted phenylpiperazines to stimulate the phosphorylation of ERK1/2 was strongly biased for the D2L His393^6.55^Ala receptor (Figure 4B). Dopamine and 7-OH-DPAT were more active at the D2L wild type receptor (Figure 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 D2L His393^6.55^Ala 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 to the D2L 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 D2L His393^6.55^Phe 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 D2L His393^6.55^Phe receptor (Figure 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; aripiprazole and FAUC321 were weak inverse agonists, whereas FAUC346 and CPD1 acted as very strong inverse agonists. The mutation His393^6.55^Phe considerably reduced the efficacy of 1,4-disubstituted phenylpiperazines.
To estimate the ability of the investigated compounds to stimulate D₂L His393⁶.⁵⁵Phe receptor-mediated phosphorylation of ERK1/2, we used discrete sample concentrations (between 1-10 µM). As expected, the mutation His393⁶.⁵⁵Phe had no influence on the efficacy of dopamine and 7-OH-DPAT (Figure 5B). Surprisingly, the mutation His393⁶.⁵⁵Phe 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 His393⁶.⁵⁵Phe with an attenuation of efficacy (from 15% at the D₂L wild type to -8% at the D₂L His393⁶.⁵⁵Phe receptor). These observations underscore the role of His393⁶.⁵⁵ in the D₂L receptor as a crucial determinant of multidimensional ligand efficacy.
DISCUSSION

The ability of the D\textsubscript{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; Klewe et al., 2008; Lane et al., 2007; Urban et al., 2007). 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 to inhibit the firing of nigral dopaminergic neurons (Mottola et al., 2002). Functional selectivity was also reported for propynorapomorphine, dinapsoline and (S)-3-PPP (Gay et al., 2004; Lane et al., 2007). The best example of functional selectivity at the D\textsubscript{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\textsubscript{2}-arrestin recruitment (Burris et al., 2002; Klewe et al., 2008; Masri et al., 2008; Urban et al., 2007). 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\textsubscript{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\textsubscript{2L} receptor, a region which 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\textsubscript{2L} Phe390\textsuperscript{6.52}Trp (Tschammer et al., 2010). The mutation of His393\textsuperscript{6.55}, which is conserved within the D\textsubscript{2}-like dopamine receptors, led to an increase in
the affinity of 1,4-disubstituted phenylpiperazines to the His3936.55Ala receptor (Ehrlich et al., 2009). To further explore this increase in affinity at the D2L His3936.55Ala receptor, the ability of 1,4-disubstituted phenylpiperazines to produce the response the D2L wild type, D2L His3936.55Ala and D2L His3936.55Phe receptors were investigated in whole cell assays, the inhibition of adenylyl cyclase and the stimulation of ERK1/2 phosphorylation was measured. Distinct signaling profiles toward adenylyl cyclase and ERK1/2 were previously reported for β1 and β2 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 both in terms of affinity and efficacy (Evans et al., 2010; Kenakin and Miller, 2010; Kenakin, 2009), 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 $\tau$, 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 D2L 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 recent molecular dynamics simulation, the aromatic substituent of the phenylpiperazine moiety of FAUC335 is in close proximity to the residue His393 of the D2 receptor (Ehrlich et al., 2009). The latest crystal structure of the highly homologous dopamine D3 receptor co-crystallized with eticlopride confirmed the interactions between the ligand and His6.55 (Chien et al., 2010).

The residue at the position 6.55 was postulated to be an important structural determinant for differentiating the pharmacological dual specificities of (-)-stepholidine (SPD) for D1 and D2 receptors; SPD operated as an agonist at D1 and an antagonist at D2 (Fu et al., 2007; Jin et al., 2002). In the D1 receptor, the position 6.55 is occupied by Asn292. Mutation of the similarly positioned His297 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 β2-adrenergic receptor, the inward movement of TM6 permits the interaction of Asn293 with agonists and changes the receptor to an active conformation (Bokoch et al., 2010; Wieland et al., 1996; Zuurmond et al., 1999). 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 for alanine in the D2L 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). Similarly, 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 (C5a) receptor on or off (Buck and Wells, 2005). As depicted in Figure 6, which presents our data in terms of decreasing power.
to induce response (decreasing 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 $\text{His}363^{6.55}$ 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 $\beta_2$-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 $\text{D}_2L$ receptor (Fu et al., 2007).

The ability of 1,4-disubstituted phenylpiperazines to stimulate $\text{D}_2L\text{His}393^{6.55}\text{Phe}$ 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 $\text{D}_2$ receptors liberates the $G\alpha$ subunit of the $G_{i/o}$ class of G-proteins that leads to the inhibition of the adenyl cyclase by the $G\alpha_i$ 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 the extracellular-signal-regulated kinase 1/2 (ERK1/2) (Choi et al., 1999; Yan et al., 1999). The $G\alpha_o$ subunit does not inhibit the adenylate cyclase (Wong et al., 1992) but itself initiates the signaling cascade resulting in the 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 ($G_o$ vs. $G_{i2}$) (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} His393^{6.55}Ala and D_{2L} His363^{6.55}Phe 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 D2 receptor, which is involved in receptor activation, will increase the success rate in designing desired biased ligands.

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AUTHORSHIP CONTRIBUTION SECTION

Nuska Tschammer participated in research design, conducted experiments, performed data analysis and wrote the manuscript. Stefan Bollinger conducted experiments and contributed new reagents. Terry Kenakin performed data analysis and contributed to the writing of the manuscript. Peter Gmeiner participated in research design, performed data analysis and contributed to the writing of the manuscript.
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Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (IC50) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099-3108.


Legends for Figures

Figure 1: Chemical structure of the ligands investigated in this study.

Figure 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 the stimulation with investigated compounds. Pooled data of three to nine experiments performed in triplicate are shown as normalized curves with error bars representing the SEM. The pEC_{50} values and efficacies are summarized in the 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 SEM. The pEC_{50} values and efficacies are summarized in the Supplemental Table 3. C) The log(τ/K_{A}) and Δlog(τ/K_{A}) values obtained with the operational model of agonism. n.a. - not available because of the antagonistic behavior of the substance in the selected signaling pathway. D) The ΔΔlog(τ/K_{A}) values as a measure of ligand bias between the pathways. The error bar represent 95% confidence interval. When the range includes zero, the ligands are not biased with the respect to the reference agonist. * - indicating the exclusive preference of the compound for the selected signaling pathway. Detailed calculations of Δlog(τ/K_{A}), ΔΔlog(τ/K_{A}) values and their 95% confidence intervals are summarized in the Supplemental Table 4.
**Figure 3: Use of the operational model of agonism to quantify the ligand-bias at the D$_{2L}$ His393$^{6.55}$Ala receptor.** The assays were performed on the D$_{2L}$ His393$^{6.55}$Ala receptor expressing CHO cells. A) The inhibition of cAMP accumulation. The cells were incubated with 20 µM forskolin and the D$_{2L}$ His393$^{6.55}$Ala receptor mediated inhibition of cAMP accumulation was measured after the stimulation with investigated compounds. Pooled data of three to nine experiments performed in triplicate are shown as normalized curves with error bars representing the SEM. The pEC$_{50}$ values and efficacies are summarized in the 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 SEM. The pEC$_{50}$ values and the efficacies are summarized in the 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. * - 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 the Supplemental Table 5.

**Figure 4: Use of the operational model of agonism to quantify the ligand-bias among the D$_{2L}$ wild type and D$_{2L}$ His393$^{6.55}$Ala receptor.** The $\Delta$log($\tau$/K$_a$) values from Figure 2C and Figure 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$_{2L}$ His393$^{6.55}$Ala receptor. The error bars represent 95% confidence interval. When the range includes zero, the ligands are not biased with respect to each other. * - 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 the 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$_{2L}$ His393$^{6.55}$Ala receptor 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$_{2L}$ His393$^{6.55}$Ala receptor in their ability to stimulate ERK1/2 phosphorylation.

**Figure 5**: The ability of investigated compounds to activate the D$_{2L}$ His393$^{6.55}$Phe receptor. A) The inhibition of cAMP accumulation. The cells were incubated with 20 µM forskolin and the D$_{2L}$ His393$^{6.55}$Phe receptor mediated inhibition of cAMP accumulation was measured after the stimulation with investigated compounds. Pooled data of three to four experiments performed in triplicate are shown as normalized curves with error bars representing the SEM. 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, aripiprazole, FAUC335, FAUC321, FAUC350, FAUC346 and CPD1 at 1 µM. The level of phosphorylated ERK1/2 was detected by ELISA. The mean values with error bars representing the SEM of three to four experiments are shown.

**Figure 6**: The D$_{2L}$ His393$^{6.55}$Ala 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$_{2L}$ His393$^{6.55}$Ala (Figure 2, Figure 3 and Supplemental Table 7).
Table 1. pKi values for the dopamine receptor antagonists and agonists on D2L wild type, D2L His3936.55Ala and D2L His3936.55Phe receptor.4

<table>
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<tr>
<th>Compound</th>
<th>pKi measured</th>
<th>pKi, for D2L wild type</th>
<th>pKi, for D2L His3936.55Ala</th>
<th>pKi, for D2L His3936.55Phe</th>
<th>ΔpKiAla/wt</th>
<th>10^6 pKiAla/wt</th>
<th>ΔpKiPhe/wt</th>
<th>10^6 pKiPhe/wt</th>
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<tr>
<td>Haloperidol</td>
<td>pK0.5</td>
<td>9.41 ± 0.03b(-0.83)c</td>
<td>9.60 ± 0.02c(-0.97)d</td>
<td>9.46 ± 0.03c(-0.90)c</td>
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<td>Buspirone</td>
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<td>Aripiprazole</td>
<td>pK0.5</td>
<td>8.26 ± 0.03b(-0.86)c</td>
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<td>8.89 ± 0.04b(-0.99)c</td>
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<td>0.56</td>
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<td>0.23</td>
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<td>FAUC335</td>
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<td>8.00 ± 0.04b(-0.84)c</td>
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<td>7.70 ± 0.04b(-0.75)c</td>
<td>-0.89</td>
<td>0.13</td>
<td>0.29</td>
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<td>CPD1</td>
<td>pK0.5</td>
<td>7.43 ± 0.02b(-0.70)c</td>
<td>9.00 ± 0.08b(-0.64)c</td>
<td>6.89 ± 0.08b(-1.18)c</td>
<td>-1.57</td>
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<td>1.17</td>
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<td>6.21 ± 0.04b(-0.44)c</td>
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<td>18</td>
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4 The affinities of investigated substances were determined on membrane preparations of stably transfected CHO cells expressing either D2L wild type, D2L His3936.55Ala or D2L His3936.55Phe receptor using [3H]spiperone displacement study. Data are derived from normalized curves of 3-6 experiments done in triplicate. Data were analyzed by non-regression and were best fit to one-site (monophasic) or two-site (biphasic) competition curves. pKi values were calculated according to Cheng and Prusoff (1973). The $10^{-6 \text{pKiAla/wt}}$ and $10^{-6 \text{pKiPhe/wt}}$ values indicate the changes in the affinity. b SEM. c Hill slope. d Fraction of high-affinity sites.
Figure 1

Haloperidol

Buspirone

Dopamine

7-OH-DPAT

Quinpirole

FAUC335

Aripiprazole

FAUC321

CPD1

FAUC350

FAUC346
**Figure 2.**

(A) Inhibition of cAMP accumulation (% of quinpirole mediated effect) vs. Test compound (log[M]).

(B) Increase in a level of phosphorylated ERK1/2 (% of quinpirole mediated effect) vs. Test compound (log[M]).

(C) Table showing Inhibition of cAMP accumulation:

<table>
<thead>
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<th>Compound</th>
<th>Log (r/K_a)</th>
<th>Δlog (r/K_a)</th>
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<td>CPD1</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
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</table>

(D) Table showing Stimulation of ERK1/2 phosphorylation:

<table>
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<th>Compound</th>
<th>Log (r/K_a)</th>
<th>Δlog (r/K_a)</th>
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<tbody>
<tr>
<td>Quinpirole</td>
<td>8.41</td>
<td>0.00</td>
</tr>
<tr>
<td>7-OH-DPAT</td>
<td>9.17</td>
<td>0.76</td>
</tr>
<tr>
<td>Dopamine</td>
<td>8.62</td>
<td>0.21</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>6.67</td>
<td>-1.74</td>
</tr>
<tr>
<td>FAUC335</td>
<td>7.29</td>
<td>-1.12</td>
</tr>
<tr>
<td>FAUC321</td>
<td>8.19</td>
<td>0.22</td>
</tr>
<tr>
<td>FAUC350</td>
<td>7.41</td>
<td>-1.00</td>
</tr>
<tr>
<td>FAUC346</td>
<td>5.91</td>
<td>-2.50</td>
</tr>
<tr>
<td>CPD1</td>
<td>5.84</td>
<td>-2.57</td>
</tr>
</tbody>
</table>

More active in cAMP pathway: Dopamine, 7-OH-DPAT, Aripiprazole, FAUC335, FAUC321, FAUC350, CPD1, FAUC346. More active in ERK1/2 pathway: Quinpirole, 7-OH-DPAT, Aripiprazole, FAUC335, FAUC321, FAUC350, CPD1, FAUC346.
Figure 3.

A) Inhibition of cAMP accumulation (% of quinpirole mediated effect) vs. Test compound (log[μM]).

B) Increase in level of phosphorylated ERK1/2 (% of quinpirole mediated effect) vs. Test compound (log[μM]).

C) Table:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log (z/Kₐ)</th>
<th>Δlog (z/Kₐ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinpirole</td>
<td>6.69</td>
<td>0.00</td>
</tr>
<tr>
<td>7-OH-DPAT</td>
<td>7.55</td>
<td>0.86</td>
</tr>
<tr>
<td>Dopamine</td>
<td>6.75</td>
<td>0.06</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>6.25</td>
<td>-0.44</td>
</tr>
<tr>
<td>FAUC335</td>
<td>7.06</td>
<td>0.38</td>
</tr>
<tr>
<td>FAUC321</td>
<td>7.78</td>
<td>1.09</td>
</tr>
<tr>
<td>FAUC350</td>
<td>7.21</td>
<td>0.52</td>
</tr>
<tr>
<td>FAUC346</td>
<td>6.96</td>
<td>0.27</td>
</tr>
<tr>
<td>CPD1</td>
<td>7.59</td>
<td>0.90</td>
</tr>
</tbody>
</table>

D) Delta log (z/Kₐ) vs. Stimulation of ERK1/2 phosphorylation.

More active in cAMP pathway
More active in ERK1/2 pathway
Figure 4.

A) ΔΔlog(1/Kd) for different compounds:

- Dopamine
- 7-OH-DPAT
- Aripiprazole
- FAUC335
- FAUC321
- FAUC350
- CPD1
- FAUC346

Significance:
- * More active on D2L H6.55A
- * More active on D2L wild type

B) ΔΔlog(1/Kd) for different compounds:

- Dopamine
- 7-OH-DPAT
- Aripiprazole
- FAUC335
- FAUC321
- FAUC350
- CPD1
- FAUC346

Significance:
- More active on D2L H6.55A
- More active on D2L wild type
Figure 6.