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Molecular Determinants of the Intrinsic Efficacy of the Antipsychotic Aripiprazole

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Abstract

Partial agonists of the dopamine D₂ receptor 18 (D₂R) have been developed to treat the symptoms of schizophrenia without causing the side effects elicited by antagonists. The receptor–ligand interactions that determine the intrinsic efficacy of such drugs, however, are poorly understood. Aripiprazole has an extended structure comprising a phenylpiperazine primary pharmacophore and a 1,2,3,4-tetrahydroquinolin-2-one secondary pharmacophore. We combined site-directed mutagenesis, analytical pharmacology, ligand fragments, and molecular dynamics simulations to

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ASSOCIATED CONTENT

Supporting Information

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identify the D₂R–aripiprazole interactions that contribute to affinity and efficacy. We reveal that an interaction between the secondary pharmacophore of aripiprazole and a secondary binding pocket defined by residues at the extracellular portions of transmembrane segments 1, 2, and 7 determines the intrinsic efficacy of aripiprazole. Our findings reveal a hitherto unappreciated mechanism for fine-tuning the intrinsic efficacy of D₂R agonists.

Graphical Abstract

The dopamine D₂ receptor (D₂R), a class A G protein-coupled receptor (GPCR), is the target of drugs that relieve symptoms of Parkinson's disease (agonists) and schizophrenia (partial agonists/antagonists).¹ The antipsychotics aripiprazole, brexpiprazole, and cariprazine are D₂R partial agonists.^{2–4} They are thought to act as functional antagonists in the striatum, where excessive dopamine activity is thought to cause positive symptoms, but to show agonist activity in the mesocortical pathway, where reduced dopamine activity is thought to be associated with negative symptoms and cognitive impairment. A partial agonist may also prevent the complete blockade of the nigrostriatal or tuberoinfundibular pathways, associated with extrapyramidal symptoms and elevated prolactin levels, respectively.⁵ However, why these partial agonists display antipsychotic efficacy while other D₂R partial agonists have failed to do so remains unclear. It has been proposed that the low level of intrinsic activity elicited by aripiprazole gives sufficient functional antagonism for antipsychotic efficacy whereas other partial agonists with higher intrinsic activity, such as bifeprunox, failed in clinical development.⁶ Furthermore, the intrinsic activity of aripiprazole is apparently sufficient to avoid motor side effects and prolactinemia.

The crystal structures of the D₂R, D₃R, and D₄R, in complex with the antagonists risiperidone, eticlopride, and nemonapride, respectively, reveal the location of an orthosteric binding site (OBS) comprised of residues that are conserved in the dopamine D₂-like receptors and are consistent with earlier findings of mutagenesis and molecular modeling studies.^{7–10} Despite the therapeutic utility of D₂R full and partial agonists, our understanding of the ligand–receptor contacts that determine the degrees of intrinsic efficacy is limited. Agonist-bound class A GPCR crystal structures reveal different patterns of agonist–receptor interactions but common structural rearrangements in the extracellular part of the transmembrane (TM) bundle near the OBS upon receptor activation.^{11,12} These are translated into larger rearrangements at the cytoplasmic side of the receptor, including translation and rotation of TM5 and TM6 and relocation of TM3 and TM7. In particular,

comparisons of class A GPCR crystal structures in active and inactive states, combined with molecular dynamics (MD) simulations, have highlighted the movement of a cluster of residues, Pro^{5.50}, Ile^{3.40}, and Phe^{6.44} (termed the “PIF motif”, Ballesteros and Weinstein numbering system¹³), along with Leu/Val^{5.51} and Trp^{6.48} on receptor activation. The reconfigurations of these residues couple the conformational changes in the binding pocket to those at the intracellular coupling interface.^{14–16}

Aripiprazole is comprised of a 4-(2,3-dichlorophenyl)-piperazine primary pharmacophore (PP) and a 1,2,3,4-tetrahydroquinolin-2-one secondary pharmacophore (SP) linked by a flexible butoxy linker. This extended structure is typical of ligands that are selective for dopamine D₂-like receptors.^{7,17} Using the D₃R crystal structure,⁷ we revealed a secondary binding pocket (SBP) that extended away from the OBS toward the extracellular ends of TM1–TM3 and TM7 and demonstrated that the interaction between this SBP and the aryl tail moiety of phenylpiperazine derivatives not only was an important determinant of subtype selectivity but could also modulate ligand efficacy through reorientation of the phenylpiperazine core within the SBP.^{18,19} Surprisingly, however, little is known about the binding mode of aripiprazole at the D₂R and how this might determine its agonist efficacy. To address this, we combined MD simulations, mutagenesis, and analytical pharmacology to quantify agonist action in terms of both efficacy (τ) and functional affinity (K_A). Together, our studies reveal that the interaction between the 1,2,3,4-tetrahydroquinolin-2-one SP and the D₂R SBP is a determinant of aripiprazole’s intrinsic efficacy.

RESULTS AND DISCUSSION

Aripiprazole and Dopamine Show Distinct Sensitivities to OBS Mutations.

To interrogate the ligand–receptor interactions involved in agonist binding and the subsequent activation of the D₂R, we mutated residues within the OBS, the SBP, and the transmission switch of the D₂R. An enzyme-linked immunosorbent assay (ELISA) revealed no significant difference between the cell surface expression levels of the mutant and wild-type (WT) receptors (Supplementary Figure 1). We then determined the effect of each mutation on the dissociation constant (pK_d) of [³H] spiperone and/or [³H]raclopride. A homologous competition binding assay revealed that none of the mutations had a significant effect on the pK_d of [³H]spiperone (Supplementary Table 2) with the exception of V91^{2.61}A, F360^{6.51}A, and F361^{6.52}A, for which no detectable binding was observed. Of these three mutants, [³H]raclopride bound V91^{2.61}A with WT affinity but was unable to bind F360^{6.51}A and F361^{6.52}A (Supplementary Table 2).

The binding affinities (K_i) of the agonists at the D₂R were determined in competition binding experiments (Table 1). To measure the functional impact of the mutations, we used inhibition of forskolin-induced cAMP production as a measure of D₂R $G\alpha_i/o$ G protein signaling. Many OBS mutations, however, abrogated the binding and/or functional activity of dopamine, which prevented us from quantifying the relative effect of these mutations on aripiprazole. We, therefore, extended our studies to ropinirole, an agonist that retained activity at many OBS mutations. We designed a sensitive cAMP assay using a low (300 nM) concentration of forskolin to give a greater dynamic range with which to quantify the deleterious effects of receptor mutants. In this assay, aripiprazole displayed a robust partial

maximal response (80%) relative to that of dopamine. This contrasts with the results of previous studies of aripiprazole using the same CHO cell background for which a much weaker relative maximal response was observed.^{2,20} Such differences reflect different receptor expression levels and assay sensitivities. Our data were fitted with an operational model of agonism to derive a transduction coefficient (τ/K_A) of all three agonists, comprised of agonist efficacy (τ) and the functional affinity of the receptor when coupled to a specific signaling pathway (K_A).²¹ Although we could not define these two separate parameters for the full agonists dopamine and ropinirole, in the case of the partial agonist aripiprazole, we could determine separate values of affinity and efficacy [$K_A = 17$ nM, and $\tau = 5$ (Table 1)].

We first investigated the role of OBS residues (Table 1). Asp114^{3,32} forms a salt bridge interaction with the positively charged nitrogen of dopaminergic ligands, and the D114^{3,32}A mutation ablates agonist and antagonist binding.²² Val^{3,33}, Cys^{3,36}, and Thr^{3,37} line the OBS in the D₂R, D₃R, and D₄R structures, respectively.^{7,9,10} V115^{3,33} A reduced the binding affinity of dopamine and aripiprazole but not that of ropinirole and decreased the transduction coefficients (τ/K_A) of all ligands (Table 1). In the case of aripiprazole, this effect was caused by a significant 8-fold decrease in efficacy (τ). C118^{3,36}A or T119^{3,37}A had little effect on binding affinity (K_i) but significantly reduced the functional effect of all ligands, causing a >50-fold decrease in transduction coefficients (τ/K_A) or abrogating activity altogether (Table 1).

The conserved TM5 serine residues have been shown to be important for agonist binding and action at all DR subtypes.^{23–27} In agreement with these previous studies, the binding affinity of dopamine was significantly reduced at S193^{5,42}A, S194^{5,43}A, and S197^{5,46}A by 120-, 4-, and 3-fold, respectively (Figure 1 and Table 1). The transduction f1 coefficient of dopamine was reduced at S193^{5,42}A (1600-fold) and S194^{5,43}A (11-fold), whereas S197^{5,46}A abolished its functional effect entirely (Figure 1 and Table 1). The binding affinity and transduction coefficient of ropinirole were also significantly reduced at S193^{5,42}A by 15- and 930-fold, respectively. S194^{5,43}A had no effect on ropinirole affinity but caused a 20-fold decrease in the transduction coefficient, whereas S197^{5,46}A had no effect. Interestingly, mutation of the TM5 serines did not decrease the efficacy (τ) of aripiprazole (Figure 1). Rather, S193^{5,42}A caused a 3-fold increase in binding affinity and a 10-fold increase in efficacy, whereas S197^{5,46}A caused a 5-fold decrease in binding affinity with no effect on the functional response (Figure 1 and Table 1).

Residues within ECL2 form part of the D₂R and D₃R OBS.^{7,28} I184^{ECL2} A significantly reduced the binding affinity and transduction coefficient of dopamine (4- and 28-fold, respectively) (Table 1). None of the ECL2 mutations affected the binding affinity, functional affinity, or efficacy of aripiprazole (Table 1).

Residues 6.51 and 6.52 interact with the substituted aromatic ring of eticlopride in the D₃R and the methoxy benzamide ring of nemonapride in the D₄R.^{7,9} None of the agonists displayed functional activity at F360^{6,51}A, and F361^{6,52} A caused a significant decrease in the transduction coefficients of both ropinirole (9-fold) and dopamine (7-fold) (Figure 1 and Table 1). Residue 6.55 has been shown to be important for agonist binding and efficacy at the D₂R and D₃R.^{7,27,29,30} H364^{6,55}A decreased the binding affinity (4-fold) and

transduction coefficient (69-fold) of dopamine and the transduction coefficient (6-fold) of ropinirole but not its affinity. The H364^{6.55}F mutation reduced the transduction coefficient of ropinirole (110-fold) and dopamine (28-fold), indicating that the imidazole side chain of His364^{6.55} is important for the agonist action of these ligands (Table 1). Residues 6.58 and 6.59 line the OBS.³⁰ In the 5HT_{2B} receptor, these residues form hydrophobic contacts with ergotamine that are important for its biased action.³¹ N367^{6.58}A caused a 3-fold decrease in the binding affinity and a 10-fold decrease in the transduction coefficient of dopamine only (Table 1). I368^{6.59}A decreased the transduction coefficient of ropinirole by 5-fold. Notably, mutation of these TM6 residues (F361^{6.52}A, H364^{6.55}A/F, N367^{6.58}A, or I368^{6.59}A) did not change the affinity or efficacy of aripiprazole (Figure 1 and Table 1). Mutation of Thr383^{7.39}, a residue shown to contribute to aminergic receptor ligand binding,⁸ did not change the binding affinity of the three agonists but decreased the transduction coefficient of ropinirole (29-fold) while it increased that of aripiprazole 5-fold.

In summary, we identified OBS residues that contribute to the efficacy of all three agonists but found mutations in ECL2 (I184^{ECL2}A), TM5 (S193^{5.42}A, S194^{5.43}A, and S197^{5.46}A), and TM6 (F361^{6.52}A, H364^{6.55}A, N367^{6.58}A, or I368^{6.59}A) that had deleterious effects on the functional effect of dopamine and ropinirole but no effect on the efficacy of aripiprazole. Differential engagements of the TM5 serines (at positions 5.42, 5.43, and 5.46) and His^{6.55} by D₂R agonists have been suggested to underlie differences in efficacy through the stabilization of distinct receptor conformations.^{27,29} In the case of aripiprazole, rather than deleterious effects, the S^{5.46}A, F^{6.52}A, and H^{6.55}A mutations caused a modest increase in efficacy. Interestingly, S^{5.42}A and S^{5.46}A mutations caused decreases in the affinity and transduction coefficient of dopamine, in agreement with previous studies, whereas the S^{5.46}A mutation had no effect on ropinirole. In a previous study, our MD simulations found that N-1 of sumanirole, an agonist that is structurally similar to ropinirole, forms a hydrogen bond with the side chain of Ser^{5.42} but no interaction with Ser^{5.46} was observed.³² Ropinirole might adopt a similar orientation, but further simulations are required to confirm this hypothesis.

Transmission Switch Residues Are Required for Agonist Action at the D₂R.

Comparison of the active and inactive structures of rhodopsin and the adenosine A_{2A}, β_2 adrenergic, and μ opioid receptors revealed rearrangement of a cluster of hydrophobic and aromatic residues (including residues 3.40, 5.50, 5.51, 6.44, and 6.48) in the TM3–TM5–TM6 motif as a common feature of class A GPCR activation.^{11,12,15} I122^{3.40} is part of the conserved P^{5.50}-I^{3.40}-F^{6.44} motif that undergoes structural rearrangement upon receptor activation to allow the outward movement of TM6. The I122^{3.40}A mutation had no significant effect on the binding affinity of the agonists but abrogated functional activity. The F202^{5.51}A mutation caused a significant reduction in the binding affinity of dopamine and aripiprazole [6- and 3-fold, respectively (Table 1)]. Aripiprazole displayed no agonism with this mutation, and ropinirole and dopamine displayed >100-fold lower transduction coefficients (Table 1). Thus, all three D₂R agonists require conformational rearrangement of transmission switch residues to exert their agonistic effect. While F202^{5.51} does not form part of the OBS, the F202^{5.51}A mutation may modulate the conformation of the OBS causing the loss of affinity of dopamine and aripiprazole but not that of ropinirole.

Interestingly, the recent D2R crystal structure obtained in complex with the antagonist risperidone included the I122^{3,40} A mutation as one of three thermostabilizing mutations.¹⁰ This mutation likely exerts its thermostabilizing effect by preventing the isomerization of the receptor into the active state.

Molecular Dynamics Simulations Reveal an Extended Pose of Aripiprazole.

To characterize and dissect the contributions of residues from the OBS and SBP to the binding pose of aripiprazole, we performed a computational modeling and simulation study of D₂R models in complex with aripiprazole. From the initial docking results, we chose several poses of aripiprazole with its quinoline moiety oriented in various directions in the extracellular vestibule (EV) of D₂R (see Methods). We then collected multiple MD trajectories for each pose (Supplementary Table 1) and sought to identify a convergent trend of the ligand dynamics in the binding site.

Similar to the partial agonists with a 2,3-diCl-phenylpiperazine PP that we have characterized previously in D₃R models,¹⁸ the PP of aripiprazole adopts a pose that is relatively parallel to the membrane and close to Ser193^{5,42} but does not form an H-bond with Ser193^{5,42}. In all our simulations, Ser197^{5,46} forms a H-bond to the backbone carbonyl of Ser193^{5,42}. Thus, the S193^{5,42} A mutation may lead to an optimized hydrophobic interaction and slightly improve the affinity, whereas the Ser197^{5,46} A mutation disrupts the local conformation of TM5 and results in a slightly decreased affinity. In such a pose, both rings of 2,3-diCl-phenylpiperazine are tightly packed with Phe360^{6,51}, and it is expected that the F360^{6,51} A mutation would destabilize the observed orientation of the phenylpiperazine. Thus, this pose of the PP within the D₂R OBS is in agreement with our mutagenesis results.

For the SP and the flexible butoxy linker, however, we found that our simulations from different starting poses and multiple trajectories converged to two distinct poses in the EV, depending on the orientation of the highly conserved Trp384^{7,40}. When Trp384^{7,40} faces the lipid as in the D₃R structure, the quinoline ring occupies a cavity at the interface among TM1, TM2, and TM7 and is in contact with Leu41^{1,39}, Val91^{2,61}, and Trp384^{7,40} (Figure 2C). In contrast, when the indole ring of Trp384^{7,40} rotates inward between the side chains of Val91^{2,61} and Leu41^{1,39}, the quinoline ring can no longer extend into this cavity but rather tilts toward ECL2 and TM3, forming a weak interaction with Glu95^{2,65} (Figure 2D). Such an inward orientation of Trp384^{7,40} is observed in most of the crystal structures of aminergic receptors.³³ While Trp384^{7,40} of the D₃R faces the lipid, that of the D₂R structure is in an intermediate position, and in our simulations, we observed that this residue can adopt both inward and outward orientations.^{7,10}

The SP of Aripiprazole Confers an Increase in Efficacy.

To explore how the interaction of the SP of aripiprazole with the D₂R SBP might influence affinity and efficacy, we characterized a series of progressively extended fragments of aripiprazole incorporating either the PP or the SP. The introduction of the alkyl or alkoxy spacers (compounds **2–4**) into the PP 2,3-dichlorophenylpiperazine fragment (DCPP, **1**) conferred 32–115-fold increases in binding affinity (Figure 3 and Table 2). Incorporation of the 1,2,3,4-tetrahydroquinolin-2-one (THQ) moiety of aripiprazole enhanced the binding

affinity by a further 22-fold compared to that of the methoxybutyl-substituted derivative (**4**). Fragments containing the SP were able to displace the radioligand only upon inclusion of an ionizable nitrogen atom within its structure (Supplementary Table 3). In functional studies, this time using a bioluminescence resonance energy transfer (BRET) biosensor to measure cAMP levels, the incorporation of alkyl or alkoxy spacers conferred a 17-fold increase in functional affinity as compared to that of DCP, although a further increase in functional affinity was not observed upon incorporation of the THQ moiety. The DCP fragment of aripiprazole displayed a weak intrinsic efficacy, in agreement with previously published data,¹⁸ an effect conferred through interaction of the PP with the OBS as shown by our MD simulations. The incorporation of a propyl linker (**2**) caused a 2-fold decrease in efficacy, whereas the butyl linker (**3**) and butoxy linker (**4**) derivatives displayed a level of efficacy similar to that of DCP (Figure 3 and Table 2). Strikingly, the incorporation of the THQ moiety (to generate aripiprazole) caused a 10-fold increase in efficacy.

In our previous study, we observed that the DCP core of R22 could be replaced with a 2-methoxyphenylpiperazine (2MeOP) core with little change in efficacy or affinity at the D2R.¹⁸ We hypothesized that addition of the 7-butoxy-1,2,3,4-tetrahydroquinolin-2-one substituent of aripiprazole to the 2MeOP core (**11**) would cause an increase in both affinity and efficacy (τ). The addition of an N-butyl substitution conferred a 32-fold increase in affinity, whereas the addition of the 7-butoxy-1,2,3,4-tetrahydroquinolin-2-one substitution (**13**) conferred a 2600-fold higher affinity than the 2MeOP core to yield an extended ligand with the same affinity as aripiprazole (Figure 2 and Table 2). Importantly, we observed that the addition of the 7-butoxy-1,2,3,4-tetrahydroquinolin-2-one substituent caused 26- and 10-fold increases in efficacy (τ) as compared to those of the 2MeOP (**11**) and the N-butyl substituent (**12**), respectively (Figure 3 and Table 2). Thus, the linking of the 7-butoxy-1,2,3,4-tetrahydroquinolin-2-one SP to the 2MeOP PP to generate a novel partial agonist³⁵⁷ results in increases in both efficacy and affinity.

Interaction with SBP Residues Determines the Efficacy of Aripiprazole.

Our results show that the interaction of the SP with the SBP contributes to the affinity and, more surprisingly, the efficacy of aripiprazole. We used mutagenesis to explore the SBP residues that contribute to this interaction. In agreement with the interaction of the SP with SBP residues, the binding affinity of aripiprazole was significantly reduced by SBP mutations W90^{2.60}A (5-fold), V91^{2.61}A (8-fold), and E95^{2.65}A (3-fold) (Table 3). The V91^{2.61}A mutation caused a 11-fold reduction in the transduction coefficient of aripiprazole, whereas the E95^{2.65}A mutation resulted in a 11-fold reduction in its functional affinity (K_A) (Figure 1 and Table 3). While the V91^{2.61}A and E95^{2.65}A mutations had no effect on the two smaller agonists, the W90^{2.60}A mutation reduced the transduction coefficients of ropinirole (14-fold) and dopamine (6-fold) and the binding affinity of dopamine (6-fold). The E95^{2.65}A, V91^{2.61}A, and L41^{1.39}A mutations did not change the affinity of the DCP fragment (Table 2). The F110^{3.28}A mutation significantly reduced the binding affinity of all three agonists and the transduction coefficients of dopamine and ropinirole but not that of aripiprazole (Table 3). The L41^{1.39}A mutation increased the binding affinity of ropinirole and aripiprazole (5-fold) but had no significant effect on the binding affinity of dopamine (Figure 1 and Table 3). Strikingly, this mutation caused a 5-fold decrease in the efficacy (τ)

of aripiprazole, whereas the transduction coefficients of the smaller agonists were not significantly changed (Table 3). Val91^{2,61} and Phe110^{3,28} are in close contact with the butoxy linker of aripiprazole in both of the SP poses obtained with our MD simulations (Figure 2), and these interactions can be correlated to the negative impact of the V91^{2,61}A or F110^{3,28}A mutation on aripiprazole affinity. We extended our MD simulations to compare the pose of aripiprazole at the WT and L41^{1,39} A mutant. The L41^{1,39} A mutation is associated with a higher propensity for inward rotation of Trp384^{7,40} (Figure 2E,F and Supplementary Figure 1), which affects the orientation of Glu95^{2,65} and Tyr37^{1,35}. Interestingly, Trp384^{7,40}, Tyr37^{1,35}, and Glu95^{2,65} form an interaction network only in the mutant simulations (Figure 2F and Supplementary Figure 1). Thus, our simulations indicate that the orientation of the SP toward ECL2 and TM3 is favored in the L41^{1,39} A mutant.

To compare our data with the functional data obtained with the various fragments of aripiprazole (Table 2), we used a BRET biosensor to measure the inhibition of cAMP. In this assay, dopamine and aripiprazole displayed significant 4- and 5-fold decreases in transduction coefficients at the L41^{1,39} A mutant, respectively (Figure 4 and Supplementary Table 4). The latter effect was caused by a 5-fold decrease in aripiprazole efficacy (τ) (Figure 4J), similar to changes observed in the Alpha-screen cAMP assay. It is noteworthy that the efficacy of aripiprazole at this mutant was equivalent to that of the DCPD fragment at the WT receptor, suggesting that the increase in efficacy conferred by the SP of aripiprazole requires Leu41^{1,39}. To determine whether the decreased transduction coefficient of dopamine at L41^{1,39} A was caused by a decrease in functional affinity or efficacy, we treated cells with increasing concentrations of phenoxybenzamine to alkylate cell surface D₂Rs prior to stimulation with an agonist. We applied the operational model of agonism to these data to determine the functional affinity and efficacy of dopamine and ropinirole (Figure 4 and Supplementary Table 4). The L41^{1,39} A mutation caused a 10-fold decrease in dopamine functional affinity (K_A) but no change in efficacy (τ) (Figure 4H,K and Supplementary Table 4). The functional affinity and efficacy of ropinirole were unaffected by this mutation (Figure 4I,L and Supplementary Table 4).

As described above, Leu41^{1,39} directly affects the rotation of Trp384^{7,40} (Figure 2). To explore the interaction between Trp384^{7,40} and Leu41^{1,39}, either Trp384^{7,40} or both residues were mutated to alanine. The action of all agonists was compromised at the double mutant because of its low level of cell surface expression (Supplementary Figure 3). The W384^{7,40} A mutation caused significant decreases in dopamine (5-fold) and ropinirole (3-fold) transduction coefficients but had no effect on that of aripiprazole (Figure 4 and Supplementary Table 4). This is consistent with our proposal that the aripiprazole pose shown in Figure 2C may be more relevant to its intrinsic efficacy, as the W384^{7,40} A mutation is unlikely to have a negative impact on this pose when Trp384 faces lipids. In addition, the preference of the aripiprazole pose in the L41^{1,39} A mutant, which is coordinated with the inward rotation of Trp384 (Figure 2F), supports the idea that the impact of this remote TM1 mutation may be partially mediated by Trp384^{7,40}.

We explored the effect of adding bulk and aromaticity to the SBP by mutating both V91^{2,61} and L41^{1,39} to phenylalanine. L41^{1,39}F had no effect. V91^{2,61}F caused 35-, 30-, and 170-fold decreases in the transduction coefficients of dopamine, ropinirole, and aripiprazole,

respectively (Figure 4F and Supplementary Table 4). This mutation caused decreases in the functional affinity (14-fold) and efficacy (11-fold) of aripiprazole. Leu41^{1.39} and Val91^{2.61} directly interact (Figure 2C). The double mutation (L41^{1.39}A/V91^{2.61}A) caused a 10-fold decrease in the transduction coefficient of dopamine and ropinirole but a much greater 49-fold decrease in that of aripiprazole, driven by a 42-fold decrease in efficacy (Figure 4I and Supplementary Table 4). In contrast, this double mutation decreased the functional affinity (Figure 4H and Supplementary Table 4) of dopamine by 5-fold and had no significant effect on dopamine efficacy.

Together, these data indicate that the direct interaction of the SP of aripiprazole with the D₂R SBP contributes to its intrinsic efficacy. The addition of the SP to the phenylpiperazine PP conferred a significant increase in efficacy, and mutations within the SBP modulated the activity of aripiprazole. The mutation of Leu41^{1.39}, a SBP residue distal to the OBS, significantly decreased the efficacy of aripiprazole in all signaling pathways but increased its binding affinity. Furthermore, the increase in efficacy conferred by the addition of the SP to the SBP was lost at the L41^{1.39} A mutant. Thus, the increase in efficacy caused by the interaction of the SP with the SBP appears to be dependent on Leu41^{1.39}. Our MD simulations predicted two distinct orientations of the SP, one in which the SP occupies the SBP (contacting Leu41^{1.39}, Val91^{473.61}, and Glu95^{2.65}) and one in which the SP extends toward TM3. Our simulations show that the L41^{1.39}A mutation promotes the latter orientation (Figure 2). The mutation of V91 and E95 also caused significant losses of aripiprazole's affinity and a functional effect, consistent with the loss of SBP interactions. We propose that the interaction of the SP with the SBP promotes a higher intrinsic efficacy whereas the orientation of the SP toward TM3 appears to be associated with a lower efficacy but a higher binding affinity. The combination of the V91^{2.61} A mutation with the L41^{1.39} A mutation, which we postulate would further promote the orientation of the SP toward TM3 over the SBP pose, caused an even greater (44-fold) loss of efficacy. In our recent studies of extended 2,3-diCl-phenylpiperazine derivatives, we found that the structures of both the SP and the linker can modulate ligand efficacy. We proposed a mechanism whereby the interaction of the linker and SP with the SBP modulated the conformation of the PP in the OBS, leading to changes in ligand efficacy.¹⁸ The relationship between distinct binding orientations of a single ligand at a receptor and efficacy has been explored in studies of extended bitopic ligands that bind the muscarinic M₂ acetylcholine receptor.³⁴ In this study, it is proposed that such ligands can bind the receptor in two distinct orientations, one that occupies the OBS and one purely allosteric mode that does not.³⁴ The relative propensity of such ligands to occupy the receptor in an orthosteric versus an allosteric orientation determined the intrinsic efficacy. In the study presented here, we find no evidence that aripiprazole can bind the D₂R in a purely allosteric mode. Rather, we propose that the PP of aripiprazole occupies the OBS in a rather stable pose in both orientations of the ligand and that the direct interaction of the SP of aripiprazole with the SBP confers an increase in efficacy. We have also shown that the interaction of the SP of a D₂R negative allosteric modulator with a similar SBP was required for allosteric pharmacology, whereas the PP of this ligand acted as a competitive antagonist.³⁵ Together with the study presented here, this illustrates that the interaction of SP of extended ligands with the SBP of the D₂R can confer

changes in pharmacology relative to that which resulted from binding of the primary pharmacophore of each ligand in the orthosteric binding site.

Mutation of SBP residues also influenced the binding and functional affinity of small orthosteric agonists not expected to interact with the SBP. The effects of these mutants upon aripiprazole compared to their effects on the smaller agonists were, however, distinct. In the case of SBP mutations that affected the action of all three agonists (V91^{2.61}F and L41^{1.39}A/V91^{2.61}A), the effect on aripiprazole was much stronger. It should be noted, however, that while the L41^{1.39}A mutation or the L41^{1.39}A/V91^{2.61}A double mutation did not affect the efficacy of dopamine or ropinirole, each caused a decrease in the functional affinity of dopamine. The functional affinity presumably reflects the affinity of dopamine for the receptor when coupled to signaling effectors.³⁶ In contrast, the binding affinity of dopamine, which was unchanged relative to that of WT, reflects the affinity of dopamine for the uncoupled state of the receptor. Dopamine cannot make direct contacts with this SBP residue when bound in the OBS. Thus, this mutation appears to modulate the affinity with which dopamine binds to a coupled receptor state but does not affect the efficiency with which it stimulates receptor-mediated G protein activation. In addition, the indirect effect of this mutation upon dopamine's functional affinity is distinct from the effect upon aripiprazole efficacy that we propose is caused by modulation of the interaction between the SP and the SBP. Nonetheless, our data indicate that residues within the SBP can influence the binding of even small agonists to the OBS. This effect is dependent upon the structure of the orthosteric agonist as the L41^{1.39}A mutation had no effect on ropinirole. This is difficult to reconcile with a global effect of this mutation, such as the impairment of the transition to an active receptor state, as one would envisage that all agonists would be affected in a similar manner. Dopamine and ropinirole were shown to display distinct sensitivities to the mutation of OBS residues; for example, the S197^{5.46}A mutation ablated dopamine's functional activity but had no effect on ropinirole. Thus, they are likely to have distinct patterns of interaction with the OBS. The mutation of L41^{1.39} may modulate the conformation of the OBS in a manner that affects the functional affinity of some but not all agonists and is dependent upon their structure and the residues they engage to exert their effect. Consistent with the idea of changes in the conformation of the SBP modulating the binding of agonists to the OBS, we have previously shown that a SP fragment of an extended D₂R ligand acted as a negative allosteric modulator and that its binding was sensitive to SBP.³⁷ Moreover, allosteric modulators of the muscarinic receptor interact with residues that align with those forming the D₂R SBP.^{38,39} A SBP defined by residues of extracellular TM1, TM2, and TM7 has also been implicated in the agonist binding and/or activation of the chemokine CCR5, nicotinic acid (GPR109A), and angiotensin 1 receptors.^{40–42} Thus, the SBP defined in this study is likely to be important for the modulation of agonist action in other GPCRs.

The Biased Agonism of Aripiprazole Is Unchanged in OBS or SBP Mutants.

Previously, we have shown that aripiprazole displays biased agonism toward inhibition of cAMP over phosphorylation of ERK1/2.^{43,44} In our pERK1/2 assay, aripiprazole displayed a maximal response of 29% of ropinirole at the WT D₂R, corresponding to an efficacy of 0.39, 12-fold lower than that observed in the cAMP assay (Figure 5 and Supplementary

Table 5). We quantified the biased agonism of dopamine and aripiprazole between inhibition of cAMP production and ERK1/2 phosphorylation using ropinirole as the reference agonist.²¹ Consistent with our previous results, aripiprazole was biased toward the inhibition of cAMP production over ERK1/2 phosphorylation whereas dopamine was not (Supplementary Table 6).⁴³ None of the OBS or SBP mutations caused a significant change in this bias. Note, however, that the window in which the deleterious effects of a mutation can be detected is smaller in the pERK1/2 assay because of the lower efficacy of aripiprazole at the WT D₂R as compared to that obtained in the cAMP assay. Accordingly, we were unable to quantify a change in bias for the mutations that abrogated aripiprazole action in the pERK1/2 assay but that also had a deleterious effect in the cAMP assay (for example, L41^{1.39}A and V91^{2.61}A). While previous studies have shown that aripiprazole does not display bias between cAMP and β -arrestin recruitment,^{43,45} we were curious to see whether L41^{1.39}A might change this. In a β -arrestin translocation assay that measures the movement of a β -arrestin-2-Venus to the cell surface, aripiprazole acted as a partial agonist at the WT D₂R [E_{max} = 86% of maximal response of ropinirole (Figure 5 and Supplementary Table 7)]. Aripiprazole displayed a significant 6-fold decrease in efficacy in the L41^{1.39}A mutant as compared to that of WT. No bias between cAMP and β -arrestin-2 translocation was observed for dopamine or aripiprazole relative to ropinirole at the WT or L41^{1.39}A D₂R (Figure 5 and Supplementary Table 7).

Conclusions.

The weak intrinsic efficacy of D₂R partial agonists such as aripiprazole is thought to determine both their antipsychotic effect and their low propensity to cause extrapyramidal side effects and hyperprolactinemia as compared to those of typical antipsychotics. Our results reveal the molecular interactions that are important for this intrinsic efficacy. Aripiprazole's structure is typical of many D₂-like DR subtype-selective ligands, namely a substituted piperazine PP and a lipophilic SP.^{7,17} Previous studies have revealed that the addition of a SP to a piperazine PP can confer gains in affinity and subtype selectivity through interaction with a SBP defined by the extracellular ends of TM1, TM2, and TM7.^{7–10,18,33,46,47} In this study, we find that the interaction of the quinalinone SP of aripiprazole with the SBP is a key determinant of the intrinsic efficacy of this drug. Addition of aripiprazole's SP to the 2,3-diCl-phenylpiperazine PP or a distinct 2-methoxyphenylpiperazine PP fragment conferred gains in both affinity and efficacy. These data, combined with our previous study that found that the interaction of an SP with a distinct indole structure with the SBP caused a decrease in intrinsic efficacy,¹⁸ provide a means of designing D₂R partial agonists with the desired intrinsic efficacy.

METHODS

Materials.

Aripiprazole was synthesized in house as previously described and shown to be >98% pure.⁴³ Ropinirole was purchased from BetaPharma Co. Ltd. (Wujiang, China) and was >98% pure as described by the supplier. All novel compounds were synthesized as described in the Supporting Information. pcDNA31-His-CAMYEL was purchased from ATCC. Dulbecco's modified Eagle's medium (DMEM), hygromycin B, and FlpIn CHO cells were purchased

from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from ThermoTrace (Melbourne, Australia). [³H]Spiperone, [³H]raclopride, Alpha-Screen reagents, Ultima gold scintillation cocktail, 384-well optiplates, and 384-well proxiplates were purchased from PerkinElmer (Boston, MA). All of the other reagents were purchased from Sigma-Aldrich (Castle Hill, Australia).

Molecular Biology and Generation of Cell Lines.

The molecular biology and generation of cell lines were performed as described previously.⁴³ Full details are given in the Supporting Information. cDNA in pcDNA3.1+ encoding the short isoform of the wild-type human dopamine D2 receptor with an N-terminal SNAP tag was obtained from Cisbio (Bagnols-sur-Cèze, France).

ELISA and Cell Signaling Assays.

The ELISA protocol, ERK1/2 phosphorylation assay, cAMP Alpha-screen assay, and BRET assays measuring intracellular cAMP and β -arrestin-2 recruitment to the plasma membrane were performed as described previously.^{43,48} Full details are given in the Supporting Information.

Membrane Preparation and Radioligand Binding Assays.

Radioligand binding assays were performed as described previously.⁴³ Full details are given in the Supporting Information.

Data Analysis.

The results were analyzed using Prism 6.0 (GraphPad Software Inc., San Diego, CA). Full details of data analysis are given in the Supporting Information. All affinity (pK_i , pK_D , or pK_A), potency (pEC_{50}), and transduction ratio [$\log(\tau/K_A)$] parameters were estimated as logarithms, where fold changes were calculated using the corresponding antilog values. We have previously demonstrated that the distribution of the antilog parameters does not conform to a normal (Gaussian) distribution whereas the logarithm is approximately Gaussian. Thus, because the application of *t* tests and analyses of variance assume a Gaussian distribution, estimating the parameters as logarithms allows valid statistical comparison. All results are expressed as means $\pm$ the standard deviation. We performed a Brown-Forsythe test (GraphPad Prism 6.0) to ensure ourselves of equal variance when such parameters are compared.

MD Simulations.

Full details of the protocol are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mutations of residues within the OBS and SBP have distinct effects on the affinity and efficacy of aripiprazole as compared to those of dopamine and ropinirole. WT and mutant D₂Rs were stably expressed in FlpIN CHO cells. The change in affinity (pK_i) of (A) ropinirole, (B) dopamine, and (C) aripiprazole for each mutant was determined in competition binding experiments. The ability to increase the concentration of each agonist to activate the WT or mutant D₂Rs was determined in an assay measuring the inhibition of cAMP production. These data were fit to an operational model of agonism, and changes in

the transduction coefficient (τ/K_A) were determined for (D) ropinirole, (E) dopamine, and (F) aripiprazole for each mutant. Changes in (G) functional affinity (pK_A) and (H) efficacy (τ) were also determined for aripiprazole. Mutations that cause significant increases (one-way analysis of variance with Dunnett's post hoc test; $p < 0.05$; blue) or decreases (red) for each parameter for the mutant receptor as compared to that of the WT are highlighted on a homology model of the D₂R.

Figure 2.

Molecular modeling and ligand docking experiments reveal that aripiprazole adopts an extended orthosteric pose at the D₂R. (A) Molecular modeling and docking experiments using a homology model of the D₂R followed by MD simulations reveal that aripiprazole adopts an extended orthosteric pose within the D₂R and interacts with residues within the OBS and SBP. (B) Within the OBS, the 2,3-diCl-phenylpiperazine PP of aripiprazole adopts a pose parallel to the membrane oriented toward TM5. Within the SBP, the 1,2,3,4-tetrahydroquinolin-2-one “tail” moiety and the flexible butoxy linker adopt two distinct poses depending on the orientation of Trp384^{7,40}. (C) When this residue faces lipids, the quinalinone ring occupies a cavity within the SBP contacting residues from TM1, TM2, and TM7. (D) When Trp384^{7,40} rotates inward, the quinalinone ring can no longer occupy the SBP but instead tilts toward TM3 and ECL2. (E and F) The L41^{1,39} A mutation increases the propensity for Trp384^{7,40} to rotate inward, allowing Trp384^{7,40}, Tyr37^{1,35}, and Glu95^{2,65} to interact.

Figure 3.

7-Butoxy-1,2,3,4-tetrahydroquinolin-2-one substitution of a phenylpiperazine core confers an increase in efficacy and affinity. Two series of substituted phenylpiperazine fragments and extended compounds were synthesized, (A) one that incorporates the 2,3-dichlorophenylpiperazine (DCPP) core that includes aripiprazole and (B) one that incorporates the 2-methoxyphenylpiperazine core (2MeOPP). The ability of increasing concentrations of each compound in (C and E) the DCPP series or (D and F) the 2MeOPP series to activate the WT D_{2S}Rs was determined through an assay measuring the inhibition

of forskolin-stimulated cAMP production using a BRET biosensor. (E and F) These data were fit to an operational model of agonism, and changes in functional affinity or efficacy were determined as compared to the that of phenylpiperazine core of each series. An asterisk indicates a significant change in the parameter as compared to that of the core of each series (one-way analysis of variance with Dunnett's post hoc test; $p < 0.05$).

Figure 4.

Mutation of SBP residues decreases the efficacy of aripiprazole but not that of dopamine. The ability of increasing concentrations of (A) aripiprazole, (B) dopamine, or (C) ropinirole to activate the WT or mutant (L41^{1.39}A, W384^{7.40}A, L41F, V91F, and L41^{1.39}A/V91^{2.61}A) SNAP-tagged D_{2S}Rs was determined in a BRET assay measuring the inhibition of forskolin-stimulated cAMP production. These data were fit to an operational model of agonism and estimates of the (D–F) transduction coefficients and (G–I) functional affinities and (J–L) efficacies at the WT and mutant receptors. An asterisk indicates a significant change in the parameter for each agonist relative to WT (one-way analysis of variance with Dunnett's post hoc test; $p < 0.05$).

Figure 5.

Mutation L41^{1.39}A decreases the intrinsic efficacy of aripiprazole at multiple signaling pathways. The ability of increasing concentrations of each agonist to activate the WT or L41^{1.39}A D_{2S}Rs was determined through an Alpha-screen assay measuring the inhibition of (A) forskolin stimulated cAMP production, (B) ERK1/2 phosphorylation, and (C) β -arrestin translocation. (D) These data were fit to an operational model of agonism, and bias factors between each pathway were determined for dopamine and aripiprazole relative to ropinirole. An asterisk indicates significant bias toward one pathway (two-tailed, unpaired Student's *t* test; *p* < 0.05).

Table 1.

Effects of Mutations in the OBS and ECL2 of the D₂R on the Affinity and Functional Activity of Selected Agonists^a

construct	ropinirole		dopamine		ariprazole			
	p <i>K</i> _i (fold)	log(<i>τ</i> / <i>K</i> _A) (fold)	p <i>K</i> _i (fold)	log(<i>τ</i> / <i>K</i> _A) (fold)	p <i>K</i> _i (fold)	log(<i>τ</i> / <i>K</i> _A) (fold)	p <i>K</i> _A (fold)	log <i>τ</i> (fold)
WT	5.18 ± 0.04 (1.0)	8.29 ± 0.06 (1.0)	4.94 ± 0.03 (1.0)	8.51 ± 0.05 (1.0)	9.92 ± 0.02 (1.0)	8.45 ± 0.06 (1.0)	7.75 ± 0.14	0.69 ± 0.15
V115 ³³³ A	4.73 ± 0.20 (2.8)	6.78 ± 0.15 [*] (33)	4.06 ± 0.15 [*] (7.7)	7.32 ± 0.12 [*] (16)	9.07 ± 0.04 [*] (7.1)	7.70 ± 0.17 [*] (5.6)	7.83 ± 0.20 (0.8)	−0.19 ± 0.04 [*] (8)
C118 ³³⁶ A	5.43 ± 0.09 (0.6)	ND	4.40 ± 0.16 [*] (3.5)	5.94 ± 0.09 [*] (370)	9.36 ± 0.03 [*] (3.6)	ND	ND	ND
T119 ³³⁷ A	5.37 ± 0.16 (0.7)	6.47 ± 0.08 [*] (67)	4.84 ± 0.04 (1.3)	ND	9.68 ± 0.10 (1.7)	ND	ND	ND
I122 ³⁴⁰ A	5.11 ± 0.04 (1.2)	ND	5.21 ± 0.10 (0.5)	ND	10.11 ± 0.09 (0.6)	ND	ND	ND
L174 ^{ECL2} A	5.65 ± 0.13 (0.3)	8.21 ± 0.09 (1.2)	5.24 ± 0.12 (0.5)	8.22 ± 0.06 (1.9)	10.16 ± 0.07 (0.6)	8.43 ± 0.19 (1.0)	7.95 ± 0.14 (0.6)	0.48 ± 0.11 (1.6)
E181 ^{ECL2} A	5.52 ± 0.03 (0.5)	7.94 ± 0.19 (2.2)	5.02 ± 0.01 (0.8)	8.63 ± 0.17 (0.8)	9.88 ± 0.05 (1.1)	8.20 ± 0.22 (1.8)	8.00 ± 0.16 (0.6)	0.19 ± 0.08 (3)
I183 ^{ECL2} A	5.50 ± 0.02 (0.5)	8.51 ± 0.18 (0.6)	5.02 ± 0.03 (0.8)	8.75 ± 0.10 (0.6)	9.50 ± 0.08 (2.7)	8.05 ± 0.24 (2.5)	7.61 ± 0.23 (1.4)	0.44 ± 0.01 (1.8)
I184 ^{ECL2} A	5.18 ± 0.06 (1.0)	7.36 ± 0.16 [*] (8.5)	4.40 ± 0.07 [*] (3.5)	7.06 ± 0.05 [*] (28)	9.45 ± 0.12 (3.0)	7.93 ± 0.05 (3.3)	7.65 ± 0.07 (1.3)	0.27 ± 0.09 (2.6)
A185 ^{ECL2} S	5.69 ± 0.03 [*] (0.3)	8.39 ± 0.08 (0.8)	5.37 ± 0.11 [*] (0.4)	8.61 ± 0.09 (0.8)	10.06 ± 0.13 (0.7)	8.65 ± 0.06 (0.6)	7.87 ± 0.15 (0.8)	0.79 ± 0.11 (0.8)
N186 ^{ECL2} A	5.01 ± 0.10 (1.5)	7.67 ± 0.07 (4.2)	4.54 ± 0.09 (2.5)	8.01 ± 0.06 (3.2)	9.60 ± 0.21 (2.1)	8.10 ± 0.20 (2.2)	7.63 ± 0.12 (1.6)	0.47 ± 0.11 (1.6)
S193 ⁵⁴² A	4.00 ± 0.05 [*] (15)	5.32 ± 0.12 [*] (930)	2.86 ± 0.09 [*] (120)	5.30 ± 0.14 [*] (1600)	10.41 ± 0.06 [*] (0.3)	8.82 ± 0.11 (0.4)	7.29 ± 0.16 (2.9)	1.53 ± 0.15 [*] (0.1)
S194 ⁵⁴³ A	5.01 ± 0.11 (1.5)	6.99 ± 0.09 [*] (20)	4.39 ± 0.08 [*] (3.6)	7.48 ± 0.12 [*] (11)	9.66 ± 0.17 (1.8)	8.42 ± 0.09 (1.1)	7.72 ± 0.07 (1.1)	0.71 ± 0.30 (1)
S197 ⁵⁴⁶ A	5.07 ± 0.04 (1.3)	8.08 ± 0.14 (1.6)	4.47 ± 0.03 [*] (3.0)	ND	9.21 ± 0.07 [*] (5.1)	8.58 ± 0.06 (0.7)	7.82 ± 0.10 (0.9)	0.80 ± 0.10 (0.8)
F202 ⁵⁵¹ A	4.91 ± 0.07 (1.9)	6.21 ± 0.21 [*] (120)	4.15 ± 0.04 [*] (6.2)	6.30 ± 0.10 [*] (160)	9.40 ± 0.10 [*] (3.3)	ND	ND	ND
F360 ⁶⁵¹ A	ND	ND	ND	ND	ND	ND	ND	ND
F361 ⁶⁵² A	ND	7.36 ± 0.15 [*] (8.6)	ND	7.65 ± 0.18 [*] (7.2)	ND	8.03 ± 0.23 (2.6)	7.29 ± 0.18 (2.8)	0.73 ± 0.13 (0.9)
H364 ⁶⁵⁵ A	5.39 ± 0.05 (0.6)	7.52 ± 0.25 [*] (5.9)	4.33 ± 0.08 [*] (4.1)	6.67 ± 0.21 [*] (69)	10.22 ± 0.03 (0.5)	8.58 ± 0.16 (0.7)	7.64 ± 0.24 (1.3)	0.89 ± 0.07 (0.6)
H364 ⁶⁵⁵ F	5.11 ± 0.09 (1.2)	6.26 ± 0.11 [*] (110)	4.63 ± 0.05 (2.1)	7.06 ± 0.07 [*] (28)	9.74 ± 0.05 (1.5)	8.43 ± 0.12 (1.0)	7.50 ± 0.14 (1.8)	0.93 ± 0.19 (0.6)

construct	ropinirole		dopamine		aripiprazole			
	p <i>K</i> _i (fold)	log(<i>τ</i> / <i>K</i> _A) (fold)	p <i>K</i> _i (fold)	log(<i>τ</i> / <i>K</i> _A) (fold)	p <i>K</i> _i (fold)	log(<i>τ</i> / <i>K</i> _A) (fold)	p <i>K</i> _A (fold)	log <i>τ</i> (fold)
N367 ^{6,58} A	5.28 ± 0.07 (0.8)	7.71 ± 0.08 (3.8)	4.45 ± 0.06 [*] (3.1)	7.51 ± 0.03 [*] (10)	9.91 ± 0.04 (1.0)	8.29 ± 0.13 (1.4)	7.93 ± 0.10 (0.7)	0.20 ± 0.04 (3.0)
I368 ^{6,59} A	5.41 ± 0.09 (0.6)	7.58 ± 0.16 [*] (5.2)	5.45 ± 0.11 [*] (0.3)	8.59 ± 0.19 (0.8)	9.62 ± 0.07 (2.0)	8.49 ± 0.10 (0.9) (0.7)	7.89 ± 0.14 (0.7)	0.60 ± 0.12 (1.2)
S380 ^{7,36} A	5.57 ± 0.01 (0.4)	8.00 ± 0.13 (2.0)	5.06 ± 0.10 (0.8)	8.68 ± 0.16 (0.7)	9.92 ± 0.10 (1.0)	8.23 ± 0.26 (1.7)	8.41 ± 0.18 (0.22)	0.87 ± 0.19 (0.66)
T383 ^{7,39} A	5.36 ± 0.04 (0.7)	6.83 ± 0.06 [*] (29)	4.87 ± 0.04 (1.2)	7.91 ± 0.10 (4)	10.13 ± 0.14 (0.6)	9.09 ± 0.07 (0.2) [*]	7.99 ± 0.10 (0.6)	1.09 ± 0.13 (0.40)

^a Binding affinity values were obtained in competition binding experiments using the radioligand [³H]spiperone. Functional affinities, efficacies, and transduction ratios were determined in an assay measuring inhibition of forskolin-induced intracellular cAMP production. Values are expressed as means ± the standard deviation from three (binding) or four (cAMP) separate experiments. ND indicates no specific binding or agonist activity could be determined.

* $p < 0.05$, significantly different from that of the wild-type receptor determined by one-way analysis of variance, with Dunnett's post

Table 2.

Binding Affinities and Functional Action of Phenylpiperazine Fragments and Extended Compounds at the D2R^a

Compound	p <i>K</i> _i ± SEM [³ H]spiperone			p <i>K</i> _i ± SEM [³ H]raclopride		cAMP (BRET Biosensor)	
	WT	L41 ^{1,39} A (fold)	E9S ^{2,65} A (fold)	WT	V91 ^{2,61} A (fold)	p <i>K</i> _A	Log τ
1	6.24 ± 0.04 [#]	6.80 ± 0.16 (0.3)	6.29 ± 0.16 (0.9)	7.45 ± 0.31	7.59 ± 0.17 (0.7)	6.49 ± 0.18	0.12 ± 0.06 [#]
2	7.74 ± 0.07 [#]	-	-	-	-	7.50 ± 0.28	-0.30 ± 0.07 [#]
3	8.30 ± 0.10 [#]	-	-	-	-	7.20 ± 0.24	-0.04 ± 0.06 [#]
4	7.70 ± 0.09 [#]	-	-	-	-	7.72 ± 0.19	0.00 ± 0.05 [#]
Asipiprazole	9.11 ± 0.12	-	-	-	-	7.41 ± 0.30	1.02 ± 0.24

Compound	$pK_i \pm \text{SEM}$ [³ H]piperone		$pK_i \pm \text{SEM}$ [³ H]raclopride		pK_A	Log τ
	WT	L41 ^{1,39} A (fold)	E95 ^{2,65} A (fold)	WT	V91 ^{2,61} A (fold)	
11	5.64 ± 0.07 [*]	6.23 ± 0.15 [*] (0.4)	6.12 ± 0.13 [*] (0.3)		6.43 ± 0.24 [*] (1.1)	-0.42 ± 0.11 [*]

	6.49 ± 0.19					
12	7.03 ± 0.05 [*]	-	-	-	7.31 ± 0.20 [*]	-0.03 ± 0.05 [*]

13	9.11 ± 0.08	10.4 ± 0.09 [*] (0.1)	9.34 ± 0.16 (0.6)	10.3 ± 0.22	8.41 ± 0.26 [*] (77)	1.00 ± 0.22

^a Binding affinity (K_i) determined by competition binding experiments using radiolabeled antagonist [³H]piperone or [³H]raclopride at WT or mutant SNAP-D₂SR. Functional affinity (K_A) and efficacy (τ) determined in an assay measuring inhibition of forskolin-stimulated cAMP production. Values are expressed as means ± the standard deviation from three separate experiments.

^{*} Values significantly different from that of the WT as determined by one-way analysis of variance (Dunnett's post hoc test; $p < 0.05$).

[#] Values significantly different from that of aripiprazole as determined by one-way analysis of variance (Dunnett's post hoc test; $p < 0.05$).

Table 3.

Effects of Mutations in the SBP of the D₂R on the Binding Affinities and Functional Activities of Selected Agonists^a

construct	ropinirole			dopamine			aripiprazole		
	p <i>K_i</i> (fold)	log(<i>pK_A</i>) (fold)	p <i>K_i</i> (fold)	p <i>K_i</i> (fold)	log(<i>pK_A</i>) (fold)	p <i>K_i</i> (fold)	log(<i>pK_A</i>) (fold)	p <i>K_A</i> (fold)	log τ (fold)
WT	5.18 ± 0.04		4.94 ± 0.03			9.92 ± 0.02			
WT [#]	5.83 ± 0.12	8.29 ± 0.06	5.78 ± 0.13	8.51 ± 0.05		9.86 ± 0.11	8.45 ± 0.06	7.75 ± 0.14	0.69 ± 0.15
L41 ^{1,39} A	5.85 ± 0.10 [*] (0.2)	8.09 ± 0.16 (1.6)	5.29 ± 0.02 (0.5)	7.89 ± 0.27 (4.2)		10.61 ± 0.04 [*] (0.2)	8.12 ± 0.16 (2.1)	8.07 ± 0.16 (0.5)	0.03 ± 0.07 [*] (4.6)
W90 ^{2,60} A	4.90 ± 0.14 (1.9)	7.14 ± 0.18 [*] (1.4)	4.17 ± 0.05 [*] (5.9)	7.71 ± 0.20 [*] (6.4)		9.18 ± 0.08 [*] (5.5)	8.13 ± 0.16 (2.1)	7.74 ± 0.10 (1.0)	0.37 ± 0.08 (2.0)
V91 ^{2,61} A [#]	6.03 ± 0.02 (0.6)	8.32 ± 0.12 (1.0)	6.03 ± 0.08 (0.6)	8.69 ± 0.20 (0.7)		8.96 ± 0.04 [*] (8.1)	7.41 ± 0.26 [*] (11)	7.29 ± 0.36 (2.8)	0.12 ± 0.22 (3.7)
E95 ^{2,65} A	5.68 ± 0.08 [*] (0.3)	8.16 ± 0.11 (1.4)	5.24 ± 0.06 (0.5)	8.22 ± 0.11 (2.0)		9.41 ± 0.06 [*] (3.2)	7.87 ± 0.15 (3.8)	6.71 ± 0.21 [*] (11)	1.16 ± 0.24 (0.3)
F110 ^{3,28} A	4.37 ± 0.15 [*] (6.5)	7.19 ± 0.13 [*] (13)	3.57 ± 0.08 [*] (24)	7.58 ± 0.19 [*] (8.6)		8.68 ± 0.06 [*] (17)	8.08 ± 0.11 (2.3)	7.30 ± 0.16 (2.8)	0.78 ± 0.10 (0.8)

^a Binding affinity values were obtained in competition binding experiments using the radioligand [³H] spiperone unless otherwise stated. Functional affinities, efficacies, and transduction ratios were determined in an assay measuring inhibition of forskolin-induced intracellular cAMP production.[#] Binding affinity values are obtained in competition binding experiments using the radioligand [³H]raclopride. Values are expressed as means ± the standard deviation from three (binding) or four (cAMP) separate experiments.^{*} *p* < 0.05, significantly different from that of the wild-type receptor determined by a one-way analysis of variance, with Dunnett's post hoc test.