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**CRITICAL SIGNALING AND LIGAND INTERACTION MECHANISMS OF
THE DOPAMINE D₁ RECEPTOR:
INSIGHT INTO EFFECTIVE PHARMACOTHERAPY**

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by
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ABSTRACT

Dopamine D₁ receptor full agonists have been efficacious in Parkinson's disease (PD) animal models and PD patients. SKF-83959 is reported to be a functionally selective dopamine D₁ receptor ligand with high bias for D₁-mediated phospholipase C (PLC) versus D₁-coupled adenylate cyclase (AC) signaling. The signaling bias of SKF-83959 is commonly accepted and proposed to explain D₁-mediated behavioral activity in PD animal models, but there is substantial (although not all unanimous) literature that failed to account for SKF-83959-mediated PLC activation. Thus, we decided to conduct an in-depth pharmacological characterization of SKF-83959. Contrary to common assumptions, SKF-83959 is a partial agonist (not an antagonist) at AC *in vitro* and *ex vivo*. In addition, it shows partial agonistic activity for β -arrestin activation. SKF-83959 failed to show D₁-mediated PLC signaling in a cellular expression system. We conclude that SKF-83959 is not a highly-biased functionally selective D₁ ligand, and that its reported behavioral effects can be explained solely by its partial D₁ agonism for canonical signaling pathway(s).

Current dopamine D₁ receptor full agonists have poor pharmacokinetic properties due to their intrinsic catechol moiety, and it is important to determine how novel non-catechol D₁ ligands might be designed. To provide a scientific platform for structure-based drug design, we investigated the molecular interactions of the D₁ receptor with several ergolines that have significant D₁ activity and oral bioavailability, but not a catechol moiety. I focused on the conserved amino acids of the D₁ receptor (T3.37, S5.42, S5.43, S5.46, F6.51, and F6.52) that are known to play a critical role in ligand interactions and/or receptor activation. Mutations to alanine (T3.37A, S5.42A, S5.43A, S5.46A, F6.51A, and F6.52A) on the D₁ receptor were basically used to examine the role of the conserved amino acids in ligand interactions.

A T3.37A mutation greatly decreased the D₁ affinity and efficacy of the ergolines. However, a hydrogen bond-conservative T3.37S mutation markedly restored the loss of D₁ affinity and efficacy suggesting the possible role of a hydrogen bond provided by

T3.37. Unexpectedly, a S5.42A mutation increased the D₁ affinity and efficacy for D₁-mediated AC activation suggesting that this mutation may induce a favorable D₁ receptor conformation for the ergolines. Although a S5.43A mutation failed to decrease the affinity of the ergolines consistently, a S5.46A mutation significantly decreased the affinity of the ergolines but to a small degree. Both the S5.43A and S5.46A mutations showed no significant effects on D₁ efficacy of the ergolines. S5.42A/S5.46A and S5.43A/S5.46A double mutations elicited equal or greater effects than those of the single mutations. An F6.51A mutation dramatically decreased the D₁ affinity of the ergolines, and an F6.52A mutation showed smaller, but significant decreases than the F6.51A mutation. The F6.51A mutation greatly decreased the ergoline efficacy for AC activation, but an aromatic-ring conserved F6.51W mutation markedly restored the D₁ affinity and efficacy. This suggests the critical role of the hydrophobic and aromatic interactions provided by F6.51. Docking simulations illustrated that B-ring nitrogen of the ergoline agonists is located close to T3.37 and S5.46. In addition, the B-ring and the D-ring of the ergoline backbone are located close to F6.52 and F6.51, respectively.

Rotigotine is another non-catechol drug that has reasonable D₁ receptor efficacy. S5.42A and S5.43A mutations greatly decreased the D₁ affinity and efficacy of rotigotine, whereas a S5.46A mutation failed to make changes suggesting that S5.42 and S5.43 may provide hydrogen bonds for rotigotine. An F6.51A mutation decreased the D₁ affinity and efficacy of rotigotine to a far greater extent than an F6.52A mutation indicating that hydrophobic and aromatic interactions of F6.51 are particularly important. Mutagenesis results with 5-OH DPAT and 7-OH DPAT also supported the interaction between the thiophene group of rotigotine and F6.51. The simulations showed that the hydroxyl group is located close to S5.42 and S5.43 and that the thiophene group interacts closely with F6.51.

In conclusion, we report that PLC activation by SKF-83959 is not a D₁-mediated response, and that it is highly likely non-specific effects occurring at supra-pharmacological concentrations. Using AC activation (not PLC activation) as a functional end-point of D₁ receptor signaling, we investigated the molecular interactions

between the D₁ receptor and non-catechol ligands (the ergolines and rotigotine). This study provides molecular mechanisms for the critical signaling and ligand interactions of the D₁ receptor that may help design novel non-catechol D₁ agonists.

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List of Abbreviations

5-OH DPAT	5-hydroxy-N,N-dipropyl-2-aminotetralin
7-OH DPAT	7-hydroxy-N,N-dipropyl-2-aminotetralin
AC	adenylate cyclase
B _{max}	maximum binding
CNS	central nervous system
D ₁	dopamine D ₁ receptor
DA	dopamine
desmethylSKF	6-chloro-7,8-hydroxy-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine
DHX	dihydroxidine; (trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydro-benzo[<i>a</i>]phenanthridine)
EC ₅₀	half maximal effective concentration
ERK	extracellular-signal-regulated kinase
GPCR	G protein-coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	3-isobutyl-1-methylxanthine
IC ₅₀	half maximal inhibitory concentration
IP1	Inositol monophosphate
IP3	Inositol triphosphate
K _{0.5}	apparent affinity constant
K _{0.5}	Apparent affinity constant
K _A	acid dissociation constant
K _D	dissociation constant
NMS	N-methylspiperone
PD	Parkinson's disease
PLC	Phospholipase C
SKF-83959	6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine
TM3	GPCR transmembrane three segment
TM5	GPCR transmembrane five segment
TM6	GPCR transmembrane six segment

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Chapter 1: Introduction to the dopamine D₁ receptor

Preface

The physiological actions of the neurotransmitter dopamine are mediated by a family of receptors that belong to the Class A G protein-coupled receptor (GPCR) superfamily. Dopamine receptors have been promising targets for several brain disorders, and there are clinically available dopamine receptor ligands for conditions such as Parkinson's disease, schizophrenia, and restless leg syndromes among other conditions. I will offer a brief overview focusing on D₁ dopamine receptor signaling to show how D₁ activation may translate to clinical benefits. This chapter will also explain the way that my research developed, in some cases unexpectedly. Because later chapters will deal with molecular interactions for the ligand-receptor complex, I will also provide brief information on the molecular forces that can work for dopamine receptors and their ligands.

Dopamine D₁ receptor signaling

A brief history of distinct dopamine receptor signaling

The first evidence for dopamine receptor signaling was the report on the increase in cyclic AMP (cAMP) and the activation of adenylate cyclase by dopamine in bovine cervical ganglion (Kebabian and Greengard, 1971). Dopaminergic drugs (e.g., amphetamine and apomorphine) induced psychotic behaviors, and hyperactive dopaminergic signaling is suggested to cause positive symptoms of schizophrenia. Clinical potency of antischizophrenic drugs was reported to correlate with their ability to bind to dopamine receptors (Creese et al., 1976; Seeman et al., 1975). Sulpride that was marketed then as a new antipsychotic medicine presumably working as a dopamine receptor antagonist actually failed to inhibit dopamine-mediated adenylate cyclase activity in rat striatum (Trabucchi et al., 1975). This led to the hypothesis of two different kinds of dopamine receptors; one stimulates adenylate cyclase, but the other has no effect on adenylate cyclase (Garau et al., 1978). Based on these reports, two types of dopamine receptors were first designated as D-1 and D-2 receptors (Kebabian and Calne, 1979). In the early 1990's, five distinct genes encoding dopamine receptors were identified in humans using molecular cloning (Dearry et al., 1990; Monsma, Jr. et al., 1989; Sokoloff et al., 1990; Sunahara et al., 1991; van Tol et al., 1991; Zhou et al., 1990). All five genes are G protein coupled receptors, and they are divided into D₁-like family (D₁ and D₅) and D₂-like family (D₂, D₃, and D₄). D₁-like receptors stimulate adenylate cyclase and produce cAMP primarily through G $\alpha_{OLF/S}$ activation, whereas D₂-like receptors often coupled with G $\alpha_{I/O}$ inhibit adenylate cyclase activity and cAMP production. Although adenylate cyclase activation has been accepted as the canonical signaling of the D₁ receptor, the D₁ receptor is also known to trigger other signaling pathways.

D₁ receptor-mediated cAMP/protein kinase A signaling

In striatum, where dopamine is deficient in Parkinson's disease patients, G α_{OLF} is highly expressed, but G α_S is less abundant; the coupling of the D₁ receptor to adenylate cyclase in striatum is primarily mediated by G α_{OLF} (Herve et al., 1993). G α_{OLF} knock-out

mice reduced dopamine-stimulated cAMP production in the striatum, and they lack the behavioral responses to the D₁ receptor stimulation supporting the essential role of G α_{OLF} in D₁ receptor signaling (Zhuang et al., 2000; Corvol et al., 2001). Although the roles of G protein $\beta\gamma$ subunit have not been extensively reported, the suppression of endogenous γ_7 subunit in HEK-293 cells is known to greatly diminish D₁-mediated adenylate cyclase activity (Wang et al., 2001). Both D₁ receptor and γ_7 subunit are selectively expressed in the striatum suggesting the potential interaction of γ_7 subunit with the D₁ receptor (Watson et al., 1994). Dopamine sensitive-adenylate cyclase is highly concentrated in striatum and named as type 5 adenylate cyclase (Glatt and Snyder, 1993). Genetic ablation of the adenylate cyclase type 5 eliminated adenylate cyclase activity in striatum stimulated by D₁ agonists (Lee et al., 2002). In addition, adenylate cyclase type 5 null-mice exhibited Parkinsonian-like motor dysfunction suggesting that adenylate cyclase type 5 is involved in motor symptoms of Parkinson's disease (Iwamoto et al., 2003). Striatal cAMP, produced by adenylate cyclase type 5, binds to the regulatory subunits of protein kinase A (PKA) that phosphorylates several proteins (e.g., dopamine and cAMP-regulated 32kDa phosphoprotein DARPP-32 and cAMP response element-binding protein CREB). Although these down-stream molecules are involved in the regulation of gene expression (Neve et al., 2004), how the gene regulation contributes to D₁-mediated behavioral effects is still unclear.

Mitogen-activated protein (MAP) kinase activation via the D₁ receptor

MAP kinases play critical roles in cell proliferation and apoptotic cell death as the downstream mediators of signal transduction from the cell surface receptors. Three kinds of MAP kinases have been identified: extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK). Although D₁-mediated p38 and JNK activation has not been reported in striatum, ERK phosphorylation is known to be triggered by D₁ receptor stimulation in striatal neurons (Brami-Cherrier et al., 2002). A selective D₁ receptor agonist SKF38393 also increased ERK1/2 phosphorylation in the prefrontal cortex as well as in cortical neurons in mice (Nagai et al., 2007). However, there are conflicting reports on D₁-mediated ERK activation in SK-N-MC neuroblastoma

cells, which endogenously express D₁ receptors. Chen et al. (2004) showed that the D₁ selective agonist SKF38393 increased ERK phosphorylation, whereas Zhen et al. (1998) failed to detect ERK phosphorylation by the same D₁ agonist. Although the mechanisms for D₁-mediated ERK activation remain to be fully elucidated, β -arrestin2 is suggested to act as a scaffold protein for ERK activation. Coimmunoprecipitation results showed the heterotrimeric complexes of the D₁ receptor, ERK, and β -arrestin2 (Chen et al., 2004). D₁ receptor knock-out mice blunted the formation of the β -arrestin2 and ERK complexes suggesting the existence of the heterotrimer (Urs et al., 2011). Nevertheless, D₁-mediated ERK phosphorylation in striatum may be dependent on PKA because co-treatment with a selective PKA inhibitor Rp-cAMP eliminated the ERK phosphorylation (Brami-Cherrier et al., 2002). Recent reports also showed the involvement of a PKA substrate DARPP-32 in D₁-mediated ERK activation (Santini et al., 2007; Santini et al., 2012). cAMP is reported to directly activate a Rap-specific guanine nucleotide-exchange factor EPAC that activates Rap GTPase and promotes MAP kinase cascade (de Rooij et al., 1998; Weissman et al., 2004). Together, these reports suggest that D₁-mediated cAMP/PKA signaling can regulate ERK activation. Because ERK activation correlates with levodopa-induced dyskinesia in the PD animal model, and because blocking ERK activation significantly decreases it (Santini et al., 2007), extensive future studies should be carried out to clearly understand the roles of D₁-mediated ERK activation in PD symptoms.

Regulation of Ca²⁺, K⁺, and Na⁺ channels by the D₁ receptor

D₁ receptors have been involved in the regulation of ion channels in striatal and prefrontal cortical neurons. Voltage-dependent Ca²⁺ channels (L-, N-, and P/Q-type) play a critical role in balancing the intracellular Ca²⁺ concentration that is a key parameter for neurotransmitter release and synaptic plasticity (Cull-Candy et al., 2006; Luebke et al., 1993; Regehr and Mintz, 1994; Takahashi and Momiyama, 1993). Activation of the D₁ receptor is known to enhance L-type, but decrease N- and P-type Ca²⁺ channel conductance in rat striatum (Surmeier et al., 1995). By acting on L-type Ca²⁺ current, D₁ receptors are reported to regulate the firing of striatal neurons depending on the level of membrane depolarization (Hernandez-Lopez et al., 1997). In addition, decreasing N- and

P-type Ca^{2+} current is reported to inhibit spike-induced Ca^{2+} influx and attenuate neurotransmitter release (Takahashi and Momiyama, 1993). A report with prefrontal cortex supports the D_1 regulation on Ca^{2+} channels showing evidence that D_1 receptors physically interact with N-type Ca^{2+} channel and inhibit Ca^{2+} current (Kisilevsky et al., 2008). In addition, the cAMP/PKA/DARPP-32 signaling cascade appears to mediate these effects on Ca^{2+} channels (Surmeier et al., 1995). Nonetheless, how D_1 -mediated Ca^{2+} current regulates neuronal activity and develops subsequent behavioral effects still remains elusive.

A study on striatal K^+ channel showed that D_1/D_2 receptor coactivation increased spike firing of striatal neurons through the inhibition of voltage-gated K^+ current (I_A) (Hopf et al., 2003). In addition, selective D_1 receptor activation is known to suppress K^+ current in prefrontal cortex by inhibiting several K^+ channels (Dong and White, 2003; Dong et al., 2004; Gorelova et al., 2002; Yang and Seamans, 1996). Although the precise D_1 signaling mechanism for K^+ channel regulation remains unclear, studies have raised possible mechanisms: the direct interaction of cAMP with K^+ channels (Dong et al., 2004) and the involvement of D_1 -mediated cAMP/PKA signaling (Dong and White, 2003). By inhibiting several K^+ channels, D_1 receptor agonists may contribute to the neuronal excitability in prefrontal cortex and striatum.

For Na^+ channels, D_1 receptor activation is reported to inhibit Na^+ current in striatum (Calabresi et al., 1987; Surmeier et al., 1992). Na^+ current inhibition induces the depression of striatal neuronal excitability, and PKA signaling was reported to be involved in this effect (Schiffmann et al., 1995). A subsequent report by Schiffmann et al. (1998) suggested a critical role of phosphorylated DARPP-32 in the inhibition of Na^+ current. A report with hippocampal neurons also suggests that D_1 -stimulated PKA activation mediates the inhibition of hippocampal Na^+ current, and that the phosphorylation of Ser573 of the Na^+ channel α subunit is critical for D_1 -mediated Na^+ current inhibition (Cantrell et al., 1997).

Overall, the D_1 receptor can modulate neuronal excitability by acting on multiple ion channels in striatum and prefrontal cortex. Although canonical cAMP/PKA signaling

seems to be involved in these effects, the mechanisms for how ion currents contribute to *in vivo* effects are still elusive and remain to be fully investigated for future studies.

Phospholipase C activation as purported D₁ receptor signaling

Studies with adenylyl cyclase type 5 null mice showed that the null mice exhibited Parkinsonian-like motor dysfunction with a great loss of adenylyl cyclase activity in striatum (Iwamoto et al., 2003; Lee et al., 2002). Interestingly, D₁ receptor-mediated motor functions were preserved in the null mice. Genetic disruption of adenylyl cyclase type 5 induced compensatory increases in other adenylyl cyclase expression and significant decreases in D₁ receptor and Gα_s expressions (Iwamoto et al., 2003). Although these compensatory changes may complicate the interpretation of the behaviors of the null mice, the involvement of other signaling was suggested for the D₁-mediated motor behaviors. Such one non-cyclase signaling for the D₁ receptor is phospholipase C (PLC) activation and subsequent Ca²⁺ elevation (Mahan et al., 1990; Undie and Friedman, 1990; Undie et al., 1994). A D₁ receptor agonist SKF38393 increased Gα_q binding to the D₁ receptor in striatum suggesting the possible role of Gα_q in D₁-mediated PLC activation (Wang et al., 1995). Calcyon as a D₁ receptor interacting protein was once suggested to mediate Gα_q-mediated intracellular Ca²⁺ release, but the report was retracted in 2006 by the authors because the main results were not reproduced (Lezcano et al., 2000; Lezcano et al., 2006). Nonetheless, a behaviorally active D₁ receptor ligand SKF-83959 that has no efficacy for adenylyl cyclase, but full efficacy for PLC activation has supported the role of D₁-mediated PLC signaling in motor function (Jin et al., 2003; Zhen et al., 2005).

Contrary to the earlier reports, the D₁ involvement in PLC signaling still remains as the subject of controversy. In addition, we found that SKF-83959 showed D₁-mediated adenylyl cyclase activation with over 35% intrinsic activity in several D₁ receptor expression systems and *in vivo* striatum and that SKF-83959 failed to stimulate D₁-mediated PLC activation (Lee et al., 2014). If SKF-83959 is a typical partial agonist for adenylyl cyclase activation, its behavioral effects can be explained by the known cyclase

signaling. Recent papers have shown that SKF-83959 stimulates PLC activation via D₁-D₂ heterodimers (Hasbi et al., 2009; Hasbi et al., 2011; Rashid et al., 2007a; Rashid et al., 2007b). However, the striatal formation of the D₁-D₂ heterodimers is also controversial because most D₁ and D₂ receptors are segregated in mice striatum (Bateup et al., 2008; Aubert et al., 2000; Gerfen et al., 1990; Thibault et al., 2013; Bertran-Gonzalez et al., 2010; Le Moine and Bloch, 1995). These lines of evidence strongly suggest that if D₁-D₂ heterodimers exist, PLC activation triggered by them would be minor and limited compared to adenylate cyclase activation. Because at micromolar concentrations SKF-83959 can bind to several GPCRs that directly mediate PLC activation (Chun et al., 2013), PLC activation by SKF-83959 is highly likely non-specific effects. Chapter 2 and 6 will deal with this matter in detail.

Potential utility of D₁ receptor functional selectivity

Functional selectivity has been used to demonstrate the different activation of independent signaling pathways mediated by one single receptor. Functionally selective ligands can act as a full agonist for one signaling pathway, but it can act as a total antagonist for the other (Mailman, 2007; Urban et al., 2007). Dopamine D₁ receptor belongs to GPCR Class A, and it signals through multiple G proteins or G protein-independent mechanisms (e.g., β -arrestin signaling). It has been difficult to study functional selectivity of the D₁ receptor due to the lack of the independent signaling that mediates clinical effects. Nevertheless, a couple of studies examined the efficacy of D₁ agonists for adenylate cyclase activation and receptor internalization (Ryman-Rasmussen et al., 2005; Ryman-Rasmussen et al., 2007). Whereas full D₁ agonists for adenylate cyclase caused significant receptor internalization, partial D₁ agonists for adenylate cyclase failed to show receptor internalization (Ryman-Rasmussen et al., 2005). In addition, Ryman-Rasmussen et al. (2007) compared two structurally different D₁ agonists that have full activity for adenylate cyclase (an isochroman A-77636 and an isoquinoline dinapsoline). A-77636 showed receptor internalization greater than dinapsoline, and it caused the receptor to be retained intracellularly much longer than dinapsoline after agonist removal. These reports indicate that although they have full D₁ activity for

adenylate cyclase, the chemically distinct full D₁ agonists can produce different intracellular D₁ trafficking.

Although the concept of functional selectivity has provided novel opportunities for the design of more desirable D₁ agonists, more studies are required to clearly understand how independent D₁ signaling contributes to *in vivo* behavioral effects. In Parkinson's disease, levodopa-induced dyskinesia is known to correlate with ERK activation (Santini et al., 2007). Although D₁-mediated cAMP/PKA signaling may be involved in ERK activation (Brami-Cherrier et al., 2002), the mechanisms for how ERK is activated are still unclear. Theoretically, the D₁ agonist with full intrinsic activity for adenylylase and partial or no intrinsic activity for ERK activation may reduce dyskinesia in Parkinson's disease. β -arrestin has been known to mediate ERK activation, and it is suggested as one of G protein-independent D₁ signaling pathways. Studies on how β -arrestin mediates dyskinesia *in vivo* may help develop novel functionally selective D₁ ligands that may have less clinical side effects.

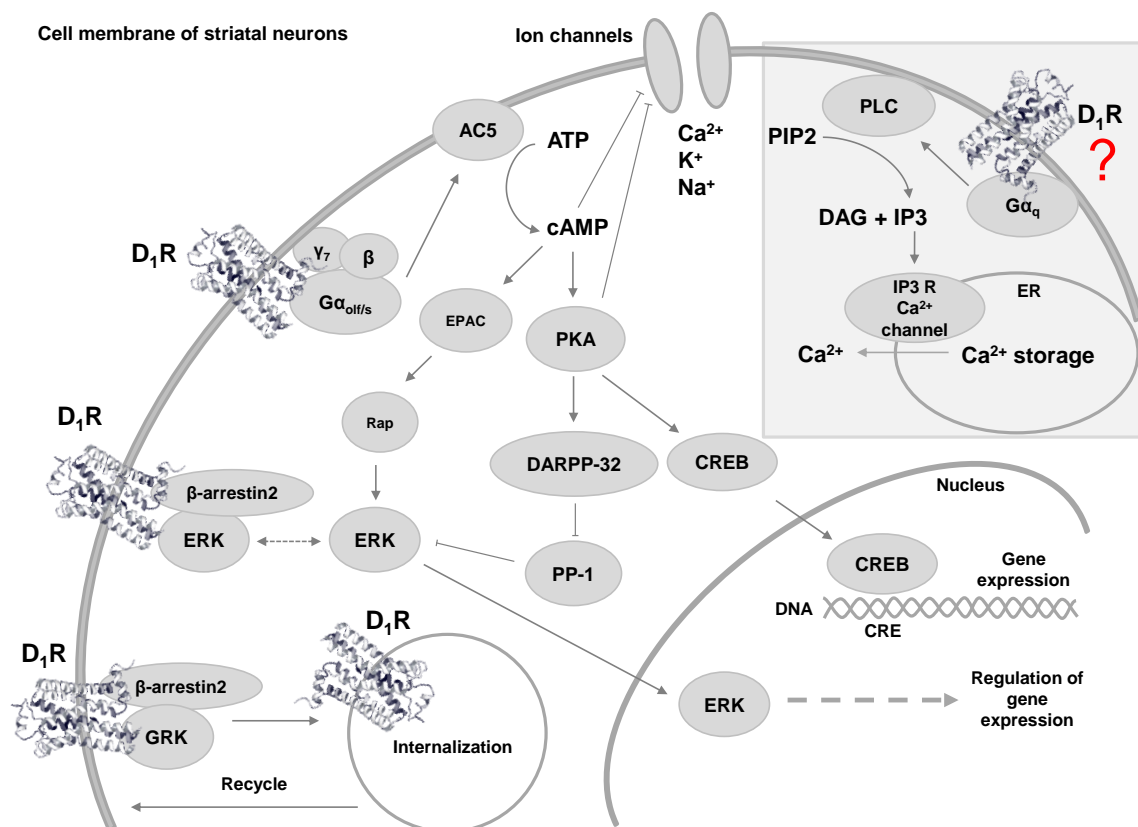


Figure 1-1. D₁ receptor signaling pathways in striatal neurons.

Activating effects are indicated with a solid line ending in an arrowhead, and inhibitory effects with a solid line ending in a bar. D₁ receptor activation inhibits Ca²⁺, K⁺, and Na⁺ currents via the ion channels mentioned in the present dissertation except the L-type Ca²⁺ channel. D₁-mediated PLC activation is still in controversy. D₁R, dopamine D₁ receptor; AC5, adenylate cyclase type 5; PKA, protein kinase A; DARPP-32, Dopamine and cAMP-related phosphoprotein 32KDa; PP-1, protein phosphatase 1; EPAC, Exchange protein activated by cAMP; Rap, a small GTPase; ERK, extracellular-signal-regulated kinase; GRK, G protein-coupled receptor kinase; CREB, cAMP response element-binding protein; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacyl glycerol; IP3, inositol 1,4,5-trisphosphate; IP3 R, IP3 receptor; ER, endoplasmic reticulum.

Molecular forces used for receptor-ligand interactions

There are four important intramolecular forces primarily working on receptor-ligand interactions: salt bridge interactions, hydrogen bonds, aromatic pi interactions, and hydrophobic interactions. I will briefly introduce these molecular interactions involved in biogenic amine GPCRs and their ligands.

Salt bridge interactions

Salt bridge interactions are an example of non-covalent interactions, and they are electrostatic interactions between two oppositely charged molecules (e.g., amino acids in proteins). The salt bridge often arises between the anionic carboxylate (RCOO^-) of aspartic acid or glutamic acid and the cationic ammonium (RNH_3^+) of lysine or the guanidinium ($\text{RNHC}(\text{NH}_2)_2^+$) of arginine. Ionizable side chains such as histidine, tyrosine, and serine can also participate depending on outside factors perturbing their pK_A . The distance between the residues participating in the salt bridge is also important, and the distance required is less than 4 Å (Kumar and Nussinov, 2002).

The conserved amino acids in biogenic amine GPCRs are known to interact with biogenic amine ligands such as dopamine, epinephrine, serotonin, and histamine. The amine part of these endogenous ligands is protonated in the physiological pH condition ($\text{pH } 7 \pm 2$), and the protonated amine is known to form salt bridge interactions with the receptors (Floresca and Schetz, 2004). The amino acid that is reported to interact with the protonated amine is the highly conserved aspartate D3.32 in transmembrane three segment (TM3) (Ballesteros et al., 2001). The Ballesteros and Weinstein nomenclature is used to indicate amino acids in a transmembrane segment (Ballesteros and Weinstein, 1995). The protonated amine has a positive charge, and aspartate D3.32 of the receptors has a negative charge within physiological pH levels. The salt bridge interactions are known as a major attraction force between biogenic amine receptors and their endogenous ligands.

The electrostatic interactions between the transmembrane segments are also known as a critical factor for receptor activation. The D(E)RY region in TM3 (aspartate D3.49 or glutamate E3.49, arginine R3.50, and tyrosine Y3.51) is highly conserved in biogenic amine receptors. Particularly, the positive charge of arginine R3.50 is reported to form an ionic lock with glutamate E6.30 in TM6 in the inactive β_2 -adrenergic receptor (Ballesteros et al., 2001). Mutations on these residues break this electrostatic interaction and increase the constitutive activity of the β_2 -adrenergic receptor (Rasmussen et al., 1999). This polar interaction maintains the inactive state of the receptor by bridging TM3

and TM6 segments and preventing TM6 outward movement. Together, the electrostatic salt bridge interactions play a major role in the activation of the biogenic amine GPCRs.

Hydrogen bonds

Hydrogen bonds are attractive interactions between polar molecules where a hydrogen atom shares electrons with a highly electronegative atom like oxygen and nitrogen. This is a particularly strong dipole-dipole attraction. A hydrogen atom attached to a relatively electronegative atom works as a hydrogen bond donor, and an electronegative atom such as oxygen or nitrogen works as a hydrogen bond acceptor. Hydrogen bonds can vary in strength depending on the atoms used for hydrogen bond donors or acceptors and the bond angle between them. Generally, a hydrogen bond is weaker than covalent or ionic bonds, but stronger than van der Waals interaction. In addition, a single hydrogen bond acceptor can take two hydrogen bond donors and form two hydrogen bonds at the same time. The “bifurcated” (split in two or 'two-forked') hydrogen bonds are used in natural molecules including reorientation of a water molecule (Laage and Hynes, 2006).

The hydrogen bond is another critical force for receptor-ligand interactions. For dopamine D₁ receptors, the catechol moiety of dopamine and all full D₁ agonists is known to form hydrogen bonds with conserved TM5 serines: S5.42, S5.43, and S5.46 (Chemel et al., 2012; Pollock et al., 1992). The hydroxyl groups of both the serines and the catechol moiety can act as hydrogen bond donors/acceptors. All of serine to alanine mutations that remove the hydroxyl group greatly reduced the D₁ affinity and activity of dopamine and catechol-type D₁ agonists (Chemel et al., 2012; Pollock et al., 1992). The hydrogen bonds between the hydroxyl groups are most common in receptor-ligand interactions. In the β_2 -adrenergic receptor, the β -hydroxyl group of epinephrine is also known to form hydrogen bonds with the side chain amine of both aspartate D3.32 and asparagine N7.39 (Ring et al., 2013). Likewise, the nitrogen atom of drugs can also participate in hydrogen bond formation evidenced by the recent report that threonine T3.37 of several serotonin 5-HT receptors forms a hydrogen bond with B-ring nitrogen of

ergotamine (Wang et al., 2013). Water molecules can also be part of the hydrogen bonds and bridge the ligand and the receptors (Cueva et al., 2011). In addition, the hydrogen bond between the ligands and S5.46 of the β_1 -adrenergic receptor is suggested to participate in receptor full activation (Warne et al., 2011). Full agonists can form hydrogen bonds with serine S5.42 and S5.46 of the β_1 -adrenergic receptor, but partial agonists only interact with serine S5.42. Together, hydrogen bonds between the ligands and the conserved TM5 serines can contribute to both the ligand affinity and efficacy for the biogenic amine GPCRs.

Aromatic pi interactions

Aromatic pi interactions are a type of non-covalent interaction that involves pi bonds. Pi bonds are covalent chemical bonds where two lobes of one involved atomic electron orbital overlap two lobes of the other involved atomic electron orbital occurring at, for example, aromatic rings. Aromatic pi interactions produce an attractive force between two aromatic rings. Two benzene dimers, for example, can have three representative conformations with aromatic pi bonds: parallel-displaced, T-shape edge-to-face, and eclipsed face-to-face (Figure 1-2). A pair of aromatic rings (dimer) is reported to preferentially take the parallel-displaced conformation, which is 1 kcal/mol more stable than a T-shaped structure (McGaughey et al., 1998). When there is a favorable superimposition of the polarized atoms in the interacting partner (e.g., between aromatic heterocycles), aromatic pi interactions frequently occur in the eclipsed face-to-face conformation.

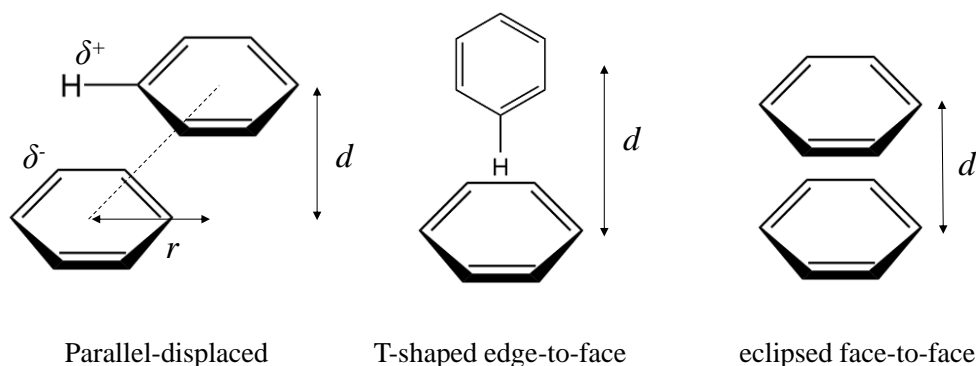


Figure 1-2. Three conformations of aromatic pi interactions at the benzene dimer.

δ^+ and δ^- indicate the polarity of electron cloud. r and d represent the horizontal and vertical distances between the centers of aromatic rings, respectively.

Aromatic pi interactions contribute to many biological events, and many known protein crystal structures have supported the critical role of aromatic pi-interactions in receptor-ligand complexes (Salonen et al., 2011). Steuber and co-workers (2007) solved the crystal structure of aldose reductase that shows favorable aromatic pi stacking interaction between the aromatic inhibitor and the side chain of tryptophan 111. The acetylcholinesterase (AChE) inhibitor Tacrine is suggested to form pi stacking interaction with the indole ring of tryptophan 84 of AChE (Rydberg et al., 2006). As intramolecular interactions, aromatic pi-stacking at transmembrane six segment (TM6) is hypothesized as a main mechanism that moves transmembrane segment for receptor activation (Shi et al., 2002). The conserved phenylalanine F6.44, tryptophan W6.48, and phenylalanine F6.51 and F6.52 of the dopamine D₁ receptor are suggested as candidate amino acids responsible for the TM6 segment movement (Floresca and Schetz, 2004). Although not yet tested with the dopamine D₁ receptor, aromatic pi interactions are suggested to play a prominent role in the ligand interaction and the activation of the D₁ receptor.

Hydrophobic interactions (hydrophobic effects)

Hydrophobic interactions are the tendency of non-polar molecules to aggregate in aqueous solution and exclude water molecules (Chandler, 2005). At the molecular level, hydrophobic interactions are important in protein folding, formation of micelles, and protein-ligand interactions. Hydrophobic amino acids like alanine, valine, leucine,

isoleucine, phenylalanine, tryptophan, and methionine consist of the hydrophobic core of the protein that is buried in the interior of the protein out of contact with water (Lesser and Rose, 1990). Minimizing their exposure to water molecules is known as one of the driving forces of protein folding (Dill, 1990). These hydrophobic interactions are also reported to make large contributions to the stability of globular proteins (Pace et al., 1996). In addition, hydrophobic interactions participate in protein-ligand interactions (Breiten et al., 2013; Snyder et al., 2011). When the hydrophobic surfaces of the protein and the ligand approach one another, the water molecules interacting with both surfaces rearrange and, in part, escape into the bulk water solution. By aggregating water molecules, the non-polar parts of the ligand and the protein can reduce the surface exposed to water and minimize the disruption of hydrogen bond network of water molecules. Simulations with molecular dynamics showed the formation of five-membered water ring at the streptavidin binding cavity supporting the role of water molecules in the supra-strong affinity of the streptavidin-biotin system (Young et al., 2007). Releasing water molecules filling in binding site of the protein into bulk is reported to correlate with peptide binding affinity (Beuming et al., 2009). Although the molecular models for the hydrophobic effect remain a subject of controversy, the hydrophobic effects initiated by the hydrogen bonds of water molecules are widely believed as a key factor for protein-ligand interactions.

Goal of my research

As this framework indicates, I began my research with the goal of understanding the molecular mechanisms by which ligands could activate the D₁ receptor. My hope was to provide new insights that would help in the structure-based drug design with two novel properties: 1) ligands with a high degree of bias for D₁ receptor signaling, and 2) non-catechol and non-toxic compounds that are orally available and of high intrinsic activity.

As I was beginning my research, there was a common belief that focused on the role of D₁ receptors in stimulating phospholipase C activity via activation of Gα_q. Of special relevance was the fact that one ligand, SKF-83959, not only fully activated PLC,

but was also a highly-biased functionally selective compound (specifically, an antagonist at D₁-mediated adenylate cyclase via G $\alpha_{OLF/S}$ activation). Obviously, this was a crucial potential lead for my planned studies on D₁ receptor functional selectivity. Thus, as detailed in the next chapter, I began studies that would provide the needed framework on D₁ receptor signaling.

Chapter 2: SKF-83959 is not a highly-biased functionally selective dopamine D₁ receptor ligand with activity at phospholipase C

Preface

The notion of discovering functionally selective ligands requires understanding the important signaling pathways triggered by a receptor. Although adenylate cyclase and β -arrestin activations are accepted pathways of the D₁ receptor, there have been reports that the D₁ receptor also signals through stimulation of phospholipase C via G α_Q . More recently, it has been proposed that at least part of this D₁-G α_Q -PLC signaling may involve a dopamine D₁-D₂ heterodimer. Most importantly, a major tool used by dozens of papers in the field was the ligand SKF-83959, widely considered to be a highly-biased functionally selective D₁ ligand that fully activates D₁-mediated phospholipase C, but acts as an antagonist at D₁-mediated adenylate cyclase. Because this was potentially important in my mechanistic studies of D₁ activation mechanisms, I needed to utilize this compound as an important reference for my studies. This led to the unexpected results in the following chapter, and to an overall commentary that comprises Chapter 6 of the dissertation.

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Abstract

SKF-83959 [6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine] is reported to be a functionally selective dopamine D₁ receptor ligand with high bias for D₁-mediated phospholipase C (PLC) versus D₁-coupled adenylate cyclase signaling. This signaling bias is proposed to explain behavioral activity in both rat and primate Parkinson's disease models, and a D₁-D₂ heterodimer has been proposed as the underlying mechanism. We have conducted an in-depth pharmacological characterization of this compound in dopamine D₁ and D₂ receptors in both rat brain and heterologous systems expressing human D₁ or D₂ receptors. Contrary to common assumptions, SKF-83959 is similar to the classical, well-characterized partial agonist SKF38393 in all systems. It is a partial agonist (not an antagonist) at adenylate cyclase *in vitro* and *ex vivo*, and is also a partial agonist in D₁-mediated β -arrestin recruitment. Contrary to earlier reports, it does not have D₁-mediated effects on PLC signaling in heterologous systems. Because drug metabolites can also contribute, its 3-N-demethylated analog was synthesized and tested. As expected from the known structure-activity relationships of the benzazepines, this compound also had high affinity for the D₁ receptor and somewhat higher intrinsic activity than the parent ligand, and might contribute to *in vivo* effects of SKF-83959. Together, these data demonstrate that SKF-83959 is not a highly-biased functionally selective D₁ ligand, and that its reported behavioral data can be explained solely by its partial D₁ agonism in canonical signaling pathway(s). Mechanisms that have been proposed based on the purported signaling novelty of SKF-83959 at PLC should be reconsidered.

Introduction

One of the major conceptual changes in pharmacology the last decade has been the awareness that some ligands, after interacting with a single molecular target, can differentially affect the signaling pathways engaged by the target. This phenomenon is now generally termed functional selectivity (Urban et al., 2007), and the degree of differential activation is termed the bias of a ligand. One of the first GPCR receptor systems in which functional selectivity was demonstrated was for dopamine receptors (Lawler et al., 1999; Mailman et al., 1998). The potential therapeutic utility of this mechanism has probably been shown most clearly by aripiprazole, a compound with a mechanism of action clearly differentiated from other approved antipsychotic drugs (Lawler et al., 1999; Shapiro et al., 2003; Mailman, 2007; Mailman and Murthy, 2010). Although aripiprazole is a partial D_2 agonist in some systems, in other systems (e.g., behavioral, electrophysiological, and GTP γ S binding) it behaves as a pure antagonist, and in others may have very high intrinsic activity (Kikuchi et al., 1995; Lawler et al., 1999; Shapiro et al., 2003). It also has markedly different potencies at different assays in the same cell systems unlike typical partial agonists (Shapiro et al., 2003; Urban et al., 2007). These reports are consistent with the hypothesis that aripiprazole is functionally selective at D_2 receptors, but are inconsistent with it being simply a partial agonist (Burris et al., 2002). Aripiprazole has a clinical profile somewhat different than earlier antipsychotics, and its D_2 functional selectivity may explain at least part of its atypicality.

Canonical signaling of D_1 -like receptors (D_1 , D_5) is thought to involve coupling to the G proteins $G_{\alpha_{OLF}}$ or G_{α_S} to stimulate adenylyl cyclase, whereas D_2 -like family receptors (D_2 , D_3 , D_4) couple to G_{α_I} or G_{α_O} to inhibit adenylyl cyclase (Mailman et al., 2001; Neve et al., 2004). Thus, the cAMP resulting from D_1 receptor activation could initiate a host of downstream cascades such as from activation of cAMP/protein kinase A (PKA) signaling. There have been data, however, that challenge this notion and postulate that behavioral effects of D_1 agonists in rodents can be due not to canonical pathways (Gnanalingham et al., 1995a; Gnanalingham et al., 1995b), but rather phospholipase C (PLC)-mediated calcium elevation (Undie et al., 1994; O'Sullivan et al., 2004; Undie and

Friedman, 1990; Andringa et al., 1999b). This has led to the hypothesis that a functionally selective D₁ ligand highly biased against cAMP signaling would have an improved therapeutic index [e.g., for antiparkinson effects (Taylor et al., 1991; Mailman et al., 2001) or cognitive enhancement (Arnsten et al., 1994)], without undesirable effects such as seizures (Starr, 1996) or rapid tolerance (Asin and Wirtshafter, 1993; Gulwadi et al., 2001).

There is a prevailing view that SKF-83959 [6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine] represents such a highly biased D₁ ligand. SKF-83959 is purported to be a biochemical antagonist at D₁-coupled adenylate cyclase. Conversely, it is proposed to stimulate phospholipase C via G α_Q based on the fact that it stimulated phosphatidylinositol-4,5-bisphosphate hydrolysis in membranes of rat frontal cortex in a D₁ receptor-dependent manner (Jin et al., 2003; Zhen et al., 2005; Panchalingam and Undie, 2001), and also induced PLC-mediated calcium elevation through the G α_Q protein in D₁-D₂ receptor heterodimer-expressing cells (Rashid et al., 2007b; Hasbi et al., 2009). SKF-83959 is behaviorally active in rat and primate Parkinson's disease (PD) models via its action at D₁ receptors (Arnt et al., 1992; Jin et al., 2003), and has behavioral activity in a variety of species that is known to be induced by D₁ full or partial agonists, but not by D₁ antagonists (Gnanalingham et al., 1995a). This has led to the hypothesis that the behavioral actions of SKF-83959 may be mediated by its unique actions at PLC/Ca²⁺ signaling at D₁-D₂ heterodimers (Rashid et al., 2007a; Hasbi et al., 2009; Downes and Waddington, 1993; Deveney and Waddington, 1995; Fujita et al., 2010; Perreault et al., 2010; Arnt et al., 1992). This widespread belief that SKF-83959 is the first highly biased D₁ ligand has led many to consider it an important probe for studying the mechanisms related to D₁ signaling (Yu et al., 2008; Zhang et al., 2007; Zhang et al., 2005; Perreault et al., 2011; Zhang et al., 2009b).

We had previously used this compound, and contrary to its widely-cited pharmacology, consistently found that SKF-83959 was a partial agonist at D₁-adenylate cyclase (Ryman-Rasmussen et al., 2005). Although one recent report offered data suggesting that in at least one system SKF-83959 may not be a highly-biased PLC

preferring ligand (Chun et al., 2013), the recent literature is replete with use of this compound for its novel properties. A rigorous reevaluation of its pharmacology in a variety of heterologous and native D₁ systems is therefore needed. In addition, the structure of SKF-83959 suggests that the N-demethylated analog (“desmethylSKF”) is a probable metabolite that might contribute to its pharmacology *in vivo*. We therefore synthesized and characterized this potential metabolite. Our data clearly show that rather than being a highly-biased functionally selective D₁ ligand, SKF-83959 actually is a typical partial agonist of low intrinsic activity much like the prototype D₁ partial agonist SKF38393. Our data suggest that many hypotheses based on the purported signaling bias must be reconsidered.

Material and Methods

Materials

Both SKF-83959 and desmethylSKF [6-chloro-7,8-hydroxy-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine] (Figure 2-1) were synthesized according to procedures described in the supplemental data (Lee et al., 2014). In addition, SKF-83959 was also purchased commercially from Sigma/RBI. Samples from both sources behaved identically. [³H]-SCH23390 and [³H]-N-methylspiperone (NMS) were purchased from Perkin-Elmer Life Sciences Inc. (Boston, MA). SKF38393, quinpirole, and SCH23390 were obtained from Sigma/RBI (Natick, MA). Ham’s F-12, penicillin, streptomycin, and geneticin (G418) were from Invitrogen Co. (Carlsbad, CA). Rat brains were either obtained from Sprague-Dawley rats euthanized according to procedures approved by the institutional IACUC committees, or purchased frozen (Pel-Freez Biologicals, Rogers, AR). Goat anti-cAMP antibody was obtained from Dr. Gary Brooker (George Washington University, Washington D.C.), and the donkey anti-goat secondary antibody was immobilized to Biomagnetic Particles (BMP) according to the manufacturer’s procedure (Polyscience, Inc. Warrington, PA) for the filtration. All other reagents and materials were from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

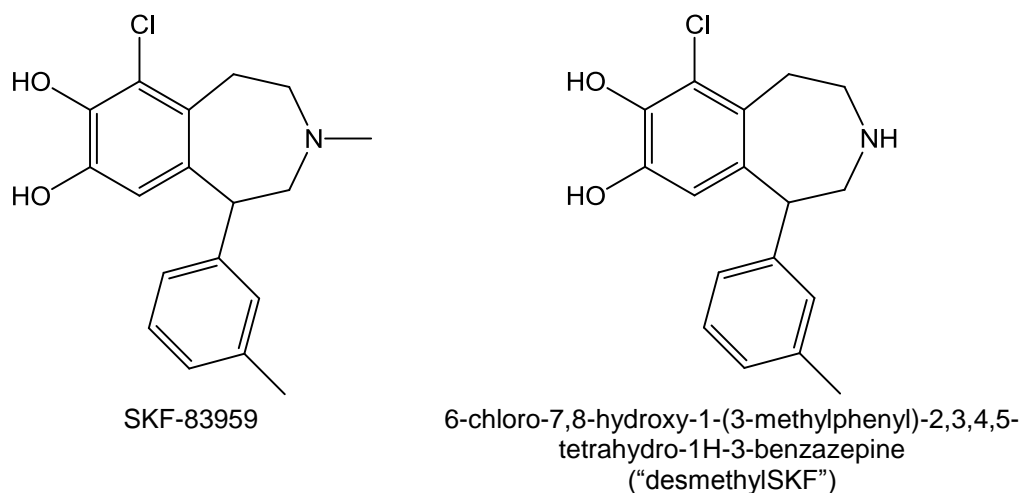


Figure 2-1. Structures of SKF-83959 and a postulated demethylated metabolite.

Receptor source

All experiments with rats were conducted under protocols approved by our Institutional Animal Care and Use Committee. Rat striatal tissue was obtained from male Sprague Dawley rats (250-400 g) obtained from Charles River Laboratories, and housed under a 12 h light/dark cycle and given food and water *ad libitum*. For experiments, the rats were briefly restrained in DecapiCones (Braintree Scientific), and the head was removed using a rat guillotine. Whole brains were removed rapidly, chilled briefly in ice-cold 0.9% (w/v) sodium chloride solution, and sliced into 1.2 mm coronal sections with the aid of a dissecting block similar to that described by Heffner et al. (1980). The striatum was dissected and frozen immediately on dry ice, and stored at -70° C until the day of the assay. In addition, two different cell lines were used for D₁ or D₂ receptor expression: HEK-293 and CHO cells.

Membrane homogenates

Transfected cells and fresh or frozen rat striata were homogenized by several manual strokes in a Wheaton Teflon-glass homogenizer in 5 mL ice-cold lysis buffer (2 mM HEPES, 2 mM EDTA, 1 mM DTT, protease inhibitor cocktail, pH 7.4). The homogenate was centrifuged at 27,000 g for 20 min, the supernatant was discarded, and

the pellet was homogenized (20 strokes) in ice-cold buffer and centrifuged again. The pellet was used as the membrane fraction for radioreceptor assays.

Radioreceptor assays

Human D₁ or D_{2L} receptor density was assessed with saturation binding assay with [³H]-SCH23390 or [³H]-NMS, respectively. For saturation binding assay, membrane homogenates in triplicate were incubated with several concentrations of a radioligand in binding buffer (50 mM HEPES, 4 mM MgCl₂, 0.1% ascorbic acid, pH 7.4) for 15 min at 37°C. The affinity of a test ligand was assessed by competitive binding assays in which triplicate wells were incubated with increasing concentrations of a test ligand and a fixed concentration of a radioligand. Non-specific binding for the D₁ or D₂ receptor was determined by parallel incubation with 1 μM SCH23390 or 1 μM haloperidol, respectively.

Adenylate cyclase assays

Essentially following a published procedure (Watts et al., 1993), striatal tissue was homogenized with eight manual strokes in a Wheaton-Teflon glass homogenizer in 5 mM HEPES buffer (pH 7.5) containing 2 mM EGTA (50 mL/g tissue). Following the addition and mixing of another 50 mL/g of HEPES buffer, a 20 μL aliquot of this tissue homogenate was added to a prepared reaction mixture (final volume of 100 μL) containing 0.5 mM ATP, 0.5 mM isobutylmethylxanthine, [³²P]ATP (0.5 μCi), 1 mM cAMP, 2 mM MgCl₂, 100 mM HEPES buffer, 2 μM GTP, and test drug(s), 10 mM phosphocreatine and 5 U creatine phosphokinase. Triplicate determinations were performed for each drug concentration. The reaction proceeded for 15 min at 30°C and was terminated by the addition of 100 μL of 3% sodium dodecyl sulfate (SDS). Proteins and much of the non-cyclic nucleotides were precipitated by addition of 300 μL each of 4.5% ZnSO₄ and 10% Ba(OH)₂. Samples were centrifuged (10,000 g for 8-9 min), and the supernatants injected on an HPLC system (Waters Z-module or RCM 8 × 10 module equipped with a C18, 10 μm cartridge). The mobile phase was 150 mM sodium acetate

(pH 5.0) with 23% methanol. A UV detector (254 nm detection) was used to quantify the unlabeled cAMP added to the samples to serve as internal standard. The radioactivity in each fraction was determined by a flow-through radiation detector (Inus Systems, Tampa, FL) using Cerenkov counting. Sample recovery was based on UV measurement of total unlabeled cAMP peak areas quantified using PE Nelson (Cupertino, CA) Model900 data collection modules and TurboChrom software.

For the studies in heterologous systems, we used two different assay methods to insure that assay issues were not affecting our results. Thus, both radioimmunoassay and the GloSensor™ cAMP system (Promega Corporation, Madison, WI) were used. Stably transfected CHO cells and transiently transfected HEK-293 cells were used for radioimmunoassay, and GloSensor™ cAMP system used transiently transfected HEK-293 cells. The procedures of radioimmunoassay have been previously published (Brown et al., 2009; Harper and Brooker, 1975). For D₂ receptor-mediated cAMP inhibition, forskolin was added in assay media to elevate the basal level of cAMP, and haloperidol (10 µM) was used to block D₂ receptor activity. The inhibition of forskolin-induced cAMP was assessed as described above.

Assessment of activation of phospholipase C by D₁ receptors

The IP-One assay was used to determine inositol phosphate accumulation (Cisbio, Bedford, MA). In 96-well plates (pre-treated with poly-L-lysine), HEK-293 cells transiently transfected with the D₁ receptor were seeded at 80,000 cells/well and incubated overnight. Drug dilutions were performed in stimulation buffer and added to the plates, which were incubated for 1 h at 37°C, 5% CO₂. Cells were then incubated in lysis buffer for 30 min. Samples were transferred to the IP-One ELISA plate, and IP1 standards, α-IP1 mAb, and competitive IP1-HRP conjugate were added. The plate was incubated for 3 h at room temperature followed by six washings. The colorimetric reaction was initiated by adding TMB (tetramethylbenzidine), and the plate was incubated for 30 min. The reaction was terminated with stop solution. The 96-well plate

was read at 450 nm/620 nm using a V_{\max} plate reader (Molecular Devices, Sunnyvale, CA).

Assessment of β -arrestin activation at D_1 or D_2 receptors

β -arrestin activation in live CHO cells was monitored using PathHunter® β -arrestin assay (DiscoverX Corporation, Fremont, CA) as a D_1 or D_2 mediated non-cyclase signaling pathway. The platform of this assay is based on ligand binding to the D_1 or D_2 receptor that triggers the recruitment of intracellular β -arrestin to the activated receptors. The receptor expression in this kit was not available from DiscoverX, but they informed us that these engineered systems stably expressing D_1 or D_2 receptors have been standardized with well-known full and partial agonists and the potency and intrinsic activity are consistent with literature reports (personal communication).

Data and statistical analysis

The receptor binding and dose-response curves were analyzed by nonlinear regression using Prism 5.0 (GraphPad Software, San Diego, CA). $K_{0.5}$ for test ligands was calculated by Cheng-Prusoff equation with IC_{50} from the binding curves (Cheng and Prusoff, 1973). Functional dose-response curves for all experiments (except D_1 -mediated β -arrestin activation) were analyzed using a sigmoidal dose-response equation with a fixed slope to obtain apparent potency (EC_{50}) and maximal intrinsic activity (E_{\max}). For D_1 -mediated β -arrestin activation, a sigmoidal dose-response equation with a variable slope was used to better represent the original data points. Student's t-tests or ANOVA with *post hoc* Tukey's test were used for the statistical differences between two or multiple groups, respectively.

Cheng-Prusoff equation

$$K_{0.5} = \frac{IC_{50}}{1 + \frac{[ligand]}{K_D}}$$

A sigmoidal dose-response equation

$$\text{Response} = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{\text{LogEC50} - [\text{Test drug}]}}$$

Results

Dopamine D₁ or D₂ receptor expressions and SKF-83959 affinity

We used several human D₁ receptor expression systems: stably transfected CHO and HEK-293 cells (0.7 pmol/mg protein and 5.2 pmol/mg protein, respectively) and transiently transfected HEK-293 cells (1.1 pmol/mg protein). D₁ receptor expression in rat striatal membranes was 1.95 pmol/mg protein. D₂ receptor expression in stably transfected CHO cells was 6.45 pmol/mg protein, which was higher than that in rat striatum (0.93 pmol/mg protein). SKF-83959 showed high affinity ($K_{0.5} = 2.5 \pm 0.2$ nM) for the D₁ receptor in rat striatum, whereas it had much lower affinity for the D₂ receptor ($K_{0.5} = 1.1 \pm 0.2$ μ M) (Table 2-1). The affinity of SKF-83959 for human D₁ or D₂ receptor expressed heterologously was similar to that in rat striatum.

Table 2-1. Summary of SKF-83959 binding affinity.

Receptor	Source, System	SKF-83959	desmethylSKF
		$[K_{0.5} \text{ (nM)}]$	
D ₁ -like	rat striatum	2.5 \pm 0.2	4.0 \pm 0.7
D ₁	human, CHO	1.1 \pm 0.1	--
D ₁	human, HEK-293	0.8 \pm 0.1	--
D ₂ -like	rat striatum	1,080 \pm 225	> 1,000
D _{2L}	human, CHO	1,080 \pm 115	--

Data are from at least three independent experiments and expressed as mean \pm SEM.

Partial intrinsic activity of SKF-83959 at D₁ receptor-mediated adenylate cyclase

The maximal dopamine response for D₁-mediated cAMP synthesis was defined as 100% intrinsic activity. SKF-83959 had $35 \pm 2\%$ intrinsic activity at cAMP production in CHO cells expressing human D₁ receptors (Figure 2-2), whereas the prototypical D₁ partial agonist SKF38393 had ca. 50% intrinsic activity. In CHO cells, the prototypical D₁ receptor antagonist SCH23390 (10 μ M) blocked cAMP elevation induced by SKF-83959 (Figure 2-3 Left) indicating that the cAMP production was a D₁ receptor-mediated response. SKF-83959 also had around 50% intrinsic activity at cAMP production in HEK-293 cells transiently expressing human D₁ receptors. When we assessed the cAMP elevation using cAMP-dependent luciferase activity in the same cells, SKF-83959 showed $42 \pm 6\%$ intrinsic activity, similar to results using radioimmunoassay (Figure 2-2). Again, the activity of SKF-83959 was significantly inhibited by SCH23390 (10 μ M) (Figure 2-3 Middle and Right panels). Inhibition by SCH23390 at Glosensor assay was not as strong as those at RIA. Whereas SCH23390 was pretreated 5 min at RIA before adding drugs, SCH23390 was co-treated with drugs at Glosensor assay. This cotreatment with agonists may be insufficient for SCH23390 to fully inhibit the effects of the agonists. Together, these results demonstrate that SKF-83959 is a partial agonist much like SKF38393 at D₁ receptor-mediated cAMP synthesis.

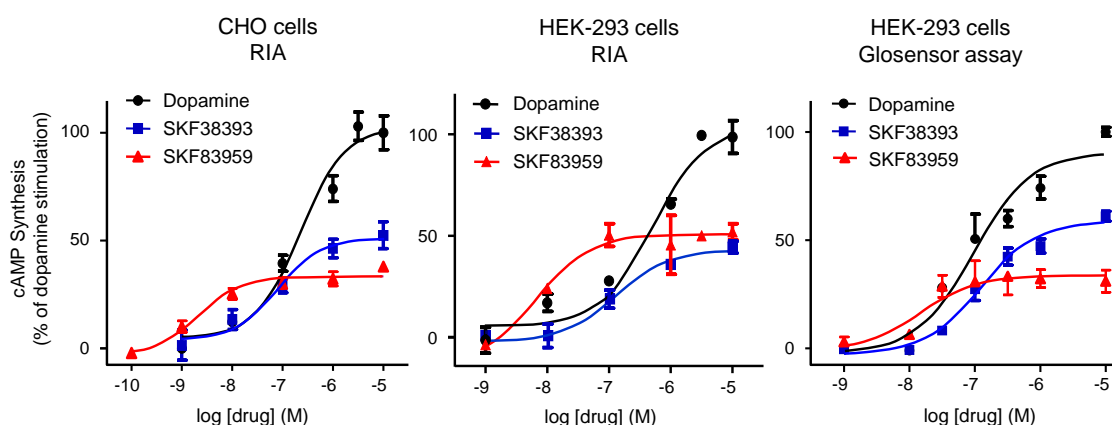


Figure 2-2. D₁-mediated adenylate cyclase activation by SKF-83959 in heterologous expression systems.

D₁ receptor activation in: (Left) CHO cells and (Middle) HEK-293 cells expressing D₁ receptors was assessed by radioimmunoassay. (Right) D₁ receptor activation in transiently transfected HEK-293 cells was also assessed by Glosensor assay. Representative curves of at least three independent experiments are shown. The points in each figure are the mean of triplicates, and error bars are SEM.

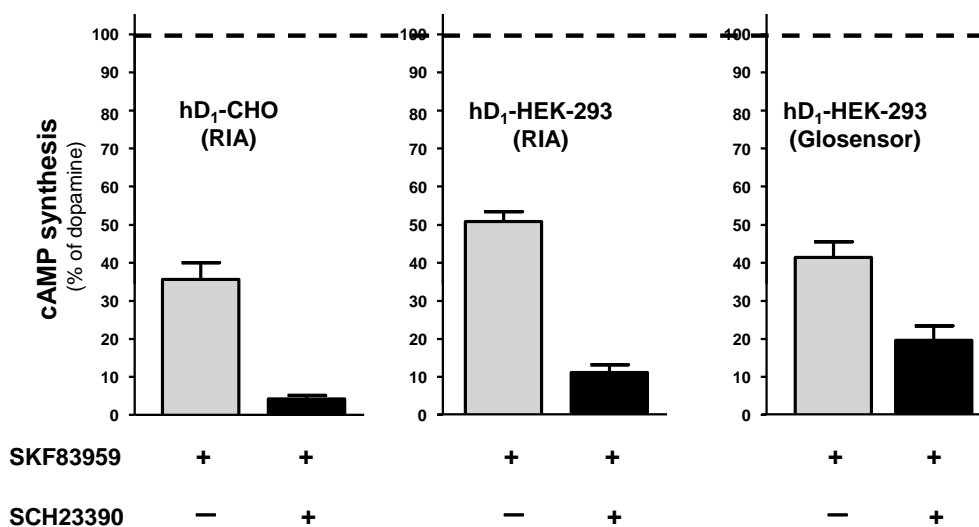


Figure 2-3. Inhibition of SKF-83959-stimulated cAMP synthesis by SCH23390 (10 μ M).

Left panel: CHO cells. Middle and right panels: HEK-293 cells. Both cell types were transfected with the human D₁ receptor as noted in the methods.

SKF-83959 fails to activate the D₂ receptor

Because SKF-83959 had some affinity for the D₂ receptor, we sought to characterize its D₂ functional effects by assessing its actions on D₂-mediated inhibition of

cAMP synthesis. SKF-83959 has no intrinsic activity whatsoever against forskolin-stimulated adenylate cyclase activity (Figure 2-4), whereas the prototypical D_2 full agonist quinpirole and the partial agonist (-)3-PPP showed the expected full and partial activity, respectively.

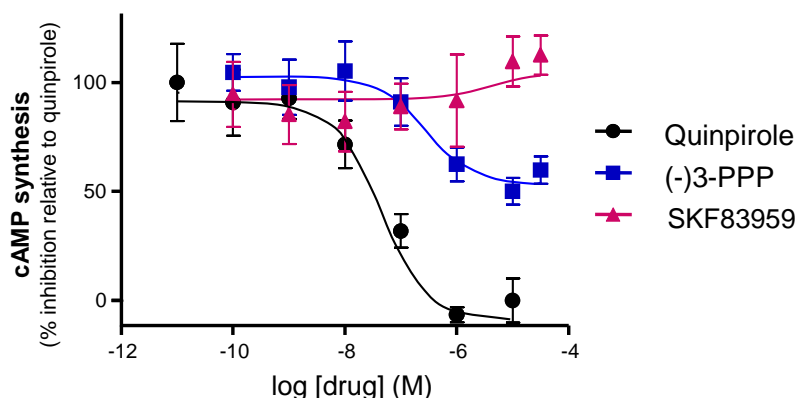


Figure 2-4. D_2 -mediated adenylate cyclase inhibition.

D_2 receptor activation was assessed by radioimmunoassay in CHO cells stably transfected with human D_{2L} receptors. Representative curves of at least three independent experiments are shown. A sigmoidal dose-response curve equation was used to fit the lines.

Potency and intrinsic activity of SKF-83959 for β -arrestin activation at D_1 receptors

G protein-coupled receptor kinases (GRKs) have been known to phosphorylate dopamine receptors, and the phosphorylated dopamine receptors can recruit β -arrestin that plays a critical role in receptor desensitization and internalization (Gainetdinov et al., 2004). β -arrestin recruitment to dopamine receptors can be a measure of non-cyclase dopamine receptor signaling. SKF-83959 was a partial agonist with $32 \pm 2\%$ intrinsic activity in D_1 -mediated β -arrestin activation (Figure 2-5 Left), slightly less than the prototypical partial agonist SKF38393. SCH23390 treatment (10 μ M) significantly reduced the effect of SKF-83959 (Figure 2-5 Right) indicating that this β -arrestin activation was mediated by the D_1 receptor.

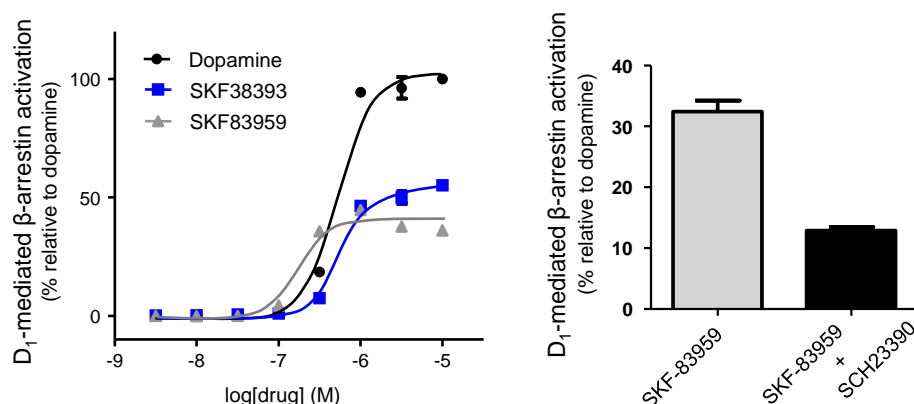


Figure 2-5. Induction of β -arrestin activation at D_1 receptors by SKF-83959.

SKF-83959 was a partial agonist at β -arrestin activation at D_1 (Left), and this was reversed by SCH23390 (Right). Representative curves of at least three independent experiments are shown. For the right panel, 10 μ M concentrations were used for both SKF-83959 and SCH23390.

Potency and intrinsic activity of SKF-83959 for β -arrestin activation at D_2 receptors

SKF-83959 showed $19 \pm 2\%$ of the maximal response of quinpirole at D_2 -mediated β -arrestin activation (Figure 2-6 Left). Consistent with its low D_2 affinity, the potency of SKF-83959 at the D_2 receptor ($1.3 \pm 0.2 \mu$ M) was much less than that at the D_1 receptor. The typical D_2 receptor antagonist haloperidol (10 μ M) completely inhibited the activity of SKF-83959 (Figure 2-6 Right). These results indicate that SKF-83959 is also a partial agonist at β -arrestin signaling mediated by either the D_1 or D_2 receptor.

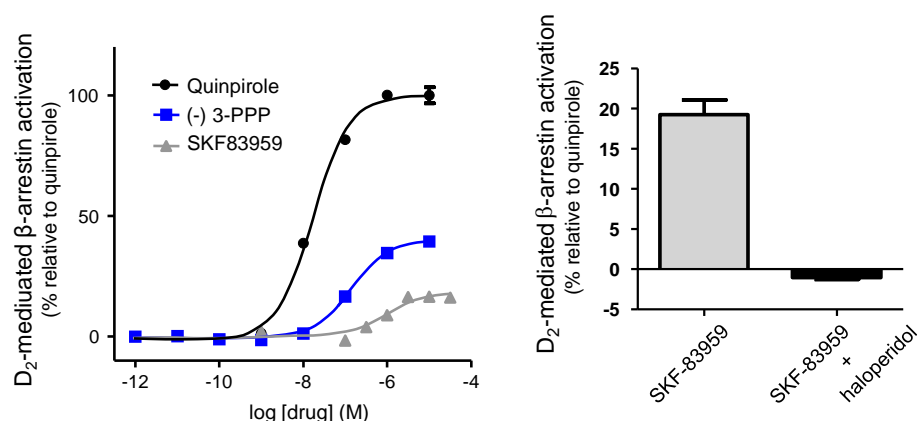


Figure 2-6. Partial agonism of SKF-83959 on induction of β -arrestin by D₂ receptors.

(Left) SKF-83959 causes ca. 20% activation, somewhat less than the known partial agonist (–)3-PPP. (Right) The D₂ antagonist haloperidol (10 μ M) completely inhibits the SKF-83959-mediated activation of β -arrestin.

Lack of SKF-83959 activity on D₁ receptor-mediated PLC stimulation

As noted in the Introduction, the major foundation for the proposed functional selectivity of SKF-83959 was its purported high bias and high intrinsic activity towards D₁-mediated PLC activation versus antagonism at D₁-mediated adenylate cyclase. Because of the potential issues that were discussed above (*vide supra*), this was examined in detail in a heterologous system using human D₁ receptor-transfected HEK-293 cells. As shown in Figure 2-7A, in hD₁-HEK-293 cells the muscarinic agonist carbachol (a positive control) caused marked stimulation of IP1 production, and the potency we observed is consistent with its known micromolar-range affinity for muscarinic receptors (Cheng et al., 2002; Jakubik et al., 1997; Wood et al., 1999). SKF-83959, however, produced no significant increase except for a slight trend at non-pharmacological concentrations (i.e., 300 μ M). Moreover, there was no difference between the amount of IP1 produced by SKF-83959 alone and in combination with the D₁ antagonist SCH23390 (10 μ M) (Figure 2-7B). Conversely, the stimulation of carbachol was blocked by the muscarinic receptor antagonist atropine (10 μ M), whereas atropine had no effect on the action of SKF-83959 (Figure 2-7C).

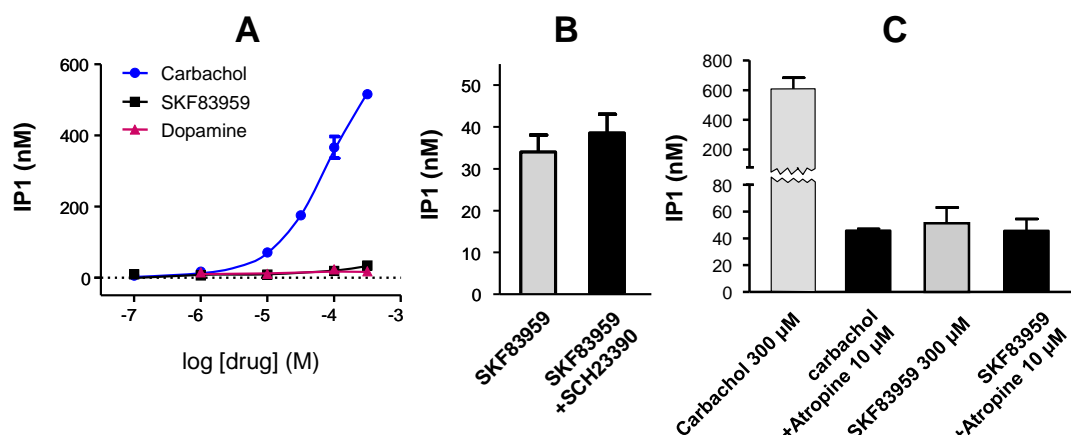


Figure 2-7. SKF-83959 does not stimulate phospholipase C activity via D₁ receptors in hD₁-HEK-293 cells.

(Panel A) SKF-83959 could not induce IP1 production in transiently transfected hD₁-HEK-293 cells. The muscarinic receptor agonist carbachol produced IP1 dose-dependently with expected potency. Representative curves of at least three independent experiments are shown. (Panel B) The slight increase seen at 300 μ M SKF-83959 was not inhibited by the D₁ antagonist SCH23390 (10 μ M). (Panel C) The stimulation by the muscarinic agonist carbachol was inhibited by the muscarinic antagonist atropine (10 μ M), and there is no effect on the action of SKF-83959 (300 μ M).

These studies were also repeated in stably transfected hD₁-HEK-293 cells using D₁ agonists from three chemical classes [benzazepines (SKF-83959 & SKF-83822), phenanthridine (dihydroxidine), or isochroman (A77636)]. None caused significant activation of PLC even at concentrations as high as 30 μ M (Figure 2-8A). In addition, the effects of both carbachol and SKF-83959 were identical in both D₁ transfected and wild-type HEK-293 cells (Figure 2-8B).

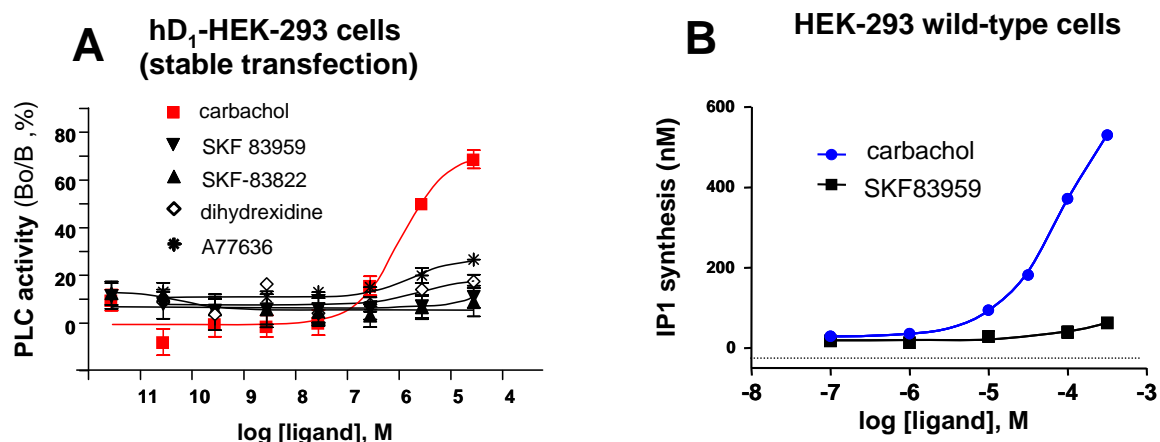


Figure 2-8. Selective D₁ agonists including SKF-83959 and dopamine fail to stimulate PLC activity at physiologically-relevant concentrations.

(Panel A) In stably transfected hD₁-HEK-293 cells, D₁ agonists from three chemical classes [benzazepines (SKF-83959 & SKF-83822), phenanthridine (dihydrexidine), or isochroman (A77636)] failed to activate PLC. (Panel B) In wild-type HEK-293 cells, carbachol caused significant PLC activation whereas SKF-83959 did not, and both compounds caused identical results to those shown in Figure 2-7A. Representative curves of at least two independent experiments are shown.

Activity of SKF-83959 and its N-demethylated analog in rat striatum

Because some of the novelty of SKF-83959 has been related to its actions *in vivo*, we also examined the pharmacology of the parent compound and a likely oxidative metabolite, the N-demethylated analog, both of which were synthesized as described by Lee et al. (2014). In rat striatal membranes, the prototypical partial agonist SKF38393 (Watts et al., 1993), SKF-83959, and its demethylated analog had high affinity for the rat D₁ receptor (Table 2-1). Interestingly, as shown in Figure 2-9, the N-demethylated analog had intrinsic activity (~60%) at D₁-mediated stimulation of adenylate cyclase significantly higher than SKF-83959 (~35%) (Student's t-test, $P < 0.05$) with both compounds having sub-micromolar potency. The stimulation of cAMP synthesis was completely blocked by SCH23390 (Figure 2-9).

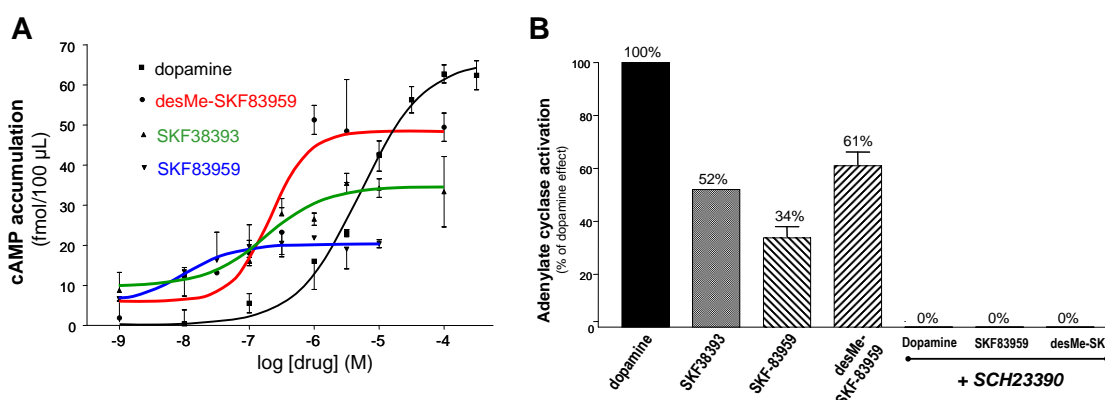


Figure 2-9. Partial agonist properties of SKF-83959 and desMe-SKF-83959 on D₁-stimulated adenylate cyclase of rat striatum.

For the panel B, the benzazepine ligands were used at 1 μ M concentrations, dopamine at 10 μ M, and SCH23390 at 10 μ M.

Discussion

There are no clinically-approved CNS penetrant, high intrinsic activity selective dopamine D₁ agonists, yet animal models have suggested that selective D₁ agonists may have utility for Parkinson's disease (Taylor et al., 1991; Mailman et al., 2001) and cognition (Arnsten et al., 1994; Schneider et al., 1994; Steele et al., 1996; Steele et al., 1997) among other disorders. For these two conditions, therapeutic efficacy of D₁ agonists has been translated into the clinic with large effect sizes predicted by preclinical models (Rosell et al., 2014; Rascol et al., 1999). Despite this support for the clinical efficacy of D₁ agonists, there are reports of serious D₁-mediated side effects that may prevent approval of a D₁ agonist, including rapid tolerance (Asin and Wirtshafter, 1993), profound hypotension (Blanchet et al., 1998), and seizures (Starr, 1996). During the past decade, it has become clear that one way of improving the pharmacological actions of a drug is to make it functionally selective at its targeted receptor(s) (Urban et al., 2007; Neve, 2009; Kenakin, 2007; Mailman, 2007). If a D₁ agonist with high bias for different D₁-mediated signaling pathways were available, it would be a useful research tool that also might overcome some of the possible limitations associated with current D₁ agonists.

SKF-83959 is purported to be such a ligand, highly-biased towards D₁ stimulation of phosphoinositide hydrolysis (via a D₁-G α _Q mechanism), but with no intrinsic activity

at $G_{OLF/S}$ mechanisms that stimulate adenylyl cyclase (Undie and Friedman, 1992; Undie et al., 1994; Gnanalingham et al., 1995b; Arnt et al., 1992). The literature is consistent with our finding that SKF-83959 has nanomolar affinity for the dopamine D_1 receptor in rat brain and the human D_1 receptor expressed heterologously (Table 2-1), and micromolar affinity for the cloned D_{2L} and rat brain D_2 -like receptor. The question, then, is what functional profile does this ligand actually have?

D_1 effects on adenylyl cyclase stimulation and β -arrestin activation

We studied D_1 -mediated cAMP synthesis using the expressed human D_1 receptor in two different cell lines and two different assays, as well as in rat brain striatal homogenates. Both heterologously and *in situ*, SKF-83959 was a partial agonist with intrinsic activity slightly less than SKF38393, the prototypical partial agonist. These studies were performed both with compound synthesized by us (structure verified chemically), as well as material obtained commercially, and with two different assays for cAMP. Identical results were obtained with all combinations. We also studied these two compounds against a second accepted signaling system, β -arrestin-mediated signaling (Shenoy and Lefkowitz, 2005; Urs et al., 2011). SKF-83959 also was a partial agonist at the D_1 receptor with intrinsic activity similar to the partial agonist SKF38393 and similar potency to its effects on adenylyl cyclase, both in the nanomolar range. Together, this is consistent evidence that SKF-83959 appears to have properties expected of a partial D_1 agonist, not a highly-biased D_1 ligand. It is important to reiterate the fact of essentially identical intrinsic activity and potency seen with the D_1 receptor in rat striatum versus the human D_1 receptor expressed heterologously. Although receptor reserve clearly can influence measured intrinsic activity (Watts et al., 1995b), the agreement between the data obtained *ex vivo* versus *in vitro* strongly suggests that our conclusions are physiologically relevant.

How does one resolve the conundrum that many consider SKF-83959 an antagonist at D_1 -cAMP signaling? A previous study from our lab also reported partial intrinsic activity for SKF-83959 in a human D_1 heterologous system (Ryman-Rasmussen

et al., 2005), and some of the published data are consistent with the current report when the data are examined (Gnanalingham et al., 1995b; Rashid et al., 2007b; Chemel et al., 2012), yet other reports clearly show that SKF-83959 completely lacks intrinsic activity at D₁-stimulated adenylate cyclase (Jin et al., 2003; Arnt et al., 1992). Further independent studies are needed to arrive at a consensus.

D₁ effects on activation of phospholipase C

The second conundrum relates to the purported stimulation of PLC signaling by SKF-83959. The current data found no evidence that SKF-83959 causes any stimulation of PLC mediated by the D₁ receptor, even at concentrations five orders-of-magnitude higher than the K_D. This clearly contrasts with numerous reports that D₁ receptors directly stimulate the phosphoinositide signaling system in murine and primate brain (Liu et al., 2009a; Liu et al., 2009b; Yu et al., 2008; Perreault et al., 2014; Felder et al., 1989a; Felder et al., 1989b; Dyck, 1990; Undie and Friedman, 1992; Vyas et al., 1992; Undie and Friedman, 1994; Pacheco and Jope, 1997; Lee et al., 2004; Banday and Lokhandwala, 2007; Liu et al., 2009a; Mizuno et al., 2012; Mahan et al., 1990; Undie and Friedman, 1990; Zhang et al., 2009b). We believe that there is a logical explanation for this lack of concordance. In examining the prior literature, the one constant was that effects of SKF-83959 on PLC signaling required concentrations of 10 μ M and higher. Although SKF-83959 is a nanomolar affinity, selective D₁ ligand, it is known that in the micromolar range it (and its congeners) have numerous off-target activities (Setler et al., 1978; Neumeyer et al., 2003; Chun et al., 2013) some of which are to systems known to be coupled to PLC. In addition, despite the plethora of reports linking D₁ agonists to PLC activation, those studies only used D₁ agonists of the phenylbenzazepine family, and generally relied on SCH23390 (another phenylbenzazepine) as the D₁ antagonist. Because SCH23390 and SKF-83959 share phenylbenzazepine backbone, SCH23390 may bind to other receptors similarly to SKF-83959, and can counteract the non-specific effects of SKF-83959. Thus, using antagonists with similar chemical structure of test agonist can increase the likelihood of confounding off-target effects.

Effects of D₁-D₂ heterodimers on phospholipase C activation

There has been an alternative hypothesis that D₁-D₂ heterodimers mediate PLC activation (Rashid et al., 2007b; Hasbi et al., 2009). However, the existence of D₁-D₂ heterodimers *in vivo* is still unclear. We noticed that most of those papers supporting this hypothesis also used the typically high SKF-83959 concentrations, and we feel that they must be approached skeptically due to the following reasons. 1) In brain the percentage of D₁ receptors that are colocalized with D₂ is very small (Le Moine and Bloch, 1995; Aubert et al., 2000; Thibault et al., 2013; Lidow and Goldman-Rakic, 1994; Gerfen et al., 1990); 2) Because colocalization is a tiny fraction of the total D₁, one would predict that the magnitude of PLC or Ca²⁺ signals would be much less than from known Gα_q receptors directly linked to PLC and expressed at high levels, but it is not; 3) Co-expressing at high levels different receptors in heterologous systems can force protein interactions that would never occur *in situ*; 4) One paper from the George group used more reasonable (but still high, 100 nM) SKF-83959 concentrations in specifically isolated D₁-D₂ coexpressing neurons (Hasbi et al., 2009), but while a Ca²⁺ response was reported, PLC was not tested directly and the response could have been secondary to other signaling; 5) A recent paper from Sibley's group failed to replicate some of the effects predicted from the George hypothesis (Chun et al., 2013). What is more important is that the heterodimer hypothesis is secondary to the first hypothesis of our manuscript: that SKF-83959 and a possible metabolite both are partial D₁ agonists and activate adenylate cyclase. Accepting the PLC hypothesis would seem to require first showing that cAMP-PKA-dependent signaling was not involved, but this was never done. Our secondary target is related to D₁ stimulation of PLC, and here one well-controlled experiment was offered and supplemented by a careful look at the literature. Together, these lines of evidence strongly suggest that PLC activation is highly likely a result of non-D₁ effects caused by the use of suprapharmacological concentrations.

Summary

To our knowledge, this is the first rigorous characterization of the pharmacology of SKF-83959 in several systems. Although we are confident in our current data, we

recognize the possibility that unknown experimental differences might explain why we feel that SKF-83959 is a typical partial agonist, rather than being a highly-biased functionally selective D₁ ligand. Our data suggest that at relevant pharmacological concentrations, SKF-83959 does not activate PLC, and we hypothesize that the prior reports were detecting off-target effects. The use of 10-300 μ M concentrations of a nanomolar affinity ligand would thereby affect many known, as well as unknown, targets. We recognize that there are many hypotheses that could explain why D₁ receptor activation might directly stimulate PLC (homomeric D₁, D₁-D₂ dimer, D₁ heteromer with some other receptor, etc.), but all of these depend on the D₁. Of particular relevance, therefore, is a study that actually compared the PLC stimulation of the 1-phenyl-3-benzazepines in wild-type and D₁ knockout mice (Friedman et al., 1997). Those authors report no difference in PLC stimulation between wild-type and D₁ knockout mice, yet rather than reject the D₁-PLC hypothesis, they postulated that it was caused by a novel, unknown D₁-like receptor (Friedman et al., 1997). This novel explanation seems extremely unlikely as there are essentially no D₅ receptors in the mouse striatum and no other GPCR (characterized or orphan) in the mouse genome that has D₁-like properties.

These data impact many studies that have examined aspects of D₁ function based on novel properties of SKF-83959 (Yu et al., 2008; Guo et al., 2013; Lee et al., 2004; Hasbi et al., 2009; Hasbi et al., 2011; Rashid et al., 2007a; Rashid et al., 2007b), including suggestions that downplay the role of cAMP signaling in behavioral actions of D₁ ligands (Arnt et al., 1992; Downes and Waddington, 1993; Gnanalingham et al., 1995a). If our assessment of the pharmacology of SKF-83959 is correct, it may affect the validity of the conclusions that were drawn from such studies. In addition, we have shown that at least one predicted metabolite of SKF-83959 has actually somewhat higher intrinsic activity than the parent molecule, offering another possible confounding mechanism when used in behavioral or physiological studies. We urge investigators to consider the current data and to review the supporting literature for themselves, before utilizing this compound for properties that it may, or may not, have.

Chapter 3: Dopamine D₁ receptor interaction with ergolines: roles of conserved TM3 threonine and TM5 serines

Preface

Although current catechol-type full D₁ agonists have a great efficacy on several CNS disorders, no full D₁ agonists are approved clinically primarily due to poor oral bioavailability. The intrinsic catechol moiety of the current full D₁ agonists can be rapidly metabolized at gut and liver by multiple enzymes (e.g., catechol-O-methyl transferases and Phase II enzymes). Thus, we focused on non-catechol scaffolds and chose the ergolines as a candidate for orally available D₁ agonists. Because structural information of the receptor-ligand complex helps design novel D₁ agonists, we investigated the molecular interactions between non-catechol ergolines and the D₁ receptor particularly through hydrogen bonds in this chapter. Because similar methods were applied for ligand interaction studies, the methods used in the Chapter 3 were elaborated separately in the Appendix section.

Abstract

Current dopamine D₁ receptor full agonists have poor pharmacokinetic properties due to their intrinsic catechol moiety in the chemical structure, and it is important to determine how novel non-catechol D₁ ligands might be designed. To provide the scientific basis for novel D₁ agonists, we investigated the molecular interactions of the D₁ receptor with non-catechol ergolines. I examined the role of the threonine (T3.37) within transmembrane (TM) three segment, which is reported to form a hydrogen bond for ergotamine at serotonin receptors. As expected, the alanine mutation on T3.37 (T3.37A) greatly decreased the affinity and efficacy of the ergolines within the concentration range we used (maximum 10 μ M), whereas the hydrogen bond-conservative serine mutation (T3.37S) acted similarly to the wild-type D₁ receptor. Based on the preliminary docking simulations, we also hypothesized that at least one of the conserved serines in TM five segment (S5.42, S5.43, and/or S5.46) would form a hydrogen bond with the non-catechol ergolines. The alanine mutation at S5.46 (S5.46A) significantly decreased the affinity of the ergolines, but not D₁ efficacy of the ergolines. Contrary to our hypothesis, the alanine mutation at S5.42 (S5.42A) greatly increased the D₁ affinity and efficacy of the ergolines. The alanine mutation at S5.43 (S5.43A) was ineffective for the ergoline interaction except the small decrease in LEK-8829 affinity. The effects of the double mutations on these serines were similar or greater than those of the individual mutations. At D₁ receptor homology models, the ergoline agonists have docking poses close to T3.37, S5.42, and S5.46 that may explain current mutagenesis results. Together, this structural information helps understand the mechanisms of how non-catechol ergolines interact with the conserved amino acids in TM3 and TM5 of the D₁ receptor.

Introduction

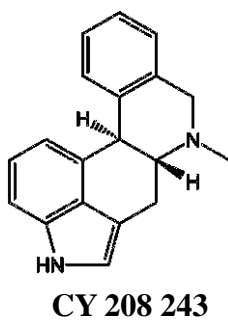
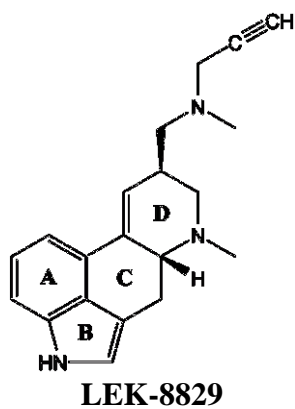
Dopamine D₁ receptor full agonists have a great deal of clinical potential as being equi-efficacious to levodopa in monotherapeutic treatment for Parkinson's disease (Blanchet et al., 1998; Rascol et al., 1999; Taylor et al., 1991), as well as being useful for cognition (Cai and Arnsten, 1997; Steele et al., 1997; Hersi et al., 1995; Arnsten et al., 1994) and drug abuse (Self et al., 1996; Haney et al., 1999). All current selective full D₁ agonists contain a catechol moiety in the chemical structure that is presumably a major factor causing their poor oral bioavailability and short duration of action (Mailman et al., 2001). Thus, non-catechol scaffolds may be useful for the effective full D₁ agonists that have favorable pharmacokinetic profile (Kvernmo et al., 2006). One such class of compounds is ergolines. The ergolines generally have more favorable oral bioavailability than catechol-type D₁ agonists. Although they are selective for D₂ receptors, they have reasonable D₁ affinity and intrinsic activity (Krisch et al., 1994; Emre et al., 1992). Thus, we decided to characterize the interaction of the D₁ receptor with non-catechol ergolines as part of receptor-ligand interaction studies. I first focused on the conserved amino acids in transmembrane three and five segments that have been reported critical for ligand interactions. Ballesteros and Weinstein nomenclature was used to indicate the conserved amino acids (Ballesteros and Weinstein, 1995).

Threonine T3.37 in transmembrane three segment (TM3) is one of the most highly conserved amino acids among Class A GPCRs for catecholamine binding (Xhaard et al., 2006). A recent report revealed a crystal structure of several serotonin receptors and suggested that T3.37 forms a hydrogen bond with ergotamine that has an ergoline backbone (Wang et al., 2013). Based on this report, we hypothesized that T3.37 may provide a critical hydrogen bond for the ergolines at the D₁ receptor. I mutated the threonine to alanine (T3.37A) to remove the hydroxyl group that works as a hydrogen bond donor/acceptor. I also mutated the threonine to serine (T3.37S) to keep the hydroxyl group and remove the methyl group. In addition, our preliminary computational simulations suggested that the ergolines have docking poses close to the conserved serines in transmembrane 5 (TM5): S5.42, S5.43, and S5.46. These serines are located in

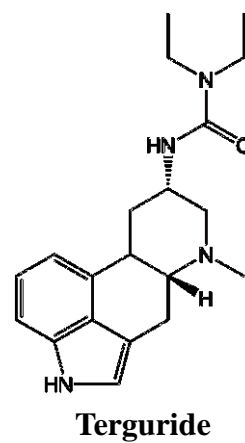
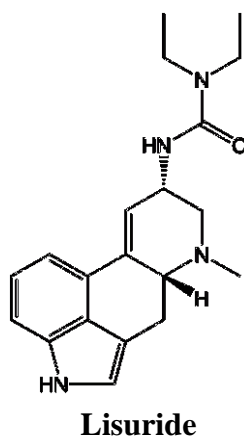
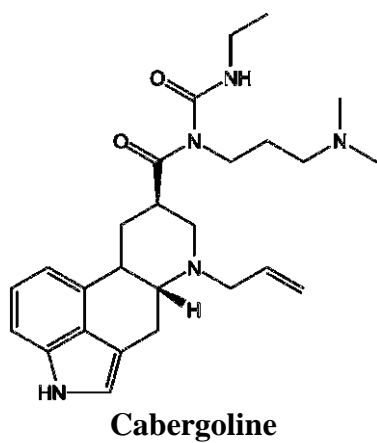
the ligand binding pocket and reported to provide critical hydrogen bonds for dopamine and other catechol-type D₁ agonists (Pollock et al., 1992; Chemel et al., 2012). S5.46 would be most critical for the interaction because S5.46 has the shortest distance and the most proper angle for hydrogen bonding in the simulations. Bach et al. (1980) supported this idea suggesting that B-ring nitrogen of the ergoline backbone may form a hydrogen bond with the TM5 conserved serines in a similar pattern to a catechol-type D₂/D₁ agonist apomorphine. Thus, in the current study each residue of the serines was mutated to alanine to investigate the roles of the hydroxyl group of the serines in hydrogen bond interactions (S5.42A, S5.43A, and S5.46A). The double mutations on the serines were also created and tested (S5.42A/S5.46A and S5.43A/S5.46A).

Based on the mutagenesis results, we also refined the docking poses of the ergoline agonists to the D₁ receptor homology models and illustrated the hypothetical interaction mechanisms for the ergolines with the conserved TM3 threonine and TM5 serines. This structural information on the receptor-ligand complex can serve as a structural basis for new drug design.

Ergoline agonists



Other ergoline ligands



Non-ergoline ligands

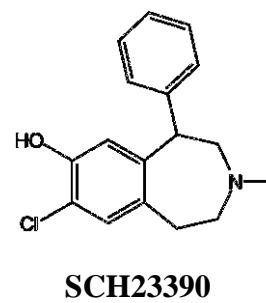
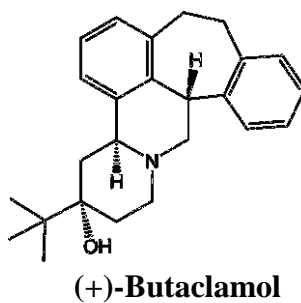


Figure 3-1. Chemical structures of the D₁ ligands tested in the study.

Each ring of the ergoline backbone is labeled the same way as that depicted in LEK-8829.

Table 3-1. Receptor expressions and K_D of SCH23390 for wild-type and mutated D_1 receptors.

Receptor	B_{max} (pmol/mg protein)	K_D of SCH23390 (nM)
	Mean \pm SEM	
Wild-type	1.0 \pm 0.2	1.0 \pm 0.1
T3.37A	0.4 \pm 0.1	14.4 \pm 0.7
T3.37S	1.5 \pm 0.6	1.9 \pm 0.2
S5.42A	0.8 \pm 0.1	10.0 \pm 1.0
S5.43A	3.4 \pm 0.3	8.1 \pm 0.4
S5.46A	1.2 \pm 0.1	0.8 \pm 0.1
S5.42A/S5.46A	0.8 \pm 0.1	11.3 \pm 0.7
S5.43A/S5.46A	2.3 \pm 0.5	14.1 \pm 0.5

B_{max} and K_D were produced using the one-site specific binding mode of non-linear regression. $N \geq 3$.

Results

Transient expressions of wild-type and mutated dopamine D_1 receptors

Wild-type human D_1 receptors were expressed at the level of 1 pmol/mg protein. The expression of other mutated receptors ranged from 0.4 to 3.4 pmol/ mg protein. Receptor expressions varied depending on the mutations. Higher amounts of DNA plasmid were used for some mutated receptors (e.g., T3.37A, S5.42A, S5.43A, and double mutations) versus the wild-type to obtain comparable expressions to the wild-type. No significant cell death was observed until 48 h after transfection.

Mutational effects of T3.37A and T3.37S on the D_1 affinity and efficacy of the ergoline agonists and DHX

Consistent with our hypothesis, the T3.37A mutation decreased the affinity of the ergoline agonists and DHX by at least 5-fold (Table 3-2 and Figure 3-2A). The hydroxyl group-conservative mutation T3.37S, however, completely restored the affinity loss. Consistent with the affinity results, the T3.37A mutation greatly decreased the cAMP production stimulated by the ergoline agonists or DHX within the concentration range we

used (maximal 10 μ M) (Figure 3-2B), whereas the T3.37S mutation markedly restored the loss of cAMP production caused by T3.37A mutation (Figure 3-2C). These data suggest that the hydroxyl group of T3.37 plays a critical role in the interaction with both the ergoline agonists and DHX through possible hydrogen bond formation. In addition, the methyl group of T3.37 was dispensable for the affinity of the ergolines. The T3.37A mutation also significantly decreased the affinity of butaclamol by 5-fold, which is a D₁ antagonist with a unique non-catechol structure (Figure 3-3).

Table 3-2. Affinity of the D₁ receptor agonists at wild-type and mutated D₁ receptors.

	pK_{0.5} (Mean \pm SEM)					
	LEK-8829		CY 208 243		Dihydropyridine	
Wild-type	6.59 \pm 0.08	(1)	6.36 \pm 0.04	(1)	6.76 \pm 0.12	(1)
T3.37A	5.58 \pm 0.12	(10.2 \downarrow)***	5.63 \pm 0.14	(5.4 \downarrow)***	4.87 \pm 0.06	(77.6 \downarrow)***
T3.37S	6.59 \pm 0.08	(1)	6.46 \pm 0.10	(1.3 \uparrow)	6.55 \pm 0.10	(1.6 \downarrow)
S5.42A	7.71 \pm 0.06	(13.2 \uparrow)***	7.45 \pm 0.07	(12.3 \uparrow)***	5.48 \pm 0.06	(19.1 \downarrow)***
S5.43A	6.21 \pm 0.07	(2.4 \downarrow)*	6.23 \pm 0.08	(1.4 \downarrow)	5.35 \pm 0.05	(25.7 \downarrow)***
S5.46A	6.15 \pm 0.09	(2.8 \downarrow)**	5.91 \pm 0.08	(2.8 \downarrow)**	5.51 \pm 0.08	(17.8 \downarrow)***
S5.42A/S5.46A	7.00 \pm 0.08	(2.6 \uparrow)*	6.78 \pm 0.05	(2.6 \uparrow)**	4.97 \pm 0.12	(61.7 \downarrow)***
S5.43A/S5.46A	5.86 \pm 0.11	(5.4 \downarrow)***	5.98 \pm 0.01	(2.4 \downarrow)*	5.40 \pm 0.08	(22.9 \downarrow)***

Fold changes in K_{0.5} are shown in the parentheses with an up or down arrow. One-way ANOVA followed by Tukey's multiple comparison test was used to show significant differences from the wild-type receptor (* P < 0.05, ** P < 0.01, *** P < 0.001). N \geq 3.

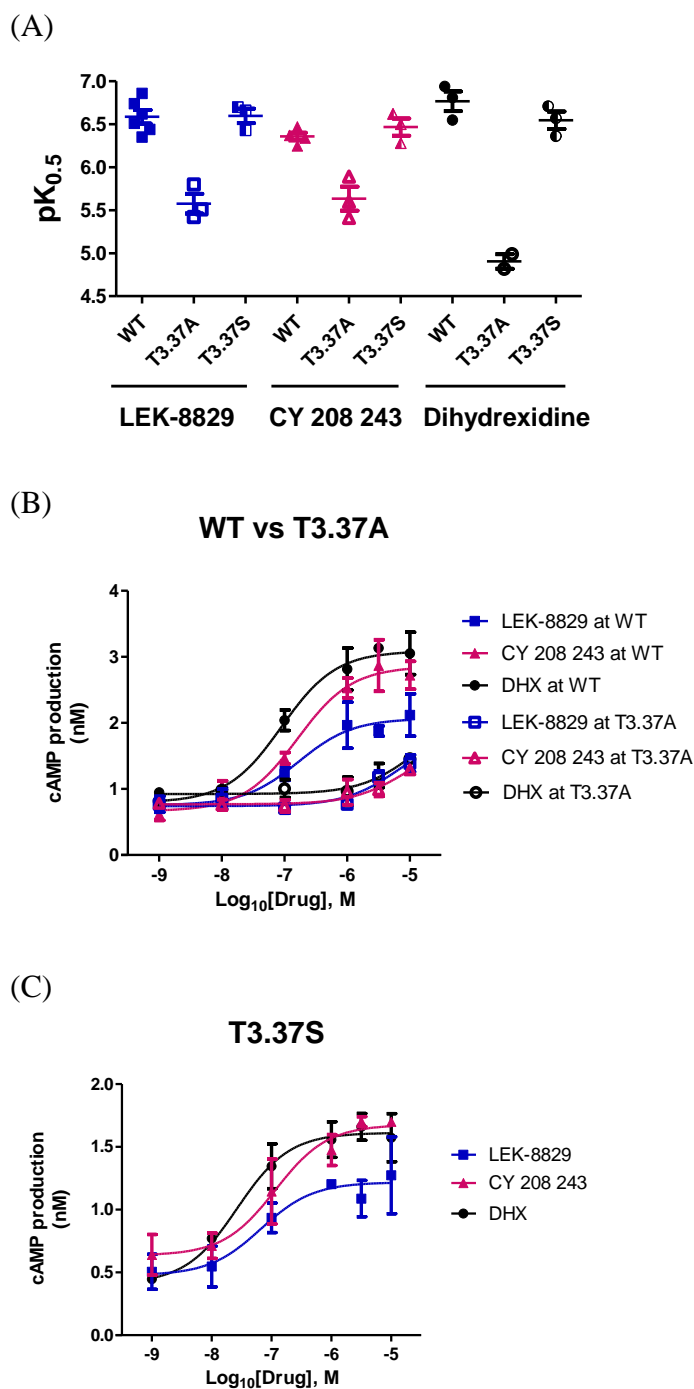


Figure 3-2. The effects of T3.37A and T3.37S mutations on D₁ affinity and efficacy of the ergoline agonists and dihydroxidine (DHX).

(A) The T3.37A mutation significantly decreased the D₁ affinity that is completely restored by the T3.37S mutation. The lines indicate the mean of pK_{0.5} from three independent experiments. (B) The T3.37A mutation greatly decreased the cAMP production mediated

by the ergoline agonists and DHX. (C) The T3.37S mutation partially restored the D₁ efficacy of the ergoline agonists and DHX.

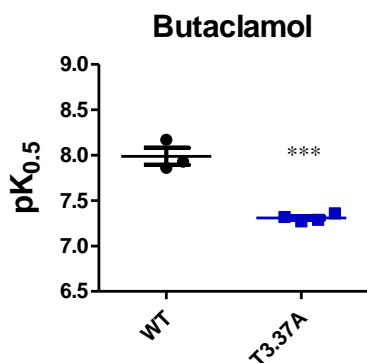


Figure 3-3. The T3.37A mutation significantly decreased the D₁ affinity of butaclamol.

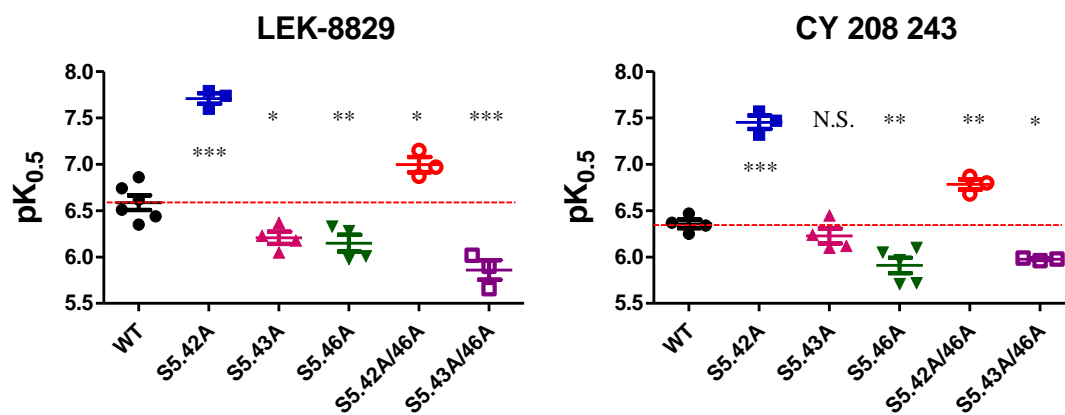
Student's t-test was used to show significant differences from the wild-type receptor (***) $P < 0.001$).

Mutational effects of the conserved TM5 serines on D₁ affinity of the ergoline agonists and DHX

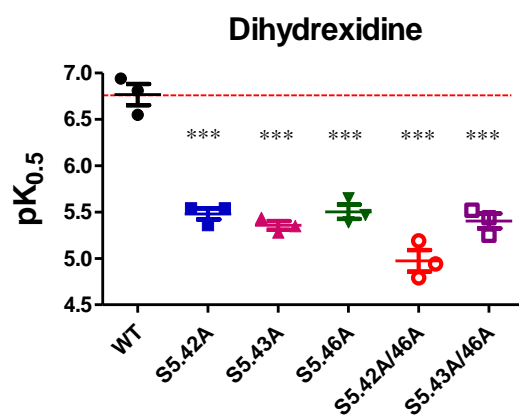
Figure 3-4 and Table 3-2 show the affinity changes of the ergoline agonists and the catechol-type full D₁ agonist DHX. Contrary to the results of the T3.37A mutation and what we hypothesized, the S5.42A mutation actually increased the affinity of the ergoline agonists by over 10-fold. The S5.43A mutation failed to change the affinity of CY 208 243, but it significantly decreased LEK-8829 by 2-fold, which is relatively a small degree compared with the affinity decrease by the T3.37A mutation. The S5.46A mutation also showed a small, but significant and consistent, 3-fold decrease in the affinity of both LEK-8829 and CY 208 243 (Figure 3-4A). S5.42A/S5.46A double mutations significantly increased the affinity of the ergoline agonists by 3-fold, but the increases were less than those caused by the S5.42A mutation alone. Although the S5.43A/S5.46A double mutations failed to make a statistical difference from the individual mutations, they decreased the affinity of LEK-8829 greater than the individual mutations. The S5.43A/S5.46A double mutations significantly decreased the affinity of CY 208 243 similarly to the S5.46A single mutation. In contrast to the mutational effects on the ergoline agonists, all of S5.42A, S5.43A and S5.46A single mutations markedly

decreased the affinity of DHX (Figure 3-4B). The S5.42A/S5.46A double mutations decreased the affinity of DHX greater than the individual mutations, whereas the S5.43A/S5.46A double mutations decreased the affinity of DHX similarly to each of the single mutations. The affinity of butaclamol was unchanged by any of the mutations suggesting that the binding pocket for butaclamol was preserved at the mutated receptors (Figure 3-4C).

(A)



(B)



(C)

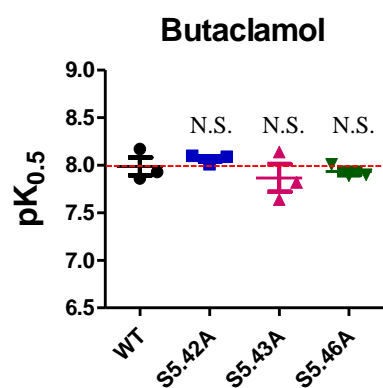


Figure 3-4. Changes in the affinity of (A) the ergoline agonists and (B) dihydrexidine by the single and double mutations on the conserved TM5 serines. (C) A D₁ antagonist butaclamol was used as a negative control.

Significant differences from the wild-type receptor are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey's multiple comparison test). N.S.; not significant.

Changes in D₁-mediated cAMP synthesis by single and double mutations on the TM5 conserved serines

Consistent with the affinity results, the S5.42A mutation greatly increased the potency of the ergoline agonists for D₁-mediated cAMP synthesis. In addition, the maximal levels of cAMP production stimulated by the ergoline agonists were markedly increased (Figure 3-5A). The cAMP synthesis mediated by the ergoline agonists was unchanged by either of the single S5.43A and S5.46A mutations (Table 3-3). Despite no significant change in the potency versus the wild-type, S5.42A/S5.46A double mutations markedly increased the maximal levels of cAMP produced by the ergoline agonists. However, these increases were less than those caused by the S5.42A mutation alone. Contrary to the effects of either the S5.43A or S5.46A single mutation, S5.43A/S5.46A double mutations significantly decreased the potency of the ergoline agonists (Figure 3-5A). In addition, the S5.43A/S5.46A double mutations considerably decreased cAMP production mediated by the ergoline agonists within the concentration range we used (maximal 10 μ M), but the effects failed to obtain statistical significance versus the wild-type (ANOVA followed by Tukey's multiple comparison test).

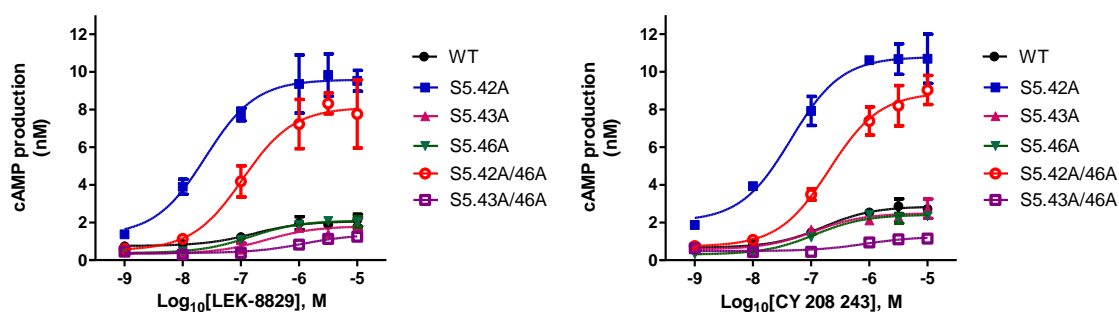
Consistent with the previous reports with DHX, the S5.42A mutation greatly decreased the potency of DHX, but it actually increased the maximal level of DHX-mediated cAMP production (Figure 3-5B). Except for S5.42A mutation, all other single and double mutations greatly diminished DHX-mediated cAMP production within the concentration range we used (maximum 10 μ M) confirming the critical role of the serines in the interaction with catechol-type D₁ agonists.

Table 3-3. cAMP production mediated by the ergoline agonists and pEC50.

	LEK-8829	CY 208 243	LEK-8829	CY 208 243
	cAMP production (nM) at 10 μ M		pEC50	
Wild-type	2.0 \pm 0.1	2.8 \pm 0.2	6.52 \pm 0.06	6.83 \pm 0.14
T3.37A	N.A.	N.A.	N.A.	N.A.
T3.37S	1.2 \pm 0.03	1.7 \pm 0.1	7.14 \pm 0.06***	6.99 \pm 0.16
S5.42A	8.1 \pm 1.0***	9.5 \pm 1.3***	7.36 \pm 0.12***	7.27 \pm 0.10*
S5.43A	1.5 \pm 0.2	2.3 \pm 0.2	6.42 \pm 0.07	6.93 \pm 0.03
S5.46A	2.1 \pm 0.3	2.3 \pm 0.3	6.64 \pm 0.11	6.61 \pm 0.13
S5.42A/S5.46A	7.0 \pm 0.6***	7.1 \pm 0.6***	6.86 \pm 0.07	6.69 \pm 0.05
S5.43A/S5.46A	1.5 \pm 0.2	1.7 \pm 0.3	5.98 \pm 0.07**	6.00 \pm 0.12***

Significant differences from the wild-type receptor are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA followed by Tukey's multiple comparison test). N.A.; not applicable. Values represent the mean \pm SEM of at least three independent experiments.

(A)



(B)

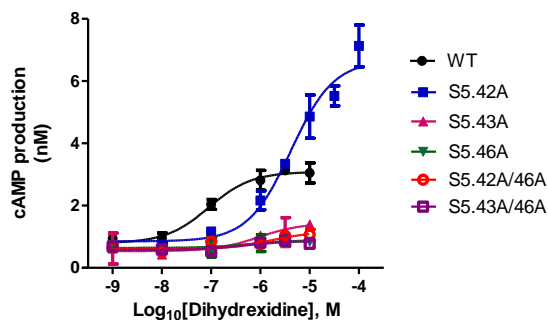


Figure 3-5. cAMP production stimulated by (A) the ergoline agonists and (B) dihydrexidine at wild-type and mutated D_1 receptors.

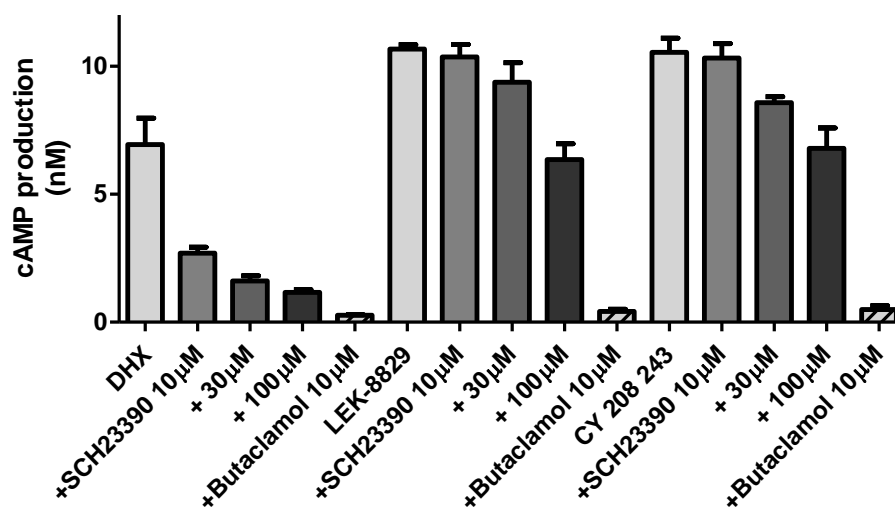
cAMP production at S5.42A and S5.42A/S5.46A mutated receptors is a D₁ receptor-mediated response

The typical D₁ antagonist SCH23390 decreased cAMP production stimulated by the ergoline agonists at the S5.42A mutated receptor dose-dependently (Figure 3-6A). However, only high concentrations of SCH23390 (over 30 μ M) inhibited the activity of the ergoline agonists by half at the S5.42A mutated receptor. Butaclamol (10 μ M), on the other hand, completely inhibited cAMP production at the S5.42A mutated receptor at the 10 μ M concentration suggesting that cAMP production is mediated by the D₁ receptor. S5.42A mutation dramatically decreased the affinity of SCH23390, and the affinity of SCH23390 for S5.42A mutated receptors became very similar to that of butaclamol (ca. 10 nM). Despite the similar affinity of SCH23390 and butaclamol, SCH23390 failed to inhibit ergoline-mediated cAMP production at the 10 μ M concentration, whereas butaclamol clearly inhibited the effects of the ergolines. I hypothesize that conformational changes by the S5.42A mutation may weaken the competitive antagonist property of SCH23390, so that SCH23390 failed to inhibit D₁-mediated cAMP production similarly to butaclamol. Although DHX-induced cAMP production was greatly decreased by 10 μ M SCH23390 at the S5.42A mutated receptor, the effect was smaller than that of butaclamol. Another possibility is that real affinity of SCH23390 for the S5.42A mutated receptor may be lower than what I obtained from saturation binding assays. Some previous reports show lower affinity of SCH23390 (e.g., K_D = 52 nM or greater) than that I obtained in the current study (K_D = 10 nM) (Chemel et al., 2012; Pollock et al., 1992). Assuming that the affinity of SCH23390 is lower than butaclamol affinity can easily explain less inhibition of SCH23390 than that of butaclamol at the same concentration.

cAMP production at the S5.42A mutated receptor was also dependent on the receptor expression. When the receptor expression was reduced, the maximal levels of cAMP production were also decreased (Figure 3-7). At the S5.42A/S5.46A double mutated receptor, SCH23390 also markedly decreased the cAMP production dose-

dependently (Figure 3-6B). Together, these results suggest that cAMP production at the S5.42A and S5.42A/S5.46A mutated receptors is a D₁ receptor-mediated response.

(A)



(B)

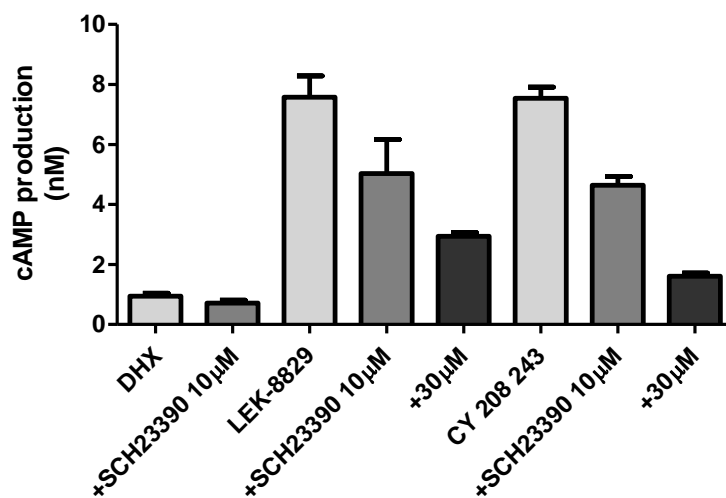


Figure 3-6. Inhibition of cAMP production by SCH23390 or butaclamol at the (A) S5.42A or (B) the S5.42A/S5.46A mutated receptors.

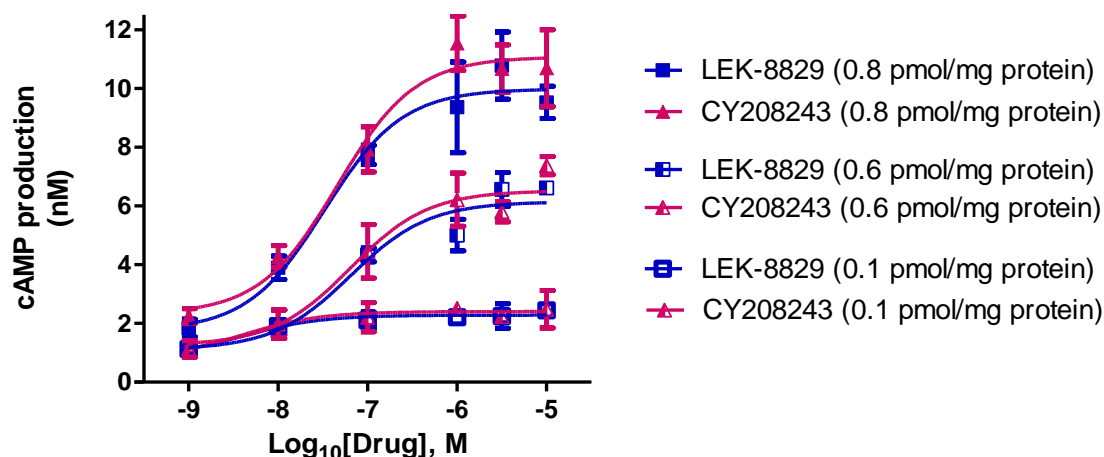


Figure 3-7. cAMP production by the ergoline agonists at S5.42A mutated receptors was dependent on the receptor expression.

Mutational effects of the conserved TM3 threonine and TM5 serines on other ergoline-type D₁ ligands

Three additional D₁ receptor ligands with the ergoline backbone (cabergoline, lisuride, and terguride) were used to confirm the mutational effects of the conserved TM3 threonine and TM5 serines. The S5.42A mutation markedly increased the affinity of these ergoline-type D₁ ligands, whereas the S5.46A mutation showed small, but significant decreases in their affinity (Figure 3-8). Consistent with the results of LEK-8829 and CY 208 243, the T3.37A mutation greatly decreased the affinity of these ergoline-type D₁ ligands. The common pattern of the mutational effects between the ergoline ligands suggest that the ergoline backbone may be involved in the interaction with the conserved TM3 threonine and TM5 serines.

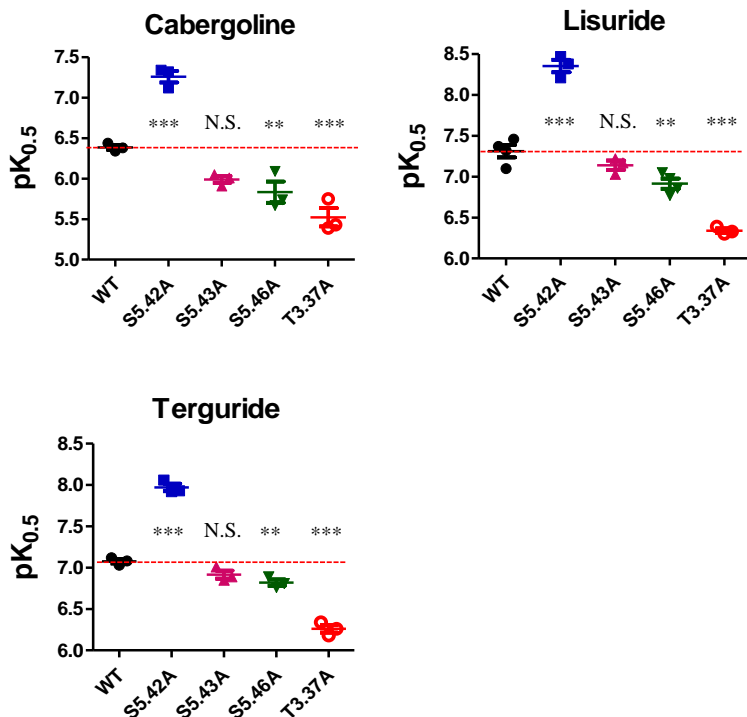


Figure 3-8. Mutational effects on the affinity of other ergoline-type D₁ ligands.

Significant differences from the wild-type are shown (** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with Tukey's multiple comparison test). N.S.; not significant.

Docking simulations with the ergoline agonists at the D₁ receptor homology models

The structure of the β_2 -adrenergic receptor representing the active or the inactive state was used as a template for the active or the inactive state of D₁ receptor homology model, respectively (Rasmussen et al., 2011a; Cherezov et al., 2007). When the D₁ receptor is activated, the TM5 segment moves toward the ligand binding pocket in the models. In particular, S5.46 turns its hydroxyl group to the ligand binding pocket. This movement seems to induce a more favorable position for S5.46 to form a hydrogen bond with B-ring nitrogen of the ergolines (Figure 3-9). LEK-8829 and CY 208 243 showed similar docking poses to the ligand-binding pocket of the D₁ receptor models. B-ring nitrogen of the ergoline agonists is located close to T3.37 and S5.46, and the distance from T3.37 is shorter than that from S5.46 suggesting that T3.37 may form a stronger hydrogen bond than S5.46. In addition, A-ring of the ergoline backbone is located close

to S5.42 generating possible steric hindrance with the hydroxyl group of S5.42. The simulations also suggested that S5.43 is located distant, over 7Å from B-ring nitrogen of the ergoline backbone.

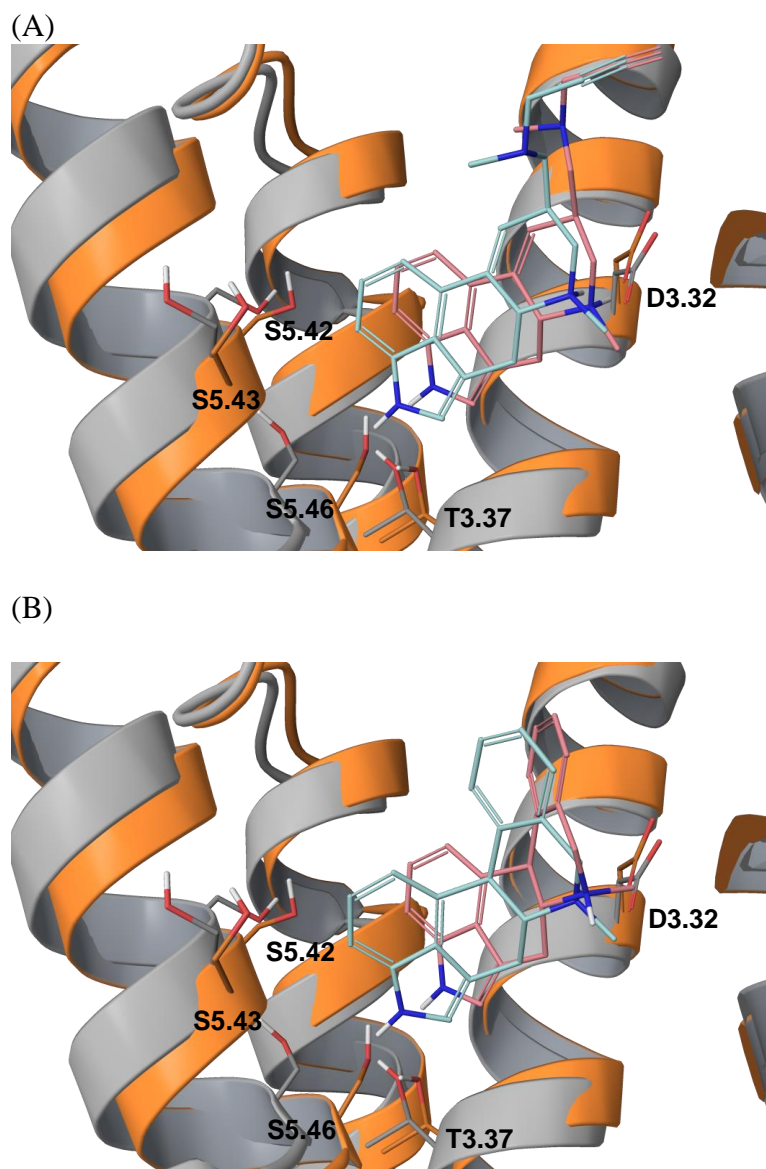


Figure 3-9. Docking poses of (A) LEK-8829 and (B) CY 208 243 at the D₁ receptor homology models.

The grey ribbons indicate the inactive-state D₁ receptor model, whereas the orange ribbons reflect the active-state D₁ receptor model. Agonists are depicted as light blue in the inactive-state model and pink in the active-state model.

Discussion

A catechol moiety has been reported as a critical component for D₁ receptor full agonists (Mottola et al., 1996), and the activation mechanisms of catechol-type D₁ agonists have been extensively investigated (Ryman-Rasmussen et al., 2007; Zhang et al., 2009a; Chemel et al., 2012; Pollock et al., 1992). The catechol moiety, however, can be rapidly metabolized by phase II enzymes which subsequently inactivate the D₁ agonists. To help establish a scientific basis for the development of orally available D₁ agonists, we chose the ergolines as a non-catechol scaffold and investigated their interaction with the D₁ receptor. This study is the first report on the mechanisms of how these ergolines interact with the conserved TM3 threonine and TM5 serines of the D₁ receptor. The current study reveals that the ergolines interact with the conserved TM3 threonine and TM5 serines quite differently than the typical catechol-type D₁ agonist DHX.

Possible hydrogen bond formation between the ergolines and the D₁ receptor

The T3.37A mutation markedly decreased the D₁ affinity and efficacy of the ergoline agonists and DHX within the concentration range we used (Table 3-2 and Figure 3-2) suggesting that T3.37 may provide a critical hydrogen bond for both the ergoline agonists and DHX. The S5.46A mutation also decreased the affinity of the ergolines significantly, but the decreases were smaller than those caused by the T3.37A mutation. Although S5.46 is reported to play a critical role in the hydrogen bond interaction with catechol-type D₁ agonists (Chemel et al., 2012; Pollock et al., 1992), the hydrogen bond provided by S5.46 for the ergolines may be weaker than that provided by T3.37. A recent report in the D₂ receptor showed that T3.37 may form an intermolecular hydrogen bond with S5.46, and that T3.37 and S5.46 may be functionally coupled during receptor activation (Daeffler et al., 2012). Likewise, T3.37 of the D₁ receptor may form an intramolecular hydrogen bond with S5.46, and both T3.37 and S5.46 may be involved together in the interaction with the ergolines. The crystal structures of ergotamine at several serotonin receptors showed that T3.37 forms a hydrogen bond with B-ring nitrogen of the ergoline backbone (Wang et al., 2013) suggesting the conserved T3.37

may be involved in hydrogen bond interactions at both dopamine and serotonin receptors. In addition to the hydrogen bond formation, there is a possibility that the T3.37A mutation may disrupt the overall ligand binding site of the D₁ receptor and decrease the affinity of the ergolines. This mutation significantly decreased the affinity of butaclamol that appears not to interact with T3.37 at docking simulations suggesting that the binding pocket for butaclamol is likely to be affected by the mutation.

Increases in the ergoline affinity by the S5.42A mutation

Whereas the S5.42A mutation greatly decreased the affinity of the catechol-type agonist DHX, it dramatically increased the affinity of the ergolines (Table 3-2, Figures 3-4 and 3-8). At the D₄ receptor, the S5.42A mutation also increased the affinity of (–)-sulpride and Ro10-4548 (Cummings et al., 2010). In addition, the S5.42A mutation in the D₂ receptor greatly increased the affinity of an ergoline-type D₂ agonist pergolide (Wilcox et al., 2000). Based on these reports, S5.42 in the D₁ receptor is unlikely to form a hydrogen bond with the ergolines. Rather, the hydroxyl group of S5.42 may produce steric hindrance for the ergolines, so that the S5.42A mutation actually increased the interaction with the ergolines. Computational simulations support the hypothesis that (–)-sulpride and Ro10-4548 are located beyond the range of possible hydrogen bond interactions with S5.42 (Cummings et al., 2010). Docking simulations with the ergoline agonists (Figure 3-9) demonstrated that A-ring of the ergoline backbone is located close to S5.42 and is likely to generate steric hindrance. Loss of the hydroxyl group at S5.42 may reduce the steric hindrance and make more favorable conditions for the ergolines to interact with the D₁ receptor.

Simulations of hydrophobic interactions suggest that A-ring of the ergolines have hydrophobic interactions with tyrosine Y5.38 and isoleucine I3.33 of the D₁ receptor. Particularly, tyrosine Y5.38 can form additional aromatic pi interaction with the A-ring. As yet undetermined, Y5.38 and I3.33 may play a role in the interaction with the A-ring of the ergoline backbone through the additional aromatic and hydrophobic interactions. Thus, the loss of the A-ring of the ergolines would decrease the affinity for the D₁

receptor. A previous report supports this idea showing that tricyclic structures containing the B-, C-, D-rings of the ergoline backbone (e.g., quinpirole) possess very weak D₁ receptor interaction, but strong dopamine D₂ receptor agonist activity (Schaus et al., 1993).

Unique roles of TM5 conserved serines in D₁-mediated cAMP production

High concentrations of DHX (over 10 μ M) increased the maximal cAMP production at the S5.42A mutated receptor (Figure 3-5B). A previous report showed a similar increase in dopamine-stimulated cAMP production at the S5.42A mutated D₁ receptor (Pollock et al., 1992). The S5.42A mutation also increased the maximal level of cAMP production stimulated by the ergoline agonists (Figure 3-5A). The basal activity of cAMP production was unchanged at the S5.42A mutated receptor. Thus, it seems that the S5.42A mutation increases the ligand-mediated D₁ receptor activation. A recent paper suggested that agonist binding in β_2 adrenergic receptor have a bit different energy landscape than that of G protein binding suggesting multiples steps in in the process of GPCR activation (Nygaard et al., 2013). I hypothesize that the S5.42A mutation may affect the conformational changes of the D₁ receptor at the activation processes and may induce conformational changes for better G protein binding and activation. If the ergoline agonists still show great D₁ efficacy at transfected S5.42A mutated D₁ receptors *in vivo*, the S5.42A mutation and the ergoline agonists together can be used a scientific tool for investigating the behavioral effects of cAMP overproduction.

The S5.43A mutation in the D₁ receptor is a naturally occurring polymorphism in human population (Al-Fulaij et al., 2008). Previous studies have demonstrated that this polymorphic S5.43A mutation dramatically decreases the interaction with catechol-type D₁ agonists (Chemel et al., 2012; Floresca and Schetz, 2004). The current study, however, shows that the S5.43A mutation failed to decrease the D₁ affinity and efficacy of the ergoline agonists (Tables 3-2 and 3-3). In fact, the ergoline agonists produced more cAMP than the catechol-type agonist DHX at the S5.43A mutated receptor. These results

suggest that the ergolines may be used as the chemical source that activates the D₁ receptor regardless of the polymorphic S5.43A mutation in an individual.

Previous reports showed that S5.46 was a critical residue for the D₁ interaction with catechol-type D₁ agonists, and that the S5.46A mutation greatly decreased the D₁ efficacy of DHX (Chemel et al., 2012; Pollock et al., 1992). In contrast to the results of catechol-type D₁ agonists, the S5.46A mutation was unable to significantly reduce D₁ efficacy of the ergoline agonists although the S5.46A mutation significantly decreased the affinity of the ergoline agonists. Future studies should probe the interaction of the conserved residues in TM6 that play a critical role in G protein binding and receptor activation (Lebon et al., 2012). These studies may provide additional information on the critical residues that contributes to the D₁ efficacy of the ergoline agonists.

Equal and greater effects of double mutations versus single mutations

S5.42A/S5.46A double mutations markedly increased the cAMP production stimulated by the ergoline agonists like the S5.42A mutation, but the potency at the double mutated receptor was considerably weaker than that at the S5.42A mutated receptor (Table 3-3 and Figure 3-5A). The potency at this double mutated receptor seems to reflect the affinity of the ergoline agonists decreased by the S5.46A mutation. I also made S5.43A/S5.46A double mutations that decreased the affinity of LEK-8829 greater than either of the S5.43A and S5.46A single mutations (Table 3-2 and Figure 3-4A). Although each of the S5.43A and S5.46A single mutations failed to decrease ergoline-mediated cAMP production, the combined double mutations decreased it considerably. This decrease did not reach statistical significance, but it suggests that at least either S5.43 or S5.46 may be necessary for the D₁ efficacy of the ergoline agonists.

Docking poses of DHX to the D₁ receptor

DHX and other full agonists are suggested to share a D₁ receptor pharmacophore, and the two hydroxyl groups of the catechol moiety are proposed as key components in the recognition and activation of the D₁ receptor (Mottola et al., 1996). Several previous reports illustrated the possible docking poses of the catechol-type D₁ agonists at the D₁

receptor homology models. Bonner et al. (2011) suggested that the meta- and para-catechol hydroxyl groups of the heterocyclic compounds directly form a hydrogen bond with S5.42 and S5.46, respectively. In this model, S5.43 did not participate in the hydrogen bond interactions, but it was located close to N6.55 suggesting possible intramolecular interaction with N6.55. Consistent with the results of Bonner et al., Cueva et al. (2011) reported that S5.42 and S5.46 form hydrogen bonds with the catechol hydroxyl groups of DHX. This study also suggests that S5.43 interacts with N6.55, and that N6.55 forms a hydrogen bond with the meta-catechol hydroxyl group via a water molecule. Malo et al. (2012) reported the docking pose of another D₁ full agonist SKF89626, and suggested that S5.43 was unable to form the hydrogen bond with the catechol groups of SKF89626. The hydroxyl group S5.43 was quite distant (5 Å) from the meta-hydroxyl group SKF89626 (Malo et al., 2012). This study also reports the hydrogen bond interaction between T3.37 and the para-catechol hydroxyl group of SKF89626. Together, T3.37, S5.42, and S5.46 of the D₁ receptor, but not S5.43 are suggested to serve as hydrogen bond donors/acceptors for the catechol group of D₁ agonists. S5.43 in the D₁ receptor seems unlikely to form a hydrogen bond with the catechol moiety of D₁ agonists, but it may form an intramolecular interaction with other residues (e.g., N6.55) and participate in ligand interactions facilitated by a water molecule.

Conclusions

In the current study, we reported that the T3.37A mutation greatly decreased both D₁ affinity and efficacy of the ergoline agonists suggesting a possible hydrogen bond formation between the ergolines and T3.37. In addition, we showed that at least five ergoline-type D₁ ligands (LEK-8829, CY 208 243, cabergoline, lisuride, and terguride) interact with S5.42 and S5.46 differently from the interactions of the catechol-type D₁ agonist DHX. This structural information assists with understanding the molecular interactions between the ergolines and the conserved amino acids of TM3 and TM5 in the D₁ receptor. This study provides a scientific basis for the development of modified ergolines that may have improved D₁ receptor interaction.

Chapter 4: Critical interaction of the dopamine D₁ receptor with ergolines: roles of conserved aromatic residues in transmembrane six segment

Preface

I have shown possible hydrogen bond interactions between the ergolines and the D₁ receptor. Particularly, T3.37 and S5.46 likely have hydrogen bonds with B-ring nitrogen of the ergolines. In addition to the hydrogen bonds, aromatic and hydrophobic interactions are also reported critical in the formation of receptor-ligand complexes. In particular, the conserved aromatic residues in TM6 are known to contribute to G protein binding and receptor activation. Because the ergolines have aromatic and heterocyclic rings in their chemical structure, we interrogated the possible aromatic and hydrophobic interactions between the ergoline and the D₁ receptor focusing on the roles of the conserved aromatic residues in TM6. Because similar methods were applied for ligand interaction studies, the methods used in the Chapter 4 were elaborated separately in the Appendix section.

Abstract

I have investigated the interaction of the dopamine D₁ receptor with ergoline-based ligands to understand how non-catechols activate this receptor. In the current study, we examined the roles of the conserved aromatic residues (F6.51 and F6.52) in transmembrane six segment (TM6), which are known to play a critical role in GPCR activation. An alanine mutation on F6.51 (F6.51A) dramatically decreased the binding affinity of the ergolines, and an alanine mutation on F6.52 (F6.52A) showed significant, but smaller decreases in the ergoline affinity than the F6.51A mutation. The potency of these ergolines for D₁-mediated cAMP synthesis was greatly diminished at the F6.51A mutated receptor within the concentration range we used (maximal 10 μ M). The F6.52A mutation also significantly reduced the potency of the ergolines (consistent with their decreased affinity), but it actually increased the maximal cAMP production. I also studied F6.51Y and F6.51W mutations that likely mimic aromatic and hydrophobic interactions of F6.51. Although the F6.51Y mutation failed to restore the affinity loss caused by the F6.51A mutation, it markedly restored the functional potency. The F6.51W mutation was essentially identical to the wild-type receptor when compared with D₁ affinity and efficacy of the ergolines. Both F6.51A and F6.52A mutations greatly decreased the affinity of the catechol-type D₁ full agonist dihydrexidine (DHX), and the affinity decreases were greater than those of the ergolines. Based on my mutagenesis results and docking simulations, we hypothesize that B-ring and the D-ring of the ergoline backbone have a docking pose close to F6.52 and F6.51, respectively. This structural information provides insight into chemical modifications of the ergolines that may induce better D₁ efficacy.

Introduction

Full D₁ agonists (e.g., dihydrexidine) have been efficacious in Parkinson's disease (PD) animal models and PD patients as well as other neurodisorders (e.g., cognitive deficit and drug addiction) (Rascol et al., 1999; Blanchet et al., 1998; Arnsten et al., 1994; Cai and Arnsten, 1997; Haney et al., 1999; Self et al., 1996). However, full D₁ agonists (e.g., dihydrexidine and ABT-431) are not clinically available primarily due to poor oral bioavailability and dose-limiting side effects (e.g., hypotension). Current full D₁ agonists have a catechol moiety that presumably results in rapid metabolism and short drug action. Although a couple of catechol-type D₁ agonists have been orally available in animals (Kebabian et al., 1992; Gulwadi et al., 2001), how the structural features contribute to oral bioavailability is still elusive. On the other hand, non-catechol dopamine agonists such as ergolines have much longer half-life and duration of action than catechol-type D₁ agonists (Kvernmo et al., 2006; Poewe, 2009). These led us to hypothesize that D₁ agonists without the catechol moiety may have improved oral bioavailability versus catechol-type D₁ agonists.

I chose ergoline derivatives as a non-catechol scaffold because the ergolines do not contain the catechol moiety and generally have longer half-life than catechol-type D₁ agonists (Blanchet, 1999; Kvernmo et al., 2006). Although they have selective interaction with serotonin and dopamine D₂ receptors (Kvernmo et al., 2006; Millan et al., 2002), they have shown D₁ partial agonistic activity (e.g., pergolide and CY 208 243) (Markstein et al., 1992). Our previous results show that T3.37 in the transmembrane three segment (TM3) may interact with the ergolines and dihydrexidine possibly through hydrogen bonds. In addition, we have investigated the interaction of the ergolines with the conserved serines (S5.42, S5.43, and S5.46) in the transmembrane five segment (TM5), which are reported to participate in hydrogen bond interactions with catechol-type D₁ ligands. Contrary to our hypothesis, a S5.42A mutation actually increased D₁ affinity and efficacy of the ergoline agonists suggesting inhibitory interaction occurs between the hydroxyl group of S5.42 and the ergolines. Although a S5.46A mutation failed to reduce the D₁ efficacy of the ergolines, it significantly decreased the affinity of

the ergolines. A S5.43A mutation failed to decrease the D₁ affinity and efficacy of the ergoline agonists consistently suggesting that S5.43 may not participate in D₁ receptor interaction with the ergolines.

I expanded the receptor-ligand interaction study to the transmembrane segment six (TM6) of the D₁ receptor, which has been extensively examined for G protein binding and receptor activation (Shi and Javitch, 2002; Cho et al., 1995; Shi et al., 2002). The recent report on the crystal structure of the β_2 -adrenergic receptor revealed that the outward movement of TM6 is critical for G protein binding (Rasmussen et al., 2011b). In the D₂ receptor, conserved five aromatic residues (F6.44, W6.48, F6.51, F6.52, and H6.55) are lined in TM6 and believed to form a hydrophobic face for ligands and trigger TM6 movement at receptor activation (Floresca and Schetz, 2004; Javitch et al., 1998). The D₁ receptor has aspartate N6.55 instead of histidine H6.55, and F6.44 of the D₁ receptor is located relatively far from the center of the ligand binding pocket. Thus, we first focused on three conserved aromatic residues (W6.48, F6.51, and F6.52) of the D₁ receptor for the interaction with the ergolines. Our mutagenesis results were used to refine hypothetical docking poses of the ergolines at the D₁ receptor models suggesting the potential utility of the ergolines for a lead skeleton of non-catechol D₁ agonists.

Results

Receptor expression and K_D of SCH23390 for wild-type and mutated D₁ receptors

No significant binding of radiolabeled SCH23390 was detected at W6.48A mutated receptors. However, the W6.48A mutated receptors were considerably expressed in transiently transfected cells indicating that W6.48A mutated receptors were successfully translated and expressed (Figure 4-1). Because the specific binding of SCH23390 was not observed, the W6.48A mutation appears to greatly diminish the affinity of radiolabeled SCH23390 for the D₁ receptor. Other mutated receptors were expressed similarly to the level of the wild-type except F6.52A mutated receptors that

were expressed considerably less than the wild-type. All the mutated receptors greatly decreased the affinity of SCH23390 at least by 7-fold (Table 4-1).

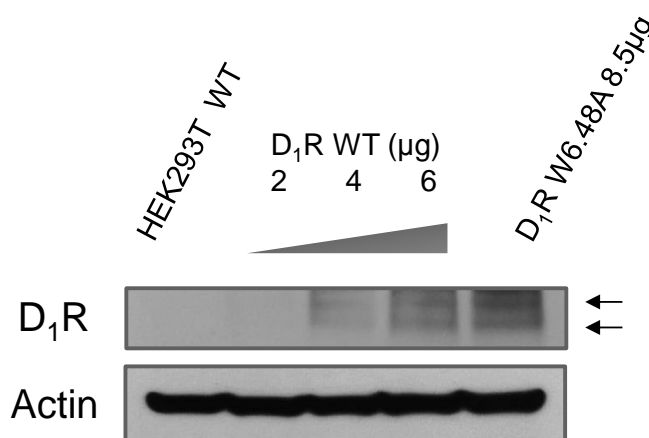


Figure 4-1. W6.48A mutated receptors were expressed in HEK-293 cells.

DNA amounts were shown for transient expression of wild-type and W6.48A mutated receptors.

Table 4-1. Receptor expression and K_D of SCH23390 for wild-type and mutated D₁ receptors.

Receptor	B_{max} (pmol/mg protein) Mean \pm SEM	K_D of SCH23390 (nM)
Wild-type	1.0 \pm 0.2	1.0 \pm 0.1
W6.48A	N.A.	N.A.
F6.51A	1.5 \pm 0.2	7.8 \pm 0.6
F6.51Y	0.8 \pm 0.1	10.2 \pm 0.7
F6.51W	0.8 \pm 0.2	10.5 \pm 0.6
F6.52A	0.4 \pm 0.03	9.6 \pm 0.5

$N \geq 3$. N.A.; not applicable. B_{max} and K_D were produced using the one-site specific binding mode of non-linear regression.

F6.51A and F6.52A mutations significantly decreased the affinity of the ergoline agonists and dihydrexidine

An F6.51A mutation dramatically decreased the affinity of the ergoline agonists and dihydrexidine, and an F6.52A mutation showed significant, but less decreases than

the F6.51A mutation (Figure 4-2A and 4-2B). The decreases in dihydrexidine affinity by the F6.51A and F6.52A mutations were much greater than those of the ergoline agonists. Aromatic ring-conservative F6.51Y and F6.51W mutations were made to test the hydrophobic and aromatic interactions provided by F6.51. Although the F6.51Y mutation failed to restore the affinity decreases of both the ergoline agonists and dihydrexidine, the F6.51W mutation markedly restored the affinity loss caused by the F6.51A mutation. The F6.51W mutation restored the affinity loss of CY 208 243 somewhat greater than that of LEK-8829. Although the F6.51A mutation significantly decreased the affinity of butaclamol, the effects of F6.51A and F6.52A mutations on butaclamol affinity were much smaller than those on the affinity of the ergoline agonists and dihydrexidine (Figure 4-2C). Table 4-2 summarizes the affinity changes of the test ligands by the mutations.

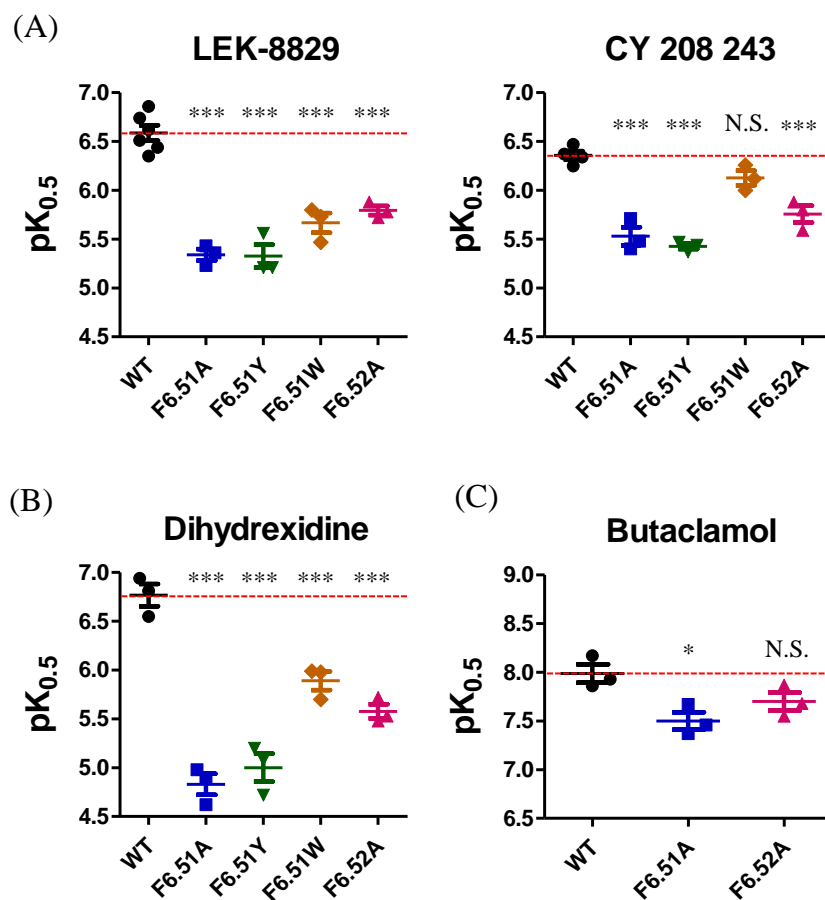


Figure 4-2. Affinity changes of (A) the ergoline agonists, (B) dihydropyridine, and (C) a D₁ antagonist butaclamol by the mutations on the conserved aromatic residues in TM6. Significant differences from the wild-type receptor are shown (* P < 0.05, *** P < 0.001; one way ANOVA with Dunnett's multiple comparison test). N.S.; not significant.

Table 4-2. Affinity changes of the ergoline agonists and dihydrexidine by the mutations on the conserved aromatic residues in TM6.

	pK _{0.5} (Mean ± SEM)					
	LEK-8829		CY 208 243		Dihydrexidine	
Wild-type	6.59 ± 0.08	(1)	6.36 ± 0.04	(1)	6.76 ± 0.12	(1)
F6.51A	5.34 ± 0.06	(17.8)***	5.53 ± 0.09	(6.8)***	4.83 ± 0.11	(85.3)***
F6.51Y	5.33 ± 0.12	(18.4)***	5.43 ± 0.03	(8.6)***	5.00 ± 0.15	(57.5)***
F6.51W	5.67 ± 0.10	(8.4)***	6.13 ± 0.07	(1.7) ^{N.S.}	5.89 ± 0.10	(7.4)***
F6.52A	5.79 ± 0.05	(6.2)***	5.76 ± 0.09	(4.0)***	5.58 ± 0.07	(15.3)***

N ≥ 3. N.S.; not significant. Fold decreases in K_{0.5} were shown in the parentheses. One-way ANOVA with Dunnett's multiple comparison test was used for statistical differences between the wild-type and the mutated receptors (*** P < 0.001).

Effects of non-conservative or conservative mutations on D₁ efficacy of the ergoline agonists and dihydrexidine

A non-conservative F6.51A mutation greatly diminished cAMP production mediated by the ergoline agonists and dihydrexidine within the concentration range we used (maximum 10 μM) (Figure 4-3). Another non-conservative F6.52A mutation also significantly decreased the potency of the ergoline agonists and dihydrexidine for cAMP production (Table 4-4). However, the maximal levels of cAMP production were actually increased at the F6.52A mutated receptor (Table 4-3). Although an aromatic ring-conservative F6.51Y mutation failed to restore the loss of the affinity caused by the F6.51A mutation, the ergoline-type D₁ agonists (10 μM) produced cAMP at the F6.51Y mutated receptor similarly to at the wild-type receptor. Another conservative F6.51W mutation mimicked the potency of the ergoline agonists and dihydrexidine at the wild-type receptors. Moreover, the F6.51W mutation considerably increased the maximal levels of cAMP production compared to the wild-type receptor.

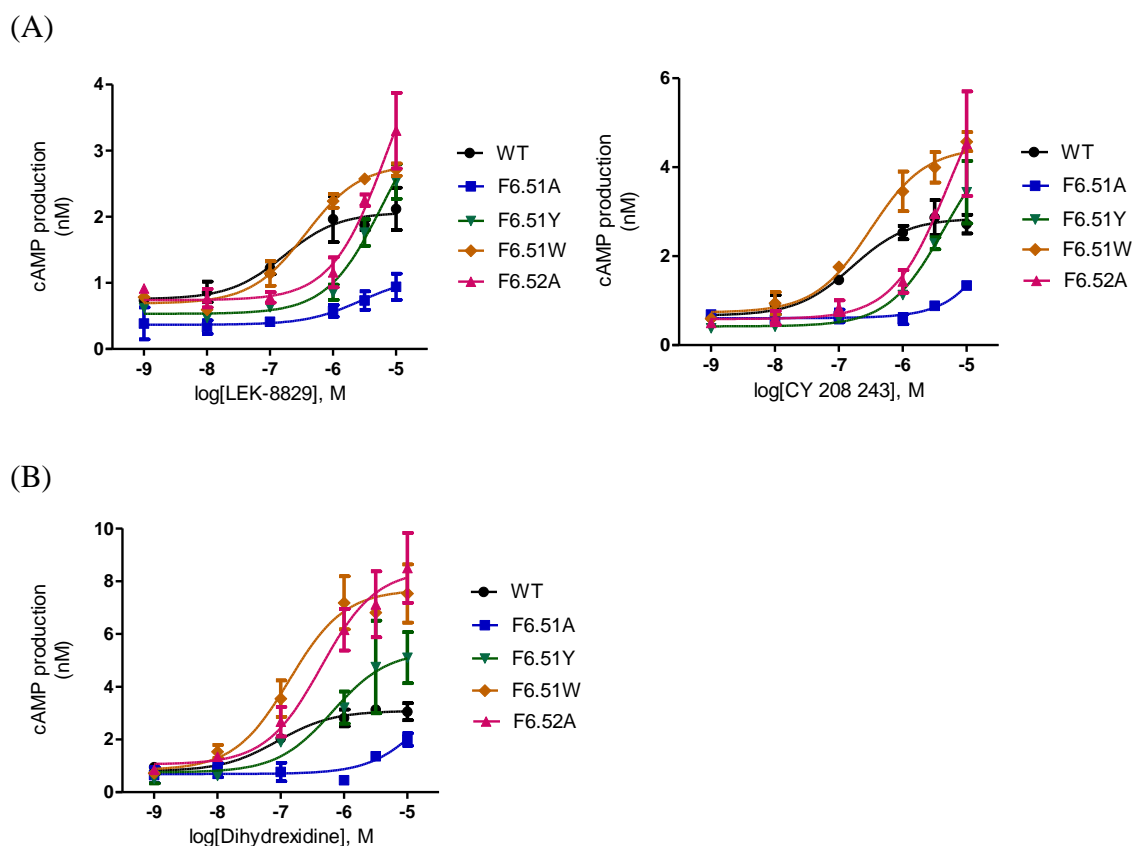


Figure 4-3. cAMP production stimulated by (A) the ergoline agonists and (B) dihydrexidine.

Table 4-3. Maximal levels of cAMP production stimulated by the ergoline agonists and dihydrexidine at the 10 μ M concentrations.

	LEK-8829	CY 208 243	Dihydrexidine
	cAMP production for 5 min (nM)		
Wild-type	2.0 \pm 0.1	2.8 \pm 0.2	2.6 \pm 0.3
F6.51A	0.9 \pm 0.1*	1.1 \pm 0.1*	1.5 \pm 0.3
F6.51Y	2.4 \pm 0.3	3.5 \pm 0.2	4.0 \pm 0.7
F6.51W	3.0 \pm 0.3*	3.7 \pm 0.5	6.7 \pm 0.6*
F6.52A	3.7 \pm 0.4***	5.6 \pm 0.9***	9.7 \pm 1.6***

Mean \pm SEM, $N \geq 3$. Significant differences from the wild-type are shown (* $P < 0.05$, *** $P < 0.001$; one-way ANOVA with Dunnett's multiple comparison test).

Table 4-4. pEC₅₀ of the ergoline agonists and dihydrexidine for D₁-mediated cAMP production.

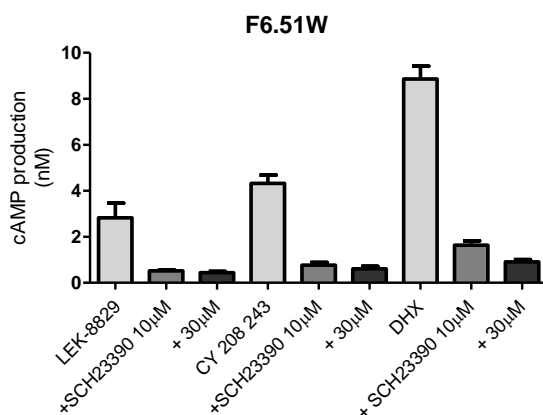
	LEK-8829	CY 208 243	Dihydrexidine
	pEC ₅₀ (M)		
Wild-type	6.52 ± 0.06	6.83 ± 0.14	7.46 ± 0.19
F6.51A	N.A.	N.A.	N.A.
F6.51Y	5.15 ± 0.08***	5.42 ± 0.17***	6.24 ± 0.06*
F6.51W	6.20 ± 0.12	6.64 ± 0.17	6.74 ± 0.14
F6.52A	5.28 ± 0.17***	5.45 ± 0.11***	6.35 ± 0.31*

Mean ± SEM, N ≥ 3. N.A.; not applicable. Significant differences from the wild-type are shown (* P <0.05, *** P <0.001; one-way ANOVA with Dunnett's multiple comparison test).

Inhibition of cAMP production by SCH23390 at F6.51W and F6.52A mutated receptors

F6.51W and F6.52A mutations greatly increased cAMP production mediated by the ergoline agonists and dihydrexidine. I examined whether these increases are mediated by the D₁ receptor. Co-treatment of the D₁ receptor antagonist SCH23390 markedly blocked the cAMP production dose-dependently suggesting that the cAMP production is a D₁ receptor-mediated response (Figure 4-4A and 4-4B). Although SCH23390 inhibited cAMP significantly at F6.51W and F6.52A mutated receptors, cAMP production by dihydrexidine at the F6.52A mutated receptor was not completely inhibited by the D₁ antagonist SCH23390 even at the 30 μM concentration.

(A)



(B)

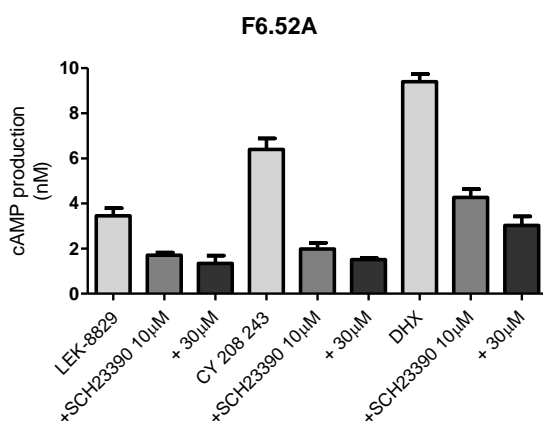


Figure 4-4. The D₁ receptor antagonist SCH23390 inhibits cAMP production at (A) F6.51W and (B) F6.52A mutated receptors.

Mutational effects of the TM6 aromatic residues on other ergoline-type D₁ ligands

Consistent with the results of LEK-8829 and CY 208 243, both F6.51A and F6.52A mutations significantly decreased the affinity of cabergoline, and the effect of F6.51A mutation was greater than that of the F6.52A mutation. As expected, the aromatic-ring conservative F6.51W mutation partially restored the affinity loss of cabergoline (Figure 4-5). Both F6.51A and F6.52A mutations significantly decreased the affinity of lisuride. The F6.52A mutation decreased the affinity of lisuride greater than

the F6.51A mutation. Interestingly, the F6.51A mutation failed to affect the affinity of terguride, but the F6.52A mutation markedly decreased it (Figure 4-5). Lisuride affinity at the F6.51W mutated receptor was similar to that at the wild-type receptor confirming the critical role of hydrophobic and aromatic interactions between the F6.51 and lisuride. Because lisuride and terguride have a very similar chemical structure, the great difference in the F6.51A mutational effects led us to further examine the interaction of F6.51 with lisuride and terguride at the D₁ receptor model.

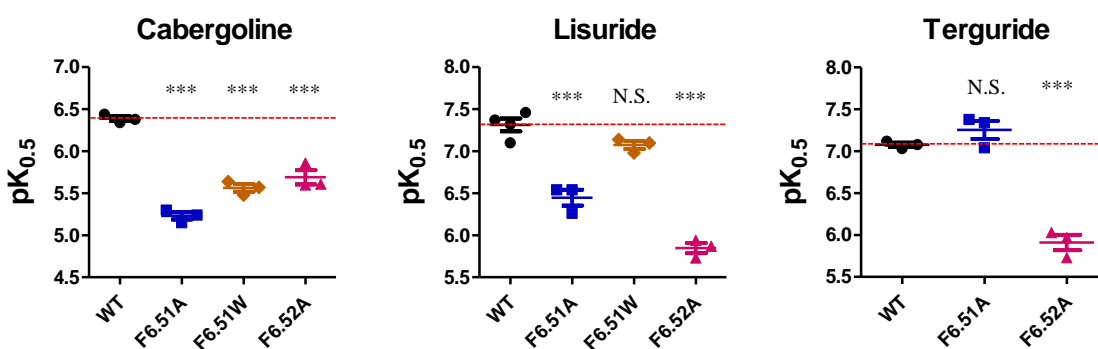


Figure 4-5. Effects of F6.51A, F6.51W, and F6.52A on the affinity of other ergoline-type D₁ ligands.

The F6.52A mutation decreased the affinity of lisuride and terguride greater than the F6.51A mutation. Significant differences from the wild-type are shown (***) P < 0.001; one way ANOVA with Dunnett's multiple comparison test). N.S.; not significant.

Docking simulations of the ergoline agonists with the active-state D₁ receptor homology model

The active-state D₁ receptor homology model was constructed using the structure of the nanobody-stabilized β_2 -adrenergic receptor that is suggested to be the active state of the receptor (PDB: 3P0G). Figure 4-6 shows that B-ring and D-ring of the ergoline backbone are located close to F6.52 and F6.51, respectively. The docking poses of the two ergoline agonists (LEK-8829 and CY 208 243) at the D₁ receptor model were very similar. The computational simulations suggested that the F6.51A mutation reduced the hydrophobic interactions between the ergoline agonists and the D₁ receptor, and that the aromatic ring-conservative F6.51W mutation markedly restored the reduction as expected (Figure 4-7).

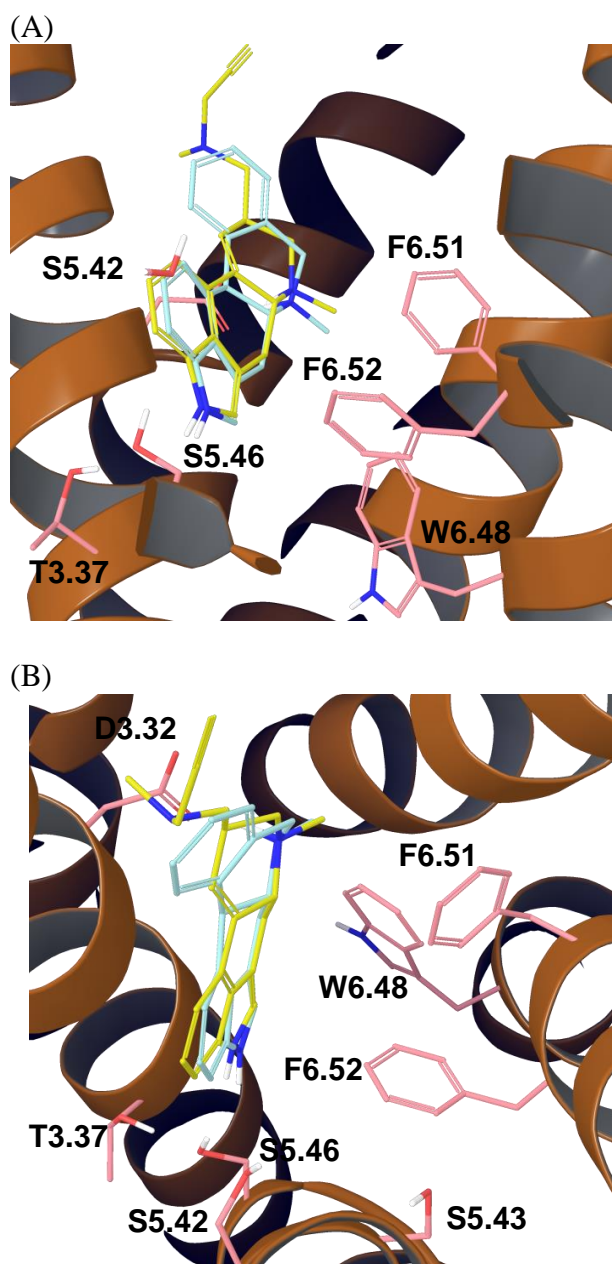


Figure 4-6. Docking poses of the ergoline agonists to the active-state D₁ receptor homology model. (A) A front view and (B) an upright view.

The orange ribbons indicate the active-state D₁ receptor model. LEK-8829 and CY 208 243 are depicted as yellow and light blue, respectively. Pink indicates the conserved residues of the active-state D₁ receptor model.

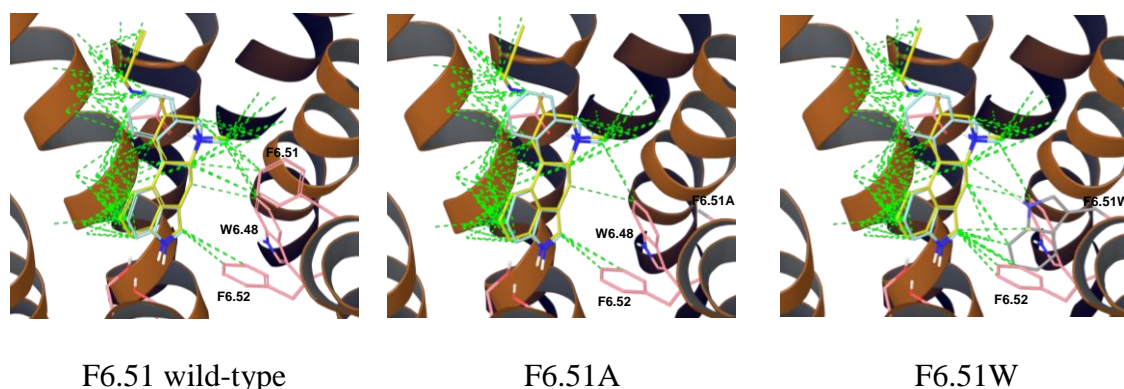


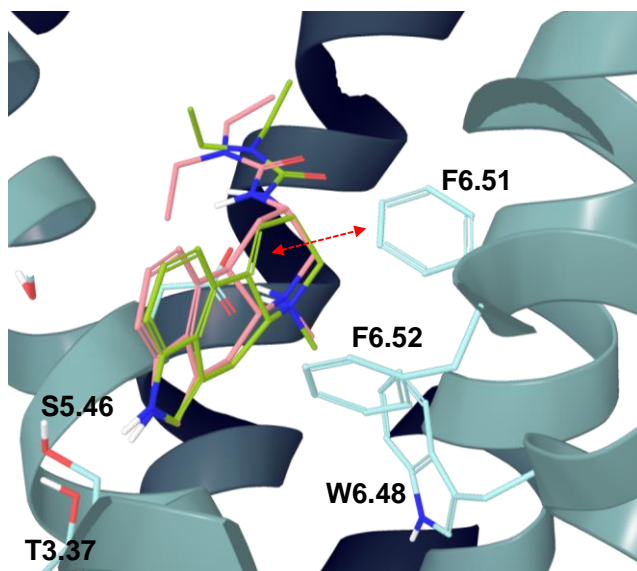
Figure 4-7. Hypothetical hydrophobic interactions between the D₁ receptor and the ergoline agonists.

The F6.51W mutation restored the loss of the hydrophobic interactions caused by the F6.51A mutation. The dotted green line shows possible hydrophobic interactions. LEK-8829 and CY 208 243 are depicted as yellow and light blue, respectively. Pink and grey indicate the wild-type and mutated residues of the active-state D₁ receptor model, respectively.

Docking simulations of lisuride and terguride with the inactive-state D₁ receptor homology model

I used the inactive-state model of the D₁ receptor for the docking poses of lisuride and terguride because they have been known as D₁ receptor antagonists or partial agonists with low intrinsic activity (Markstein et al., 1992; Fici et al., 1997). The only difference in the chemical structure between lisuride and terguride is that lisuride has a carbon-carbon double bond in the D-ring, whereas terguride has a carbon-carbon single bond. The docking poses of lisuride and terguride were very similar, and the D-rings of both lisuride and terguride were located close to F6.51 (Figure 4-8) suggesting that the interaction with F6.51 may contribute to the affinity changes by the F6.51A mutation. Lisuride may interact with the aromatic ring of F6.51 stronger than terguride due to its carbon-carbon double bond. Terguride has no intrinsic activity, but lisuride is a partial D₁ agonist with low intrinsic activity (Figure 4-9). Thus, compared with terguride the additional interaction between lisuride and F6.51 may contribute to the D₁ intrinsic activity of lisuride.

(A)



(B)

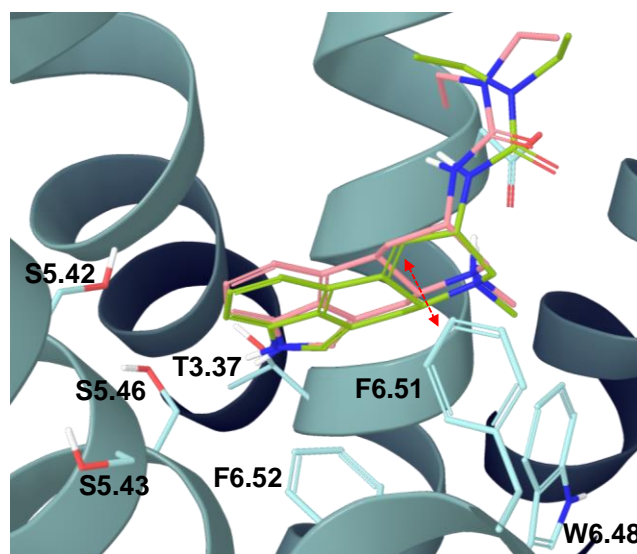


Figure 4-8. Docking simulations with lisuride and terguride at the inactive-state D₁ receptor homology model from (A) a front view and (B) an upright view.

Lisuride and terguride are depicted as green and pink, respectively. Blue indicates the conserved residues of the inactive-state D₁ receptor model.

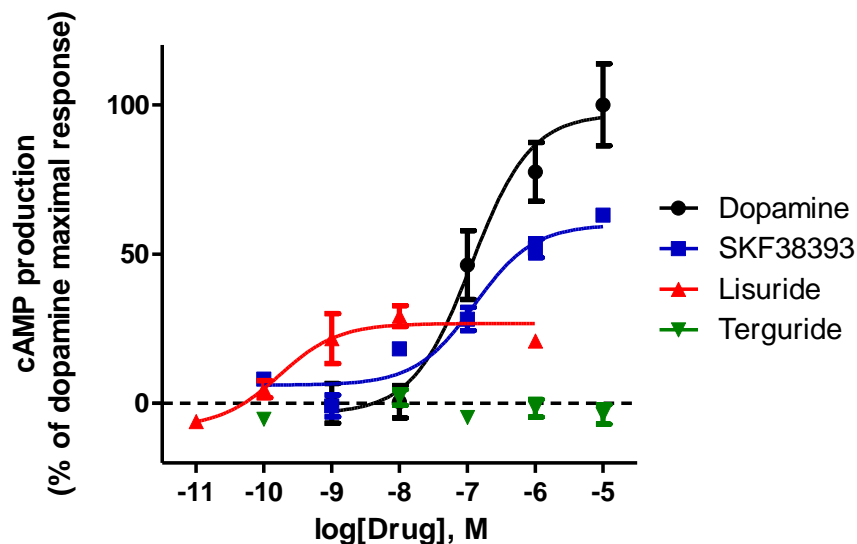


Figure 4-9. D₁-mediated cAMP production by lisuride and terguride at CHO cells stably transfected with human D₁ receptors.

SKF38393 was used as the typical partial D₁ agonist. Representative curves are shown from two independent experiments.

Discussion

The ligand binding pocket of GPCRs for biogenic amines is mainly comprised of the conserved residues in TM3, TM5, TM6, and TM7 (Shi and Javitch, 2002). The crystal structure of the β_2 -adrenergic receptor bound with G_{α_s} protein shows that the outward movement of the TM6 segment is necessary for G protein binding (Rasmussen et al., 2011b). Although the molecular mechanisms for the outward movement of TM6 are still unclear, interactions between conserved TM6 aromatic residues are suggested to be the main force for the TM6 movement and G protein binding (Shi et al., 2002). For Class A GPCRs, highly conserved aromatic residues (e.g., W6.48, F6.51, and F6.52) have been suggested as the residues that participate in these aromatic interactions (Floresca and Schetz, 2004). In the dopamine D₁ receptor, the current study suggests that F6.51 and F6.52 interact with D-ring and B-ring of the ergoline backbone through possible hydrophobic and aromatic interactions, respectively. As the first mutagenesis report on the TM6 aromatic residues of the D₁ receptor, our results provide structural

information on how the ergolines interact with the conserved aromatic residues of TM6 in the D₁ receptor.

Critical interaction of F6.51 and F6.52 with the ergoline agonists and dihydrexidine

An F6.51A mutation greatly decreased the D₁ affinity and efficacy of both the ergoline agonists and dihydrexidine within the concentration range we used (maximal 10 μ M). The F6.51A mutation also significantly decreased the affinity of cabergoline and lisuride. Although this mutation significantly, but moderately decreased the affinity of the D₁ receptor antagonist butaclamol, it had no effect on the affinity of another D₁ antagonist terguride. The order of affinity decreases was a full agonist dihydrexidine > partial agonists (LEK-8829, CY 208 243, cabergoline, and lisuride) > antagonists (butaclamol and terguride). This suggests that F6.51 may form stronger interaction with the full agonists than the partial agonists or antagonists, and that F6.51 may be a critical residue for D₁ receptor activation. An aromatic ring-conservative F6.51W mutation markedly restored the loss of the D₁ affinity and efficacy of dihydrexidine, LEK-8829, and CY 208 243 caused by the F6.51A mutation. Computational simulations suggest that the F6.51W mutation restored the hydrophobic interactions with the ergolines decreased by the F6.51A mutation. The critical role of F6.51 was also reported in the D₂ receptor interaction. An F6.51A mutation in the D₂ receptor greatly decreased the affinity of the ergoline-type D₂ full agonist bromocriptine (Cho et al., 1995).

An F6.52A mutation significantly decreased the affinity and functional potency of the ergoline agonists and dihydrexidine, but less than the F6.51A mutation. Unexpectedly, the F6.52A mutation actually increased the maximal cAMP produced by the ergoline agonists and dihydrexidine. Although the interaction of F6.52 may contribute to the D₁ receptor affinity, the loss of the phenyl group on F6.52 may be favorable for D₁ receptor activation. For example, several single point mutations have been reported to enhance the basal GPCR activity by interrupting intramolecular interaction and inducing a high energy state (Cotecchia et al., 2003). Although the F6.52A mutation failed to affect the basal activity of the D₁ receptor, the removal of the phenyl ring at F6.52 may

induce the D₁ receptor conformation that is more favorable for the agonist-mediated D₁ activation.

Consistent with my results, an F6.52A mutation at the serotonin 5-HT_{2A} receptor greatly impaired the binding of lisuride and ergonovine (Choudhary et al., 1995). A recent paper reported the crystal structures of 5-HT_{1B} and 5-HT_{2B} serotonin receptors bound with ergotamine (Wang et al., 2013), and the docking poses of ergotamine at the serotonin receptors were similar to those of the ergoline agonists at the D₁ receptor model shown in the current study. These suggest that the several conserved amino acids in TM6 of both the dopamine and serotonin receptors commonly contribute to the interaction with the ergolines.

Different interaction of F6.51 with lisuride and terguride

Lisuride and terguride share most of their chemical structure, and the only difference is that the presence or absence of the carbon-carbon double bond in D-ring of the ergoline backbone. Despite the chemical similarity, different pharmacological profiles between lisuride and terguride have been reported at several receptors. Lisuride acts as a weak partial agonist at serotonin 5-HT_{2A} receptor, whereas terguride behaves as a silent antagonist (Kekewska et al., 2012; Kurrasch-Orbaugh et al., 2003). At the histamine H₁ receptor, lisuride has over 10-fold higher binding affinity for the guinea pig H₁ receptor than terguride (Pertz et al., 2006). Lisuride is a low intrinsic activity partial agonist for D₁-mediated cAMP synthesis (Fici et al., 1997), whereas terguride was known to be a D₁ receptor antagonist (Markstein et al., 1992). I also examined the partial intrinsic activity of lisuride and an antagonistic property of terguride for D₁-mediated cAMP production (Figure 4-9). Figure 4-5 shows that lisuride has slightly higher affinity than terguride for the D₁ receptor (54 nM Vs 84 nM). In addition, the F6.51A mutation greatly decreased the affinity of lisuride, but not terguride suggesting lisuride and terguride may have different interaction with F6.51. Computational docking simulations illustrate that the D-ring double bond of lisuride is located close to F6.51. Taken together, we hypothesize that lisuride may have additional interaction with F6.51 compared with terguride, and this

interaction may provide a scientific mechanism for how lisuride has higher D₁ affinity and efficacy than terguride.

The role of W6.48 in GPCR activation

In the ligand-binding pocket of the D₂ receptor, five conserved aromatic residues (F6.44, W6.48, F6.51, F6.52, and H6.55) in the TM6 segment are believed to form hydrophobic contacts. These residues can generate steric clashes that reorient neighboring aromatics (Floresca and Schetz, 2004). In particular, the movement of tryptophan W6.48 from perpendicular to parallel orientation was observed in rhodopsin activation, and this is suggested to trigger the movement of TM6 (Lin and Sakmar, 1996). Although the hypothesis of “W6.48 movement” has not yet been directly tested for the D₁ or D₂ receptor, a mutagenesis study with the D₂ receptor shows that a W6.48C mutation greatly reduced the affinity of D₂ antagonists such as [³H]-methylspiperone and [³H]-sulpride (Javitch et al., 1998). In the present study, no binding of [³H]-SCH23390 was detected for the W6.48A mutated D₁ receptor. Although the W6.48A mutated D₁ receptors were significantly expressed in the transfected cells (Figure 4-1), the mutation appears to greatly decrease the binding affinity of [³H]-SCH23390 for the D₁ receptor. These suggest that W6.48 of the D₁ or D₂ receptor at least contributes to the ligand interactions.

However, the conformations of W6.48 were almost the same at the active- and the inactive-state D₁ receptor homology models. This is because, for the homology models, we used the crystal structures of the β_2 -adrenergic receptors as templates where the conformations of W6.48 were unchanged at the active and inactive states of the receptor (Rasmussen et al., 2011a). A recent mutagenesis study with serotonin 5-HT₄ receptors showed that the rotamer change of W6.48 contributed to stabilizing the receptor in the presence of synthetic agonists, but it was not involved in the receptor activation mediated by an endogenous ligand serotonin (Pellissier et al., 2009). More recently, active-state GPCRs were reported to have multiple conformations (Fowler et al., 2012). Thus, we hypothesize that the rotamer conformational change of W6.48 represents one type of the

multiple mechanisms for GPCR activation and may be unrelated to the D₁ receptor activation.

Interestingly, the computational simulations suggest that tryptophan 7.43 (W7.43) forms strong hydrophobic interactions with D-ring of the ergolines particularly at the active state D₁ receptor model (data not shown). In contrast, at the inactive D₁ receptor model W7.43 shows no hydrophobic interactions with the ergoline agonists. In addition, the hydrophobic accessory parts of the ergoline agonists (the moiety out of the ergoline backbone) are suggested to form hydrophobic interactions with phenylalanine 7.35 (F7.35) and valine 7.39 (V7.39) in TM7. These suggest that the amino acids in TM7 such as F7.35, V7.39, and W7.43 may participate in the interaction with the ergolines. Thus, the interaction of these conserved TM7 residues should be investigated for future studies.

Insight into chemical modifications on the ergoline-type D₁ agonists

Full D₁ receptor activation is required to show satisfactory improvement in Parkinson's disease symptoms (Emre et al., 1992). Because the ergoline derivatives used in the present study are all partial D₁ agonists, the ergolines should be chemically modified to enhance their D₁ efficacy. There have been several reports on structure-activity relationship with the ergolines. The methyl group on D-ring nitrogen of the ergoline backbone was replaced with an ethyl or a propyl group to examine if the longer alkyl groups may affect the D₁ receptor interaction. Addition of an ethyl or a propyl group on CY 208 243 failed to enhance the D₁ efficacy and potency (Markstein et al., 1992). Lysergic acid diethylamide (LSD) has the ergoline backbone and is reported to have interactions with dopamine receptors. Although adding an ethyl group on D-ring nitrogen of LSD slightly increased D₁ efficacy, an allyl group was unable to make any significant changes (Watts et al., 1995a). Moreover, addition of a bulky group on D-ring nitrogen of LSD dramatically reduced the affinity for the D₁ and D₂ receptor (Thoma et al., 2009). Bases on these reports, adding an alkyl or a bulky group to D-ring nitrogen of the ergoline backbone may not warrant the improvement of D₁ receptor efficacy.

On the other hand, the necessary hydrophobic region of the ergoline backbone has been targeted for chemical modifications. Seiler et al. (1993) showed that the loss of the benzene ring attached to D-ring of CY 208 243 reduced its D₁ efficacy over 50% with an over 10-fold potency decrease. This suggests that the benzene ring attached to D-ring of the ergoline backbone plays a critical role in D₁ receptor potency and activation. The mutagenesis results and computational simulations support the importance of the hydrophobic D-ring of the ergoline backbone that is suggested to interact with F6.51 of the D₁ receptor. In addition, Mantegani et al. (1999) reported that the ergoline derivatives with an additional dioxopiperazine moiety attached to D-ring showed strong D₁ agonistic activity. Although more structural information is required, adding a hydrophobic moiety on D-ring of the ergoline backbone may enhance interaction with F6.51 and D₁ receptor activation.

Conclusions

We investigated the interaction of the D₁ receptor with the ergolines as a candidate skeleton for non-catechol full D₁ agonists. The mutagenesis results strongly suggest the hydrophobic and aromatic interactions occurring at the conserved F6.51 and F6.52 and the ergoline backbone. Computational simulations support this idea that F6.51 and F6.52 interact with D-ring and B-ring of the ergoline backbone, respectively. This structural information provides a scientific basis for the chemical modifications of the ergolines that may serve as novel non-catechol full D₁ agonists.

Chapter 5: Interaction of rotigotine with the dopamine D₁ receptor: roles of the conserved amino acids in transmembrane five and six segments

Preface

In addition to the ergolines, rotigotine is another non-catechol scaffold that has reasonable D₁ affinity and efficacy. Rotigotine has a unique chemical structure with one hydroxyl group and one thiophene group on the 2-aminotetralin backbone. I interrogated the molecular interactions between the D₁ receptor and rotigotine using the mutations on the conserved amino acids in TM5 and TM6. Two more 2-aminotetralins (5-OH DPAT and 7-OH DPAT) were also used to test the critical role of the thiophene group of rotigotine in D₁ interaction. Because similar methods were applied for ligand interaction studies, the methods used in the Chapter 5 were elaborated separately in the Appendix section.

Abstract

Rotigotine is a dopamine D₂/D₃ receptor agonist clinically used as symptomatic therapy for Parkinson's disease. Although all available D₁ receptor full agonists have a catechol moiety that causes poor oral bioavailability, rotigotine does not contain a catechol moiety but has partial agonistic D₁ activity. The molecular mechanisms by which rotigotine activates the D₁ receptor may provide leads to novel non-catechol D₁ agonists. Thus, we investigated the interaction of the D₁ receptor with rotigotine. Mutations to alanine were made on the D₁ receptor in the conserved serines (S5.42A, S5.43A, and S5.46A) in the transmembrane five segment, as well as the conserved aromatic residues (F6.51A and F6.52A) in the transmembrane six segment that have been reported critical for ligand interactions and GPCR activation. S5.42A and S5.43A single mutations greatly decreased the D₁ affinity and efficacy of rotigotine within the concentration range we used (maximal 10 μ M), whereas a S5.46A mutation failed to make significant changes suggesting S5.42 and S5.43 may form hydrogen bonds with rotigotine. S5.42A/S5.43A double mutations decreased the rotigotine affinity similarly to either the S5.42A or S5.43A single mutation. An F6.51A mutation decreased the affinity of rotigotine to a far greater extent than an F6.52A mutation suggesting that hydrophobic and aromatic interactions of F6.51 are particularly important. An aromatic-ring conservative F6.51W mutation significantly restored the affinity of rotigotine decreased by the F6.51A mutation. In addition, the F6.51A mutation greatly diminished cAMP production mediated by rotigotine, but F6.51W and F6.51Y mutations partially recovered the loss of D₁ efficacy. The F6.51A mutation failed to affect the affinity of 5-OH DPAT and 7-OH DPAT that have no thiophene group strongly suggesting that the thiophene of rotigotine contributes to the interaction with F6.51. These molecular interactions between rotigotine and the D₁ receptor may provide structural information useful for the development of novel non-catechol D₁ agonists.

Introduction

The main hallmark of Parkinson's disease (PD) is degeneration of dopaminergic cells in substantia nigra par compacta (Damier et al., 1999; Hassler R, 1938). A dopamine precursor levodopa has been a gold standard medicine for PD symptoms. However, long term treatment of levodopa has shown the decreases in efficacy and the increases in side effects (e.g., dyskinesias) due to the massive loss of dopaminergic cells as the disease progresses (Marsden and Parkes, 1976; Spencer and Wooten, 1984; Nutt, 1987). Direct activation of dopamine receptors has been pursued as alternative therapy to levodopa. In the early 1990's, molecular cloning studies identified five kinds of dopamine receptors: D₁-like (D₁ and D₅) and D₂-like (D₂, D₃, and D₄) receptors (Sunahara et al., 1991; Zhou et al., 1990; van Tol et al., 1991; Monsma, Jr. et al., 1989; Sokoloff et al., 1990). Previous reports primarily focused on D₂ receptor activation as the molecular target of levodopa because D₂ antagonists showed similar PD symptoms (e.g., extrapyramidal side effects) (Barnes and McPhillips, 1999; Creese et al., 1976; Serrano, 1981). Several D₂ receptor agonists have been developed and clinically approved for PD patients (Calne, 1999; Hobson et al., 1999). Although D₂ selective agonists significantly improve PD symptoms, D₂ receptor activation alone failed to show antiparkinson effects equal to levodopa's efficacy (Parkinson Study Group, 2000).

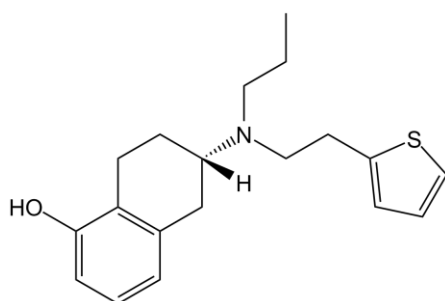
The dopamine D₁ receptor is the most abundant dopamine receptor type in striatum (De Keyser J., 1993; Meador-Woodruff et al., 1996). Full activation of the D₁ receptor has shown satisfactory improvement of the PD symptoms equal to levodopa in animal models and PD patients (Blanchet et al., 1998; Rascol et al., 1999; Taylor et al., 1991). Although full D₁ agonists (e.g., dihydrexidine and ABT-431) have the potential for effective PD therapy, they are not clinically available due to several side effects and pharmacokinetic issues. When full D₁ agonists are given intravenously due to their poor oral bioavailability, dopamine receptor activation in peripheral tissues is suggested as the main cause for the side effect hypotension. A catechol structure in currently available full D₁ agonists is known to cause rapid metabolism in gut and liver and subsequent poor bioavailability (Mailman et al., 2001; Myohanen et al., 2010; Nissinen et al., 1988).

Although in some cases D₁ full agonists with a catechol moiety (A77636, dinapsoline, and doxanthrine) are orally effective in PD animal models (Gulwadi et al., 2001; Kebebian et al., 1992; McCorvy et al., 2012), it is still elusive which structural features contribute to their oral bioavailability. However, dopamine agonists without a catechol moiety generally have higher oral bioavailability than those with a catechol moiety (e.g., ergolines and approved D₂ agonists). Thus, we focused on non-catechol skeletons for the development of novel D₁ agonists with reasonable bioavailability.

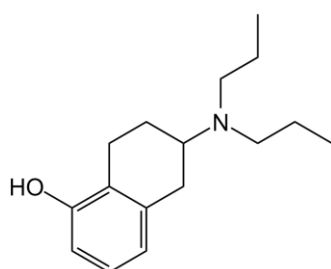
Rotigotine is a clinically approved D₂/D₃ selective full agonist for PD patients. It also has shown reasonable D₁ receptor affinity and efficacy (Scheller et al., 2009). The chemical structure of rotigotine is different than those of the available full D₁ agonists as it does not have a catechol moiety. Although rotigotine is not metabolized by the enzymes targeting a catechol moiety, its oral bioavailability is very low due to the first pass effect at gastrointestinal tracts (Swart and De Zeeuw, 1992). When it is delivered transdermally, however, the bioavailability of rotigotine approaches 37 % without rapid metabolism (Cawello et al., 2009). Although the first pass effect induces poor oral bioavailability of rotigotine, its unique structure as a D₁ agonist led us to hypothesize that rotigotine may serve as a chemical backbone for novel non-catechol D₁ agonists.

To provide a scientific basis for how rotigotine interacts with the D₁ receptor, we investigated the roles of the conserved serines in TM5 and the aromatic amino acids in TM6 of the D₁ receptor (Floresca and Schetz, 2004). Previous studies on the conserved TM5 serines (S5.42, S5.43, and S5.46) showed that the mutations to alanine greatly decreased the interaction with D₁ agonists and that the serines may provide hydrogen bonds for the catechol moiety of the D₁ agonists (Bonner et al., 2011; Chemel et al., 2012; Malo et al., 2012; Pollock et al., 1992). Because rotigotine has one hydroxyl group that can form hydrogen bonds with the conserved TM5 serines, I hypothesized that the conserved TM5 serines will play a role in rotigotine interaction through hydrogen bonds. In addition to the conserved TM5 serines, the conserved TM6 aromatic residues of the β_2 -adrenergic receptor have been suggested to involve TM6 movement and G protein binding by generating steric clashes between the aromatic rings (Shi et al., 2002). Among

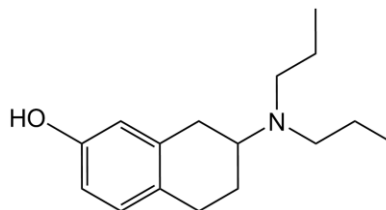
these aromatic residues, F6.44, W6.48, F6.51 and F6.52 are conserved in the D₁ receptor (Floresca and Schetz, 2004). Because F6.44 are located far from the ligand binding pocket relatively to other residues, and because the mutation on W6.48 caused an experimental issue (refer to Chapter 4), I focused on the roles of F6.51 and F6.52 in rotigotine interaction. In addition, the thiophene group of rotigotine is highly likely to contribute to the D₁ efficacy of rotigotine through the interaction with the conserved TM6 aromatic residues. Thus, I test the hypothesis that the conserved TM6 aromatic residues will critically interact with the thiophene group of the rotigotine through hydrophobic and aromatic interactions. I also used two aminotetralins (5-OH DPAT and 7-OH DPAT) that lack the thiophene group to further examine the role of the thiophene group of rotigotine in D₁ receptor interaction. If our hypotheses are correct, I expect the affinity and efficacy of rotigotine would be greatly decreased by the mutations on the residues possibly by removing the molecular interactions (e.g., hydrogen bonds or hydrophobic/aromatic interactions).



Rotigotine



5-OH DPAT (Racemate)



7-OH DPAT (Racemate)

Figure 5-1. Chemical structures of the D₁ ligands used in this chapter.

Results

Receptor expression and the affinity of SCH23390 at wild-type and mutated D₁ receptors

I made single and double point mutations on the conserved TM5 serines and the TM6 aromatic residues of the D₁ receptor. Table 5-1 shows the mutated receptor expressions used in this study. S5.43A and S5.43A/S5.46A mutated receptors were expressed 3.6-fold and 2.3-fold higher than the wild-type, respectively. In contrast, both S5.42A/S5.43A and F6.52A mutated receptors were expressed 2.5-fold less than the wild-type. Other mutated receptors were expressed similarly to the wild-type receptor. The affinity of SCH23390 was greatly decreased by all of the mutations except the

S5.46A mutation by which the affinity of SCH23390 was unchanged. K_D of SCH23390 for each of mutated receptors was used for $K_{0.5}$ calculation of test ligands.

Table 5-1. Wild-type and mutated D_1 receptor expression and the affinity of SCH23390.

Receptor	B_{max} (pmol/mg protein) Mean \pm SEM	K_D of SCH23390 (nM)
Wild-type	1.0 \pm 0.2	1.0 \pm 0.1
S5.42A	0.8 \pm 0.1	10.0 \pm 1.0
S5.43A	3.4 \pm 0.3	8.1 \pm 0.4
S5.46A	1.2 \pm 0.1	0.8 \pm 0.1
S5.42A/S5.43A	0.4 \pm 0.07	14.7 \pm 0.7
S5.42A/S5.46A	0.8 \pm 0.2	11.3 \pm 0.7
S5.43A/S5.46A	2.3 \pm 0.5	14.1 \pm 0.5
F6.51A	1.5 \pm 0.2	7.8 \pm 0.6
F6.51Y	0.8 \pm 0.1	10.2 \pm 0.7
F6.51W	0.8 \pm 0.2	11.8 \pm 0.7
F6.52A	0.4 \pm 0.03	9.6 \pm 0.5

$N \geq 3$. B_{max} and K_D were produced using the one-site specific binding mode of non-linear regression.

Changes in D_1 affinity and efficacy of rotigotine by the mutations on the TM5 conserved serines

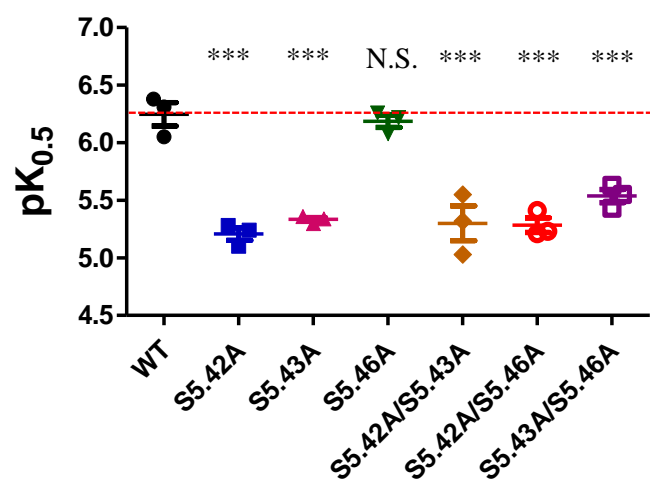
Table 5-2 and Figure 5-2A summarize the affinity changes of rotigotine by the single and double mutations on the conserved TM5 serines. S5.42A and S5.43A mutations greatly decreased the affinity of rotigotine by over 7-fold, whereas a S5.46A mutation failed to make a significant change. The effects of all of S5.42A/S5.43A, S5.42A/S5.46A, and S5.43A/S5.46A double mutations were similar to those of the S5.42A and S5.43A single mutations. Consistent with the affinity results, the S5.42A and S5.43A mutations dramatically reduced the rotigotine efficacy for D_1 -mediated cAMP production (Figure 5-2B), and the S5.46A mutation had no effect on rotigotine efficacy. At all of the double mutated receptors, rotigotine failed to produce cAMP significantly within the concentration range we used (maximum 10 μ M).

Table 5-2. Changes in the D₁ affinity of rotigotine by the mutations.

	pK_{0.5} (Mean ± SEM)
Wild-type	6.25 ± 0.10 (1)
S5.42A	5.21 ± 0.05 (10.6)***
S5.43A	5.33 ± 0.02 (7.8)***
S5.46A	6.19 ± 0.05 (1.1)
S5.42A/S5.43A	5.30 ± 0.15 (9.4)***
S5.42A/S5.46A	5.28 ± 0.06 (8.9)***
S5.43A/S5.46A	5.54 ± 0.06 (4.9)***
F6.51A	5.28 ± 0.08 (9.0)***
F6.51Y	5.47 ± 0.08 (5.8)***
F6.51W	5.82 ± 0.03 (2.6)**
F6.52A	5.86 ± 0.08 (2.4)*

Fold decreases in K_{0.5} were shown in the parentheses. One-way ANOVA with Dunnett's multiple comparison test was used to show significantly differences from the wild-type receptor (* P < 0.05, ** P < 0.01, *** P < 0.001). N ≥ 3.

(A)



(B)

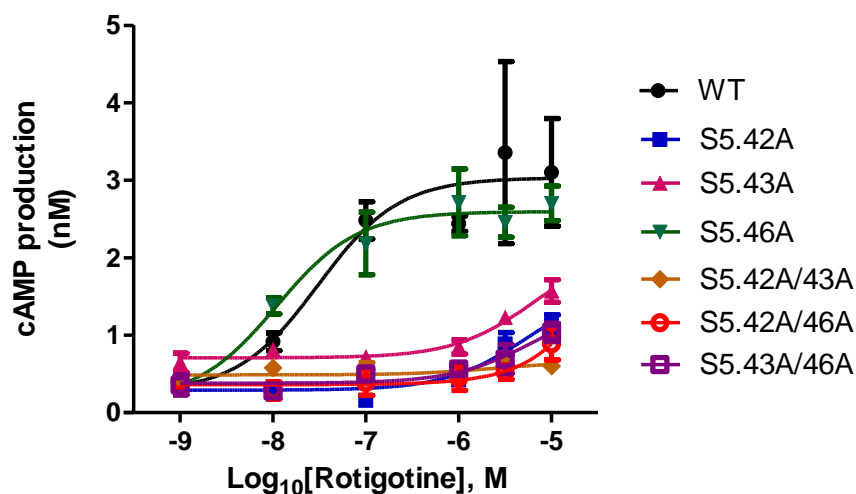


Figure 5-2. Changes in the D₁ affinity and efficacy of rotigotine by the single and double mutations on the conserved TM5 serines.

(A) pK_{0.5} of rotigotine, (B) D₁-mediated cAMP production stimulated by rotigotine.

Effects of S5.42A/S5.43A double mutations on the affinity of the catechol-type D₁ agonist dihydrexidine

Although each of S5.42A and S5.43A mutations greatly decreased the affinity of rotigotine, the S5.42A/S5.43A double mutations were unable to decrease rotigotine affinity greater than the single mutations (Figure 5-2A). I further examined the effect of S5.42A/S5.43A double mutations on the affinity of the catechol-type D₁ agonist

dihydropyridine. Although dihydropyridine markedly lost D_1 receptor affinity by either of the S5.42A and S5.43A mutations, the S5.42A/S5.43A double mutations failed to decrease dihydropyridine affinity significantly greater than the individual S5.42A and S5.43A mutations (Figure 5-3). Together, the S5.42A/S5.43A double mutations mimicked the effects of the single mutations on the affinity of both rotigotine and dihydropyridine.

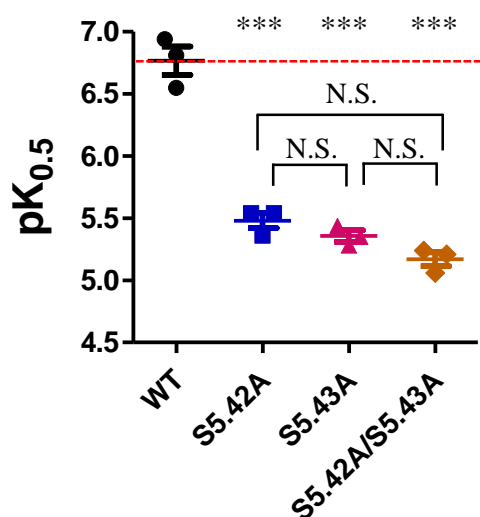


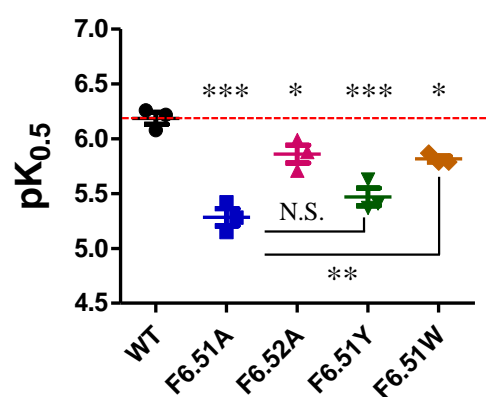
Figure 5-3. Change of dihydropyridine affinity by the S5.42A/S5.43A double mutations.

Changes in D_1 affinity and efficacy of rotigotine by the mutations on the conserved TM6 aromatic residues

F6.51A and F6.52A mutations significantly decreased the affinity of rotigotine by 9-fold and 2.4-fold, respectively (Figure 5-4A). The effect of the F6.51A mutation was significantly greater than that of the F6.52A mutation ($P < 0.001$, ANOVA with Tukey's multiple comparison test) suggesting F6.51 is particularly important for the rotigotine interaction. Interestingly, aromatic ring-conservative F6.51Y and F6.51W mutations partially restored the affinity loss of rotigotine caused by the F6.51A mutation. The F6.51W mutation restored rotigotine affinity greater than the F6.51Y mutation. Rotigotine lost most of D_1 efficacy for cAMP production by the F6.51A mutation (Figure 5-4B). The F6.51Y mutation fully restored the maximal level of cAMP production at the 10 μ M concentration, and the F6.51W mutation seems to further increase the cAMP

production versus the wild-type receptor. The potency of rotigotine was partially restored by both of the aromatic ring-conservative mutations. Consistent with the affinity results, the F6.51W mutation restored the potency of rotigotine greater than the F6.51Y mutation. Although the F6.52A mutation significantly decreased the potency of rotigotine, cAMP production by 10 μ M rotigotine was actually higher than the level at the wild-type receptor (Table 5-3).

(A)



(B)

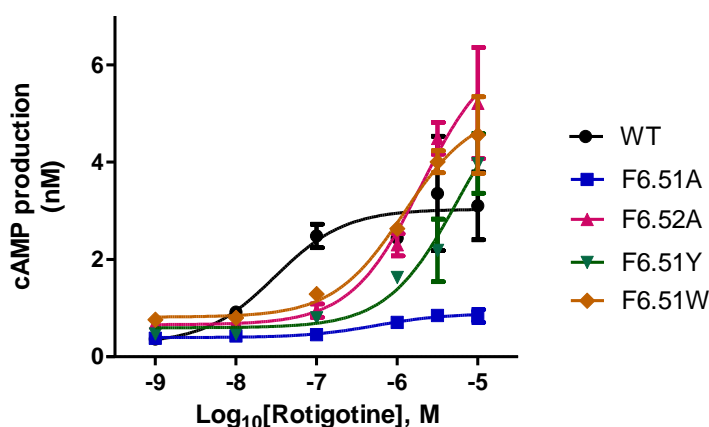


Figure 5-4. Changes in D₁ affinity and efficacy of rotigotine by the mutations on the conserved TM6 aromatic residues.

(A) pK_{0.5} of rotigotine, (B) D₁-mediated cAMP production stimulated by rotigotine.

Table 5-3 cAMP production mediated by 10 μ M rotigotine and pEC50.

	cAMP production (nM)	pEC50 (M)
Wild-type	2.6 \pm 0.4	7.30 \pm 0.16
S5.42A [†]	1.2 \pm 0.1	N.A.
S5.43A	1.6 \pm 0.2	N.A.
S5.46A	2.6 \pm 0.1	7.48 \pm 0.21
S5.42A/S5.43A	0.9 \pm 0.1*	N.A.
S5.42A/S5.46A [†]	1.3 \pm 0.4	N.A.
S5.43A/S5.46A [†]	1.2 \pm 0.1	N.A.
F6.51A [†]	1.1 \pm 0.2	N.A.
F6.51Y	3.6 \pm 0.4	5.44 \pm 0.15***
F6.51W	3.7 \pm 0.5	5.83 \pm 0.13***
F6.52A	5.3 \pm 1.0***	5.93 \pm 0.14***

Significant differences from the wild-type receptor are shown (* $P < 0.05$, *** $P < 0.001$; one-way ANOVA with Dunnett's multiple comparison test). Mean \pm SEM, $N \geq 3$. [†] $N=2$.

Molecular interactions of rotigotine at the D₁ receptor models

I constructed inactive- and active-state D₁ receptor homology models with the crystal structures of the β_2 -adrenergic receptor using PRIME 3.0 in MAESTRO 2012 package. Based on the mutagenesis results, the best docking pose of rotigotine was selected and shown in Figure 5-5. Except for S5.46, the positions of the conserved amino acids in TM5 and TM6 were not considerably changed by receptor activation. Although S5.46 moves towards the ligand binding pocket at the active-state D₁ model, the S5.46 appears far from the hydroxyl group of rotigotine. The hydroxyl group of rotigotine is located close to S5.42 and S5.43, especially in the active-state D₁ receptor model. F6.51 and F6.52 were close to the thiophene group and the aminotetralin backbone of rotigotine, respectively. Based on mutagenesis and simulation results, the thiophene group of rotigotine appears to move toward F6.51 and W6.48 when the D₁ receptor is activated. Figure 5-6 shows hydrophobic interactions of rotigotine at the active-state D₁ receptor model. Loss of the aromatic benzene ring of F6.51 reduced the hydrophobic

interactions between rotigotine and the D₁ receptor, but these interactions were considerably restored by the aromatic-ring conservative F6.51W mutation.

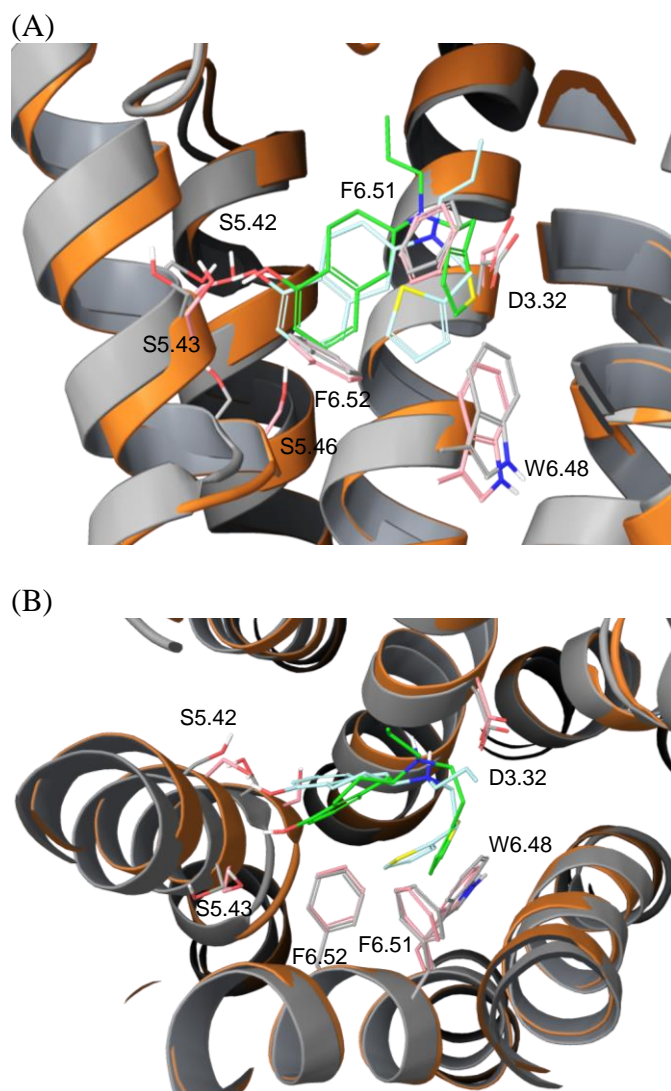


Figure 5-5. Docking poses of rotigotine at the D₁ receptor homology models.

(A) A front view, (B) an upright view. Grey; the inactive-state D₁ receptor model, Orange; the active-state D₁ receptor model, Light blue; rotigotine in the inactive-state model, Green; rotigotine in the active-state model.

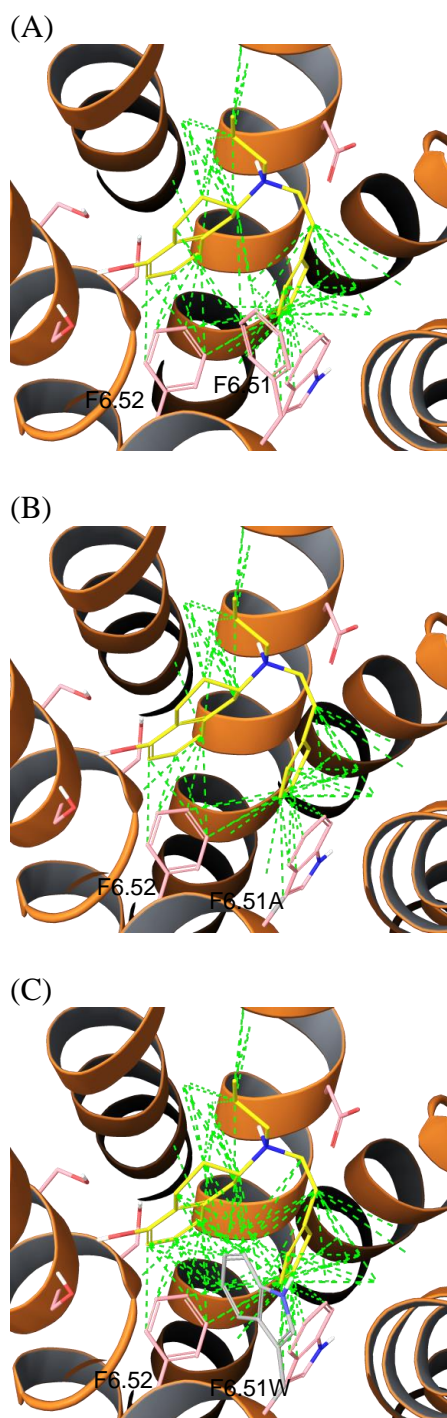


Figure 5-6. Changes in the hydrophobic interactions by F6.51A and F6.51W mutations.

(A) The wild-type D₁ receptor, (B) the F6.51A mutated receptor, (C) the F6.51W mutated receptor. Hydrophobic interactions between the active D₁ receptor model and rotigotine (yellow) were shown with green dotted lines.

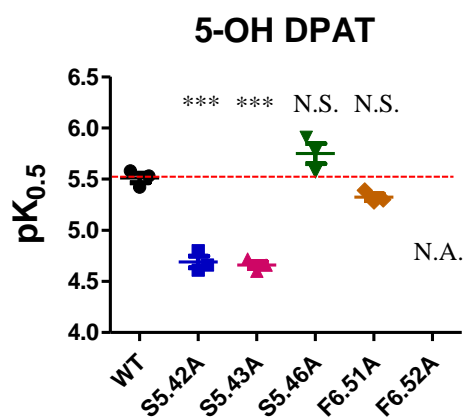
Interactions of the D₁ receptor with other 2-aminotetralin D₁ ligands: 5-OH DPAT and 7-OH DPAT

Racemate 5-OH DPAT and 7-OH DPAT (Figure 5-1) were used to investigate the role of the thiophene group of rotigotine in D₁ receptor interaction. 5-OH DPAT has the chemical backbone and the hydroxyl group same as those of rotigotine, but it does not have the thiophene group. The affinity of 5-OH DPAT for the D₁ receptor was considerably lower than that of rotigotine suggesting the significant role of the thiophene group in D₁ receptor interaction. As expected, S5.42A and S5.43A mutations greatly decreased the affinity of 5-OH DPAT, but a S5.46A mutation has no effect (Figure 5-7A). An F6.51A mutation failed to significantly affect the 5-OH DPAT affinity supporting the interaction between the thiophene group and F6.51. However, an F6.52A mutation greatly decreased the affinity of 5-OH DPAT for the D₁ receptor (Figure 5-7A). In fact, we could not calculate $K_{0.5}$ of 5-OH DPAT at the F6.52A mutated receptor because the whole curve of the competitive binding was not obtained within the concentration range we used (maximum 300 μ M). Docking simulations with the inactive D₁ receptor model suggest that the 5-OH DPAT is located at the receptor similarly to rotigotine (Figure 5-7B).

7-OH DPAT has a hydroxyl group that locates in the different position versus 5-OH DPAT. Due to the different position of the hydroxyl group, we hypothesized that 7-OH DPAT would interact with S5.46 greater than S5.42 and S5.43. Unexpectedly, the S5.46A mutation failed to make any affinity change, but the S5.42A and S5.43A mutations significantly decreased the affinity of 7-OH DPAT (Figure 5-8A). Affinity decreases of 7-OH DPAT by the S5.42A and S5.43A mutations were similar to those of 5-OH DPAT and rotigotine. Docking simulations suggest that 7-OH DPAT has a flip-flop conformation and that the hydroxyl group of 7-OH DPAT is located closer to S5.42 and S5.43 than S5.46 (Figure 5-8B). When 7-OH DPAT approaches the D₁ receptor from the outside of the ligand binding pocket, the hydroxyl group of the flip-flop conformation of 7-OH DPAT may interact with S5.42 and S5.43 before it reaches S5.46. I also hypothesize that this precedent interaction with S5.42 and S5.43 may prevent 7-OH

DPAT from moving down to S5.46 that locates at the bottom of the ligand binding pocket.

(A)



(B)

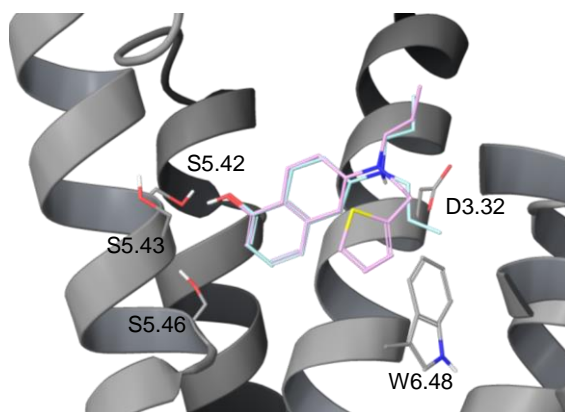
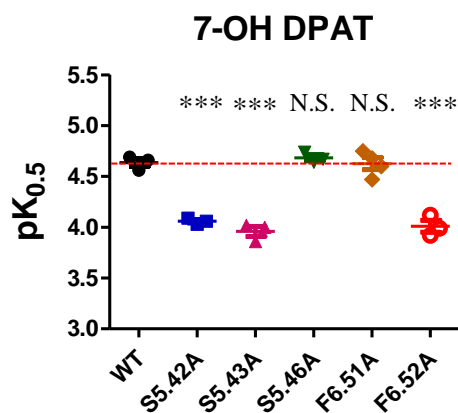


Figure 5-7. Effects of the mutations on the D₁ affinity of 5-OH DPAT.

(A) D₁ affinity changes of 5-OH DPAT. N.A.; non-available due to the lack of the whole competitive binding curve within the concentration range we used (maximum 300 μ M). (B) Docking pose comparison of 5-OH DPAT with rotigotine. Purple and light blue colors indicate rotigotine and 5-OH DPAT, respectively.

(A)



(B)

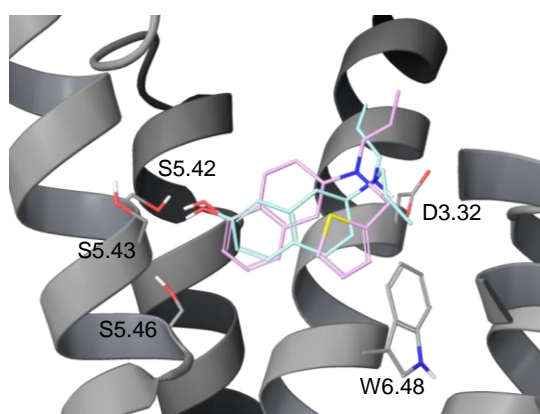


Figure 5-8. Effects of the mutations on the D₁ affinity of 7-OH DPAT.

(A) D₁ affinity changes of 7-OH DPAT. (B) Docking pose comparison of 7-OH DPAT with rotigotine. Purple and light blue colors indicate rotigotine and 7-OH DPAT, respectively.

Discussion

The aminotetralin derivative N-0437 (2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin) was known as a potent D₂ receptor agonist (Van der Weide et al., 1986; Beaulieu et al., 1984). N-0437 was a racemic mixture, and the (–)-enantiomer of N-0437 was reported to have greater potency and efficacy for the D₂ receptor than its (+)-enantiomer (Van der Weide et al., 1987; Timmerman et al., 1989; Belluzzi et al., 1994). The (–)-enantiomer of N-0437 became known as N-0923, and later it has another name

rotigotine. Because N-0437 has selective affinity and efficacy for the D₂ over the D₁ receptor, the molecular interactions of aminotetralin derivatives have been focused on the D₂ receptor (Cho et al., 1995; Mansour et al., 1992). Nevertheless, rotigotine was reported to bind to the D₁ receptor with moderate affinity and activate adenylate cyclase in the D₁ receptor expression systems (Scheller et al., 2009). In addition, aminotetralin derivatives with 5,6-dihydroxy groups were reported to activate D₁-mediated adenylate cyclase in retina homogenates with equi-potency to dopamine (Beaulieu et al., 1984). Based on its unique chemical structure and the D₁ receptor efficacy, rotigotine may be useful as a non-catechol skeleton for the development of novel D₁ agonists. In this chapter, I report that the conserved serine S5.42 and S5.43 and the conserved aromatic residue F6.51 of the D₁ receptor critically interact with the hydroxyl group and the thiophene group of rotigotine, respectively. This structural information helps understand how non-catechol rotigotine interacts with the D₁ receptor and may provide insights into the design of novel non-catechol D₁ agonists.

Hydrogen bond interactions between rotigotine and the D₁ receptor

Conserved serine S5.43 and S5.46 have been investigated as possible hydrogen bond participants for meta- and para-hydroxyl groups of the catecholamines at the α_2 - and β_2 -adrenergic receptors (Strader et al., 1989; Wang et al., 1991). For the D₁ or D₂ dopamine receptor, mutagenesis studies on the conserved TM5 serines (S5.42, S5.43, and S5.46) suggested that hydrogen bonds provided by the serines play a critical role in the affinity and efficacy of catechol-type D₁ agonists (Chemel et al., 2012; Floresca and Schetz, 2004; Pollock et al., 1992). Several studies demonstrated the hydrogen bonds between the catechol moiety and S5.42 and S5.46 of the D₁ receptor (Bonner et al., 2011; Cueva et al., 2011; Malo et al., 2012). Rotigotine has a hydroxyl group that corresponds to the catechol moiety of the known D₁ agonists. A study using radiolabeled N-0437 suggested that the hydroxyl group of N-0437 may form a hydrogen bond with one of the S5.43 and S5.46 of the D₂ receptor (Mansour et al., 1992). Although S5.43A and S5.46A single mutations failed to affect the D₂ receptor affinity of N-0437, N-0437 showed no specific binding for the D₂ receptor with S5.43A/S5.46A double mutations (Mansour et

al., 1992). Racemate 5-hydroxy-N,N-dipropyl-2-aminotetralin (5-OH DPAT) has the same chemical backbone as rotigotine, but it does not have the thiophene group. The loss of the hydroxyl group in 5-OH DPAT markedly decreased the potency for D₂-mediated inhibition of prolactin release suggesting the significant role of the hydroxyl group in D₂ receptor interaction (Beaulieu et al., 1984). Our mutagenesis results also suggest that S5.42 and S5.43 of the D₁ receptor, but not S5.46 may form hydrogen bonds with the hydroxyl group of rotigotine. The hydroxyl group of rotigotine can form bifurcated hydrogen bonds with S5.42 and S5.43. Interestingly, S5.42A/S5.43A double mutations decreased the affinity of rotigotine equal to either the S5.42A or the S5.43A single mutation. Based on these results, I hypothesize that the bifurcated hydrogen bonds exist only when both S5.42 and S5.43 are available, and that either the S5.42 or S5.43 alone may be unable to form a hydrogen bond with the rotigotine.

Critical interactions of the aromatic residues in TM6 with rotigotine

The conserved TM6 aromatic amino acids (e.g., W6.48, F6.51 and F6.52) have been known as the key interaction sites for the activation of dopamine receptors and other GPCRs (Floresca and Schetz, 2004; Shi and Javitch, 2002). Hydrophobic and aromatic interactions between these aromatic amino acids are hypothesized as the main force for TM6 movement and G protein binding (Shi et al., 2002). Although the molecular interactions of rotigotine with the D₁ receptor have not been previously reported, one paper showed that the F6.51A or F6.52A mutated D₂ receptor was unable to bind [³H]-N-0437 (Cho et al., 1995). This suggests that the conserved TM6 aromatic residues of the D₂ receptor play a critical role in rotigotine interaction.

The aminotetralin backbone and thiophene (thienyl) moiety of rotigotine are likely to produce hydrophobic and aromatic interactions with these aromatic residues. The affinity of racemate 5-OH DPAT for the D₂ receptor was relatively lower than that of N-0437 (Glase et al., 1995; van Vliet et al., 1996; Rodenhuis et al., 2000) suggesting that the thiophene group of rotigotine is responsible for the high affinity for the D₂ receptor. In addition, the replacement of the thiophene group of N-0437 with the phenyl moiety

retained D₂ affinity and agonistic activity similarly to those of N-0437 (Beaulieu et al., 1984; Van der Weide et al., 1986). This suggests that hydrophobic and aromatic interactions provided by the phenyl ring can compensate for the role of the thiophene in the D₂ receptor interaction. In the current study, the mutagenesis results suggest that the thiophene group of rotigotine critically interacts with F6.51 possibly through hydrophobic and aromatic interactions. The results that the affinity of 5-OH DPAT and 7-OH DPAT was unchanged by the F6.51A mutation also support the interactions between the thiophene group of rotigotine and F6.51. The aromatic ring of F6.51 and the heterocyclic ring of rotigotine can have a conjugated planar structure presumably using delocalized aromatic pi-electrons. Although the attraction force of aromatic pi-interactions is weaker than that of the hydrogen bond, the aromatic pi-interactions may play a significant role in D₁ receptor interaction with rotigotine (Marsili et al., 2008).

I was unable to obtain the affinity of rotigotine for the W6.48A mutated D₁ receptor due to extremely low specific binding of [³H]-SCH23390 at the mutated receptor. However, the docking simulations suggested the thiophene group of rotigotine is located in proximity of hydrophobic interactions with W6.48 (Figure 5-5). Thus, the role of W6.48 in rotigotine interaction can be pursued as an interesting research topic. In addition, the roles of the conserved TM7 residues of the D₁ receptor (e.g., NPXXY motif) remain to be investigated for better understanding of additional interactions between the D₁ receptor and rotigotine.

Insights into the modifications of rotigotine for better D₁ receptor interaction

Among monohydroxy 2-aminotetralins, 5-OH DPAT showed the highest affinity for the D₁ receptor. 7-OH DPAT and 8-OH DPAT have relatively low affinity for the D₁ receptor suggesting the position of the hydroxyl group seems to contribute to D₁ receptor interaction. In addition, (S)-5-OH DPAT was more potent for dopamine receptor activation than (R)-5-OH DPAT (Seiler and Markstein, 1984; van Vliet et al., 1996) suggesting the chirality on the aminotetralin backbone is also critical for the D₁ receptor interaction. Likewise, (S)-enantiomer rotigotine (N-0923) has higher D₁ receptor affinity

than (R)-enantiomer N-0924 (Belluzzi et al., 1994). Moreover, 5,6-dihydroxy DPAT showed higher potency than monohydroxy 5-OH DPAT for D₁-mediated cAMP production (Beaulieu et al., 1984) as well as for D₂-mediated emetic response (McDermid et al., 1976). Although having two hydroxyl groups in the aminotetralin backbone may provide better interaction with the D₁ receptor, the resulting catechol moiety can be easily metabolized by multiple enzymes such as catechol-O-methyl transferases and Phase II conjugation enzymes. When the 5-hydroxyl group of N-0437 was modified or removed, the affinity for both D₁ and D₂ receptors was greatly weakened *in vitro* (Jansen et al., 1991) suggesting the necessity of the intact 5-OH group for dopamine receptor interactions. Several studies have reported the dopamine receptor activity of the pyridine or thiophene analogs of 5-OH DPAT. Glase et al. (1995) replaced the phenolic portion of 5-OH DPAT with pyridine and tested for D₂ or D₃ receptor interaction. All pyridine substitutions used in the study failed to improve binding and efficacy of 5-OH DPAT for the D₂ and D₃ receptor (Glase et al., 1995). In addition, Rodenhuis et al. (2000) replaced the phenol moiety of 5-OH DPAT with a thiophene moiety, and tetrahydrobenzo[b]thiophenes used in the study also failed to gain better affinity for D₂ and D₃ receptors than that of original 5-OH DPAT. Although the pyridine and thiophene substitutes for the phenolic part of 5-OH DPAT have not been tested for D₁ receptor interaction, previous results with the D₂ receptor suggest that those modifications on 5-OH DPAT would not warrant the improvement of the D₁ receptor interaction.

The thiophene group of rotigotine was modified and tested for better dopamine receptor interaction. Chemical modifications that remove the thiophene group and add a heterocyclic ring next to aminotetralin backbone actually increased the affinity for the D₁ receptor (Risgaard et al., 2014). Although the increase was small and the modified chemical still has higher affinity for the D₂ receptor versus D₁, these results provide evidence that chemical modifications at the thiophene group may improve D₁ receptor interaction of rotigotine. The mutagenesis results and docking simulations also show that the thiophene group of rotigotine is located close to the F6.51 that is suggested as a

critical site for receptor activation. Thus, the modifications on the thiophene group that enhance the hydrophobic and aromatic interactions with F6.51 may produce novel D₁ agonists that show better D₁ interaction than rotigotine.

Conclusions

In the current study, we report the molecular interaction of the D₁ receptor with rotigotine focusing on the conserved amino acids in TM5 and TM6. Although rotigotine is a D₂ and D₃ selective agonist, the D₁ receptor interaction with rotigotine can provide structural knowledge on how non-catechol scaffolds activate the D₁ receptor. The results suggested that the hydroxyl group and the thiophene group of rotigotine may be critical for D₁ receptor interaction via hydrogen bonds (with S5.42 and S5.43) and hydrophobic and aromatic interactions (with F6.51), respectively. D₁ receptor agonists without a catechol moiety can show better pharmacokinetic properties than current catechol-type D₁ agonists, and full D₁ agonists with reasonable oral bioavailability may enhance the utility of D₁ receptor agonists in clinic. The structural information from the current study may serve as a scientific basis for the design of novel non-catechol D₁ agonists.

Chapter 6: Dopamine D₁ receptor signaling: Does Gα_Q-phospholipase C actually play a role?

Preface

Functional selectivity has opened a new era for GPCR drug development in that selective signaling activation mediated by one receptor type may help design more desirable drugs by enhancing efficacy and reducing side effects. As was discussed in the Introduction to Chapter 2, it was of special interest that SKF-83959 has been widely considered a highly-biased functionally selective D₁ ligand that fully activates D₁-mediated phospholipase C, but is an antagonist at D₁-mediated adenylate cyclase. As the studies in Chapter 2 were being finished, I felt that there was an opportunity to do a critical review of the whole issue of D₁ activation of PLC, especially as it impacted the notion of special signaling properties of D₁-D₂ heterodimers that has been extant in papers including many in high impact journals. This chapter is a result of that effort.

[This chapter was my contribution to a *Perspective in Pharmacology* that has been accepted publication in the *Journal of Pharmacology and Experimental Therapeutics*. Sang-Min Lee, Yang Yang, and Richard B. Mailman. Dopamine D₁ receptor signaling: Does Gα_Q-phospholipase C actually play a role? *Journal of Pharmacology and Experimental Therapeutics*. S-ML was the lead author, with substantial contributions from the co-authors.]

Abstract

Despite numerous studies showing therapeutic potential, no central dopamine D₁ receptor ligand has ever been approved because of potential limitations such as hypotension, seizures, and tolerance. Functional selectivity has been widely recognized as providing a potential mechanism to develop novel therapeutics from existing targets, and a highly-biased functionally selective D₁ ligand might overcome some of the past limitations. SKF-83959 is reported to be a highly biased D₁ ligand, having full agonism at D₁-mediated activation of phospholipase C (PLC) signaling (via G α_Q) and antagonism at D₁-mediated adenylylate cyclase signaling (via G $\alpha_{OLF/S}$). For this reason, numerous studies have used this compound to elucidate the physiological role of D₁-PLC signaling, including a novel molecular mechanism (G α_Q -PLC activation via D₁:D₂ heterodimers). There is, however, contradictory literature that suggests that SKF-83959 is actually a partial agonist at both D₁-mediated adenylylate cyclase and β -arrestin recruitment. Moreover, the D₁-mediated of PLC stimulation has also been questioned. This perspective reviews 30 years of relevant literature, and proposes that the data strongly favor alternate hypotheses, first that SKF-83959 is a typical D₁ partial agonist, and second, that the reported activation of PLC by SKF-83959 and related 1-phenyl-3-benzazepines likely is due to off-target effects, not actions at D₁ receptors. If these hypotheses are supported by future studies, it would suggest that caution should be used regarding the role of PLC and downstream pathways in D₁ signaling.

The potential utility of a functional selective D₁ ligand

Animal models have suggested the utility of selective D₁ agonists for a variety of therapeutic targets including Parkinson's disease (Taylor et al., 1991; Mailman et al., 2001; Mailman and Nichols, 1998), cognition (Arnsten et al., 1994; Schneider et al., 1994; Steele et al., 1996; Steele et al., 1997; Goldman-Rakic et al., 2004; Toda and Abi-Dargham, 2007; Rosell et al., 2014), and other disorders. In fact, for Parkinson's disease, the efficacy of D₁ agonists in animal models has translated into clinical trials with large effect sizes similar to those seen in the preclinical models (Rascol et al., 2001; Rascol et al., 1999). A recent preliminary clinical study (Rosell et al., 2014) also reported significant effects on working memory consistent with predictions from preclinical models (Arnsten et al., 1994; Schneider et al., 1994; Steele et al., 1996; Steele et al., 1997). Despite this support for the clinical efficacy of D₁ agonists, there are reports of serious D₁-mediated side effects that may prevent approval of a D₁ agonist, including rapid tolerance (Asin and Wirtshafter, 1993; Gulwadi et al., 2001), profound hypotension (Blanchet et al., 1998), and seizures (Starr, 1996).

During the past decade, it has become clear that one way of improving a pharmacological profile is to discover functionally selective ligands for the target receptor (Urban et al., 2007; Neve, 2009; Kenakin, 2007; Mailman, 2007). Functional selectivity describes the property of some ligands to interact with a single receptor and differentially affect the signaling pathways engaged by that target. The degree to which the ligand differentially affects signaling pathways is termed the bias of the ligand. The first use of the term functional selectivity in the context of a single receptor was with dopamine receptor ligands (Mailman et al., 1998; Lawler et al., 1999; Mailman et al., 1998), and the earliest therapeutic utility of this mechanism was suggested by aripiprazole, a D₂-preferring compound with a mechanism of action clearly differentiated from earlier approved antipsychotic drugs (Kikuchi et al., 1995; Lawler et al., 1999; Shapiro et al., 2003; Mailman, 2007; Mailman and Murthy, 2010). Thus, if a D₁ agonist with high bias for D₁-mediated signaling pathways were available, it would be an

excellent investigational tool, and it also might overcome some of the possible limitations sometimes thought to be obligatory with D₁ agonists.

SKF-83959, the first functionally selective D₁ ligand?

Dopamine receptors were originally classified on the basis of their coupling to adenylate cyclase (Kebabian et al., 1972; Garau et al., 1978; Kebabian and Calne, 1979), and this canonical signaling of the D₁-like receptors (D₁, D₅) is thought to involve coupling to the G proteins G α_{OLF} or G α_S (Neve et al., 2004; Mailman and Huang, 2007). Thus, the cAMP resulting from D₁ receptor activation could initiate a host of downstream cascades such as engaged by the activation of cAMP/protein kinase A (PKA) signaling. The importance of this pathway was challenged, however, with reports that the behavioral effects of some D₁ agonists were unrelated to cAMP/PKA signaling (Gnanalingham et al., 1995a; Gnanalingham et al., 1995b), but rather involved non-cAMP-mediated signaling including phospholipase C (PLC)-mediated calcium elevation (Undie et al., 1994; O'Sullivan et al., 2004; Andringa et al., 1999b; Undie and Friedman, 1990). This has led to the hypothesis that a functionally selective D₁ ligand highly biased against cAMP signaling might have an improved therapeutic index.

Much of this research has focused on SKF-83959 [6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine] because it was purported to be a highly biased D₁ ligand that was a biochemical antagonist at D₁-coupled adenylate cyclase, but a full agonist at D₁-stimulated PLC via G α_Q (Jin et al., 2003; Zhen et al., 2005; Rashid et al., 2007b; Hasbi et al., 2009; Panchalingam and Undie, 2001) (see Figure 6-1 for structures of SKF-83959 and other relevant ligands). SKF-83959 is behaviorally active in rat and primate PD models via its action at D₁ receptors (Arnt et al., 1992; Jin et al., 2003), and it has behavioral activity in several animal species that is known to be induced by D₁ full or partial agonists, but not by D₁ antagonists (Gnanalingham et al., 1995a).

The concatenation of these data led to the hypothesis that the behavioral actions of SKF-83959 were independent of D₁-mediated cAMP signaling, but depended on D₁

actions at PLC/calcium signaling (Rashid et al., 2007a; Hasbi et al., 2009; Downes and Waddington, 1993; Deveney and Waddington, 1995; Fujita et al., 2010; Perreault et al., 2010), possibly mediated by D₁-D₂ heterodimers (Rashid et al., 2007a; Hasbi et al., 2009; Perreault et al., 2010; Chun et al., 2013). This would mean that SKF-83959 is the first highly-biased functionally selective D₁ ligand (Undie and Friedman, 1992; Undie et al., 1994; Gnanalingham et al., 1995b; Arnt et al., 1992), and would make it an important probe for studying mechanisms related to D₁ signaling (Yu et al., 2008; Zhang et al., 2007; Zhang et al., 2005; Perreault et al., 2011; Liu et al., 2009a; Liu et al., 2009b; Perreault et al., 2014; Zhang et al., 2009b) as if often explicitly stated “*SKF 83959 robustly stimulat[es] the D₁-D₂ heteromer-mediated calcium signal and [does] not activat[e] adenylyl cyclase by the D₁-D₁ homomer*” (Perreault et al., 2014); SKF-83959 is “*a selective PI-linked D₁-like receptor agonist*” (Zhang et al., 2009b); and there are “*potential stimulant-antagonist actions, as observed with SKF-83959*” (Neumeyer et al., 2003).

Some years ago Pacheco and Jope (1997) noted some contradictions in the literature, but speculated that “*variations in experimental methods likely contribute to the differing results*” although no attempts to discern what these experimental issues might be. They themselves reported that “*dopamine D₁ receptors directly stimulate the [phosphoinositide] signaling system*” in human brain (Pacheco and Jope, 1997). In noting such inconsistencies, we had offered a skeptical view of the hypothesis that SKF-83959 was a highly biased functionally selective ligand (Huang et al., 2001). Yet the increasing numbers of papers whose data depend in large part on the highly-biased signaling of SKF-83959 have made it important to re-examine the pharmacology of this compound. In evaluating the evidence for SKF-83959 being a highly biased ligand, one must also consider the role of D₁-PLC signaling. We have chosen to do this by formulating a series of hypotheses that together form the basis for accepted dogma about the direct role for D₁ receptors in stimulating Gα_Q-PLC and more recently, the role of D₁-D₂ heterodimers in affecting PLC/Ca²⁺-mediated signaling.

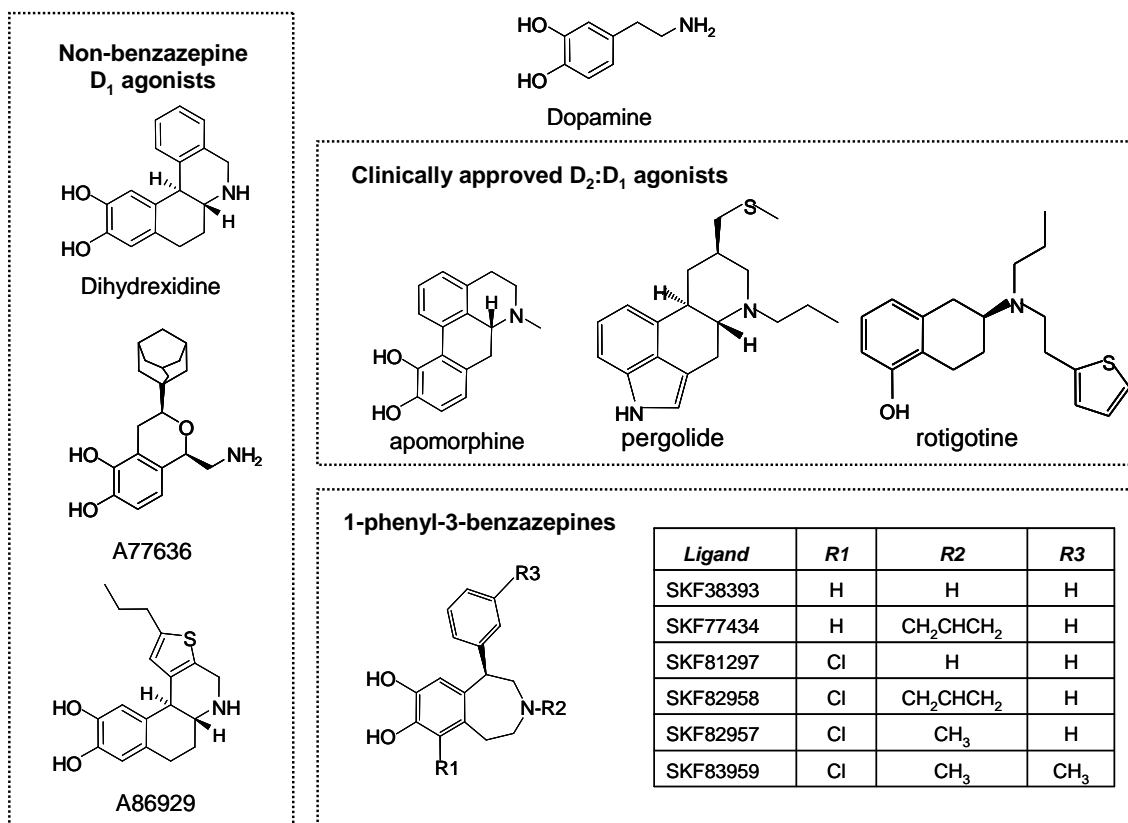


Figure 6-1. Research ligands and drugs that have properties relevant to these issues.

Hypothesis: SKF-83959 is a selective high affinity D₁ ligand

There is universal agreement that SKF-83959 has nanomolar affinity for both rat and human D₁ receptors - heterologously expressed or *in situ* (Lee et al., 2014; Arnt et al., 1992; Chun et al., 2013), and also that the ligand has micromolar affinity for D₂ receptors (Arnt et al., 1992; Chun et al., 2013; Lee et al., 2014) where it is an antagonist at cAMP signaling and a low-intrinsic activity partial agonist at β -arrestin recruitment (Lee et al., 2014). Similar D₁:D₂ selectivity with a wide range of D₁ intrinsic activities has been shown for other ligands of the 1-phenyl-3-benzazepine family (Setler et al., 1978; Weinstock et al., 1985; Neumeyer et al., 2003; Iorio et al., 1983). Because the D₂ affinities of most 1-phenyl-3-benzazepines are in the micromolar range, this provides an obvious off-target mechanism when high concentrations or doses are used experimentally. Ligands of this family also have measurable affinity for other neuroreceptors. Andringa et al. (1999a) reported that SKF-83959 had micromolar affinity

for all α -adrenoreceptors and the norepinephrine transporter, and Neumeyer et al. (2003) noted that it also had affinity for both 5-HT_{2A} (K_I = 88 nM) as well as the α_2 -adrenergic (K_I ~ 3,000 nM) receptors. More recently, Chun et al. (2013) reported the results of a broader screen, reporting micromolar affinity for dozens of GPCRs, including K_I < 10 μ M for many receptors that couple directly to G α_Q such as the 5-HT_{2A} and 5-HT_{2C} serotonin, α_1 -adrenergic, H₁ histamine, and M₅ muscarinic receptors, among others. Thus, the literature shows that although SKF-83959 has its highest affinity for D₁/D₅ receptors, at micromolar concentrations it will interact both with D₂-like dopamine receptors and a variety of other targets. This has implications discussed below.

Hypothesis: SKF-83959 has no intrinsic activity at D₁-mediated stimulation of adenylate cyclase

Although purported to have no intrinsic activity at D₁-mediated adenylate cyclase, we previously used SKF-83959 and found it to be a partial agonist (Ryman-Rasmussen et al., 2005). Because of the continuing use of this ligand for its “novel” properties, we recently did a rigorous evaluation of SKF-83959 pharmacology in a variety of heterologous and native D₁ systems, studying both the parent ligand and a potential N-demethylated metabolite, 6-chloro-1-(m-tolyl)-2,3,4,5-tetrahydro-1H-benzo[d]azepine-7,8-diol, that might contribute to pharmacological effects *in vivo* (Lee et al., 2014). Both *in vitro* and *ex vivo*, SKF-83959 was a partial agonist at D₁-mediated adenylate cyclase in two different cell lines transfected with the human D₁ receptor, as well as in rat striatal homogenates. Its potency and affinity were very similar in all of these systems. The functional effects were inhibited by SCH23390, as well as by structurally dissimilar D₁ antagonists, and SKF-83959 had no effect in untransfected cells. As noted above, while D₁ selective, SKF-83959 also had micromolar affinity for D₂ receptors, where it was an antagonist at cAMP signaling (Lee et al., 2014).

In some cases, the published data actually agree with the findings of Lee et al. (2014). As an example, Gnanalingham et al. (1995b) reported that SKF-83959 did not cause significant stimulation of adenylate cyclase, yet their data clearly show measurable

(~50%) intrinsic activity, with some data points marked as significant. In addition to our previous study showing >40% intrinsic activity in an hD₁ heterologous system (Ryman-Rasmussen et al., 2005), Chemel et al. (2012) reported high intrinsic activity in an over-expressed human D₁ receptor system. Although Rashid et al. (2007b) showed a “flat” dose-response curve for SKF-83959, only relative activity units were provided making it impossible to know the level of basal synthesis. The lowest SKF-83959 concentration tested was 10 nM (Rashid et al., 2007b) making it possible that maximal stimulation had already been caused. Without either basal synthesis levels or lower concentrations, the only valid conclusion from those data is that SKF-83959 was not a full agonist. There are, however, a few studies reporting no intrinsic activity for SKF-83959 for which obvious experimental or interpretational confounds are not evident (Jin et al., 2003; Arnt et al., 1992). It will be useful for other laboratories using this compound to independently determine whether or not SKF-83959 has measurable intrinsic activity in canonical assays.

Signaling via β -arrestin also represents an important non-G protein signaling pathway (Shenoy and Lefkowitz, 2005), one that can be evoked by D₁ receptors (Urs et al., 2011). SKF-83959 also is a partial agonist at the D₁-mediated β -arrestin activation, with intrinsic activity similar to the partial agonist SKF38393 and with similar potency as seen with adenylate cyclase activation (Lee et al., 2014). Importantly, its potency in both D₁-mediated adenylate cyclase and β -arrestin assays was in the nanomolar range, similar to its affinity (Lee et al., 2014). Interestingly, at the D₂ receptor, SKF-83959 had no intrinsic activity at adenylate cyclase, but modest agonist activity at D₂-mediated β -arrestin activation (Lee et al., 2014). At both of these D₁-mediated functions, the potency of SKF-83959 was, again, similar to its affinity, a point of relevance to the following section. Thus, the weight of the evidence suggests that SKF-83959 is a typical partial D₁ agonist, not a highly-biased, non-cyclase preferring D₁ ligand.

Hypothesis: SKF-83959 activates phospholipase C via a D₁-Gα_Q mechanism

Although SKF-83959 may have partial agonist properties at Gα_{OLF/S}-mediated stimulation of cAMP, it may still be useful as a moderately-biased functionally selective ligand if it were a full agonist in activating PLC. Some years ago, we briefly reviewed the evidence for D₁ linkage to PLC (Huang et al., 2001), and suggested flaws with that hypothesis, yet there has been a continued use of this compound based on this property. We recently investigated whether D₁ signaling was mediated by PLC activation. There was no significant stimulation caused by SKF-83959 in either D₁-transfected or wild-type HEK-293 cells. Conversely, in both wild-type and D₁-transfected cells, the positive Gα_Q control carbachol markedly stimulated IP1 synthesis with a potency consistent with its affinity, and the response was completely blocked by atropine.

In attempting to resolve the discrepancy between these recent data (Lee et al., 2014) and a sizable literature, we carefully reviewed the experimental protocols. Strikingly, in all prior publications reporting direct D₁ stimulation of PLC activity, the concentrations of SKF-83959 or other 1-phenyl-3-benzazepines required to elicit these effects were in the high micromolar/low millimolar range (Felder et al., 1989a; Felder et al., 1989b; Dyck, 1990; Undie and Friedman, 1992; Vyas et al., 1992; Undie and Friedman, 1994; Pacheco and Jope, 1997; Lee et al., 2004; Banday and Lokhandwala, 2007; Liu et al., 2009a; Mizuno et al., 2012; Mahan et al., 1990; Undie and Friedman, 1990; Zhang et al., 2009b). No rationale was offered for use of SKF-83959 and chemically-related benzazepines at concentrations far greater than their nanomolar K_D's. For SKF-83959 in particular, this raises a conundrum. The functional potency reported for D₁-mediated PLC activation is about four orders-of-magnitude greater than either its D₁ affinity or its D₁ potency for stimulating cAMP synthesis or activating β-arrestin.

Earlier we summarized the off-target activity of SKF-83959 and other 1-phenyl-3-benzazepines, noting the many receptors and transporters whose K_D's are in the 1-10 μM micromolar range. Thus, when concentrations of 100-300 μM are used, these (as well as other) targets are probably engaged unintentionally. It would seem prudent that there be

tests of the obvious alternate hypothesis that activation of PLC, when found, is mediated by one or several of these off-target receptors. In fact, some data already support this latter hypothesis. Yu et al. (1996) reported that 5 μ M fenoldopam (an SKF-83959 analog that has high D₁ intrinsic activity) increased PLC activity, but only by 30-50% and that this increase was mediated by cAMP, not direct PLC activation. Similarly, Jin et al. (2003) reported that the D₁ receptor linked to PLC was found in striatum, hippocampus, frontal cortex, and cerebellum. Since neither D₁ binding sites nor D₁ mRNA are found in the cerebellum, this suggests an off-target, rather than D₁, mechanism. Finally, in almost every study using pharmacological antagonism of D₁-PLC effects, the sole antagonist employed was the structurally similar 1-phenyl-3-benzazepine SCH23390 (Felder et al., 1989a; Felder et al., 1989b; Dyck, 1990; Undie and Friedman, 1992; Vyas et al., 1992; Undie and Friedman, 1994; Pacheco and Jope, 1997; Lee et al., 2004; Banday and Lokhandwala, 2007; Liu et al., 2009a; Mizuno et al., 2012; Mahan et al., 1990; Undie and Friedman, 1990; Zhang et al., 2009b). Yet when structurally diverse antagonists were used, a very different picture emerges. For example, Jin et al. (2003) reported that the antagonist cis-flupenthixol was only half as effective as SCH23390 in blocking purported D₁-PLC activation, moreover, the D₁/D₂ antagonist (+)butaclamol failed to block these effects at all.

In our view, the most compelling evidence comes from the study of Friedman et al. (1997), who found that the PLC-activation *ex vivo* was unaffected by knockout of the D₁ receptor. Rather than interpreting this as evidence for a non-D₁ action of the 1-phenyl-3-benzazepines, Friedman et al. (1997) proposed that there was a novel D₁-like receptor that was responsible, despite inconsistencies with both the known expression of the D₅ receptor, and the likely properties of then-orphan receptors in the mouse genome. Recalling that every published study over the last three decades failed to find D₁-mediated PLC activation at theoretical fractional occupancies even of 95%, there seems compelling support for the alternate hypothesis that off-target effects were the actual mechanism. The original idea has, however, morphed into a newer second hypothesis that

D₁-D₂ heterodimers actually activate the Gα_Q-PLC-Ca²⁺ cascade. Although we feel the foundation for this novel hypothesis was flawed, it must be evaluated on its merits.

Hypothesis: D₁-D₂ heterodimer-mediated activation of Gα_Q-PLC-Ca²⁺ by D₁ selective ligands is an important signaling mechanism

George and coworkers have recognized the discrepancy between functional potencies of SKF-83959 and its affinity, and hypothesized that whereas D₁ receptors alone (monomer and/or homodimer) could not stimulate PLC signaling, this would occur via co-activation of a D₁-D₂ heterodimer in a Gα_Q protein-dependent manner (Lee et al., 2004; Hasbi et al., 2009; Hasbi et al., 2011; Rashid et al., 2007a; Rashid et al., 2007b). Their view is that the limiting factor is the μM affinity of SKF-83959 for the D₂ receptor (Rashid et al., 2007b; Lee et al., 2014). There are several lines of evidence, however, that are inconsistent with this D₁-D₂ hypothesis.

First, if the effects are dependent on D₂ receptors, it is unclear how one can conclude the occupancy of the D₁ receptor is important as the D₁ receptor is essentially saturated at the concentrations when there is even partial fractional occupancy of the D₂. In addition, the reports of PLC activation in either kidney or brain (Felder et al., 1989a; Felder et al., 1989b; Dyck, 1990; Undie and Friedman, 1992; Vyas et al., 1992; Undie and Friedman, 1994; Pacheco and Jope, 1997; Lee et al., 2004; Banday and Lokhandwala, 2007; Liu et al., 2009a; Mizuno et al., 2012; Mahan et al., 1990; Undie and Friedman, 1990; Zhang et al., 2009b) show no effect at concentrations that would essentially saturate both D₁ and D₂ receptors (e.g., 10 μM). The rare reports showing functional Ca²⁺ effects at more reasonable concentrations also have anomalies. For example, one study reported essentially a quantal-like response for SKF-83959 in which no significant effects were seen at 30 nM concentrations, but a maximal effect at 100 nM (see Figure 1B in Hasbi et al., 2009). This type of dose-response behavior is inconsistent with a Gα-type of mechanism that almost always yields graded response.

More recently, it has been suggested that there is a unique role of D₁:D₂ heterodimers in subsets of co-expressing striatal neurons (Perreault et al., 2012; Perreault

et al., 2014). Although this is a change from the original hypothesis of direct D₁-Gα_Q-PLC activation by SKF-83959, it is important to consider its merits. One line of evidence that argues against the D₁-D₂ heterodimer hypothesis is that Chun et al. (2013) failed to find the predicted calcium signaling. Secondly, in HEK-293 cells, SKF-83959 is an antagonist at D₂-mediated adenylate cyclase and a low intrinsic activity partial agonist at D₂-mediated β-arrestin recruitment (Lee et al., 2014), inconsistent with a dopamine-like physiological effect. Thirdly, there was no PLC activation by SKF-83959 in HEK-293 cells co-expressing D₁ and D₂ receptors (Lee et al., 2014). Finally, although there may be some co-expressing D₁ and D₂ cells (especially early in development), the vast majority of adult striatal neurons [where D₁-PLC activation was first reported (Undie and Friedman, 1992; Friedman et al., 1993; Undie and Friedman, 1990)] are highly segregated. Medium spiny neurons of the direct pathway are largely D₁-expressing, those of the indirect pathway largely D₂-expressing, and such segregation also occurs elsewhere in brain (Harrison et al., 1990; Bateup et al., 2008; Aubert et al., 2000; Deng et al., 2006; Rappaport et al., 1993; Matamales et al., 2009; Scibilia et al., 1992; Bertran-Gonzalez et al., 2010; Gerfen et al., 1990; Le Moine and Bloch, 1995). In summary, while the D₁-D₂ heterodimer hypothesis is enticing, there is a significant body of evidence that suggests it may not be valid.

Hypothesis: D₁-PLC signaling of SKF-83959 causes novel behavioral effects

One purportedly novel characteristic of SKF-83959 is its D₁-like behavioral activity in the absence of the ability to activate adenylate cyclase. This has been reported in both murine (Arnt et al., 1992; Downes and Waddington, 1993) and primate species (Gnanalingham et al., 1995a), and has been used as *prima facie* functional evidence for the importance of the D₁-Gα_Q-PLC and/or D₁-D₂ heterodimer-Gα_Q-PLC-Ca²⁺ signaling pathway. There are, however, two lines of evidence that suggest that this hypothesis may not be true. These relevant *in vivo* studies have relied solely on the 1-phenyl-3-benzazepine family of D₁ agonists that like SKF-83959, are behaviorally effective at quite low doses. For example, Arnt et al. (1992) reported that SKF-83959 caused

maximal turning in the unilateral 6-OHDA model at a dose of 0.3 $\mu\text{mol/kg}$, whereas Deveney et al. (1995) found that yet lower doses were active in evoking D_1 behaviors in unlesioned rats. Yet even assuming no metabolism (and these catechols are rapidly metabolized), this yields a predicted concentration at the D_1 receptor far lower than required *in vitro* to activate either the hypothesized D_1 -PLC or D_1 - D_2 heterodimer systems. More recently, Medvedev et al. (2013) concluded that PLC β via D_1 mechanisms regulated forward locomotion in mice, yet the sole experimental selective D_1 agonist, SKF81297, was used at doses of 10 mg/kg, far higher than the 0.001-0.3 mg/kg known to show typical D_1 -like behavioral activity (Vermeulen et al., 1994; Diaz and Castellanos, 2006; Cai and Arnsten, 1997). A parsimonious deduction would seem to be that D_1 -like behavioral effects are irrelevant to these purported PLC signaling mechanisms requiring near-millimolar concentrations.

Another important potential confound also relates to pharmacokinetic factors. For example, a classic phase I metabolic reaction is N-demethylation, and SKF-83959 has the chemical properties that suggest it would be a substrate for this type of CYP-mediated reaction (see Figure 6-1). Recently, we demonstrated that the N-demethylated product of SKF-83959 actually has somewhat greater D_1 intrinsic activity than the parent compound (Lee et al., 2014). Thus, even if one still posits that the behavioral effects of SKF-83959 cannot involve adenylate cyclase signaling, the possibility that all of its behavioral actions might be explained by one or more active metabolites must be addressed.

Reconciliation of the role of D_1 receptors and $G\alpha_Q$ -PLC activation

Popper (1959) has written *“Whenever the ‘classical’ system of the day is threatened by the results of new experiments which might be interpreted as falsifications..., the system will appear unshaken to the conventionalist. He will explain away the inconsistencies which may have arisen; perhaps by blaming our inadequate mastery of the system. Or he will eliminate them by suggesting ad hoc the adoption of certain auxiliary hypotheses, or perhaps of certain corrections to our measuring instruments.”* It is with due irony that this seems to encapsulate the issues addressed in

this Perspective. We have not sought to be provocative, but rather to follow the notion that it is appropriate, and even desirable, to be skeptical (not cynical) about hypotheses (or even theories or laws). Devising experiments to disprove a hypothesis actually honors the original conceptualization if those experiments fail to do so. Thus, the fundamental question is whether SKF-83959, or indeed any other D_1 agonist, causes PLC activation via actions at the D_1 receptor. We have encapsulated the issues at hand into three inter-related hypotheses illustrated in the cartoon in Figure 6-2.

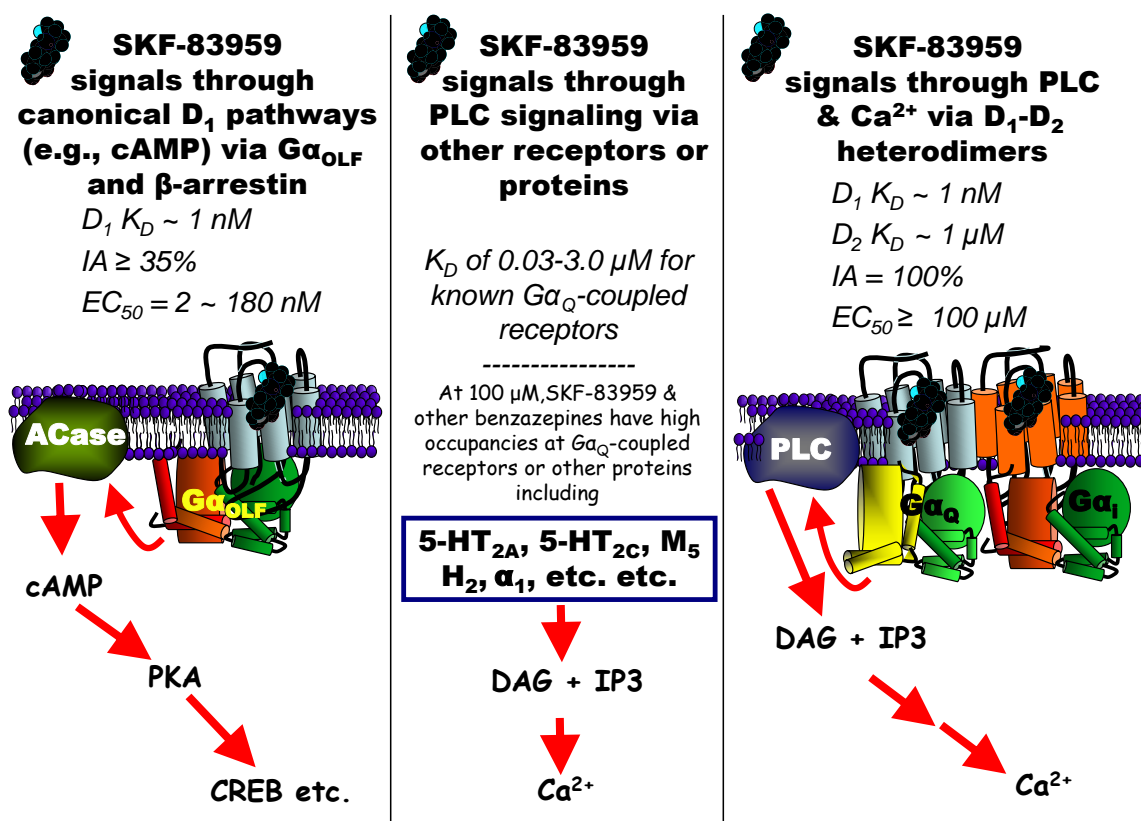


Figure 6-2. Schematic representation of three hypotheses related to D_1 signaling by SKF-83959.

Activating effects are indicated with a solid line ending in an arrowhead. K_D , dissociation constant; IA , intrinsic activity; EC_{50} , half maximal effective concentration; D_1 , dopamine D_1 receptor; ACase, adenylate cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CREB, cAMP response element-binding protein; 5-HT_{2A} and 5-HT_{2C}, 5-hydroxytryptamine serotonin receptor subtype 2A and 2C; M₅, muscarinic acetylcholine receptor subtype 5; H₂, histamine receptor subtype 2; α_1 , adrenergic receptor subtype 1; PLC, phospholipase C; DAG, diacyl glycerol; IP3, inositol 1,4,5-trisphosphate.

The first hypothesis (Figure 6-2, left panel) deals with whether SKF-83959 is highly biased because of a lack of activity at canonical signaling pathways, a property used to explain why D₁-PLC signaling was important for behavioral/physiological effects of SKF-83959 and related compounds. As we have reviewed, the weight of the literature (although not unanimous) suggests that the compound is actually a partial agonist at D₁ receptors in both heterologous systems and in the striatum, with activity in at least three D₁-mediated pathways: adenylate cyclase; β -arrestin; and receptor internalization (Ryman-Rasmussen et al., 2005; Lee et al., 2014; Gnanalingham et al., 1995b; Chemel et al., 2012). Thus, above and beyond the hypothesis that the D₁ receptor engages G α_Q -PLC, SKF-83959 would, at most, have modest bias, and canonical mechanisms would have to be ruled out before assigning physiological/behavioral effects to PLC signaling.

The second hypothesis (Figure 6-2, center panel) posits that PLC-mediated signaling is an important mechanism for SKF-83959 and other D₁ agonists. The studies in both kidney and brain that have supported a role of D₁ activation of PLC have been consistent in the use of ligands only from the 1-phenyl-3-benzazepine family, and have employed these agonists at concentrations orders of magnitude higher either than their K_D or than their functional potency in canonical assays. Moreover, they generally used only the structurally similar SCH23390 as an antagonist (see references cited earlier). Unfortunately, the use of suprapharmacological concentrations of SKF-83959 and failure to consider non-D₁ mechanisms is not unique to studies of PLC signaling (Yu et al., 2008; Guo et al., 2013). It is unclear why off-target actions, an obvious factor in pharmacological designs, have not generally been considered, especially as many of the likely targets are G α_Q -linked receptors.

To complicate matters, numerous studies failing to show D₁ mediated stimulation of phosphoinositide hydrolysis (Kelly et al., 1988; Rubinstein and Hitzemann, 1990; Kimura et al., 1995; Demchyshyn et al., 1995; Pedersen et al., 1994; Lee et al., 2014; Dearry et al., 1990) have often been ignored. Indeed, Wallace and Claro (1990) noted that dopamine (at concentrations that stimulate cAMP synthesis) actually inhibited muscarinic stimulation of phosphoinositide hydrolysis. The most telling piece of data is the fact that

PLC activation by SKF-83959 and other benzazepines was absolutely preserved in tissue from mice that had the D₁ receptor knocked out genetically (Friedman et al., 1997). Together, the available data suggests that the second hypothesis in Figure 6-2 is false -- SKF-83959 and other 1-phenyl-3-benzazepines do not stimulate PLC activity via the D₁ receptor.

The third hypothesis (Figure 6-2, right panel) posits a critical role for D₁-D₂ heterodimers, and is the one of greatest current interest because of the novel underlying molecular mechanism. In Popper's words, it may be considered as a "correction" of the initial hypothesis (Figure 6-2, center panel). Although it was based originally on the D₁-PLC mechanism and the use of SKF-83959, it has now taken on a life of its own because of the hypothesized unique role of D₁:D₂ heterodimers in subsets of co-expressing striatal neurons (Perreault et al., 2012; Perreault et al., 2014). We have reviewed the available data that we feel can be construed as weakening this hypothesis. Above and beyond the experimental issues [including our failure to be able to see evidence of PLC signaling in D₁-D₂ co-transfected HEK cells (Lee et al., 2014)], there is the question of the degree to which D₁-D₂ colocalization occurs in the mature nervous system. Thus, our view is that hypothesis 3 is also false. Some may view this conclusion as premature, yet a recent paper concluded "*.... the mechanism of D₁R/D₂R-mediated calcium signaling involves more than receptor-mediated Gq protein activation, may largely involve downstream signaling pathways, and may not be completely heteromer-specific. In addition, SKF-83959 may not exhibit selective activation of D₁-D₂ heteromers, and its significant cross-reactivity to other receptors warrants careful interpretation*" (Chun et al., 2013). Unfortunately, this may be interpreted by some as simply being an experimental difference between investigators. A more skeptical view is that an extensive literature has already provided adequate evidence to falsify all of these prevailing hypotheses, much as we suggested years ago (Huang et al., 2001).

Conclusions

Although we have tried to offer a critical review of the available literature, there are several issues that we could not address. An obvious one with a pharmacological basis has to do with receptor reserve, a well-known mechanism that can affect interpretation of functional studies and might be thought to be a reason for the disparate literature. Yet, it is important to remember that much of the PLC hypothesis has been based on studies done *ex vivo* using brain or kidney tissue where presumably all investigators were dealing with both physiologically-relevant and similar receptor reserve. Thus, the huge difference in D₁ potencies between canonical functional assays and PLC activation is unlikely to involve receptor reserve. Moreover, the near-millimolar concentrations of D₁ agonists required to cause PLC activation *ex vivo* never will be achieved *in vivo* when administering D₁ dopamine agonists to animals at behaviorally active concentrations (Mottola et al., 2002; Arnsten et al., 1994; Schneider et al., 1994; Taylor et al., 1991).

In addition to our recent work (Lee et al., 2014), there are other experiments that might be useful if one felt that the available data were not adequately conclusive. The two most obvious are those with a pharmacological basis. First, future studies should take advantage of the structurally diverse D₁ agonists and antagonists that are available (e.g., Figure 6-1). Second, when there is a question of off-target engagement, studies done *in vivo* or *ex vivo* should measure drug concentrations in tissue. In addition, a host of molecular approaches might also be useful. The most challenging relate to the idea that D₁-D₂ heterodimers are the mechanistic key. Although we feel that this hypothesis is not well-supported, others may feel further studies are required to reach firm conclusions. Techniques such as proximity ligation assays (Trifilieff et al., 2011) can be used to look for molecular proximity between D₁ and D₂ in brain slices, and bioluminescence resonance energy transfer (BRET) can be used to determine if G α_Q is activated by the D₁ alone or when complexed with D₂ (Urizar et al., 2011). Indeed, there are recent data using such approaches that failed to find evidence for either D₁ agonist engagement of G α_Q , or molecular proximity of D₁ and D₂ in mouse brain (Jonathan Javitch et al.,

personal communication). Such data, while “negative,” are compelling evidence for the perspectives we have offered.

There may be substantial impact to resolution of this controversy. The potential clinical utility of D₁ agonists has slowly been receiving human validation (Rosell et al., 2014; Rascol et al., 1999), although the development of an approvable drug has been inhibited by issues including pharmacokinetics, seizures, and hypotension. A functionally selective D₁ agonist could theoretically have advantages (Mailman, 2007), yet whereas some existing D₁ ligands do have some degree of functional selectivity (Lewis et al., 1998; Ryman-Rasmussen et al., 2005; Ryman-Rasmussen et al., 2007), no compound has yet been shown to have sufficient signaling bias to translate into meaningful pharmacological differences. As we conclude here, none of the known 1-phenyl-3-benzazepine compounds is a highly biased D₁ ligand, contrary to what has been hypothesized. It is timely that the field should focus on the substantial scientific issues that remain to be elucidated about D₁ function and their impact on drug discovery, and not be distracted by mechanisms that may be artificial.

Chapter 7: Summary of research, implications, and future research directions

Preface

In early chapters, I introduced the area in which my research was to be conducted. I provided the results about common belief and our hypotheses on functional selectivity at the D₁ receptor, and about ligand interaction mechanisms for non-catechol scaffolds. My research has established the critical D₁ signaling that is useful for the explanation of its behavioral effects and further can be adopted for the development of functionally selective D₁ ligands. The common belief on PLC-preferring SKF-83959 was greatly weakened by our results, and PLC activation is unlikely to be a solid D₁-mediated signaling. In later chapters, for the first time I have provided a scientific basis for novel non-catechol full D₁ agonists suggesting the molecular mechanism for how non-catechol scaffolds interact with the conserved amino acids in TM3, TM5, and TM6 of the D₁ receptor. This chapter will discuss the implications toward novel D₁ agonists and future research directions that may overcome the limitations of the current study.

Critical cell signaling for the development of novel D₁ agonists

Since dopamine receptors are reported to stimulate adenylate cyclase (AC) and increase intracellular cAMP (Kebabian and Greengard, 1971), G $\alpha_{OLF/S}$ -adenylate cyclase activation has been a canonical signaling pathway for the dopamine D₁ receptor. For the D₂ receptor, which is another major dopamine receptor type in brain, inhibition of AC activity and subsequent reduction of cAMP have been used to assess the intrinsic activity of D₂ receptor agonists. Although regulation of AC activity has been the main signaling mechanism of the D₁ or D₂ receptors, several reports suggest that non-AC signaling may be stimulated by the dopamine receptor and important for its clinical benefits (Undie et al., 1994; O'Sullivan et al., 2004; Andringa et al., 1999b; Undie and Friedman, 1990; Gnanalingham et al., 1995b; Gnanalingham et al., 1995a). G α_Q -phospholipase C activation has been used as one such non-cyclase signaling mechanism mediated by D₁ receptors (Felder et al., 1989a; Felder et al., 1989b; Dyck, 1990; Undie and Friedman, 1992; Vyas et al., 1992; Undie and Friedman, 1994; Pacheco and Jope, 1997; Banday and Lokhandwala, 2007; Liu et al., 2009a; Zhang et al., 2009b; Lee et al., 2004). Because some of the experiments supporting D₁-mediated PLC activation were not well-designed and because we have failed to reproduce some of previous results, we interrogated whether PLC activation is truly mediated by the D₁ receptor. In many reports that suggest PLC activation occurs in striatum by dopamine and selective D₁ agonists, we have found that D₁ agonist concentrations used for PLC stimulation were much higher than the K_D for the D₁ receptor and the EC₅₀ for adenylate cyclase activation. Among the D₁ agonists, we focused on SKF-83959 because it has been known as the first PLC-preferring highly biased D₁ ligand. Although SKF-83959 has high affinity for the D₁ receptor (ca. 1 nM), the EC₅₀ for PLC activation was at least four orders of magnitude higher than its affinity (Jin et al., 2003). In addition, SKF-83959 was reported as a chemical antagonist for D₁-mediated AC activation and a full agonist for D₁-mediated PLC activation. Friedman et al. (1997) reported that D₁ agonists still showed PLC activation in D₁ knock-out mice, whereas AC activation was totally eliminated. This evidence from the D₁ knock-out study led us to reexamine the D₁ pharmacology of SKF-83959 more skeptically.

We investigated D₁ signaling of SKF-83959 in cellular expression systems and striatal tissues. Contrary to what was commonly assumed, SKF-83959 was a typical partial agonist for D₁-mediated AC activation and failed to stimulate D₁-mediated PLC activation. Furthermore, we examined the activity of SKF-83959 for a non-cyclase signaling pathway β -arrestin recruitment. SKF-83959 showed partial agonistic activity similarly to the typical D₁ partial agonist SKF38393. More interestingly, we synthesized a possible demethylated form of SKF-83959 (desmethylSKF) and found that the metabolite also has higher intrinsic activity for AC activation than the parent compound. Although we cannot fully explain the reasons for these discrepancies between the previous reports and our current data, our results strongly suggest that SKF-83959 is a D₁ partial agonist both for AC activation and β -arrestin recruitment, but has no intrinsic activity for D₁-mediated PLC activation. Moreover, many reports are also consistent with our results (Kelly et al., 1988; Rubinstein and Hitzemann, 1990; Kimura et al., 1995; Demchyshyn et al., 1995; Pedersen et al., 1994; Dearth et al., 1990). Thus, hypotheses and conclusions based on the assumption that SKF-83959 is a PLC-preferring D₁ ligand should be reconsidered. Partial intrinsic activity for AC signaling should be addressed when evaluating D₁ receptor efficacy of SKF-83959. We hope that our results will be confirmed by other independent research groups in the near future.

Insights into non-catechol full D₁ agonists from the molecular interaction studies

Although partial D₁ agonists (e.g., SKF38393 and CY 208 243) have failed to show satisfactory effects on Parkinson's disease (PD) (Emre et al., 1992; Braun et al., 1987), full D₁ agonists (e.g., dihydrexidine and ABT-431) have clearly shown clinical efficacy for PD equal to the gold-standard therapeutic medicine, levodopa (Blanchet et al., 1998; Rascol et al., 1999). One of the main issues for full D₁ agonists is poor oral bioavailability presumably caused by an intrinsic catechol moiety. Although the catechol moiety was suggested to be a critical part of full D₁ agonists (Mottola et al., 1996), there are non-catechol scaffolds that have reasonable D₁ affinity and efficacy suggesting that

full D₁ agonists without a catechol moiety may be developed (Krisch et al., 1994; Emre et al., 1992; Scheller et al., 2009).

Although the ergolines and rotigotine are selective for D₂ or D₃ receptors, I have chosen them as non-catechol scaffolds due to their partial D₁ agonistic activity. The ergolines are reported to have longer half-life than catechol-type D₁ agonists and reasonable oral bioavailability (Kvernmo et al., 2006). Although rotigotine itself was unable to show high oral bioavailability due to rapid metabolism in liver (first-pass effects), its transdermal patch formulation has shown over 37% bioavailability and the plasma concentration of rotigotine lasts consistently over 24 hours (Cawello et al., 2009). Because molecular interactions of the receptor-ligand complex can provide insights to new drug design (Ehrlich et al., 2009; Tschammer et al., 2011), I investigated the interaction between the D₁ receptor and these two non-catechol scaffolds (ergolines and rotigotine). I focused on the conserved threonine in TM3, serines in TM5, and aromatic residues in TM6 that are located in the ligand-binding pocket and highly likely to play a role in receptor-ligand interaction (Floresca and Schetz, 2004; Shi et al., 2002; Shi and Javitch, 2002). Adenylate cyclase activation was used as a measure of the D₁ intrinsic activity for these non-catechol scaffolds.

In Chapter 3, I found that the mutation of threonine T3.37 to alanine (T3.37A) greatly decreased the D₁ affinity and efficacy of the ergoline agonists, and that the mutation of the TM5 conserved serine S5.46 to alanine (S5.46A) showed a significant but small decrease in the affinity of the ergolines. Docking simulations suggest that B-ring nitrogen of the ergoline backbone is located close to both T3.37 and S5.46 providing possible hydrogen bond interactions between the ergoline backbone and the D₁ receptor. Unexpectedly, the alanine mutation of the conserved serine S5.42 (S5.42A) markedly increased the D₁ affinity and efficacy of the ergoline agonists. S5.42 appears to generate steric hindrance for the A-ring of the ergolines, but docking simulations show that the A-ring can additionally interact with Y5.38 through hydrophobic and aromatic interactions. The S5.42A mutation may be able to provide more room for the A-ring of the ergoline backbone to interact with Y5.38. This suggests a possible mechanism for the great

increases in the affinity and efficacy of the ergolines at the S5.42A mutated receptor. For chemical modifications on the A-ring, I propose that replacing the A-ring with 5-membered ring heterocyclic compounds such as pyrrole, furan, and thiophene may reduce steric hindrance with the hydroxyl group of S5.42, but can keep the hydrophobic and aromatic interactions with Y5.38. These modifications may generate the ergoline-type D₁ ligand with better affinity and/or efficacy. Because the A-ring can form hydrophobic and aromatic interactions with Y5.38, removing the entire A-ring from the ergoline backbone would decrease D₁ interaction with the ergolines.

In addition to hydrogen bonds, hydrophobic and aromatic interactions also play a critical role in receptor-ligand interaction. In Chapter 4, I found that the conserved phenylalanine F6.51 to alanine mutation (F6.51A) greatly decreased the D₁ affinity and efficacy of the ergoline agonists. The mutagenesis results and simulations with lisuride and terguride also support the hypothesis that interaction with F6.51 may be critical for D₁ receptor efficacy. Docking simulations suggest that F6.51 and F6.52 are positioned closely to the D-ring and the B-ring of the ergoline backbone, respectively. I propose that chemical modifications on the D-ring that enhance the interaction with F6.51 may produce ergoline-type D₁ agonists with higher D₁ intrinsic activity. In case of CY 208 243, introducing a double bond to the D-ring may increase D₁ intrinsic activity of CY 208 243. Other modifications can focus on the accessory hydrophobic ring of CY 208 243 and add, for example, a methyl or an ethyl groups. These modifications may increase the interaction with TM6 or TM7 amino acids by providing additional hydrophobic contacts. In the current dissertation, I have not investigated the roles of conserved TM7 residues that can form hydrophobic interactions with the accessory ring of CY 208 243. Future studies that include molecular interactions with TM7 residues would provide more information on chemical modifications of the ergolines.

Chapter 5 investigated the molecular interactions of another non-catechol scaffold rotigotine. I have found that S5.42A and S5.43A mutations greatly decreased the D₁ affinity and efficacy. Docking simulations show that the hydroxyl group of rotigotine is located close to S5.42 and S5.43 suggesting the possible formation of bifurcated

hydrogen bonds. In addition, the F6.51A mutation also markedly decreased the D₁ affinity and efficacy of rotigotine. Mutagenesis results with two 2-aminotetralins (5-OH DPAT and 7-OH DPAT) that do not have the thiophene group also showed the critical role of the thiophene group in D₁ receptor interaction. Although rotigotine is reported to have full agonistic properties in the D₁ receptor expression system (Scheller et al., 2009), my preliminary data suggest that rotigotine showed partial D₁ agonistic activity like CY 208 243 (ca. 80% of dopamine maximal response). Thus, chemical modifications on the thiophene group that increase the interaction with F6.51 may generate aminotetralin D₁ ligands with enhanced D₁ efficacy. I propose that adding a methyl or an ethyl group to the thiophene ring may increase the hydrophobic contacts to F6.51. In addition, adding ring structures such as benzene, pyrrole, furan or thiophene to the propyl group of rotigotine may enhance the molecular interaction with TM6 and TM7 residues. Although these bulky groups may decrease the D₁ receptor interaction by producing steric hindrance with other amino acids, they can contribute to additional hydrophobic and aromatic interactions with the D₁ receptor. Together, our results strongly suggest that there are critical residues for the interactions of ergolines and rotigotine, and that alternations of these molecular interactions can regulate the D₁ affinity and efficacy of the non-catechol ligands. The chemical modifications I suggest in this section may be useful for the synthesis of novel non-catechol D₁ agonists with full intrinsic activity.

Future research directions for clinically available selective full D₁ agonists

Although non-catechol full D₁ agonists may overcome the poor pharmacokinetic properties of current full D₁ agonist, there are several issues that need to be resolved prior to the clinical approval of full non-catechol D₁ agonists.

Rapid tolerance to D₁ receptor activation

Contrary to the D₂ agonists that do not develop tolerance to chronic receptor activation, the key issue of D₁ receptor full agonists has been that it induces rapid tolerance and that the beneficial effects last no more than several days. The best example is the case of A77636 that has a unique isochroman structure (Ryman-Rasmussen et al.,

2007; Lin et al., 1996). Although the internalized D₁ receptors are rapidly recycled to the membrane within 1 h of removal of dopamine, A77636 caused the receptor to be retained intracellularly up to 48 h after agonist removal (Ryman-Rasmussen et al., 2007). Computational simulations suggest that the isochroman structure may stick to the D₁ receptor at the additional site out of the ligand binding pocket (Ryman-Rasmussen et al., 2007). Although the unique isochroman structure may explain the increase in receptor occupancy and the subsequent desensitization to D₁ receptor signaling, other full D₁ agonists also caused behavioral tolerance in 6-OHDA-lesioned rats after administration for several days (Britton et al., 1991; Blanchet et al., 1996) and especially when administered continuously over 24 h (Gulwadi et al., 2001). Although some desensitization of the effects was observed in one experiment, interestingly once or twice treatment of dinapsoline a day retained the behavioral effects for 14 days (Gulwadi et al., 2001). In addition, our preliminary results also showed that when dinapsoline was injected intermittently and D₁ receptor occupancy lasted no more than 20 h, it still exhibited significant D₁-mediated behavioral effects on the next day. Together, these suggest that duration of D₁ receptor occupancy may be critical for the D₁ receptor tolerance and that D₁ agonists with an intermediate half-life (e.g., 3 ~ 5 h) may retain their clinical efficacy for several days without showing rapid tolerance. Thus, the range of appropriate half-life should be tested, and drug regimen should be carefully considered to avoid continuous receptor occupancy and rapid tolerance. More research is also needed to clearly understand the molecular mechanisms for the D₁ receptor tolerance.

Side effects of D₁ receptor activation

One typical side effect of full D₁ agonists is hypotension. This side effect was the main reason for the discontinuation of the previous clinical study with dihydrexidine (Blanchet et al., 1998). In the pilot human trial that administered dihydrexidine intravenously during one hour, peripheral D₁ receptors in the kidney and blood vessels were activated by dihydrexidine and suggested as the mechanism leading to the severe hypotension (Amenta et al., 1995). However, one paper showed that subcutaneous injection of a single 20 mg dose of dihydrexidine is safe and tolerated in patients with

schizophrenia (George et al., 2007). Because lower doses of dihydrexidine are required for the improvement of cognitive and memory ability than those for PD symptoms, the hypotension caused by D₁ agonists may not be an issue for schizophrenic patients. Future studies should establish a safe therapeutic dose range of full D₁ agonists and address this issue prior to the clinical use of full D₁ agonists.

Production of seizures has been another issue for full D₁ agonists. Although dihydrexidine did not cause seizures in normal rats (Darney, Jr. et al., 1991), other D₁ agonists (e.g., A68930 and A77636) have been reported to induce seizures at higher doses than those showing behavioral effects (DeNinno et al., 1991; Keabian et al., 1992). Although some reports suggest that selective D₁ agonists are proconvulsant (e.g., lowering the seizure threshold) (al-Tajir et al., 1990; Starr, 1996), other receptors (e.g., D₂ receptors, α_2 -adrenergic receptors, and serotonin receptors) are also involved in inducing seizures and these full D₁ agonists may interact with them when used at high doses. Because the mechanisms involved in seizures are not fully understood, future studies should reveal the role of the D₁ receptor in seizures and establish the safety margin of D₁ agonists that does not elicit seizures and seizure-like behaviors.

Concerns on non-catechol scaffolds

One concern about using non-catechol scaffolds as full D₁ agonists is that they have relatively low D₁ receptor selectivity over D₂-like and other biogenic amine receptors. All selective D₁ full agonists have a catechol moiety that is, at least to date, suggested to be necessary for D₁ receptor selectivity. The ergolines and rotigotine used as non-catechol scaffolds in the current dissertation have selective D₂-like receptor interaction than D₁, and some of ergolines show full D₂ receptor activity. Based on previous interaction profiles, full D₁ non-catechol agonists (if any) are expected to have the interactions with other dopamine receptor subtypes (e.g., D₂ receptors). A recent paper, however, showed that adding a spacer that sticks outside the actual orthosteric binding pocket towards the extracellular loops greatly increased the dopamine D₅ receptor selectivity versus D₁ (Troger et al., 2014). In addition, a couple of reports

suggest that ligand interaction with one glycine in the extracellular loop 1 is critical for dopamine D₂ or D₃ subtype selectivity (Michino et al., 2013; Troger et al., 2014). These molecular interactions may provide clues to dopamine D₁ receptor selectivity versus other subtypes. More research is still required to clearly understand the molecular mechanism for dopamine receptor subtype selectivity.

D₁-mediated non-AC signaling for novel D₁ full agonists

The definition of “D₁ full agonist” has been based on its efficacy in canonical D₁ signaling AC activation. Recent reports also suggest that other signaling pathways are also activated by the D₁ receptor (Chen et al., 2004; Urs et al., 2011; Del'guidice et al., 2011), and such signaling (e.g., β -arrestin signaling) may be important for the action of the D₁-mediated behavioral effects. In Chapter 2, I showed that phospholipase C activation that has been purported as non-AC signaling is not a D₁-mediated response, but a non-specific effect occurring at suprapharmacological concentrations of phenylbenzazepines. For non-AC signaling, the D₁ receptor is known to recruit β -arrestin to the receptor that plays a role in D₁-mediated ERK activation (Chen et al., 2004; Urs et al., 2011; Del'guidice et al., 2011). In addition, the D₁ receptor can mediate other G protein-independent signaling pathways (e.g., ion channel activation). For the D₂ receptor, Free et al. (2014) recently discovered and characterized G protein signaling-biased agonists that inhibit β -arrestin recruitment, but activates G protein signaling (e.g., cAMP regulation). There is a possibility that partial agonists and antagonists for AC signaling may have different intrinsic activity for non-AC signaling. Although how non-AC signaling contributes to the behavioral effects of the D₁ receptor is still elusive, non-AC signaling pathway(s) (excluding erroneous PLC activation) should be considered for the development of full D₁ agonists and tested for novel D₁ agonists.

Other research topics for the development of full D₁ agonists

Finally, an obvious topic of future research is to investigate the possibility of allosteric ligands as a way to accomplish long-term goals for which I focused on orthosteric compounds in this dissertation. To this date, no good allosteric ligand has

been reported yet for any of the dopamine receptors. Based on the anecdotal reports from several investigators who had tried to search for such ligands and consideration of the informal data from the research groups expert in this type of study, we felt that screening for allosteric modulators would not be an attractive topic. It may be that after a non-catechol and selective D₁ agonist is discovered, there would be utility for discovery of a functionally selective allosteric modulator that might attenuate D₁-side effects that might occur (e.g., hypotension).

More directly, I have not discussed the discovery of selective ligands for the D₁ receptor versus the D₅ receptor, which is very similar to the D₁ and also triggers a G $\alpha_{OLF/S}$ -AC-cAMP signaling cascade. Although there are reports of drugs with modest selectivity, neither antagonist nor agonist adequately selective for these two very similar receptors has been found. D₁ or D₅ receptor selective agonists or antagonists would be great pharmacological tools to investigate the functional roles of each of D₁ and D₅ receptors. Activation of D₁ or D₅ receptors may have different roles in functions that can be targeted for more desirable drugs (e.g., reducing off-target effects). In addition, the molecular interaction studies with D₁ or D₅ selective ligands may provide a mechanism for how D₁ and D₅ receptor selectivity can be achieved by the ligands. One route towards such a ligand is to expand my studies into the D₅ area. Furthermore, there would be some more of the interesting future research topics that could be pursued based on this dissertation.

Appendix

Summary of the methods used in this work

Reagents and materials

Cell culture media, flasks and dishes were purchased from VWR (West Chester, PA). Trypsin and penicillin/streptomycin were purchased from Invitrogen (Grand Island, NY). UniFilter-96 GF/B RIA filter plates, Microscint™ 20, [³H]-SCH23390, and [³H]-N-methylspiperone were obtained from Perkin-Elmer Life Sciences Inc. (Boston, MA). SKF38393, quinpirole, SCH23390, (+)-butaclamol, 7-OH DPAT, and fetal bovine serum were purchased from Sigma Aldrich (St. Louis, MO). LEK-8829 was a gift from Dr. Marko Živin, and CY 208 243, cabergoline, lisuride, terguride, and rotigotine were purchased from Tocris Bioscience (Ellisville, MO). Dihydropyridine was synthesized according to published procedures (Brewster et al., 1990). 5-OH DPAT was obtained from Pfizer through Compound Transfer Program (Peapack, NJ).

Mutations of human dopamine D₁ receptors

The human dopamine D₁ receptor containing an HA tag was cloned from a human cDNA library and the human D₁ receptor was confirmed by DNA sequencing. The DNA plasmid vector that carries the wild-type dopamine D₁ receptor was used as a template for creating mutated D₁ receptors. Primers for mutated receptors were designed using the Quikchange Primer Design program (Stratagene, La Jolla, CA) and synthesized by Invitrogen (Carlsbad, CA). Single point mutations were introduced using the Quikchange™ kit (Stratagene, La Jolla, CA). Double mutations were made using the single mutated D₁ receptor as a template and the primers required for the other mutation. After mutagenesis, the particular mutations of the mutated D₁ receptors were confirmed by DNA sequencing. The verified DNA plasmids for the wild-type and the mutated D₁ receptors were stored at 4°C until their use for transfection.

Receptor source

HEK-293 cells were grown in 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 50 U/mL of penicillin and 50 µg/mL of streptomycin supplemented with 10% fetal bovine serum. HEK-293 cells were transiently transfected with human D₁ receptors using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA) in OptiMEM medium according to the manufacturer's protocol or using calcium phosphate precipitation (Kingston et al., 2003). Transiently transfected cells were used for radioreceptor and functional assays 48 h after transfection. For stable transfection, the cells were split into new plates 24 h after transfection, and every two or three days the culture media were replaced with fresh selection media containing 500 µg/mL G418. When stably transfected colonies were visible, the entire plate was resuspended and transferred to a new plate. These cells were maintained in DMEM (Hyclone Laboratories, Inc., South Logan, UT) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 500 µg/mL G418. CHO cells stably expressing the human D₁ or D_{2L} receptor were maintained in Ham's F-12 medium (Hyclone Laboratories, Inc., South Logan, UT) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 500 µg/mL G418.

Fresh or frozen rat striata were homogenized by several manual strokes in a Wheaton Teflon-glass homogenizer in 8 mL ice-cold 50 mM HEPES buffer with 4.0 mM MgCl₂ (pH 7.4). Tissue was centrifuged at 27,000 g for 20 min, the supernatant was discarded, and the pellet was homogenized (20 strokes) and resuspended in ice-cold buffer and centrifuged again. D₁ receptors were labeled with [³H]-SCH23390, and D₂ receptors were labeled with [³H]-N-methylspiperone. Total and non-specific bindings were defined, and saturation and competitive binding assays with rat striata were performed as described below.

Radioligand binding assay and K_{0.5} calculation

Expressions of D₁ or D_{2L} receptors and affinity of ligands were determined by saturation and competitive binding assays according to the published procedures (Gay et

al., 2004; Watts et al., 1993; Mottola et al., 2002; Kilts et al., 2002; Ryman-Rasmussen et al., 2005). The total protein concentrations of membrane preparations used in the saturation binding assay were determined using BioRad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). The competition curves of the receptor binding experiments were analyzed by the one-site binding equation of nonlinear regression using Prism 5.0 (GraphPad Software, San Diego, CA). $K_{0.5}$ values for test ligands were calculated by using Cheng-Prusoff equation with IC_{50} from sigmoid curves (Cheng and Prusoff, 1973).

cAMP radioimmunoassay

Transiently transfected HEK-293 cells were seeded in 48-well plates at a density of 10^5 cells per well and grown overnight. After aspirating the cell culture media, fresh assay media (serum-free media containing 25 mM HEPES, 500 μ M IBMX, 100 μ M propranolol, and 0.1% ascorbic acid) containing various concentrations of test compounds were added. The plates were incubated for 5 min at 37°C, and then the cells were rinsed with fresh assay media. After adding 0.1M HCl to each well, cAMP production was quantified using a modified radioimmunoassay according to the previously reported method (Brown et al., 2009).

Dopamine D₁ receptor homology model and ligand docking simulation

The crystal structure of a nanobody-stabilized active state of the β_2 -adrenergic receptor (PDB ID: 3P0G) was used as a template for the active-state D₁ receptor homology model. For the inactive-state D₁ receptor homology model, the β_2 -adrenergic receptor bound with the inverse agonist carazolol (PDB ID: 2RH1) was used. D₁ receptor homology models were constructed using PRIME 3.0 (Schrödinger, Inc., Portland, OR). The chemical structures of the test ligands were obtained from the PUBCHEM database, and the three dimensional coordinates were generated using Epik 2.3 in Schrodinger suite 2012. Docking simulation was performed with Maestro GLIDE (Schrödinger, Inc., Portland, OR). The most reliable docking poses were selected based on their energy level and visual inspection. Because the highly conserved D3.32 has been known to provide a

strong salt bridge for the biogenic amine neurotransmitters (Xhaard et al., 2006), the interaction between the amine nitrogen in the ligands and the conserved D3.32 of the D₁ receptor was considered when selecting docking poses (Floresca and Schetz, 2004).

The nomenclature suggested by Ballesteros and Weinstein (Ballesteros and Weinstein, 1995) was used to indicate the conserved amino acids in transmembrane segments of the D₁ receptor. According to this nomenclature, the most conserved single amino acid in each TM segment of the class A GPCRs is labeled as X.50 where X indicates the TM segment number. The positions of all other amino acids in the same TM segment are numbered incrementally or decrementally according to the relative position to X.50 (Ballesteros and Weinstein, 1995; Ballesteros et al., 2001).

Statistical analysis

Significant differences in binding affinity ($pK_{0.5}$) and functional values (maximal cAMP production and pEC_{50}) between wild-type and mutated D₁ receptors were analyzed using analysis of variance (ANOVA) with *post hoc* Tukey's test or Dunnett's test for multiple comparisons.

References

- Al-Fulaij MA, Ren Y, Beinborn M and Kopin AS (2008) Pharmacological analysis of human D1 AND D2 dopamine receptor missense variants. *J Mol Neurosci* **34**:211-223.
- al-Tajir G, Chandler CJ, Starr BS and Starr MS (1990) Opposite effects of stimulation of D1 and D2 dopamine receptors on the expression of motor seizures in mouse and rat. *Neuropharmacology* **29**:657-661.
- Amenta F, Ferrante F and Ricci A (1995) Pharmacological characterisation and autoradiographic localisation of dopamine receptor subtypes in the cardiovascular system and in the kidney. *Hypertens Res* **18 Suppl 1**:S23-S27.
- Andringa G, Drukarch B, Leysen JE, Cools AR and Stoof JC (1999a) The alleged dopamine D₁ receptor agonist SKF 83959 is a dopamine D₁ receptor antagonist in primate cells and interacts with other receptors. *Eur J Pharmacol* **364**:33-41.
- Andringa G, Stoof JC and Cools AR (1999b) Sub-chronic administration of the dopamine D(1) antagonist SKF 83959 in bilaterally MPTP-treated rhesus monkeys: stable therapeutic effects and wearing-off dyskinesia. *Psychopharmacology (Berl)* **146**:328-334.
- Arnsten AF, Cai JX, Murphy BL and Goldman-Rakic PS (1994) Dopamine D1 receptor mechanisms in the cognitive performance of young adult and aged monkeys. *Psychopharmacology (Berl)* **116**:143-151.
- Arnt J, Hyttel J and Sanchez C (1992) Partial and full dopamine D1 receptor agonists in mice and rats: relation between behavioural effects and stimulation of adenylate cyclase activity in vitro. *Eur J Pharmacol* **213**:259-267.
- Asin KE and Wirtshafter D (1993) Effects of repeated dopamine D₁ receptor stimulation on rotation and c-fos expression. *Eur J Pharmacol* **235**:167-168.
- Aubert I, Ghorayeb I, Normand E and Bloch B (2000) Phenotypical characterization of the neurons expressing the D1 and D2 dopamine receptors in the monkey striatum. *J Comp Neurol* **418**:22-32.
- Bach NJ, Kornfeld EC, Jones ND, Chaney MO, Dorman DE, Paschal JW, Clemens JA and Smalstig EB (1980) Bicyclic and tricyclic ergoline partial structures. Rigid 3-(2-aminoethyl)pyrroles and 3- and 4-(2-aminoethyl)pyrazoles as dopamine agonists. *J Med Chem* **23**:481-491.
- Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U and Javitch JA (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J Biol Chem* **276**:29171-29177.

- Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci* **25**:366-428.
- Banday AA and Lokhandwala MF (2007) Oxidative stress reduces renal dopamine D1 receptor-Gq/11alpha G protein-phospholipase C signaling involving G protein-coupled receptor kinase 2. *Am J Physiol Renal Physiol* **293**:F306-F315.
- Barnes TR and McPhillips MA (1999) Critical analysis and comparison of the side-effect and safety profiles of the new antipsychotics. *Brit J Psychiatr Suppl* 34-43.
- Bateup HS, Svenningsson P, Kuroiwa M, Gong S, Nishi A, Heintz N and Greengard P (2008) Cell type-specific regulation of DARPP-32 phosphorylation by psychostimulant and antipsychotic drugs. *Nat Neurosci* **11**:932-939.
- Beaulieu M, Itoh Y, Tepper P, Horn AS and Keabian JW (1984) N,N-disubstituted 2-aminotetralins are potent D-2 dopamine receptor agonists. *Eur J Pharmacol* **105**:15-21.
- Belluzzi JD, Domino EF, May JM, Bankiewicz KS and McAfee DA (1994) N-0923, a selective dopamine D2 receptor agonist, is efficacious in rat and monkey models of Parkinson's disease. *Mov Disord* **9**:147-154.
- Bertran-Gonzalez J, Herve D, Girault JA and Valjent E (2010) What is the Degree of Segregation between Striatonigral and Striatopallidal Projections? *Front Neuroanat* **4**.
- Beuming T, Farid R and Sherman W (2009) High-energy water sites determine peptide binding affinity and specificity of PDZ domains. *Protein Sci* **18**:1609-1619.
- Blanchet PJ (1999) Rationale for use of dopamine agonists in Parkinson's disease: review of ergot derivatives. *Can J Neurol Sci* **26 Suppl 2**:S21-S26.
- Blanchet PJ, Fang J, Gillespie M, Sabounjian L, Locke KW, Gammans R, Mouradian MM and Chase TN (1998) Effects of the full dopamine D1 receptor agonist dihydrexidine in Parkinson's disease. *Clin Neuropharmacol* **21**:339-343.
- Blanchet PJ, Grondin R, Bedard PJ, Shiosaki K and Britton DR (1996) Dopamine D₁ receptor desensitization profile in MPTP-lesioned primates. *Eur J Pharmacol* **309**:13-20.
- Bonner LA, Laban U, Chemel BR, Juncosa JJ, Lill MA, Watts VJ and Nichols DE (2011) Mapping the catechol binding site in dopamine D(1) receptors: synthesis and evaluation of two parallel series of bicyclic dopamine analogues. *ChemMedChem* **6**:1024-1040.
- Brami-Cherrier K, Valjent E, Garcia M, Pages C, Hipkind RA and Caboche J (2002) Dopamine induces a PI3-kinase-independent activation of Akt in striatal neurons: a new route to cAMP response element-binding protein phosphorylation. *J Neurosci* **22**:8911-8921.
- Braun A, Fabbrini G, Mouradian MM, Serrati C, Barone P and Chase TN (1987) Selective D-1 dopamine receptor agonist treatment of Parkinson's disease. *J Neural Transm* **68**:41-50.

- Breiten B, Lockett MR, Sherman W, Fujita S, Al-Sayah M, Lange H, Bowers CM, Heroux A, Krilov G and Whitesides GM (2013) Water networks contribute to enthalpy/entropy compensation in protein-ligand binding. *J Am Chem Soc* **135**:15579-15584.
- Brewster WK, Nichols DE, Riggs RM, Mottola DM, Lovenberg TW, Lewis MH and Mailman RB (1990) trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[a]phenanthridine: a highly potent selective dopamine D1 full agonist. *J Med Chem* **33**:1756-1764.
- Britton DR, Kebabian JW and Curzon P (1991) Rapid reversal of denervation supersensitivity of dopamine D1 receptors by l-dopa or a novel dopamine D1 receptor agonist, A68930. *Eur J Pharmacol* **200**:89-93.
- Brown JT, Kant A and Mailman RB (2009) Rapid, semi-automated, and inexpensive radioimmunoassay of cAMP: Application in GPCR-mediated adenylate cyclase assays. *J Neurosci Methods* **177**:261-266.
- Burris KD, Molski TF, Xu C, Ryan E, Tottori K, Kikuchi T, Yocca FD and Molinoff PB (2002) Aripiprazole, a novel antipsychotic, is a high-affinity partial agonist at human dopamine D2 receptors. *J Pharmacol Exp Ther* **302**:381-389.
- Cai JX and Arnsten AF (1997) Dose-dependent effects of the dopamine D1 receptor agonists A77636 or SKF81297 on spatial working memory in aged monkeys. *J Pharmacol Exp Ther* **283**:183-189.
- Calabresi P, Mercuri N, Stanzione P, Stefani A and Bernardi G (1987) Intracellular studies on the dopamine-induced firing inhibition of neostriatal neurons in vitro: evidence for D1 receptor involvement. *Neuroscience* **20**:757-771.
- Calne DB (1999) Differentiation of dopamine agonists and their role in the treatment of Parkinson's disease. *J Neural Transm Suppl* **56**:185-192.
- Cantrell AR, Smith RD, Goldin AL, Scheuer T and Catterall WA (1997) Dopaminergic modulation of sodium current in hippocampal neurons via cAMP-dependent phosphorylation of specific sites in the sodium channel alpha subunit. *J Neurosci* **17**:7330-7338.
- Cawello W, Braun M and Boekens H (2009) Absorption, disposition, metabolic fate, and elimination of the dopamine agonist rotigotine in man: administration by intravenous infusion or transdermal delivery. *Drug Metab Dispos* **37**:2055-2060.
- Chandler D (2005) Interfaces and the driving force of hydrophobic assembly. *Nature* **437**:640-647.
- Chemel BR, Bonner LA, Watts VJ and Nichols DE (2012) Ligand-specific roles for transmembrane 5 serine residues in the binding and efficacy of dopamine D(1) receptor catechol agonists. *Mol Pharmacol* **81**:729-738.
- Chen J, Rusnak M, Luedtke RR and Sidhu A (2004) D1 dopamine receptor mediates dopamine-induced cytotoxicity via the ERK signal cascade. *J Biol Chem* **279**:39317-39330.

- Cheng K, Khurana S, Chen Y, Kennedy RH, Zimniak P and Raufman JP (2002) Lithocholylcholine, a bile acid/acetylcholine hybrid, is a muscarinic receptor antagonist. *J Pharmacol Exp Ther* **303**:29-35.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.
- Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**:1258-1265.
- Cho W, Taylor LP, Mansour A and Akil H (1995) Hydrophobic residues of the D2 dopamine receptor are important for binding and signal transduction. *J Neurochem* **65**:2105-2115.
- Choudhary MS, Sachs N, Uluer A, Glennon RA, Westkaemper RB and Roth BL (1995) Differential ergoline and ergopeptine binding to 5-hydroxytryptamine_{2A} receptors: ergolines require an aromatic residue at position 340 for high affinity binding. *Mol Pharmacol* **47**:450-457.
- Chun LS, Free RB, Doyle TB, Huang XP, Rankin ML and Sibley DR (2013) D1-D2 dopamine receptor synergy promotes calcium signaling via multiple mechanisms. *Mol Pharmacol* **84**:190-200.
- Corvol JC, Studler JM, Schonn JS, Girault JA and Herve D (2001) $G_{\alpha(olf)}$ is necessary for coupling D1 and A2a receptors to adenylyl cyclase in the striatum. *J Neurochem* **76**:1585-1588.
- Cotecchia S, Fanelli F and Costa T (2003) Constitutively active G protein-coupled receptor mutants: implications on receptor function and drug action. *Assay Drug Dev Technol* **1**:311-316.
- Creese I, Burt DR and Snyder SH (1976) Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. *Science* **192**:481-483.
- Cueva JP, Gallardo-Godoy A, Juncosa JI, Vidi PA, Lill MA, Watts VJ and Nichols DE (2011) Probing the steric space at the floor of the D1 dopamine receptor orthosteric binding domain: 7 α -, 7 β -, 8 α -, and 8 β -methyl substituted dihydrexidine analogues. *J Med Chem* **54**:5508-5521.
- Cull-Candy S, Kelly L and Farrant M (2006) Regulation of Ca²⁺-permeable AMPA receptors: synaptic plasticity and beyond. *Curr Opin Neurobiol* **16**:288-297.
- Cummings DF, Ericksen SS, Goetz A and Schetz JA (2010) Transmembrane segment five serines of the D4 dopamine receptor uniquely influence the interactions of dopamine, norepinephrine, and Ro10-4548. *J Pharmacol Exp Ther* **333**:682-695.
- Daeffler KN, Lester HA and Dougherty DA (2012) Functionally important aromatic-aromatic and sulfur- π interactions in the D2 dopamine receptor. *J Am Chem Soc* **134**:14890-14896.

- Damier P, Hirsch EC, Agid Y and Graybiel AM (1999) The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. *Brain* **122** (Pt 8):1437-1448.
- Darney KJ, Jr., Lewis MH, Brewster WK, Nichols DE and Mailman RB (1991) Behavioral effects in the rat of dihydrexidine, a high-potency, full-efficacy D1 dopamine receptor agonist. *Neuropsychopharmacology* **5**:187-195.
- De Keyser J. (1993) Subtypes and localization of dopamine receptors in human brain. *Neurochem Int* **22**:83-93.
- de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A and Bos JL (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396**:474-477.
- Dearry A, Gingrich JA, Falardeau P, Fremeau RT, Jr., Bates MD and Caron MG (1990) Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature* **347**:72-76.
- Del'guidice T, Lemasson M and Beaulieu JM (2011) Role of Beta-arrestin 2 downstream of dopamine receptors in the Basal Ganglia. *Front Neuroanat* **5**:58.
- Demchyshyn LL, Sugamori KS, Lee FJ, Hamadanizadeh SA and Niznik HB (1995) The dopamine D1/D2 receptor. Cloning and characterization of three pharmacologically distinct D1-like receptors from Gallus domesticus. *J Biol Chem* **270**:4005-4012.
- Deng YP, Lei WL and Reiner A (2006) Differential perikaryal localization in rats of D1 and D2 dopamine receptors on striatal projection neuron types identified by retrograde labeling. *J Chem Neuroanat* **32**:101-116.
- DeNinno MP, Schoenleber R, MacKenzie R, Britton DR, Asin KE, Briggs C, Trugman JM, Ackerman M, Artman L and Bednarz L (1991) A68930: a potent agonist selective for the dopamine D1 receptor. *Eur J Pharmacol* **199**:209-219.
- Deveney AM and Waddington JL (1995) Pharmacological characterization of behavioural responses to SK&F 83959 in relation to 'D1-like' dopamine receptors not linked to adenylyl cyclase. *Br J Pharmacol* **116**:2120-2126.
- Diaz HR and Castellanos FX (2006) Differential effects of a selective dopamine D1-like receptor agonist on motor activity and c-fos expression in the frontal-striatal circuitry of SHR and Wistar-Kyoto rats. *Behav Brain Funct* **2**:18.
- Dill KA (1990) Dominant forces in protein folding. *Biochemistry* **29**:7133-7155.
- Dong Y, Cooper D, Nasif F, Hu XT and White FJ (2004) Dopamine modulates inwardly rectifying potassium currents in medial prefrontal cortex pyramidal neurons. *J Neurosci* **24**:3077-3085.

- Dong Y and White FJ (2003) Dopamine D1-class receptors selectively modulate a slowly inactivating potassium current in rat medial prefrontal cortex pyramidal neurons. *J Neurosci* **23**:2686-2695.
- Downes RP and Waddington JL (1993) Grooming and vacuous chewing induced by SK&F 83959, an agonist of dopamine 'D1-like' receptors that inhibits dopamine-sensitive adenylyl cyclase. *Eur J Pharmacol* **234**:135-136.
- Dyck LE (1990) Effects of dopamine on phosphoinositide hydrolysis in slices of rat striatum and cortex. *Neurochem Int* **17**:77-82.
- Ehrlich K, Gotz A, Bollinger S, Tschammer N, Bettinetti L, Harterich S, Hubner H, Lanig H and Gmeiner P (2009) Dopamine D2, D3, and D4 selective phenylpiperazines as molecular probes to explore the origins of subtype specific receptor binding. *J Med Chem* **52**:4923-4935.
- Emre M, Rinne UK, Rascol A, Lees A, Agid Y and Lataste X (1992) Effects of a selective partial D1 agonist, CY 208-243, in de novo patients with Parkinson disease. *Mov Disord* **7**:239-243.
- Felder CC, Blecher M and Jose PA (1989a) Dopamine-1-mediated stimulation of phospholipase C activity in rat renal cortical membranes. *J Biol Chem* **264**:8739-8745.
- Felder CC, Jose PA and Axelrod J (1989b) The dopamine-1 agonist, SKF 82526, stimulates phospholipase-C activity independent of adenylate cyclase. *J Pharmacol Exp Ther* **248**:171-175.
- Fici GJ, Wu H, VonVoigtlander PF and Sethy VH (1997) D1 dopamine receptor activity of anti-parkinsonian drugs. *Life Sci* **60**:1597-1603.
- Floresca CZ and Schetz JA (2004) Dopamine receptor microdomains involved in molecular recognition and the regulation of drug affinity and function. *J Recept Signal Transduct Res* **24**:207-239.
- Fowler JC, Bhattacharya S, Urban JD, Vaidehi N and Mailman RB (2012) Receptor conformations involved in dopamine D(2L) receptor functional selectivity induced by selected transmembrane-5 serine mutations. *Mol Pharmacol* **81**:820-831.
- Free RB, Chun LS, Moritz AE, Miller B, Doyle TB, Conroy JL, Padron A, Meade JA, Xiao J, Hu X, Dulcey AE, Han Y, Duan L, Titus S, Bryant-Genevier M, Barnaeva E, Ferrer M, Javitch JA, Beuming T, Shi L, Southall N, Marugan JJ and Sibley DR (2014) Discovery and Characterization of a G Protein-biased Agonist that Inhibits beta-arrestin Recruitment to the D2 Dopamine Receptor. *Mol Pharmacol*.
- Friedman E, Butkerait P and Wang HY (1993) Analysis of receptor-stimulated and basal guanine nucleotide binding to membrane G proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Analytical Biochemistry* **214**:171-178.

- Friedman E, Jin LQ, Cai GP, Hollon TR, Drago J, Sibley DR and Wang HY (1997) D1-like dopaminergic activation of phosphoinositide hydrolysis is independent of D1A dopamine receptors: evidence from D1A knockout mice. *Mol Pharmacol* **51**:6-11.
- Fujita S, Kiguchi M, Kobayashi M, Kinsella A, Koshikawa N and Waddington JL (2010) Assessment of jaw movements by magnetic sensor in relation to topographies of orofacial behaviour in freely moving rats: Studies with the dopamine D(1)-like receptor agonists SKF 83822 vs SKF 83959. *Eur J Pharmacol* **632**:39-44.
- Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ and Caron MG (2004) Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci* **27**:107-144.
- Garau L, Govoni S, Stefanini E, Trabucchi M and Spano PF (1978) Dopamine receptors: pharmacological and anatomical evidences indicate that two distinct dopamine receptor populations are present in rat striatum. *Life Sci* **23**:1745-1750.
- Gay EA, Urban JD, Nichols DE, Oxford GS and Mailman RB (2004) Functional selectivity of D2 receptor ligands in a Chinese hamster ovary hD2L cell line: evidence for induction of ligand-specific receptor states. *Mol Pharmacol* **66**:97-105.
- George MS, Molnar CE, Grenesko EL, Anderson B, Mu Q, Johnson K, Nahas Z, Knable M, Fernandes P, Juncos J, Huang X, Nichols DE and Mailman RB (2007) A single 20 mg dose of dihydrexidine (DAR-0100), a full dopamine D(1) agonist, is safe and tolerated in patients with schizophrenia. *Schizophr Res* **93**:42-50.
- Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ, Jr. and Sibley DR (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**:1429-1432.
- Glase SA, Corbin AE, Pugsley TA, Heffner TG and Wise LD (1995) Synthesis and dopaminergic activity of pyridine analogs of 5-hydroxy-2-(di-n-propylamino)tetralin. *J Med Chem* **38**:3132-3137.
- Glatt CE and Snyder SH (1993) Cloning and Expression of An Adenylyl Cyclase Localized to the Corpus Striatum. *Nature* **361**:536-538.
- Gnanalingham KK, Erol DD, Hunter AJ, Smith LA, Jenner P and Marsden CD (1995a) Differential anti-parkinsonian effects of benzazepine D₁ dopamine agonists with varying efficacies in the MPTP-treated common marmoset. *Psychopharmacol* **117**:275-286.
- Gnanalingham KK, Hunter AJ, Jenner P and Marsden CD (1995b) Stimulation of adenylate cyclase activity by benzazepine D-1 dopamine agonists with varying efficacies in the 6-hydroxydopamine lesioned rat--relationship to circling behaviour. *Biochem Pharmacol* **49**:1185-1193.
- Goldman-Rakic PS, Castner SA, Svensson TH, Siever LJ and Williams GV (2004) Targeting the dopamine D₁ receptor in schizophrenia: insights for cognitive dysfunction. *Psychopharmacology (Berl)*.

- Gorelova N, Seamans JK and Yang CR (2002) Mechanisms of dopamine activation of fast-spiking interneurons that exert inhibition in rat prefrontal cortex. *J Neurophysiol* **88**:3150-3166.
- Gulwadi AG, Korpinen CD, Mailman RB, Nichols DE, Sit SY and Taber MT (2001) Dinapsoline: characterization of a D₁ dopamine receptor agonist in a rat model of Parkinson's disease. *J Pharmacol Exp Ther* **296**:338-344.
- Guo L, Zhao J, Jin G, Zhao B, Wang G, Zhang A and Zhen X (2013) SKF83959 Is a Potent Allosteric Modulator of Sigma-1 Receptor. *Mol Pharmacol* **83**:577-586.
- Haney M, Collins ED, Ward AS, Foltin RW and Fischman MW (1999) Effect of a selective dopamine D₁ agonist (ABT-431) on smoked cocaine self-administration in humans. *Psychopharmacology (Berl)* **143**:102-110.
- Harper JF and Brooker G (1975) Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'0 acetylation by acetic anhydride in aqueous solution. *J Cyclic Nucleotide Res* **1**:207-218.
- Harrison MB, Wiley RG and Wooten GF (1990) Selective localization of striatal D₁ receptors to striatonigral neurons. *Brain Res* **528**:317-322.
- Hasbi A, Fan T, Alijaniam M, Nguyen T, Perreault ML, O'Dowd BF and George SR (2009) Calcium signaling cascade links dopamine D₁-D₂ receptor heteromer to striatal BDNF production and neuronal growth. *Proc Natl Acad Sci U S A* **106**:21377-21382.
- Hasbi A, O'Dowd BF and George SR (2011) Dopamine D₁-D₂ receptor heteromer signaling pathway in the brain: emerging physiological relevance. *Mol Brain* **4**:26.
- Hassler R (1938) Zur pathologie der paralysis agitans und des postenzephlitischen parkinsonismus. *J Psychol Neurol* **48**:387-476.
- Heffner TG, Hartman JA and Seiden LS (1980) A rapid method for the regional dissection of the rat brain. *Pharmacol Biochem Behav* **13**:453-456.
- Hernandez-Lopez S, Bargas J, Surmeier DJ, Reyes A and Galarraga E (1997) D₁ receptor activation enhances evoked discharge in neostriatal medium spiny neurons by modulating an L-type Ca²⁺ conductance. *J Neurosci* **17**:3334-3342.
- Hersi AI, Rowe W, Gaudreau P and Quirion R (1995) Dopamine D₁ receptor ligands modulate cognitive performance and hippocampal acetylcholine release in memory-impaired aged rats. *Neuroscience* **69**:1067-1074.
- Herve D, Levi-Strauss M, Marey-Semper I, Verney C, Tassin JP, Glowinski J and Girault JA (1993) G_{olf} and G_s in rat basal ganglia: possible involvement of G_{olf} in the coupling of dopamine D₁ receptor with adenylyl cyclase. *J Neurosci* **13**:2237-2248.
- Hobson DE, Pourcher E and Martin WR (1999) Ropinirole and pramipexole, the new agonists. *Can J Neurol Sci* **26 Suppl 2**:S27-S33.

- Hopf FW, Cascini MG, Gordon AS, Diamond I and Bonci A (2003) Cooperative activation of dopamine D1 and D2 receptors increases spike firing of nucleus accumbens neurons via G-protein betagamma subunits. *J Neurosci* **23**:5079-5087.
- Huang X, Lawler CP, Lewis MM, Nichols DE and Mailman RB (2001) D1 dopamine receptors. *Int Rev Neurobiol* **48**:65-139.
- Iorio LC, Barnett A, Leitz FH, Houser VP and Korduba CA (1983) SCH 23390, a potential benzazepine antipsychotic with unique interactions on dopaminergic systems. *J Pharmacol Exp Ther* **226**:462-468.
- Iwamoto T, Okumura S, Iwatsubo K, Kawabe J, Ohtsu K, Sakai I, Hashimoto Y, Izumitani A, Sango K, Ajiki K, Toya Y, Umemura S, Goshima Y, Arai N, Vatner SF and Ishikawa Y (2003) Motor dysfunction in type 5 adenylyl cyclase-null mice. *J Biol Chem* **278**:16936-16940.
- Jakubik J, Bacakova L, El-Fakahany EE and Tucek S (1997) Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. *Mol Pharmacol* **52**:172-179.
- Jansen JM, den D, I, Rollema H, Swart PJ, Tepper PG, De Vries JB and Horn AS (1991) Pharmacological profile of non-hydroxylated and ether derivatives of the potent D2-selective agonist N-0437. *Naunyn Schmiedebergs Arch Pharmacol* **343**:134-142.
- Javitch JA, Ballesteros JA, Weinstein H and Chen J (1998) A cluster of aromatic residues in the sixth membrane-spanning segment of the dopamine D2 receptor is accessible in the binding-site crevice. *Biochemistry* **37**:998-1006.
- Jin LQ, Goswami S, Cai G, Zhen X and Friedman E (2003) SKF83959 selectively regulates phosphatidylinositol-linked D₁ dopamine receptors in rat brain. *J Neurochem* **85**:378-386.
- Kebabian JW, Britton DR, DeNinno MP, Perner R, Smith L, Jenner P, Schoenleber R and Williams M (1992) A-77636: a potent and selective dopamine D1 receptor agonist with antiparkinsonian activity in marmosets. *Eur J Pharmacol* **229**:203-209.
- Kebabian JW and Calne DB (1979) Multiple receptors for dopamine. *Nature* **277**:93-96.
- Kebabian JW and Greengard P (1971) Dopamine-sensitive adenylyl cyclase: possible role in synaptic transmission. *Science* **174**:1346-1349.
- Kebabian JW, Petzold GL and Greengard P (1972) Dopamine-sensitive adenylyl cyclase in caudate nucleus of rat brain, and its similarity to the "dopamine receptor". *Proc Natl Acad Sci U S A* **69**:2145-2149.
- Kekewska A, Gornemann T, Jantschak F, Glusa E and Pertz HH (2012) Antiserotonergic properties of terguride in blood vessels, platelets, and valvular interstitial cells. *J Pharmacol Exp Ther* **340**:369-376.

- Kelly E, Batty I and Nahorski SR (1988) Dopamine receptor stimulation does not affect phosphoinositide hydrolysis in slices of rat striatum. *J Neurochem* **51**:918-924.
- Kenakin T (2007) Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. *Trends Pharmacol Sci* **28**:407-415.
- Kikuchi T, Tottori K, Uwahodo Y, Hirose T, Miwa T, Oshiro Y and Morita S (1995) 7-(4-[4-(2,3-Dichlorophenyl)-1-piperazinyl]butyloxy)-3,4-dihydro-2(1H)-quinolinone (OPC-14597), a new putative antipsychotic drug with both presynaptic dopamine autoreceptor agonistic activity and postsynaptic D2 receptor antagonistic activity. *J Pharmacol Exp Ther* **274**:329-336.
- Kilts JD, Connery HS, Arrington EG, Lewis MM, Lawler CP, Oxford GS, O'Malley KL, Todd RD, Blake BL, Nichols DE and Mailman RB (2002) Functional selectivity of dopamine receptor agonists. II. Actions of dihydrexidine in D2L receptor-transfected MN9D cells and pituitary lactotrophs. *J Pharmacol Exp Ther* **301**:1179-1189.
- Kimura K, Sela S, Bouvier C, Grandy DK and Sidhu A (1995) Differential coupling of D1 and D5 dopamine receptors to guanine nucleotide binding proteins in transfected GH4C1 rat somatomammotrophic cells. *J Neurochem* **64**:2118-2124.
- Kingston RE, Chen CA and Rose JK (2003) Calcium phosphate transfection. *Curr Protoc Mol Biol* **Chapter 9**:Unit.
- Kisilevsky AE, Mulligan SJ, Altier C, Iftinca MC, Varela D, Tai C, Chen L, Hameed S, Hamid J, Macvicar BA and Zamponi GW (2008) D1 receptors physically interact with N-type calcium channels to regulate channel distribution and dendritic calcium entry. *Neuron* **58**:557-570.
- Krisch I, Bole-Vunduk B, Pepelnak M, Lavric B, Ocvirk A, Budihna MV and Sket D (1994) Pharmacological studies with two new ergoline derivatives, the potential antipsychotics LEK-8829 and LEK-8841. *J Pharmacol Exp Ther* **271**:343-352.
- Kumar S and Nussinov R (2002) Close-range electrostatic interactions in proteins. *Chembiochem* **3**:604-617.
- Kurrasch-Orbaugh DM, Watts VJ, Barker EL and Nichols DE (2003) Serotonin 5-hydroxytryptamine 2A receptor-coupled phospholipase C and phospholipase A2 signaling pathways have different receptor reserves. *J Pharmacol Exp Ther* **304**:229-237.
- Kvernmo T, Hartter S and Burger E (2006) A review of the receptor-binding and pharmacokinetic properties of dopamine agonists. *Clin Ther* **28**:1065-1078.
- Laage D and Hynes JT (2006) A molecular jump mechanism of water reorientation. *Science* **311**:832-835.
- Lawler CP, Prioleau C, Lewis MM, Mak C, Jiang D, Schetz JA, Gonzalez AM, Sibley DR and Mailman RB (1999) Interactions of the novel antipsychotic aripiprazole (OPC-14597) with dopamine and serotonin receptor subtypes. *Neuropsychopharmacology* **20**:612-627.

- Le Moine C and Bloch B (1995) D1 and D2 dopamine receptor gene expression in the rat striatum: sensitive cRNA probes demonstrate prominent segregation of D1 and D2 mRNAs in distinct neuronal populations of the dorsal and ventral striatum. *J Comp Neurol* **355**:418-426.
- Lebon G, Warne T and Tate CG (2012) Agonist-bound structures of G protein-coupled receptors. *Curr Opin Struct Biol* **22**:482-490.
- Lee KW, Hong JH, Choi IY, Che Y, Lee JK, Yang SD, Song CW, Kang HS, Lee JH, Noh JS, Shin HS and Han PL (2002) Impaired D2 dopamine receptor function in mice lacking type 5 adenylyl cyclase. *J Neurosci* **22**:7931-7940.
- Lee SM, Kant A, Blake D, Murthy V, Boyd K, Wyrick SJ and Mailman RB (2014) SKF-83959 is not a highly-biased functionally selective D dopamine receptor ligand with activity at phospholipase C. *Neuropharmacology*.
- Lee SP, So CH, Rashid AJ, Varghese G, Cheng R, Lanca AJ, O'Dowd BF and George SR (2004) Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal. *J Biol Chem* **279**:35671-35678.
- Lesser GJ and Rose GD (1990) Hydrophobicity of amino acid subgroups in proteins. *Proteins* **8**:6-13.
- Lewis MM, Watts VJ, Lawler CP, Nichols DE and Mailman RB (1998) Homologous desensitization of the D1A dopamine receptor: efficacy in causing desensitization dissociates from both receptor occupancy and functional potency. *J Pharmacol Exp Ther* **286**:345-353.
- Lezcano N, Mrzljak L, Eubanks S, Levenson R, Goldman-Rakic P and Bergson C (2000) Dual signaling regulated by calcyon, a D1 dopamine receptor interacting protein. *Science* **287**:1660-1664.
- Lezcano N, Mrzljak L, Levenson R and Bergson C (2006) Retraction. *Science* 1681.
- Lidow MS and Goldman-Rakic PS (1994) A common action of clozapine, haloperidol, and remoxipride on D1- and D2-dopaminergic receptors in the primate cerebral cortex. *Proc Natl Acad Sci U S A* **91**:4353-4356.
- Lin CW, Bianchi BR, Miller TR, Stashko MA, Wang SS, Curzon P, Bednarz L, Asin KE and Britton DR (1996) Persistent activation of the dopamine D₁ receptor contributes to prolonged receptor desensitization: studies with A-77636. *J Pharmacol Exp Ther* **276**:1022-1029.
- Lin SW and Sakmar TP (1996) Specific tryptophan UV-absorbance changes are probes of the transition of rhodopsin to its active state. *Biochemistry* **35**:11149-11159.
- Liu J, Wang F, Huang C, Long LH, Wu WN, Cai F, Wang JH, Ma LQ and Chen JG (2009a) Activation of phosphatidylinositol-linked novel D1 dopamine receptor contributes to the calcium mobilization in cultured rat prefrontal cortical astrocytes. *Cell Mol Neurobiol* **29**:317-328.

- Liu J, Wang W, Wang F, Cai F, Hu ZL, Yang YJ, Chen J and Chen JG (2009b) Phosphatidylinositol-linked novel D(1) dopamine receptor facilitates long-term depression in rat hippocampal CA1 synapses. *Neuropharmacology* **57**:164-171.
- Luebke JI, Dunlap K and Turner TJ (1993) Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron* **11**:895-902.
- Mahan LC, Burch RM, Monsma FJ, Jr. and Sibley DR (1990) Expression of striatal D1 dopamine receptors coupled to inositol phosphate production and Ca²⁺ mobilization in *Xenopus* oocytes. *Proc Natl Acad Sci USA* **87**:2196-2200.
- Mailman R, Huang X and Nichols DE (2001) Parkinson's disease and D1 dopamine receptors. *Curr Opin Investig Drugs* **2**:1582-1591.
- Mailman RB (2007) GPCR functional selectivity has therapeutic impact. *Trends Pharmacol Sci* **28**:390-396.
- Mailman RB and Huang X (2007) Dopamine receptor pharmacology. *Handb Clin Neurol* **83**:77-105.
- Mailman RB and Murthy V (2010) Third generation antipsychotic drugs: partial agonism or receptor functional selectivity? *Curr Pharm Des* **16**:488-501.
- Mailman RB and Nichols DE (1998) Dopamine D1 receptor agonists as antiparkinson drugs. *Trends Pharmacol Sci* **19**:255-256.
- Mailman RB, Nichols DE, Lewis MM, Blake BL and Lawler CP (1998) Functional Effects of Novel Dopamine Ligands: Dihydroxidine and Parkinson's Disease as a First Step, in *Dopamine Receptor Subtypes: From Basic Science to Clinical Application* (Jenner P and Demirdemar R eds) pp 64-83, IOS Stockton Press.
- Malo M, Brive L, Luthman K and Svensson P (2012) Investigation of D(1) receptor-agonist interactions and D(1)/D(2) agonist selectivity using a combination of pharmacophore and receptor homology modeling. *ChemMedChem* **7**:483-94, 338.
- Mansour A, Meng F, Meador-Woodruff JH, Taylor LP, Civelli O and Akil H (1992) Site-directed mutagenesis of the human dopamine D2 receptor. *Eur J Pharmacol* **227**:205-214.
- Markstein R, Seiler MP, Jatón A and Briner U (1992) Structure activity relationship and therapeutic uses of dopaminergic ergots. *Neurochem Int* **20 Suppl**:211S-214S.
- Marsden CD and Parkes JD (1976) "On-off" effects in patients with Parkinson's disease on chronic levodopa therapy. *Lancet* **1**:292-296.
- Marsili S, Chelli R, Schettino V and Procacci P (2008) Thermodynamics of stacking interactions in proteins. *Phys Chem Chem Phys* **10**:2673-2685.

- Matamales M, Bertran-Gonzalez J, Salomon L, Degos B, Deniau JM, Valjent E, Herve D and Girault JA (2009) Striatal medium-sized spiny neurons: identification by nuclear staining and study of neuronal subpopulations in BAC transgenic mice. *PLoS One* **4**:e4770.
- McCorvy JD, Watts VJ and Nichols DE (2012) Comparison of the D(1) dopamine full agonists, dihydrexidine and doxanthrine, in the 6-OHDA rat model of Parkinson's disease. *Psychopharmacology (Berl)* **222**:81-87.
- McDermid JD, McKenzie GM and Freeman HS (1976) Synthesis and dopaminergic activity of (+/-)-, (+)-, and (-)-2-dipropylamino-5-hydroxy-1,2,3,4-tetrahydronaphthalene. *J Med Chem* **19**:547-549.
- McGaughey GB, Gagne M and Rappe AK (1998) pi-Stacking interactions. Alive and well in proteins. *J Biol Chem* **273**:15458-15463.
- Meador-Woodruff JH, Damask SP, Wang J, Haroutunian V, Davis KL and Watson SJ (1996) Dopamine receptor mRNA expression in human striatum and neocortex. *Neuropsychopharmacology* **15**:17-29.
- Medvedev IO, Ramsey AJ, Masoud ST, Bermejo MK, Urs N, Sotnikova TD, Beaulieu JM, Gainetdinov RR and Salahpour A (2013) D1 dopamine receptor coupling to PLCbeta regulates forward locomotion in mice. *J Neurosci* **33**:18125-18133.
- Michino M, Donthamsetti P, Beuming T, Banala A, Duan L, Roux T, Han Y, Trinquet E, Newman AH, Javitch JA and Shi L (2013) A single glycine in extracellular loop 1 is the critical determinant for pharmacological specificity of dopamine D2 and D3 receptors. *Mol Pharmacol* **84**:854-864.
- Millan MJ, Maiofiss L, Cussac D, Audinot V, Boutin JA and Newman-Tancredi A (2002) Differential actions of antiparkinson agents at multiple classes of monoaminergic receptor. I. A multivariate analysis of the binding profiles of 14 drugs at 21 native and cloned human receptor subtypes. *J Pharmacol Exp Ther* **303**:791-804.
- Mizuno K, Kurokawa K and Ohkuma S (2012) Dopamine D1 receptors regulate type 1 inositol 1,4,5-trisphosphate receptor expression via both AP-1- and NFATc4-mediated transcriptional processes. *J Neurochem* **122**:702-713.
- Monsma FJ, Jr., McVittie LD, Gerfen CR, Mahan LC and Sibley DR (1989) Multiple D2 dopamine receptors produced by alternative RNA splicing. *Nature* **342**:926-929.
- Mottola DM, Kilts JD, Lewis MM, Connery HS, Walker QD, Jones SR, Booth RG, Hyslop DK, Piercey M, Wightman RM, Lawler CP, Nichols DE and Mailman RB (2002) Functional selectivity of dopamine receptor agonists. I. Selective activation of postsynaptic dopamine D2 receptors linked to adenylate cyclase. *J Pharmacol Exp Ther* **301**:1166-1178.
- Mottola DM, Laiter S, Watts VJ, Tropsha A, Wyrick SD, Nichols DE and Mailman RB (1996) Conformational analysis of D1 dopamine receptor agonists: pharmacophore assessment and receptor mapping. *J Med Chem* **39**:285-296.

- Myohanen TT, Schendzielorz N and Mannisto PT (2010) Distribution of catechol-O-methyltransferase (COMT) proteins and enzymatic activities in wild-type and soluble COMT deficient mice. *J Neurochem* **113**:1632-1643.
- Nagai T, Takuma K, Kamei H, Ito Y, Nakamichi N, Ibi D, Nakanishi Y, Murai M, Mizoguchi H, Nabeshima T and Yamada K (2007) Dopamine D1 receptors regulate protein synthesis-dependent long-term recognition memory via extracellular signal-regulated kinase 1/2 in the prefrontal cortex. *Learn Mem* **14**:117-125.
- Neumeyer JL, Kula NS, Bergman J and Baldessarini RJ (2003) Receptor affinities of dopamine D1 receptor-selective novel phenylbenzazepines. *Eur J Pharmacol* **474**:137-140.
- Neve KA (2009) *Functional Selectivity of G Protein-Coupled Receptor Ligands*. Humana, New York.
- Neve KA, Seamans JK and Trantham-Davidson H (2004) Dopamine receptor signaling. *J Rec Sign Transd Res* **24**:165-205.
- Nissinen E, Tuominen R, Perhoniemi V and Kaakkola S (1988) Catechol-O-methyltransferase activity in human and rat small intestine. *Life Sci* **42**:2609-2614.
- Nutt JG (1987) On-off phenomenon: relation to levodopa pharmacokinetics and pharmacodynamics. *Ann Neurol* **22**:535-540.
- Nygaard R, Zou Y, Dror RO, Mildorf TJ, Arlow DH, Manglik A, Pan AC, Liu CW, Fung JJ, Bokoch MP, Thian FS, Kobilka TS, Shaw DE, Mueller L, Prosser RS and Kobilka BK (2013) The dynamic process of beta(2)-adrenergic receptor activation. *Cell* **152**:532-542.
- O'Sullivan GJ, Roth BL, Kinsella A and Waddington JL (2004) SK&F 83822 distinguishes adenylyl cyclase from phospholipase C-coupled dopamine D1-like receptors: behavioural topography. *Eur J Pharmacol* **486**:273-280.
- Pace CN, Shirley BA, McNutt M and Gajiwala K (1996) Forces contributing to the conformational stability of proteins. *FASEB J* **10**:75-83.
- Pacheco MA and Jope RS (1997) Comparison of [3H]phosphatidylinositol and [3H]phosphatidylinositol 4,5- biphosphate hydrolysis in postmortem human brain membranes and characterization of stimulation by dopamine D1 receptors. *J Neurochem* **69**:639-644.
- Panchalingam S and Undie AS (2001) SKF83959 exhibits biochemical agonism by stimulating [(35S)]GTP gamma S binding and phosphoinositide hydrolysis in rat and monkey brain. *Neuropharmacology* **40**:826-837.
- Parkinson Study Group (2000) Pramipexole vs levodopa as initial treatment for Parkinson disease: A randomized controlled trial. Parkinson Study Group. *JAMA* **284**:1931-1938.

- Pedersen UB, Norby B, Jensen AA, Schiødt M, Hansen A, Suhr JP, Scheideler M, Thastrup O and Andersen PH (1994) Characteristics of stably expressed human dopamine D1a and D1b receptors: atypical behavior of the dopamine D1b receptor. *Eur J Pharmacol* **267**:85-93.
- Pellissier LP, Sallander J, Campillo M, Gaven F, Queffeuilou E, Pillot M, Dumuis A, Claeysen S, Bockaert J and Pardo L (2009) Conformational toggle switches implicated in basal constitutive and agonist-induced activated states of 5-hydroxytryptamine-4 receptors. *Mol Pharmacol* **75**:982-990.
- Perreault ML, Fan T, Alijanian M, O'Dowd BF and George SR (2012) Dopamine D1-D2 receptor heteromer in dual phenotype GABA/glutamate-coexpressing striatal medium spiny neurons: regulation of BDNF, GAD67 and VGLUT1/2. *PLoS One* **7**:e33348.
- Perreault ML, Hasbi A, Alijanian M, Fan T, Varghese G, Fletcher PJ, Seeman P, O'Dowd BF and George SR (2010) The dopamine D1-D2 receptor heteromer localizes in dynorphin/enkephalin neurons: increased high affinity state following amphetamine and in schizophrenia. *J Biol Chem* **285**:36625-36634.
- Perreault ML, Hasbi A, O'Dowd BF and George SR (2011) The dopamine D1-D2 receptor heteromer in striatal medium spiny neurons: evidence for a third distinct neuronal pathway in Basal Ganglia. *Front Neuroanat* **5**:31.
- Perreault ML, Hasbi A, O'Dowd BF and George SR (2014) Heteromeric dopamine receptor signaling complexes: emerging neurobiology and disease relevance. *Neuropsychopharmacology* **39**:156-168.
- Pertz HH, Gornemann T, Schurad B, Seifert R and Strasser A (2006) Striking differences of action of lisuride stereoisomers at histamine H1 receptors. *Naunyn Schmiedeberg's Arch Pharmacol* **374**:215-222.
- Poewe W (2009) Treatments for Parkinson disease--past achievements and current clinical needs. *Neurology* **72**:S65-S73.
- Pollock NJ, Manelli AM, Hutchins CW, Steffey ME, MacKenzie RG and Frail DE (1992) Serine mutations in transmembrane V of the dopamine D₁ receptor affect ligand interactions and receptor activation. *J Biol Chem* **267**:17780-17786.
- Popper K (1959) *The Logic of Scientific Discovery*. Routledge Classics (2002 Reissue), London.
- Rappaport MS, Sealfon SC, Prikhozhan A, Huntley GW and Morrison JH (1993) Heterogeneous distribution of D1, D2 and D5 receptor mRNAs in monkey striatum. *Brain Res* **616**:242-250.
- Rascol O, Blin O, Thalamas C, Descombes S, Soubrouillard C, Azulay P, Fabre N, Viallet F, Lafnizegger K, Wright S, Carter JH and Nutt JG (1999) ABT-431, a D1 receptor agonist prodrug, has efficacy in Parkinson's disease. *Ann Neurol* **45**:736-741.
- Rascol O, Nutt JG, Blin O, Goetz CG, Trugman JM, Soubrouillard C, Carter JH, Currie LJ, Fabre N, Thalamas C, Giardina WJ and Wright S (2001) Induction by dopamine D₁ receptor

- agonist ABT-431 of dyskinesia similar to levodopa in patients with Parkinson's disease. *Arch Neurol* **58**:249-254.
- Rashid AJ, O'Dowd BF, Verma V and George SR (2007a) Neuronal Gq/11-coupled dopamine receptors: an uncharted role for dopamine. *Trends Pharmacol Sci* **28**:551-555.
- Rashid AJ, So CH, Kong MM, Furtak T, El-Ghundi M, Cheng R, O'Dowd BF and George SR (2007b) D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. *Proc Natl Acad Sci USA* **104**:654-659.
- Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, DeVree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI and Kobilka BK (2011a) Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* **469**:175-180.
- Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK and Kobilka BK (2011b) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* **477**:549-555.
- Rasmussen SG, Jensen AD, Liapakis G, Ghanouni P, Javitch JA and Gether U (1999) Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6. *Mol Pharmacol* **56**:175-184.
- Regehr WG and Mintz IM (1994) Participation of multiple calcium channel types in transmission at single climbing fiber to Purkinje cell synapses. *Neuron* **12**:605-613.
- Ring AM, Manglik A, Kruse AC, Enos MD, Weis WI, Garcia KC and Kobilka BK (2013) Adrenaline-activated structure of beta2-adrenoceptor stabilized by an engineered nanobody. *Nature* **502**:575-579.
- Risgaard R, Jensen M, Jorgensen M, Bang-Andersen B, Christoffersen CT, Jensen KG, Kristensen JL and Puschl A (2014) Synthesis and SAR study of a novel series of dopamine receptor agonists. *Bioorg Med Chem* **22**:381-392.
- Rodenhuis N, Timmerman W, Wikstrom HV and Dijkstra D (2000) Thiophene analogs of naphthoxazines and 2-aminotetralins: bioisosteres with improved relative oral bioavailability, as compared to 5-OH-DPAT. *Eur J Pharmacol* **394**:255-263.
- Rosell DR, Zaluda LC, McClure MM, Strike KS, Barch DM, Harvey PD, Girgis RR, Hazlett EA, Mailman RB, Abi-Dargham A, Lieberman JA and Siever LJ. Effects of the D₁ dopamine receptor agonist dihydrexidine (DAR-0100A) on working memory in schizotypal personality disorder. *Neuropsychopharmacol.* under review. 2014.
- Rubinstein JE and Hitzemann RJ (1990) Further evidence against the coupling of dopamine receptors to phosphoinositide hydrolysis in rat striatum. *Biochem Pharmacol* **39**:1965-1970.

- Rydberg EH, Brumshtein B, Greenblatt HM, Wong DM, Shaya D, Williams LD, Carlier PR, Pang YP, Silman I and Sussman JL (2006) Complexes of alkylene-linked tacrine dimers with *Torpedo californica* acetylcholinesterase: Binding of Bis5-tacrine produces a dramatic rearrangement in the active-site gorge. *J Med Chem* **49**:5491-5500.
- Ryman-Rasmussen JP, Griffith A, Oloff S, Vaidehi N, Brown JT, Goddard WA, III and Mailman RB (2007) Functional selectivity of dopamine D(1) receptor agonists in regulating the fate of internalized receptors. *Neuropharmacology* **52**:562-575.
- Ryman-Rasmussen JP, Nichols DE and Mailman RB (2005) Differential activation of adenylate cyclase and receptor internalization by novel dopamine D1 receptor agonists. *Mol Pharmacol* **68**:1039-1048.
- Salonen LM, Ellermann M and Diederich F (2011) Aromatic rings in chemical and biological recognition: energetics and structures. *Angew Chem Int Ed Engl* **50**:4808-4842.
- Santini E, Feyder M, Gangarossa G, Bateup HS, Greengard P and Fisone G (2012) Dopamine- and cAMP-regulated phosphoprotein of 32-kDa (DARPP-32)-dependent activation of extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin complex 1 (mTORC1) signaling in experimental parkinsonism. *J Biol Chem* **287**:27806-27812.
- Santini E, Valjent E, Usiello A, Carta M, Borgkvist A, Girault JA, Herve D, Greengard P and Fisone G (2007) Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesia. *J Neurosci* **27**:6995-7005.
- Schaus JM, Kornfeld EC, Titus RD, Nichols CL, Huser DL, Clemens JA, Smalstig EB and Fuller RW (1993) Progress in the medicinal chemistry of dopaminergic agents. *Drug Des Discov* **9**:323-332.
- Scheller D, Ullmer C, Berkels R, Gwarek M and Lubbert H (2009) The in vitro receptor profile of rotigotine: a new agent for the treatment of Parkinson's disease. *Naunyn Schmiedeberg's Arch Pharmacol* **379**:73-86.
- Schiffmann SN, Desdouts F, Menu R, Greengard P, Vincent JD, Vanderhaeghen JJ and Girault JA (1998) Modulation of the voltage-gated sodium current in rat striatal neurons by DARPP-32, an inhibitor of protein phosphatase. *Eur J Neurosci* **10**:1312-1320.
- Schiffmann SN, Lledo PM and Vincent JD (1995) Dopamine D1 receptor modulates the voltage-gated sodium current in rat striatal neurones through a protein kinase A. *J Physiol* **483** (Pt 1):95-107.
- Schneider JS, Sun ZQ and Roeltgen DP (1994) Effects of dihydrexidine, a full dopamine D-1 receptor agonist, on delayed response performance in chronic low dose MPTP-treated monkeys. *Brain Res* **663**:140-144.
- Scibilia RJ, Lachowicz JE and Kilts CD (1992) Topographic nonoverlapping distribution of D1 and D2 dopamine receptors in the amygdaloid nuclear complex of the rat brain. *Synapse* **11**:146-154.

- Seeman P, Chau-Wong M, Tedesco J and Wong K (1975) Brain receptors for antipsychotic drugs and dopamine: direct binding assays. *Proc Natl Acad Sci U S A* **72**:4376-4380.
- Seiler MP, Floersheim P, Markstein R and Widmer A (1993) Structure-activity relationships in the trans-hexahydroindolo[4,3-ab]phenanthridine ("benzergoline") series. 2. Resolution, absolute configuration, and dopaminergic activity of the selective D1 agonist CY 208-243 and its implication for an "extended rotamer-based dopamine receptor model". *J Med Chem* **36**:977-984.
- Seiler MP and Markstein R (1984) Further characterization of structural requirements for agonists at the striatal dopamine D2 receptor and a comparison with those at the striatal dopamine D1 receptor. Studies with a series of monohydroxyaminotetralins on acetylcholine release from rat striatum. *Mol Pharmacol* **26**:452-457.
- Self DW, Barnhart WJ, Lehman DA and Nestler EJ (1996) Opposite modulation of cocaine-seeking behavior by D1- and D2-like dopamine receptor agonists. *Science* **271**:1586-1589.
- Sergio Mantegani, Emanuele Arlandini, Tiziano Bandiera, Daniela Borghi, Enzo Brambilla, Carla Caccia, Maria Antonietta Cervini, Paolo Cremonesi, Robert Albert McArthur, Gabriella Traquandi and Mario Varasi (1999) D1 Agonist and/or D2 antagonist dopamine receptor properties of a series of ergoline derivatives: a structure-activity study. *Eur J Med Chem* **34**:107-124.
- Serrano AC (1981) Haloperidol -- its use in children. *J Clin Psychiatry* **42**:154-156.
- Setler PE, Sarau HM, Zirkle CL and Saunders HL (1978) The central effects of a novel dopamine agonist. *Eur J Pharmacol* **50**:419-430.
- Shapiro DA, Renock S, Arrington E, Sibley DR, Chiodo LA, Roth BL and Mailman RB (2003) Aripiprazole, a novel atypical antipsychotic drug with a unique and robust pharmacology. *Neuropsychopharmacology* **28**:1400-1411.
- Shenoy SK and Lefkowitz RJ (2005) Seven-transmembrane receptor signaling through beta-arrestin. *Sci STKE* **2005**:cm10.
- Shi L and Javitch JA (2002) The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu Rev Pharmacol Toxicol* **42**:437-467.
- Shi L, Liapakis G, Xu R, Guarnieri F, Ballesteros JA and Javitch JA (2002) Beta2 adrenergic receptor activation. Modulation of the proline kink in transmembrane 6 by a rotamer toggle switch. *J Biol Chem* **277**:40989-40996.
- Snyder PW, Mecinovic J, Moustakas DT, Thomas SW, III, Harder M, Mack ET, Lockett MR, Heroux A, Sherman W and Whitesides GM (2011) Mechanism of the hydrophobic effect in the biomolecular recognition of arylsulfonamides by carbonic anhydrase. *Proc Natl Acad Sci U S A* **108**:17889-17894.

- Sokoloff P, Giros B, Martres MP, Bouthenet ML and Schwartz JC (1990) Molecular cloning and characterization of a novel dopamine receptor (D₃) as a target for neuroleptics. *Nature* **347**:146-151.
- Spencer SE and Wooten GF (1984) Altered pharmacokinetics of L-dopa metabolism in rat striatum deprived of dopaminergic innervation. *Neurology* **34**:1105-1108.
- Starr MS (1996) The role of dopamine in epilepsy. *Synapse* **22**:159-194.
- Steele TD, Hodges DB, Jr., Levesque TR and Locke KW (1997) D1 agonist dihydrexidine releases acetylcholine and improves cognitive performance in rats. *Pharmacol Biochem Behav* **58**:477-483.
- Steele TD, Hodges DB, Jr., Levesque TR, Locke KW and Sandage BW, Jr. (1996) The D1 agonist dihydrexidine releases acetylcholine and improves cognition in rats. *Ann N Y Acad Sci* **777**:427-430.
- Steuber H, Heine A and Klebe G (2007) Structural and thermodynamic study on aldose reductase: nitro-substituted inhibitors with strong enthalpic binding contribution. *J Mol Biol* **368**:618-638.
- Strader CD, Candelore MR, Hill WS, Sigal IS and Dixon RA (1989) Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. *J Biol Chem* **264**:13572-13578.
- Sunahara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, van Tol HH and Niznik HB (1991) Cloning of the gene for a human dopamine D₅ receptor with higher affinity for dopamine than D₁. *Nature* **350**:614-619.
- Surmeier DJ, Bargas J, Hemmings HC, Jr., Nairn AC and Greengard P (1995) Modulation of calcium currents by a D1 dopaminergic protein kinase/phosphatase cascade in rat neostriatal neurons. *Neuron* **14**:385-397.
- Surmeier DJ, Eberwine J, Wilson CJ, Cao Y, Stefani A and Kitai ST (1992) Dopamine receptor subtypes colocalize in rat striatonigral neurons. *Proc Natl Acad Sci U S A* **89**:10178-10182.
- Swart PJ and De Zeeuw RA (1992) Extensive gastrointestinal metabolic conversion limits the oral bioavailability of the dopamine D2 agonist N-0923 in freely moving rats. *Pharmazie* **47**:613-615.
- Takahashi T and Momiyama A (1993) Different types of calcium channels mediate central synaptic transmission. *Nature* **366**:156-158.
- Taylor JR, Lawrence MS, Redmond DE, Jr., Elsworth JD, Roth RH, Nichols DE and Mailman RB (1991) Dihydrexidine, a full dopamine D1 agonist, reduces MPTP-induced parkinsonism in monkeys. *Eur J Pharmacol* **199**:389-391.

- Thibault D, Loustalot F, Fortin GM, Bourque MJ and Trudeau LE (2013) Evaluation of D1 and D2 dopamine receptor segregation in the developing striatum using BAC transgenic mice. *PLoS One* **8**:e67219.
- Thoma G, Baenteli R, Lewis I, Wagner T, Oberer L, Blum W, Glickman F, Streiff MB and Zerwes HG (2009) Special ergolines are highly selective, potent antagonists of the chemokine receptor CXCR3: discovery, characterization and preliminary SAR of a promising lead. *Bioorg Med Chem Lett* **19**:6185-6188.
- Timmerman W, Dubocovich ML, Westerink BH, De Vries JB, Tepper PG and Horn AS (1989) The enantiomers of the dopamine agonist N-0437: in vivo and in vitro effects on the release of striatal dopamine. *Eur J Pharmacol* **166**:1-11.
- Toda M and Abi-Dargham A (2007) Dopamine hypothesis of schizophrenia: making sense of it all. *Curr Psychiatry Rep* **9**:329-336.
- Trabucchi M, Longoni R, Fresia P and Spano PF (1975) Sulpiride: a study of the effects on dopamine receptors in rat neostriatum and limbic forebrain. *Life Sci* **17**:1551-1556.
- Trifilieff P, Rives ML, Urizar E, Piskorowski RA, Vishwasrao HD, Castrillon J, Schmauss C, Slattman M, Gullberg M and Javitch JA (2011) Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum. *Biotechniques* **51**:111-118.
- Troger T, Langenberg M, Zhong S, Ambrosini D and Enzensperger C (2014) Fishing for Accessory Binding Sites at GPCRs with 'Loop-Hooks' - An Approach towards Selectivity? Part I. *Chem Biodivers* **11**:197-208.
- Tschammer N, Bollinger S, Kenakin T and Gmeiner P (2011) Histidine 6.55 is a major determinant of ligand-biased signaling in dopamine D2L receptor. *Mol Pharmacol* **79**:575-585.
- Undie AS and Friedman E (1990) Stimulation of a dopamine D₁ receptor enhances inositol phosphates formation in rat brain. *J Pharmacol Exp Ther* **253**:987-992.
- Undie AS and Friedman E (1992) Selective dopaminergic mechanism of dopamine and SKF38393 stimulation of inositol phosphate formation in rat brain. *Eur J Pharmacol* **226**:297-302.
- Undie AS and Friedman E (1994) Inhibition of dopamine agonist-induced phosphoinositide hydrolysis by concomitant stimulation of cyclic AMP formation in brain slices. *J Neurochem* **63**:222-230.
- Undie AS, Weinstock J, Sarau HM and Friedman E (1994) Evidence for a distinct D₁-like dopamine receptor that couples to activation of phosphoinositide metabolism in brain. *J Neurochem* **62**:2045-2048.
- Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M and Mailman RB (2007)

- Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**:1-13.
- Urizar E, Yano H, Kolster R, Gales C, Lambert N and Javitch JA (2011) CODA-RET reveals functional selectivity as a result of GPCR heteromerization. *Nat Chem Biol* **7**:624-630.
- Urs NM, Daigle TL and Caron MG (2011) A dopamine D1 receptor-dependent beta-arrestin signaling complex potentially regulates morphine-induced psychomotor activation but not reward in mice. *Neuropsychopharmacology* **36**:551-558.
- Van der Weide J, De Vries JB, Tepper PG and Horn AS (1986) Pharmacological profiles of three new, potent and selective dopamine receptor agonists: N-0434, N-0437 and N-0734. *Eur J Pharmacol* **125**:273-282.
- Van der Weide J, De Vries JB, Tepper PG and Horn AS (1987) In vitro binding of the very potent and selective D-2 dopamine agonist, [3H]N-0437 to calf caudate membranes. *Eur J Pharmacol* **134**:211-219.
- van Tol HH, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB and Civelli O (1991) Cloning of the gene for a human dopamine D₄ receptor with high affinity for the antipsychotic clozapine. *Nature* **350**:610-614.
- van Vliet LA, Tepper PG, Dijkstra D, Damsma G, Wikstrom H, Pugsley TA, Akunne HC, Heffner TG, Glase SA and Wise LD (1996) Affinity for dopamine D₂, D₃, and D₄ receptors of 2-aminotetralins. Relevance of D₂ agonist binding for determination of receptor subtype selectivity. *J Med Chem* **39**:4233-4237.
- Vermeulen RJ, Drukarch B, Sahadat MC, Goosen C, Wolters EC and Stoof JC (1994) The dopamine D₁ agonist SKF 81297 and the dopamine D₂ agonist LY 171555 act synergistically to stimulate motor behavior of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned parkinsonian rhesus monkeys. *Mov Disord* **9**:664-672.
- Vyas SJ, Eichberg J and Lokhandwala MF (1992) Characterization of receptors involved in dopamine-induced activation of phospholipase-C in rat renal cortex. *J Pharmacol Exp Ther* **260**:134-139.
- Wallace MA and Claro E (1990) A novel role for dopamine: inhibition of muscarinic cholinergic-stimulated phosphoinositide hydrolysis in rat brain cortical membranes. *Neurosci Lett* **110**:155-161.
- Wang C, Jiang Y, Ma J, Wu H, Wacker D, Katritch V, Han GW, Liu W, Huang XP, Vardy E, McCorvy JD, Gao X, Zhou XE, Melcher K, Zhang C, Bai F, Yang H, Yang L, Jiang H, Roth BL, Cherezov V, Stevens RC and Xu HE (2013) Structural basis for molecular recognition at serotonin receptors. *Science* **340**:610-614.
- Wang CD, Buck MA and Fraser CM (1991) Site-directed mutagenesis of alpha 2A-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol Pharmacol* **40**:168-179.

- Wang HY, Undie AS and Friedman E (1995) Evidence for the coupling of Gq protein to D1-like dopamine sites in rat striatum: possible role in dopamine-mediated inositol phosphate formation. *Mol Pharmacol* **48**:988-994.
- Wang Q, Jolly JP, Surmeier JD, Mullah BM, Lidow MS, Bergson CM and Robishaw JD (2001) Differential dependence of the D1 and D5 dopamine receptors on the G protein gamma 7 subunit for activation of adenylylcyclase. *J Biol Chem* **276**:39386-39393.
- Warne T, Moukhametzianov R, Baker JG, Nehme R, Edwards PC, Leslie AG, Schertler GF and Tate CG (2011) The structural basis for agonist and partial agonist action on a beta(1)-adrenergic receptor. *Nature* **469**:241-244.
- Watson JB, Coulter PM, Margulies JE, de Lecea L, Danielson PE, Erlander MG and Sutcliffe JG (1994) G-protein gamma 7 subunit is selectively expressed in medium-sized neurons and dendrites of the rat neostriatum. *J Neurosci Res* **39**:108-116.
- Watts VJ, Lawler CP, Fox DR, Neve KA, Nichols DE and Mailman RB (1995a) LSD and structural analogs: pharmacological evaluation at D1 dopamine receptors. *Psychopharmacology (Berl)* **118**:401-409.
- Watts VJ, Lawler CP, Gilmore JH, Southerland SB, Nichols DE and Mailman RB (1993) Dopamine D1 receptors: efficacy of full (dihydroxidine) vs. partial (SKF38393) agonists in primates vs. rodents. *Eur J Pharmacol* **242**:165-172.
- Watts VJ, Lawler CP, Gonzales AJ, Zhou QY, Civelli O, Nichols DE and Mailman RB (1995b) Spare receptors and intrinsic activity: studies with D1 dopamine receptor agonists. *Synapse* **21**:177-187.
- Weinstock J, J.P.Hieble and J.W.Wilson (1985) The chemistry and pharmacology of 3-benzazepine derivatives. *Drugs Future* **10**:645-696.
- Weissman JT, Ma JN, Essex A, Gao Y and Burstein ES (2004) G-protein-coupled receptor-mediated activation of rap GTPases: characterization of a novel Galpha_i regulated pathway. *Oncogene* **23**:241-249.
- Wilcox RE, Huang WH, Brusniak MY, Wilcox DM, Pearlman RS, Teeter MM, DuRand CJ, Wiens BL and Neve KA (2000) CoMFA-based prediction of agonist affinities at recombinant wild type versus serine to alanine point mutated D2 dopamine receptors. *J Med Chem* **43**:3005-3019.
- Wood MD, Murkitt KL, Ho M, Watson JM, Brown F, Hunter AJ and Middlemiss DN (1999) Functional comparison of muscarinic partial agonists at muscarinic receptor subtypes hM1, hM2, hM3, hM4 and hM5 using microphysiometry. *Br J Pharmacol* **126**:1620-1624.
- Xhaard H, Rantanen VV, Nyronen T and Johnson MS (2006) Molecular evolution of adrenoceptors and dopamine receptors: implications for the binding of catecholamines. *J Med Chem* **49**:1706-1719.

- Yang CR and Seamans JK (1996) Dopamine D1 receptor actions in layers V-VI rat prefrontal cortex neurons in vitro: modulation of dendritic-somatic signal integration. *J Neurosci* **16**:1922-1935.
- Young T, Abel R, Kim B, Berne BJ and Friesner RA (2007) Motifs for molecular recognition exploiting hydrophobic enclosure in protein-ligand binding. *Proc Natl Acad Sci U S A* **104**:808-813.
- Yu PY, Eisner GM, Yamaguchi I, Mouradian MM, Felder RA and Jose PA (1996) Dopamine D1A receptor regulation of phospholipase C isoform. *J Biol Chem* **271**:19503-19508.
- Yu Y, Wang JR, Sun PH, Guo Y, Zhang ZJ, Jin GZ and Zhen X (2008) Neuroprotective effects of atypical D1 receptor agonist SKF83959 are mediated via D1 receptor-dependent inhibition of glycogen synthase kinase-3 beta and a receptor-independent anti-oxidative action. *J Neurochem* **104**:946-956.
- Zhang H, Ma L, Wang F, Chen J and Zhen X (2007) Chronic SKF83959 induced less severe dyskinesia and attenuated L-DOPA-induced dyskinesia in 6-OHDA-lesioned rat model of Parkinson's disease. *Neuropharmacology* **53**:125-133.
- Zhang J, Xiong B, Zhen X and Zhang A (2009a) Dopamine D1 receptor ligands: where are we now and where are we going. *Med Res Rev* **29**:272-294.
- Zhang X, Zhou Z, Wang D, Li A, Yin Y, Gu X, Ding F, Zhen X and Zhou J (2009b) Activation of phosphatidylinositol-linked D1-like receptor modulates FGF-2 expression in astrocytes via IP3-dependent Ca²⁺ signaling. *J Neurosci* **29**:7766-7775.
- Zhang ZJ, Jiang XL, Zhang SE, Hough CJ, Li H, Chen JG and Zhen XC (2005) The paradoxical effects of SKF83959, a novel dopamine D1-like receptor agonist, in the rat acoustic startle reflex paradigm. *Neurosci Lett* **382**:134-138.
- Zhen X, Goswami S and Friedman E (2005) The role of the phosphatidylinositol-linked D1 dopamine receptor in the pharmacology of SKF83959. *Pharmacol Biochem Behav* **80**:597-601.
- Zhen X, Uryu K, Wang HY and Friedman E (1998) D1 dopamine receptor agonists mediate activation of p38 mitogen-activated protein kinase and c-Jun amino-terminal kinase by a protein kinase A-dependent mechanism in SK-N-MC human neuroblastoma cells. *Mol Pharmacol* **54**:453-458.
- Zhou QY, Grandy DK, Thambi L, Kushner JA, van Tol HH, Cone R, Pribnow D, Salon J, Bunzow JR and Civelli O (1990) Cloning and expression of human and rat D₁ dopamine receptors. *Nature* **347**:76-80.
- Zhuang X, Belluscio L and Hen R (2000) G(olf)alpha mediates dopamine D1 receptor signaling. *J Neurosci* **20**:RC91.

Curriculum Vitae
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Publications and Manuscripts

1. **Lee SM**, Andrew Kant, Daniel Blake, Vishakantha Murthy, Kevin Boyd, Steven J. Wyrick, Richard B. Mailman. SKF-83959 is not a highly biased functionally selective D₁ ligand: Do D₁ receptors directly stimulate phospholipase C? *Neuropharmacology* 2014 Jun 12. pii: S0028-3908(14)00218-4. doi: 10.1016/j.neuropharm.2014.05.042. [Epub ahead of print] PMID: 24929112
2. **Lee SM**, Yang Yang, Richard B. Mailman. Dopamine D₁ receptor signaling: Does Gα_q-phospholipase C actually play a role? *Journal of Pharmacology and Experimental Therapeutics* (In press)
3. Shin DW, Kim SN, **Lee SM**, Lee WJ, Song MJ, Park SM, Lee TR, Baik JH, Kim HG, Hong JH, Noh MS. (2009) (-)-Catechin promotes adipocyte differentiation in human bone marrow mesenchymal stem cells through PPAR gamma transactivation. *Biochemical Pharmacology* 77(1):125-33.
4. **Lee SM**, Kim CW, Kim JK, Shin HJ, Baik JH. (2008) GCG-rich tea catechins are effective in lowering cholesterol and triglyceride concentrations in hyperlipidemic rats. *Lipids* 43(5):419-29.
5. Kim MS, **Lee SM**, Kim WD, Ki SH, Moon A, Lee CH, Kim SG. (2007) Gα_{12/13} basally regulates p53 through Mdm4 expression. *Molecular Cancer Research* 5(5):473-84.

Honors and Awards

- Student Travel Award** from Parkinson's Disease Foundation Inc., 2014.
- Travel Grant** from 3rd World Parkinson Congress, 2013.
- Graduate Student Travel Award** from ASPET, 2013.
- Summer Student Fellowship** from Parkinson's Disease Foundation Inc., 2012.
- University Graduate Fellowship** from Pennsylvania State University, 2009-2012.