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Molecular determinants of the intrinsic efficacy of the antipsychotic aripiprazole.

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Abstract

Partial agonists of the dopamine D_2 receptor (D_2R) 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 identify the D_2R-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 determine the intrinsic efficacy of aripiprazole. Our findings reveal a hitherto unappreciated mechanism through which to fine-tune the intrinsic efficacy of D_2R agonists.

Introduction

The dopamine D_2 receptor (D_2R), 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 D_2R 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 avoid the complete blockade of the nigrostriatal or tuberoinfundibular pathways, associated with extrapyramidal symptoms and elevated prolactin levels, respectively[5]. However, it remains unclear why these partial agonists display antipsychotic efficacy, while other D_2R partial agonists have failed to do so. 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 prolactinaemia.

The crystal structures of the D_2R, D_3R and D_4R - 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 D_2-like receptors, and are consistent with earlier findings of mutagenesis and molecular modelling studies[7-10]. Despite the therapeutic utility of D_2R full and partial agonists, our understanding of the ligand-receptor contacts that determine 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 system[13]) 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 D_2-like receptors[7,17]. Using the D_3R crystal structure[7], we revealed a secondary binding pocket (SBP) that extended away from the OBS towards the extracellular ends of TMs 1, 2, 3 and 7, and demonstrated that the interaction between this SBP and the aryl tail moiety of phenylpiperazine derivatives was not only 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 D_2R 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 D_2R 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 D_2R, we mutated residues within the OBS, the SBP and the transmission switch of the D_2R. 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 [^{3}H]spiperone and/or [^{3}H]raclopride. A homologous competition binding assay revealed that none of the mutations had a significant effect on the pK_d of [^{3}H]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, [^{3}H]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 D_2R 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 D_2R $G\alpha_{i/o}$ G protein signalling. 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 to previous studies of aripiprazole using the same CHO cell background for which a much lower relative maximal response was observed[2,20]. Such differences reflect different receptor expression levels and assay sensitivity. 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 signalling 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, τ = 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 mutation D114$^{3.32}$A ablates agonist and antagonist binding[22]. Val$^{3.33}$, Cys$^{3.36}$ and Thr$^{3.37}$ line the OBS in the D_2R, D_3R and D_4R structures[7,9,10]. V115$^{3.33}$A reduced the binding affinity of dopamine and aripiprazole but not 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 greater than 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, Table 1). The transduction 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, Table 1). The binding affinity and transduction coefficient of ropinirole were also significantly reduced at S193$^{5.42}$A by 15-fold and 930-fold, respectively. S194$^{5.43}$A had no effect on ropinirole affinity but caused a 20-fold decrease in 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 upon the functional response (Figure 1, Table 1).

Figure 1

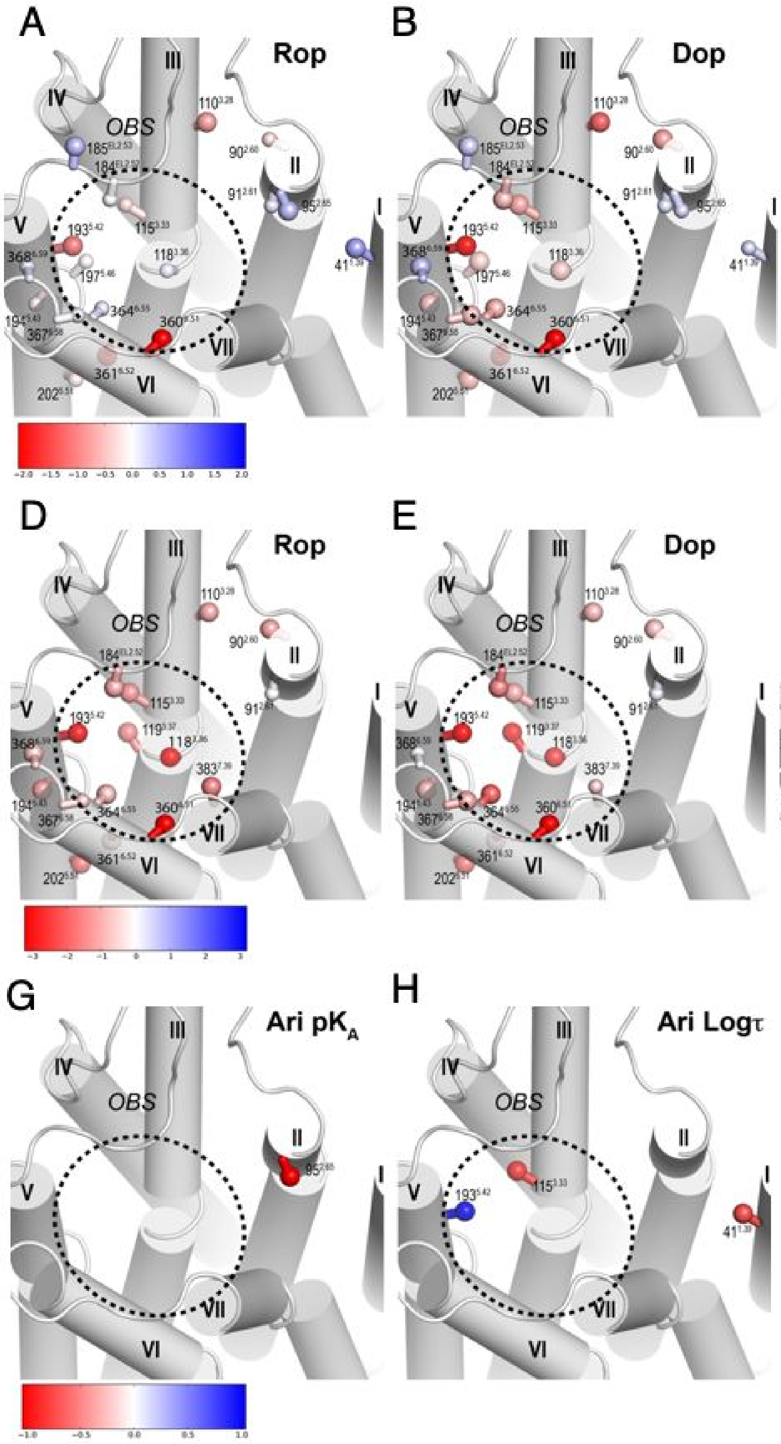


Figure 1: Mutation of residues within the OBS and SBP have distinct effects upon the affinity and efficacy of aripiprazole as compared to dopamine and ropinirole. WT and mutant D_2Rs were stably expressed in FlpIN CHO cells. The change in affinity (pK_i) of ropinirole (**A**), dopamine (**B**) and aripiprazole (**C**) at each mutant was determined in

competition binding experiments. The ability of increasing concentrations of each agonist to activate the WT or mutant D_2Rs was determined in an assay measuring the inhibition of cAMP production. These data were fit to an operational model of agonism and changes in transduction coefficient (τ/K_A) were determined for ropinirole (**D**), dopamine (**E**) and aripiprazole (**F**) at each mutant. Changes in functional affinity (pK_A, **G**) and efficacy (τ, **H**) were also determined for aripiprazole. Mutations that cause significant increases (one-way ANOVA with Dunnett's post hoc test, $P < 0.05$, blue) or decreases (red) for each parameter at the mutant receptor as compared to WT are highlighted on a homology model of the D_2R.

Residues within ECL2 form part of the D_2R and D_3R OBS[7,28]. I184^{ECL2}A significantly reduced the binding affinity and transduction coefficient of dopamine (4-fold 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 D_3R and the methoxy benzamide ring of nemonapride in the D_4R[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) (Figure1, Table 1). Residue 6.55 has been shown to be important for agonist binding and efficacy at the D_2R and D_3R[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, 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 D_2R agonists have been suggested to underlie differences in efficacy through the stabilisation of distinct receptor conformations[27,29]. In the case of aripiprazole, rather than deleterious effects, the mutations S$^{5.46}$A, F$^{6.52}$A and H$^{6.55}$A caused a modest increase in efficacy. Interestingly, S$^{5.42}$A and S$^{5.46}$A caused decreases in the affinity and transduction coefficient of dopamine in agreement with previous studies, whereas S$^{5.46}$A had no effect on ropinirole. In a previous study our MD simulations found that the 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$^{5.46}$ is 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 3.40, 5.50, 5.51, 6.44, and 6.48) in TM3–TM5–TM6 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 on receptor activation to allow the outward movement of TM6. I122$^{3.40}$A had no significant effect on the binding affinity of the agonists but abrogated functional activity. F202$^{5.51}$A caused a significant reduction in the binding affinity of dopamine and aripiprazole (6-fold and 3-fold respectively, Table 1). Aripiprazole displayed no agonism at this mutant and ropinirole and dopamine displayed more than 100-fold lower transduction coefficients (Table 1). Thus, all three D_2R 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 may modulate the conformation of the OBS causing the loss of affinity of dopamine and aripiprazole but not ropinirole. Interestingly, the recent D_2R crystal structure obtained in complex with the antagonist risperidone included I122$^{3.40}$A as one of three thermostabilizing mutations[10]. This mutation likely exerts its thermostabilizing effect by preventing the isomerisation of the receptor into the active state.

Molecular dynamic 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 modelling and simulation study of D_2R models in complex with aripiprazole. From the initial docking results, we chose several aripiprazole poses with its quinoline moiety oriented in various directions in the extracellular vestibule (EV) of D_2R (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.

Tables

Table 1. The effect of mutations in the OBS and ECL2 of the D_2R on the affinity and functional activity of selected agonists.

Construct	Ropinirole		Dopamine		Aripiprazole			
	pK_i (fold Δ)	Log(τ/K_A) (fold Δ)	pK_i (fold Δ)	Log(τ/K_A) (fold Δ)	pK_i (fold Δ)	Log(τ/K_A) (fold Δ)	pK_A (fold Δ)	Logτ (fold Δ)
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$^{3.33}$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$^{3.36}$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$^{3.37}$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$^{3.40}$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	7.67 ±0.07	4.54 ± 0.09	8.01 ± 0.06	9.60 ± 0.21	8.10 ± 0.20	7.63 ± 0.12	0.47 ± 0.11
	(1.5)	(4.2)	(2.5)	(3.2)	(2.1)	(2.2)	(1.6)	(1.6)
S193$^{5.42}$A	4.00 ± 0.05*	5.32 ± 0.12*	2.86 ± 0.09*	5.30 ± 0.14*	10.41 ± 0.06*	8.82 ± 0.11	7.29± 0.16	1.53 ± 0.15*
	(15)	(930)	(120)	(1600)	(0.3)	(0.4)	(2.9)	(0.1)
S194$^{5.43}$A	5.01 ± 0.11	6.99 ± 0.09*	4.39 ± 0.08*	7.48 ± 0.12*	9.66 ± 0.17	8.42 ± 0.09	7.72 ± 0.07	0.71 ± 0.30
	(1.5)	(20)	(3.6)	(11)	(1.8)	(1.1)	(1.1)	(1)
S197$^{5.46}$A	5.07 ± 0.04	8.08 ± 0.14	4.47 ± 0.03*	ND	9.21 ± 0.07*	8.58 ± 0.06	7.82 ± 0.10	0.80 ± 0.10
	(1.3)	(1.6)	(3.0)		(5.1)	(0.7)	(0.9)	(0.8)
F202$^{5.51}$A	4.91 ± 0.07	6.21 ± 0.21*	4.15 ± 0.04*	6.30 ± 0.10*	9.40 ± 0.10*	ND	ND	ND
	(1.9)	(120)	(6.2)	(160)	(3.3)			
F360$^{6.51}$A	ND	ND	ND	ND	ND	ND	ND	ND
F361$^{6.52}$A	ND	7.36 ± 0.15*	ND	7.65 ± 0.18*	ND	8.03 ± 0.23	7.29 ± 0.18	0.73 ± 0.13
		(8.6)		(7.2)		(2.6)	(2.8)	(0.9)
H364$^{6.55}$A	5.39 ± 0.05	7.52 ± 0.25*	4.33 ± 0.08*	6.67 ± 0.21*	10.22 ± 0.03	8.58 ± 0.16	7.64 ± 0.24	0.89 ± 0.07
	(0.6)	(5.9)	(4.1)	(69)	(0.5)	(0.7)	(1.3)	(0.6)
H364$^{6.55}$F	5.11 ± 0.09	6.26 ± 0.11*	4.63 ± 0.05	7.06 ± 0.07*	9.74 ± 0.05	8.43 ± 0.12	7.50 ± 0.14	0.93 ± 0.19
	(1.2)	(110)	(2.1)	(28)	(1.5)	(1.0)	(1.8)	(0.6)
N367$^{6.58}$A	5.28 ± 0.07	7.71 ± 0.08	4.45 ± 0.06*	7.51 ± 0.03*	9.91 ± 0.04	8.29 ± 0.13	7.93 ± 0.10	0.20 ± 0.04
	(0.8)	(3.8)	(3.1)	(10)	(1.0)	(1.4)	(0.7)	(3.0)
I368$^{6.59}$A	5.41 ± 0.09	7.58 ± 0.16*	5.45 ± 0.11*	8.59 ± 0.19	9.62 ± 0.07	8.49 ± 0.10	7.89 ± 0.14	0.60 ± 0.12
	(0.6)	(5.2)	(0.3)	(0.8)	(2.0)	(0.9)	(0.7)	(1.2)
S380$^{7.36}$A	5.57 ± 0.01	8.00 ± 0.13	5.06 ± 0.10	8.68 ± 0.16	9.92 ± 0.10	8.23 ± 0.26	8.41 ± 0.18	0.87 ± 0.19
	(0.4)	(2.0)	(0.8)	(0.7)	(1.0)	(1.7)	(0.22)	(0.66)
T383$^{7.39}$A	5.36 ± 0.04	6.83 ± 0.06*	4.87 ± 0.04	7.91 ± 0.10 (4)	10.13 ± 0.14	9.09 ± 0.07	7.99 ± 0.10	1.09 ± 0.13
	(0.7)	(29)	(1.2)		(0.6)	(0.2)*	(0.6)	(0.40)

Binding affinity values were obtained in competition binding experiments using the radioligand [^{3}H]spiperone. Values of functional affinity, efficacy and transduction ratios were determined in an assay measuring inhibition of forskolin-induced intracellular cAMP production. Values are expressed as mean ± S.D. from three (binding) or four (cAMP) separate experiments. ND = no specific binding or agonist activity could be determined. *$P < 0.05$, significantly different from the wild-type receptor determined by a one-way ANOVA, Dunnett post-hoc test.

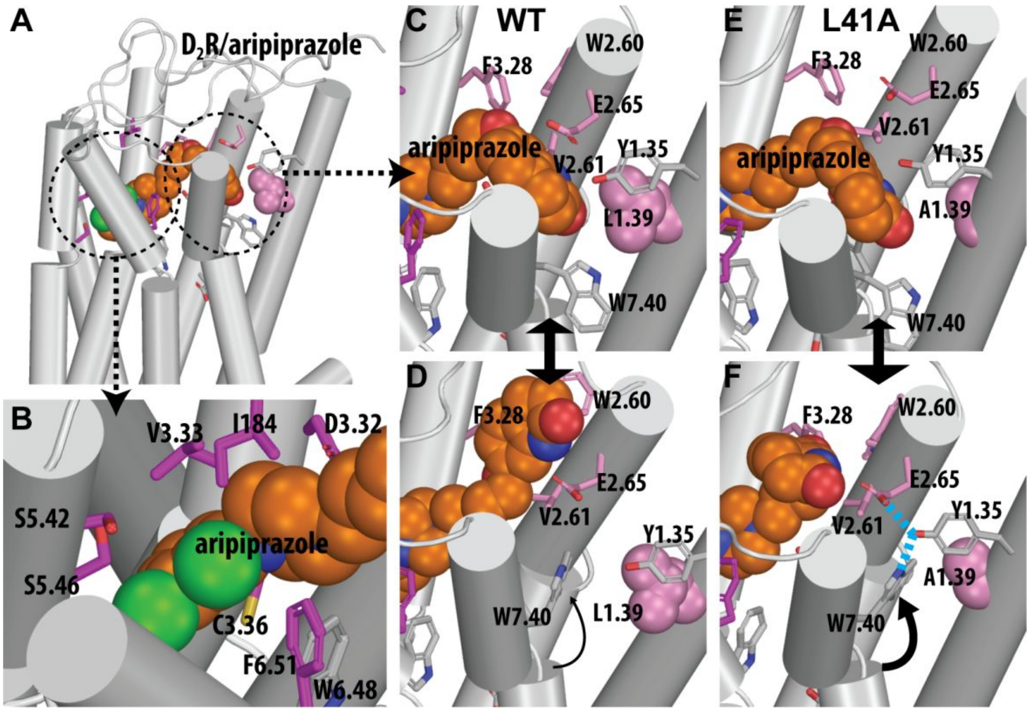


Figure 2: Molecular modeling and ligand docking experiments reveal that aripiprazole adopts an extended orthosteric pose at the D_2R. **A**) Molecular modeling and docking experiments using a homology model of the D_2R followed by MD simulations reveal that aripiprazole adopts an extended orthosteric pose within the D_2R 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 towards TM5. Within the SBP, the 1,2,3,4-tetrahydroquinolin-2-one "tail" moiety and the flexible butoxy linker adopt two distinct poses dependent upon 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, 2 & 7. **D**) When $Trp384^{7.40}$ rotates inward the quinalinone ring can no longer occupy the SBP but instead tilts towards TM3 and ECL2. **E & F**) The $L41^{1.39}A$ mutation increases the propensity for $Trp384^{7.40}$ to rotate inwards, allowing $Trp384^{7.40}$, $Tyr37^{1.35}$ and $Glu95^{2.65}$ to interact.

Similar to the partial agonists with a 2,3-diCl-phenylpiperazine PP that we have characterized previously in D_3R models[18], the PP of aripiprazole adopts a pose that is relatively parallel to the membrane, and in close vicinity 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 Ser197$^{5.46}$A mutation disrupts the local conformation of TM5 and results in 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 D_2R OBS is in agreement with our mutagenesis results.

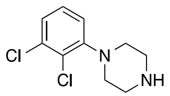
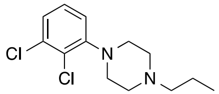
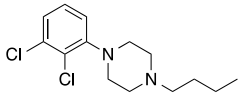
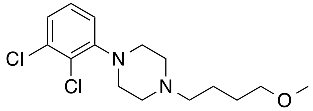
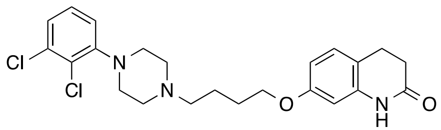
For the SP and the flexible butoxy linker, however, we found that our simulations from different starting poses and multiple trajectories converged to two distinct poses in the EV, depending on the orientation of the highly conserved Trp384$^{7.40}$. When Trp384$^{7.40}$ faces lipid as in the D_3R structure, the quinoline ring occupies a cavity at the interface between 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 sidechains 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 the Trp384$^{7.40}$ of the D_3R faces lipid, that of the D_2R-structure is in an intermediate position and in our simulations, we observed that this residue can adopt both the inward and outward orientation[7,10].

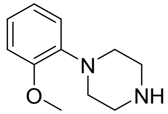
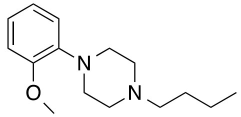
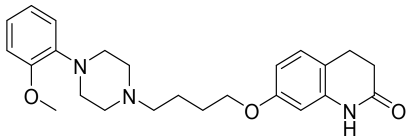
The SP of aripiprazole confers an increase in efficacy

To explore how the interaction of the SP of aripiprazole with the D_2R 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**) to the PP 2,3-dichloropheylpiperazine fragment (DCPP, **1**) conferred 32 to 115-fold increases in binding affinity (Figure 3, 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 the methoxybutyl substituted derivative (**4**). Fragments containing the SP were only able to displace the radioligand upon inclusion of an ionisable nitrogen atom within its structure (Supplementary Table 3). In functional studies, this time using a BRET biosensor to measure cAMP levels, the incorporation of alkyl or alkoxy spacers conferred up to 17-fold increase in functional affinity as compared to DCPP, although a further increase in functional affinity was not observed with the incorporation of the THQ moiety. The DCPP fragment of aripiprazole displayed 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 similar level of efficacy as DCPP (Figure 3, 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 DCPP core of R22 could be replaced with a 2-methoxyphenylpiperazine (2MeOPP) core with little change in efficacy or affinity at the D_2R[18]. We hypothesized that addition of the 7-butoxy-1,2,3,4-tetrahydroquinolin-2-one substituent of aripiprazole to the 2MeOPP 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 2MeOPP core to yield an extended ligand with the same affinity as aripiprazole (Figure 2, Table 2). Importantly, we observed that the addition of the 7-butoxy-1,2,3,4-tetrahydroquinolin-2-one substituent caused a 26-fold and 10-fold increase in efficacy (τ) as compared to the 2MeOPP (**11**) and the N-butyl substituent (**12**) respectively (Figure 3, Table 2). Thus, the linking of the 7-butoxy-1,2,3,4-tetrahydroquinolin-2-one SP to the 2MeOPP PP to generate a novel partial agonist results in both increases in efficacy and affinity.

Table 2. Binding affinities and functional action of phenylpiperazine fragments and extended compounds at the D_2R.

	pK_i ± SEM [3H]spiperone			pK_i ± SEM [3H]raclopride		cAMP (BRET Biosensor)	
Compound	**WT**	**L41$^{1.39}$A (fold)**	**E95$^{2.65}$A (fold)**	**WT**	**V91$^{2.61}$A (fold)**	**pK_A**	**Logτ**
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#
Aripiprazole 	9.11 ± 0.12	-	-	-	-	7.41 ± 0.30	1.02 ± 0.24
11	5.64 ± 0.07*	6.23 ± 0.15* (0.4)	6.12 ± 0.13* (0.3)	6.49 ± 0.19	6.43 ± 0.24 (1.1)	6.51± 0.42*	-0.42 ± 0.11*

							
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)	8.56 ± 0.26	1.00 ± 0.22

Binding affinity (K_i) determined by competition binding experiments using radiolabelled antagonist [^{3}H]spiperone or [^{3}H]raclopride at WT or mutant SNAP-D_{2S}R. Functional affinity (K_A) and efficacy (τ) determined in an assay measuring inhibition of forskolin-stimulated cAMP production. Values are expressed as mean ± S.D. from three separate experiments. *Values significantly different compared to WT as determined by one-way ANOVA (Dunnett's post hoc test, $p < 0.05$). #Values significantly different from aripiprazole as determined by one-way ANOVA (Dunnett's post-hoc test) ($p < 0.05$).

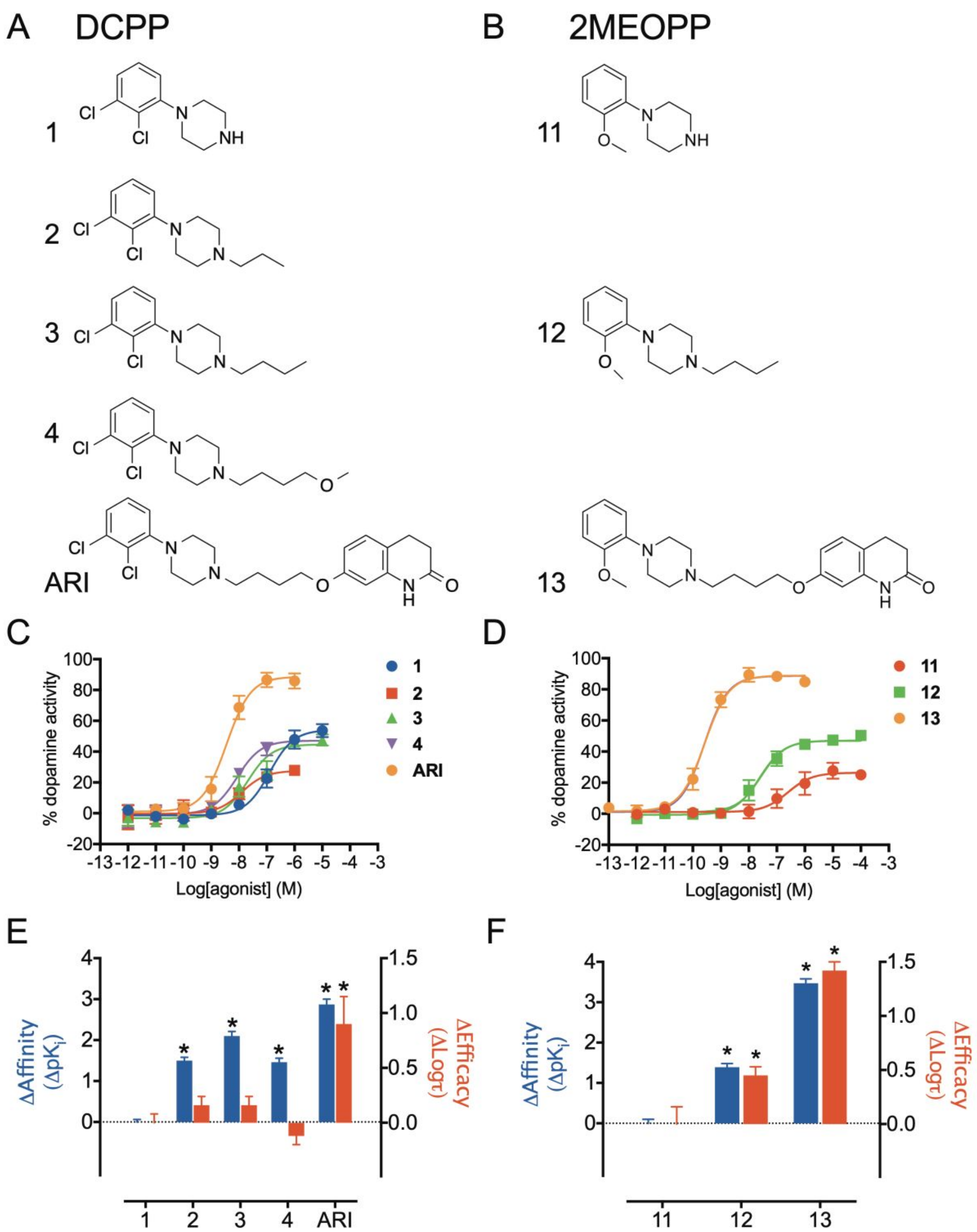


Figure 3: The 7-butoxy-1,2,3,4-tetrahydroquinolin-2-one substitution of a phenylpiperazine core confers an increase in efficacy and affinity.

Two series of substituted phenylpiperazine fragments and extended compounds were synthesized, one that incorporates the 2,3-dichloropheylpiperazine (**A**, DCPP) core that includes aripiprazole, and one that incorporates the 2-methoxyphenylpiperazine core (2MeOPP, **B**). The ability of increasing concentrations of each compound in the DCPP series (**C**, **E**) or the 2MeOPP series (**D**, **F**) to activate the WT $D_{2S}Rs$ was determined through an assay measuring the inhibition of forskolin-stimulated cAMP production using a BRET biosensor.

These data were fit to an operational model of agonism (**E**, **F**) and changes in functional affinity or efficacy were determined as compared to the phenylpiperazine core of each series. *significant change in parameter as compared to that of the core of each series (one-way ANOVA with Dunnett's post hoc test, $P < 0.05$).

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). V91$^{2.61}$A caused a 11-fold reduction in the transduction coefficient of aripiprazole, whereas E95$^{2.65}$A resulted in a 11-fold reduction of its functional affinity (K_A, Figure 1, Table 3). While V91$^{2.61}$A and E95$^{2.65}$A had no effect on the two smaller agonists, W90$^{2.60}$A reduced the transduction coefficients of ropinirole (14-fold) and dopamine (6-fold), and the binding affinity of dopamine (6-fold). The mutations E95$^{2.65}$A, V91$^{2.61}$A and L41$^{1.39}$A did not change the affinity of the DCPP fragment (Table 2). F110$^{3.28}$A significantly reduced the binding affinity of all three agonists, and the transduction coefficients of dopamine and ropinirole but not aripiprazole (Table 3). The mutation L41$^{1.39}$A increased the binding affinity of ropinirole and aripiprazole (5-fold) but had no significant effect on the binding affinity of dopamine (Figure 1, 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 V91$^{2.61}$A or F110$^{3.28}$A 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, 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, Supplementary Figure 1). Thus, our simulations indicate that the orientation of the SP towards ECL2 and TM3 is favoured in the L41$^{1.39}$A mutant.

In order to make comparisons 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-fold and 5-fold decreases in transduction coefficients at the L41$^{1.39}$A mutant, respectively (Figure 4, 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 Alphascreen cAMP assay. Of note, the efficacy of aripiprazole at this mutant was equivalent to that of the DCPP fragment at the WT receptor suggesting that the efficacy gain 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 D_2Rs prior to stimulation with agonist. We applied the operational model of agonism to these data to determine the functional affinity and efficacy of dopamine and ropinirole (Figure 4, Supplementary Table 4). The mutation L41$^{1.39}$A caused a 10-fold decrease in dopamine functional affinity (K_A) but no change in efficacy (τ) (Figure 4H & K, Supplementary Table 4). The functional affinity and efficacy of ropinirole was unaffected by this mutation (Figure 4I & L, Supplementary Table 4).

Table 3. The effect of mutations in the SBP of the D_2R on the binding affinities and functional activity of selected agonists.

Construct	Ropinirole		Dopamine		Aripiprazole			
	pK_i (fold Δ)	$Log(\tau/K_A)$ (fold Δ)	pK_i (fold Δ)	$Log(\tau/K_A)$ (fold Δ)	pK_i (fold Δ)	$Log(\tau/K_A)$ (fold Δ)	pK_A (fold Δ)	$Log\tau$ (fold Δ)
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* (14)	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)

Binding affinity values were obtained in competition binding experiments using the radioligand [^{3}H]spiperone unless otherwise stated. Values of functional affinity, efficacy 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 [^{3}H]raclopride. Values are expressed as mean ± S.D. from three (binding) or four (cAMP) separate experiments. ND = no specific binding or agonist activity could be determined. *P<0.05, significantly different from the wild-type receptor determined by a one-way ANOVA, Dunnett post-hoc test.

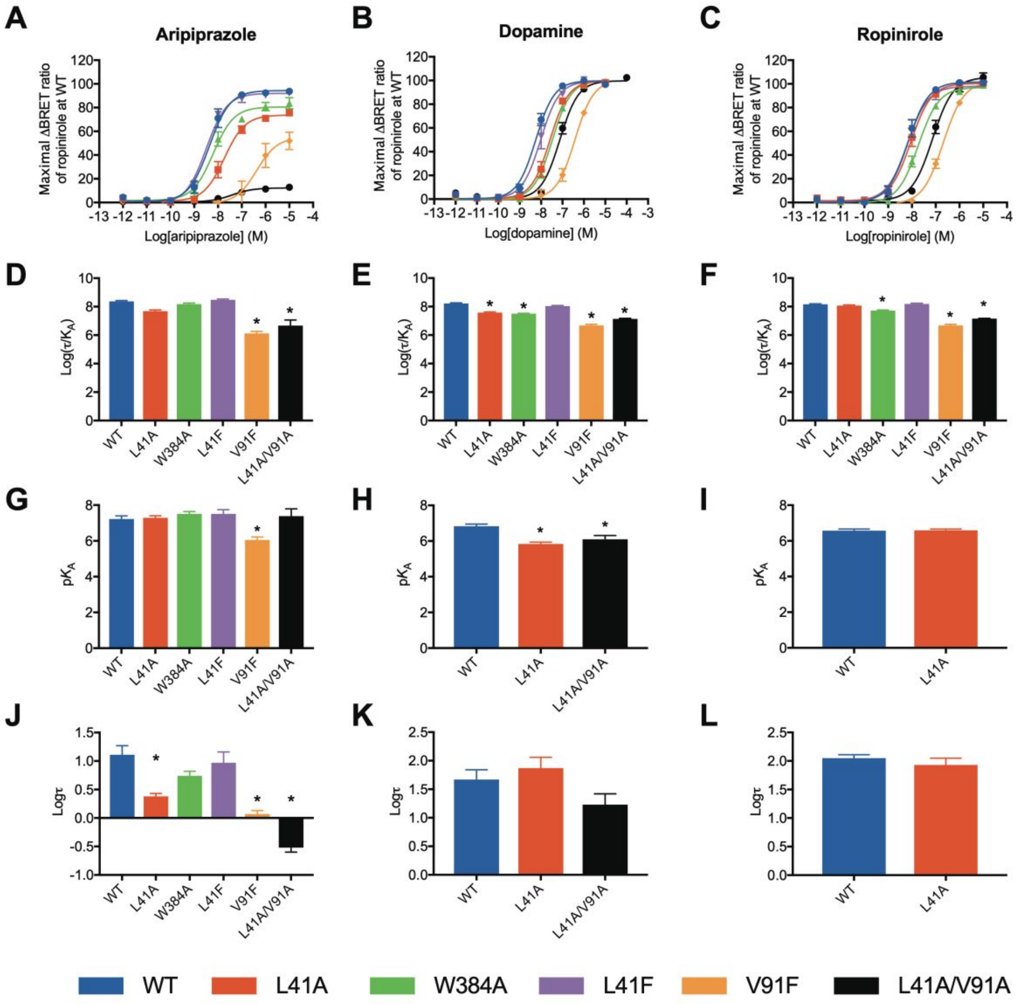


Figure 4: Mutation of SBP residues decrease the efficacy of aripiprazole but not dopamine

The ability of increasing concentrations of aripiprazole (**A**), dopamine (**B**) or ropinirole (**C**) 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 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 in transduction coefficient (**D-F**), and the functional affinity (**G-I**) and efficacy (**J-L**) at the WT and mutant receptors. *significant changes in parameter for each agonist relative to WT (one-way ANOVA with Dunnet's post-hoc test, $P < 0.05$).

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 cell surface expression (Supplementary Figure 3). W384$^{7.40}$A caused significant decreases in dopamine (5-fold) and ropinirole (3-fold) transduction coefficients but had no effect on aripiprazole (Figure 4, 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 mutation of W384$^{7.40}$A 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-fold, 30-fold and 170-fold decreases in the transduction coefficients of dopamine, ropinirole and aripiprazole respectively (Figure 4F, Supplementary Table 4). This mutation caused both a decrease 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 (L41$^{1.39}$A/V91$^{2.61}$A) mutant caused a 10-fold decrease in the transduction coefficient of dopamine and ropinirole but a much greater 49-fold decrease for aripiprazole, driven by a 42-fold decrease in efficacy (τ, Figure 4I, Supplementary Table 4). In contrast this double mutation decreased the functional affinity (K_A, Figure 4H, 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 D_2R 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 signalling 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 efficacy increase gained through 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$^{2.61}$ and Glu95$^{2.65}$) and one in which the SP extends towards TM3. Our simulations show that L41$^{1.39}$A promotes the latter orientation (Figure 2). The mutation of V91$^{2.61}$ and E95$^{2.65}$ also caused significant losses of aripiprazole's affinity and functional effect, consistent with the loss of SBP interactions. We propose that the interaction of the SP with the

SBP promotes higher intrinsic efficacy whereas the orientation of the SP towards TM3 appears to be associated with lower efficacy but higher binding affinity. The combination of V91$^{2.61}$A with L41$^{1.39}$A, which we postulate would further promote the orientation of the SP towards 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 M_2 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 intrinsic efficacy. In this present study we find no evidence that aripiprazole can bind the D_2R 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 D_2R 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 present study this illustrates that the interaction of SP of extended ligands with the SBP of the D_2R can confer changes in pharmacology relative to that 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, L41$^{1.39}$A/V91$^{2.61}$A), the effect on aripiprazole was much greater. It should be noted, however, while L41$^{1.39}$A or the double mutation L41$^{1.39}$A/V91$^{2.61}$A did not affect the efficacy of dopamine or ropinirole, they caused a decrease in the functional affinity of dopamine. Functional affinity presumably reflects the affinity of dopamine for the receptor when coupled to signalling effectors[36]. In contrast, the binding affinity of dopamine, which was unchanged relative to 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. Further, 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 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 interaction patterns 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 D_2R 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 to those forming the D_2R SBP[38,39]. A SBP defined by extracellular TMs 1, 2 and 7 residues, 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 modulation of agonist action in other GPCRs.

The biased agonism of aripiprazole is unchanged at OBS or SBP mutants

Previously, we have shown that aripiprazole displays biased agonism towards 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 D_2R, corresponding to a value of efficacy (τ) of 0.39, 12-fold lower than that observed in the cAMP assay (Figure 5, 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 towards 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 to detect the deleterious effects of a mutation is smaller in the pERK1/2 assay because of the lower efficacy (τ) of aripiprazole at the WT D_2R 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 D_2R (E_{max} = 86% of maximal response of ropinirole, Figure 5, Supplementary Table 7). Aripiprazole displayed a significant 6-fold decrease in efficacy (τ) at the L41$^{1.39}$A mutant as compared to the 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 D_2R (Figure 5, Supplementary Table 7).

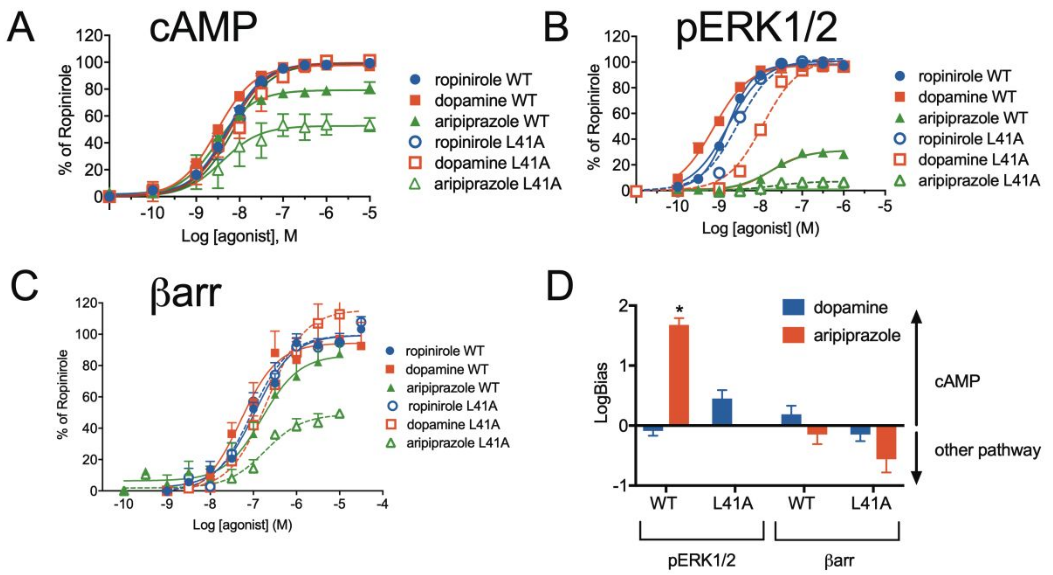


Figure 5: The mutation L41$^{1.39}$A decreases the intrinsic efficacy of aripiprazole at multiple signalling pathways.

The ability of increasing concentrations of each agonist to activate the WT or L41$^{1.39}$A D_{2S}Rs was determined through an Alphascreen^{TM} assay measuring the inhibition of forskolin-stimulated cAMP production (**A**), ERK1/2 phosphorylation (**B**) and β arrestin translocation (**C**). **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. *significant bias towards one pathway (two-tailed, un-paired Students t-test, $P < 0.05$).

Conclusions

The weak intrinsic efficacy of D_2R partial agonists such as aripiprazole is thought to determine both their antipsychotic effect and low propensity to cause extrapyramidal side effects and hyperprolactinaemia as compared to typical antipsychotics. Our results reveal the molecular interactions important for this intrinsic efficacy. Aripiprazole's structure is typical of many D_2-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 TMs1, 2 and 7[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], provides a means to design D_2R partial agonists with 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 >98% pure as described by the supplier. All novel compounds were synthesized as described in the supplementary methods. The pcDNA3L-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, VIC, Australia). [^{3}H]Spiperone, [^{3}H]Raclopride, AlphaScreen 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, NSW, Australia).

Molecular Biology and Generation of Cell Lines

Molecular biology and generation of cell lines were performed as described previously[43]. Full details are given in the supplementary methods. 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-Ce`ze, France).

ELISA and Cell signalling assays:

The ELISA protocol, ERK1/2 Phosphorylation Assay, cAMP Alphascreen^{TM} Assay and Bioluminescence Resonance Energy Transfer (BRET) assays measuring intracellular cAMP and β-arrestin-2 recruitment to the plasma membrane assay were performed as described previously[43,48]. Full details are given in the supplementary methods.

Membrane Preparation and radioligand binding assays.

Radioligand binding assays were performed as described previously[43]. Full details are given in the supplementary methods.

Data analysis

The results were analysed using Prism 6.0 (GraphPad Software Inc., San Diego, CA). full details of data analysis are given in the supplementary methods. 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, since the application of t tests and analyses of variance assume Gaussian distribution, estimating the parameters as logarithms allows valid statistical comparison. All results are expressed as the mean ± S.D. We performed a Brown-Forsythe test (Graphpad prism 6) to assure ourselves of equal variance when such parameters are compared.

MD simulations

Full details of the protocol are provided in supplementary methods.

Supporting Information

Supplementary Methods including chemical synthesis, Supplementary Figures 1-3, Supplementary Tables 1-7, Supplementary References. This material is available free of charge *via* the internet at http://pubs.acs.org.

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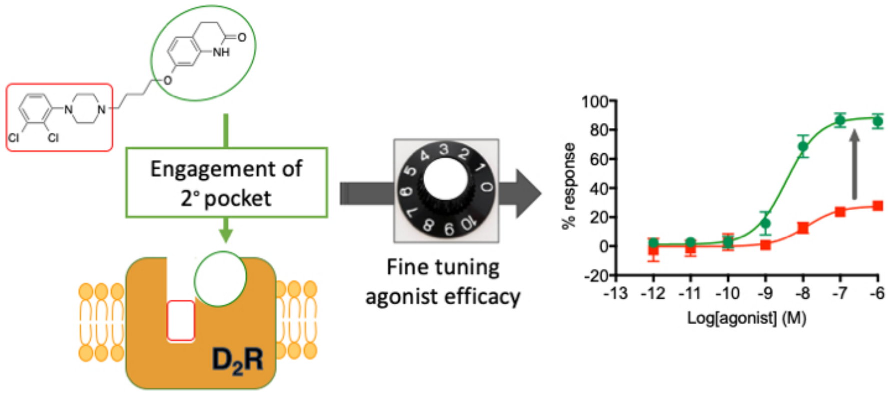

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