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Role of the fourth transmembrane domain and the second extracellular loop in structure-activity
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Role of the fourth transmembrane domain and the second extracellular loop in
structure-activity relationships in D1-like dopaminergic receptors

By

Nancy Ogden

This thesis is submitted as a partial fulfillment of the M.Sc program in
Pharmacology

Department of Cellular and Molecular Medicine

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Abstract

The mammalian D1-like dopaminergic receptors are composed of two subtypes, D1R (or D1A) and D5R (or D1B), which belong to the large family of heptahelical G protein-coupled receptors (GPCRs). D1R and D5R receptors have been characterized pharmacologically via their ability to couple to $G\alpha_s$ and activate AC. Against the high overall amino acid sequence homology of the two D1-like dopaminergic receptors, the divergent primary structure of the extracellular loops, as well as discrete residues within the exofacial end of the transmembrane (TM) regions provide new targets for the assessment of structure-activity relationships represented by D1R and D5R. While residues within the second extracellular loop (EL2) of a number of GPCRs have been the subject of site-directed mutagenesis, a broader understanding of the structure-activity role of EL2 as a whole has yet to be formed. In the present study, chimeric proteins have been engineered to swap the EL2 of D1R and D5R receptors, both with and without the exchange of the divergent amino acid in the exofacial end of TM4. An assessment of the functional phenotype of these chimeric proteins using radioligand binding and whole cell cAMP studies has provided the first identification of D1-like structural determinants constraining wild-type receptor function, and of D1-like determinants responsible for ligand selectivity ratios. Also, EL2 and the variant exofacial TM4 residue of D1R and D5R are identified as direct regulators of phenylbenzazepine binding, while further evidence is presented for multiple D1-like receptor active states. In general, the TM4/EL2 junction region functions differently in D1R and D5R, and interacts differently with agonists versus inverse agonists. Furthermore, subtype-specific differences in the binding and receptor activation by non-phenylbenzazepine agonists dihydrexidine and A77636 had yet to be assessed in D1R/D5R-expressing HEK293 cells. Here, we show that these two agonists are indeed, to different degrees, D5R-selective, and that they interact differently than dopamine with D1-like active conformations, as probed via the TM4/EL2 chimeras.

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List of Abbreviations

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine	MPTP
[³ H]-cAMP/Total [³ H]-Adenine	CA/TU
3-isobutyl-1-methyl xanthine	IBMX
50% maximal activation	EC50
6-hydroxy-dopamine	6-OHDA
A kinase anchoring proteins	AKAP
AC	AC
Adenosine-1 receptor	A1
Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	AMPA
Analysis of variance	ANOVA
Aromatic amine decarboxylase	AAD
Attention deficit/hyperactivity disorder	ADHD
Catechol-O-methyl transferase	COMT
Constitutively active mutant	CAM
Correlated mutation analysis	CMA
Cyclic adenosine monophosphate	cAMP
Cytoplasmic tail	CT
Ser162 of D1R(TM4)	TM4A
Asn179 of D5R(TM4)	TM4B
Dimethyl sulfoxide	DMSO
Dissociation constant	K _d

Dopamine	DA
Dopamine- and cAMP-Regulated phosphoprotein of M _r 32 000	DARPP-32
Dopamine receptor interacting protein	DRIP
Dopamine transporter	DAT
Extracellular loop	EL
Fetal bovine serum	FBS
G protein coupled receptor	GPCR
GPCR regulating kinase	GRK
GDP/GTP exchange factor	GEF
GTPase activating protein	GAP
HEPES-buffered saline	HBS
Human embryonic kidney cells	HEK 293
Inhibition constant	K _i
Inositol 1,4,5-triphosphate	InsP ₃
Interleukin-6	IL-6
Intracellular loop	IL
Long-term depression	LTD
Long-term potentiation	LTP
Medium spiny	MS
Minimum essential medium	MEM
Monoamine oxidase	MAO
Nitric oxide	NO
Nitric oxide synthase	NOS

N-methyl-D-aspartate	NMDA
Obsessive-compulsive disorder	OCD
Parkinson's disease	PD
Phosphate buffered saline	PBS
Phosphodiesterase 1B1	PDE 1B1
PLC	PLC
Polymerase chain reaction	PCR
Post-synaptic density protein 95 kD	PSD-95
Prefrontal cortex	PFC
Protein kinase A	PKA
Protein kinase C	PKC
Receptor expression	R
Regulator of G protein signaling	RGS
Second extracellular loop	EL2
EL2 of D1R	EL2A
EL2 of D5R	EL2B
Serotonin	5HT
Signal transducer and activator of transcription-3	STAT-3
Standard error	S.E.
Structure-activity relationship	SAR
TM (domain)	TM
Vesicular monoamine transporter-2	VMAT-2
γ -amino butyric acid	GABA

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Chapter 1: Introduction

1.1 Receptor theory

At the turn of the 20th century, John Langley and Paul Ehrlich first proposed the concepts of cellular “receptive substances” or “side-chains” responsible for discriminating between multiple incoming biochemical signals [1]. The paradigm shift from “receptors” as a concept used to describe the behaviour of biological tissues, to receptors as quantifiable biochemical entities took decades, and required the development of molecular methods enabling the study of ligand-receptor interactions directly, through radioligand binding and membrane lipid reconstruction, rather than through downstream endpoints such as muscle contraction and secretion [2]. In the last twenty years, characterization and manipulation of cell-surface receptor proteins, now categorized into families according to structure, function, and phylogenetics, have dominated the biological and medical sciences.

Overall, receptors are composed of a limited number of structural motifs, allowing extracellular regions to interact with selective affinity with the array of incoming ligands, and intracellular domains to perform a selective (but possibly multifunctional) role upon receptor activation. The complexity of cellular functions is mediated by fluxes of expression and desensitization of subtly distinct receptors able, through chemical structure, to translate extracellular signals to cytoplasmic components through both direct and crosstalking signals. Cell-surface receptors developed along with the emergence of multicellular

organisms and the necessity for endocrine signaling. With the development of nematodes and flies, four distinct types of receptors developed. Some, such as the epidermal growth factor (a non-receptor tyrosine kinase), adopted enzymatic functions. Ionotropic multiunit ligand-gated ion channels, receptors with membrane transport function, interact with a wide range of neurotransmitters- nicotinic, GABA, glutamate, aspartate, glycine-modulating membrane potential. Receptors with intrinsic transcriptional activity, such as Delta/Notch, also emerged in this era. Finally, and central to the work of this thesis, metabotropic receptors, including G protein-coupled receptors (GPCRs), interact with downstream effectors upon activation [3]. Rationalizing structure-activity relationships in any one of these receptor families, and in GPCRs in particular, is of use in the study of pathology and therapeutics.

1.2 G protein-coupled receptors (GPCRs)

Over 800 human genes code for members of the GPCR family, one of the largest families in the human genome, half of which still remains orphaned [2, 4]. All GPCRs exhibit seven transmembrane domains (TM) linked by three extracellular loops (EL) and three intracellular loops (IL). The range of functions spanned by this protein family covers every physiological realm: the special senses (olfaction, vision, nociception), synaptic neurotransmission (adrenergic, cholinergic, dopaminergic, glutaminergic), chemotaxis (eicosanoid), and hormone signaling (antidiuretic hormone, gonadotropin releasing hormone, lutenizing hormone). As such, an enormous range of ligands interacts with GPCRs, and 40% of

pharmaceuticals currently on the market function by altering GPCR-dependent signaling pathways. Generally, GPCR interaction with a specific ligand triggers interaction with an intermediate heterotrimeric GTP-binding proteins, or G proteins, subtypes of which modulate the activity of effectors such as adenylyl cyclase (AC) or phospholipase C (PLC) [5]. As G protein subtypes are each coupled to a range of GPCRs, the flux of myriad biochemicals triggered by physiological processes can be integrated into a concerted effect on overlapping signaling pathways [2, 6-14]. Three as yet partially defined and interwoven concepts are currently at the center of research in GPCR function: the description and prediction of receptor conformation under varying conditions, the synaptic interaction between GPCRs and G proteins, and the ability of GPCRs to adopt multiple active states, both ligand-selectively and ligand-independently [15].

1.2.1 Familial classification

GPCRs across species are grouped by similarity into three large subfamilies- A. Rhodopsin/adrenergic, B. Glucagon/VIP/Calcitonin, C. Metabotropic/Calcium- and three small subfamilies- D. Yeast pheromone STE2, E. Yeast pheromone STE3, F. Slime mold cAMP receptors [7]. When the human genome alone is analyzed phylogenetically, an alternate grouping scheme emerges: G. Glutamate (15 members), R. Rhodopsin (701 members), A. Adhesion (24 members), F. Frizzled (24 members), and S. Secretin (15 members) [16].

1.2.2 The bovine rhodopsin model

The conserved TM structure among GPCRs suggests that a common activating mechanism may exist, accounting for the fact that the coupling of hundreds of GPCRs converge on only several dozen common G proteins and are desensitized by several common kinases [17]. Because of the innate difficulties in crystallizing TM proteins combined with the existence of multiple GPCR conformations, the only GPCR X-ray structure available at this date is that of bovine rhodopsin [18]. The primary structural features of this receptor are well within the average parameters of the GPCR family as a whole, and so structural extrapolations from rhodopsin to other GPCRs, particularly those found within the rhodopsin subfamily (A or R), are possible [17]. Within transmembrane regions (TMs) it has been shown that given a greater than 25% similarity in amino acid sequence, secondary and tertiary structural similarities can be expected; below 25% amino acid sequence similarity and proteins diverge drastically in structural features of higher order [19]. Less is known about modeling loop regions than TM regions and among GPCRs it is in these areas that primary structures diverge the most. Comparisons between the bovine rhodopsin loop crystal structure and other GPCR loop regions must be made keeping this in mind. Furthermore, rhodopsin differs from other GPCRs, including others in the rhodopsin subfamily A(R) itself, in that its ligand retinal is permanently bound, and activation occurs via isomerization of retinal upon interaction with UV light. **Therefore, the added complexity of diffusible chemical ligands does not exist for rhodopsin [20].**

It has been suggested that structural mimicry may explain the probable persistence of similarity in GPCR secondary structures despite divergent primary patterns [21]. A common ancestor may have diverged sufficiently to develop ligand selectivity, which allowed interpretation of extracellular signals, all the while maintaining a common activation pattern through conformational changes in TM regions.

In addition to the canonical seven TMs, the rhodopsin crystal structure revealed an eighth helical region lying parallel to the intracellular membrane, between the seventh TM domain and a palmitoylation site. Also, the second extracellular loop (EL2) exhibits considerable secondary structure, in addition to the previously characterized EL2-TM3 disulfide bond common to most GPCRs [18].

Antiparallel sheets allow the loop to be in contact with the retinal binding site, interesting given that the composition and length of EL2 among GPCRs vary considerably [17, 22]. While peptide GPCRs are well known to interact with ligand via the extracellular regions, the possibility that these regions of aminergic receptors do as well has not been explored thoroughly [23].

1.2.3 GPCR function

1.2.3.1 GPCR ligand interactions and activation

The Law of Mass Action currently defines the way by which GPCRs are thought to interact with incoming ligands. GPCRs exist in at least two conformations: the

inactive state R, and the active state R*. Classically, the active state is stabilized by stimulatory ligands (agonists), while inhibitory antagonists hinder the binding of agonists; therefore, the proportion of receptors in one state or the other at a given point in time is ligand-dependent. However, and as will be discussed throughout this introduction, it is becoming increasingly clear that the R/R* ratio is modified by an increasingly complex variety of variables. The central challenge in the field of GPCRs is to elucidate the regulation of the inactive and active receptor forms. Some information is available regarding basic chemical changes a receptor undergoes upon activation. In rhodopsin and potentially aminergic GPCRs as well, light activation or agonist binding disrupts a salt bridge between a TM3 glutamic acid and a TM7 lysine, residues conserved in Family A(R) GPCRs [24, 25]. It was first shown in β -adrenergic receptors that the series of serine residues found in the fifth TM of all catecholamine receptors each have roles to play in agonist and antagonist binding, likely through hydrogen bonding to the hydroxyl catechol substituents, while the aromatic ring interacts with TM6 aromatic residues (Figure 1, dopamine) [26].

A common pattern among aminergic GPCRs has been elucidated for conformational changes associated with the switch between active and inactive receptor states. In the conserved TM3 DRY motif an ionic bond between a TM3 Asp and Arg is thought to constrain the orientation of TM3 Arg in a position compatible with the inactive state. Protonation of the TM3 Asp provides a release allowing movement of TM6 away from TM3, necessary for interaction

with the α subunit of a G protein and the formation of the active state [13, 27]. In the active state, the TM3 Arg interacts with the acidic side chain of a TM7 Asp, and is stabilized by a TM3 Ile/Val [21]. Through studies binding a fluorescent probe to a cysteine at the IL3/TM6 border of β_2 AR, it is known that the cytoplasmic end of TM6 is involved in agonist-induced receptor activation: four residues in this region end up in a more polar environment in the active state, accounted for by a clockwise rotation of TM6 towards TM4 [20, 28-30].

Complex intramolecular GPCR interactions arise from the close tertiary structure of these large proteins, and require that a synoptic view of GPCR activation be taken [31]. Although residues necessary for amine binding have been found in the TMs, 50% of interhelical inactivating mutations are found in the EL2 and EL3 regions [22]. Also, activating mutations are not localized to receptor regions directly interacting with G protein, indicating that many receptor regions contribute to the formation of the active conformation of GPCRs [4] [22]. For example, a single point mutation of asparagine to aspartic acid in the TM2 of GnRH receptor results in a nonfunctional receptor. This drastic effect is recovered by engineering a reciprocal aspartic acid to asparagines mutation in TM7, illustrating the structural interdependence that exists within the GPCR tertiary structure [32]. Furthermore, external components play a role in determining GPCR conformation: the activation state of G proteins themselves, modified by nucleotides, impacts the state of an associated GPCR relative to ligand binding [33].

The intramolecular nature of GPCRs therefore allows many allosteric ligands to indirectly affect receptor function, through conformational changes favouring G protein coupling. Clearly, many ligand actions may fall into the allosteric category: in fact any ligand that does not involve direct competition at the classical ligand binding site. Receptors theoretically may involve numerous binding sites linked to activation of selected signaling pathways. They may selectively accommodate a range of ligands, or a common ligand depending on ligand affinity and concentration [6, 34-36].

1.2.3.2 Coupling to GTP-binding proteins

Heterotrimeric G proteins include α , β , and γ subunits. There exists a range of subtypes of each of these subunits (20 α , 5 β , 12 γ), which mediate GPCR signaling, allowing a range of effects to occur depending on the given conditions. It is thought that the α subunit determines the signaling pathway, so that α_s activates AC [37], α_i inhibits AC, α_q activates PLC, and α_{12} and α_{13} , among other roles, couple to the activation of guanine exchange factors, as well as modulate the Na/H exchanger [38-41]. The classical GPCR signaling pathway involves an extracellular ligand, which upon binding to and activation of membrane bound GPCR receptor, triggers a GPCR interaction with the α subunit of the heterotrimeric G protein, forming a ternary complex [13]. The intrinsic guanine nucleotide exchange factor (GEF) activity of GPCRs catalyzes the replacement of GDP by GTP on the $G\alpha$ subunit, leading to conformational changes allowing

the dissociation of G protein subunits into α and $\beta\gamma$ subunits, which then individually interact with effector molecules. The G protein coupling process is inactivated by hydrolysis of the α -bound GTP to α -bound GDP, catalyzed by the intrinsic GTPase activity of $G\alpha$ and regulated by external GTPase activating proteins (GAPs), followed by the reassociation with the $\beta\gamma$ subunit to reform the heterotrimeric G protein complex. These steps are facilitated by a Regulators of G protein Signaling (RGS) family of proteins [42, 43].

Members of the RGS family, scaffolding proteins such as A-kinase anchoring proteins (AKAPs), Homer, and arrestins are increasingly being shown to be involved in facilitating novel G protein coupling modes [44]. In general these proteins act to bring the receptor in contact with effector molecules, to maintain the selectivity of the activated signaling pathway by recruiting specific effectors and by compartmentalizing the action of the activated signaling pathway [13]. As a result, GPCR-G protein-effector interactions are not likely to be solely ruled by concentration-based thermodynamics such as the Law of Mass action, rather by catalytically motivated, regionally subdivided molecular orchestrations [45].

All of the possible combinations of G protein subunits in a heterotrimeric whole provide the ability for GPCR signaling pathways to be subtly modified. In addition, many GPCRs are known to be promiscuous in their coupling, interacting with more than one $G\alpha$ subunit and therefore impacting more than one intracellular signaling pathway [46, 47]. GPCR coupling *in vivo* can be expected to

depend on relative amounts of ligand and G protein subunits, as well as to be impacted by organizing factors including cytoskeleton, lipid composition and RGS [46, 48]. Given the varying propensity of GPCRs to form the conformation required for G protein coupling, in certain cases GPCRs may be able to activate downstream effectors independently of the presence of ligands, as discussed further below. Similarly, certain GPCRs may favour a conformation disallowing interaction with G protein. Furthermore, single point mutations have been shown to facilitate a GPCR conformation increasing ligand-independent G protein activation relative to wild-type [25], while other GPCR mutations inhibit G protein activation [49].

1.2.3.3 Desensitization and internalization

In addition to G protein inactivation by GTP hydrolysis discussed above, GPCR function is kept in check at many points, including ligand binding, membrane trafficking, G protein interaction, and effector availability. Modes of inactivation range from rapid dampening effects by receptor phosphorylation and internalization, to long-term degradation or downregulation of gene expression. At the receptor level, phosphorylation of the third intracellular loop and cytoplasmic tail by second messenger-dependent kinases (PKA and PKC) or GPCR kinases (GRKs) triggers a complex desensitization process. GRKs include a region homologous to that of RGS in the N-terminal region responsible for GPCR binding. GRKs are targeted to the membrane either by a G $\beta\gamma$ binding domain, allowing for recruitment by this G protein subunit upon activation, or by a

phospholipid binding farnysyl or palmitoyl moiety. Arrestins bind either to sites of GRK phosphorylation or to sites made available by GRK phosphorylation. Arrestin binding both disallows further GPCR interaction with G proteins and targets the GPCR for endocytosis via clathrin-coated vesicles, which are internalized. The vesicle-bound receptors are either rapidly recycled to the membrane via endosomes enriched in phosphatase activity, degraded in lysosomes, or slowly recycled via endosomes. In some cases MAPK signaling pathways are activated only upon receptor internalization, a finding which emphasizes that the role of internalization is one of receptor resensitization and trafficking rather than desensitization [50-53].

1.2.3.4 Pharmacological descriptions

1.2.3.4.1 Models of ligand-GPCR-G protein interactions

Agonists, partial agonists, and inverse agonist drugs differentially affect the ability of GPCRs to couple to G proteins. This is thought to occur through differential stabilization of inactive receptor conformation R or active receptor conformation R*. Agonists stabilize the GPCR in an active state, while inverse agonists stabilize the inactive state, and antagonists competitively inhibit activation without affecting the distribution of conformational states. The extent to which ligands interact with GPCRs can, based on the Law of Mass Action, be described through parameters including affinity, efficacy, and potency. However, studies of GPCR signaling show that in certain cases, either ligand or G protein are not necessary for downstream signaling, and that a given GPCR is often able to

couple to more than one G protein [54-57]. In order to describe conformational changes associated with these findings, the extended ternary complex model has been developed [14] as well as a cubic model [58] (Figure 2). The latter allows all permutations and combinations of ligand, GPCR and G protein in both inactive and active states. The extent to which one of these conformations is favoured depends on the nature of each component in the triad. While these models formalize what is currently understood regarding GPCR activation, they do not account for the infinite number of transition states available to the receptor proteins, each of which may contribute to the kinetic nature of cellular GPCR interactions. Furthermore, as discussed further below, these models, and signaling pathways studied in isolation, are unable to discern the presence of multiple active states [10].

1.2.3.4.2 Ligand affinity

An unoccupied receptor exists in equilibrium between inactive and active conformations. This equilibrium is affected by the presence of ligand, the extent to which depends both on the chemical affinity of the ligand for receptor and on the receptor conformation favoured by and therefore stabilized by the ligand. The resulting receptor-ligand equilibrium constant is therefore the product of the ratio between active and inactive receptor, and the ratio of ligand occupied active and inactive receptor. In other words an effective ligand affinity is the average value of affinity for each receptor conformation, normalized for the relative proportions of each conformation [26]. The interaction of both inactive and active

receptor, free or ligand-bound, with G protein can also be described thermodynamically, and ligands will differentially skew the natural affinity of receptor, active or inactive, for G protein, stabilizing multiple ligand-specific conformations for one given GPCR, as discussed below.

1.2.3.4.3 Efficacy and potency

The combined effects of ligand on the ratio of inactive receptor to active receptor, and of the affinity of that receptor population for G protein results in the systematic, measurable parameter ligand potency, visually, the midpoint of a dose response curve. Differing from *clinical efficacy*, the therapeutically useful activity of a compound, ligand *intrinsic efficacy*, is related to the ratio of R^*/R , as well as the G protein affinity for effector. Therefore, ligand efficacy is a measure of the efficiency with which ligand-activated GPCR is able to modulate a downstream signaling pathway, and so is dependent on the degree of receptor desensitization in a given system. Agonists are said to have positive efficacy, antagonists zero efficacy, and inverse agonists negative efficacy. Given that potency and efficacy are dependent on the extent of three interactions- receptor-ligand, receptor-G protein, and G protein-effector- it is possible, for example, for a ligand to promote the receptor active state, but inhibit the formation of the active receptor-G protein complex [56]. The wide range of binding-coupling fingerprints seen for ligand-receptor-G protein ensembles is biologically required to allow distinct receptor subtypes to interact with their common endogenous

ligands with different potencies, efficacies, and effectors, resulting in the nuanced modulation of downstream signaling pathways.

1.2.3.5 Consequences of multiple GPCR conformations

The ternary complex model predicts that GPCRs are capable of existing in both an active and inactive state, and that these two states are each able to interact with ligand and/or G protein. In this way, the ternary complex model is able to account for *constitutive activity*, discussed below. However, it is now becoming clear that more than two GPCR conformations (R and R*) with varying affinities for a range of G proteins contribute to the activation and signaling profile of these receptors, also discussed below in terms of *agonist-trafficking* and *protean agonism*.

1.2.3.5.1 Constitutive activation

Basal levels of G protein coupling in the absence of agonist is a naturally occurring phenomenon, one which defines the function of inverse agonist, those compounds which decrease levels of basal activity by stabilizing the inactive R GPCR conformation, favouring the dissociation of receptor from G protein [59]. Constitutively active mutants receptors (CAMs) are also characterized by increased ligand-independent activity compared to wild-type, increased agonist affinity, and increased partial agonist efficacy, factors consistent with an increased proportion of R* [60]. While it might follow that inverse agonists,

thought to stabilize and shift the equilibrium towards inactive R, would show decreased affinity at CAMs relative to wild-type, in a D2 dopaminergic CAM, no decrease in inverse agonist binding was observed. This suggests that in at least the case of D2 dopamine receptors, inverse agonists bind to both active and inactive receptor conformations, and upon binding effect the redistribution of receptor conformation to the R inactive state [61]. CAM studies indicate that it is the inactive receptor conformation which has evolved to be tightly constrained, dependent on discrete, single residue interactions within the molecule, while the active conformation is more easily attained energetically, but less stable [8].

Constitutively active wild-type and mutant receptors not only represent the active conformation of the receptor, useful for studying receptor structure-activity relationships [62], but also are receptors with deep therapeutic consequences. Numerous constitutively activating mutations have been associated with disease, and receptors are overexpressed in tumours where the cumulative effects of constitutive activation could be substantial. Inverse agonist treatment for these aberrant constitutively active systems may be a more subtle mode than antagonist treatment, one more selectively targeted to dysfunctional constitutively activated systems [57]. However, application of inverse agonists has been shown to result in long-term upregulation of target GPCRs as well as to affect signaling components downstream of the target constitutively active GPCR, resulting in upregulation of $G\alpha$ subunits [63-69].

Many therapeutic compounds originally assumed to be antagonists have since been shown to in fact be inverse agonists. Detection methods cannot currently be expected to always identify inverse agonist-induced decreases in basal activity, particularly in systems in which endogenous levels of agonist may exist [15]; however, it is possible to definitively identify antagonists by showing them to have zero efficacy in assays conducted in known constitutively active systems, or by applying guanine nucleotides, which increase inverse agonist affinity by increasing the proportion of receptors in the R state. However, these effects are small, and cannot always be used to categorize drugs [57]. Nevertheless, given that neutral ligands (ie. antagonists) must be able to bind with equal affinity to a range of receptor conformations (at the very least, to the active and inactive receptor), and that ligands are generally expected to change receptor conformation upon binding, true antagonists are likely a rare breed [56, 70].

1.2.3.5.2 Agonist-specific GPCR activity/ ligand-selective efficacy

Just as it is now known that a ligand-free active R^* state can be attained by certain GPCRs, mounting evidence suggests that individual activating ligands can stabilize a discrete GPCR active state, different from that attained by binding by other agonists. The ternary complex model, based on the existence of R and R^* GPCR conformations, suggests that receptor activation is unidirectional, and that all agonists change receptor conformation via this one route, so that the magnitude of ligand efficacy may vary, but in a manner independent of the downstream effector pathway [67]. In recent years, the quality of agonist efficacy

at a single GPCR has been shown to be divergent. Reversals of efficacy rank for ligands at different effectors have been observed, a finding which requires that different G protein/effector-specific GPCR active states be available for differential stabilization by agonists [67, 71, 72]. Furthermore, it is possible to dissociate ligand efficacy from induction of desensitization, a finding not tenable in the classical view of GPCR signaling. This novel ligand-signaling relationship has been called agonist-trafficking, or ligand-specific activation [15, 73, 74].

It is possible that different agonists favour GPCR conformations that present alternate facets of the receptor to the cytosol, in turn favouring interaction with one G protein and associated signaling pathway over another [75]. The question still exists whether ligands stabilize existing GPCR conformations emerging from an energy landscape, or whether ligands actualize conformations through conformational change after binding. The existence of constitutive activity provides evidence for the former mode, while agonist-trafficking supports the latter, and there is no theoretical reason why the two modes should be exclusionary [76]. It has been suggested that given the existence of agonist-specific G protein coupling, ligands may be more accurately thought to act on receptor-G protein complexes, rather than receptors alone [10]. From this angle, drug specificity may be now considered to be achieved not only through selective receptor affinity, but also through the selective activation of one receptor-coupled signaling pathway [67].

1.2.3.5.3 Protean ligands

Protean ligands are those ligands that act as either partial agonists or inverse agonists depending on the nature of the system to which they are applied [15, 77]. Binding of a partial agonist may produce a receptor-ligand complex with lower affinity for G protein than the affinity of the fraction of constitutively active, ligand-free receptors for G protein. In a system exhibiting considerable basal activity, such a ligand would act as an inverse agonist, decreasing the proportion of ligand free- R^* . On the other hand, in a system not disposed to producing R^* , the ligand will act as a partial agonist. This duality, along with the ability of GPCRs to couple to multiple G proteins discussed above, requires that the categorization of ligands into classical agonist/antagonist groups be done with care, and with more subdivision.

1.3 Aminergic neurotransmission

Neurons in the CNS are defined by the major neurotransmitter released upon excitation. Major neurotransmitters include γ -aminobutyric acid (GABA), norepinephrine, acetylcholine, glutamate, serotonin, and dopamine.

Postsynaptic receptors interacting with these released neurotransmitters signal in an overall excitatory or inhibitory fashion, so that GABAergic neurons are inhibitory, glutaminergic receptors are excitatory, and others depend on the nature of the major postsynaptic GPCR subtype. Co-release of neurotransmitters has been observed, and cross-talk between neurotransmitter pathways can be expected [78]. Although isolated behavioural links have been

made to each of these neurotransmitter pathways, their holistic roles have yet to be determined, partly because of large gaps in understanding regarding the way in which neurotransmitter receptors function. This project focuses on contributing to our understanding of the role of the dopaminergic pathway through the study of dopamine receptors.

1.3.1 Dopaminergic pathways

Composing half of all catecholamine neurotransmitters in the central nervous system, dopaminergic neurotransmission is key to cognitive function and movement. Dopaminergic neurons are concentrated in the substantia nigra pars compacta and the ventral tegmental area of the midbrain [79]. As such, dopamine acts to modulate the neuronal activity of terminal regions rather than directly relay information in the brain [80].

Dopamine (Figure 1) in the central nervous system is a slow-acting neurotransmitter released in a number of firing patterns: single spike, both regular and random, burst firing, in which higher levels of DA and cotransmitters are released activating early immediate genes, and tonic dopamine release [81, 82]. Phasic components of dopamine transmission regulate distinct functions. A fast (100-300 ms) is involved in reward prediction error firing while slow (s-min) transmission is linked to lordosis, movement and stress [82].

Five principal anatomical pathways can be outlined for dopamine neurotransmission:

1. *Nigrostriatal pathway*

Striatal dopaminergic signaling regulates both motor and cognitive activities [83].

The predominantly GABAergic striatal neurons integrate both glutaminergic inputs from the corticostriatal pathway and dopaminergic projections from the substantia nigra pars compacta to the dorsal striatum, composed of the caudate nucleus and putamen. Two GABAergic output pathways extending either directly to the substantia nigra pars reticularis, or indirectly, via the globus pallidus and subthalamic nucleus act to enhance and inhibit neural signaling, respectively, modulating locomotion. Branches of these two pathways are targets for Parkinson's and Huntington's diseases, dystonias, Tourette's syndrome, as well as the extrapyramidal side-effects of antipsychotic drugs.

2. *Mesolimbic pathway*

The limbic system is the seat of emotion, olfaction, and memory. Dopaminergic neurons in the mesolimbic pathway project from the ventral tegmental area to the nucleus accumbens in the ventral striatum. Dopamine also acts in the hippocampus, olfactory tubercle, amygdala, implicating this neurotransmitter in reward and motivational impulses, the dysregulation of which leads to addiction and novelty-seeking, as well as certain aspects of schizophrenia.

3. *Mesocortical pathway*

Dopaminergic neurons projecting from the ventral tegmental area to the frontal cortex are responsible for working memory and attention span, capabilities disrupted in schizophrenia and attention deficit/hyperactivity disorder [80].

Dopaminergic neurons also extend to the cortex from raphe's nucleus. Cortical networks are innervated by the subcortex via a number of other neurotransmitters. Cholinergic neurons extend from Meynert's basal nucleus, noradrenergic neurons from the locus coeruleus, and histaminergic neurons from the posterior hypothalamus [82], and so the dopaminergic neurons of the mesocortical pathway do not function in isolation.

4. *Tuberoinfundibular pathway*

The presence of dopaminergic neurons in the hypothalamus implicates dopamine in the regulation of hormone release from the pituitary. Dopamine inhibits prolactin release from the anterior pituitary [84]. Prolactin secreting tumours in the pituitary gland can be treated with dopamine receptor-specific ligands [85] [84].

5. *Periphery*

In non-neural tissue, dopamine is not converted to norepinephrine and acts in an autocrine or paracrine manner [86]. Dopamine plays a role in haemodynamics and epithelial transport, central in the regulation of extracellular cellular fluid volume and blood pressure [87]. Defective dopamine signaling has also been

shown to lead to hypertension [12, 86, 88]. Dopamine modulates epinephrine/norepinephrine release in the medulla of the adrenal gland and mediates vasodilation in the media of blood vessels. In the kidney, dopamine increases renal blood flow and filtration rates in the glomerulus of the kidney, and inhibits the resorption of sodium in the proximal tubule. In the GI tract, dopaminergic neurons modulate fluid and sodium intake.

1.4 Dopamine receptor subtypes

To establish reliable and dynamic synaptic transmission necessary for the formation of distinct signaling pathways, neurotransmitters act at post-synaptic receptors with specificity, and in a fashion that can be modified depending on environmental stimuli, a phenomenon known as synaptic plasticity [89]. To this end, neurotransmitters interact with a range of receptor subtypes whose cellular and subcellular location, concentration and active conformation equilibrium determine the extent of interaction under the current conditions.

In 1979, Kebabian identified two subgroups of receptors that interact differentially with AC upon dopamine stimulation. Since then, five dopamine receptor genes have been cloned, and are grouped into D1-like (D1R (D1A) and D5R (D1B)) and D2R-like (D2R, D3R and D4R) subtypes based on primary sequence similarity and G protein-coupling properties [12, 90]. Two D5R pseudogenes have been identified with premature stop codons [91]. D2R also exists as short and long

isoforms resulting from alternative splicing, and alternative splice variants of D3 and multiple polymorphic D4 alleles have been identified [92-94].

1.4.1 Structural homology of dopamine receptor subtypes

As with all GPCRs, dopamine receptor subtypes diverge significantly in extracellular and cytoplasmic regions, presumably allowing subtype-specific ligand and/or G protein coupling properties. As outlined in Jarvie and Caron (1993) [95], the primary amino acid sequence of D1R and D5R are 60% identical overall, and 82% identical in TMs. D2R and D3R are 60 % identical, 77% in membrane regions, while D4R is 40% identical to D2R and D3R and 55% in TM regions. D1R and D5R are approximately 35% identical to D2R, D3R, and D4R, and about 45% in TM regions.

1.4.1.1 Localization of dopamine receptor subtypes

As outlined in Table 1, dopamine receptor subtypes are expressed both independently as well as being colocalized in brain tissues [96]. In the absence of subtype-specific drugs, the overlap in expression patterns complicates the discernment of subtype function. This project focuses on human D1R and D5R.

D1R and D5R dopamine receptors are found both pre and post-synaptically, though most research has revealed information regarding their post-synaptic function [80, 97]. D1R is the most widely distributed of all five dopamine receptor

subtypes [98]. In the striatum, 70% of D1-like radioligand binding can be attributed to immunologically precipitated D1R receptors [96]. D1R is highly expressed in the prefrontal cortex, striatal medium spiny neurons, nucleus accumbens, the substantia nigra pars reticularis, and the caudate nucleus. Although the expression pattern of D5R is less concentrated than D1R in the cortex, striatum, and nucleus accumbens, D5R is more highly expressed than D1R in the hippocampus, striatal interneurons, the hypothalamus (linked to circadian rhythms and female rodent sexual behaviour), and in adrenal tissues.

D2R (long) and D3R receptors exist post-synaptically on target cell bodies and dendrites providing inhibition of neuron firing, and D2R (short) as a presynaptic autoreceptor on dopaminergic axon terminals, providing autoregulation of DA synthesis and synaptic release [80]. In the striatum, D2R is the most highly expressed dopamine receptor subtype [99]. D2R is also expressed in the ventral tegmental area and substantia nigra pars compacta, nucleus accumbens (ventral striatum), globus pallidus (striatal output structure), olfactory tubercle structures and in the limbic cortices [82]. D3R is densely expressed in the Islands of Calleja, as well as the amygdala, while D4R is highly expressed in the frontal cortex, amygdala, hippocampus, hypothalamus, and mesencephalon [12, 100, 101].

1.4.2 Function of D1-like dopamine receptors

1.4.2.1 Subcellular localization of D1-like receptors

Subcellular compartmentalization of receptors allows a further level of input specificity and selectivity of effector coupling in addition to subtype-specific cellular localization, and ligand and G protein affinities. As with D2R, D1R receptors are generally found in dendritic spines of cerebral cortical pyramidal neurons and striatum, while D5R receptors are enriched in the soma and dendritic shafts [83, 97, 102]. Immunoelectron microscopy has shown that D5R receptors are subcellularly localized to reticular specializations called subcellular cisterns, along with the 1,4,5 inositol triphosphate receptor. It is not known whether this clustering is influenced by extracellular signals or cytosolic influences [103].

In addition to classical neurotransmission across closed synaptic clefts facilitating a tight molecular chain of events, open dopaminergic synaptic clefts have also been observed, which allow the transmission of dopaminergic signals by the gradient diffusion of neurotransmitter to receptors on proximal and remote nonsynaptic membranes through the extracellular fluid, a process called volume transmission [104]. As such, elucidating the subcellular distribution of dopamine receptor subtypes is relevant to understanding their physiological roles.

1.4.2.2 D1-like physiology and pathophysiology

Dopamine neurotransmission is central to neuropsychiatric disorders including Parkinson's Disease (PD), schizophrenia, attention deficit/hyperactivity disorder (ADHD), and addiction [83, 102]. A broad range of drugs currently used to treat diseases including PD, schizophrenia, and ADHD are thought to interact with and/or modulate dopaminergic pathways, but their direct targets are still not clear [82].

Despite differences in structure and function, no subtype specific drugs are as yet available for D1R and D5R receptors, limiting both structure-activity relationship (SAR) studies and in vivo investigations into the physiology of dopaminergic receptor subtypes. In the absence of subtype-specific drugs, mice models isolating components of physiological pathways have provided information describing the separate roles and interactions of D1-like receptor subtypes D1R and D5R [83, 88, 102, 105-107]. Alternative techniques include antisense oligonucleotide infusions and ribozymal targeting of transcriptional regulators of target genes [108-110].

1.4.2.2.1 D1-like receptors in the cerebrum

The basal ganglia comprise the striatum, and the output structures of the globus pallidus, the subthalamic nucleus, and the substantia nigra. The striatum can be divided into two anatomically continuous portions: the ventral striatum, or the nucleus accumbens, and the dorsal striatum, made up of the caudate nucleus

and putamen. The striatum as a whole receives two major dopaminergic pathways: the corticostriatal pathway projects from the ventral tegmental area to the nucleus accumbens, while the dorsal striatum receives information from the PFC via the substantia nigra pars compacta. 70-75% of dopaminergic neurons are found in the substantia nigra pars compacta and the ventral tegmental area [79]. D1-like receptors are not found in the ventral tegmental area, but D5R has been shown to be expressed in the substantia nigra pars compacta, indicating D1-like receptors may have a presynaptic role in specific synaptic pathways [111]. D2R and D1R are both expressed in the nucleus accumbens and dorsal striatum [82]. Outputs from the striatum fall into two classes: GABAergic projections to the globus pallidus expressing the cotransmitter enkephalin, and projections to the substantia nigra pars reticularis expressing substance P and dynorphin. D1R and, to a lesser extent, D5R are expressed in the latter projections, while D2-like receptors are largely coexpressed with enkephalin. D1-like receptors are also found coexpressed with D2-like receptors in a subset of striatal post-synaptic neurons [82].

Striatum and movement

The direct nigrostriatal dopaminergic pathway extends from the substantia nigra pars compacta to striatal D1-like receptors that stimulate inhibitory GABAergic projections extending to the substantia nigra pars reticularis, decreasing GABAergic output to the thalamus, and increasing glutaminergic output to the cerebral cortex, ultimately facilitating movement. In addition, an indirect

nigrostriatal dopaminergic pathway contributes to movement. Via excitatory glutaminergic projections to the globus pallidus, the subthalamic nucleus negatively regulates D2R-mediated inhibitory GABAergic outputs from the striatum to the globus pallidus, decreasing cortical stimulation by thalamic glutaminergic neurons. *c-fos* expression in the subthalamic nucleus is increased by D1-like agonists, in either the absence or presence of inhibiting D2-like agonists. Therefore, it is likely that an input to the subthalamic nucleus additional to that from the globus pallidus has yet to be identified [112].

There is conflicting evidence as to the dopamine receptor subtypes found in the subthalamic nucleus, but likely D5R is the dominant form: D1-like receptors control calcium conductance necessary for burst firing of burst competent subthalamic nucleus neurons and D1R knockout mice show similar burst firing to wild-type, implicating D5R in shaping this firing pattern [105]. D2R is coexpressed with enkephalin in subthalamic nucleus GABAergic medium spiny projections to the globus pallidus, as well as being coexpressed with D1-like receptors (ie. D5R) in a subset of neurons [82].

D1-like receptors and movement

In rats, the D1R-specific antagonist SCH23390 produces a time and dose dependent attenuation of spontaneous motor control as measured by horizontal movement, rearing, and stereotypy times. Drug-specific effects of D1-like antagonists are seen in wall-seeking behaviour: at high doses SCH23990 evokes

an earlier onset, while another D1R-specific antagonist SKF83566 attenuates wall-seeking behaviour (thigmotaxis). D2-like antagonists produce similar behavioural consequences, but over a different time course [113]. SCH23390 is less effective in reducing motor activity in D1R knockout mice, suggesting that D1R acts permissively on D5R, and that when activated in the same cell, D1R and D5R would produce a synergistic effect via coupling to G_{α_s} . In different neuronal populations, however, simultaneously activated D1R and D5R may act in opposition to one another. Alternatively, D1R receptors might only produce a stimulatory effect when acting in concert with D5R [83, 102].

Rotational activity was increased by the D1R-specific agonist SKF38393 in unilaterally 6-OHDA-lesioned rats treated with D5R antisense oligonucleotides, indicating that D5R may play an inhibitory role in locomotion, perhaps due to expression in the subthalamic nucleus [112, 114]. D1-like agonist-stimulated AC activation in the striatum is actually upregulated on the lesioned side relative to control, suggesting either that D1-like receptors are upregulated in response to neural degeneration, or that a pool of receptors unassociated with AC are drawn on after lesion [115].

Studies with benzophenanthridine derivative dopaminergic agonists such as the prototype dihydrexidine have begun to discern the relative roles of pre- and post-synaptic D1-like and D2-like receptors in movement. The N-propyl analogue of the dopaminergic agonist dihydrexidine is D2R selective. While vacuous

chewing was thought to be a feature of D1R-stimulation, this behaviour was induced by N-propyl-dihydroxidine, along with D2R-associated behaviours such as yawning and inhibition of locomotion. Inhibition of locomotion and yawning were originally thought to be the result of activation of presynaptic D2R receptors, but in the case of N-propyl-dihydroxidine, no changes in synaptic dopamine release were observed [116].

D1R knockout mice exhibit increased locomotion, and less potent inhibition of movement by the D1-like antagonist SCH23390 than wild-type, while D5R knockout mice show increases in horizontal activity. D1R may play a permissive role towards D5R [83, 88, 102]. Rearing from standing, usually an indicator of motivation, is reduced in both D1R and D5R, but this may be due to a lack of motor coordination rather than a lack of desire. Similarly, hyperactive grooming is uncoordinated and is missing the pattern observed in wild-type mice [117]. While D1R knockout mice are unresponsive to the hyperactive effects of SKF 81297, indicating a major phenotype, D5R knockout mice do not exhibit statistically significant decreases in D1-like agonist effects, suggesting that D5R plays a smaller role than D1R in locomotion. This is consistent with limited striatal D5R expression [118]. However, D1R knockout mice have implicated D5R receptors in potentiating burst-firing via cAMP/PKA and L-type calcium channel pathways in the subthalamic nucleus, supported by *in situ* hybridization [105]. Deep-brain stimulation of the subthalamic nucleus has been shown to

have beneficial effects on parkinsonian symptoms, suggesting that D5R-selective drugs, via their interaction in this region of the brain, may be of therapeutic use.

Parkinson's disease (PD)

Physiological correlations to PD symptoms are reviewed in Brooks 2003 [119].

The pathophysiology of Parkinson's disease revolves around an 80% loss of dopaminergic neurons in the nigrostriatal pathway, those extending between the substantia nigra pars compacta and the striatum, a loss correlated with the severity of akinesia and rigidity. Amphetamine-evoked cortical dopamine release is maintained in the Parkinsonian brain, indicating that these neurons are not compromised. Cognitive dysfunction may actually precede the motor symptoms of PD; low dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, a dopaminergic neurotoxin) lesions are thought to mimic this early disease stage [120]. D1R and D2R levels decrease naturally with age, so that aged monkeys have also been used as models for PD. Positron emission topography studies have shown that a correlation exists between dopamine transporter activity, regulating the synaptic clearance of dopamine, and PD symptoms.

Parkinsonian resting tremors are associated with a loss of 5HT receptors from the raphe nucleus. In L-dopa treatment, a "honeymoon phase" of symptom regression is followed by increased motor fluctuations correlated with decreases in striatal dopamine.

The oral dyskinesia induced by the non-selective dopaminergic agonist apomorphine is blocked by the D1-like antagonist SCH23390, but not by D2-like antagonists. Furthermore, the D1-like agonist A77636 induces oral dyskinesia when infused into the subthalamic nucleus. This all suggests that D1R and more likely D5R, given its localization in the subthalamic nucleus, discussed above, are directly responsible for PD dyskinesias [121].

Anti-parkinsonian drugs

Four dopaminergic agonists are currently approved for the treatment of Parkinson's disease: bromocriptine (D2 selective), pergolide (non-selective), and D2/D3 selective non-ergot derivatives pramipexole and ropinirole. Agonist side effects include nausea, orthostasis, somnolence and hallucinations [122]. As a precursor of dopamine through aromatic amine decarboxylase (AAD), L-dopa as a PD therapeutic is generally thought to be inactive itself, efficiently crossing the blood-brain barrier by aromatic amino acid transporters. To avoid biotransformation before reaching target regions of the brain, L-dopa is often prescribed with carbidopa, an inhibitor of AAD in the periphery [123]. It has recently been suggested, however that L-dopa itself acts as a neurotransmitter [124]. A different set of drawbacks is associated with L-dopa treatment: dyskinesias, shortened life span, psychiatric problems, and short duration responses, side effects which are sometimes worse than the disease itself [125]. As L-dopa must compete with dietary amino acids for transport across the gut epithelium and the blood-brain barrier, its pharmacokinetics are poorly

reproducible and free radicals are generated. It is not yet agreed as to which mode of treatment, agonist or precursor, is superior, although there is suggestion that agonists provide neuroprotection [122].

Inhibiting monoamine oxidase (MAO), which breaks down synaptic neurotransmitters, can also enhance dopamine levels in the striatum. Selegiline is selective for MAO-B, avoiding contraindications relating to tyramine seen with non-selective MAO inhibitors. Finally, amantadine, an antiviral agent against influenza A has mild action in treating Parkinson's disease, possibly via interaction with stimulatory glutamate receptors. Chronic high frequency subthalamic nucleus stimulation has also been shown to reduce dyskinesias by inactivating the subthalamic nucleus neuronal activity upregulated in PD [121, 125]. D5R receptors in burst-competent subthalamic nucleus neurons are likely involved in mediating the chronic pulsatile stimulation by short-lived L-dopa that leads to dyskinesias.

Striatum and learning

The striatum integrates dopaminergic inputs from the substantia nigra and ventral tegmental area, as well as cortical glutaminergic projections. 95% of striatal neurons are medium spiny (MS) GABAergic projection neurons; as such they give rise to the majority of outputs upon dopaminergic stimulation from substantia nigra pars compacta and ventral tegmental area afferents [126]. The minority of

striatal neurons consist of aspiny interneurons, which likely hold inhibitory control of striatal output [126]. These can be divided into four classes:

- 1) Large cholinergic interneurons display large bodies, wide dendritic trees and extensive axonal fields. They relay inputs from dopaminergic afferents to projection neurons. D2-like agonists reduce, while D1-like agonists stimulate acetylcholine release. This release has been shown to be mediated specifically by D5R receptors [83, 102, 127]
- 2) Parvalbumin containing GABAergic interneurons compose 3-5% of striatal cells and are larger than medium spiny neurons. They mediate cortical inputs and their gap junctions allow the integration of inputs to multiple neurons. They are fast firing and produce short duration action potentials evoked by dopamine and mediated by D5R, inhibiting projection neurons [128].
- 3) Calretinin-containing GABAergic interneurons have not been shown to be linked to dopamine-dependent pathways.
- 4) Nitric oxide synthase (NOS)-containing interneurons compose 1-2% of striatal neurons. They receive cortical glutaminergic inputs, as well as cholinergic and dopaminergic innervation and synapse themselves with medium spiny neurons, feeding forward from the cortex in a low-threshold-spiking manner. Their extensive axonal arboration allows impacts up to a mm away from the cell body and express the most identified transmitters

of all striatal neurons, notably nitric oxide (NO). D5R has been shown to be solely expressed NOS-positive striatal neurons [83, 97, 102].

Interneurons are generally thought to play a feed forward role in striatal inhibition via the integration of subthreshold membrane potentials.

D1-like receptors and synaptic plasticity

Memory and learning are linked to use-dependent changes in synaptic efficacy through the repetitive stimulation of excitatory pathways, which results in either the long-term potentiation (LTP) or long-term depression (LTD) of synaptic transmission by gene expression or synapse formation. This phenomenon has been observed in the striatum, hippocampus (the neural substrate for some forms of memory), visual cortex and cerebellum (associated with motor learning) [129, 130]. D1-like antagonists prevent both LTD and LTP modes of neuronal signaling involved in repetitive learning [83, 102, 131]. While LTP is PKA-dependent, LTD is NO/cGMP dependent, suggesting that LTD is mediated through NOS-positive interneurons, and LTP through medium spiny neurons [132].

Long-term synaptic potentiation (LTP)

D1-like agonists attenuate GABAergic lateral inhibition between principal cells in the nucleus accumbens [133]. In D1R knockout mice, neural plasticity is affected in terms of decreases in LTP, measured through recordings of striatal spiny

neurons during activation of corticostriatal glutaminergic terminals. The application of D1-like specific antagonist further affects plasticity by reducing LTD, implicating D5R-receptors in this pathway, as discussed further below. Using antisense knockdown of D1R, D5R has been shown to be responsible for regulating acetylcholine release in the hippocampus [134]. D1-like agonists induce LTP in hippocampal CA1 neurons, while hippocampal acetylcholine release is decreased in D5R knockout mice, with no effect by D1-like agonist SKF38393, suggesting a tonic role for D5R, but not D1R in regulating this hippocampal LTP pathway.

It is possible, in the absence of initiating high frequency synaptic stimulation, for a late phase of LTP to occur. With D1-like antagonists and in D1R knockout mice, hippocampal LTP is decreased, as well as the persistence of LTP, characterized by PKA-dependent late protein synthesis. Although NMDA and dopamine activation synergistically increase cAMP levels, it is controversial as to whether dopamine agonists trigger the PKA-dependent late protein synthesis phase of LTP, or whether they play a modulatory role [135, 136].

Long-term synaptic depression (LTD)

LTD occurs when slow, weak stimulation of presynaptic neurons causes a reduction in postsynaptic sensitivity [83, 102]. In the nigrostriatal dopaminergic pathway, activation of D1-like receptors in the somato-dendritic region of low-threshold-spiking, NO-positive interneurons stimulates the release of GABA and

nitric oxide, producing LTD, a form of synaptic plasticity that reduces the input sensitivity of medium spiny neurons [83, 102, 126, 129, 130, 137]. NO is well known as an endothelium derived relaxing factor upregulating guanylyl cyclase in vascular smooth muscle, producing vasodilation. In the striatum, NO released from interneurons likely controls local blood flow and has been shown to be responsible for LTD [137]. As a diffusing neurotransmitter, NO modulates the response of surrounding neurons to neurotransmitters by, for example, modifying NMDA receptors [138]. When applied exogenously to the striatum, NO increases acetylcholine, GABA and glutamate release [129, 130]. Calcium/calmodulin binds to NOS, which is activated following increases in intracellular calcium levels coupled to D1-like receptors [138]. Striatal projection neurons are characterized by high guanylyl cyclase levels, making them a target for NO released by interneurons.

D2R autoreceptors are thought to attenuate NOS activation by suppressing glutamate release from corticostriatal terminals. D2R/D3R antagonists increase, while the D1-like antagonist SCH23390 decreases striatal NOS activity, invoking the possibility that the two dopamine receptor subclasses act simultaneously and oppositely at striatal interneurons [138]. Moreover, after repetitive stimulation of the glutaminergic corticostriatal pathway, striatal LTD is blocked by D1R and D2R antagonists, indicating that coactivation of both D1-like and D2-like receptors is required for striatal LTD [129]. The use of a selective D5R antibody in the striatal neurons of D1R knockout mice showed that D5R alone is

expressed in NOS-positive interneurons [83, 102] and therefore D5R is likely responsible for facilitating LTD.

Other proteins are known to be involved in D1-like mediation of synaptic plasticity. Post-synaptic density protein 95 (PSD95)-mediated LTP is invoked by super-sensitivity to drugs sometimes seen after chronic treatment or addiction [139]. Dopamine- and cAMP-Regulated phosphoprotein of M_r 32 000 (DARPP-32), inhibits protein phosphatase-1, a regulator of the phosphorylation state of both NMDA (LTP) and AMPA (LTD) glutamate receptors, is highly expressed in medium spiny neurons, and is a substrate for protein kinase G. Similar to when D1-like receptors are antagonized, both LTP and LTD in DARPP-32 knockout mice are ablated [132], suggesting a roles in a common signaling pathway regulating synaptic plasticity for D1-like receptors and DARPP-32.

D1-like receptors and reward-related learning

Striatal dopaminergic projections to the nucleus accumbens and prefrontal cortex have been imparted a prime motivational role, and are a critical substrate for reward-related learning in the CNS [140]. 75% of rodent dopaminergic neurons are activated when hidden food is touched. In contrast, 14% of dopaminergic neurons, from the same pool activated by reward, respond to adverse stimuli, suggesting that dopaminergic neurons may be involved in mediating specifically rewarding stimuli. The CNS relationship between stimulus and reward (or reinforcer) contributes to learning, depending on the predictability of the reward.

During learning, reward occurrence can be said to be better than predictable, as a pattern has yet to be identified. As learning a task progresses, or a stimulus becomes familiar, the predictability of the reward associated with stimulus increases, and dopaminergic activation upon receipt of reward, or reward-predicting stimuli decreases. In the case that a reward is obtained out of pattern, dopaminergic activation occurs. A shift in dopaminergic response occurs during learning, from response to reward receipt to response to the stimulus predicting the reward. Once a behaviour has been learned, reward occurrence is predictable, and no further learning from the stimulus/reward relationship is achieved. Reciprocally, if the reward expected through conditioning is not provided after stimulus, or the normal time-course of reward-receipt is deviated from, disrupting the relationship between stimulus and reward, dopaminergic neural activity is depressed, and will eventually ablate any learning already achieved [119]. Activation of AC via D1-like receptors has been implicated in reward-related learning [141].

The ability of D5R knockouts to find a hidden raft suggests that their spatial learning is intact [118]. In contrast, D1R knockout mice show decreased spatial learning abilities, pointing to different roles for D1R and D5R in this form of learning, although it is possible that alternate pathways are favoured in these chronically and globally altered mice [142]. Normal fear conditioning is achieved in D1R and D5R knockout mice, indicating that the involved hippocampal and amygdalal pathways are intact, but extinction of fear conditioning is compromised

in D1R knockout mice [143]. When adverse shocks are applied to rats after stimulus, increased dopamine release is observed in the PFC but not the nucleus accumbens. Once conditioned to these adverse stimuli, an increase in striatal dopamine release, and a decrease in PFC dopamine release, is observed on stimulus. Therefore, dopaminergic neurons report rewards relative to their predictability, and so are involved in conditional learning [140]. More specifically, the striatal dopaminergic neurons reinforce learning while cortical dopaminergic neurons are involved in identifying novel circumstances.

D1-like receptors and addiction

Addictive drugs interfere with D1-like dopaminergic modulation of learning and reward functions based in the nucleus accumbens [126]. Cocaine, amphetamines, and dopamine all decrease synaptic transmission between prefrontal cortex and the nucleus accumbens via presynaptic D1-like receptors [144]. Amphetamines increase response to conditioned rewards, modulated by D1-like agonists and antagonists [145]. The D1-like antagonist SCH23390 applied to the substantia nigra and ventral tegmental area in rodents blocks cocaine-induced reward response, while long-term cocaine administration results in decreased D1-like receptor densities [146-148]. Addictive drug-specific D1-like receptor-dependent activation of immediate early gene expression, for example *c-fos*, has been shown in the striatum, possibly mediated by cAMP responsive elements [81]. D1R knockout mice show decreased voluntary alcohol consumption than wild-type, indicating a role for D1R in motivating

alcohol consumption [149], and using antisense nucleotides in the nucleus accumbens, D5R, but not D1R, has been shown to contribute to detection of cocaine [110]. Therefore, D1-like receptors play an inhibitory role in drug-seeking behaviour.

D1-like agonists with varying efficacies at dopamine receptors may act as addiction therapeutics, as their activities may depend on the condition of drug-modulated dopamine tone [150]. For example, the D1-like agonist SKF83959 has been shown to reduce self-administration of cocaine in monkeys. In both the presence and absence of cocaine, an inhibitor of catechol-O-methyl transferase (COMT), SKF83959 acts as an antagonist, reducing behavioural affects of stimulant abuse without producing full agonist-induced hypertension, or the motor effects of antagonists, such as catalepsy. SKF83959 is also an α_2 antagonist, a receptor linked with the expression of catalepsy [151].

1.4.2.2.2 D1-like receptors in the cerebral cortex

Dopamine inputs to the prefrontal cortex integrate neuronal signals, forming working memory and the basis for memory fields [152]. D1R is expressed to a greater extent than D5R in the cerebral cortex. More specifically, D1R is found in both the frontal (dorsolateral, pre-) and limbic cortices, on distal dendritic spines of pyramidal neurons in conjunction with glutamate synapses, perhaps gating glutaminergic transmission, on terminals of glutaminergic projections, and on postsynaptic GABAergic neurons, producing feed forward inhibition [120]. This

multi-functional distribution of D1R may explain the inverted U dose response curve seen for dopamine effects on working memory [153]. D1R is colocalized with adenosine-1 (A1) receptors on cell bodies, dendrites and spines of cortical neurons, and the two form heteroreceptors facilitating antagonistic cross-talk [154]. In pyramidal neurons, L-type calcium channels are colocalized with D5R. D1-like agonists instigate the transient augmentation of calcium hump potentials, similar to the regulation of burst-firing in the subthalamic nucleus [105].

Stimulation of dopamine receptors decreases the spontaneous firing rate of neurons in the prefrontal cortex, through differential interactions with AMPA and NMDA receptors. However, during short-term working memory processing, D1R-dependent increases in prefrontal cortex neural firing have been seen, accompanied by an increase in both intracellular calcium and PKA, likely increasing phosphorylation of glutamate receptors. This is perhaps a similar mechanism to the long-term potentiation mediated by D1-like receptors at hippocampal- prefrontal cortex and corticostriatal synapses [152].

Schizophrenia

Schizophrenia, an idiopathic psychiatric disorder, is characterized by disordered thinking, withdrawal, delusions, and hallucinations and affects 1% of the world's population. Adoption studies have confirmed that it is genetic, not intrafamilial factors, which are responsible for familial aggregation of schizophrenia susceptibility, which is then exacerbated by environmental factors. However, no

single major locus has yet been identified [155]. The dopamine hypothesis of schizophrenia has guided research in this area, based on the correlation between D2-like binding affinities for clinically effective neuroleptic compounds. Given that D1-like agonists improve working memory, a central deficit in the pathology of schizophrenia, these receptors are likely also involved as well [156].

Schizophrenia and cortical D1-like receptors

The clinical outcome of schizophrenia is correlated with the extent to which cognitive functions, and underlying working memory, are compromised. Working memory, measured as the ability to manipulate information briefly, arises from the prefrontal cortex (PFC), a region of the brain which has been shown to be altered in schizophrenic patients [157]. In primates, the mesocortical dopaminergic pathway has been shown to be required from working memory. In untreated schizophrenic patients, radioligand binding studies show that D1-like receptor expression is increased in the PFC, and the degree of increase relative to control is a predictor of decreases in working memory performance [99]. In this study, no change in striatal D1-like expression was observed. If this were a primary phenomenon, one would expect that D1-like antagonists would improve working memory; clinically this is not the case. Therefore, upregulation of D1-like receptors in the PFC may be secondary to decreased presynaptic dopaminergic function. This sequence of events is supported by the improvement in working

memory induced by dopaminergic agonists, and the decreases in working memory seen in DA-depleted systems.

DARPP-32 levels are decreased in the schizophrenic PFC, while, calcyon, a D1-like adaptor protein which facilitates coupling via $G\alpha_q$, activating phosphoinositide hydrolysis by PLC, is upregulated in the PFC of schizophrenics compared to control subjects, as is the second messenger inositol triphosphate [156, 158, 159]. As an increase in a neuronal marker is unusual in a disease such as schizophrenia defined by disorganization of neuronal pathways, it could be that the calcyon upregulation is secondary to other neurochemical decreases. It has also been shown that overactivation of PKC, activated by PLC-phosphoinositide hydrolysis second messengers is linked to disruptions of working memory, while PKC levels are decreased in the cortex of schizophrenic patients [160].

Schizophrenia and striatal D1-like receptors

In contrast to the cortex, dopamine levels are increased in schizophrenic subcortical areas. Increased striatal dopamine activity has been correlated with disruption in the PFC in schizophrenia, where an increase in striatal ^{18}F -DOPA uptake at presynaptic terminals is inversely coupled with PFC neuronal activation in schizophrenic patients, as measured by increased blood flow during task completion. A correlation also exists between PFC neuronal integrity and amphetamine-induced striatal DA release [161]. While D2R is the most highly expressed receptor subtype in the striatum, conflicting evidence exists for the

role of D2R in schizophrenia: PET studies show no changes in D2 levels in the striatum in schizophrenia, but D2R receptor occupancy studies have measured increases in D2R stimulation in schizophrenia [99, 162]. A relationship exists between striatal D1-like binding potency and the severity of hallucinatory symptoms [120].

Antipsychotic drugs

It is possible that the deficit-based symptoms of schizophrenia arise from PFC hypodopaminergia, and the psychotic symptoms from subcortical hyperdopaminergia. Drugs currently used to treat psychiatric illnesses including severe psychoses, anxiety, mania, and depression are not disease-specific; it is not known which physiological pathways are modified in each disorder. In the case of mood disorders, all drugs currently prescribed fit into the monoamine hypothesis of mood disorder, which is based on links between depletion of aminergic neurotransmitters in the forebrain and depression. In the case of antipsychotic compounds, a broad correlation exists between clinical efficacy and antagonistic affinity for D2R-like dopaminergic receptors [163]. However, success in treating schizophrenia varies widely from patient to patient, and many as yet unexplained side-effects are induced by current antipsychotics. Characterization of other dopaminergic receptors, such as D1-like receptors, will provide insight and possibly novel therapies for the disease. Indeed,

observations of drug action itself have been a driving force behind understanding the physiological basis of schizophrenia.

Tricyclic compounds (Figure 1C) make up the majority of clinically useful anti-psychotic drugs, such as phenothiazines (eg. thioridazine) and thioxanthenes (eg. thiothixene, flupenthixol). These include an efficacy-increasing, electron-withdrawing substituent, and either an aliphatic (low potency), piperidine (decreased extrapyramidal effects), or piperazine (potent, decreased sedation, increased extrapyramidal effects) side chain. The size of the amino N-alkyl substituent is correlated with activity, while decreasing the space between the side-chain amino group and the central ring provides tricyclic compounds with antihistaminergic and anticholinergic properties in addition to their antidopaminergic properties.

Dibenzapines, tricyclic compounds that include a central 7-member ring, and an electron-withdrawing group found close to side-chain nitrogen atoms, are known as atypical antipsychotics, in that they induce few extrapyramidal, locomotory side effects. In general, these compounds have a low affinity for dopamine receptors. Clozapine, has a higher affinity for D4R over other dopamine receptors, has been useful in patients unresponsive to other drugs. It increases PFC dopamine release to a greater extent than in subcortical regions, in contrast to haloperidol [101, 164].

A range of heterocyclic compounds (haloperidol, spiperone, fluspirilene, butaclamol) for which few patterns in structure can be identified, may act as potent antipsychotics, sedatives, or as treatment for Tourette's-related tics [123].

Attention deficit/hyperactivity disorder (ADHD)

The prefrontal cortex guides behaviour based on working memory, for which D1-like receptors play a central role [165] [117]. ADHD is characterized by inattention, hyperactivity and impulsivity. The cognitive disorder associated with ADHD is thought, like in schizophrenia, to arise from hypodopaminergia in the cortex, with the hyperactive phenotype from hyperdopaminergia in the striatum. The widely used treatment by methylphenidate antagonizes the presynaptic dopamine transporter (DAT), upregulated in ADHD, effectively increasing dopamine levels in the synapse. This therapy is thought to decrease the background noise of DA firing and increase the signal-to-noise ratio, enhancing task-related neuronal firing [166]. It is also possible that serotonin dysregulation, like in schizophrenia, is involved in the pathology of ADHD [80].

1.4.2.2.3 Peripheral D1-like function

In the periphery, D1R stimulates PLC pathways in a calcyon dependent manner. This increases renin secretion and natriuresis, providing a mechanism for hypertension seen in D1R knockout mice [167, 168]. Genetic hypertension is due not to a decrease in D1-like receptor expression, but to a decrease in D1-like receptor function, ie. uncoupling to AC in the renal proximal tubule and medullary thick ascending limb of Henle [87]. This reduced activity produces a decreased

inhibition of Na,K-ATPase by PKA and PKC [87]. In contrast, D5R inhibits PLC in the periphery. Hypertension measured in D5R knockout mice has been linked to an increase in pituitary oxytocin-dependent sensitization of vasopressin and α adrenergic receptors in the adrenal medulla, increasing sympathetic outflow [82].

In human essential hypertension, dopamine is excreted at abnormal levels as a result of high blood pressure. At low doses, dopamine has a vasodilatory effect, reducing blood pressure, while at high doses vasoconstriction is observed [169]. Vasodilation may occur through an inhibition in NE release via D2R-like and α_2 autoreceptors. In the retina, exogenous dopamine stimulation decreases choroidal vascular resistance, and effect blocked by SCH23390, indicating that D1-like receptors are responsible [170].

1.4.2.2.4 Dopamine genetic links to disease

CNS enervation by glutaminergic neurons is more widespread than dopaminergic neurons [80], but dopamine-related dysfunction is more often implicated in disease. This could be because polymorphic gene components of glutaminergic pathways are too disruptive to survive evolution. Mutations to components of dopaminergic pathways on the other hand are not necessarily life threatening, allowing the emergence of diseased systems.

Alteration of a complex contingent of genes associated with dopaminergic neurotransmission may be the basis for the neuropsychiatric disorders discussed above. There is crossover between the synthesis pathways of dopamine,

norepinephrine, epinephrine, and serotonin. For example, tyrosine is the synthetic precursor of both norepinephrine and dopamine; both neurotransmitters are implicated in depression, anxiety, and obsessive-compulsive disorders (OCDs), and gene mutations can be expected to affect both pathways [80].

Other gene products in which mutations may affect dopaminergic neurotransmission include vesicular monoamine transporter 2 (VMAT2), which replenishes dopamine stored in vesicles at nerve terminals with newly synthesized and recycled dopamine, DAT, which carries out the majority of dopamine reuptake at the synapse, and COMT and MAO-A, which play a minor role in dopamine clearance.

D2R gene polymorphisms have been correlated with schizophrenic persecution disorder, dystonias, alcoholism (based in a reward deficiency syndrome, a core behavioural deficit in ADHD, Tourette's syndrome, OCD, autism, and gambling, [80]. Interestingly, this increase in reward seeking is in opposition to the D2R knockout phenotype in which alcohol preference is reduced. This suggests that the D2R polymorphism produces a more active receptor phenotype. D4R is the receptor subtype for which atypical antipsychotics such as clozapine have a high affinity. Nevertheless, a null D4 deletion produces no compromised neuropsychiatric features [80]. No significant correlation has been made between nonsense and missense D5R mutations and schizophrenia [171].

Only one association has been made between D1R polymorphic genes with disease, that of genetic risk for the inattention phenotype (as compared to the hyperactive/impulsive component) of ADHD [117]. As discussed above, D1R receptors play a central role in working memory, necessary for concentration. It has not been established whether this D1R haplotype is an indicator for susceptibility to working memory deficits as well. D4R, D5R and DAT genes have been repeatably linked to ADHD, although with relatively low risks, invoking the involvement of other genes in the disease [172].

40% of Parkinson's patients experience hallucinatory episodes. An attempt was made to link DA receptor polymorphisms to this tendency, without significant outcome [173]. In early onset PD, torsin A, a protein highly expressed in dopaminergic neurons is mutated. PD patients treated with L-dopa eventually develop dystonic dyskinesias and the non-pyramidal side-effects of antipsychotic drugs include dystonias. Several D5R alleles have been identified and statistically significant susceptibility links to blepharospasm and cervical dystonia have been made [174]. These polymorphisms are not in the coding region of D5R, and allele identification has not been replicated. This suggests that a common functional D5R variant exists which has yet to be identified [174].

1.5 Molecular biology of D1-like dopamine receptors

1.5.1 Dopaminergic molecular signaling pathways

Although D1R and D5R both activate AC via the G protein subunit $G\alpha_s$, substantial pharmacological differences exist between the two subtypes when expressed in heterologous cells, which, in addition to their divergent localization and *in vivo* function, discussed above, suggest that the development of subtype-specific drugs will be of therapeutic use. D1R-expressing cells exhibit a higher dopamine-mediated maximal activation of AC (efficacy), and antagonist/inverse agonist affinity than D5R-expressing cells. On the other hand, D5R-expressing cells display higher constitutive activity, agonist affinity and potency. Because of their striking functional and pharmacological differences, albeit a high degree of identity in their primary structures (notably TM regions) (Figure 3), D1R and D5R represent a unique receptor system in which to study SARs involved in ligand binding and activation processes. Study of these two receptors may also help elucidate the molecular basis of multiple G_s -linked receptors for one natural ligand [175].

D1-like dopamine receptors are most widely coupled to AC and formation of the second messenger cAMP via $G\alpha_s$. Expanded D1-like receptor functionality is lent by their ability to mediate other signaling pathways, both cAMP-dependent and independent, such as arachadonic acid production, K^+ efflux, and Na^+ , K^+ -ATPase activation [176-178]. The selective impact D1-like dopamine receptors have on *in vivo* signaling can be expected to depend on a combination of 1) G

protein subunit selection, 2) direct or cross-talk interactions with other receptors such as ionotropic NMDA and GABA, or 3) interactions with adaptor proteins favouring coupling to one effector over another [108, 156, 179].

1.5.1.1 D1-like G protein subtype interactions

Both D1R and D5R are known to interact with G protein subunits triggering signaling pathways other than that of $G\alpha_s$, such as $G\alpha_q$ and $G\alpha_{12/13}$ [47, 167, 168, 180-183]. The cytosolic complement of second messengers may also influence the coupling of GPCRs to alternate signaling pathways, as will ligand-specific activated receptor conformations [89, 184]. For example, when PKC is activated in CHO cells transfected with D2R, dopamine activation is switched from coupling through $G\alpha_i$ to inhibition of AC to stimulation of arachadonic acid release [89]. This expansion of signaling partners allows multiple parallel intracellular signals arising from one receptor subtype, just as multiple receptor subtypes allow a single neurotransmitter to couple to multiple pathways [89].

Although it is clear that D1R and D5R both couple to AC, subtype-specific impacts on cellular signaling may arise through differential interactions with intermediate pathway components. For example, the γ_7 G protein subunit (one of 12) is most highly expressed in medium spiny neurons of the striatum, as are D1R and AC. A ribozyme approach targeting this subunit in D1R-expressing cells, but not D5R-expressing cells, showed a reduction in agonist-stimulated D1R activity relative to inactive ribozyme [108].

The bulk of information currently available for D1-like dopaminergic receptors focuses on their G protein coupling impacting two major signaling pathways, the AC pathway and the phospholipase C/phosphoinositide hydrolysis pathway.

1.5.1.1.1 AC (AC) /PKA

G α_s and G α_{olf} (88% homologous) are currently known to be the main α -subtypes with which D1-like dopamine receptors interact, causing stimulation of AC and activation of the PKA phosphorylation pathway. Both cAMP and PKA regulate downstream components such as DARPP-32 via phosphorylation [185]. D1-like activation of AC can trigger PKA-dependent activation of PLC that results in the inhibition of renal Na⁺, K⁺-ATPase [186].

1.5.1.1.2 PLC/IP₃

40% of dopamine receptors in the striatum, and a portion in the amygdala have been shown to not be coupled via AC [187, 188], and behavioural effects of D1-like agonists are not correlated with their efficacy in stimulating AC, suggesting that at least a portion of dopamine receptors are coupled to another pathway in vivo [115, 184, 189]. The major alternative pathway is likely the phosphoinositide pathway [190-192]. D1R can stimulate PLC, and thus intracellular calcium release, through an interaction with a G α_q -like protein in the striatum, hippocampus, amygdala and frontal cortex, possibly coupled by the calcyon adaptor protein [180, 193-196]. G α_{15} and G α_{16} have also been shown to couple

D1R to PLC [47]. However, D1-like stimulation of phosphoinositide hydrolysis via SKF38393 in a hippocampus-neuroblastoma fusion cell line and in D1R knockout mice was shown to be independent of D1R, suggesting that D5R may be involved [197, 198].

D1R has also been co-immunoprecipitated with $G\alpha_i$ [181]. D5R has been not been co-immunoprecipitated with $G\alpha_i$ but with $G\alpha_z$ [167, 168], and in the kidney, D5R receptors colocalize and interact in an agonist-dependent manner with $G\alpha_{12}$ and $G\alpha_{13}$. While D1-like agonists inhibit the sodium/hydrogen exchanger in the proximal tubule via $G\alpha_s$, $G\alpha_{12, 13}$ have a stimulatory influence through a cAMP-independent PKA pathway [86]. D1-like activation of PLC has also been shown in the kidney [199].

Therefore, via different G protein affinities and possibly subcellular compartmentalization, D1R and D5R interact with different G protein subunits, effectively coupling each receptor subtype to different signaling pathways both in the kidney and the CNS. These pharmacological features contribute to the distinct signaling properties of D1R and D5R.

1.5.1.2 Homo and heterodimerization of D1-like receptors

Subtype-specific signaling may also be achieved via receptor dimerization. GPCRs are known to form homodimers, as well as heterodimers both with metabotropic receptors and ligand gated channels [154, 200-203]. In some

cases dimers can exist basally (constitutively), or form upon receptor stimulation; the latter has yet to be shown for D1-like dopamine receptors. D1R receptors dimerize with NMDA ionotropic glutamate receptors in two modes, one of which decreases the activity of NMDA receptors, and the other which decreases NMDA-mediated cell death [204, 205]. There is decreased expression of NMDAR1 subunit in D1R knockout striatal neurons and NMDA activation enhances D1R-mediated cAMP accumulation by recruiting D1R to the membrane [205, 206]. Activated D5R, but not D1R interacts with the γ_2 subunit of GABA_A [202]. Both D1-like receptors and GABAergic activity in the cortex are dysregulated in schizophrenia, suggesting that dysregulation of dopamine dimerization partners may be a pathological root of the disease.

In both the case of NMDA and GABA dimerization, interactions involved cytoplasmic tail regions of the D1-like dopamine receptors and inhibition of NMDA and GABA receptors via D1R or D5R is possibly achieved by receptor internalization [207]. Differences in D1-like receptor interactions with other receptors add a level of complexity to understanding the subtype-specific role of D1R and D5R, and of dopamine as a neurotransmitter.

1.5.1.3 Novel D1-like receptor interacting proteins

In addition to interactions with G proteins and dimerization with other receptors, interactions of D1-like dopamine receptors with increasing numbers of cellular proteins, collectively known as Dopamine Receptor Interacting Proteins (DRIPs)

are being identified [208, 209]. *Calcyon* is coexpressed with D1R in pyramidal neurons of the prefrontal cortex, and in medium spiny neurons of the caudate nucleus. It has been shown to couple dopamine receptors to G_{α_q} -mediated stimulation of calcium-dependent signaling [195, 196, 210]. Part of this modulation is due to the regulation of the D1R affinity state by calcyon upon priming by G_q -coupled receptor agonists [210]. *DRiP78* is an ER membrane-bound protein involved in regulating D1R receptor trafficking [211] via a FxxxFxxxF domain in helix 8 of N1R. *Neurofilament-M* (a major element of the cytoskeleton) interacts with the third intracellular loop of D1R, and to a lesser extent, of D5R [212], reducing cell surface expression, ligand binding, maximal activation and receptor desensitization. DARPP-32, a protein phosphatase-1 inhibitor, is activated upon phosphorylation by PKA, ultimately activating the inositol triphosphate (InsP_3) receptor, which is required for calcium influx mediated by D1-like receptors [192]. AKAPs have been shown to bind to GPCRs, scaffolding their signaling partners, and in tandem with PSD-95 couple D1-like activated PKA to phosphorylation of AMPA, modulating AMPA surface expression [213]. *Phosphodiesterase 1B1* (PDE1B1), a calmodulin-dependent calcium-activated enzyme known to downregulate cAMP signals, shows enriched expression in the brain, in a pattern matching that of D1-like receptors, suggesting that PDE1B1 may interact specifically with D1-like signaling pathways [214]. *Calcineurin*, a protein phosphatase, is involved in the dephosphorylation of D1R, and has been coimmunoprecipitated with D1R together with PKA [215]. Finally, *arrestins*, in addition to their role in events leading to desensitization of G

protein coupling have also been shown to link GPCRs to G protein independent signaling pathways involving non-receptor tyrosine kinase Src [44, 216-218].

1.5.2 Structure-activity relationships of D1-like dopamine receptors

1.5.2.1 *In vitro* methods of study

Techniques currently available for probing the role of an individual neurotransmitter receptor in the CNS are diverse and allow very different aspects of the protein to be assessed. The use of recombinant proteins, chimeras, single nucleotide permutations, or the substituted cysteine accessibility method, in well-understood and reproducible cell lines allows the study of a receptor under closely controlled conditions. However, given the importance of agonist-selective activity and receptor-interacting proteins, physiologically relevant contributors to GPCR signaling are likely missing in these *in vitro* systems, possibly allowing for incorrect conclusions [219]; information from such studies is limited to very direct interactions, between receptor and ligand for example. Nevertheless, measures of radioligand binding, and uptake and metabolism of radiolabelled substrates are fundamental tools to study SARs in GPCRs. Cellular assays with chimeric receptors allow the study of signaling components such as ligand affinity, G protein coupling (via either GTP-binding or cAMP accumulation assays) receptor cross-talk, dimerization, and the role of receptor-interacting proteins.

Often, rhodopsin-based models of GPCRs are used to direct mutational analysis of a given GPCR; this has the drawback of propagating a model-based

hypothesis [4]. Alternatively, random mutagenesis approaches may allow the uncovering of structural features not predicted by the rhodopsin structure. Also, the use of correlated mutation analysis (CMA) with a family such as the aminergic GPCRs allows the model-independent prediction that, for example, the IL2 and IL3 are important for activation. Computer models can therefore be considered to provide structural hypotheses for observed mutational data [21, 46].

If sufficient ligands in a chemical class are studied via competition radioligand binding, it is possible to correlate the chemical nature of the ligands with trends in binding affinity, and thus comment on the role of a receptor region in binding. Factors including size, rigidity, aromaticity, and the ability of ligand substituents to compete with protein residues in intramolecular interactions, may cause subtle shifts in binding affinity between wild-type and chimeric receptors. Evolutionary comparisons between receptors can also yield structure-activity information: it has, for example, been suggested that dopamine receptors may have evolved from purine nucleotide receptors, given similarities in dopaminergic agonist/antagonists and ATP/GTP structures, and the fact that dopamine receptors regulate cAMP production [220]. Ligand specific conformations must also be taken into consideration, and may result in different efficacies, both with respect to magnitude and nature of coupling through a range of effector pathways.

Both *in vivo* and *in vitro* studies will be forwarded by the development of receptor specific drugs, which will in turn leading to more effectively targeted therapeutic tools. In the present study, an *in vitro* recombinant chimeric approach to studying D1-like SARs has been taken. Structure-activity studies have been carried out on a number of mutant and chimeric D1R/D5R proteins which provide information regarding ligand binding sites, and G protein coupling determinants of D1R and D5R [221-227].

1.5.2.2 D1-like ligand binding sites

In a number of GPCRs including the dopamine D1R receptor, Ser199(TM5) is required for agonist binding, as well as partial agonist and inverse agonist binding. Ser202(TM5) was required only for dopamine binding, while the substitution of Ser198(TM5) by alanine abolished all ability to bind radioligand [228]. As these serines are conserved in D5R, it is not expected that they will play a role in pharmacological differences between the two receptors. In D1-like receptors, a Trp(TM7) is involved in antagonist binding, and a Asp(TM2) and a cysteine and a serine in TM3 are involved in both agonist and antagonist binding. Studies with D1R/D2 chimeric receptors showed that TM6 and TM7 are required for D1R dopamine binding, to a greater extent than for phenylbenzazepine binding [229]. Chimeric D1R and D5R studies swapping the cytoplasmic tail (CT) region showed a switch in dopamine affinity [222]; part of this effect can be attributed to the fourth intracellular loop (IL4), otherwise known as helix 8 [227]. As these are intracellular receptor components, the contribution of an

extracellular determinant in D1-like agonist binding remains to be established.

EL3 has been shown to partially modulate D1R/D5R dopamine affinity, and so is involved in ligand recognition, although other receptor components may well be involved [224]. Furthermore, structural components accounting for differences in D1R and D5R binding affinity for other classes of agonists and inverse agonists have yet to be determined.

1.5.2.3 D1-like G protein coupling determinants

As with ligand binding, previous studies investigating the activation of D1R/D5R chimeric receptors indicate that distinct receptor functions depend not on isolated protein regions but rather on interactions between distinct receptor structural determinants. For example, the higher constitutive activity displayed by D5R relative to D1R is a central difference in the pharmacological profiles of these two subtypes. As in α_1 and β_2 adrenergic receptors, the distinct constitutive activity of D1R and D5R is in part due to intramolecular interactions provided by Phe264(D1R-IL3) and Ile288(D5R-IL3) [75] [221]. As predicted by the partial switch in D1R and D5R constitutive activity achieved by single and double point mutations in IL3, other receptor regions have been implicated in the regulation of the R* conformation of D1-like receptors: when either the cytoplasmic tail or the IL4 portion alone are swapped between D1R and D5R, a full switch in constitutive activity relative to wild-type receptors is observed, while the EL3 partially modulates constitutive activity [222, 224, 227]. The cytoplasmic tail and EL3 also play interactive roles in other facets of D1-like G protein coupling function. Swapping the CT or IL4 alone between D1R and D5R induces a

general decrease in potency, mediated by the absence of appropriate EL3 sequences of D1R and D5R, respectively. Chimeras swapping the EL3 showed a complete reversal of dopamine efficacy while the cytoplasmic tail interferes with this effect [226, 227]. The cytoplasmic tail has also been shown to mediate the greater efficacy of partial agonists at D5R relative to D1R [230, 231]

Overall, studies using chimeric D1R and D5R receptors indicate that GPCRs are capable of adopting a variety of active conformations which can selectively modulate one pharmacological property or another. Studies from our lab suggest that receptor components beyond a region encompassing EL3, TM6, TM7, and the CT must be involved in regulating ligand binding and G protein coupling properties.

1.5.2.4 D1-like extracellular regions

The high TM homology in the primary structures of D1R and D5R (Figure 3) suggests that the divergent extracellular portions of D1R and D5R may likely contribute significantly to differences in ligand binding affinity and G protein-coupling (constitutive and agonist potency and efficacy). Furthermore, while extracellular regions are well-known to be involved in ligand binding by peptide receptors [7], studies of other small-ligand GPCRs indicate that extracellular regions may be similarly involved in both antagonist binding, and likely in the formation of an active receptor conformation, and thus in modulation of agonist

binding as well. The analysis of chimeric D1-like receptor binding and activity is an effective approach in determining the contribution of extracellular regions.

1.5.2.4.1 The exofacial end of the fourth TM domain

D1R and D5R exhibit the most primary sequence similarity in their TMs (Figure 3), accounting for the 82% identity (95% conserved residues) between the two subtypes [95, 232]. TM4 is the most divergent of the seven TM domains; specifically, one residue located in proximity of the putative extracellular end of the helix, which may in fact have access to the extracellular milieu upon activation of the receptor, is a serine in D1R and an asparagine in D5R. These are both uncharged polar residues, but the amide Asn is larger and has the lowest amino pK_a (8.72) of all amino acids. The hydroxyl-bearing serine is phosphorylatable, although this is likely only relevant for intracellular regions.

It is interesting to note that not only do serine and asparagines represent a point divergent residues in TM4, but EL2 of D1R also includes more serines than EL2 of D5R, and EL2 of D5R more asparagines than EL2 of D1R (Figure 3). The concentration of a particular amino acid in one region indicates perhaps the evolution of a specific electrostatic environment, a wall or ring of a given chemical quality, or the availability of more than one residue able to perform a single role, such as to accommodate ligands of varying types. My research will determine the importance of this divergence in TM4 and its potential impact on the function of the EL2.

In rhodopsin and D2R, TM4 has been found to form the interface in the receptor homodimers [18, 233, 234]. However, the specific TM4 residues participating in the formation of this interface remains to be investigated.

1.5.2.4.2 Second extracellular loop (EL2)

As shown in Figure 3, the primary structure of the EL2 of D1R and D5R is highly dissimilar. The EL2 of D5R, at 41 amino acids, is the second longest of all GPCRs. The EL2 of the C3a anaphylatoxin receptor is 171 amino acids long although only short regions flanking the TM domains have been shown to be necessary to basic receptor function [235]. As stated above, SARs indicate that structural determinants outside of the TM6/EL3/TM7/CT region must be involved in D1-like subtype-specific ligand binding and G protein coupling functions. Then, the EL2 may play a key structural role in establishing D1-like subtype-specific functional properties.

EL2 binding studies

A review of the literature finds that the role of the EL2 in agonist and antagonist affinity, receptor trafficking and activation has been studied in a number of small molecule GPCRs (reviewed [36]). In the muscarinic m2 receptor, three acidic EL2 residues are responsible for binding the allosteric antagonist gallamine [35]. In the human 5HT_{1D}, the EL2 is involved in antagonist binding [236], and differences in antagonist binding seen in the canine 5-HT_{1D} receptor are linked to

an EL2 uncharged, polar glutamine [179]. In adenosine A₃, an EL2 lysine is required for antagonist, but not agonist binding [237]. Antibodies targeted to the EL2 of α_1 , β_1 , M₂, and β_2 receptors act as agonists [200] [238]. Three carboxy terminal EL2 residues have been linked to antagonist binding in $\alpha_{1a/b}$ receptors, allowing speculation that the binding site may be more extracellular than that of agonists [239]. Negatively charged residues in the EL2 of the adenosine A_{2A} receptor have been shown to be required for agonist and antagonist binding [240]. When ligand binding regions of prostacyclin and thromboxane receptors, each with different physiological consequences, are compared, some EL2 residues are necessary for ligand binding by both receptors, while other necessary EL2 residues are unique to each [241]. While these studies may help the interpretation of the role of D1-like EL2s, the EL2 of D1R and D5R are much less conserved than any of other receptor subtypes whose structure-activity relationships have been probed by the construction of chimeric proteins, and so can be expected to play a larger role in establishing D1-like phenotypes.

EL2-TM interactions

Beyond interacting directly with incoming ligands, the EL2 can be expected to determine the orientation of at least its flanking TM4 and TM5 regions. Studies of the adenosine A_{2A} receptor [240] and molecular simulations of the parathyroid hormone receptor predicted that EL2 interacts with the TM bundle. The crystal structure of rhodopsin [18, 31] definitively revealed this secondary structure of the EL2: rather than jutting out into the extracellular environment, the N-terminal

end of this loop, through β -sheets, lies parallel to the membrane, giving much of it closer proximity to the TMs than had been previously shown for GPCRs.

Regulation of agonist binding by EL2 may be facilitated through interaction with TM domains. Hydrophobic EL2 residues might favour interaction with TM domains, be involved in binding cofactor ions, or modulate the assembly of TM regions. Recently, the results of a substituted cysteine accessibility method study of the EL2 of D2R showed that discrete EL2 residues may be involved in binding through interactions with the TM [242]. This type of secondary structure becomes all the more interesting with respect to D1R and D5R, as the two subtypes are highly divergent in their EL2 regions, both in terms of primary structure and size. As the EL2 of D1R is shorter than D5R at 26 aa, it can be expected to be less flexible, possibly less complex, and thus have fewer modes than D5R by which interaction with TM domains is possible. Such distinct primary and secondary structures may account for differences in ligand binding and G protein coupling between D1R and D5R.

Conserved structural determinants in D1R and D5R EL2

The conserved cysteine

A disulfide bond exists between the consistently conserved cysteines in EL2 and TM3 of most GPCRs, likely playing an architectural role necessary for ligand binding and receptor coupling to G proteins [243, 244]. Alternately, it has been

hypothesized that the conserved disulfide cysteine between TM3 and EL2 is cleaved during binding of agonist: many GPCRs include an aspartic acid within several amino acids from the conserved cysteine in EL2 [245]; the protonatable nitrogen in ligands like dopamine may interact with the aspartic acid in a way which allows disulfide bond cleavage.

Site-directed mutagenesis of TM3 and EL2 cysteines has been carried out in a number of receptors [240, 242-244, 246-249]. In opioid receptors, mutants in which disulfide cysteines were replaced by either alanine or serine (providing an alcohol group which theoretically would maintain any hydrogen-bonding present in the wild-type protein, while eliminating covalent interactions associated with cysteine) were trafficked to the membrane but unable to bind any radioligands tested. No difference was seen in the pharmacological profile of the serine compared to alanine mutant proteins, suggesting that the cysteine group in EL2 of opioid receptors is not involved in ligand binding via hydrogen bonds [243]. In contrast, in the human calcium-sensing receptor, mutation of EL2/TM3 disulfide cysteines ablated cell-surface receptor expression, while in the gonadotropin receptor, a naturally occurring mutation of the conserved EL2 cysteine by tyrosine results in no detectable ligand binding, and 90% reduction in inositol phosphate production, while substitution by serine reduced activity by only 50% [249] [250, 251].

NCDSSL motif

While the ELs of D1R and D5R exhibit much less homology than their TMs, both subtypes include a NCDSSL motif presenting a concentration of polar and charged residues around the disulfide cysteine, at the carboxyl end of EL2. D1-like dopamine receptors are the only GPCRs to contain this motif, but a BLAST search of this string reveals that STAT-3 (Signal Transducer and Activator of Translation-3), a transcription factor associated with the interleukin-6 receptor (IL-6) (NP_64805.1), is the only non-D1-like protein in the NCBI database (October 14, 2004) incorporating the motif. The STAT-3 region including the NCDSSL motif is involved in DNA binding [252].

Glycosylation

Karpa has shown that N-glycosylation of at least one of the two N-X-S/T motifs in the EL2 of D5R receptors is required for sorting to the membrane [253]. The EL2 of D1R also has a N-X-S/T motif, but it plays no role in targeting of the protein to the membrane.

General amino acid composition of the EL2 of D1R and D5R

EL2 of D5R contains considerably more proline and tryptophan residues than EL2 of D1R (Figure 3). These two aromatic amino acids provide bulkiness, rigidity and possibly kinks to protein primary structure [254]. A bulky, kinked

motif provided by proline and tryptophan might constrain D5R in a conformation unattainable by the shorter EL2 of D1R.

1.5.3 Drugs with activity at D1-like dopaminergic receptors

1.5.3.1 Agonists

Seven agonists are considered in the present research: dopamine, dihydrexidine, A77636 (non-phenylbenzazepines), SCH23390, SKF83959, SKF38393, and fenoldopam (phenylbenzazepines) (Figure 1). All of these ligands exhibit three features which presently define the D1-like pharmacophore: para and meta hydroxyl groups, a basic nitrogen group, and an accessory ring system [255]. Extrapolating features of pharmacophore maps to trends in D1R selectivity suggests that D1-like binding may be favoured by increased height of the nitrogen atom above the plane of the catechol ring, and by increased symmetry of the N atom relative to the two catechol hydroxyl groups [256]. The relative selectivity of this class of agonists for D1R and D2R depends exquisitely on the nature of the accessory ring and N-substituents [116]. For example, behavioural potency and efficacy of benzazepine agonists (eg. SKF83959) in 6-OHDA lesioned rats is increased by a 3N-alkyl substituent [115].

A77636 and dihydrexidine (Figure 1A) are full AC agonists in vivo and have been shown to improve working memory in dopamine lesioned monkeys.

Dihydrexidine also exhibits efficacy at D2 receptors [257]. Both A77636 and dihydrexidine have a tethered accessory ring component lending a more rigid

structure than phenylbenzazepines. This results in fewer low-energy conformations, and possibly simpler structure-activity relationships. The human D1R/D5R selectivity of these two agonists remains to be clarified [258].

Phenylbenzazepines are the original class of D1R/D5R-specific dopaminergic ligands (Figure 1B). SCH23390 and SKF83959 exhibit differing efficacies depending on the system to which they are applied. D1R displays higher SCH23390 and SKF83959 affinity than D5R, a feature typical of antagonists. In vivo, SCH23390 is an antagonist of both the AC and phosphoinositide hydrolysis pathways, while SKF83959 has no AC positive efficacy in vivo, but stimulates phosphoinositide hydrolysis in vivo probably via G_{α_q} , possibly specifically via D5R [259-261]. However, in HEK293 cells, SCH23390 acts as a weak partial agonist, and in PC12 cells it has been shown that SKF83959 inhibits AC [175, 261]. SKF38393 is a partial agonist in vivo, but its actions depend on the nature of the system being studied [262]: in HEK293 cells, SKF38393 acts as a full agonist. As a D1-like agonist, fenoldopam increases renal perfusion through vasodilation of D1R-rich vascular beds [263]. A structural explanation for differences in the pharmacology of ligands from the phenylbenzazepine class interact with D1-like receptors has not yet been determined.

1.5.3.2 Inverse agonists

As discussed above, the distinction between antagonists and inverse agonists has until recently been undetectable. Furthermore, it is possible that

antipsychotic drugs, also discussed above, may differentially act as antagonists and inverse agonists at the various receptors involved. It was first in HEK293 cells that the negative efficacy of antipsychotic drugs at D1-like receptors was identified for butaclamol and flupenthixol [175]. In this Master's project, five inverse agonistic antipsychotics are considered: cis-flupenthixol, thioridazine, thiothixene, (+)-butaclamol, and fluspirilene (Figure 1C). All five are typical antipsychotics, although thioridazine and thiothixene have in fact been shown to be antagonists (neutral ligands) at the 5HT_{2C} receptor, but inverse agonists at the 5HT_{2A} [264]. Therefore, the efficacy of these ligands depends on the nature of the receptor itself.

1.5.3.3 Drug-specific behavioural effects

As is predicted by the existence of multiple receptor active conformations, it can no longer be assumed that all agonists or inverse agonists will impact a common signaling pathway or pathways. Substantial evidence exists for functional selectivity by receptor ligands, where a single compound may act as an agonist by one functional measure, as an antagonist by another, or have not alter the system at all [10, 15, 73, 74, 76, 216, 265-267]. This phenomenon may be attributed to direct effector activation or inhibition, or alternately, cross-talk between differentially activated pathways, and can be expected to result in drug-specific behavioural effects, adding a level of complexity to the interpretation of SAR studies intended to elucidate GPCR subtype specific roles in vivo.

The behavioural effects of SKF 83822, a D1-like agonist which stimulates only the AC signaling cascade, and SKF83959, a D1-like agonist which stimulates only the phosphoinositide hydrolysis pathway, differ from that of other agonists, such as SKF38393 which is not specific in mediating G protein coupling [184, 260, 261]. In rodents, four distinct behavioural endpoints have been studied, and are differentially affected by phenylbenzazepine agonists. Intense grooming, triggered by SKF38393 or by the specific activation of PLC-mediated phosphoinositide hydrolysis by SKF83959, does not arise from SKF83822 treatment. However, tonic convulsions and explosive hyperlocomotion are behaviours unique to SKF83822 agonism. On the other hand, sniffing, locomotion and rearing are affected by both SKF83822 and SKF83959 indicating that these behaviours arise from different neuronal pathways than grooming and hyperlocomotion. Vacuous jaw movements are likely mediated by a phosphoinositide hydrolysis-dependent pathway, as inactivation of AC-coupled dopamine receptors in the striatum (approximately 75%) by EEDQ has no effect on these agonist-stimulated behaviours [268].

It is likely that cross-talk exists between the AC and phosphoinositide hydrolysis pathways, as both pathways exist in the striatum and drugs activating only one route have been shown to inactivate the other [115, 190, 191]. Also, the role of multiple D1-like receptors in intercepting and differentially triggering signaling pathways has not yet been assessed.

1.5.4 Objectives of study and hypothesis

The high degree of homology in TMs of D1R and D5R receptors leads to similar pharmacological profiles for each receptor subtype, but differences in these profiles can likely be attributed to divergent extracellular regions. Also, previous SAR studies indicate that regions outside of the TM bundle are responsible for subtype-specific phenotypes. The different cellular and subcellular distribution of D1R and D5R suggests that the two play different roles in the CNS, and that subtype specific ligands may provide better-targeted therapies for neuropsychiatric disorders.

We hypothesize, based on the low degree of primary sequence identity, that the EL2 of D1R and D5R receptors plays a role in modulating the D1-like subtype-specific functional features (agonist and antagonist binding, and/or G-protein coupling activity), possibly in tandem with the divergent TM4 residues Ser162(D1R-TM4) and Asn179(D5R-TM4).

Objective 1: To study the functional characteristics of chimeric D1R and D5R receptors swapping Ser162(D1R-TM4) and Asn179(D5R-TM4) (Figure 4) to establish a role for these divergent residues in conferring ligand binding affinity and G protein coupling activity.

Objective 2: To study the functional characteristics of chimeric D1R and D5R receptors swapping the EL2 (Figure 4) to establish a role for this region in conferring ligand binding affinity and G protein coupling activity.

Objective 3: To study the functional characteristics of chimeric D1R and D5R receptors swapping both the EL2 and Ser162(D1R-TM4) and Asn179(D5R-TM4) (Figure 4) to establish a combined role for these structural determinants in conferring ligand binding affinity and G protein coupling activity.

Chapter 2: Materials and Methods

2.1 Construction of chimeric human D1R and D5R receptors

All six TM4/EL2 D1R and D5R chimeras were constructed by gene splicing, using a polymerase chain reaction (PCR)-based overlap extension method (Perkin Elmer GeneAmp PCR system 2400) and D1R and D5R templates subcloned in the expression vector pCMV5 [269] (Figure 5). The first stage of PCR included one cycle of denaturation (94 °C, 3 min.), annealing (42 °C, 1 min.), and extension with Taq Polymerase (Invitrogen, Burlington, Canada) (72 °C, 3 min.), followed by 25 cycles of denaturation (94 °C, 45 sec.), annealing (42 °C, 1 min.), and extension (72 °C, 1 min.), terminated by one cycle of annealing (42 °C, 1 min.) and extension (72 °C, 8 min.). The overlap stage of PCR included one cycle of denaturation (94 °C, 3 min.), annealing (42 °C, 1 min.), and extension (72 °C, 10 min.) of overlapping template fragments, followed by 20 cycles of denaturation (94 °C, 45 sec.), annealing (42 °C, 1 min.), and extension (72 °C, 1 min.) with flanking primers, terminated by one cycle of annealing (42 °C, 1 min.) and extension (72 °C, 8 min.). Complete reactions were kept at 4 °C. Primers were from Sigma-Genosys (Oakville, Ontario, Canada) (Table 2).

Chimeras were named so that A and B identify components of D1R and D5R, respectively. TM4A and TM4B represent the residues at the exofacial end of TM4, Ser 162 of D1R and Asn 179 of D5R, respectively, and TE represents both the TM4 residue and EL2. Therefore, D1RTM4B and D5RTM4A chimeras swap

only the TM4 residues, D1REL2B and D5REL2A chimeras swap only the EL2, and D1RTEB and D5RTEA chimeras swap both the TM4 residue and EL2.

2.1.1 D1RTM4B

Two overlapping DNA fragments were generated in the first round of PCR, one encoding the D1R region between a TM2 Bgl II restriction site and EL2, with an EcoR I site appended 5', the other encoding the region between a TM5 Hind III restriction site and TM4, with an EcoR I site appended 3'. Each overlapping fragment encodes an Asn162 in TM4, replacing the wild-type D1R Ser162, as well as a diagnostic silent mutation encoding a Mfe I restriction site. Using D1R as template, the primers D1AEL2B-1 and D1AEL2B-7 amplified the 5' fragment and the primers D1AEL2B-8 and D1AEL2B-9 generated the 3' fragment (Table 2, Figure 5A).

2.1.2 D5RTM4A

Two overlapping DNA fragments were generated in the first round of PCR, one encoding the region 5' of D5R in pCMV5 to the carboxyl-terminal end of EL2 of D5R, the other encoding the region 3' of D5R in pCMV5 to the carboxyl-terminal end of TM4. Each overlapping fragment encodes a Ser179 in TM4, replacing the wild-type D5R Asn179, as well as a diagnostic silent mutation encoding an Apa I restriction site. Using D5R as template, the primers pCMV5 5' (forward) and D5-TM4SerA-R amplified the 5' fragment and the primers D5-TM4SerA-F and pCMV5 B (reverse) generated the 3' fragment. Two subcloning steps were used

in the case of this chimera, first into pSK+ using Xba I restriction sites, followed by ligation in pSK-D5R via Sty I restriction sites. Finally, the construct was subcloned to pCMV5-D5R using Kpn I and HinD III sites (Table 2, Figure 5A).

2.1.3 D1RTEB

Two overlapping DNA fragments were generated in the first round of PCR, one encoding the D1R region between a TM2 Bgl II restriction site and the carboxyl-terminal end of EL2 of D5R, with an EcoR I site appended 5', the other encoding the D5R EL2 region, flanked by D1R TM4 and TM5 sequences, with an EcoR I site appended 3'. Each overlapping fragment encodes an Asn162 in TM4, replacing the wild-type Ser162 of D1R, as well as a diagnostic silent mutations encoding Mfe I and BseR I restriction site. Using D1R as template, the primers D1AEL2B-1 and D1AEL2B-5 amplified the 5' fragment, and using the D5R as template, the primers D1AEL2B-6 and D1AEL2B-4 generated the 3' fragment (Figure 5C, Table 1).

2.1.4 D5RTEA

Two overlapping DNA fragments were generated in the first round of PCR, one encoding the D5R region between a TM2 Sty I restriction site and TM4, with an EcoR I site appended 5', the other encoding the EL2 of D5R region, flanked by D1R TM4 and TM5 sequences, with an EcoR I site appended 3'. Endogenous BstX I and Apal restriction sites were used to identify this chimera relative to the other chimeras. Using D1R as template, the primers D1BEL2A-1 and D1BEL2A-

2 amplified the 5' fragment, and using the D5R as template, the primers D1BEL2A-3 and D1BEL2A-4 generated the 3' fragment (Figure 5C, Table 1).

2.1.5 D1REL2B

Two overlapping DNA fragments were generated in the first round of PCR, one encoding the D1R region between a TM2 Bgl II restriction site and TM4, with an EcoR I site appended 5', the other encoding the EL2 of D5R region, flanked by D1R TM4 and TM5 sequences, with an EcoR I site appended 3'. The 3' fragment includes a diagnostic silent mutation encoding a BseR I restriction site. Using D1R as template, the primers D1AEL2B-1 and D1AEL2B-2 amplified the 5' fragment and the primers D1AEL2B-3 and D1AEL2B-4 generated the 3' fragment (Figure 5B, Table 1).

2.1.6 D5REL2A

Using D5RTEA as template, no overlap PCR was necessary for the construction of D5REL2A. One DNA fragment was generated with the primers D5EL2A-5 and D1BEL2A-4, encoding the EL2 of D1R region flanked by D5R sequences from a TM3 Sty I restriction site to a TM6 Sty I restriction site, with EcoR I sites appended 5' and 3'. The fragment encodes a diagnostic silent mutation encoding a Mfe I restriction site (Figure 5B, Table 1).

Each amplified fragment was separated on a 1% agarose gel, and the band was cut out and isolated using the QIAEX II gel extraction method (Qiagen, Valencia, CA). Dilutions of overlap fragment pairs were subjected to overlap PCR using

appropriate flanking primers. Except in the case of D5RTM4A, discussed above, EcoR I restriction sites were used to subclone the overlap fragments into pSK+ vector (dephosphorylated with calf intestinal alkaline phosphatase (MBI Fermentas Inc., Burlington, Canada)), followed by subcloning into pCMV5 expression vector linearized using restriction enzymes specific to each chimera, listed above, and ligated with T4 ligase (New England Biolabs, Pickering, Canada), listed above. Chimeric sequences were confirmed using dideoxy Sequenase version 2.0 from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada) or automated sequencing (Applied Biosystems 3730DA Analyzer, Ottawa Genome Centre, Ottawa, Ontario, Canada). Chimeric DNA in pCMV5 was prepared in concentrated form (1-3 $\mu\text{g/mL}$) using the Qiagen Maxiprep kit.

2.2 Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37 °C and 5% CO₂ in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (10 $\mu\text{g/mL}$) (Invitrogen, Burlington, Ontario, Canada). Cells (passage 38-52) were seeded in 100 mm dishes (2.5×10^6 /dish) and 24 hours later transiently transfected with 5 μg DNA using the calcium phosphate method [270]. When less than 5 μg receptor DNA per dish was required, total DNA added per condition was equalized by adding empty pCMV5 expression vector. 900 μL sterile milli-Q water, 10 μg DNA and 100 μL 2.5 M CaCl₂ were added to a 13 mL plastic tube. 1 mL of 2 $\times$ HEPES-buffered saline (HBS), pH 7.1 (0.28 M

NaCl, 0.05 M HEPES, pH 7.0, and 1.5 mM Na₃PO₄, pH 7.1) was added dropwise to the DNA-calcium solution. After gentle mixing, two 1mL aliquots were removed from the tube, each spread dropwise over one 100-mm dish (5µg/dish). Transfection by the DNA-calcium phosphate precipitate was allowed to proceed overnight prior to reseeding.

2.3 Membrane preparation

Transfected cells were washed with phosphate buffered saline (PBS), trypsinized, and reseeded in 150 mm dishes. After 48 hours, transfected cells were washed with PBS, scraped with lysis buffer (10mM Tris- HCl, pH 7.4, 5 mM EDTA), and centrifuged at 40 000 x g for 20 minutes at 4 °C. The resulting pellet was resuspended in lysis buffered using a Brinkmann Polytron at 17000 rpm for 15 seconds. After a second centrifugation and resuspension in lysis buffer, the membrane suspension was either used immediately (saturation studies) or frozen at -80 °C for use later (competition studies).

2.4 Drugs

N-[methyl-³H]-SCH23390, [³H]-adenine, and [¹⁴C]-cAMP were from Amersham Pharmacia Biotech (Baie d'Urf, Quebec, Canada). Dopamine (dissolved in ascorbic acid), dihydrexidine (dissolved in ethanol), A77636 (dissolved in ethanol), SCH23390 (dissolved in water), SKF38393 (dissolved in water), fenoldopam (dissolved in ascorbic acid), flupenthixol (dissolved in water), thiothixene (dissolved in water), thioridazine (dissolved in water), fluspirilene (dissolved in water), (+)-butaclamol (dissolved in water), and 1-methyl-3-

isobutylxanthine (IBMX) (dissolved in dimethyl sulfoxide (DMSO)) were from Sigma/Research Biochemicals International (Oakville, Ontario, Canada). SKF83959 (dissolved in 50% DMSO) was a kind gift of Dr. Neumeyer, McLean Hospital, Belmont, US).

2.5 Receptor binding assays

100 μ L of membrane resuspended in binding buffer (final concentrations in the binding assay: 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 4 mM MgCl₂, 1.5 mM CaCl₂, and 1 mM EDTA) was used per sample of 500 μ L. For saturation studies, fresh membrane was incubated with a range of N-[methyl-³H]-SCH23390 concentrations (0.01-15 nM). For competition studies, frozen membrane was thawed on ice and incubated with a fixed N-[methyl-³H]-SCH23390 concentration, between 0.6 and 1.2 nM, depending on the K_d value determined in saturation studies, and a range of concentrations of competing ligand. Non-specific binding was determined by measuring SCH23390 binding in the presence of 10 μ M flupenthixol (dissolved in milli-Q). Samples were incubated for 90 minutes at room temperature, followed by rapid harvesting through glass filters (GF/C, Whatman). Filters were washed three times with washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl). Filters were immersed in 5 mL scintillant fluid (Amersham Biosciences, Buckinghamshire, England) and bound radioactivity was determined using a scintillation counter (Beckmann Coulter, LS6500). Protein concentrations were determined using the Bio-Rad assay kit with bovine serum albumin as standard.

2.6 Whole cell cAMP accumulation assays

24 hours after HEK293 cells were seeded in 100 mm dishes they were transfected, as described above, with a range of wild-type and chimeric DNA volumes (μg), normalizing the membrane expression of each receptor (D1R, 0.0125-5; D5R, 0.0175-5 ; D1RTM4B, 0.005-5; D5RTM4A, 0.03-5 ; D1REL2B, 0.05-5; D5REL2A, 0.0075-5; D1RTEB, 0.025-5; D5RTEA, 0.015-5). When less than 5 μg of receptor DNA was added, empty pCMV5 expression was included to normalize the total volume of DNA added. Transfected cells were reseeded in 6- or 12-well dishes 18-24 hours after transfection. 24 hours later, the cells were metabolically labeled with 1-2 μCi [^3H]-adenine in fresh minimal essential media containing 5% FBS and 100 $\mu\text{g}/\text{mL}$ gentamicin and incubated for approximately 18 hours. The radioactive media was then aspirated and the cells bathed in 20 mM HEPES-buffered minimal essential media containing 1mM 1-methyl-3-isobutylxanthine (cAMP phosphodiesterase), ascorbic acid if necessary (to prevent oxidation of dopamine), and a range of agonist concentrations (0-10 μM). Cells were stimulated for 30 minutes at 37 °C. At the end of incubation the dishes were aspirated on ice and cells were lysed with 1 mL of stop solution (2.5% perchloric acid, 1 mM cAMP, [^{14}C -cAMP]) for 30 minutes at 4 °C. Samples were transferred to tubes containing 100 μL of 4.2 M KOH for neutralization, and precipitates were pelleted by centrifugation at 1500 rpm at 4 °C for 10 minutes. Sequential chromatography using Dowex (AG 50W-X4) and alumina columns was used to isolate [^3H]-cAMP [271]. [^3H]-cAMP (CA) over total [^3H]-adenine (TA) was determined as a measure of relative AC activity (CA/TU).

2.7 Curve fitting and statistics

Graphpad Prism 4.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used to non-linearly fit saturation and competition binding isotherms using the one-site binding equation, providing maximal binding ($B_{\max}$ or R) and dissociation constant (K_d) values, and to perform statistical computations. K_d values are presented as geometric means, and the standard error (S.E.) of the averaged logarithmic transform of K_d by the geometric average [272]. All other values are reported as arithmetic means. Homogeneity of variance was determined by Bartlett's test, and either the one-way analysis of variance test (ANOVA) with the Tukey multiple comparisons test, or the one-sample t-test were used to determine statistical differences

Graphpad Prism 4.0 was also used to fit average AC dose response curves, sharing the Hill Slope parameter across all conditions, providing basal and maximal activation parameters, and 50% maximal activation (EC_{50}) values. Simultaneous dose-response curve fitting was also performed sharing EC_{50} or maximal parameters, in order to determine if the best-fitted parameters for each condition were significantly different from each other. Statistically significant differences were declared when $p < 0.05$.

Chapter 3: Results

3.1 [³H]-SCH23390 binding at wild-type and chimeric D1-like receptors

The role of the EL2 and exofacial TM4 region of D1R and D5R in controlling D1-like structure-activity relationships was probed using a chimeric receptor approach. To assess whether chimeric D1R and D5R retain their ligand binding function, the equilibrium dissociation constant (K_d) and the maximal binding capacity (R) of [³H]-SCH23390, a D1-like ligand, were determined using saturation studies (Figure 6, Table 3). SCH23390 has been originally described as an antagonist but when its efficacy is revisited in heterologous expression systems (e.g. COS-7 and HEK293 cells) it behaves as a partial agonist.

In the present study, all saturation curves were best-fitted to a one-site binding model indicative of the presence of one homogenous class of binding sites on membranes derived from cells expressing wild-type and chimeric receptors (Figure 6). Raw K_d and R values are found in Table 1, while Figure 8 and Figure 9 display the results of these saturation curves relative to wild-type D1R or D5R. K_d and R values of [³H]-SCH23390 suggest that D1R and D5R chimeras retain the proper protein folding required for high affinity ligand binding and high cellular expression observed in cells expressing wild-type receptors (Table 3). Indeed, all chimeras under study express at high levels (>5pmol/mg protein). In spite of the high receptor expression displayed by all chimeras, we show that [³H]-SCH23390 has a lower R value in cells expressing D1R chimeras harboring the EL2 of D5R (D1REL2B and D1RTEB) in comparison to cells expressing the wild-type D1R

(Table 3, Figure 8). In contrast, a trend for increased R values was observed in cells expressing D5R chimeras with the EL2 of D1R (D5REL2A and D5RTEA) (Figure 9). However, these differences are not statistically significant relative to cells expressing the wild-type D5R.

On the one hand, swapping EL2 results in a full switch in [³H]-SCH23390 affinities across the wild-type D1R and D5R (D1REL2B and D5REL2A in Table 3 and Figure 8). On the other hand, exchanging the variant residue located in the exofacial end of TM4 leads to chimeric receptors (D1RTM4B and D5RTM4A in Table 3 and Figure 8) displaying further affinity “discrepancy” for [³H]-SCH23390 relative to the difference measured for wild-type D1R and D5R. The swapping of both EL2 and TM4 in chimeric receptors (D1RTEB and D5RTEA) ablates subtype distinction by [³H]-SCH23390. Overall, results obtained with these chimeric D1-like receptors suggest that EL2 and TM4 play an important role in ligand binding and subtype-selectivity of D1-like receptors. In the next series of experiments, we performed competition studies with chemically different classes of dopaminergic ligands to further assess the role of EL2 and TM4 in regulating D1-like structure-activity relationships involved in the binding of agonists and inverse agonists (antipsychotic drugs).

3.2 Role of EL2 and exofacial end of TM4 in D1-like subtype-specific agonist and inverse agonist binding

The affinity (as indexed by K_i values relative to [^3H]-SCH23390-bound receptors) of seven agonists at wild-type and chimeric receptors was assessed using competition curves (Figure 7). Among the tested agonists, dopamine and two rigid dopaminergic pharmacophores, dihydrexidine, and A77636, are similar in their inclusion of catechol hydroxyl groups and an amine [255]. In contrast to dopamine, the two rigid D1-like synthetic ligands include an accessory binding component (a benzene in dihydrexidine and an adamantane group in A77636, which likely add rigidity), which is thought to interact with the exofacial end of TM6 [255] (Figure 1). Our results show that these three agonists exhibit greater than four-fold selectivity for D5R over D1R (Table 4, Figure 10). Four substituted phenylbenzazepine agonists were also considered in our competition studies. SCH23390 and SKF83959 are two-fold selective for D1R, while SKF38393 and fenoldopam are two-fold selective for D5R (Table 6, Figure 12). The binding of five inverse agonists was also tested, which show between two- and ten-fold selectivity for D1R (Table 8, Figure 14). These include three tricyclic antipsychotics: flupenthixol, thiothixene and thioridazine; and two heterocyclic antipsychotics: fluspirilene and (+)-butaclamol. All competition curves were best-fitted to a one-site binding model (Figure 7). Non-phenylbenzazepine, phenylbenzazepine, and inverse agonist binding affinities are expressed as raw K_i values (Table 4, Table 6, and Table 8), as a percentage of ligand affinity for the wild-type parent (Figure 10, Figure 11, Figure 12, Figure 13, Figure 14,

Figure 15), and as selectivity ratios between pairs of chimeras (Table 5, Table 7, Table 9). Selectivity ratios are a useful assessment of the role of a given receptor component in determining subtype-specific characteristics.

3.2.1 Study of agonist binding

3.2.1.1 Role of the TM4 and EL2 of D1R in agonist binding

Our results show that Asn179 of D5R tends to increase dopamine, dihydrexidine and A77636 affinity at D1RTM4B, while EL2 of D5R functions in D1REL2B to reduce the affinity of these three agonists (Table 4, Figure 10). When both of these receptor regions are exchanged, the resulting chimera (D1RTEB) displays an affinity for the three agonists that is increased relative to D1REL2B (Table 4, Figure 10). These results indicate an interference or antagonism exerted by Asn179 of D5R on the effect induced by EL2 of D5R. In a similar fashion, Asn179 of D5R increases the affinity of all four phenylbenzazepine ligands at D1RTM4B, while EL2 of D5R decreases the affinity of D1REL2B for these compounds (Table 6, Figure 12). At D1RTEB, a pharmacological phenotype reminiscent of the wild-type D1R is observed, supporting the antagonistic role exerted by Asn179 of D5R in the D1RTEB chimera.

3.2.1.2 Role of the TM4 and EL2 of D5R in agonist binding

Our competition studies show that the D5R chimera harboring Ser162 of D1R exhibits a decreased affinity for dopamine, dihydrexidine or A77636 (D5RTM4A

in Table 4, Figure 11). In contrast, EL2 of D1R functions in D5REL2A to slightly increase the affinity of these three agonists. At the D5RTEA chimera, the EL2 of D1R-induced spatial relationships rule over effects induced by Ser162 of D1R alone (compare D5RTEA and D5RTM4A in Table 4, Figure 11). Additionally, our studies demonstrate that Ser162 of D1R substantially reduces the affinity of all four phenylbenzazepine ligands at D5RTM4A, while EL2 of D1R considerably increases the binding affinity of D5REL2A (Table 6, Figure 13). At D5RTEA, an increased phenylbenzazepine affinity is observed, as with D5EL2A, again indicating that the effect induced by EL2 of D1R on the binding conformation of the chimera dominates that of Ser162 of D1R seen alone in D5RTM4A (Table 6, Figure 13).

Overall, results obtained with agonist binding at wild-type and chimeric D1R and D5R suggest distinct determinants involved in the binding of the non-phenylbenzazepine and phenylbenzazepine classes of agonists. We also noted that within these two classes the extent of affinity change relative to wild-type (Figure 10, Figure 11, Figure 12, Figure 13) is not the same for each agonist tested, suggesting a differential modulation of the binding determinants for these drugs by EL2 and TM4. We reasoned that these findings might have a significant impact on the selectivity ratio of agonist binding displayed at highly homologous receptors, such as D1R and D5R. A comparison of the effect of swapping a receptor region on the selectivity ratios of ligand affinity gives an indication of the role of a particular receptor region in controlling D1-like subtype-

specific phenotypes. Therefore, in the next section, we assess the influence of the EL2 and TM4 swapping on selectivity ratios of agonists.

3.2.1.3 TM4/EL2 and agonist selectivity ratios

Our results show that an exchange of EL2 between D1R and D5R leads to chimeric receptors displaying greater selectivity ratios for dopamine, dihydrexidine and A77636 (compare WT and EL2 in Table 5). While the swapping of the exofacial end of TM4 has no effect on the selectivity ratio of dopamine, a slight decrease in selectivity ratios for dihydrexidine and A77636 is observed. In the case of the four phenylbenzazepines tested, wild-type D5R/D1R selectivity ratios can be grouped in two classes divided by ligand preference for D1R or D5R. SCH23390 and SKF83959 (Class I) are more selective for D1R, while SKF39393 and fenoldopam (Class II) are more selective for D5R (Table 7). Swapping of the EL2 region deepens the selectivity ratio of SKF383393 and fenoldopam (Class II) while switching fully (SCH23390) or partially (SKF83959) the selectivity ratios of Class I phenylbenzazepines (EL2 in Table 7). In contrast, swapping of the exofacial end of TM4 intensifies the selectivity ratios of Class I phenylbenzazepines whereas selectivity ratios for Class II phenylbenzazepines are fully switched (TM4 in Table 7). Our studies show also that exchange of both EL2 and TM4 mediates these antagonistic effects on chimeras, leading to selectivity ratios for agonists that are closer to values of wild-type receptors (TE in Table 5 and Table 7). Furthermore, while a common trend in changes in affinity is seen across a chemical class of agonist (Table 4, Table 6), our results

indicate that EL2 and TM4 have differential effects on selectivity ratios within a chemical class (Table 5, Table 7). For example, the combined effect of EL2 and TM4 increases the selectivity ratio of dihydrexidine and dopamine, but not that of A77636 (TE in Table 5).

3.2.2 Study of inverse agonists binding

3.2.2.1 Role of the TM4 and EL2 of D1R in inverse agonist binding

As with phenylbenzazepine agonists, K_i values of all inverse agonists tested in our competition studies have a propensity towards an increased affinity at D1RTM4B (Table 8, Figure 14). These inverse agonists differ from agonists however, in that EL2 of D5R also causes a small increase in antipsychotic drug affinity, except in the case of thiothixene (D1REL2B in Table 8, Figure 14). In D1RTEB, the effects of EL2 of D5R and Asn179 of D5R are additive, except in the case of (+)-butaclamol, for which a return to the wild-type phenotype is seen (Table 8, Figure 14).

3.2.2.2 Role of the TM4 and EL2 of D5R in inverse agonist binding

As with agonists, we measured a substantially reduced inverse agonist affinity at D5RTM4A with all the drugs tested (Table 8, Figure 15). In contrast to D1R-based chimeras (Figure 14), a parallel loss in inverse agonist affinity at D5REL2A is only observed with flupenthixol, thioridazine and (+)-butaclamol, while

thiothixene and fluspirilene display a small but not significant increased affinity for this chimera (Figure 15). In the D5RTEA chimera, with the exception of fluspirilene, for which the effect of Ser162 of D1R dominates that of EL2 of D1R, EL2 of D1R interferes with the large decrease in inverse agonist affinity induced by Ser162 of D1R alone (Figure 15).

3.2.2.3 TM4/EL2 and inverse agonist selectivity ratios

Swapping EL2 between D1R and D5R generally increases the chimeric selectivity ratios for inverse agonists, relative to wild-type (EL2 in Table 9). When the variant residue found in exofacial end of TM4 is swapped, an even larger increase in selectivity ratio is seen (TM4 in Table 9). When both receptor components are exchanged simultaneously, the magnitude of selectivity ratios (except for fluspirilene) is reminiscent of EL2 swap alone, suggesting an antagonistic effect of TM4 and EL2 (TE in Table 9).

3.3 Delineating the role of EL2 and TM4 of D1R and D5R in G protein coupling properties

The role of the EL2 and the exofacial end of TM4 in G protein coupling was assessed by whole cell cAMP assays on HEK293 cells transfected with wild-type or chimeric expression constructs in the absence or presence of increasing concentrations of dopamine, dihydrexidine, or A77636. These studies have allowed us to determine three parameters for receptor ability to couple to AC: agonist-independent (constitutive) activity, agonist-mediated maximal stimulation

of AC (efficacy), and potency, as indexed by EC_{50} , the agonist concentration at which 50% of maximal stimulation of cAMP accumulation is achieved.

3.3.1 Role of EL2 and TM4 in D1-like receptor constitutive activity

Figure 16A describes constitutive activation of AC elicited in cells expressing wild-type or chimeric receptors. To account for differences in receptor expression, data were normalized against respective R values obtained for wild-type and chimeric receptors, and relative to D1R (Figure 16B).

Ser162 of D1R causes an increase in constitutive activity of D5RTM4A, relative to wild-type; this is not mirrored in cells expressing D1RTM4B (Figure 16B). No significant difference in constitutive activity is noted for D1REL2B or D5REL2A when compared to their respective wild-type receptors. As seen with our ligand binding studies, effects elicited on constitutive activity of the D5RTM4A are prevented by swapping Ser162 and EL2 of D1R simultaneously (D5RTEA in Figure 16B).

3.3.2 Role of EL2 and TM4 of D1-like receptors in regulating agonist-mediated activation of AC

3.3.2.1 Role of EL2 and TM4 of D1-like receptors in agonist efficacy

Parameters of agonist-stimulated maximal activation of cAMP production were extracted from dose-response curves fitted by a non-linear regression using

shared slope factors (Hill coefficient), or from single-point experiments performed exclusively to assess agonist-mediated maximal activation of AC. Best-fitted top values from dose-response curves represent agonist-mediated maximal activation of AC. In single-point experiments, agonist-mediated maximal activation of AC was determined using a 10 μ M concentration of agonist. Three agonists were assessed- dopamine, dihydrexidine, and A77636- and were found to have similar abilities for maximally activating wild-type D1R and D5R. As reported previously for dopamine, the three drugs under study elicit, in D1R-expressing cells, higher maximal activation of AC (approximately 25%) than D5R-expressing cells (Figure 17).

Our data show that the variant residue of the exofacial end of TM4 (Ser162 of D1R and Asn179 of D5R) modulates the efficacy of each agonist differently (D1RTM4B and D5RTM4A in Figure 17, Figure 18, Figure 19). For dopamine, a full switch is seen between D1RTM4B and D5RTM4A (Figure 17). For dihydrexidine, only D1RTM4B shows decreased efficacy; D5RTM4A remains similar to wild-type D5R (Figure 18). In contrast no change in A77636 efficacy is seen at D1RTM4B but this drug gains efficacy at D5RTM4A (Figure 19). Importantly, these results cannot be explained by receptor expression levels, as R values measured for these experiments were similar.

Our results also indicate a non-reciprocal role of EL2 in regulating agonist efficacy in cells expressing D1R or D5R. The efficacy of the three agonists is

further enhanced or unchanged in cells expressing D1REL2B or D5REL2A, respectively (Figure 17, Figure 18, Figure 19). These results suggest that EL2 of D5R promotes a greater stabilization of the R* conformation of D1REL2B by agonists. A combined exchange of EL2 and the divergent TM4 residue, as indexed with D1RTEB and D5RTEA chimeras, ablates the effects seen on efficacy of these agonists in cells expressing TM4 single-point mutants (Figure 17, Figure 18, Figure 19). Results obtained in cells expressing D1REL2B or D5REL2A are essentially recapitulated in cells expressing D1RTEB or D5RTEA, suggesting that EL2 plays a predominant role over TM4 in controlling agonist efficacy (Figure 17, Figure 18, Figure 19). However, in the case of dihydrexidine, efficacy is further enhanced at D1RTEB (Figure 18).

3.3.3 Role of EL2 and TM4 of D1-like receptors in agonist potency

Agonist-dependent G protein coupling properties of D1-like receptors can be further assessed via the measurement of agonist potency for AC activation, known to be approximately ten-fold greater in D5R- than D1R-expressing cells (~1 nM versus ~10 nM). To delineate the role of EL2 and exofacial end of TM4, dose-response curves for dopamine or dihydrexidine were carried out in cells expressing wild-type or chimeric receptors (Figure 20). EC₅₀ (nM) values were calculated by non-linear curve-fitting of dose-response curves. The statistical significance of relative EC₅₀ shifts was determined by comparing the statistical F value obtained using a simultaneous curve fitting approach comparing unconstrained (no sharing of EC₅₀ value) versus constrained (sharing same EC₅₀ value) parameters (Figure 21, Figure 22).

Results obtained from these experiments show that Asn179 of D5R imparts a more “relaxed” conformation to the receptor as demonstrated by the increased potency of dihydrexidine in cells expressing D1RTM4B relative to cells expressing D1R (Figure 22). Interestingly, dopamine potency in the same transfected cells is not significantly altered (Figure 21). Meanwhile, potency data show that Ser162 of D1R constrains receptor conformation relative to D5R against dopamine activation (D5RTM4A in Figure 21B). Ser162 of D1R does not introduce the same constraint on dihydrexidine potency (D5RTM4A in Figure 22).

Cells expressing D1REL2B display substantially reduced dopamine and dihydrexidine potency relative to cells expressing D1R (Figure 21, Figure 22). In contrast, D5REL2A-expressing cells display increased dihydrexidine potency while dopamine potency remains essentially unchanged relative to D5R-expressing cells (Figure 21, Figure 22). Agonist potencies measured in cells expressing the chimera harboring both EL2 and Asn179 of D5R (D1RTEB) suggest a receptor coupling conformation close to D1R (Figure 21, Figure 22). In an opposite manner, cells expressing D5RTEA (the chimera harboring EL2 and Ser162 of D1R) exhibit dopamine potency similar to D5REL2A-expressing cells (Figure 21), whereas dihydrexidine potency is significantly increased (Figure 22). These results suggest a reciprocal modulation by EL2 and TM4 of agonist-selective receptor conformations for G protein coupling.

Chapter 4: Discussion

D1R and D5R, despite their close primary sequence similarity, notably in the TM regions, exhibit different pharmacological profiles, and thus different physiological roles [175]. It is of interest to account for these differences structurally. A number of functionally important residues have been identified, in both TM and intracellular loop regions for the extensively studied β_2 adrenergic receptor [14, 20, 27, 28, 30, 273-281]. Amino acid sequence alignments show that the important TM residues involved in β_2 -adrenergic receptor function are conserved in D1-like receptors, indicating that other D1-like receptors regions must be responsible for their subtype-specific ligand binding and G protein coupling properties. The as yet unstudied role of the EL2 of D1-like receptors may include broadly modulating the tertiary structure of D1R and D5R. The present study probes the roles of the divergent EL2 region in D1R and D5R structure-activity relationships through engineered chimeric proteins D1REL2B and D5REL2A, swapping the EL2 between wild-type receptors. Divergent TM4 residues Ser162 of D1R and Asn179 of D5R are possibly extracellular in some or all receptor conformations. As such, the construction of D1RTM4B and D5RTM4A chimeras, which swap the single variant residue of the exofacial end of TM4, along with D1RTEB and D5RTEA, which swap both components between D1R and D5R, was also carried out.

While studies of the EL2 of other small molecule GPCRs have generally focused on the ligand binding role of this region, direct and allosteric, in addition to ligand

binding affinities, the present study probes the contribution of EL2 and the exofacial end of TM4 to D1-like G protein coupling properties [34, 35, 62, 236-240, 242, 247, 249, 282-285].

4.1 Selectivity ratios

Theoretically, it is possible to identify a specific ligand feature responsible for the differential interactions of similar ligands with D1-like receptor subtypes, ie. subtype specificity. The four phenylbenzazepine agonists tested by competition studies- SCH23390, SKF83959, SKF38393, and fenoldopam, differ in catechol, benzyl, and N-substituents. These subtle differences amount to changes in wild-type D1R and D5R selectivity [286], and possibly in ligand efficacy. For example, SCH23390 and SKF83959 (Class I) include an N-methyl substituent, unlike SKF38393 and fenoldopam (Class II) (Figure 1); this may contribute to the greater D1R affinity for SCH23390 and SKF83959 (Table 6). This type of switch has been shown previously [27, 255, 256, 286, 287]. MCL203, which includes a 3'-CH₃ substituent, is selective for D1R, while MCL212 differs only by the inclusion instead of a 4'-CH₃, but shows higher D5R affinity (Figure 1). Similarly, fenoldopam includes a 4'-OH and displays greater D5R affinity, while SKF83959 has a 3'-CH₃ and has greater D1R affinity [286] (Figure 1, Table 6).

Just as the analysis of wild-type selectivity ratios can be rationalized by identifying key ligand features, shifts in the selectivity ratios of chimera pairs may be attributed to specific ligand binding features provided by the swapped receptor

structural determinants (Table 5, Table 7, Table 9). The EL2 therefore is at least partially responsible for the selectivity ratio of SCH23390 and SKF83959, perhaps due to a positive interaction of EL2 of D1R with the N-CH₃ group possessed by both. Similarly, Asn179 of D5R perhaps allows a favourable interaction with the electron-withdrawing hydroxyl and sulfate groups of SCH38393 and fenoldopam, respectively. Interestingly, each of these receptor elements interact in the same way with all four phenylbenzazepines, indicating that it is the relative impact of the exofacial end of TM4 and EL2 which determines the wild-type selectivity. Furthermore, reuniting EL2 and TM4 elements ablates SCH23390 and SKF83959 selectivity, while further enhancing that of SKF38393 and fenoldopam. This suggests that the EL2 conformation involved in ligand binding is modified by the TM4 residue.

In the case of non-phenylbenzazepine agonists (dopamine, dihydrexidine, A77636) (Table 5) as well as inverse agonists (Table 9), a swapping of EL2, with or without the variant residue found in the exofacial end of TM4, causes an increase in selectivity ratio. Therefore EL2 may function as a molecular switch to balance the overall level of constraint or flexibility of each D1-like receptor subtype.

4.2 Constraining features in a “relaxed protein”, and “relaxing” features in a constrained protein

Based on the higher basal activity, agonist affinity, and potency, D5R is considered to be a less constrained receptor than D1R [175]. It is hypothesized that constitutively active receptors (wild-type D5R or constitutively active D1R mutants) are energetically unstable relative to their quiescent counterpart wild-type D1R, facilitating the adoption of an active R^* state as a type of default. It follows that the maintenance of the inactive receptor state R , by D1R for example, has stricter structural requirements than the maintenance of R^* , favoured by D5R. Mutagenesis studies using chimeras and single-point mutations have provided support to the idea that D5R-derived regions or residues tend generally to confer on chimeric/mutant D1R a more “relaxed”, or less constrained conformation, as indexed by agonist affinity and G protein coupling properties (constitutive activity, efficacy, potency). As for D1R-derived regions or residues, the opposite trend is observed i.e. chimeric/mutant D5R displays a less “relaxed” or more constrained conformation. In both cases, however, some receptor components must balance the constraining or relaxing nature of the receptor, in order for the receptor to intercept and produce signals in tune with its environment. This work represents the first identification of constraining structural determinants in D5R, and of “relaxing” structural determinants in D1R. All of the binding affinity (except the phenylbenzazepine pairs discussed above), constitutive activation and potency shifts observed in D1-like chimeras swapping the TM4/EL2 region are in opposition to the nature of the

wild-type source of the region swapped. The TM4/EL2 junction residues Ser162 of D1R and Asn179 of D5R constrain and relax the binding conformation, respectively. Moreover, Ser162 of D1R increases the constitutive activity of D5R. EL2 of D1R and EL2 of D5R impart to receptor conformation relaxing and constraining determinants, respectively, for agonist affinity and potency (Table 4, Table 6, Figure 21, Figure 22). In contrast, EL2 of D1R and EL2 of D5R exert constraining and relaxing effects, respectively, on the binding conformation for inverse agonist affinity (Table 8, Figure 16).

One explanation for the opposite trends in these chimeras views EL2 acting as a lid, controlling ligand accessibility to the binding site, a paradigm suggested for both rhodopsin and P2Y₄ [18, 62]. In the case of D1-like receptors, for which subtype-specific differences in the agonist binding pocket are currently not known, if EL2 acts as a lid, either one creating a ligand diffusion barrier, or one stabilizing the ligand-receptor complex, it can be imagined that, for example, the greater agonist affinity exhibited by wild-type D5R relative to D1R might be due to EL2 of D5R reducing the dissociation rate (k_{off}) of agonists. In the D1REL2B or D1RTEB setting, such a “trapping” property of EL2 of D5R may act to block/impede agonist entry, while the shorter EL2 of D1R may facilitate ligand entry to D5REL2A and D5RTEA. The fact that in D1RTEB, Asn179 of D5R interferes with the EL2 of D5R-induced decrease in agonist binding suggests that the dependence of EL2 of D5R on the Asn179 of D5R residue is due to a role for this residue in re-situating EL2 to facilitate agonist entry (k_{on}) (Figure 10, Figure

12). In contrast, the agonist binding profiles of D5REL2A and D5RTEA are identical, suggesting that the “relaxing” determinant involved in the regulation of ligand entry by the smaller loop is not altered by the TM4 residue (Figure 11, Figure 13).

For efficacy alone, swapping components of the TM4/EL2 junction causes trends in keeping with the wild-type origin (Figure 17, Figure 18, Figure 19). In particular, a full switch in dopamine efficacy was induced by Ser162 of D1R/Asn179 of D5R (Figure 17), while partial trends were seen for the other two agonists tested, dihydrexidine and A77636 (Figure 18, Figure 19). It is important to bear in mind that efficacy can also depend on the “desensitizing machinery” in a given cellular system. Therefore, our results may also indicate that for the endogenous agonist, these TM4 residues are directly involved in controlling phosphorylation and desensitization, two physiological processes regulating receptor-G protein-effector interactions. This may occur through modulation of TM orientations that effectively transfers a signal from the EL to IL regions, affecting receptor activation. Further studies would be required to test whether these chimera display distinct desensitization and phosphorylation properties.

4.3 A permissive role for Asn179 of D5R in ligand binding

Overall, agonist and inverse agonist affinities are drastically reduced at D5RTM4A, a chimera in which Asn179 of D5R is replaced by Ser162 of D1R (Table 4, Table 6, Table 8). These results might suggest that Asn179 of D5R is

key to D5R ligand binding in general, and in particular to inverse agonists and phenylbenzazepine agonists. Alternatively, Ser162 of D1R may be a constraining structural determinant, restricting ligand binding at D5RTM4A. However, ligand binding affinity shifts for D1RTM4B do not show that Ser162 of D1R is as important as Asn179 of D5R for creating the ligand binding conformation, nor does Asn179 of D5R impart a greatly relaxed phenotype to D1R, as shown by modest increases in agonist and inverse agonist affinities at D1RTM4B (Table 4, Table 6, Table 8). These results indicate that the presence of the Asn179 of D5R residue in the context of D5R is required to create the receptor conformation allowing ligand binding. It is possible that Asn179 of D5R is required for proper TM positioning, or for the maintenance of EL2 of D5R secondary structure, and that in its absence, ligands of all types are hindered from entering the receptor binding site. The lesser role for Ser162 of D1R in D1R may be masked by other receptor components in D1RTM4B, or Ser162 of D1R may in fact be less important to D1R function than Asn179 of D5R is to D5R.

As revealed by sequence alignment, GPCRs that share the majority of the D1-like TM4 amino acid sequence tend to diverge, as do D1R and D5R, at the TM4/EL2 junction, suggesting that these divergent residues, including Ser162 of D1R and Asn179 of D5R, may contribute to distinct receptor phenotypes (Figure 4). The cytoplasmic tail of D1R and D5R has been shown to be involved in agonist binding [222], and the IL4 component of CT in inverse agonist binding [227]. As these regions are not in direct contact with extracellular ligand, an

allosteric relationship between EL2/TM4 and CT may trigger the receptor conformation most likely to bind agonists and inverse agonists. Chimeras swapping both the EL2 and CT would elucidate such a relationship, and might be expected to balance the roles of each structural determinant, ultimately exhibiting no shift in agonist affinity.

4.4 EL2 plays distinct roles in agonist and inverse agonist binding

EL2 of D5R significantly decreases receptor affinity for all seven agonists tested, while EL2 of D1R increases agonist affinity, to a slightly larger extent for phenylbenzazepines than dopamine, dihydrexidine or A77636 (Table 4, Table 6). In contrast EL2 has smaller, opposite effects on inverse agonist binding (Table 8). This is surprising, as it has been postulated that antagonistic compounds may occupy binding sites closer to extracellular regions than agonists. Numerous studies support this: $\alpha_{1a/b}$, A_{2a} , 5HT_{1D}, A_3 antagonist binding is modulated by residues in the EL2, as well as to allosteric modulators of antagonist binding, while in the peptide-binding gonadotropin receptor (GnRH), mutations within EL2 convey activating G protein coupling characteristics to antagonist binding [62, 200, 236, 238-240, 242, 283, 285].

It is also interesting to observe that the magnitude of the EL2 of D1R effect on D5R within an agonist class is fairly consistent (Figure 11, Figure 13), regardless of differences in ligand chemical structure, while that of EL2 of D5R on D1R differs across an agonist class (Figure 11, Figure 13). This is also seen with

D1RTEB, albeit to a lesser extent. This suggests that EL2 of D5R may be in more direct contact with agonists than EL2 of D1R, and therefore binding affinities are subject to more subtle differences in ligand chemical structure. Different gradients of the magnitude of affinity shift are also elicited by EL2 of D5R (non-phenylbenzazepine agonist>phenylbenzazepine>inverse agonist) (Figure 11, Figure 13, Figure 15) and EL2 of D1R (phenylbenzazepine>non-phenylbenzazepine agonist=inverse agonist) (Figure 10, Figure 12, Figure 14). Therefore, the EL2s of D1R and D5R receptors play opposing relaxing and constraining roles in ligand binding, as discussed above, these roles are of different magnitude depending on ligand class, and this region generally interacts differently with agonists versus inverse agonists.

4.5 Interaction of TM4 and EL2

Replacing Asn179 of D5R by Ser162 of D1R induces the most substantial effects on receptor function, inhibitory of ligand binding and agonist potency (Table 4, Table 6, Table 8), and facilitatory for constitutive activity (Figure 16) and agonist efficacy (Figure 17, Figure 18, Figure 19). All of these effects are dominated by the EL2 of D1R profile in D5RTEA. The same is not true for shifts seen with D1REL2B and D1RTEB: depending on the parameter assessed, Asn179 of D5R and EL2 of D5R interact antagonistically or additively. These results suggest that, firstly, the TM4 and EL2 junction regions interact differently in D1R and D5R, and secondly, for each subtype, all ligand classes may not interact the same way with the TM4/EL2 junction region, facilitating the formation of agonist-specific active conformations.

EL2 of D5R depends on Asn179 of D5R

The results obtained in this study can be explained by postulating that EL2 of D5R is dependent on Asn179 of D5R for proper positioning and/or folding. EL2 of D5R is dissociated from Asn179 of D5R in two chimeras: D1AEL2B and D5RTM4A. Both D1AEL2B and D5RTM4A bind agonists with greatly decreased affinity (Table 4, Table 6). D5RTM4A also binds inverse agonists with substantially decreased affinity (Table 8). This indicates that EL2 of D5R interacts with D1R in a way that “discourages” ligand binding, as discussed above. It is possible that D5R-like ligand binding via EL2 of D5R requires the presence of Asn179 of D5R. However, D1REL2B shows increased affinity for inverse agonists. This may suggest that the replacement of the constraining EL2 of D1R in D1R may have a positive impact on inverse agonist affinity at D1R.

EL2 of D5R and Asn179 of D5R are reunited in the chimeras D1RTEB and D5RTEA. In D1RTEB, the negative repercussions of EL2 of D5R separated from Asn179 of D5R in D1REL2B are compensated for in agonist binding (Figure 10, Figure 12), while inverse agonist affinity is further enhanced relative to D1REL2B (Figure 14). Similarly, in D5RTEA, the D5REL2A binding profile for both agonists and inverse agonists is regained (Figure 11, Figure 13, Figure 15). It can be concluded that decrease in ligand affinities at D5RTM4A and D1REL2B is due to the absence of Asn179 of D5R. EL2 of D1R is not similarly dependent

on the presence of Ser162 of D1R, as evidenced by modest affinity shifts at D5REL2A and D1RTM4B, in which EL2 of D1R is separated from Ser162 of D1R (Table 4, Table 6, Table 8).

Rationalizing the EL2/TM4 relationship

The nature of the TM4/EL2 interaction in either D1R or D5R may be one of a number of types. As discussed in the introduction, EL2 of D1R includes more serine residues than EL2 of D5R, matching Ser162 of D1R, and EL2 of D5R includes many asparagines residues, complementing Asn179 of D5R (Figure 4). Thus, the dependence of EL2 of D5R ligand affinity on Asn179 of D5R may be due to the formation of a region defined by the amide side groups of these asparagines. According to the present data, a similar interaction between EL2 of D1R/TM4 serine hydroxyl side-chains is not as important for ligand affinity at D1R. Through these interactions, Asn179 of D5R may be necessary for proper orientation of the bulky and long EL2 of D5R. EL2 of D1R is shorter and has fewer aromatic residues than EL2 of D5R, and so formation of secondary structure within the loop may be less defined, or less dependent on intramolecular interactions, and thus less dependent on Ser162 of D1R (Figure 4).

As assessed in rhodopsin by x-ray structure [18], and in other GPCRs by less direct methods [31, 240], the C-terminal side of EL2 has been shown to interact with the TM bundle, rather than simply with the extracellular environment. In this

way, and in contrast to the “lid” hypothesis discussed above, EL2 may act as a “plug” against incoming ligand, or actually form part of the binding pocket. Given the considerable differences in primary structure and size between EL2 of D1R and EL2 of D5R (Figure 4), the way in which this putative interaction occurs for D1R and D5R may be very different. If a TM4/EL2 “plug” interaction were a feature of the inactive state, as speculated for the rhodopsin model, increases in agonist affinity observed at D5REL2A are perhaps a feature of an inability to form the “plugged” inactive state facilitated by the larger EL2 of D5R. This would be in line with the purported role of EL2 of D5R in constraining receptor conformation as indexed by agonist affinities at D1REL2B.

Given the uncertainty involved in modeling membrane domains, the possibility remains that Ser162 of D1R and Asn179 of D5R interact to a certain degree, or even solely in the extracellular environment. However, this eventuality does not lead to an alternative rationalization of the present results.

4.6 D5RTM4A as a constitutively active mutant (CAM) GPCR

Receptor mutants that display increased basal activity relative to wild-type, as does D5RTM4A, are thought to exist in a more relaxed conformation, so that the active R* state becomes more easily obtained. Based on experimental evidence and computer simulations, the classical paradigm for constitutively active GPCRs relative to a more “quiescent” GPCR is: increased affinity and potency of full agonists, decreased affinity of inverse agonists and increased efficacy of partial

agonists. Our results show that D5TM4A exhibits increased constitutive activity relative to wild-type D5R (Figure 16) with a decreased affinity for inverse agonists (Table 8) as predicted by the classical paradigm of GPCR constitutive activation. Unexpectedly, agonists also display decreased affinity and potency at this constitutively active chimera (Table 4, Table 6, Figure 21, Figure 22). Although the activity of partial agonists was not assessed here, the efficacy of three full agonists- dopamine, dihydrexidine, and A77636- is increased at D5RTM4A (Figure 17, Figure 18, Figure 19). Therefore, our results suggest a new paradigm in constitutive activation of GPCRs whereby a GPCR can adopt a conformation that increases the proportion of receptor molecules in the R* state, while displaying a lower affinity for ligands in general, resulting also in lower agonist potency. Recently, a swapping of IL4 of D1R and D5R was shown to induce a full switch in constitutive activity, with no switch in inverse agonist affinity [227]. Combined, these studies clearly show that there are many receptor active conformations attainable, each defined by different ligand binding and G protein coupling profiles.

4.7 Receptor expression

EL2 of D5R decreased D1R receptor expression in the absence and presence of Asn179 of D5R, as indexed by R values for N-[methyl-3H]-SCH23390 binding (Table 3, Figure 8). This may be explained by alterations in intracellular trafficking or structural stability of these chimeras. It has been shown that glycosylation of D5R, but not D1R, is necessary for receptor trafficking. Two of

these glycosylation sites are found in the EL2 of D5R, (only one is found in EL2 of D1R) and seem to function in tandem with another on the N-terminal end of D5R [253]. The swapping of these glycosylation sites in D1R may disallow normal membrane trafficking of D1REL2B and D1RTEB. Further studies will further elucidate this possibility.

4.8 Conclusions

In conclusion, this work represents the first identification of D1-like structural determinants constraining wild-type receptor function, and of D1-like determinants responsible for ligand selectivity ratios. Also, EL2 and the variant exofacial TM4 residue of D1R and D5R are identified as direct regulators of phenylbenzazepine binding, while further evidence is presented for multiple D1-like receptor active states. In general, the TM4/EL2 junction region functions differently in D1R and D5R, and interacts differently with agonists versus inverse agonists. Subtype-specific differences in the binding and receptor activation by non-phenylbenzazepine agonists dihydrexidine and A77636 had yet to be assessed in D1R/D5R-expressing HEK293 cells. Here, we show that these two agonists are indeed, to different degrees, D5R-selective, and that they interact differently than dopamine with D1-like active conformations, as probed via the TM4/EL2 chimeras.

Based on these results, understanding the role of the EL2 region in D1R and D5R subtype specific differences would benefit from further structure-activity

studies. Single point mutations of serines in EL2 of D1R and asparagines in EL2 of D5R would shed light on the specific role of these duplicated residues.

Swapping EL2 and EL3, or EL2 and CT simultaneously between D1R and D5R may reveal the way in which the EL2 region constrains or relaxes the receptor as a whole [222, 224].

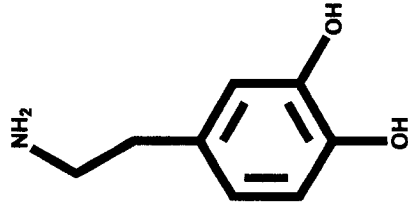
Figure 1: Chemical structures of D1-like ligands

A. Non-phenylbenzazepine agonists

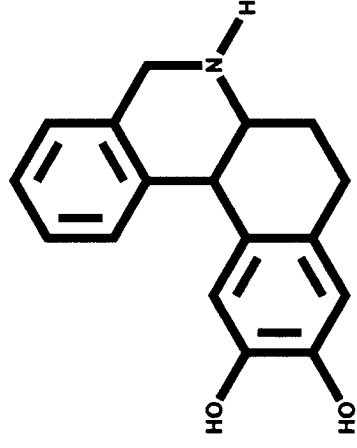
B. Phenylbenzazepine agonists

C. Inverse agonists (antipsychotics)

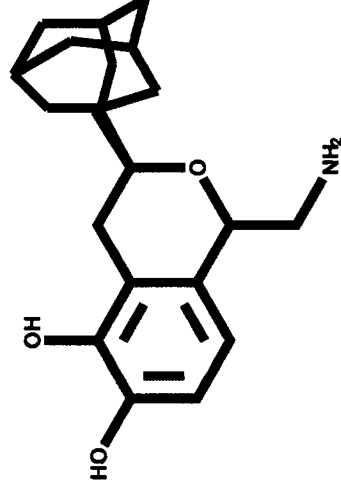
A.



Dopamine

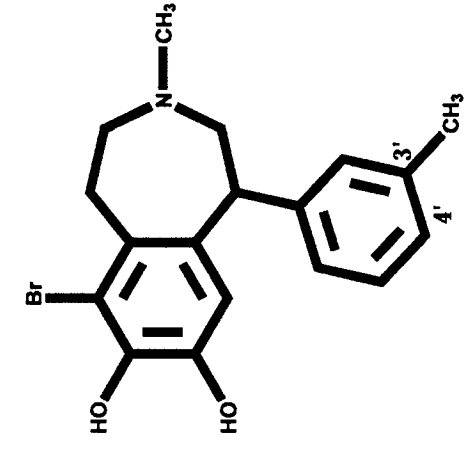


Dihydroxidine

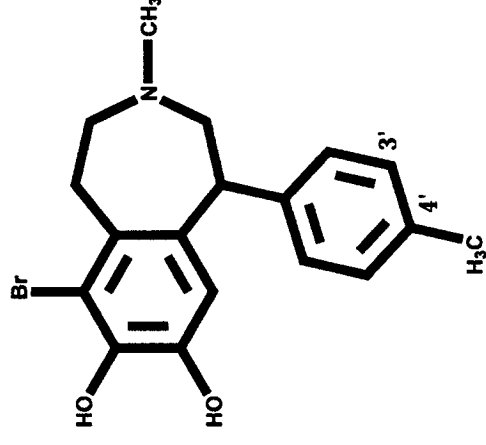


A77636

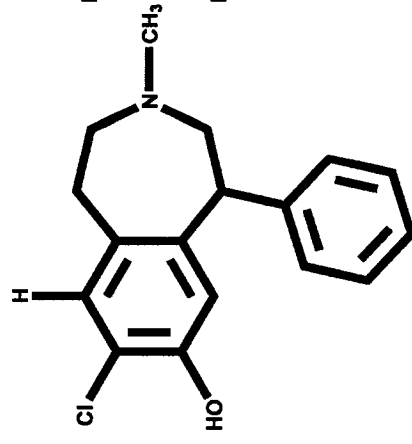
B.



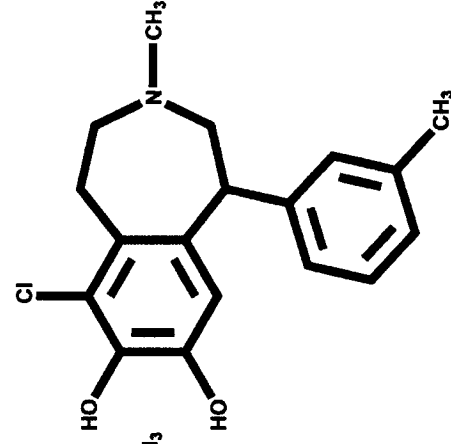
MCL 203



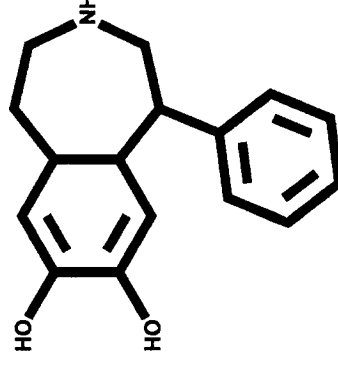
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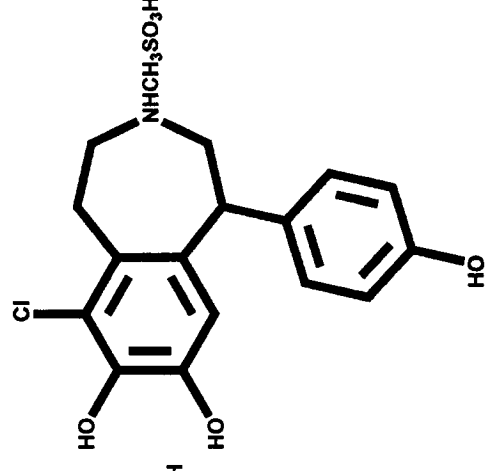
SCH23390



SKF83959



SKF38393



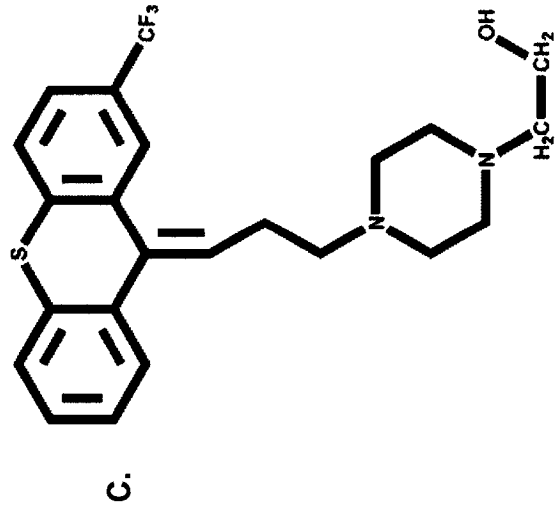
Fenoldopam

Figure 2: Two models of GPCR activation

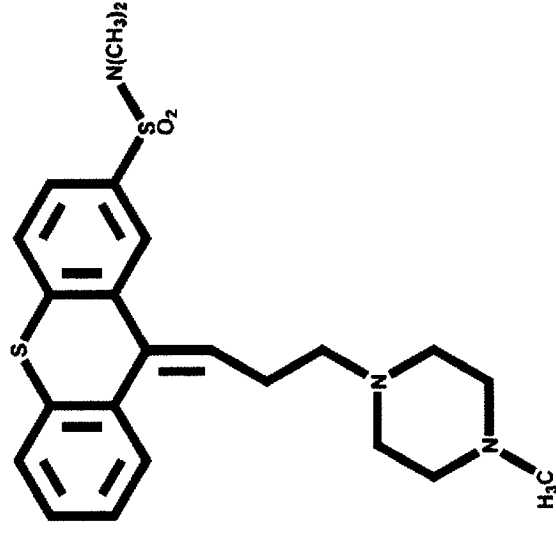
A. Extended ternary complex model. Assumes that only the active receptor R_a , bound or unbound by ligand A, interacts with G protein.

B. Cubic ternary complex model. Allows inactive receptor R_i to interact with G protein.

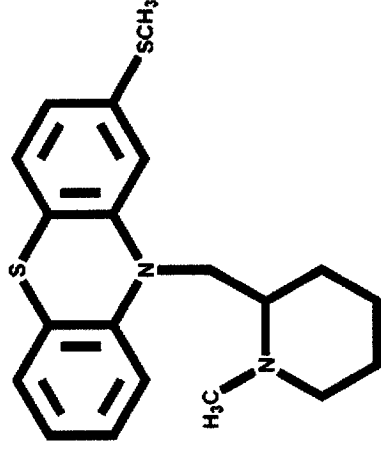
After Kenakin 1995 [73]



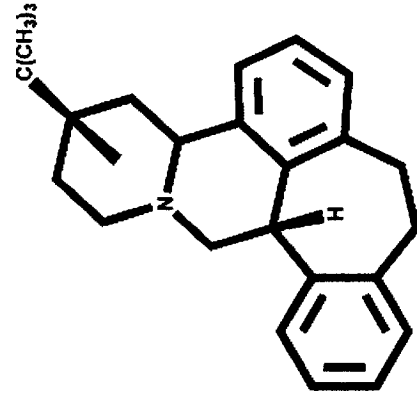
cis-Flupenthixol



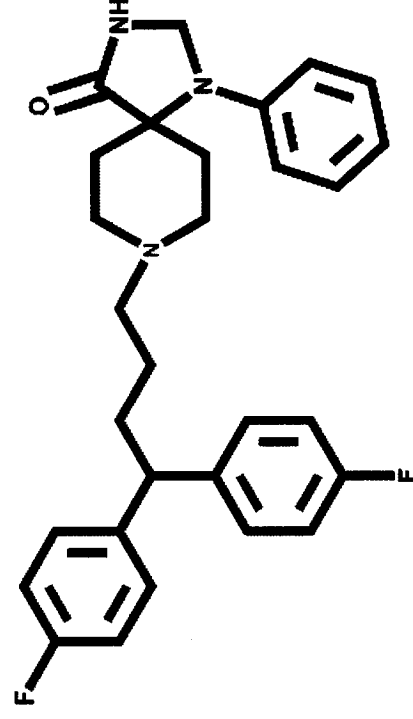
Thiothixene



Thioridazine



(+)-Butaclamol



Fluspirilene

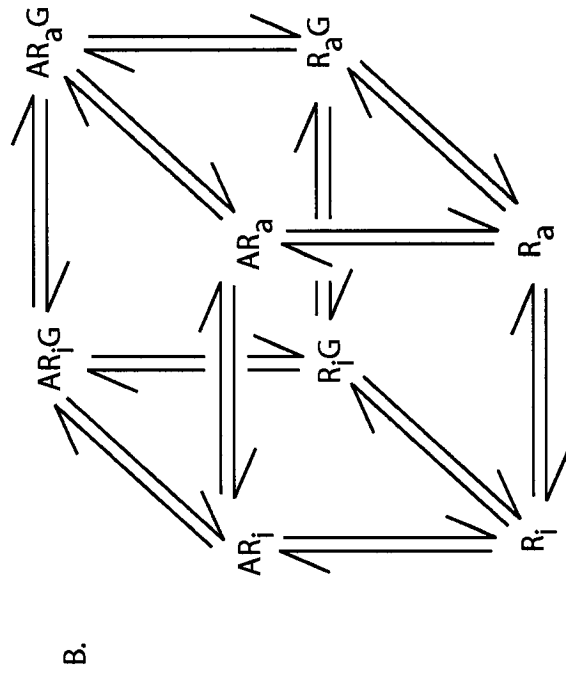
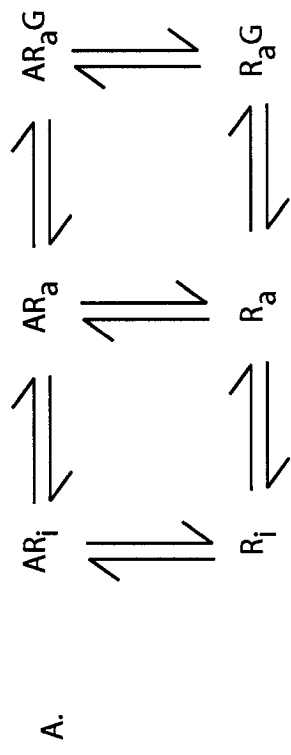
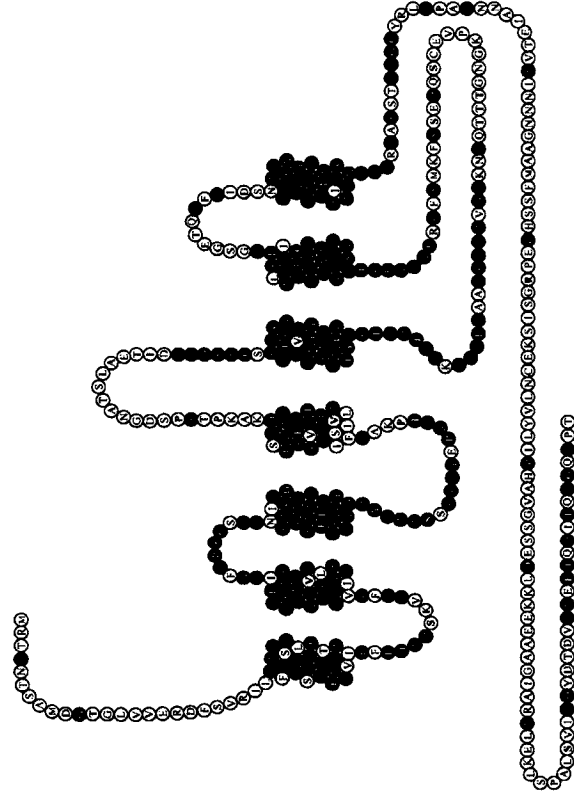


Figure 3: Primary and putative secondary structure of D1R and D5R
Black circles indicate conserved D1-like residues. White circles indicate divergent residues.

D1R



D5R

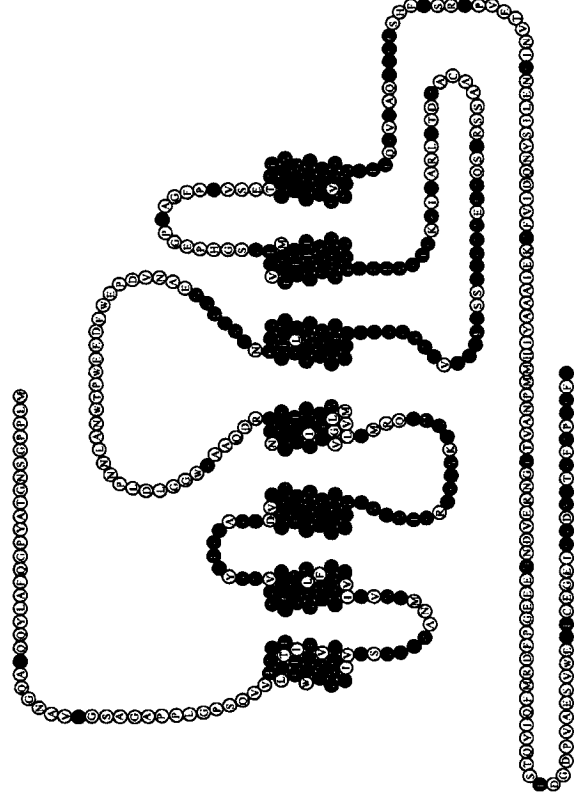


Figure 4: TM4/EL2 chimeric D1-like receptors

- A. Primary sequence of TM4, EL2, and TM5 of D1R and D5R. Asterisks indicate conserved residue.
- B. Putative secondary structure of wild-type and TM4/EL2 chimeric D1-like receptors. Black circles indicate D1R residues; red circles indicate D5R residues.

TM4

EL2

TM5

human D1R AAFILISVAWTLISVLI SFIPVQLSWHKAKPTS-----PSD-GNATSLAE-----TIDNCDSSLRSTYAISSSVISFYIPVAIMIVITYTRI

human D5R MALVMVGLAWTLISILISFIPVQLNWHRDQAASWGGLDLP MNLANWTPWEEPFWE PDVNAENCDS LNR TYAISSSVISFYIPVAIMIVITYTRI

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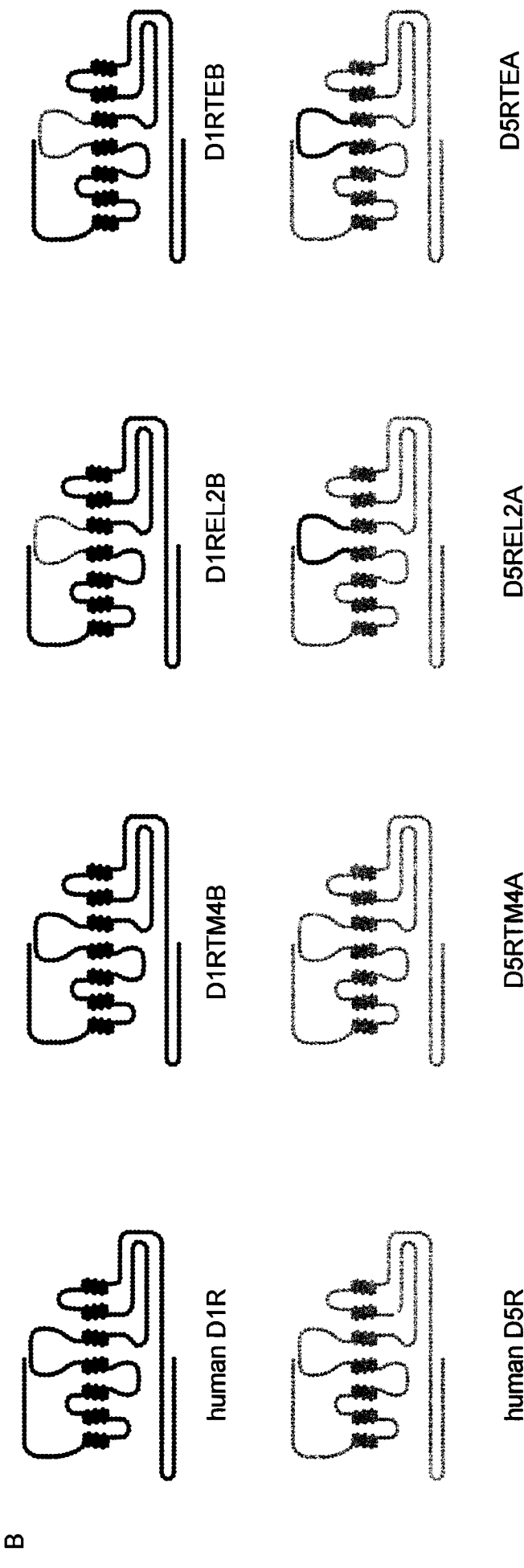


Figure 5: Schematic of chimera construction by overlap PCR

Blue indicates vector DNA; black indicates D1R DNA; red indicates D5R DNA;
semi-arrows indicate primers; italics indicate diagnostic restriction site.

A. Construction of D1RTM4B and D5RTM4A

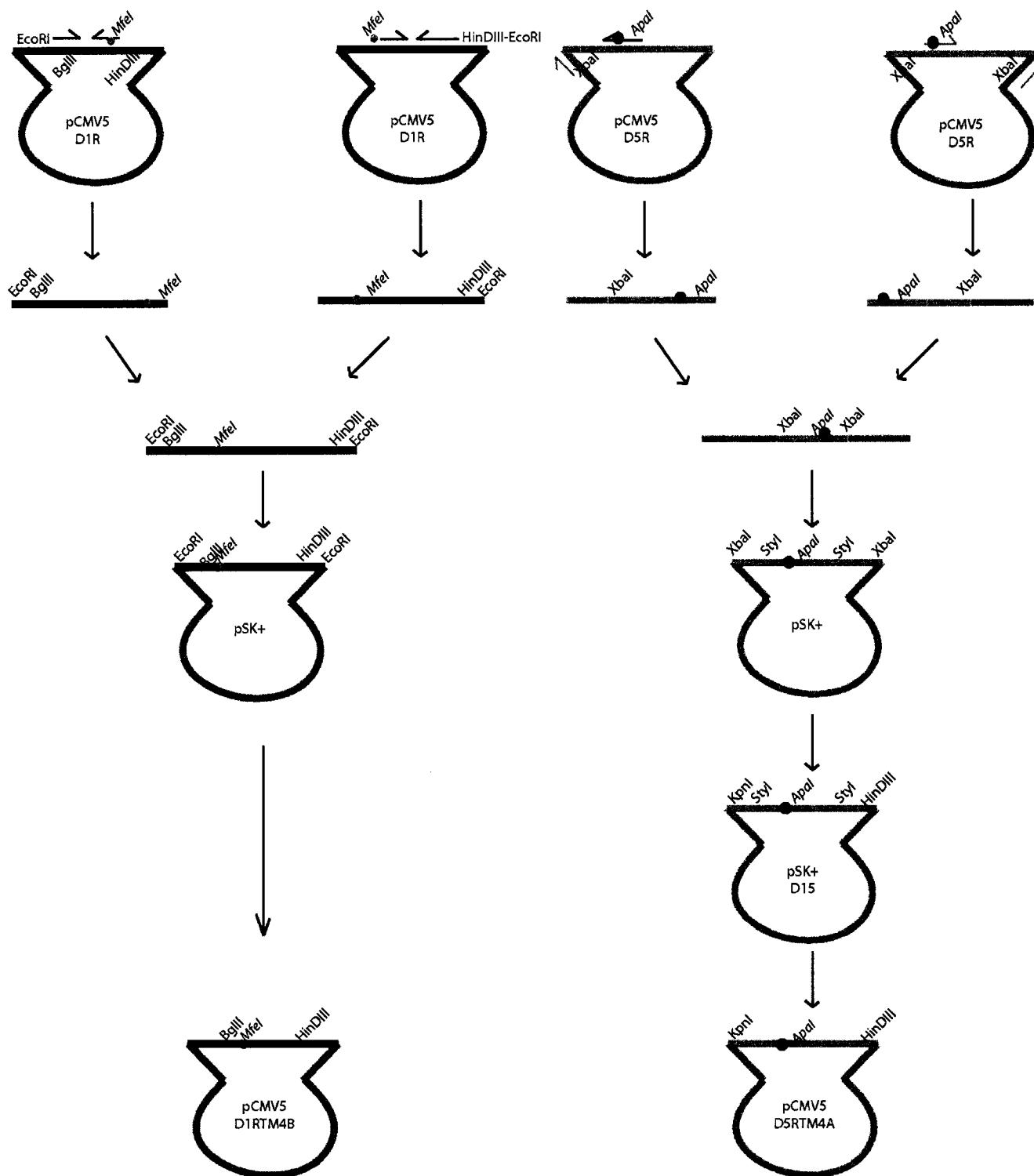
B. Construction of D1REL2B and D5REL2A

C. Construction of D1RTEB and D5RTEA

A.

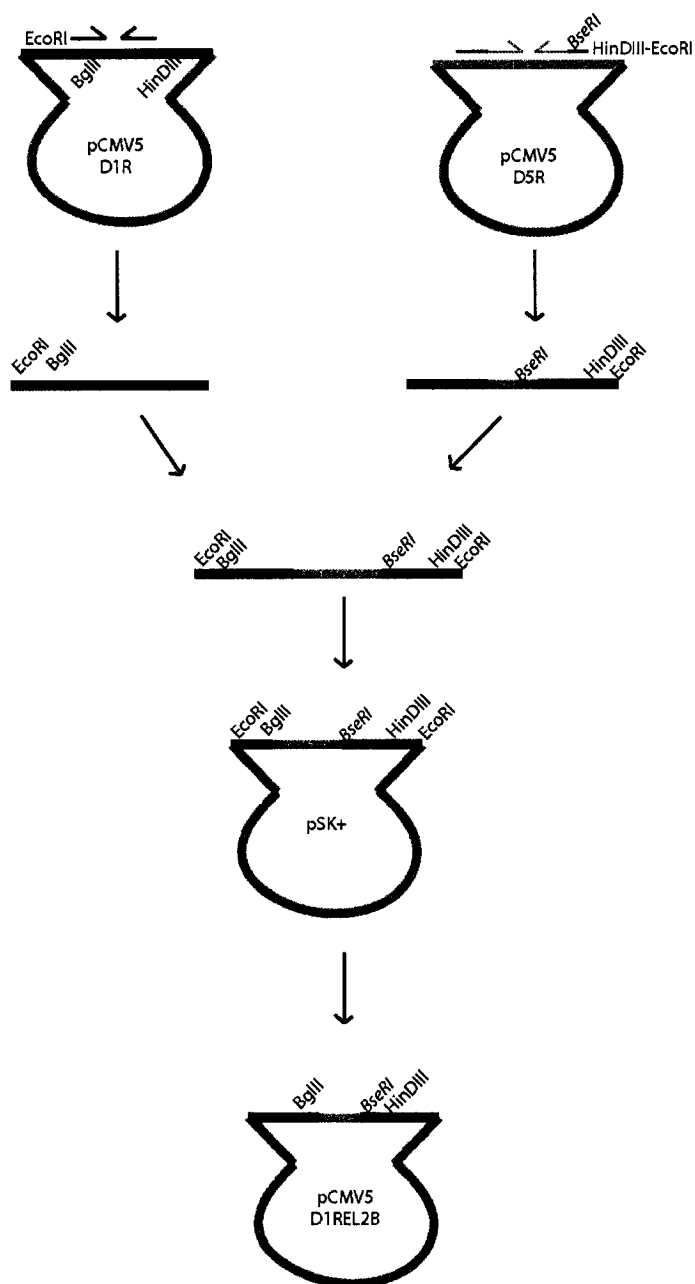
D1RTM4B

D5RTM4A

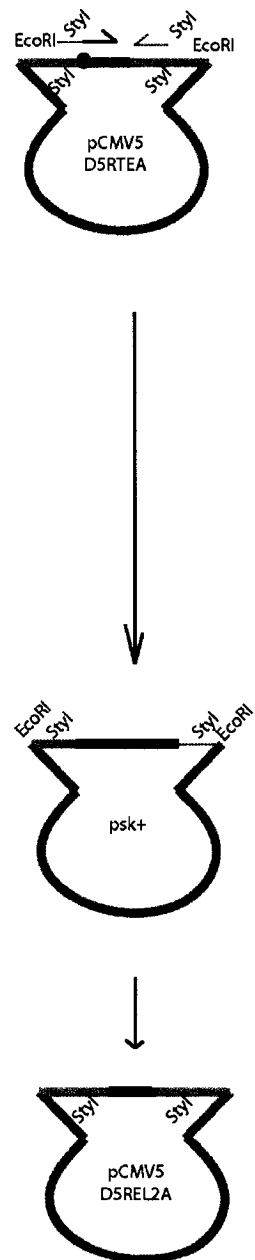


B.

D1REL2B

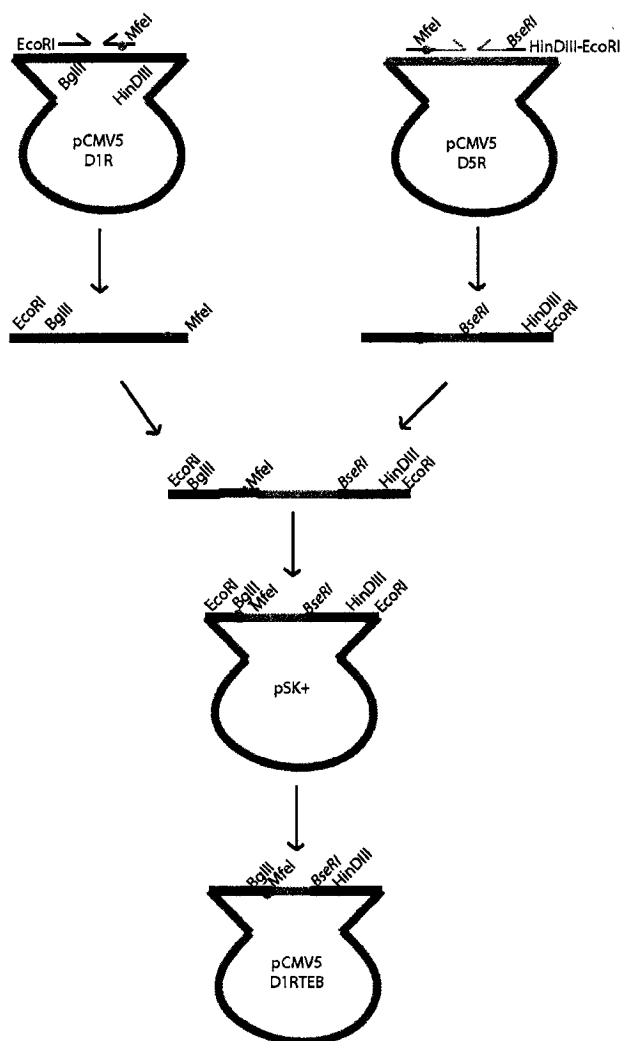


D5REL2A



C.

D1RTEB



D5RTEA

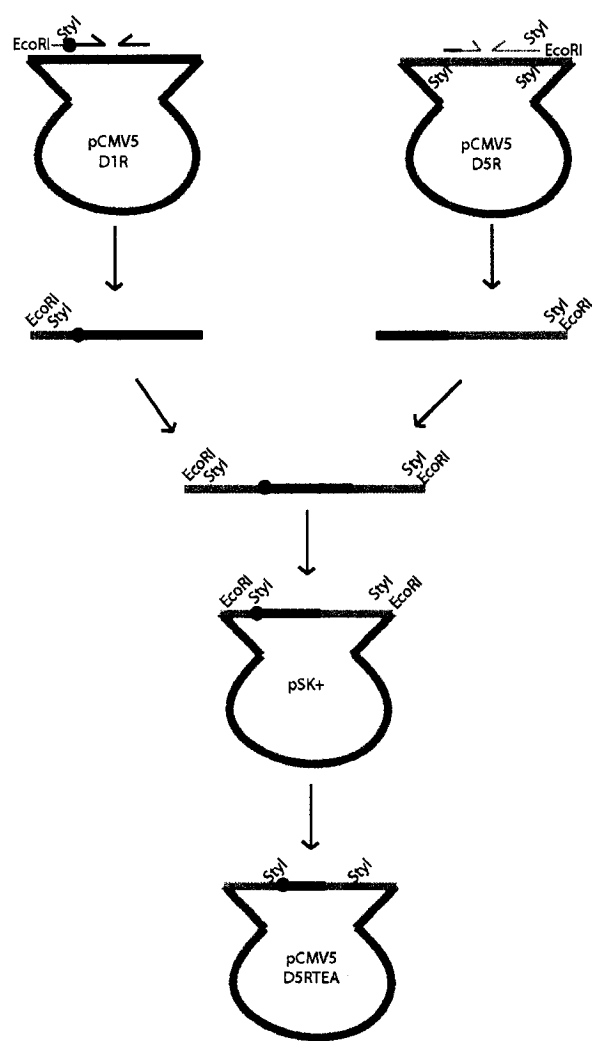


Figure 6: Representative saturation curves

NS represents non-specific binding.

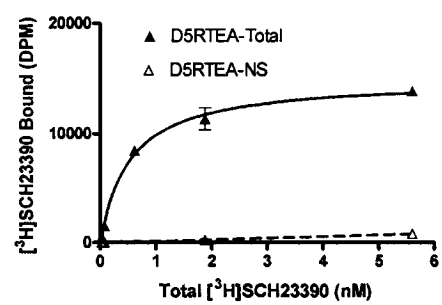
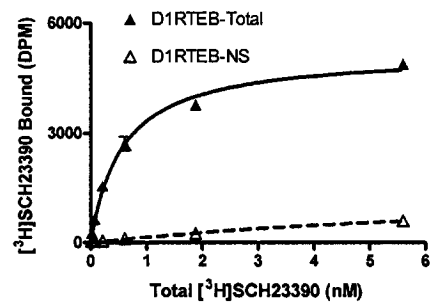
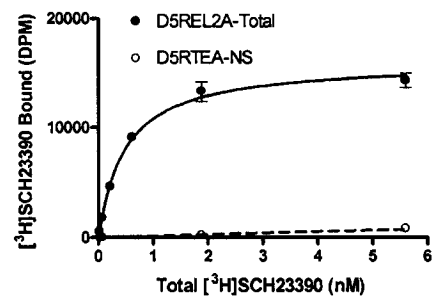
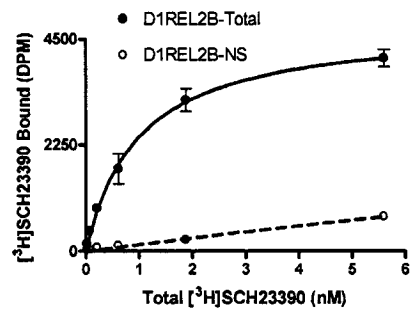
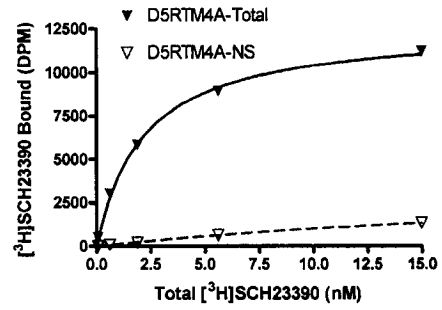
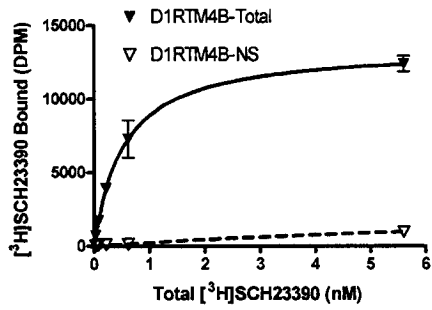
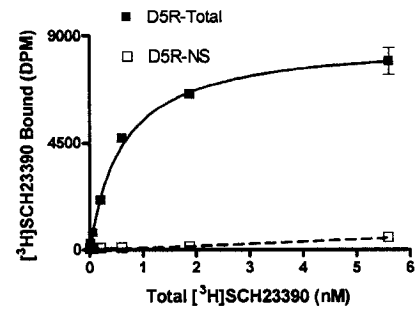
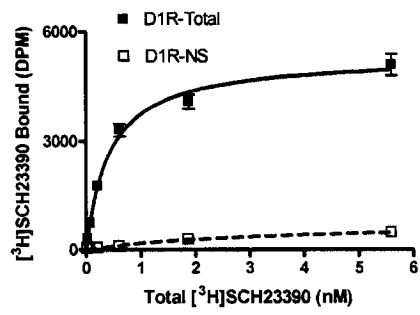


Figure 7: Representative competition curves (dopamine)

Dotted black and red lines indicate wild-type D1R and D5R curves, respectively.

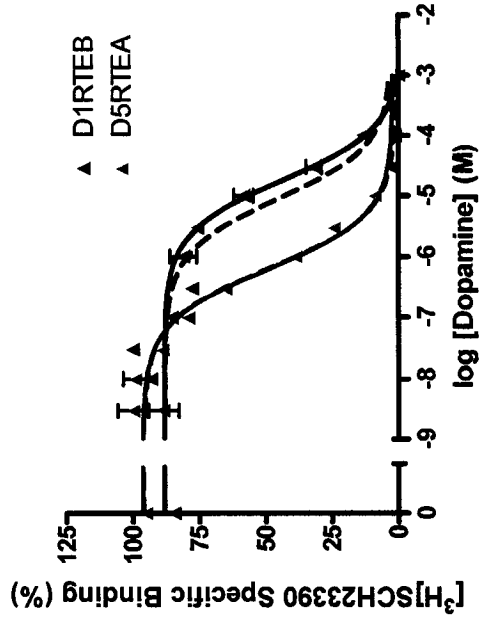
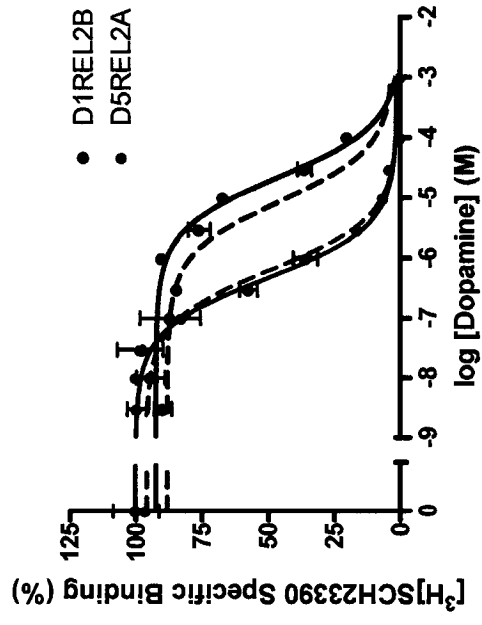
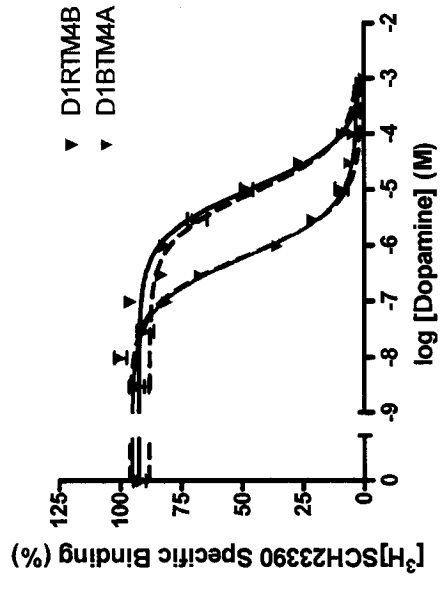
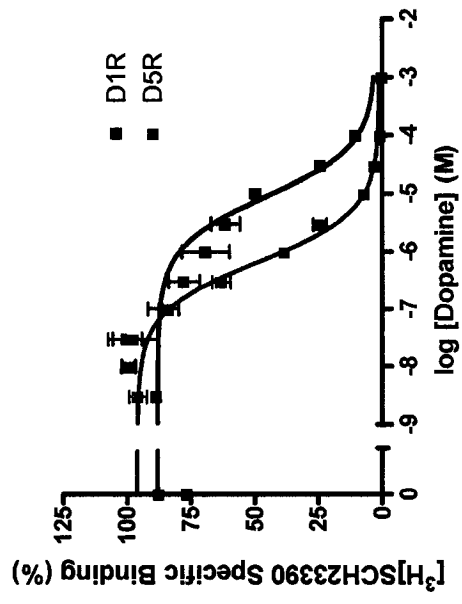
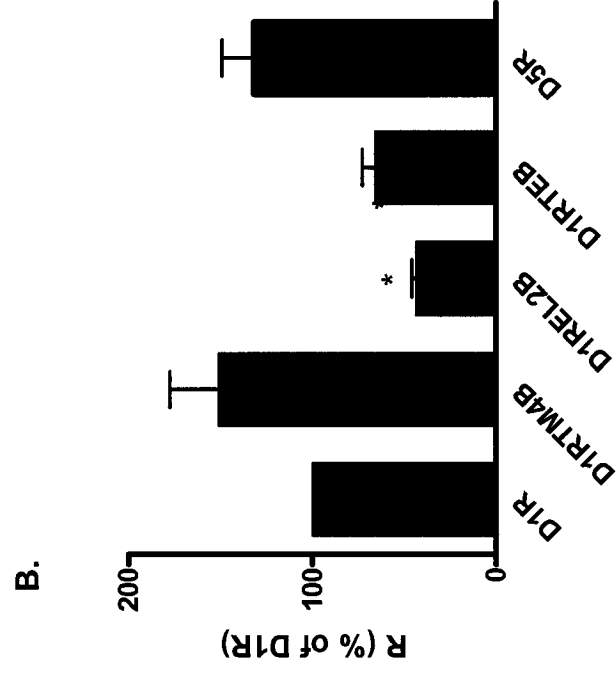
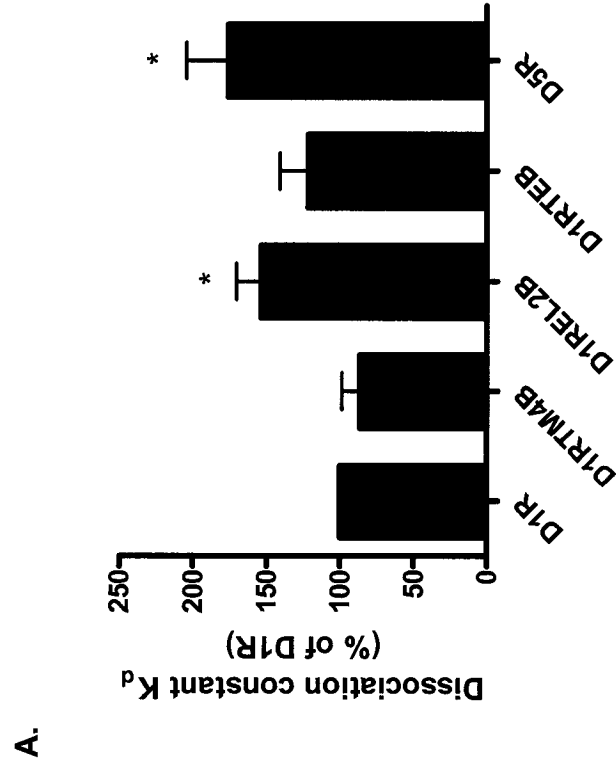


Figure 8: D1-like chimeric dissociation constants (A) and R values (B) as % of D1R
*, $p < 0.05$ relative to D1R.



**Figure 9: D5R-like chimeric dissociation constants (A) and R values (B) as % of D5R
#, $p < 0.05$ relative to D5R.**

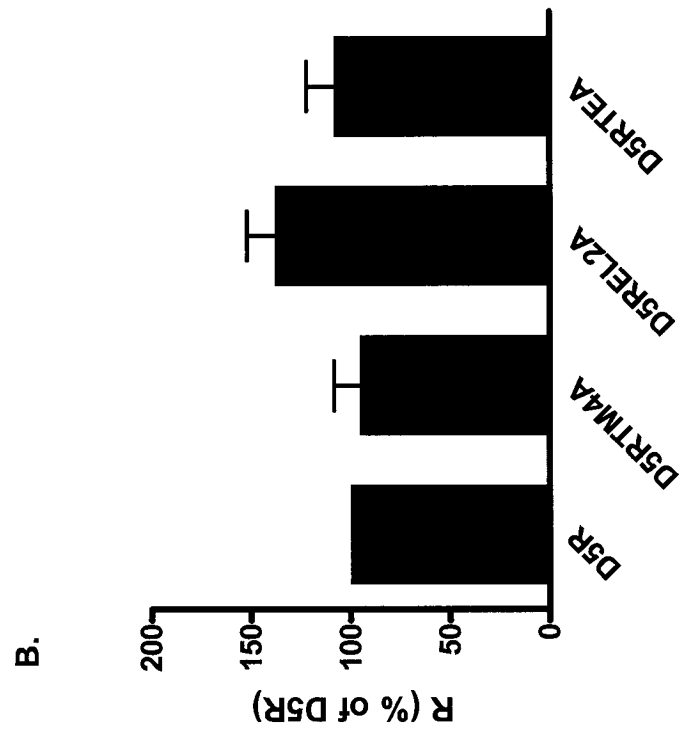
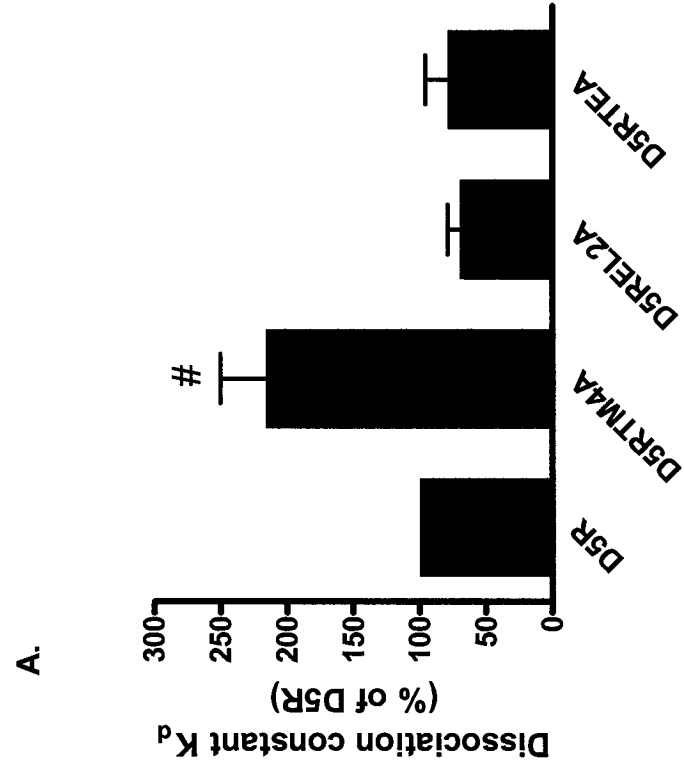


Figure 10: D1R-like chimeric non-phenylbenzazepine inhibition constants, K_i , as % of D1R
Expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. *, $p < 0.05$ relative to D1R.

- A. Dopamine
- B. Dihydroxidine
- C. A77636

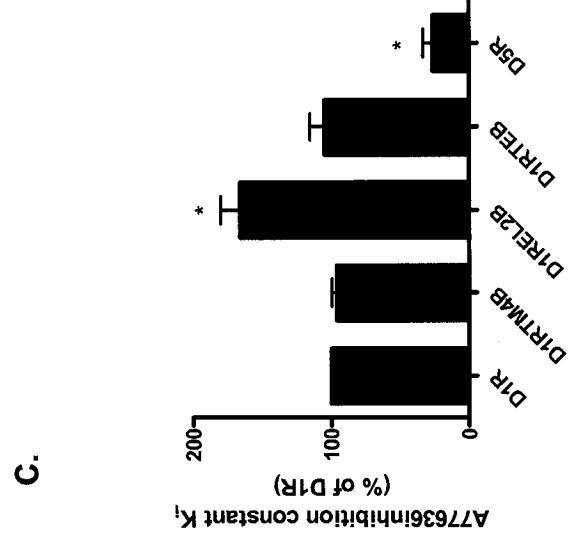
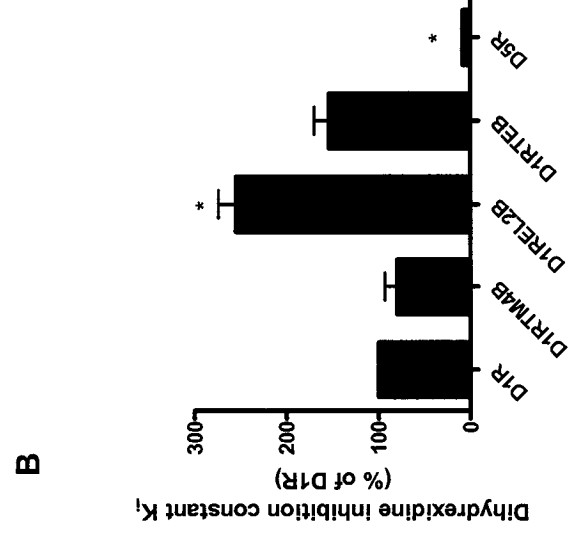
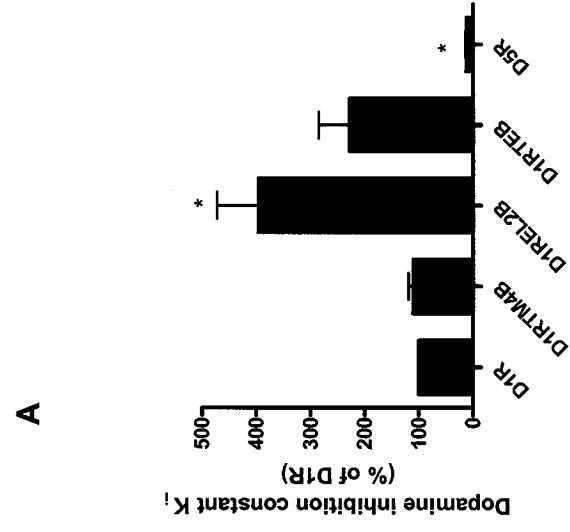


Figure 11: D5R-like chimeric non-phenylbenzazepine inhibition constants, K_i as % of D5R

Expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. #, $p < 0.05$ relative to D5R.

A. Dopamine

B. Dihydropyridine

C. A77636.

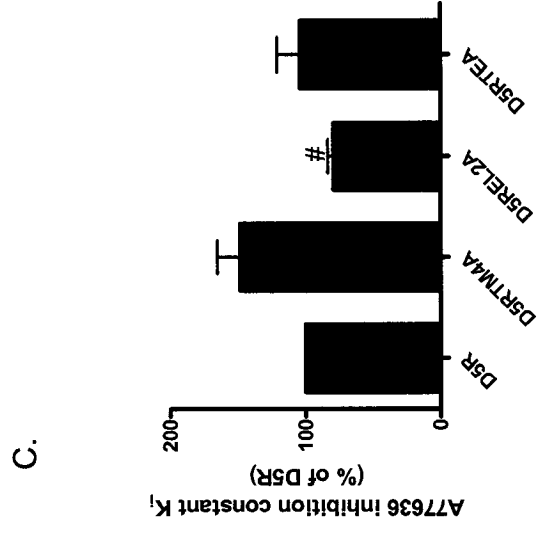
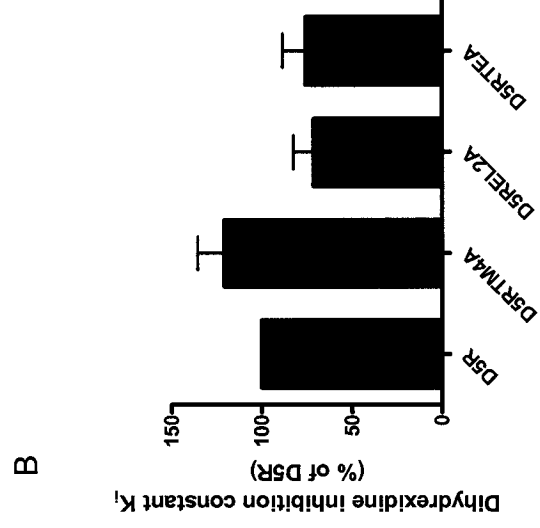
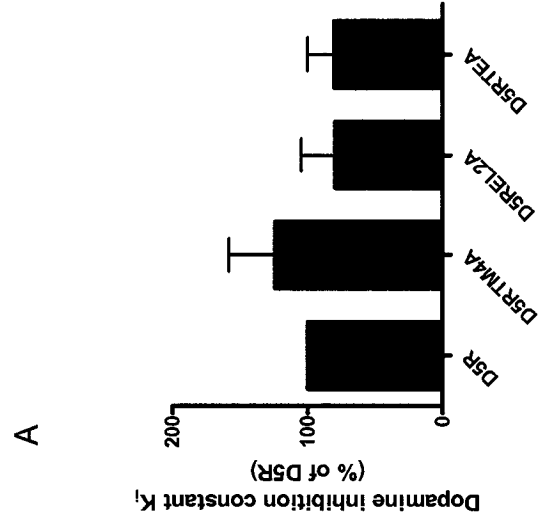
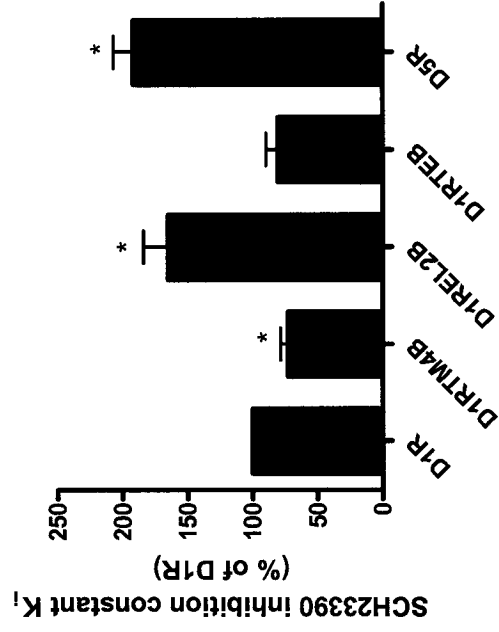


Figure 12: D1R-like chimeric phenylbenzazepine inhibition constants, K_i , as % of D1R

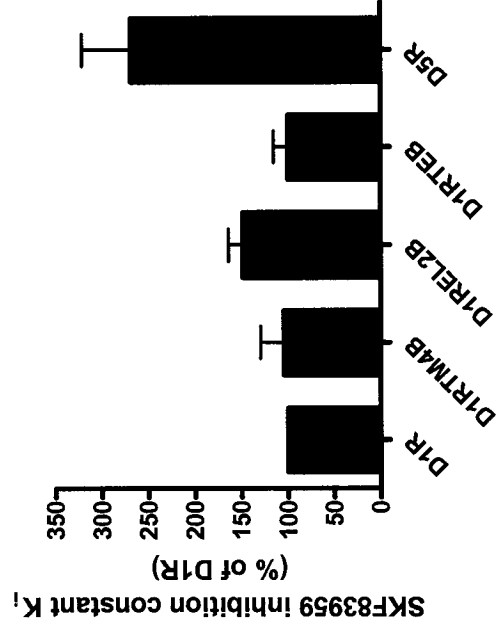
Expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. *, $p < 0.05$ relative to D1R.

- A. SCH23390
- B. SKF83959
- C. SKF38393
- D. Fenoldopam.

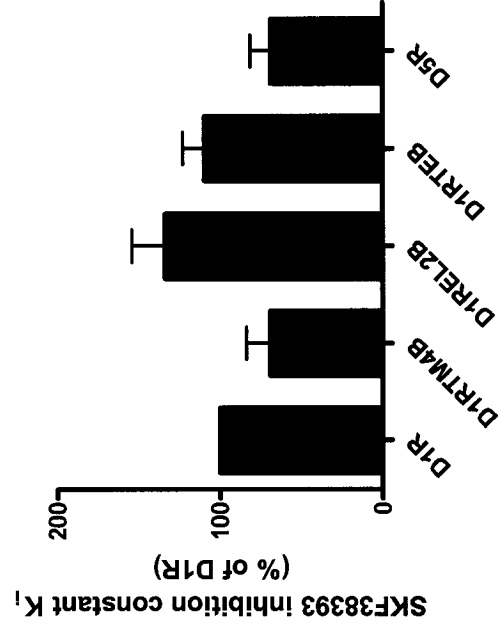
A.



B.



C.



D.

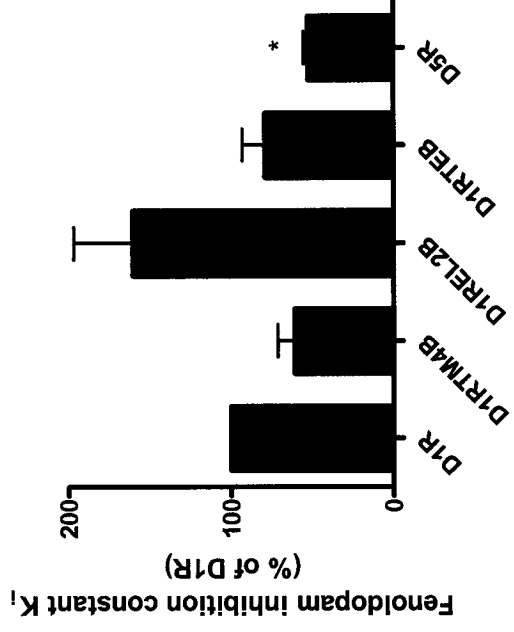


Figure 13: D5R-like chimeric phenylbenzazepine inhibition constants, K_i , as % of D5R

Expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. #, $p < 0.05$ relative to D5R.

- A. SCH23390
- B. SKF83959
- C. SKF38393
- D. Fenoldopam.

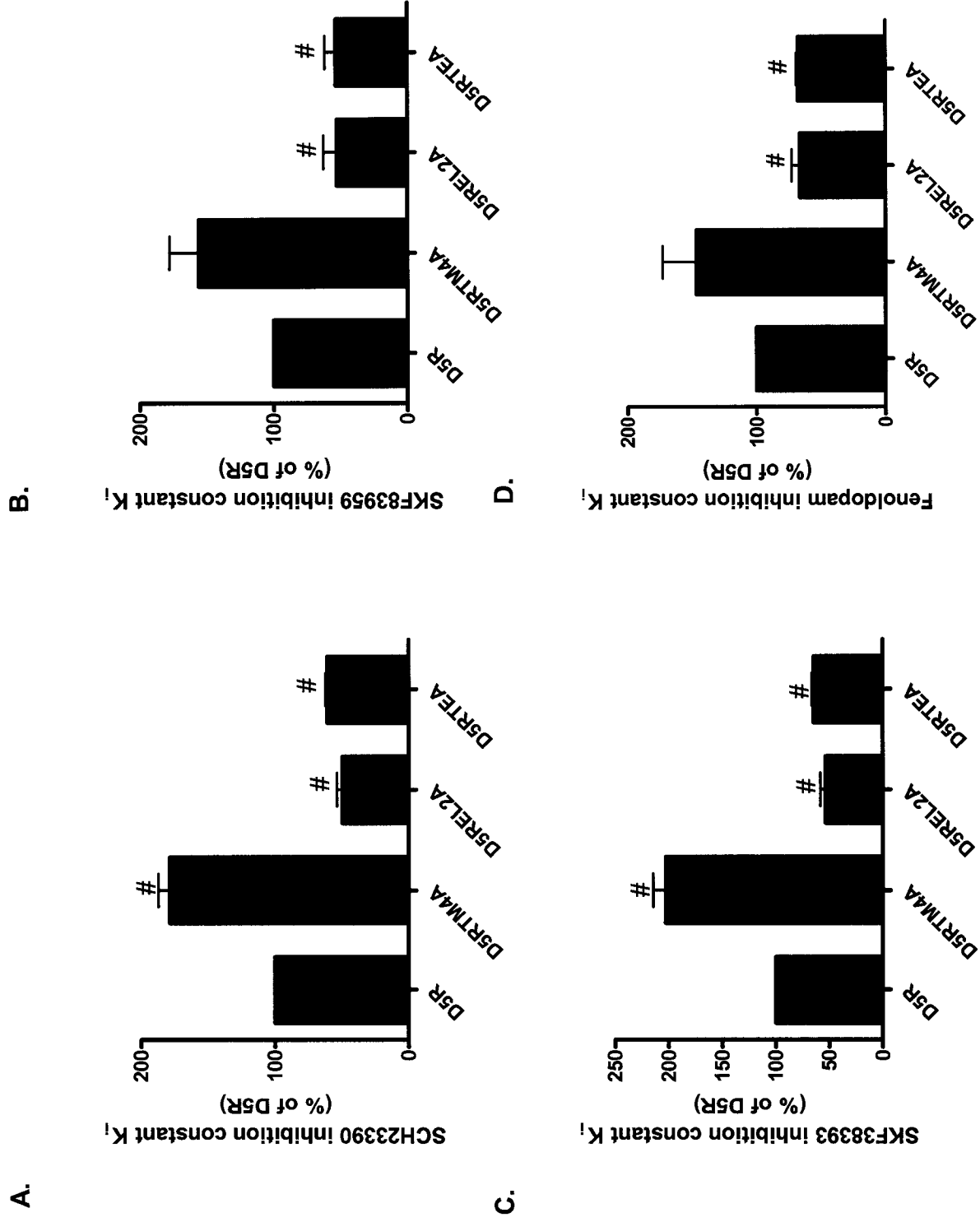


Figure 14: D1R-like chimeric inverse agonist inhibition constants, K_i , as % of D1R

Expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. *, $p < 0.05$ relative to D1R.

- A. Flupenthixol
- B. Thiothixene.
- C. Thioridazine
- D. Fluspirilene
- E. Butaclamol.

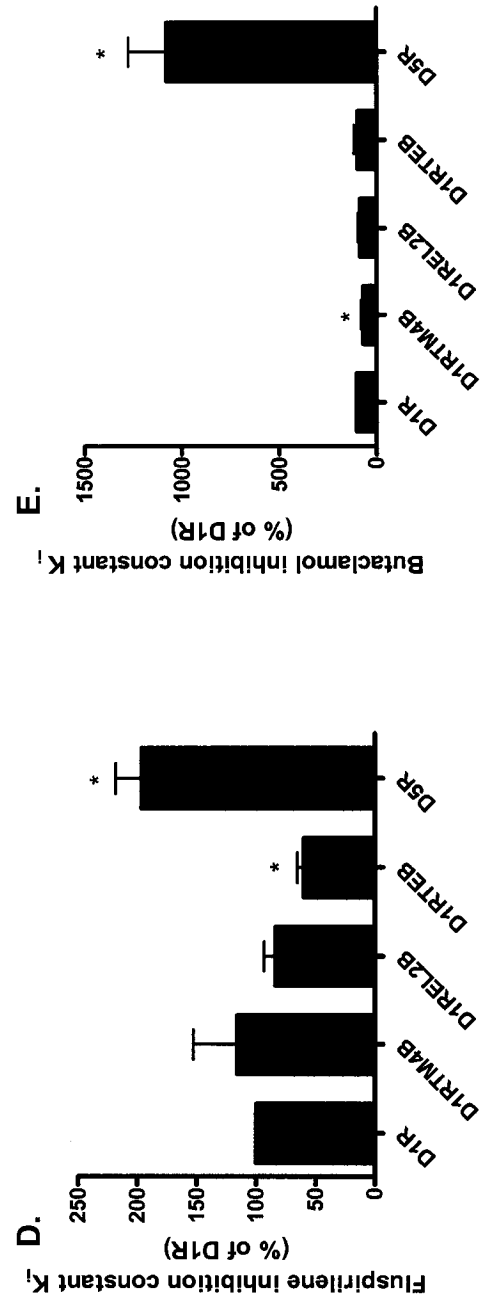
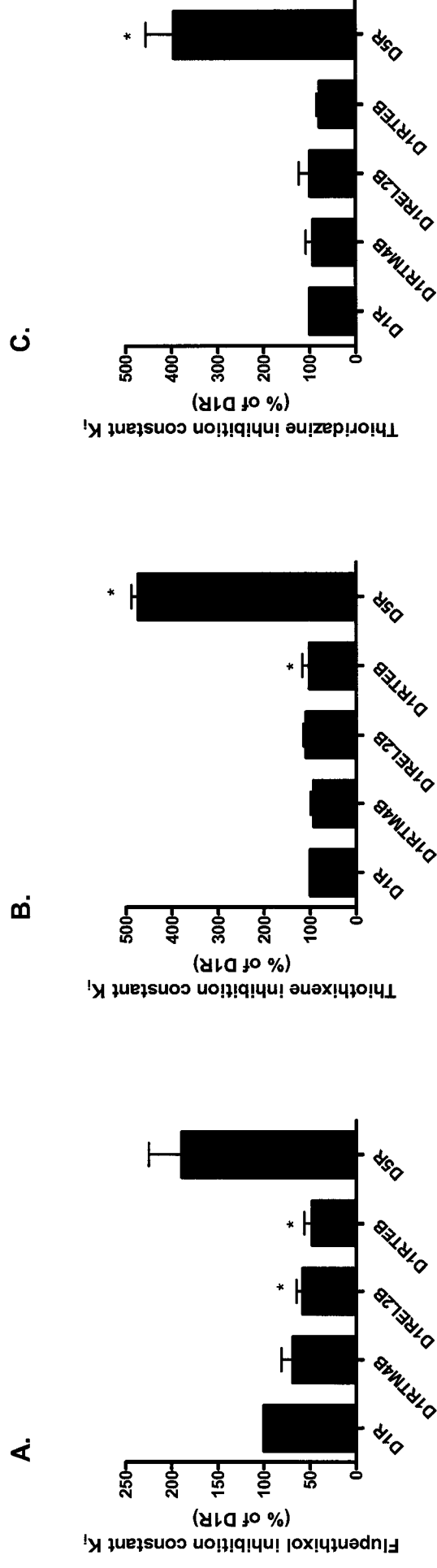


Figure 15: D5R-like chimeric inverse agonist inhibition constants, K_i , as % of D5R

Expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. #, $p < 0.05$ relative to D5R.

- A. Flupenthixol
- B. Thiothixene
- C. Thioridazine
- D. Fluspirilene
- E. Butaclamol.

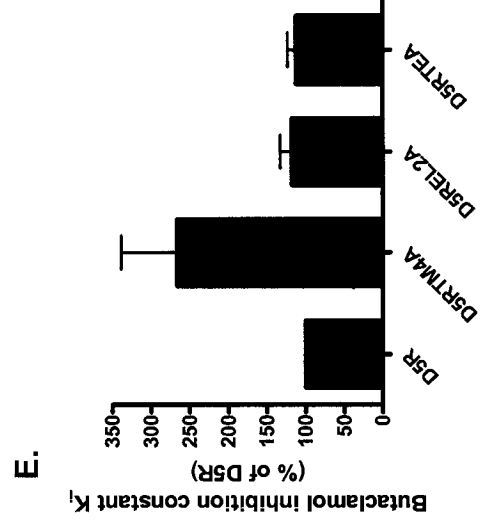
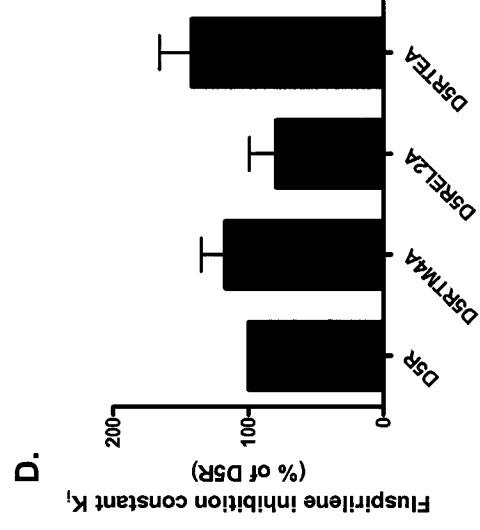
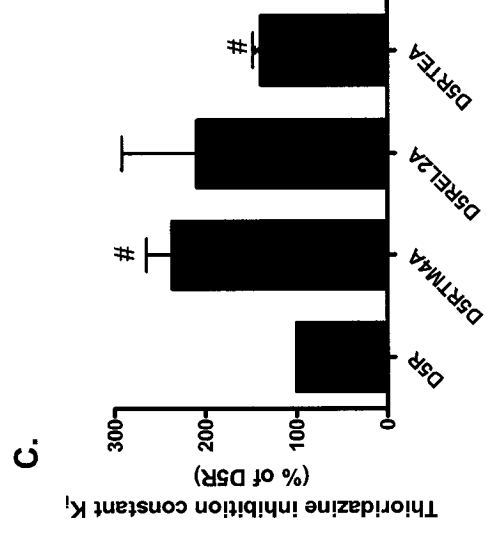
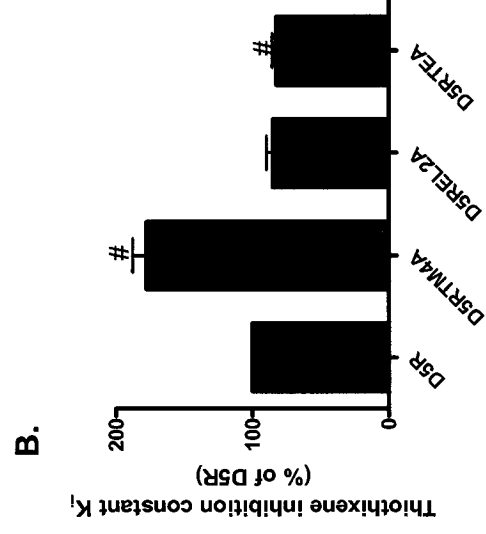
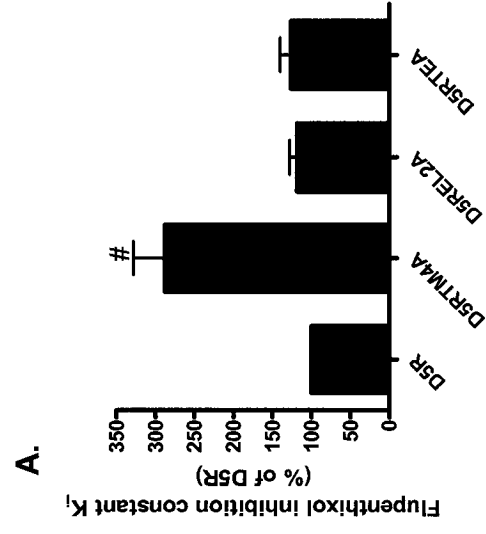


Figure 16: Agonist-independent (constitutive) activation of wild-type and chimeric D1-like receptors expressed in HEK 293 cells

Constitutive activation of AC expressed as the arithmetic means $\pm$ S.E. of 3 experiments done in triplicate determinations in 6-well dishes. One-way ANOVA with Tukey post-test determined statistical significance.

*, $p < 0.05$ relative to D1R; #, $p < 0.05$ relative to D5R.

A. Representative example of a constitutive activation experiment. The R values expressed in pmol/mg protein were as follows: 0.99 (D1R), 1.39 (D5R), 0.68 (D1RTM4B), 0.56 (D1REL2B), 0.63 (D1RTEB), 3.19 (D5RTM4A), 0.70 (D5REL2A), 0.92 (D5RTEA),

B. Constitutive activity values corrected for R and relative to D1R. The constitutive activity values were as follows: 1 (D1R), 2.46 ± 0.18 (D5R), 1.17 ± 0.12 (D1RTM4B), 1.16 ± 0.14 (D1REL2B), 1.06 ± 0.09 (D1RTEB), 4.70 ± 0.36 (D5RTM4A), 2.49 ± 0.196 (D5REL2A), 3.00 ± 0.22 (D5RTEA).

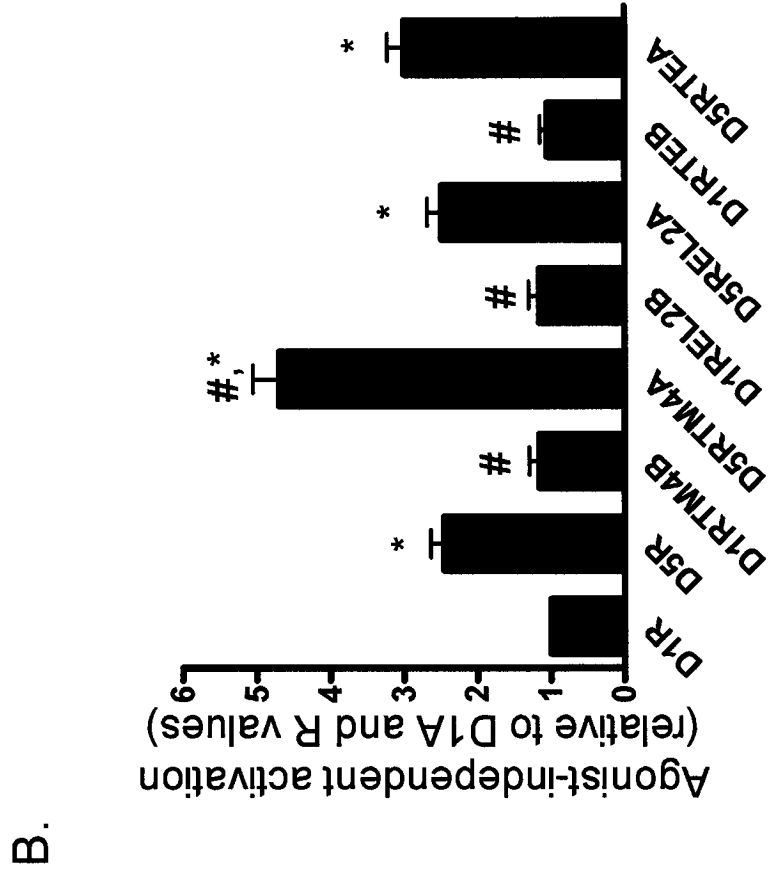
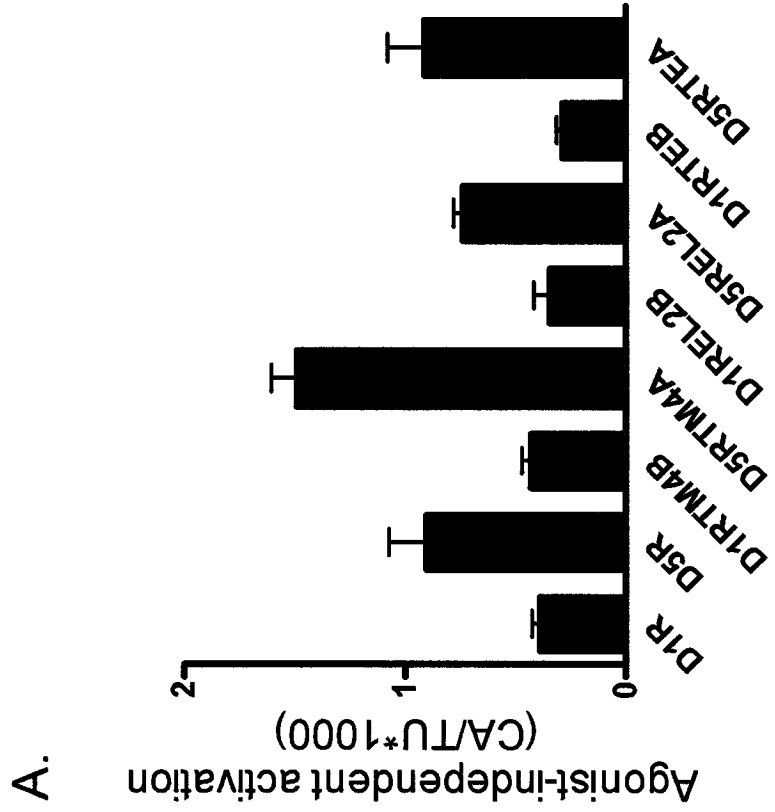


Figure 17: Dopamine mediated maximal stimulation of cAMP accumulation by wild-type and chimeric receptors

HEK 293 cells were transfected with wild-type or chimeric receptor expression constructs per dish, to achieve equal expression. Maximal activation values at 10 μ M dopamine were determined from dose-response curves carried out in 12-well dishes, and are expressed as the arithmetic mean $\pm$ S.E. of 4 experiments carried out in triplicate. *, $p < 0.05$ relative to D1R; #, $p < 0.05$ relative to D5R. The R values of N-[methyl- 3 H]SCH23390 in pmol/mg of membrane proteins (expressed as arithmetic mean $\pm$ S.E.) were as follows: 2.72 $\pm$ 0.52 (D1R), 3.56 $\pm$ 0.45 (D5R), 2.84 $\pm$ 0.42 (D1RTM4B), 3.60 $\pm$ 0.52 (D5RTM4A), 2.71 $\pm$ 0.25 (D1REL2B), 3.37 $\pm$ 3.88 $\pm$ 0.3 (D5REL2A), 3.37 $\pm$ 0.89 (D1RTEB), and 4.23 $\pm$ 0.77 (D5RTEA).

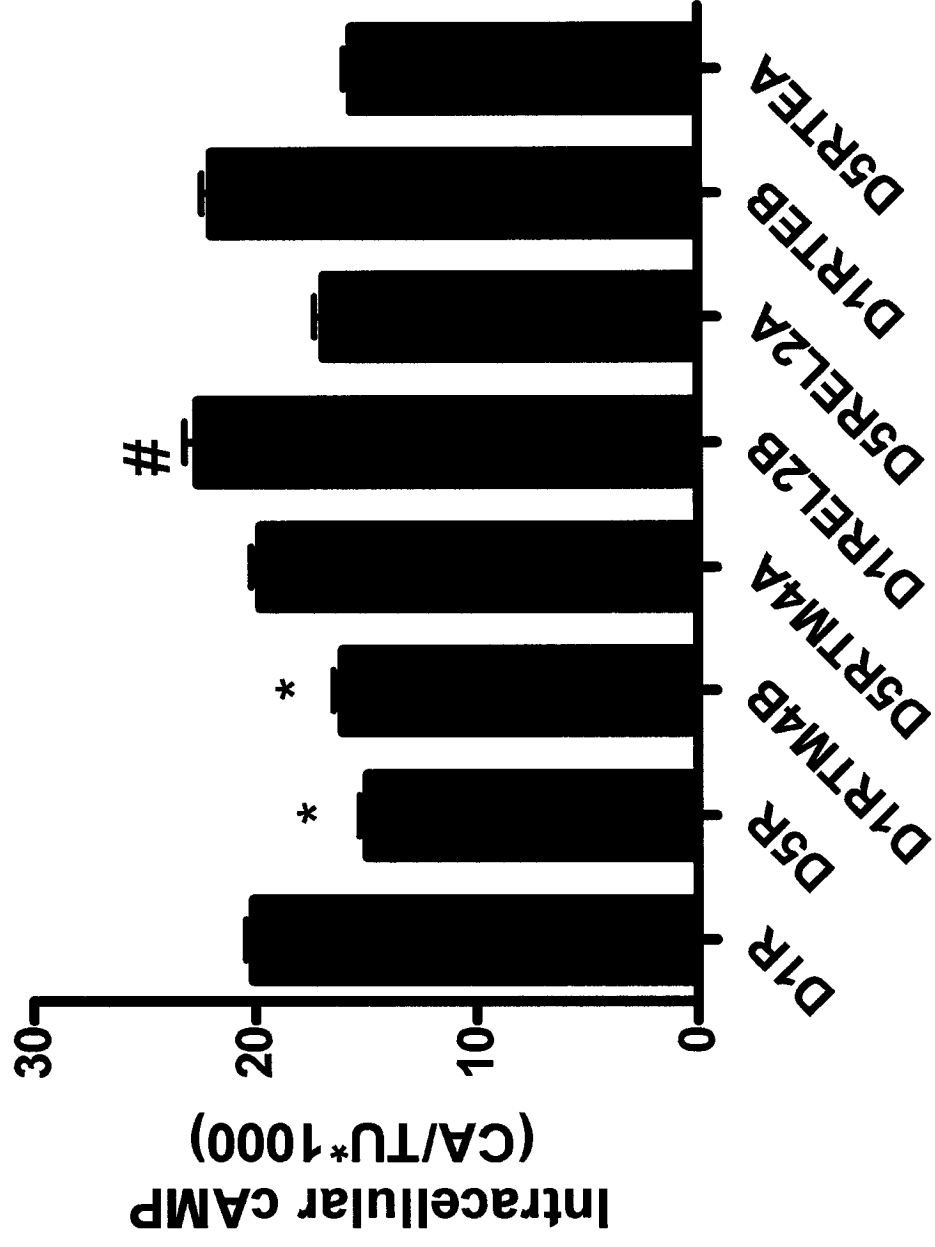


Figure 18: Dihydropyridine mediated maximal stimulation of cAMP accumulation by wild-type and chimeric receptors

HEK 293 cells were transfected with wild-type or chimeric receptor expression constructs per dish, to achieve equal expression. Maximal activation values at 10 μ M dihydropyridine were determined from dose-response curves carried out in 12-well dishes, and are expressed as the arithmetic mean $\pm$ S.E. of 4 experiments carried out in triplicate. *, $p < 0.05$ relative to D1R; #, $p < 0.05$ relative to D5R. The R values of N-[methyl- 3 H]SCH23390 in pmol/mg of membrane proteins (expressed as arithmetic mean $\pm$ S.E.) were as follows: 2.91 $\pm$ 0.68 (D1R), 2.21 $\pm$ 0.4 (D5R), 3.68 $\pm$ 1.27 (D1RTM4B), 1.99 $\pm$ 0.40 (D5RTM4A), 2.23 $\pm$ 0.52 (D1REL2B), 2.03 $\pm$ 0.41 (D5REL2A), , 2.68 $\pm$ 0.46 (D1RTEB), and 3.25 $\pm$ 0.77 (D5RTEA).

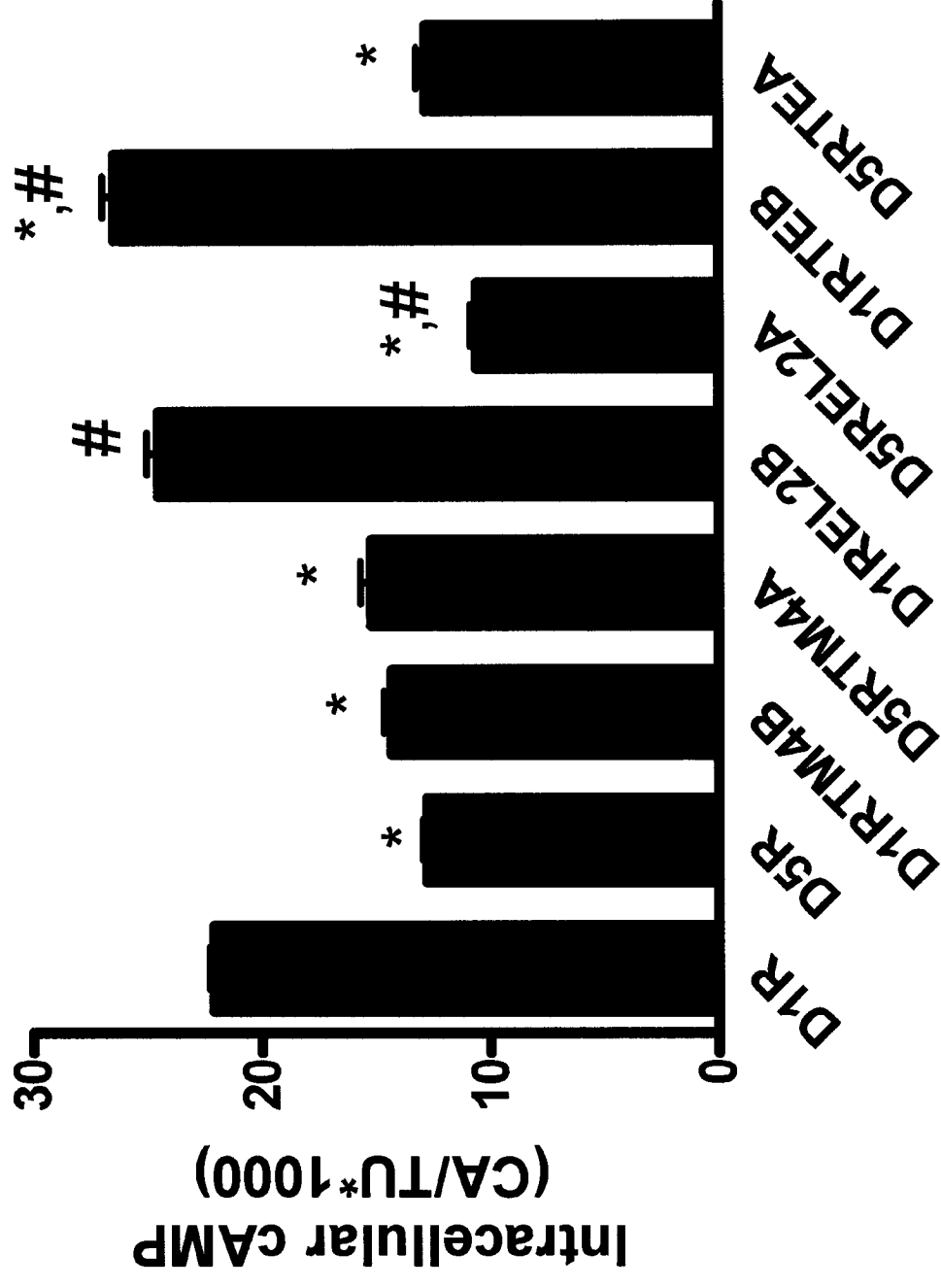


Figure 19: A77636 mediated maximal stimulation of cAMP accumulation by wild-type and chimeric receptors

HEK 293 cells were transfected with wild-type or chimeric receptor expression constructs per dish, to achieve equal expression. Maximal activation values at 10 μ M A77636 were determined from experiments carried out in 6-well dishes, and are expressed as the arithmetic mean $\pm$ S.E. of 2 experiments carried out in triplicate. *, $p < 0.05$ relative to D1R; #, $p < 0.05$ relative to D5R. The R values of [3 H]SCH23390 in pmol/mg of membrane proteins (expressed as arithmetic mean $\pm$ S.E.) were 0.93 $\pm$ 0.69 (D1R), 1.03 $\pm$ 0.75 (D5R), 0.7 $\pm$ 0.42 (D1RTM4B), 1.10 $\pm$ 0.87 (D5RTM4A), 0.88 $\pm$ 0.45 (D1REL2B), 0.93 $\pm$ 0.35 (D5REL2A), 1.40 $\pm$ 1.01 (D1RTEA), and 0.49 $\pm$ 0.97 (D5RTEA).

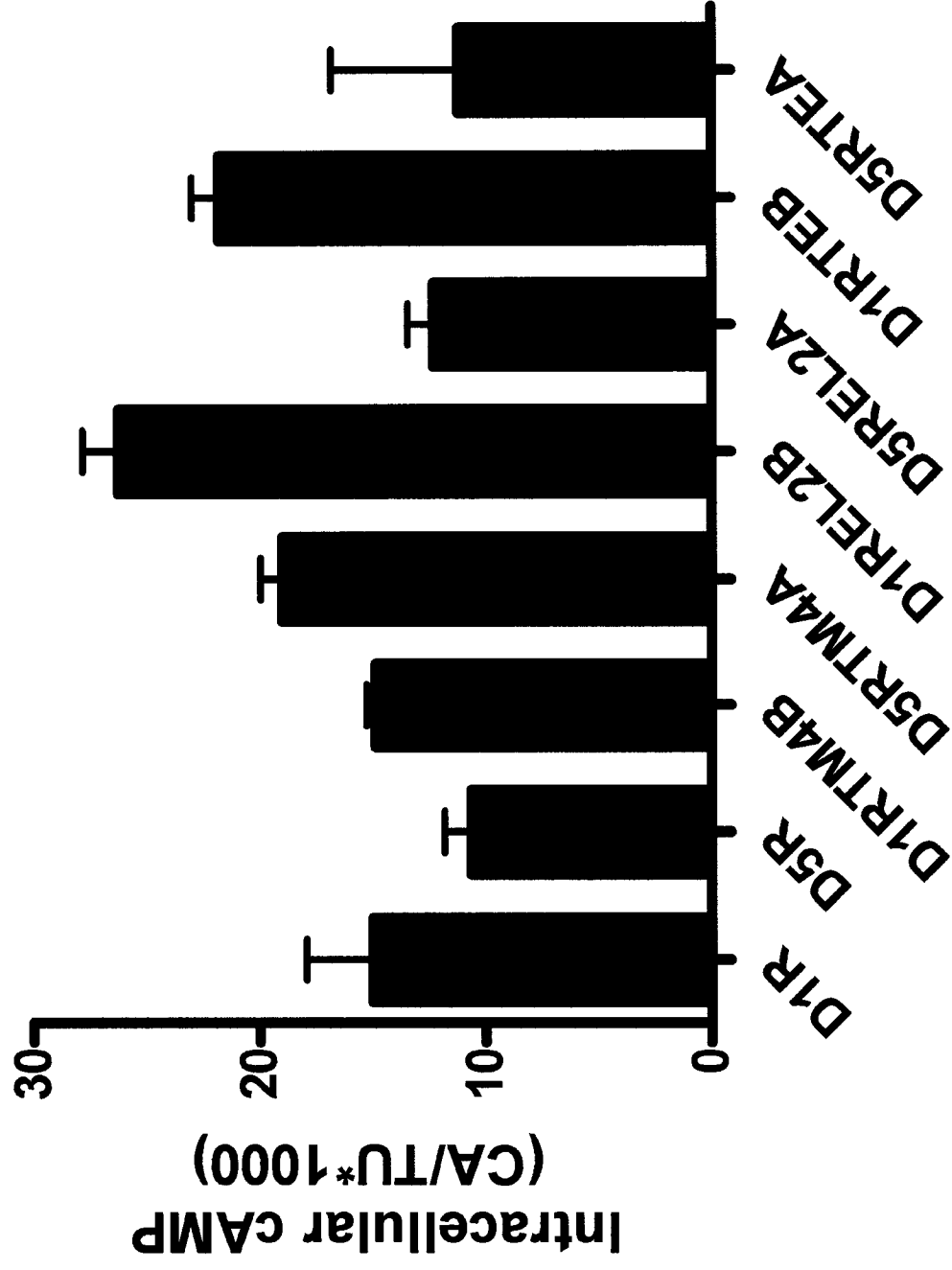


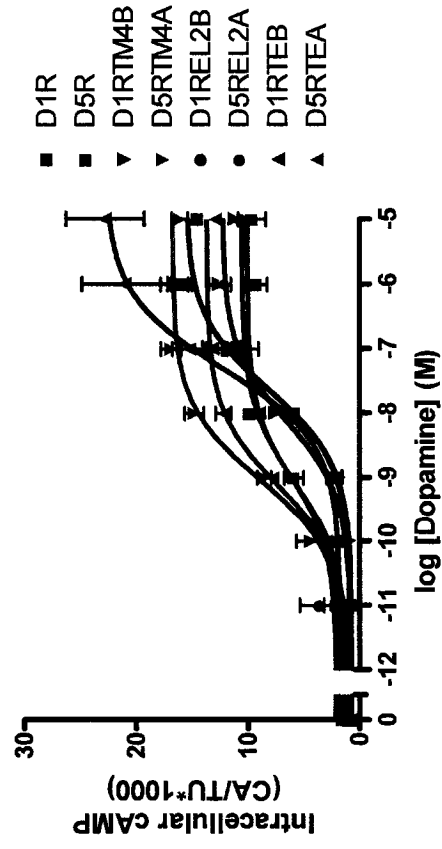
Figure 20: Dose-response curves for dopamine- and dihydrexidine-mediated stimulation of cAMP accumulation by wild-type and chimeric receptors

Expressed as [^3H]cAMP (CA) over the total amount of [^3H]adenine uptake (TU) $\times$ 1000. Average curves of 3-4 experiments were fit using Graphpad Prism V.4.0.

A. HEK 293 cells were transfected with DNA at concentrations providing the following receptor expression in pmol/mg membrane protein: 2.72 ± 0.52 (D1R), 3.56 ± 0.45 (D5R), 2.84 ± 0.42 (D1RTM4B), 3.60 ± 0.52 (D5RTM4A), 2.71 ± 0.25 (D1REL2B), 3.37 ± 0.3 (D5REL2A), 3.37 ± 0.89 (D1RTEB), and 4.23 ± 0.77 (D5RTEA). As described under "Materials and Methods", intracellular cAMP levels were measured in triplicate in 12-well dishes in the absence or presence of increasing concentrations of dopamine.

B. HEK 293 cells were transfected with DNA at concentrations providing the following receptor expression in pmol/mg membrane protein: 1.98 ± 0.45 (D1R), 1.85 ± 0.51 (D5R), 2.06 ± 1.01 (D1RTM4B), 1.67 ± 0.513 (D5RTM4A), 1.58 ± 0.5 (D1REL2B), 1.52 ± 0.39 (D5REL2A), 2.08 ± 0.4 (D1RTEB), 2.50 ± 0.92 (D5RTEA). As described under "Materials and Methods", intracellular cAMP levels were measured in triplicate in 12-well dishes in the absence or presence of increasing concentrations of dihydrexidine.

A.



B.

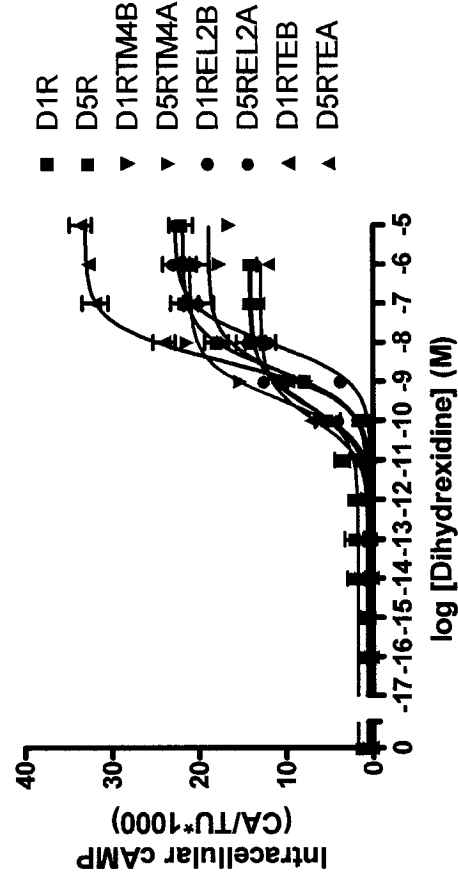
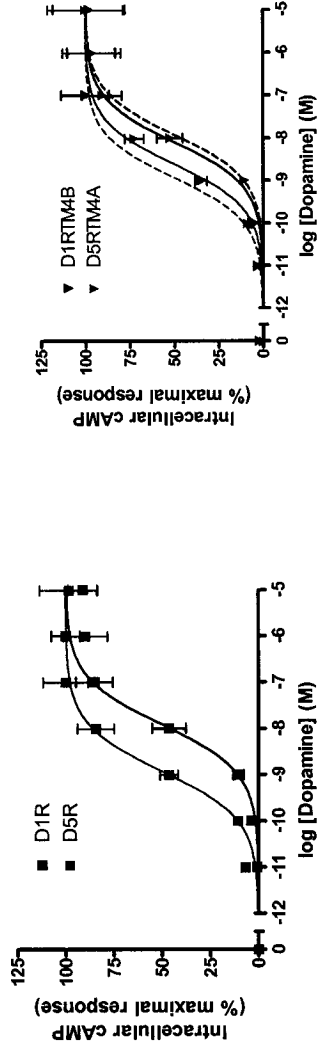


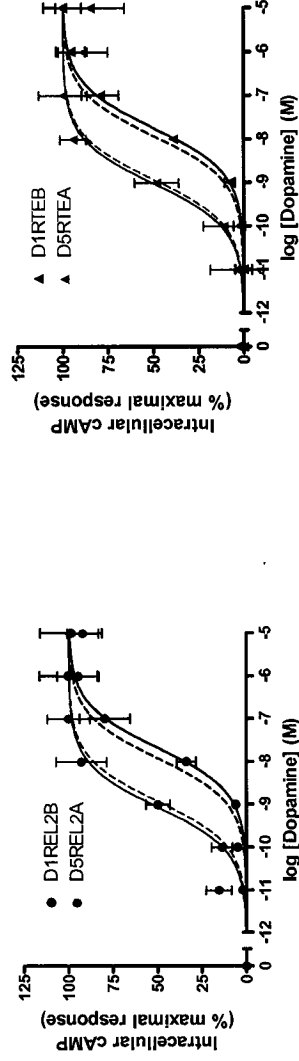
Figure 21: Analysis of dopamine dose-response curves for wild-type and chimeric receptors

A. Dopamine dose-response curves expressed as the percentage of maximal stimulation after subtraction of baseline value. Dotted black and red lines indicate wild-type D1R and D5R curves, respectively.

B. Table of dopamine EC50 values. Statistical significance was determined using unconstrained and constrained simultaneous curve fitting. *, $p < 0.05$ relative to D1R. #, $p < 0.05$ relative to D5R.



A.



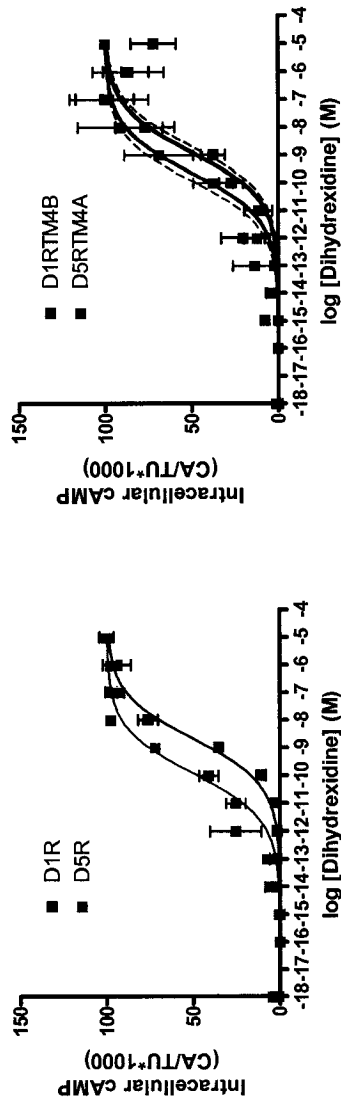
B.

	EC50 (nM)	95% confidence interval		EC50 (nM)	95% confidence interval
D1R	11.0	8.50 to 14.1	D5R	1.16(*)	0.69 to 1.95
D1RTM4B	8.71(#)	5.64 to 13.4	D5RTM4A	2.34(*#)	1.39 to 3.93
D1REL2B	22.9(*#)	15.57 to 33.8	D5REL2A	0.99(*)	0.54 to 1.80
D1RTEB	17.4(*#)	13.10 to 23.2	D5RTEA	0.83(*)	0.48 to 1.45

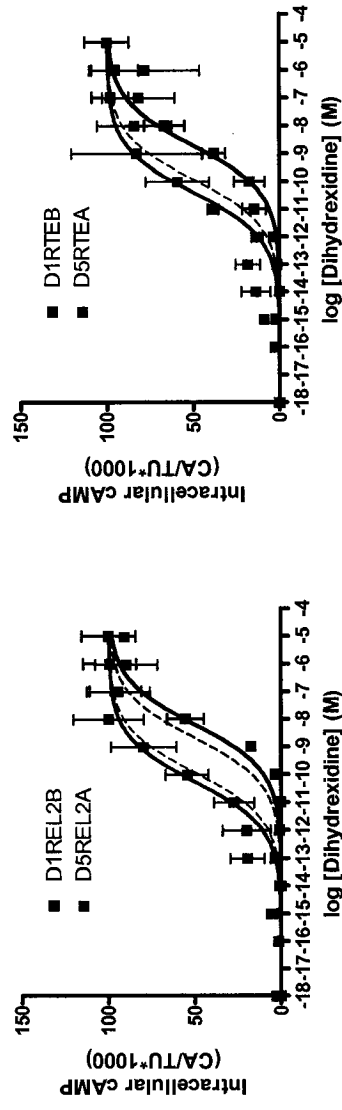
Figure 22: Analysis of dihydrexidine dose-response curves for wild-type and chimeric receptors

A. Dihydrexidine dose-response curves expressed as the percentage of maximal stimulation after subtraction of baseline value. Dotted black and red lines indicate wild-type D1R and D5R curves, respectively.

B. Table of dihydrexidine EC50 values. Statistical significance was determined using unconstrained and constrained simultaneous curve fitting. *, $p < 0.05$ relative to D1R. #, $p < 0.05$ relative to D5R.



A.



B.

	EC50 (nM)	95% confidence interval		EC50 (nM)	95% confidence interval
D1R	2.19	1.90 to 2.53	D5R	0.20(*)	0.16 to 0.24
D1RTM4B	1.22(#)	0.75 to 1.98	D5RTM4A	0.24(*)	0.13 to 0.42
D1REL2B	7.71(*#)	5.61 to 10.59	D5REL2A	0.072 (*)	0.04 to 0.13
D1RTEB	2.72(#)	1.93 to 3.84	D5RTEA	0.025(*#)	0.01 to 0.05

Table 1: Localization of dopamine receptors in the central nervous system

Subgroup	D1-like receptors		D2-like receptors		
Subtype	D1R/D1A	D5R/D1B	D2R	D3R	D4R
Localization	Cortex Striatum Nucleus accumbens Amygdala	Cortex Striatum Hippocampus Hypothalamus Amygdala Nucleus accumbens	Striatum Olfactory Tubercle Nucleus accumbens Hypothalamus Pituitary Amygdala	Nucleus accumbens Amygdala Islands of Calleja	Cortex Hippocampus Amygdala Hypothalamus
G protein Subunit	G α_s -like		G α_i -like		
Effector	Adenylyl cyclase: $\uparrow$ cAMP		AC: $\downarrow$ cAMP		

Table 2: PCR Primer sequences

Superscript: restriction site

Italics: overlap region

Underline: annealing region

Bold: engineered nucleotide

D1A-El2B-1 (F)	5'-CGGAA TTC ^{EcoR} I A GAT CT ^{Bgl} II C TTG GTG G-3'
D1A-El2B-2 (R)	5'-GAG CTG CAC TGG GAT GAA GGA-3'
D1A-El2B-3 (F)	5'-ATC CCA GTG CAG CTC AGC TGG CAC AGG GAC CAG GCG GCC TCT-3'
D1A-El2B-4 (R)	5'-CG GAA TTC ^{EcoR} I AA GCT T ^{Hind} III AT TAC CGA GGA G(A) ^{BseR} I GA GAT GGC GTA GGT-3'
D1A-El2B-5 (F)	5'-CCTGTGC CA ATT G ^{Mfe} I AG CTG CAC TGG GAT GAA GGA-3'
D1A-El2B-6 (R)	5'-ATC CCA GTG CAG CTC AAT(C) TG ^{Mfe} I G CAC AGG GAC CAG GCG GCC TCT-3'
D1A-El2B-7	5'-CTTGTGC CA ATT G ^{Mfe} I AG CTG CAC TGG GAT GAA GGA-3'
D1A-El2B-8 (F)	5'-ATC CCA GTG CAG CTC AAT(C) TG ^{Mfe} I G CAC AAG-3'
D1A-El2B-9 (R)	5'-CG GAA TTC ^{EcoR} I AA GCT T ^{Hind} III AT TAC AGA GGA-3'
D1B-El2A-1 (F)	5'-CGGAATTC ^{EcoR} I CCTTGG ^{Sly} I TCATGTGCGCCTGGCATGGACCTTGTCCATCTCCTCATCTCCTTCATC-3'
D1B-El2A-2 (R)	5'-GCTGGAGTCACAGTTGTCTA-3'
D1B-El2A-3 (F)	5'-TAGACAACTGTGACTCCAGCCTCAGCCGAACCTACGCCATC-3'
D1B-El2A-4 (R)	5'-CGGAATTC ^{EcoR} I CCTTGG ^{Sly} I TCTCCTTCTTGAT-3'
D1B-El2A-5 (F)	5'-CGGAATTC ^{EcoR} I CCTTGG ^{Sly} I TCATGTGCGCCTGGCATGGACCTTGTCCATCTCCTCATCTCCTTCATCCCGAGTGCAGCTCAATTG ^{Mfe} I GCA CAAGG;3'
D1B-El2A-4 (R)	CGGAATTC ^{EcoR} I CCTTGG ^{Sly} I TCTCCTTCTTGAT
PCM5 5' (F)	5'-TACGGTGGGAGG;3'
D1B-TM4SerA-R (R)	5'-AAGAGGCCGCCCT GGG(T)CCC ^{Apa} I TGTGCCAGCTGAGCTGGACCGGAATG-3'
D1B-TM4SerA-F (F)	5'-CA TTCGGTCCAGCTCAGCTGGCACAGGGC(A)CC ^{Apa} I AGCGGGCCTCTT-3'
PCM5 B (R)	5'-TGCAACTTAATTTTATTA-3'

Table 3: Dissociation constants and receptor expression for wild-type and chimeric receptors

Dissociation constants (K_d , nM) are expressed as the geometric mean $\pm$ S.E. of 6 experiments carried out in duplicate.

Receptor expression (R, pmol/mg protein) is expressed as the arithmetic mean $\pm$ S.E. of 6 experiments.

*, $p < 0.05$ relative to D1R. #, $p < 0.05$ relative to D5R.

	D1R	D5R	D1RTM4B	D5RTM4B	D1REL2A	D5REL2A	D1RTEB	D5RTEA
Kd	0.44	0.71	0.36	1.44	0.65	0.47	0.49	0.50
	±	±	±	±	±	±	±	±
	0.02	0.04	0.02	0.06	0.04	0.01	0.03	0.03
R	17.2	19.5	25.0	21.0	7.5	26.7	10.8	22.2
	±	±	±	±	±	±	±	±
	2.4	2.2	4.3	3.5	1.0	4.2	1.0	3.1

Table 4: Inhibition constants of non-phenylbenzazepine agonists at wild-type and chimeric receptors

Inhibition constants (K_i , nM) are expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. *, $p < 0.05$ relative to D1R; #, $p < 0.05$ relative to D5R.

	D1R	D5R	D1RTM4B	D5RTM4A	D1REL2B	D5REL2A	D1RTEB	D5RTEA
Dopamine	3631	399	3962	422	13142	266	5309	281
	±	±	±	±	±	±	±	±
	124	38	118	20	757	10	1794	14
Dihydroxidine		*	#	*	*,#	*	#	*
	371	32	290	38	940	23	567	24
	±	±	±	±	±	±	±	±
A77636	20	3	8	3	80	3	41	3
		*	#	*	#	*	#	*
	16.7	4.0	15.8	5.9	27.2	3.2	17.1	4.1
	±	±	±	±	±	±	±	±
	0.3	0.5	0.1	0.5	0.7	0.4	0.8	0.6
		*	#	*	#	*	#	*

Table 5: Non-phenylbenzazepine selectivity ratios (D5R/D1R)

Expressed as the geometric mean +/- S.E. of 3-6 experiments carried out in duplicate. *, $p < 0.05$ relative to wild-type.

	WT	TM4	EL2	TE
Dopamine	0.12	0.11	0.022	0.03
	±	±	±	±
	0.02	0.01	0.004	0.005
Dihydropyridine			*	*
	0.088	0.14	0.024	0.042
	±	±	±	±
A77636	0.004	0.02	0.002	0.003
			*	
	0.27	0.39	0.12	0.29
	±	±	±	±
	0.05	0.06	0.02	0.08

Table 6: Inhibition constants of phenylbenzazepines at wild-type and chimeric receptors

Inhibition constants (K_i , nM) are expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. *

$p < 0.05$ relative to D1R; #, $p < 0.05$ relative to D5R.

	D1R	D5R	D1RTM4B	D5RTM4A	D1REL2B	D5REL2A	D1RTEB	D5RTEA
SCH23390	0.32	0.61	0.23	1.08	0.51	0.29	0.43	0.37
	±	±	±	±	±	±	±	±
	0.01	0.01	0.01	0.03	0.02	0.01	0.06	0.01
SKF83959	1.81	4.66	1.76	7.14	2.65	2.35	1.76	2.43
	±	±	±	±	±	±	±	±
	0.12	0.12	0.10	0.29	0.13	0.23	0.04	0.15
SKF38393	115	76.8	76.0	155	151	40.8	124	49.7
	±	±	±	±	±	±	±	±
	8.8	0.6	5.2	2.9	12.2	1.3	2.9	0.4
Fenoldopam	38.0	20.0	22.4	28.4	57.6	13.1	29.0	13.6
	±	±	±	±	±	±	±	±
	3.0	1.1	0.9	0.6	1.6	0.3	1.1	0.8
		*			#	*		*

Table 7: Phenylbenzazepine selectivity ratios (D5R/D1R)

Expressed as the geometric mean +/- S.E. of 3-6 experiments carried out in duplicate. *, $p < 0.05$ relative to wild-type.

	WT	TM4	EL2	TE
SCH23390	1.92	4.70	0.60	1.19
	±	±	±	±
	0.15	0.18	0.08	0.25
SKF83959	2.69	4.25	0.98	1.43
	±	±	±	±
	0.37	0.65	0.23	0.17
SKF38393	0.69	2.07	0.29	0.40
	±	±	±	±
	0.09	0.16	0.05	0.02
Fenoldopam	0.53	1.29	0.23	0.48
	±	±	±	±
	0.02	0.12	0.02	0.06

Table 8: Inhibition constants of inverse agonists at wild-type and chimeric receptors

Inhibition constants (K_i , nM) are expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. *, $p < 0.05$ relative to D1R; #, $p < 0.05$ relative to D5R.

	D1R	D5R	D1RTM4B	D5RTM4B	D1REL2A	D5REL2A	D1RTEB	D5RTEA
cis-Flupenthixol	5.01 ± 0.88	8.52 ± 0.78	3.06 ± 0.42	23.3 ± 1.35	2.78 ± 0.39	9.89 ± 0.76	2.18 ± 0.32	10.4 ± 0.62
Thiothixene	59.3 ± 2.2	280 ± 6.4 *	54.5 ± 2.0 #	497 ± 4.6 #,*	64.8 ± 3.0 #	238 ± 9.7 *	59.3 ± 1.7 #	231 ± 1.8 *
Thioridazine	43 ± 3	166 ± 6 *	39 ± 3 #	388 ± 29 *	40 ± 2 #	218 ± 24 *	34 ± 2 #	231 ± 12 *
Fluspirilene	632 ± 35	1226 ± 45	530 ± 45	1407 ± 131	524 ± 17	1168 ± 81	377 ± 13 #	1690 ± 67 *
(+)-Butaclamol	2.65 ± 0.28	26.3 ± 2.75 *	1.69 ± 0.14 #	59.4 ± 2.41 *	2.15 ± 0.28 #	29.7 ± 1.56 *	2.41 ± 0.12 #	28.8 ± 2.00 *

Table 9: Inverse agonist selectivity ratios (D5R/D1R)

Expressed as the geometric mean $\pm$ S.E. of 3-6 experiments carried out in duplicate. *, $p < 0.05$ relative to wild-type.

	WT	TM4	EL2	TE
cis-Flupenthixol	1.90	9.50	3.86	5.63
	±	±	±	±
	0.36	2.78 [*]	0.64 [*]	1.54 [*]
Thioridazine	3.95	10.1	9.43	6.81
	±	±	±	±
	0.43	0.73	3.27	0.22
Thiothixene	4.73	9.16	3.67	3.92
	±	±	±	±
	0.11	0.46 [*]	0.03 [*]	0.20
Fluspirilene	1.96	2.68	2.28	4.61
	±	±	±	±
	0.15	0.18	0.22	0.50 [*]
(+) - Butaclamol	10.8	38.2	16.9	12.5
	±	±	±	±
	1.98	6.98 [*]	4.45	1.73

Chapter 5: Bibliography

1. Maehle, A.H., Prull, C.R., and Halliwell, R.F., *The emergence of the drug receptor theory*, in *Nat Rev Drug Discov*. 2002.
2. Lefkowitz, R.J., *Historical review: a brief history and personal retrospective of seven-transmembrane receptors*, in *Trends Pharmacol Sci*. 2004.
3. Ben-Shlomo, I., Yu Hsu, S., Rauch, R., Kowalski, H.W., and Hsueh, A.J., *Signaling receptome: a genomic and evolutionary perspective of plasma membrane receptors involved in signal transduction*, in *Sci STKE*. 2003.
4. Decaillot, F.M., Befort, K., Filliol, D., Yue, S., Walker, P., and Kieffer, B.L., *Opioid receptor random mutagenesis reveals a mechanism for G protein-coupled receptor activation*, in *Nat Struct Biol*. 2003.
5. May, D.C., Ross, E.M., Gilman, A.G., and Smigel, M.D., *Reconstitution of catecholamine-stimulated adenylate cyclase activity using three purified proteins*, in *J Biol Chem*. 1985.
6. Christopoulos, A. and Kenakin, T., *G protein-coupled receptor allostereism and complexing*, in *Pharmacol Rev*. 2002.
7. Gether, U., *Uncovering molecular mechanisms involved in activation of G protein-coupled receptors*, in *Endocr Rev*. 2000.
8. Gether, U., Asmar, F., Meinild, A.K., and Rasmussen, S.G., *Structural basis for activation of G-protein-coupled receptors*, in *Pharmacol Toxicol*. 2002.

9. Kobilka, B., *Agonist binding: a multistep process*, in *Mol Pharmacol*. 2004.
10. Leff, P., Scaramellini, C., Law, C., and McKechnie, K., *A three-state receptor model of agonist action*, in *Trends Pharmacol Sci*. 1997.
11. Lefkowitz, R.J., *The superfamily of heptahelical receptors*, in *Nat Cell Biol*. 2000.
12. Missale, C., Nash, S.R., Robinson, S.W., Jaber, M., and Caron, M.G., *Dopamine receptors: from structure to function*, in *Physiol Rev*. 1998.
13. Pierce, K.L., Premont, R.T., and Lefkowitz, R.J., *Seven-transmembrane receptors*, in *Nat Rev Mol Cell Biol*. 2002.
14. Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R.J., *A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model*, in *J Biol Chem*. 1993.
15. Kenakin, T., *Inverse, protean, and ligand-selective agonism: matters of receptor conformation*, in *Faseb J*. 2001.
16. Fredriksson, R., Lagerstrom, M.C., Lundin, L.G., and Schioth, H.B., *The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints*, in *Mol Pharmacol*. 2003.
17. Mirzadegan, T., Benko, G., Filipek, S., and Palczewski, K., *Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin*, in *Biochemistry*. 2003.

18. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., Yamamoto, M., and Miyano, M., *Crystal structure of rhodopsin: A G protein-coupled receptor*, in *Science*. 2000.
19. Wilson, C.A., Kreychman, J., and Gerstein, M., *Assessing annotation transfer for genomics: quantifying the relations between protein sequence, structure and function through traditional and probabilistic scores*, in *J Mol Biol*. 2000.
20. Ghanouni, P., Steenhuis, J.J., Farrens, D.L., and Kobilka, B.K., *Agonist-induced conformational changes in the G-protein-coupling domain of the beta 2 adrenergic receptor*, in *Proc Natl Acad Sci U S A*. 2001.
21. Ballesteros, J., Kitanovic, S., Guarnieri, F., Davies, P., Fromme, B.J., Konvicka, K., Chi, L., Millar, R.P., Davidson, J.S., Weinstein, H., and Sealfon, S.C., *Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor*, in *J Biol Chem*. 1998.
22. Karnik, S.S., Gogonea, C., Patil, S., Saad, Y., and Takezako, T., *Activation of G-protein-coupled receptors: a common molecular mechanism*, in *Trends Endocrinol Metab*. 2003.
23. Berthold, M. and Bartfai, T., *Modes of peptide binding in G protein-coupled receptors*, in *Neurochem Res*. 1997.

24. Porter, J.E., Hwa, J., and Perez, D.M., *Activation of the $\alpha 1b$ -adrenergic receptor is initiated by disruption of an interhelical salt bridge constraint*, in *J Biol Chem.* 1996.
25. Rao, V.R. and Oprian, D.D., *Activating mutations of rhodopsin and other G protein-coupled receptors*, in *Annu Rev Biophys Biomol Struct.* 1996.
26. Liapakis, G., Chan, W.C., Papadokostaki, M., and Javitch, J.A., *Synergistic contributions of the functional groups of epinephrine to its affinity and efficacy at the $\beta 2$ adrenergic receptor*, in *Mol Pharmacol.* 2004.
27. Strader, C.D., Candelore, M.R., Hill, W.S., Sigal, I.S., and Dixon, R.A., *Identification of two serine residues involved in agonist activation of the β -adrenergic receptor*, in *J Biol Chem.* 1989.
28. Gether, U., Lin, S., Ghanouni, P., Ballesteros, J.A., Weinstein, H., and Kobilka, B.K., *Agonists induce conformational changes in transmembrane domains III and VI of the $\beta 2$ adrenoceptor*, in *Embo J.* 1997.
29. Ghanouni, P., Gryczynski, Z., Steenhuis, J.J., Lee, T.W., Farrens, D.L., Lakowicz, J.R., and Kobilka, B.K., *Functionally different agonists induce distinct conformations in the G protein coupling domain of the $\beta 2$ adrenergic receptor*, in *J Biol Chem.* 2001.
30. Ghanouni, P., Schambye, H., Seifert, R., Lee, T.W., Rasmussen, S.G., Gether, U., and Kobilka, B.K., *The effect of pH on $\beta(2)$ adrenoceptor function. Evidence for protonation-dependent activation*, in *J Biol Chem.* 2000.

31. Rolz, C., Pellegrini, M., and Mierke, D.F., *Molecular characterization of the receptor-ligand complex for parathyroid hormone*, in *Biochemistry*. 1999.
32. Zhou, W., Flanagan, C., Ballesteros, J.A., Konvicka, K., Davidson, J.S., Weinstein, H., Millar, R.P., and Sealfon, S.C., *A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor*. *Mol Pharmacol*, 1994. **45**(2): p. 165-70.
33. Seifert, R., Gether, U., Wenzel-Seifert, K., and Kobilka, B.K., *Effects of guanine, inosine, and xanthine nucleotides on beta(2)-adrenergic receptor/G(s) interactions: evidence for multiple receptor conformations*, in *Mol Pharmacol*. 1999.
34. Gao, Z.G., Kim, S.K., Gross, A.S., Chen, A., Blaustein, J.B., and Jacobson, K.A., *Identification of essential residues involved in the allosteric modulation of the human A(3) adenosine receptor*, in *Mol Pharmacol*. 2003.
35. Leppik, R.A., Miller, R.C., Eck, M., and Paquet, J.L., *Role of acidic amino acids in the allosteric modulation by gallamine of antagonist binding at the m2 muscarinic acetylcholine receptor*, in *Mol Pharmacol*. 1994.
36. Shi, L. and Javitch, J.A., *The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop*, in *Annu Rev Pharmacol Toxicol*. 2002.

37. Northup, J.K., Sternweis, P.C., Smigel, M.D., Schleifer, L.S., Ross, E.M., and Gilman, A.G., *Purification of the regulatory component of adenylate cyclase*. Proc Natl Acad Sci U S A, 1980. **77**(11): p. 6516-20.
38. Lin, X., Voyno-Yasenetskaya, T.A., Hooley, R., Lin, C.Y., Orlowski, J., and Barber, D.L., *Galpha12 differentially regulates Na⁺-H⁺ exchanger isoforms*. J Biol Chem, 1996. **271**(37): p. 22604-10.
39. Kurose, H., *Galpha12 and Galpha13 as key regulatory mediator in signal transduction*. Life Sci, 2003. **74**(2-3): p. 155-61.
40. Bokoch, G.M., Katada, T., Northup, J.K., Ui, M., and Gilman, A.G., *Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase*. J Biol Chem, 1984. **259**(6): p. 3560-7.
41. Smrcka, A.V., Hepler, J.R., Brown, K.O., and Sternweis, P.C., *Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq*. Science, 1991. **251**(4995): p. 804-7.
42. De Vries, L., Zheng, B., Fischer, T., Elenko, E., and Farquhar, M.G., *The regulator of G protein signaling family*, in *Annu Rev Pharmacol Toxicol*. 2000.
43. Ross, E.M. and Wilkie, T.M., *GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins*, in *Annu Rev Biochem*. 2000.

44. Shenoy, S.K. and Lefkowitz, R.J., *Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling*. *Biochem J*, 2003. **375**(Pt 3): p. 503-15.
45. Hall, R.A. and Lefkowitz, R.J., *Regulation of G protein-coupled receptor signaling by scaffold proteins*, in *Circ Res*. 2002.
46. Horn, F., van der Wenden, E.M., Oliveira, L., AP, I.J., and Vriend, G., *Receptors coupling to G proteins: is there a signal behind the sequence?*, in *Proteins*. 2000.
47. Offermanns, S. and Simon, M.I., *G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C*, in *J Biol Chem*. 1995.
48. Holst, B., Hastrup, H., Raffetseder, U., Martini, L., and Schwartz, T.W., *Two active molecular phenotypes of the tachykinin NK1 receptor revealed by G-protein fusions and mutagenesis*, in *J Biol Chem*. 2001.
49. Wilbanks, A.M., Laporte, S.A., Bohn, L.M., Barak, L.S., and Caron, M.G., *Apparent loss-of-function mutant GPCRs revealed as constitutively desensitized receptors*, in *Biochemistry*. 2002.
50. Bohm, S.K., Grady, E.F., and Bunnett, N.W., *Regulatory mechanisms that modulate signalling by G-protein-coupled receptors*, in *Biochem J*. 1997.
51. Ferguson, S.S., *Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling*, in *Pharmacol Rev*. 2001.

52. Kohout, T.A. and Lefkowitz, R.J., *Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization*, in *Mol Pharmacol*. 2003.
53. Pitcher, J.A., Freedman, N.J., and Lefkowitz, R.J., *G protein-coupled receptor kinases*, in *Annu Rev Biochem*. 1998.
54. Beinborn, M., Ren, Y., Blaker, M., Chen, C., and Kopin, A.S., *Ligand function at constitutively active receptor mutants is affected by two distinct yet interacting mechanisms*, in *Mol Pharmacol*. 2004.
55. Chalmers, D.T. and Behan, D.P., *The use of constitutively active GPCRs in drug discovery and functional genomics*, in *Nat Rev Drug Discov*. 2002.
56. Kenakin, T., *Efficacy as a vector: the relative prevalence and paucity of inverse agonism*, in *Mol Pharmacol*. 2004.
57. Seifert, R. and Wenzel-Seifert, K., *Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors*, in *Naunyn Schmiedebergs Arch Pharmacol*. 2002.
58. Weiss, J.M., Morgan, P.H., Lutz, M.W., and Kenakin, T.P., *The cubic ternary complex receptor-occupancy model. III. resurrecting efficacy*. *J Theor Biol*, 1996. **181**(4): p. 381-97.
59. Costa, T. and Herz, A., *Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins*. *Proc Natl Acad Sci U S A*, 1989. **86**(19): p. 7321-5.

60. Kjelsberg, M.A., Cotecchia, S., Ostrowski, J., Caron, M.G., and Lefkowitz, R.J., *Constitutive activation of the alpha. 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation*, in *J Biol Chem*. 1992.
61. Wilson, J., Lin, H., Fu, D., Javitch, J.A., and Strange, P.G., *Mechanisms of inverse agonism of antipsychotic drugs at the D(2) dopamine receptor: use of a mutant D(2) dopamine receptor that adopts the activated conformation*, in *J Neurochem*. 2001.
62. Herold, C.L., Qi, A.D., Harden, T.K., and Nicholas, R.A., *Agonist versus antagonist action of ATP at the P2Y4 receptor is determined by the second extracellular loop*, in *J Biol Chem*. 2004.
63. Lee, T.W., Cotecchia, S., and Milligan, G., *Up-regulation of the levels of expression and function of a constitutively active mutant of the hamster alpha1B-adrenoceptor by ligands that act as inverse agonists*, in *Biochem J*. 1997.
64. MacEwan, D.J. and Milligan, G., *Inverse agonist-induced up-regulation of the human beta2-adrenoceptor in transfected neuroblastoma X glioma hybrid cells*. *Mol Pharmacol*, 1996. **50**(6): p. 1479-86.
65. Smit, M.J., Leurs, R., Alewijnse, A.E., Blauw, J., Van Nieuw Amerongen, G.P., Van De Vrede, Y., Roovers, E., and Timmerman, H., *Inverse agonism of histamine H2 antagonist accounts for upregulation of spontaneously active histamine H2 receptors*, in *Proc Natl Acad Sci U S A*. 1996.

66. Milligan, G. and Bond, R.A., *Inverse agonism and the regulation of receptor number*, in *Trends Pharmacol Sci*. 1997.
67. Berg, K.A., Maayani, S., Goldfarb, J., Scaramellini, C., Leff, P., and Clarke, W.P., *Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus*, in *Mol Pharmacol*. 1998.
68. Nagaraja, S., Iyer, S., Liu, X., Eichberg, J., and Bond, R.A., *Treatment with inverse agonists enhances baseline atrial contractility in transgenic mice with chronic beta2-adrenoceptor activation*, in *Br J Pharmacol*. 1999.
69. Berg, K.A., Stout, B.D., Cropper, J.D., Maayani, S., and Clarke, W.P., *Novel actions of inverse agonists on 5-HT_{2C} receptor systems*, in *Mol Pharmacol*. 1999.
70. Rossier, O., Abuin, L., Fanelli, F., Leonardi, A., and Cotecchia, S., *Inverse agonism and neutral antagonism at alpha(1a)- and alpha(1b)-adrenergic receptor subtypes*, in *Mol Pharmacol*. 1999.
71. Gay, E.A., Urban, J.D., Nichols, D.E., Oxford, G.S., and Mailman, R.B., *Functional selectivity of D2 receptor ligands in a Chinese hamster ovary hD2L cell line: evidence for induction of ligand-specific receptor states*, in *Mol Pharmacol*. 2004.
72. Brink, C.B., Wade, S.M., and Neubig, R.R., *Agonist-directed trafficking of porcine alpha(2A)-adrenergic receptor signaling in Chinese hamster ovary*

- cells: l-isoproterenol selectively activates G(s)*, in *J Pharmacol Exp Ther*. 2000.
73. Kenakin, T., *Agonist-receptor efficacy. II. Agonist trafficking of receptor signals*, in *Trends Pharmacol Sci*. 1995.
 74. Kenakin, T., *Agonist-specific receptor conformations*, in *Trends Pharmacol Sci*. 1997.
 75. Cotecchia, S., Exum, S., Caron, M.G., and Lefkowitz, R.J., *Regions of the alpha 1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function*, in *Proc Natl Acad Sci U S A*. 1990.
 76. Kilts, J.D., Connery, H.S., Arrington, E.G., Lewis, M.M., Lawler, C.P., Oxford, G.S., O'Malley, K.L., Todd, R.D., Blake, B.L., Nichols, D.E., and Mailman, R.B., *Functional selectivity of dopamine receptor agonists. II. Actions of dihydrexidine in D2L receptor-transfected MN9D cells and pituitary lactotrophs*, in *J Pharmacol Exp Ther*. 2002.
 77. Prather, P.L., *Inverse agonists: tools to reveal ligand-specific conformations of G protein-coupled receptors*, in *Sci STKE*. 2004.
 78. Devoto, P., Flore, G., Pani, L., and Gessa, G.L., *Evidence for co-release of noradrenaline and dopamine from noradrenergic neurons in the cerebral cortex*, in *Mol Psychiatry*. 2001.

79. Grillner, P. and Mercuri, N.B., *Intrinsic membrane properties and synaptic inputs regulating the firing activity of the dopamine neurons*, in *Behav Brain Res*. 2002.
80. Chen, L. and Zhuang, X., *Transgenic mouse models of dopamine deficiency*, in *Ann Neurol*. 2003.
81. Graybiel, A.M., Moratalla, R., and Robertson, H.A., *Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum*, in *Proc Natl Acad Sci U S A*. 1990.
82. Viggiano, D., Ruocco, L.A., and Sadile, A.G., *Dopamine phenotype and behaviour in animal models: in relation to attention deficit hyperactivity disorder*, in *Neurosci Biobehav Rev*. 2003.
83. Centonze, D., Grande, C., Saulle, E., Martin, A.B., Gubellini, P., Pavon, N., Pisani, A., Bernardi, G., Moratalla, R., and Calabresi, P., *Distinct roles of D1 and D5 dopamine receptors in motor activity and striatal synaptic plasticity*, in *J Neurosci*. 2003.
84. Ben-Jonathan, N. and Hnasko, R., *Dopamine as a prolactin (PRL) inhibitor*, in *Endocr Rev*. 2001.
85. Colao, A., di Sarno, A., Pivonello, R., di Somma, C., and Lombardi, G., *Dopamine receptor agonists for treating prolactinomas*, in *Expert Opin Investig Drugs*. 2002.

86. Zheng, S., Yu, P., Zeng, C., Wang, Z., Yang, Z., Andrews, P.M., Felder, R.A., and Jose, P.A., *Galpha12- and Galpha13-protein subunit linkage of D5 dopamine receptors in the nephron*, in *Hypertension*. 2003.
87. Jose, P.A., Eisner, G.M., and Felder, R.A., *Regulation of blood pressure by dopamine receptors*, in *Nephron Physiol*. 2003.
88. Hollon, T.R., Bek, M.J., Lachowicz, J.E., Ariano, M.A., Mezey, E., Ramachandran, R., Wersinger, S.R., Soares-da-Silva, P., Liu, Z.F., Grinberg, A., Drago, J., Young, W.S., 3rd, Westphal, H., Jose, P.A., and Sibley, D.R., *Mice lacking D5 dopamine receptors have increased sympathetic tone and are hypertensive*, in *J Neurosci*. 2002.
89. Di Marzo, V., Vial, D., Sokoloff, P., Schwartz, J.C., and Piomelli, D., *Selection of alternative G-mediated signaling pathways at the dopamine D2 receptor by protein kinase C*, in *J Neurosci*. 1993.
90. Kebabian, J.W. and Calne, D.B., *Multiple receptors for dopamine*. *Nature*, 1979. **277**(5692): p. 93-6.
91. Grandy, D.K., Zhang, Y.A., Bouvier, C., Zhou, Q.Y., Johnson, R.A., Allen, L., Buck, K., Bunzow, J.R., Salon, J., and Civelli, O., *Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes*, in *Proc Natl Acad Sci U S A*. 1991.
92. Giros, B., Sokoloff, P., Martres, M.P., Riou, J.F., Emorine, L.J., and Schwartz, J.C., *Alternative splicing directs the expression of two D2 dopamine receptor isoforms*, in *Nature*. 1989.

93. Van Tol, H.H., Wu, C.M., Guan, H.C., Ohara, K., Bunzow, J.R., Civelli, O., Kennedy, J., Seeman, P., Niznik, H.B., and Jovanovic, V., *Multiple dopamine D4 receptor variants in the human population*, in *Nature*. 1992.
94. Sokoloff, P., Giros, B., Martres, M.P., Andrieux, M., Besancon, R., Pilon, C., Bouthenet, M.L., Souil, E., and Schwartz, J.C., *Localization and function of the D3 dopamine receptor*. *Arzneimittelforschung*, 1992. 42(2A): p. 224-30.
95. Jarvie, K.R. and Caron, M.G., *Heterogeneity of dopamine receptors*, in *Adv Neurol*. 1993.
96. Ciliax, B.J., Nash, N., Heilman, C., Sunahara, R., Hartney, A., Tiberi, M., Rye, D.B., Caron, M.G., Niznik, H.B., and Levey, A.I., *Dopamine D(5) receptor immunolocalization in rat and monkey brain*, in *Synapse*. 2000.
97. Bergson, C., Mrzljak, L., Smiley, J.F., Pappy, M., Levenson, R., and Goldman-Rakic, P.S., *Regional, cellular, and subcellular variations in the distribution of D1 and D5 dopamine receptors in primate brain*, in *J Neurosci*. 1995.
98. Freneau, R.T., Jr., Duncan, G.E., Fornaretto, M.G., Dearry, A., Gingrich, J.A., Breese, G.R., and Caron, M.G., *Localization of D1 dopamine receptor mRNA in brain supports a role in cognitive, affective, and neuroendocrine aspects of dopaminergic neurotransmission*, in *Proc Natl Acad Sci U S A*. 1991.

99. Okubo, Y., Suhara, T., Suzuki, K., Kobayashi, K., Inoue, O., Terasaki, O., Someya, Y., Sassa, T., Sudo, Y., Matsushima, E., Iyo, M., Tateno, Y., and Toru, M., *Decreased prefrontal dopamine D1 receptors in schizophrenia revealed by PET*, in *Nature*. 1997.
100. Landwehrmeyer, B., Mengod, G., and Palacios, J.M., *Dopamine D3 receptor mRNA and binding sites in human brain*, in *Brain Res Mol Brain Res*. 1993.
101. Van Tol, H.H., Bunzow, J.R., Guan, H.C., Sunahara, R.K., Seeman, P., Niznik, H.B., and Civelli, O., *Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine*, in *Nature*. 1991.
102. Centonze, D., Grande, C., Usiello, A., Gubellini, P., Erbs, E., Martin, A.B., Pisani, A., Tognazzi, N., Bernardi, G., Moratalla, R., Borrelli, E., and Calabresi, P., *Receptor subtypes involved in the presynaptic and postsynaptic actions of dopamine on striatal interneurons*, in *J Neurosci*. 2003.
103. Paspalas, C.D. and Goldman-Rakic, P.S., *Microdomains for dopamine volume neurotransmission in primate prefrontal cortex*, in *J Neurosci*. 2004.
104. Zoli, M., Torri, C., Ferrari, R., Jansson, A., Zini, I., Fuxe, K., and Agnati, L.F., *The emergence of the volume transmission concept*. *Brain Res Brain Res Rev*, 1998. **26**(2-3): p. 136-47.

105. Baufreton, J., Garret, M., Rivera, A., de la Calle, A., Gonon, F., Dufy, B., Bioulac, B., and Taupignon, A., *D5 (not D1) dopamine receptors potentiate burst-firing in neurons of the subthalamic nucleus by modulating an L-type calcium conductance*, in *J Neurosci*. 2003.
106. Dumartin, B., Jaber, M., Gonon, F., Caron, M.G., Giros, B., and Bloch, B., *Dopamine tone regulates D1 receptor trafficking and delivery in striatal neurons in dopamine transporter-deficient mice*, in *Proc Natl Acad Sci U S A*. 2000.
107. Gainetdinov, R.R., Bohn, L.M., Sotnikova, T.D., Cyr, M., Laakso, A., Macrae, A.D., Torres, G.E., Kim, K.M., Lefkowitz, R.J., Caron, M.G., and Premont, R.T., *Dopaminergic supersensitivity in G protein-coupled receptor kinase 6-deficient mice*, in *Neuron*. 2003.
108. Wang, Q., Jolly, J.P., Surmeier, J.D., Mullah, B.M., Lidow, M.S., Bergson, C.M., and Robishaw, J.D., *Differential dependence of the D1 and D5 dopamine receptors on the G protein gamma 7 subunit for activation of adenylylcyclase*, in *J Biol Chem*. 2001.
109. Sanada, H., Jose, P.A., Hazen-Martin, D., Yu, P.Y., Xu, J., Bruns, D.E., Phipps, J., Carey, R.M., and Felder, R.A., *Dopamine-1 receptor coupling defect in renal proximal tubule cells in hypertension*, in *Hypertension*. 1999.

110. Filip, M., Thomas, M.L., and Cunningham, K.A., *Dopamine D5 receptors in nucleus accumbens contribute to the detection of cocaine in rats.* J Neurosci, 2000. **20**(19): p. RC98.
111. Khan, Z.U., Gutierrez, A., Martin, R., Penafiel, A., Rivera, A., and de la Calle, A., *Dopamine D5 receptors of rat and human brain.* Neuroscience, 2000. **100**(4): p. 689-99.
112. Svenningsson, P. and Le Moine, C., *Dopamine D1/5 receptor stimulation induces c-fos expression in the subthalamic nucleus: possible involvement of local D5 receptors,* in *Eur J Neurosci.* 2002.
113. Meyer, M.E., Cottrell, G.A., Van Hartesveldt, C., and Potter, T.J., *Effects of dopamine D1 antagonists SCH23390 and SK&F83566 on locomotor activities in rats,* in *Pharmacol Biochem Behav.* 1993.
114. Dziewczapolski, G., Menalled, L.B., Garcia, M.C., Mora, M.A., Gershanik, O.S., and Rubinstein, M., *Opposite roles of D1 and D5 dopamine receptors in locomotion revealed by selective antisense oligonucleotides,* in *Neuroreport.* 1998.
115. Gnanalingham, K.K., Hunter, A.J., Jenner, P., and Marsden, C.D., *Stimulation of adenylate cyclase activity by benzazepine D-1 dopamine agonists with varying efficacies in the 6-hydroxydopamine lesioned rat--relationship to circling behaviour,* in *Biochem Pharmacol.* 1995.

116. Smith, H.P., Nichols, D.E., Mailman, R.B., and Lawler, C.P., *Locomotor inhibition, yawning and vacuous chewing induced by a novel dopamine D2 post-synaptic receptor agonist*, in *Eur J Pharmacol.* 1997.
117. Misener, V.L., Luca, P., Azeke, O., Crosbie, J., Waldman, I., Tannock, R., Roberts, W., Malone, M., Schachar, R., Ickowicz, A., Kennedy, J.L., and Barr, C.L., *Linkage of the dopamine receptor D1 gene to attention-deficit/hyperactivity disorder*, in *Mol Psychiatry.* 2004.
118. Holmes, A., Hollon, T.R., Gleason, T.C., Liu, Z., Dreiling, J., Sibley, D.R., and Crawley, J.N., *Behavioral characterization of dopamine D5 receptor null mutant mice*, in *Behav Neurosci.* 2001.
119. Brooks, D.J., *PET studies on the function of dopamine in health and Parkinson's disease*, in *Ann N Y Acad Sci.* 2003.
120. Goldman-Rakic, P.S., Castner, S.A., Svensson, T.H., Siever, L.J., and Williams, G.V., *Targeting the dopamine D1 receptor in schizophrenia: insights for cognitive dysfunction*, in *Psychopharmacology (Berl).* 2004.
121. Mehta, A., Thermos, K., and Chesselet, M.F., *Increased behavioral response to dopaminergic stimulation of the subthalamic nucleus after nigrostriatal lesions*, in *Synapse.* 2000.
122. Wooten, G.F., *Agonists vs levodopa in PD: the thrill of whitha*, in *Neurology.* 2003.

123. J.G.Hardman, L.E.L., ed. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. ed. L.E.L. J.G.Hardman. 1996, McGraw-Hill: New York.
124. Misu, Y., Kitahama, K., and Goshima, Y., *L-3,4-Dihydroxyphenylalanine as a neurotransmitter candidate in the central nervous system*. *Pharmacol Ther*, 2003. **97**(2): p. 117-37.
125. Fraix, V., Pollak, P., Van Blercom, N., Xie, J., Krack, P., Koudsie, A., and Benabid, A.L., *Effect of subthalamic nucleus stimulation on levodopa-induced dyskinesia in Parkinson's disease*, in *Neurology*. 2000.
126. Centonze, D., Bracci, E., Pisani, A., Gubellini, P., Bernardi, G., and Calabresi, P., *Activation of dopamine D1-like receptors excites LTS interneurons of the striatum*, in *Eur J Neurosci*. 2002.
127. Aosaki, T., Kiuchi, K., and Kawaguchi, Y., *Dopamine D1-like receptor activation excites rat striatal large aspiny neurons in vitro*, in *J Neurosci*. 1998.
128. Bracci, E., Centonze, D., Bernardi, G., and Calabresi, P., *Dopamine excites fast-spiking interneurons in the striatum*, in *J Neurophysiol*. 2002.
129. Calabresi, P., Maj, R., Mercuri, N.B., and Bernardi, G., *Coactivation of D1 and D2 dopamine receptors is required for long-term synaptic depression in the striatum*, in *Neurosci Lett*. 1992.

130. Calabresi, P., Maj, R., Pisani, A., Mercuri, N.B., and Bernardi, G., *Long-term synaptic depression in the striatum: physiological and pharmacological characterization*, in *J Neurosci*. 1992.
131. Kerr, J.N. and Wickens, J.R., *Dopamine D-1/D-5 receptor activation is required for long-term potentiation in the rat neostriatum in vitro*, in *J Neurophysiol*. 2001.
132. Calabresi, P., Gubellini, P., Centonze, D., Picconi, B., Bernardi, G., Chergui, K., Svenningsson, P., Fienberg, A.A., and Greengard, P., *Dopamine and cAMP-regulated phosphoprotein 32 kDa controls both striatal long-term depression and long-term potentiation, opposing forms of synaptic plasticity*, in *J Neurosci*. 2000.
133. Taverna, S., Canciani, B., and Pennartz, C.M., *Dopamine D1-receptors modulate lateral inhibition between principal cells of the nucleus accumbens*, in *J Neurophysiol*. 2004.
134. Hersi, A.I., Kitaichi, K., Srivastava, L.K., Gaudreau, P., and Quirion, R., *Dopamine D-5 receptor modulates hippocampal acetylcholine release*, in *Brain Res Mol Brain Res*. 2000.
135. Mockett, B.G., Brooks, W.M., Tate, W.P., and Abraham, W.C., *Dopamine D1/D5 receptor activation fails to initiate an activity-independent late-phase LTP in rat hippocampus*, in *Brain Res*. 2004.
136. Swanson-Park, J.L., Coussens, C.M., Mason-Parker, S.E., Raymond, C.R., Hargreaves, E.L., Dragunow, M., Cohen, A.S., and Abraham, W.C., *A*

- double dissociation within the hippocampus of dopamine D1/D5 receptor and beta-adrenergic receptor contributions to the persistence of long-term potentiation*, in *Neuroscience*. 1999.
137. Calabresi, P., Gubellini, P., Centonze, D., Sancesario, G., Morello, M., Giorgi, M., Pisani, A., and Bernardi, G., *A critical role of the nitric oxide/cGMP pathway in corticostriatal long-term depression*, in *J Neurosci*. 1999.
 138. Morris, B.J., Simpson, C.S., Mundell, S., Maceachern, K., Johnston, H.M., and Nolan, A.M., *Dynamic changes in NADPH-diaphorase staining reflect activity of nitric oxide synthase: evidence for a dopaminergic regulation of striatal nitric oxide release*, in *Neuropharmacology*. 1997.
 139. Yao, W.D., Gainetdinov, R.R., Arbuckle, M.I., Sotnikova, T.D., Cyr, M., Beaulieu, J.M., Torres, G.E., Grant, S.G., and Caron, M.G., *Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity*, in *Neuron*. 2004.
 140. Schultz, W., *Getting formal with dopamine and reward*, in *Neuron*. 2002.
 141. Beninger, R.J. and Miller, R., *Dopamine D1-like receptors and reward-related incentive learning*. *Neurosci Biobehav Rev*, 1998. **22**(2): p. 335-45.
 142. El-Ghundi, M., Fletcher, P.J., Drago, J., Sibley, D.R., O'Dowd, B.F., and George, S.R., *Spatial learning deficit in dopamine D(1) receptor knockout mice*. *Eur J Pharmacol*, 1999. **383**(2): p. 95-106.

143. El-Ghundi, M., O'Dowd, B.F., and George, S.R., *Prolonged fear responses in mice lacking dopamine D1 receptor*. Brain Res, 2001. **892**(1): p. 86-93.
144. Nicola, S.M., Kumbian, S.B., and Malenka, R.C., *Psychostimulants depress excitatory synaptic transmission in the nucleus accumbens via presynaptic D1-like dopamine receptors*, in *J Neurosci*. 1996.
145. Sutton, M.A. and Beninger, R.J., *Psychopharmacology of conditioned reward: evidence for a rewarding signal at D1-like dopamine receptors*, in *Psychopharmacology (Berl)*. 1999.
146. Quinlan, M.G., Sharf, R., Lee, D.Y., Wise, R.A., and Rinaldi, R., *Blockade of substantia nigra dopamine D1 receptors reduces intravenous cocaine reward in rats*, in *Psychopharmacology (Berl)*. 2004.
147. Moore, R.J., Vinsant, S.L., Nader, M.A., Porrino, L.J., and Friedman, D.P., *Effect of cocaine self-administration on striatal dopamine D1 receptors in rhesus monkeys*. Synapse, 1998. **28**(1): p. 1-9.
148. Lidow, M.S., Elsworth, J.D., and Goldman-Rakic, P.S., *Down-regulation of the D1 and D5 dopamine receptors in the primate prefrontal cortex by chronic treatment with antipsychotic drugs*. J Pharmacol Exp Ther, 1997. **281**(1): p. 597-603.
149. El-Ghundi, M., George, S.R., Drago, J., Fletcher, P.J., Fan, T., Nguyen, T., Liu, C., Sibley, D.R., Westphal, H., and O'Dowd, B.F., *Disruption of dopamine D1 receptor gene expression attenuates alcohol-seeking behavior*. Eur J Pharmacol, 1998. **353**(2-3): p. 149-58.

150. Ariens, E.J., *Intrinsic activity: partial agonists and partial antagonists*. J Cardiovasc Pharmacol, 1983. **5 Suppl 1**: p. S8-15.
151. Platt, D.M., Rowlett, J.K., and Spealman, R.D., *Dissociation of cocaine-antagonist properties and motoric effects of the D1 receptor partial agonists SKF 83959 and SKF 77434*, in *J Pharmacol Exp Ther*. 2000.
152. Gonzalez-Islas, C. and Hablitz, J.J., *Dopamine enhances EPSCs in layer II-III pyramidal neurons in rat prefrontal cortex*, in *J Neurosci*. 2003.
153. Muly, E.C., 3rd, Szigeti, K., and Goldman-Rakic, P.S., *D1 receptor in interneurons of macaque prefrontal cortex: distribution and subcellular localization*, in *J Neurosci*. 1998.
154. Gines, S., Hillion, J., Torvinen, M., Le Crom, S., Casado, V., Canela, E.I., Rondin, S., Lew, J.Y., Watson, S., Zoli, M., Agnati, L.F., Verniera, P., Lluís, C., Ferre, S., Fuxe, K., and Franco, R., *Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes*, in *Proc Natl Acad Sci U S A*. 2000.
155. Berry, N., Jobanputra, V., and Pal, H., *Molecular genetics of schizophrenia: a critical review*, in *J Psychiatry Neurosci*. 2003.
156. Koh, P.O., Bergson, C., Undie, A.S., Goldman-Rakic, P.S., and Lidow, M.S., *Up-regulation of the D1 dopamine receptor-interacting protein, calcyon, in patients with schizophrenia*, in *Arch Gen Psychiatry*. 2003.
157. Abi-Dargham, A., Mawlawi, O., Lombardo, I., Gil, R., Martinez, D., Huang, Y., Hwang, D.R., Keilp, J., Kochan, L., Van Heertum, R., Gorman,

- J.M., and Laruelle, M., *Prefrontal dopamine D1 receptors and working memory in schizophrenia*, in *J Neurosci*. 2002.
158. Lidow, M.S., *Calcium signaling dysfunction in schizophrenia: a unifying approach*, in *Brain Res Brain Res Rev*. 2003.
 159. Bai, J., He, F., Novikova, S.I., Undie, A.S., Dracheva, S., Haroutunian, V., and Lidow, M.S., *Abnormalities in the dopamine system in schizophrenia may lie in altered levels of dopamine receptor-interacting proteins*, in *Biol Psychiatry*. 2004.
 160. Vianna, M.R., Barros, D.M., Silva, T., Choi, H., Madche, C., Rodrigues, C., Medina, J.H., and Izquierdo, I., *Pharmacological demonstration of the differential involvement of protein kinase C isoforms in short- and long-term memory formation and retrieval of one-trial avoidance in rats*, in *Psychopharmacology (Berl)*. 2000.
 161. Meyer-Lindenberg, A., Miletich, R.S., Kohn, P.D., Esposito, G., Carson, R.E., Quarantelli, M., Weinberger, D.R., and Berman, K.F., *Reduced prefrontal activity predicts exaggerated striatal dopaminergic function in schizophrenia*, in *Nat Neurosci*. 2002.
 162. Abi-Dargham, A., Rodenhiser, J., Printz, D., Zea-Ponce, Y., Gil, R., Kegeles, L.S., Weiss, R., Cooper, T.B., Mann, J.J., Van Heertum, R.L., Gorman, J.M., and Laruelle, M., *Increased baseline occupancy of D2 receptors by dopamine in schizophrenia*. *Proc Natl Acad Sci U S A*, 2000. 97(14): p. 8104-9.

163. Hyttel, J., Larsen, J.J., Christensen, A.V., and Arnt, J., *Receptor-binding profiles of neuroleptics*. Psychopharmacology Suppl, 1985. **2**: p. 9-18.
164. Youngren, K.D., Inglis, F.M., Pivrotto, P.J., Jedema, H.P., Bradberry, C.W., Goldman-Rakic, P.S., Roth, R.H., and Moghaddam, B., *Clozapine preferentially increases dopamine release in the rhesus monkey prefrontal cortex compared with the caudate nucleus*, in *Neuropsychopharmacology*. 1999.
165. Goldman-Rakic, P.S., *Cellular basis of working memory*. Neuron, 1995. **14**(3): p. 477-85.
166. Durston, S., *A review of the biological bases of ADHD: what have we learned from imaging studies?*, in *Ment Retard Dev Disabil Res Rev*. 2003.
167. Sidhu, A., Kimura, K., Uh, M., White, B.H., and Patel, S., *Multiple coupling of human D5 dopamine receptors to guanine nucleotide binding proteins Gs and Gz*, in *J Neurochem*. 1998.
168. Sidhu, A., Kumar, U., Uh, M., and Patel, S., *Diminished expression of renal dopamine D1A receptors in the kidney inner medulla of the spontaneously hypertensive rat*, in *J Hypertens*. 1998.
169. Brodde, O.E., *Vascular dopamine receptors: Demonstration and characterization by in vitro studies*. Life Sci, 1982. **31**(4): p. 289-306.
170. Reitsamer, H.A., Zawinka, C., and Branka, M., *Dopaminergic vasodilation in the choroidal circulation by d1/d5 receptor activation*, in *Invest Ophthalmol Vis Sci*. 2004.

171. Sobell, J.L., Lind, T.J., Sigurdson, D.C., Zald, D.H., Snitz, B.E., Grove, W.M., Heston, L.L., and Sommer, S.S., *The D5 dopamine receptor gene in schizophrenia: identification of a nonsense change and multiple missense changes but lack of association with disease*. Hum Mol Genet, 1995. 4(4): p. 507-14.
172. Manor, I., Corbex, M., Eisenberg, J., Gritsenko, I., Bachner-Melman, R., Tyano, S., and Ebstein, R.P., *Association of the dopamine D5 receptor with attention deficit hyperactivity disorder (ADHD) and scores on a continuous performance test (TOVA)*, in Am J Med Genet. 2004.
173. Wang, J., Zhao, C., Chen, B., and Liu, Z.L., *Polymorphisms of dopamine receptor and transporter genes and hallucinations in Parkinson's disease*, in Neurosci Lett. 2004.
174. Brancati, F., Valente, E.M., Castori, M., Vanacore, N., Sessa, M., Galardi, G., Berardelli, A., Bentivoglio, A.R., Defazio, G., Girlanda, P., Abbruzzese, G., Albanese, A., and Dallapiccola, B., *Role of the dopamine D5 receptor (DRD5) as a susceptibility gene for cervical dystonia*, in J Neurol Neurosurg Psychiatry. 2003.
175. Tiberi, M. and Caron, M.G., *High agonist-independent activity is a distinguishing feature of the dopamine D1B receptor subtype*, in J Biol Chem. 1994.

176. Piomelli, D., Pilon, C., Giros, B., Sokoloff, P., Martres, M.P., and Schwartz, J.C., *Dopamine activation of the arachidonic acid cascade as a basis for D1/D2 receptor synergism*, in *Nature*. 1991.
177. Laitinen, J.T., *Dopamine stimulates K⁺ efflux in the chick retina via D1 receptors independently of adenylyl cyclase activation*, in *J Neurochem*. 1993.
178. Bertorello, A.M., Hopfield, J.F., Aperia, A., and Greengard, P., *Inhibition by dopamine of (Na⁺)+K⁺)ATPase activity in neostriatal neurons through D1 and D2 dopamine receptor synergism*, in *Nature*. 1990.
179. Wurch, T. and Pauwels, P.J., *Coupling of canine serotonin 5-HT(1B) and 5-HT(1D) receptor subtypes to the formation of inositol phosphates by dual interactions with endogenous G(i/o) and recombinant G(alpha15) proteins*, in *J Neurochem*. 2000.
180. Jin, L.Q., Wang, H.Y., and Friedman, E., *Stimulated D(1) dopamine receptors couple to multiple Galpha proteins in different brain regions*, in *J Neurochem*. 2001.
181. Sidhu, A., Sullivan, M., Kohout, T., Balen, P., and Fishman, P.H., *D1 dopamine receptors can interact with both stimulatory and inhibitory guanine nucleotide binding proteins*, in *J Neurochem*. 1991.
182. Kimura, K., Sela, S., Bouvier, C., Grandy, D.K., and Sidhu, A., *Differential coupling of D1 and D5 dopamine receptors to guanine nucleotide binding*

- proteins in transfected GH4C1 rat somatomammotrophic cells*, in *J Neurochem.* 1995.
183. Zhuang, X., Belluscio, L., and Hen, R., *G(olf)alpha mediates dopamine D1 receptor signaling*, in *J Neurosci.* 2000.
 184. O'Sullivan, G.J., Roth, B.L., Kinsella, A., and Waddington, J.L., *SK&F 83822 distinguishes adenylyl cyclase from phospholipase C-coupled dopamine D1-like receptors: behavioural topography*, in *Eur J Pharmacol.* 2004.
 185. Svenningsson, P., Nishi, A., Fisone, G., Girault, J.A., Nairn, A.C., and Greengard, P., *DARPP-32: an integrator of neurotransmission*, in *Annu Rev Pharmacol Toxicol.* 2004.
 186. Gomes, P. and Soares-da-Silva, P., *Role of cAMP-PKA-PLC signaling cascade on dopamine-induced PKC-mediated inhibition of renal Na(+)-K(+)-ATPase activity*, in *Am J Physiol Renal Physiol.* 2002.
 187. Battaglia, G., Norman, A.B., Hess, E.J., and Creese, I., *Functional recovery of D1 dopamine receptor-mediated stimulation of rat striatal adenylate cyclase activity following irreversible receptor modification by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ): evidence for spare receptors*, in *Neurosci Lett.* 1986.
 188. Leonard, S.K., Anderson, C.M., Lachowicz, J.E., Schulz, D.W., Kilts, C.D., and Mailman, R.B., *Amygdaloid D1 receptors are not linked to stimulation of adenylate cyclase*. *Synapse*, 2003. **50**(4): p. 320-33.

189. Apostolakis, E.M., Garai, J., Fox, C., Smith, C.L., Watson, S.J., Clark, J.H., and O'Malley, B.W., *Dopaminergic regulation of progesterone receptors: brain D5 dopamine receptors mediate induction of lordosis by D1-like agonists in rats*, in *J Neurosci*. 1996.
190. Undie, A.S. and Friedman, E., *Inhibition of dopamine agonist-induced phosphoinositide hydrolysis by concomitant stimulation of cyclic AMP formation in brain slices*, in *J Neurochem*. 1994.
191. Undie, A.S., Weinstock, J., Sarau, H.M., and Friedman, E., *Evidence for a distinct D1-like dopamine receptor that couples to activation of phosphoinositide metabolism in brain*, in *J Neurochem*. 1994.
192. Tang, T.S. and Bezprozvanny, I., *Dopamine receptor-mediated Ca(2+) signaling in striatal medium spiny neurons*, in *J Biol Chem*. 2004.
193. Pacheco, M.A. and Jope, R.S., *Comparison of [3H]phosphatidylinositol and [3H]phosphatidylinositol 4,5-bisphosphate hydrolysis in postmortem human brain membranes and characterization of stimulation by dopamine D1 receptors*, in *J Neurochem*. 1997.
194. Wang, H.Y., Undie, A.S., and Friedman, E., *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*, 1995. 48(6): p. 988-94.

195. Lezcano, N. and Bergson, C., *D1/D5 dopamine receptors stimulate intracellular calcium release in primary cultures of neocortical and hippocampal neurons*, in *J Neurophysiol.* 2002.
196. Lezcano, N., Mrzljak, L., Eubanks, S., Levenson, R., Goldman-Rakic, P., and Bergson, C., *Dual signaling regulated by calcyon, a D1 dopamine receptor interacting protein*, in *Science.* 2000.
197. Friedman, E., Jin, L.Q., Cai, G.P., Hollon, T.R., Drago, J., Sibley, D.R., and Wang, H.Y., *D1-like dopaminergic activation of phosphoinositide hydrolysis is independent of D1A dopamine receptors: evidence from D1A knockout mice*, in *Mol Pharmacol.* 1997.
198. Jin, L.Q., Cai, G., Wang, H.Y., Smith, C., and Friedman, E., *Characterization of the phosphoinositide-linked dopamine receptor in a mouse hippocampal-neuroblastoma hybrid cell line*, in *J Neurochem.* 1998.
199. Felder, C.C., Blecher, M., and Jose, P.A., *Dopamine-I-mediated stimulation of phospholipase C activity in rat renal cortical membranes*, in *J Biol Chem.* 1989.
200. Mijares, A., Lebesgue, D., Wallukat, G., and Hoebeke, J., *From agonist to antagonist: Fab fragments of an agonist-like monoclonal anti-beta(2)-adrenoceptor antibody behave as antagonists*, in *Mol Pharmacol.* 2000.
201. Ferre, S., Torvinen, M., Antoniou, K., Irenius, E., Civelli, O., Arenas, E., Fredholm, B.B., and Fuxe, K., *Adenosine A1 receptor-mediated modulation*

- of dopamine D1 receptors in stably cotransfected fibroblast cells*, in *J Biol Chem*. 1998.
202. Liu, F., Wan, Q., Pristupa, Z.B., Yu, X.M., Wang, Y.T., and Niznik, H.B., *Direct protein-protein coupling enables cross-talk between dopamine D5 and gamma-aminobutyric acid A receptors*, in *Nature*. 2000.
 203. Terrillon, S. and Bouvier, M., *Roles of G-protein-coupled receptor dimerization*. EMBO Rep, 2004. **5**(1): p. 30-4.
 204. Lee, F.J., Xue, S., Pei, L., Vukusic, B., Chery, N., Wang, Y., Wang, Y.T., Niznik, H.B., Yu, X.M., and Liu, F., *Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor*, in *Cell*. 2002.
 205. Pei, L., Lee, F.J., Moszczynska, A., Vukusic, B., and Liu, F., *Regulation of dopamine D1 receptor function by physical interaction with the NMDA receptors*, in *J Neurosci*. 2004.
 206. Scott, L., Kruse, M.S., Forssberg, H., Brismar, H., Greengard, P., and Aperia, A., *Selective up-regulation of dopamine D1 receptors in dendritic spines by NMDA receptor activation*. Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1661-4.
 207. Salter, M.W., *D1 and NMDA receptors hook up: expanding on an emerging theme*, in *Trends Neurosci*. 2003.

208. Bergson, C., Levenson, R., Goldman-Rakic, P.S., and Lidow, M.S., *Dopamine receptor-interacting proteins: the Ca(2+) connection in dopamine signaling*, in *Trends Pharmacol Sci*. 2003.
209. Bockaert, J., Fagni, L., Dumuis, A., and Marin, P., *GPCR interacting proteins (GIP)*, in *Pharmacol Ther*. 2004.
210. Lidow, M.S., Roberts, A., Zhang, L., Koh, P.O., Lezcano, N., and Bergson, C., *Receptor crosstalk protein, calcyon, regulates affinity state of dopamine D1 receptors*, in *Eur J Pharmacol*. 2001.
211. Bermak, J.C., Li, M., Bullock, C., and Zhou, Q.Y., *Regulation of transport of the dopamine D1 receptor by a new membrane-associated ER protein*, in *Nat Cell Biol*. 2001.
212. Kim, O.J., Ariano, M.A., Lazzarini, R.A., Levine, M.S., and Sibley, D.R., *Neurofilament-M interacts with the D1 dopamine receptor to regulate cell surface expression and desensitization*, in *J Neurosci*. 2002.
213. Swayze, R.D., Lise, M.F., Levinson, J.N., Phillips, A., and El-Husseini, A., *Modulation of dopamine mediated phosphorylation of AMPA receptors by PSD-95 and AKAP79/150*, in *Neuropharmacology*. 2004.
214. Polli, J.W. and Kincaid, R.L., *Expression of a calmodulin-dependent phosphodiesterase isoform (PDE1B1) correlates with brain regions having extensive dopaminergic innervation*. *J Neurosci*, 1994. **14**(3 Pt 1): p. 1251-61.

215. Adlersberg, M., Hsiung, S.C., Glickstein, S.B., Liu, K.P., Tamir, H., and Schmauss, C., *Regulation of dopamine D-receptor activation in vivo by protein phosphatase 2B (calcineurin)*. J Neurochem, 2004. **90**(4): p. 865-73.
216. Azzi, M., Charest, P.G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M., and Pineyro, G., *Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors*, in *Proc Natl Acad Sci U S A*. 2003.
217. Luttrell, L.M., Ferguson, S.S., Daaka, Y., Miller, W.E., Maudsley, S., Della Rocca, G.J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D.K., Caron, M.G., and Lefkowitz, R.J., *Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes*. Science, 1999. **283**(5402): p. 655-61.
218. Heuss, C. and Gerber, U., *G-protein-independent signaling by G-protein-coupled receptors*, in *Trends Neurosci*. 2000.
219. Lawler, C.P., Prioleau, C., Lewis, M.M., Mak, C., Jiang, D., Schetz, J.A., Gonzalez, A.M., Sibley, D.R., and Mailman, R.B., *Interactions of the novel antipsychotic aripiprazole (OPC-14597) with dopamine and serotonin receptor subtypes*, in *Neuropsychopharmacology*. 1999.
220. Williams, W.R., Pugh, W.J., and Nicholls, P.J., *Receptor regulatory properties evident in the molecular similarity of dopamine receptor ligands and purine nucleotides*, in *J Pharm Pharmacol*. 2002.

221. Charpentier, S., Jarvie, K.R., Severynse, D.M., Caron, M.G., and Tiberi, M., *Silencing of the constitutive activity of the dopamine D1B receptor. Reciprocal mutations between D1 receptor subtypes delineate residues underlying activation properties*, in *J Biol Chem*. 1996.
222. Jackson, A., Iwasiow, R.M., and Tiberi, M., *Distinct function of the cytoplasmic tail in human D1-like receptor ligand binding and coupling*, in *FEBS Lett*. 2000.
223. Chaar, Z.Y., Jackson, A., and Tiberi, M., *The cytoplasmic tail of the D1A receptor subtype: identification of specific domains controlling dopamine cellular responsiveness*, in *J Neurochem*. 2001.
224. Iwasiow, R.M., Nantel, M.F., and Tiberi, M., *Delineation of the structural basis for the activation properties of the dopamine D1 receptor subtypes*, in *J Biol Chem*. 1999.
225. Jackson, A., Iwasiow, R.M., Chaar, Z.Y., Nantel, M.F., and Tiberi, M., *Homologous regulation of the heptahelical D1A receptor responsiveness: specific cytoplasmic tail regions mediate dopamine-induced phosphorylation, desensitization and endocytosis*, in *J Neurochem*. 2002.
226. Tumova, K., Iwasiow, R.M., and Tiberi, M., *Insight into the mechanism of dopamine D1-like receptor activation. Evidence for a molecular interplay between the third extracellular loop and the cytoplasmic tail*, in *J Biol Chem*. 2003.

227. Tumova, K., Zhang, D., and Tiberi, M., *Role of the fourth intracellular loop of D1-like dopaminergic receptors in conferring subtype-specific signaling properties*, in *FEBS Lett.* 2004.
228. Pollock, N.J., Manelli, A.M., Hutchins, C.W., Steffey, M.E., MacKenzie, R.G., and Frail, D.E., *Serine mutations in transmembrane V of the dopamine D1 receptor affect ligand interactions and receptor activation*, in *J Biol Chem.* 1992.
229. MacKenzie, R.G., Steffey, M.E., Manelli, A.M., Pollock, N.J., and Frail, D.E., *A D1/D2 chimeric dopamine receptor mediates a D1 response to a D2-selective agonist*, in *FEBS Lett.* 1993.
230. Sugamori, K.S., Hamadanizadeh, S.A., Scheideler, M.A., Hohlweg, R., Vernier, P., and Niznik, H.B., *Functional differentiation of multiple dopamine D1-like receptors by NNC 01-0012*, in *J Neurochem.* 1998.
231. Sugamori, K.S., Scheideler, M.A., Vernier, P., and Niznik, H.B., *Dopamine D1B receptor chimeras reveal modulation of partial agonist activity by carboxyl-terminal tail sequences*, in *J Neurochem.* 1998.
232. Jarvie, K.R., Tiberi, M., Silvia, C., Gingrich, J.A., and Caron, M.G., *Molecular cloning, stable expression and desensitization of the human dopamine D1b/D5 receptor*, in *J Recept Res.* 1993.
233. Guo, N., Hwang, D.R., Lo, E.S., Huang, Y.Y., Laruelle, M., and Abi-Dargham, A., *Dopamine depletion and in vivo binding of PET D1 receptor*

- radioligands: implications for imaging studies in schizophrenia*, in *Neuropsychopharmacology*. 2003.
234. Guo, W., Shi, L., and Javitch, J.A., *The fourth transmembrane segment forms the interface of the dopamine D2 receptor homodimer*, in *J Biol Chem*. 2003.
 235. Chao, T.H., Ember, J.A., Wang, M., Bayon, Y., Hugli, T.E., and Ye, R.D., *Role of the second extracellular loop of human C3a receptor in agonist binding and receptor function*, in *J Biol Chem*. 1999.
 236. Wurch, T., Colpaert, F.C., and Pauwels, P.J., *Chimeric receptor analysis of the ketanserin binding site in the human 5-Hydroxytryptamine1D receptor: importance of the second extracellular loop and fifth transmembrane domain in antagonist binding*, in *Mol Pharmacol*. 1998.
 237. Gao ZG, C.A., Barak D, Kim SK, Muller CE, Jacobson KA., *Identification by site-directed mutagenesis of residues involved in ligand recognition and activation of the human A3 adenosine receptor.*, in *J Biol Chem*. 2002.
 238. Bkaily, G., El-Bizri, N., Bui, M., Sukarieh, R., Jacques, D., and Fu, M.L., *Modulation of intracellular Ca²⁺ via L-type calcium channels in heart cells by the autoantibody directed against the second extracellular loop of the alpha1-adrenoceptors*, in *Can J Physiol Pharmacol*. 2003.
 239. Zhao, M.M., Hwa, J., and Perez, D.M., *Identification of critical extracellular loop residues involved in alpha 1-adrenergic receptor subtype-selective antagonist binding*, in *Mol Pharmacol*. 1996.

240. Kim, J., Jiang, Q., Glashofer, M., Yehle, S., Wess, J., and Jacobson, K.A., *Glutamate residues in the second extracellular loop of the human A2a adenosine receptor are required for ligand recognition*, in *Mol Pharmacol.* 1996.
241. Ruan, K.H., Wu, J., So, S.P., and Jenkins, L.A., *Evidence of the residues involved in ligand recognition in the second extracellular loop of the prostacyclin receptor characterized by high resolution 2D NMR techniques*, in *Arch Biochem Biophys.* 2003.
242. Shi, L. and Javitch, J.A., *The second extracellular loop of the dopamine D2 receptor lines the binding-site crevice*, in *Proc Natl Acad Sci U S A.* 2004.
243. Zhang, P., Johnson, P.S., Zollner, C., Wang, W., Wang, Z., Montes, A.E., Seidleck, B.K., Blaschak, C.J., and Surratt, C.K., *Mutation of human mu opioid receptor extracellular "disulfide cysteine" residues alters ligand binding but does not prevent receptor targeting to the cell plasma membrane*, in *Brain Res Mol Brain Res.* 1999.
244. Frandberg, P.A., Doufexis, M., Kapas, S., and Chhajlani, V., *Cysteine residues are involved in structure and function of melanocortin 1 receptor: Substitution of a cysteine residue in transmembrane segment two converts an agonist to antagonist*, in *Biochem Biophys Res Commun.* 2001.
245. Brandt, W., Golbraikh, A., Tager, M., and Lendeckel, U., *A molecular mechanism for the cleavage of a disulfide bond as the primary function of*

- agonist binding to G-protein-coupled receptors based on theoretical calculations supported by experiments*, in *Eur J Biochem*. 1999.
246. Cook, J.V. and Eidne, K.A., *An intramolecular disulfide bond between conserved extracellular cysteines in the gonadotropin-releasing hormone receptor is essential for binding and activation*, in *Endocrinology*. 1997.
247. Hoffmann, C., Moro, S., Nicholas, R.A., Harden, T.K., and Jacobson, K.A., *The role of amino acids in extracellular loops of the human P2Y₁ receptor in surface expression and activation processes*, in *J Biol Chem*. 1999.
248. Noda, K., Saad, Y., Graham, R.M., and Karnik, S.S., *The high affinity state of the beta 2-adrenergic receptor requires unique interaction between conserved and non-conserved extracellular loop cysteines*, in *J Biol Chem*. 1994.
249. Ray, K., Ghosh, S.P., and Northup, J.K., *The role of cysteines and charged amino acids in extracellular loops of the human Ca(2+) receptor in cell surface expression and receptor activation processes*, in *Endocrinology*. 2004.
250. Bedecarrats, G.Y., Linher, K.D., Janovick, J.A., Beranova, M., Kada, F., Seminara, S.B., Michael Conn, P., and Kaiser, U.B., *Four naturally occurring mutations in the human GnRH receptor affect ligand binding and receptor function*, in *Mol Cell Endocrinol*. 2003.
251. Bedecarrats, G.Y., Linher, K.D., and Kaiser, U.B., *Two common naturally occurring mutations in the human gonadotropin-releasing hormone*

- (GnRH) receptor have differential effects on gonadotropin gene expression and on GnRH-mediated signal transduction, in J Clin Endocrinol Metab.* 2003.
252. Becker, S., Groner, B., and Muller, C.W., *Three-dimensional structure of the Stat3beta homodimer bound to DNA*, in *Nature*. 1998.
 253. Karpa, K.D., Lidow, M.S., Pickering, M.T., Levenson, R., and Bergson, C., *N-linked glycosylation is required for plasma membrane localization of D5, but not D1, dopamine receptors in transfected mammalian cells*, in *Mol Pharmacol*. 1999.
 254. Cho, W., Taylor, L.P., and Akil, H., *Mutagenesis of residues adjacent to transmembrane prolines alters D1 dopamine receptor binding and signal transduction*, in *Mol Pharmacol*. 1996.
 255. Mottola, D.M., Laiter, S., Watts, V.J., Tropsha, A., Wyrick, S.D., Nichols, D.E., and Mailman, R.B., *Conformational analysis of D1 dopamine receptor agonists: pharmacophore assessment and receptor mapping*, in *J Med Chem*. 1996.
 256. Brusniak, M.Y., Pearlman, R.S., Neve, K.A., and Wilcox, R.E., *Comparative molecular field analysis-based prediction of drug affinities at recombinant D1A dopamine receptors*, in *J Med Chem*. 1996.
 257. Knoerzer, T.A., Watts, V.J., Nichols, D.E., and Mailman, R.B., *Synthesis and biological evaluation of a series of substituted*

- benzo[a]phenanthridines as agonists at D1 and D2 dopamine receptors*, in *J Med Chem.* 1995.
258. Grubbs, R.A., Lewis, M.M., Owens-Vance, C., Gay, E.A., Jassen, A.K., Mailman, R.B., and Nichols, D.E., *8,9-dihydroxy-1,2,3,11b-tetrahydrochromeno[4,3,2,-de]isoquinoline (dinoxylene), a high affinity and potent agonist at all dopamine receptor isoforms*, in *Bioorg Med Chem.* 2004.
 259. Zhen, X., Goswami, S., Abdali, S.A., Gil, M., Bakshi, K., and Friedman, E., *Regulation of Cyclin-Dependent Kinase 5 and Calcium/Calmodulin-Dependent Protein Kinase II by Phosphatidylinositol-Linked Dopamine Receptor in Rat Brain*. *Mol Pharmacol*, 2004. **66**(6): p. 1500-7.
 260. Panchalingam, S. and Undie, A.S., *SKF83959 exhibits biochemical agonism by stimulating [(35)S]GTP gamma S binding and phosphoinositide hydrolysis in rat and monkey brain*, in *Neuropharmacology.* 2001.
 261. Jin, L.Q., Goswami, S., Cai, G., Zhen, X., and Friedman, E., *SKF83959 selectively regulates phosphatidylinositol-linked D1 dopamine receptors in rat brain*, in *J Neurochem.* 2003.
 262. Pifl, C., Reither, H., and Hornykiewicz, O., *Lower efficacy of the dopamine D1 agonist, SKF 38393, to stimulate adenylyl cyclase activity in primate than in rodent striatum*. *Eur J Pharmacol*, 1991. **202**(2): p. 273-6.

263. Nichols, A.J., Ruffolo, R.R., Jr., and Brooks, D.P., *The pharmacology of fenoldopam*. Am J Hypertens, 1990. 3(6 Pt 2): p. 116S-119S.
264. Herrick-Davis, K., Grinde, E., and Teitler, M., *Inverse agonist activity of atypical antipsychotic drugs at human 5-hydroxytryptamine_{2C} receptors*, in *J Pharmacol Exp Ther*. 2000.
265. Mottola, D.M., Kilts, J.D., Lewis, M.M., Connery, H.S., Walker, Q.D., Jones, S.R., Booth, R.G., Hyslop, D.K., Piercey, M., Wightman, R.M., Lawler, C.P., Nichols, D.E., and Mailman, R.B., *Functional selectivity of dopamine receptor agonists. I. Selective activation of postsynaptic dopamine D₂ receptors linked to adenylate cyclase*, in *J Pharmacol Exp Ther*. 2002.
266. Kenakin, T., *Agonist-receptor efficacy. I: Mechanisms of efficacy and receptor promiscuity*, in *Trends Pharmacol Sci*. 1995.
267. Kenakin, T., *Protean agonists. Keys to receptor active states?*, in *Ann N Y Acad Sci*. 1997.
268. Undie, A.S., Berki, A.C., and Beardsley, K., *Dopaminergic behaviors and signal transduction mediated through adenylate cyclase and phospholipase C pathways*, in *Neuropharmacology*. 2000.
269. Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R., *Site-directed mutagenesis by overlap extension using the polymerase chain reaction*. Gene, 1989. 77(1): p. 51-9.

270. Gorman, C.M., *DNA Cloning: A Practical Approach*, D.M. Glover, Editor. 1985, IRL Press: Oxford. p. 143–190.
271. Johnson, R.A. and Salomon, Y., *Assay of adenylyl cyclase catalytic activity*. Methods Enzymol, 1991. **195**: p. 3-21.
272. De Lean, A., Hancock, A.A., and Lefkowitz, R.J., *Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes*. Mol Pharmacol, 1982. **21**(1): p. 5-16.
273. Barak, L.S., Tiberi, M., Freedman, N.J., Kwatra, M.M., Lefkowitz, R.J., and Caron, M.G., *A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration*, in *J Biol Chem*. 1994.
274. Barak, L.S., Menard, L., Ferguson, S.S., Colapietro, A.M., and Caron, M.G., *The conserved seven-transmembrane sequence NP(X)2,3Y of the G-protein-coupled receptor superfamily regulates multiple properties of the beta 2-adrenergic receptor*, in *Biochemistry*. 1995.
275. Gether, U., Lin, S., and Kobilka, B.K., *Fluorescent labeling of purified beta 2 adrenergic receptor. Evidence for ligand-specific conformational changes*, in *J Biol Chem*. 1995.
276. Gether, U., Ballesteros, J.A., Seifert, R., Sanders-Bush, E., Weinstein, H., and Kobilka, B.K., *Structural instability of a constitutively active G*

- protein-coupled receptor. Agonist-independent activation due to conformational flexibility*, in *J Biol Chem*. 1997.
277. Kobilka, B., Gether, U., Seifert, R., Lin, S., and Ghanouni, P., *Examination of ligand-induced conformational changes in the beta2 adrenergic receptor*, in *Life Sci*. 1998.
 278. Liggett, S.B., Caron, M.G., Lefkowitz, R.J., and Hnatowich, M., *Coupling of a mutated form of the human beta 2-adrenergic receptor to Gi and Gs. Requirement for multiple cytoplasmic domains in the coupling process*, in *J Biol Chem*. 1991.
 279. Nasman, J., Jansson, C.C., and Akerman, K.E., *The second intracellular loop of the alpha2-adrenergic receptors determines subtype-specific coupling to cAMP production*, in *J Biol Chem*. 1997.
 280. Rasmussen, S.G., Jensen, A.D., Liapakis, G., Ghanouni, P., Javitch, J.A., and Gether, U., *Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6*, in *Mol Pharmacol*. 1999.
 281. Strader, C.D., Sigal, I.S., Candelore, M.R., Rands, E., Hill, W.S., and Dixon, R.A., *Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function*, in *J Biol Chem*. 1988.

282. Goold, C.P., Usdin, T.B., and Hoare, S.R., *Regions in rat and human parathyroid hormone (PTH) 2 receptors controlling receptor interaction with PTH and with antagonist ligands*, in *J Pharmacol Exp Ther.* 2001.
283. Olah, M.E., Jacobson, K.A., and Stiles, G.L., *Role of the second extracellular loop of adenosine receptors in agonist and antagonist binding. Analysis of chimeric A1/A3 adenosine receptors*, in *J Biol Chem.* 1994.
284. Nanevycz, T., Wang, L., Chen, M., Ishii, M., and Coughlin, S.R., *Thrombin receptor activating mutations. Alteration of an extracellular agonist recognition domain causes constitutive signaling*, in *J Biol Chem.* 1996.
285. Ott, T.R., Troskie, B.E., Roeske, R.W., Illing, N., Flanagan, C.A., and Millar, R.P., *Two mutations in extracellular loop 2 of the human GnRH receptor convert an antagonist to an agonist*, in *Mol Endocrinol.* 2002.
286. Neumeyer, J.L., Kula, N.S., Bergman, J., and Baldessarini, R.J., *Receptor affinities of dopamine D1 receptor-selective novel phenylbenzazepines*, in *Eur J Pharmacol.* 2003.
287. Klabunde, T. and Hessler, G., *Drug design strategies for targeting G-protein-coupled receptors*, in *Chembiochem.* 2002.