Structure and Function of Dopamine Receptors: Ligand Binding and Arrestin Binding

By

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CERTIFICATE OF APPROVAL

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This dissertation is dedicated,
in loving memory, to
Daqin Lan

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LIST OF ABBREVIATIONS

cAMP	cyclic AMP; adenosine 3',5'-cyclic monophosphate
CMF-PBS	calcium- and magnesium-free phosphate-buffered saline
CPPMA	(3-[4-(4-chlorophenyl)piperazin-1-yl]methyl-1*H*-pyrrolo[2,3-b] pyridine (chlorophenylpiperazinyl methyl-azaindole)
D_2-A4	IYIV212-215A4 mutant of the rat dopamine D_{2L} receptor
D_2-IC2	second intracellular loop of the rat dopamine D_{2L} receptor
D_2-IC3	third intracellular loop of the rat dopamine D_{2L} receptor
D_3-IC2	second intracellular loop of the rat dopamine D_3 receptor
D_3-IC3	third intracellular loop of the rat dopamine D_3 receptor
EL2	second extracellular loop
ERK	extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
G protein	heterotrimeric GTP-binding protein
GRK	G protein-coupled receptor kinase
GST	glutathione-S-transferase
HEK	human embryonic kidney
IC	intracellular loop
SCH23390	*R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine
TH	tyrosine hydroxylase
TM	transmembrane helix
YM09151-2	*cis*-*N*-[1-benzyl-2-methyl-pyrrolidin-3-yl]-5-chloro-2-methoxy-4-methylaminobenzamide

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ABSTRACT

Investigations of how ligands specifically bind to dopamine receptors and how receptor-interacting proteins associate with dopamine receptors are important to the development of novel therapeutic strategies and the understanding of function of the central nervous system. **The goals of my dissertation are to identify the structural determinants of pharmacological specificity between D_1 and D_2 dopamine receptors and to identify the arrestin-binding sites of D_2 and D_3 dopamine receptors.*** To achieve the first goal, I hypothesized that pharmacological differentiation between D_1 and D_2 dopamine receptors results from interactions of selective ligands with nonconserved residues lining the binding pocket. Amino acid residues in the D_2 receptor were mutated to the corresponding aligned residues in the D_1 receptor and *vice versa* and the receptors were expressed in human embryonic kidney (HEK) 293 cells. The affinities of the mutant receptors for D_1- and D_2-selective antagonists were determined by radioligand binding, followed by receptor homology modeling and ligand docking. **I identified not only two residues that contribute to differential ligand binding profiles by interacting directly with ligands, but also one residue that affects D_1/D_2 pharmacological selectivity presumably by affecting the overall shape of binding pocket.** To investigate the arrestin-binding sites of D_2 and D_3 dopamine receptors, first, the second and third intracellular loops (IC2 and IC3) of both receptor subtypes were examined for their direct interactions with purified arrestin-2 and-3 using glutathinone-S-transferase (GST) pull-down assays. Secondly, chimeric, truncation, and substitution mutants were constructed to test their ability to bind purified arrestin-3. **Data from these studies indicate that higher binding affinity of arrestin-3 to D_2 receptor over D_3**

* D_1, D_2, D_3, D_4 and D_5 with subscripted numerals, genetic subtypes
D1 or D1-like and D2 or D2-like, pharmacological subtypes

receptor might be at least partly attributed to Lys149 in the carboxyl-terminal half of D_2-IC2, and that the extreme N-terminus of D_2-IC3 is particularly important for arrestin binding. Finally, the potential arrestin binding sites were further characterized by expressing mutant receptors in HEK 293 cells followed by examination of receptor internalization, translocation of arrestin-3 to the plasma membrane, and radioligand binding. **These functional studies implied that the sequence IYIV212-215 at the N-terminus of D_2-IC3 plays a specific role in the binding of arrestin.**

PREFACE

In accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health & Science University of Portland, Oregon, I have prepared my dissertation consisting of a general introduction, three chapters of original data, a chapter regarding methodological concerns, and a discussion and conclusion chapter. Each data chapter includes an abstract, introduction, materials and methods, results, and discussion section. References are listed alphabetically, and follow the format of *Molecular Pharmacology*.

Papers Representative of This Work

Lan H, Durand CJ, Teeter MM, Neve KA (2006) Structural determinants of pharmacological specificity between D_1 and D_2 dopamine receptors. Mol. Pharmacol. 69:185-194

Lan H, Liu Y, Gurevich VV, Neve KA. Identification of arrestin-binding determinants on D2-like dopamine receptors. Mol Pharmacol. (to be submitted)

Abstracts Representative of This Work

Lan H, Liu Y, Gurevich VV, Neve KA. *In vitro* binding of the second and third intracellular segments of dopamine D_2 and D_3 receptors to arrestin-2 and -3. 2005 Experimental Biology Meeting Abstr. (April 2005, San Diego, CA)

Lan H, Neve KA, Durand CJ, Teeter MM. A residue within the third transmembrane segment is important for dopamine D_1/D_2 receptor selectivity. 2002 SFN Meeting Abstr. (November 2002, Orlando, FL)

DuRand CJ, Teeter MM, Lan H, Neve KA. Modeling critical factors in D_1/D_2 receptor selective binding. DOPAMINE 2002 Abstr. (July 2002, Portland, OR)

I. INTRODUCTION

Dopamine is the most abundant catecholamine neurotransmitter in the central nervous system, where it controls a broad spectrum of functions including locomotor activity, emotion, cognition, reward, food intake, and endocrine regulation. Dopaminergic dysfunction is associated with several neuropsychiatric and endocrine disorders such as parkinsonism, schizophrenia, attention deficit hyperactivity disorder, Tourette's syndrome, hyperprolactinemia, and drug abuse. Outside the central nervous system, dopamine also plays multiple roles. Cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function, and gastrointestinal motility are all modulated by dopamine. More recent evidence indicates that dopamine is linked to pathological conditions such as hypertension and congestive heart failure.

Dopamine functions by binding to and activating 7-transmembrane integral proteins, G protein-coupled receptors (GPCRs) (Fig. 1-5), which then transduce the signal across the cell membrane and initiate various signaling events involving a variety of intracellular components, such as heterotrimeric G proteins, G protein-coupled receptor kinases (GRKs), and arrestins. Dopamine occupancy induces a conformational change in the GPCR, which in turn binds the cognate G protein, and meanwhile, specifically recruits GRKs and is phosphorylated by GRKs. Subsequently, arrestins bind to the phosphorylated receptor, causing dissociation of the G protein and receptor desensitization. Arrestins also link the receptor to the endocytic machinery and thus initiate receptor internalization.

This introductory section contains background information on dopamine as a neurotransmitter, molecular biology of the D1-like and D2-like subfamilies of dopamine receptors, and arrestins as GPCR-interacting proteins, and more specificially the ligand binding pocket and GPCR determinants for arrestin binding. In addition, the introduction will focus on

first, the role of critical amino acids in the binding of ligands, and second, the phenomenon of receptor internalization and the role of arrestin in the internalization of dopamine receptors.

DOPAMINE

Historical Perspective

Dopamine was not recognized as a neurotransmitter until the late 1950s. Before that time, dopamine had been generally believed to be just a precursor to norepinephrine. Administration of reserpine depletes the intracellular stores of norepinephrine (Carlsson et al., 1957). Carlsson and his colleagues hypothesized that treatment with L-DOPA, an intermediate in the biosynthesis of norepinephrine, would be able to restore this amine. This hypothesis turned out to be wrong. However, unexpectedly, several lines of evidence demonstrated that dopamine is not only an intermediate for norepinephrine biosynthesis, but, importantly, a normal brain constituent playing a biological role (Carlsson, 1960; Seiden and Carlsson, 1964; Carlsson, 2001). First, administration of L-DOPA did not restore brain norepinephrine level. Instead, an accumulation of dopamine in the brain was induced. Second, the accumulation of dopamine in the brain was accompanied by behavioral response, a reversal of reserpine's effects. Finally, the distribution patterns for norepinephrine and dopamine are different, with norepinephrine concentrated in the hypothalamus whereas dopamine is concentrated in the basal ganglia. Later discoveries confirmed that dopamine is a major neurotransmitter participating in numerous neuronal processes, and that dysfunction of dopaminergic systems leads to a variety of diseases. The pioneering work of Carlsson and his peers established the role of dopamine as a neurotransmitter, and laid the groundwork for intensive studies on the sites of action of dopamine, namely the dopamine receptors.

Dopaminergic Neuronal Pathways

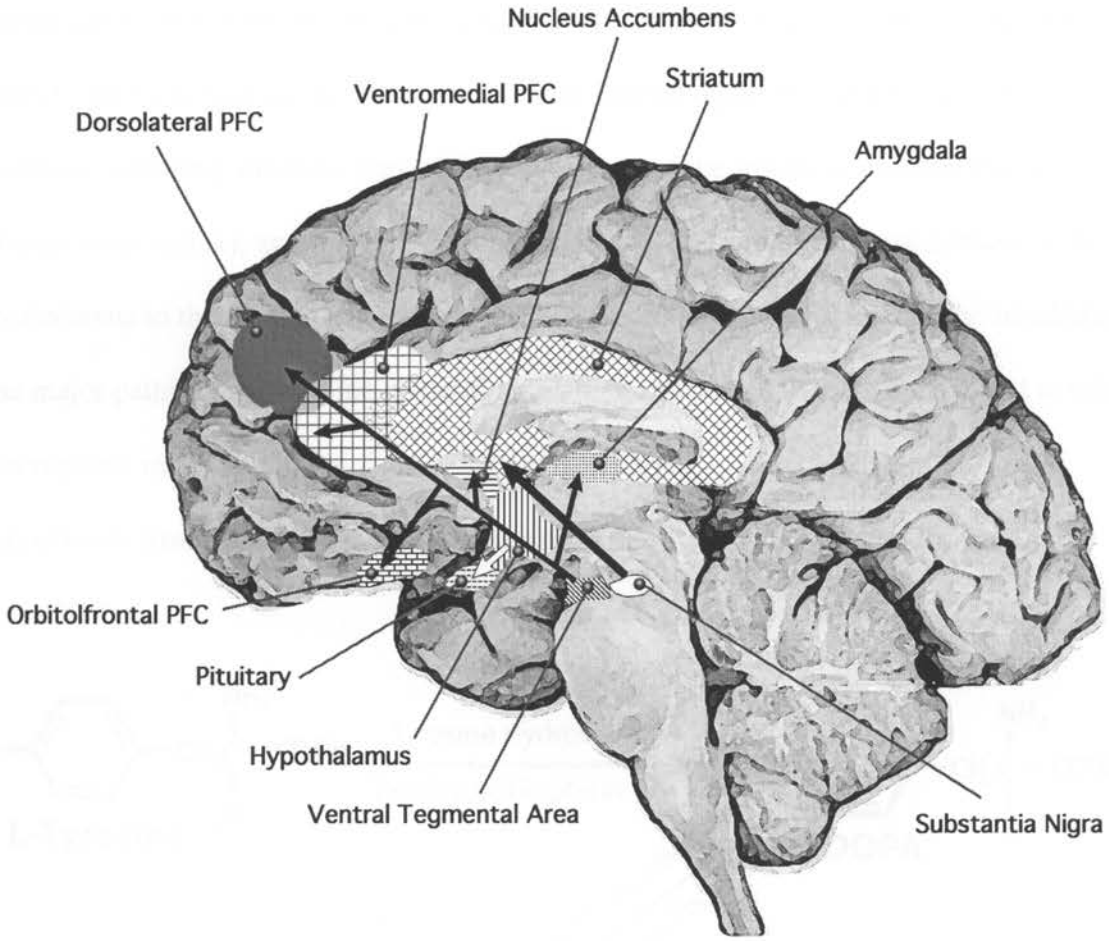

Fig. 1-1 Dopaminergic neuronal pathways in the human brain. A semi-schematic mid-saggital view of the brain shows the three major dopamine pathways: Nigrostriatal (thick arrow), mesolimbocortical (thin arrow) and tuberoinfundibular (white arrow) pathways. Some of the structures shown here cannot actually be seen in a mid-saggital section.

Dopamine is widely distributed throughout the brain, but highly concentrated in the basal ganglia. In fact, dopamine accounts for about half of the total catecholamines (comprised of dopamine, norepinephrine and epinephrine) in the brain and more than 80% of dopamine in the brain is in the basal ganglia. Several techniques have been used to map dopamine-containing neurons and the pathways of their axons to the target regions, including histofluorescence,

immunohistochemistry, mRNA mapping by *in situ* hybridization, and dopamine uptake. The principal dopaminergic fiber systems in the brain are divided into 3 groups (Fig. 1-1): the nigrostriatal system, from the substantia nigra pars compacta to the caudate nucleus and putamen; the mesolimbocortical system, from the ventral tegmental area to the nucleus accumbens, olfactory tubercle, amygdala, and regions of the frontal cortex (prefrontal, cingulate, and entorhinal cortex); and the tuberoinfundibular system, from the arcuate nucleus of the hypothalamus to the median eminence and the intermediate lobe of the pituitary. In addition to these major pathways, dopamine-containing neurons and terminals have been found in other brain regions, in the neural retina, and in the spinal cord.

Biosynthesis, Storage, Release and Metabolism

Biosynthesis

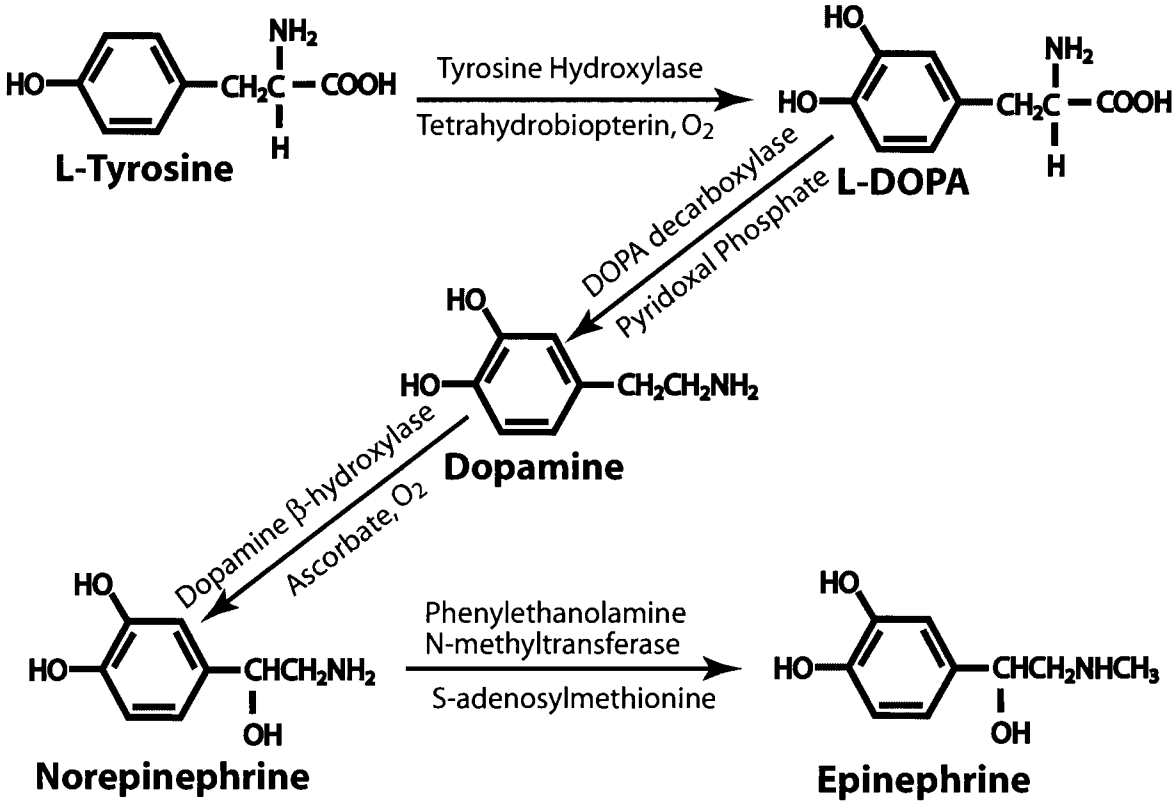

Fig. 1-2 Biosynthetic pathway for dopamine and other catecholamines.

The initial step in the biosynthesis of dopamine is the facilitated transport of the amino acid L-tyrosine from blood into brain. L-tyrosine is a nonessential amino acid that the body synthesizes from L-phenylalanine. Dietary sources of L-tyrosine are principally derived from animal and vegetable proteins. Once tyrosine is in the neuron, the enzyme tyrosine hydroxylase (TH) uses it and molecular oxygen as its substrates and biopterin as its cofactor (Fig. 1-2). TH catalyzes the addition of a hydroxyl group to the *meta* position of tyrosine, thus forming L-DOPA. This is the rate-limiting step. Next, L-DOPA is converted to dopamine by DOPA decarboxylase (also called aromatic amino acid decarboxylase), a pyridoxine-dependent enzyme. In dopamine-containing neurons, this enzyme is the final step in the pathway. However, for neurons that synthesize norepinephrine or epinephrine, dopamine β-hydroxylase and phenylethanolamine N-methyltransferase catalyze the subsequent biosynthetic step(s).

Regulation of TH activity determines dopamine levels. Several mechanisms are involved. First of all, the end product dopamine has a negative feedback on TH by competing against the biopterin cofactor for a binding site on the enzyme (Fig. 1-3); in contrast, neuronal activity disinhibits the enzyme, resulting from the release of dopamine and a decrease in cytoplasmic concentration. More importantly, nerve impulse flow in dopaminergic terminals causes an increase in activity of TH by phosphorylation of the enzyme, mediated by protein kinase C, adenosine 3',5'-cyclic monophosphate (cAMP) -dependent protein kinase, and Ca^{2+}/calmodulin-dependent protein kinases. Moreover, it is well-recognized now that prejunctional D2-like dopamine receptors, dopamine autoreceptors (D_2 and/or D_3 receptors), play a role in the inhibition of dopamine synthesis (Fig. 1-3). Stimulation of dopamine autoreceptors by dopamine released from the nerve terminal results in reduction of cAMP level, a decrease in the activity of cAMP-dependent protein kinase, and inhibition of TH. Alternatively, dopamine autoreceptors

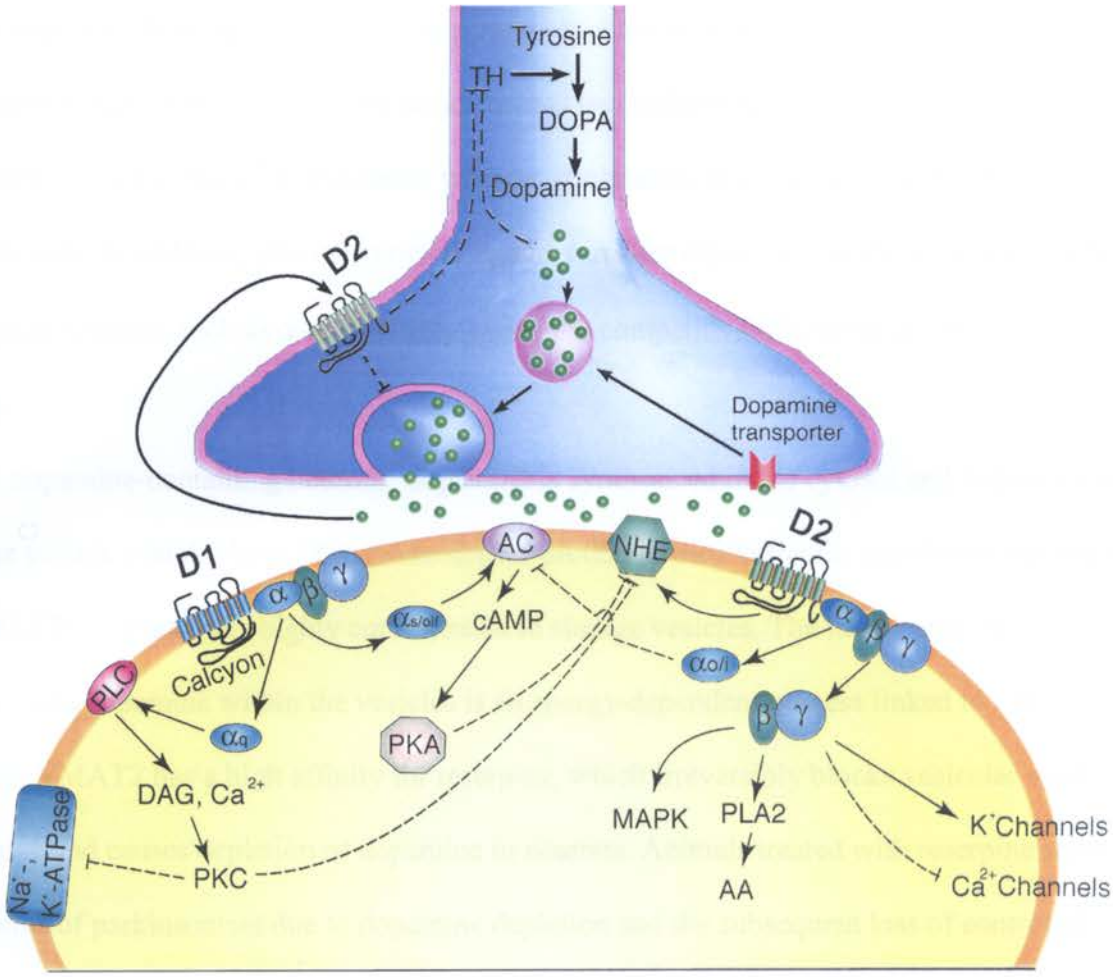

Fig. 1-3 Model of a dopaminergic synaptic structure. The presynaptic nerve terminal displays dopamine synthesis, release, reuptake and D_2 autoreceptor feedback inhibition of dopamine synthesis and release. On the postsynaptic side, major signaling pathways of D_1 and D_2 receptors are illustrated. A solid arrow indicates a stimulatory effect whereas a dashed line indicates an inhibitory effect. The diagram is a schematic representation and is not intended to imply that the two dopamine receptor subtypes are found in a single cell. Abbreviations: AA, arachidonic acid; AC, adenylate cyclase; cAMP, adenosine 3'5'-cyclic monophosphate; DAG, diacylglycerol; GTP, guanosine triphosphate; MAPK, mitogen-activated protein kinase; NHE, Na^+/H^+ exchanger; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PLA, phospholipase A; PLC, phospholipase C. (adapted from Neve et al., 2004 with modifications)

mediate an inhibition of inward calcium currents and an increase of outward potassium currents, leading to decreased phosphorylation of TH and inhibition of TH activity. In the long term, many

factors regulate TH at transcriptional and translational levels. For instance, neurotransmitters and neurotrophic factors that increase the intraneuronal level of cAMP increase TH mRNA levels, which results in increased TH synthesis, whereas stimulation of dopamine autoreceptors plays an opposite role. In addition, pharmacological agents can alter dopamine synthesis. For example, analogs of tyrosine, such as α-methyl-p-tyrosine, are competitive inhibitors of TH.

Storage

In dopamine-containing neurons, dopamine is synthesized in the cytosol and is then taken up into the storage vesicles by a 12-transmembrane protein termed vesicular membrane transporter 2 (VMAT2). Dopamine is highly concentrated in storage vesicles. The mechanism that concentrates dopamine within the vesicles is an energy-dependent process linked to a H^+-ATPase. VMAT2 has a high affinity for reserpine, which irreversibly blocks vesicular uptake of dopamine and causes depletion of dopamine in neurons. Animals treated with reserpine show symptoms of parkinsonism due to dopamine depletion and the subsequent loss of control of extrapyramidal motor function.

Release

The release of dopamine is calcium-dependent. When an action potential reaches the nerve terminal, Ca^{2+} channels open, allowing an influx of $Ca^{2+}$and subsequently additional release of Ca^{2+} from intracellular stores; increased intracellular Ca^{2+} promotes the fusion of vesicles with the neuronal membrane, resulting in the release of dopamine into the synaptic cleft. The released dopamine has a negative feedback on its own release by acting at presynaptic dopamine autoreceptors, which presumably mediate hyperpolarization of the nerve terminal by increasing potassium conductance (Fig. 1-3). A variety of biogenic amines, including tyramine and amphetamine, can also release dopamine but by a mechanism that is Ca^{2+}-independent. Due to

the broad substrate specificity of VMAT2, these drugs can be transported into and displace dopamine from storage vesicles, resulting in leakage of dopamine from the nerve terminals.

Reuptake and Metabolism

Synaptic effects of dopamine are terminated by binding of this molecule to a specific transporter protein on the outer membrane of the terminals, the dopamine transporter, which transports dopamine back into the terminals (Fig. 1-3). This reuptake is an energy-dependent and also Na^+- and Cl^--dependent process. Drugs that abolish the Na^+ gradient such as ouabain, which inhibits Na^+, K^+-ATPase, or veratridine, which opens Na^+ channels, inhibit this reuptake process. A number of other drugs including tricyclic antidepressants, cocaine, and amphetamine, can inhibit this process too but by directly targeting the dopamine transporter. However, drugs like reserpine, which blocks vesicular uptake of dopamine, have no effects on dopamine reuptake.

Dopamine is sequentially deaminated by monoamine oxidase (MAO) and O-methylated by catechol-O-methyltransferase (COMT) or *vice versa*, depending on the site of metabolism (Fig. 1-4). MAO acts on both intraneuronal and extraneuronal dopamine, whereas COMT only acts on the latter. The major metabolites of dopamine include 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Spinal fluid concentrations of HVA are indicators of the rate of dopamine synthesis in the brain. Patients with Parkinson's disease show a decrease of HVA in the cerebrospinal fluid.

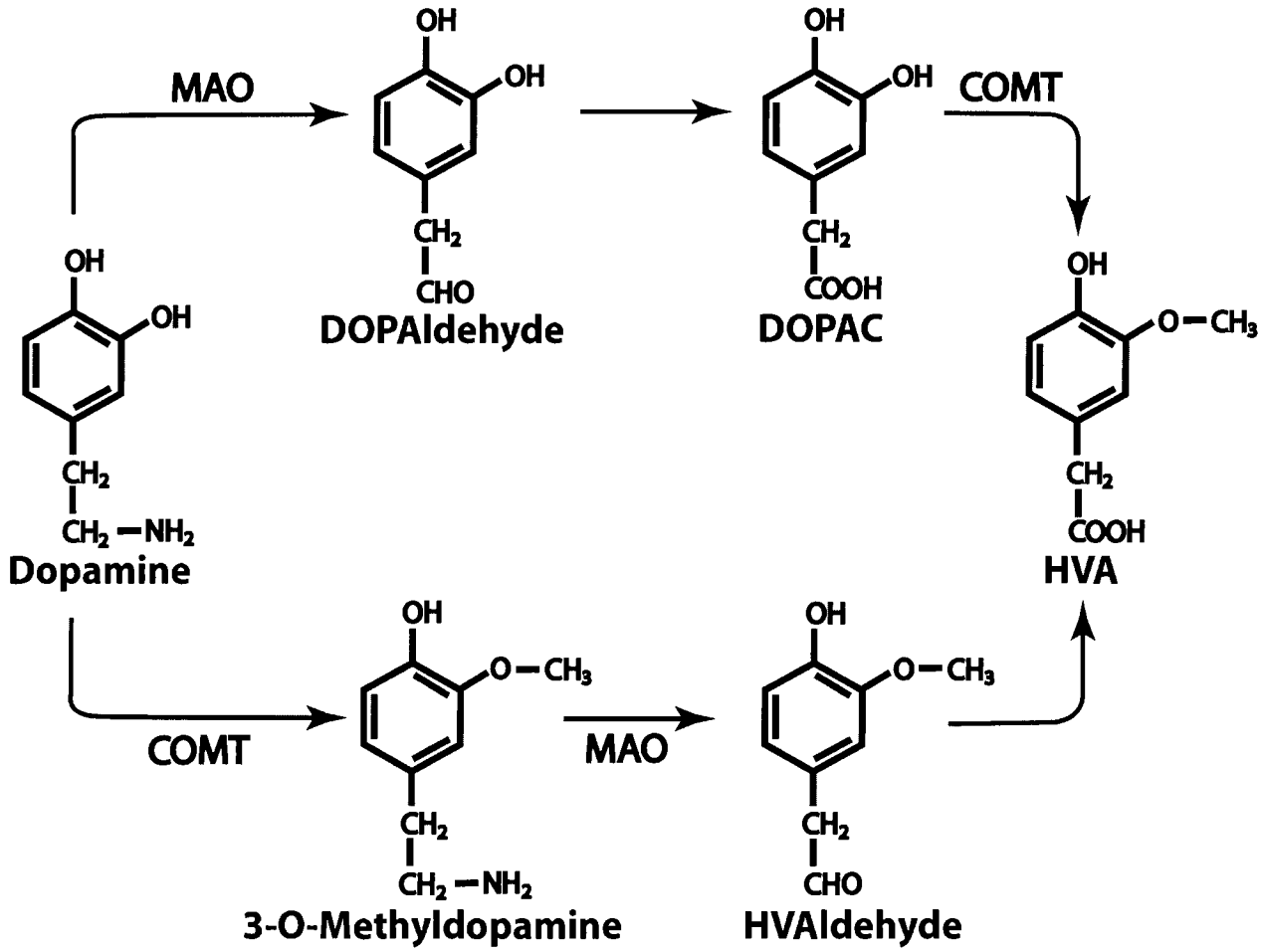

Fig. 1-4 Dopamine and its major metabolites. Dopamine is sequentially deaminated and *O*-methylated or *vice versa*, depending on the site of metabolism. *MAO,* monoamine oxidase; *COMT,* catechol-*O*-methyltransferase; *HVA,* homovanillic acid; *DOPAC,* 3,4-dihydroxyphenylacetic acid (adapted from Basic Neurochemistry, 6th Ed. with modifications).

THE DOPAMINE RECEPTORS*

Classification

The receptor concept, originally termed "receptive substance", was first introduced by J.N.

* D_1, D_2, D_3, D_4 and D_5 with subscripted numerals, genetic subtypes
D1 or D1-like and D2 or D2-like, pharmacological subtypes

Langley in 1909 (Langley, 1909). Although dopamine was discovered to be a neurotransmitter in the late 1950s, the existence of dopamine receptors was not confirmed until 1972, when Brown and Makman found that dopamine stimulation resulted in the activation of adenylate cyclase in retinal homogenate and in the formation of cAMP in intact retina (Brown and Makman, 1972). In a 1979 review, Kebabian and Calne first proposed that there are two subfamilies of receptors for dopamine, D1 and D2 (Kebabian and Calne, 1979). The D1 receptor was defined as the receptor linked to the stimulation of adenylate cyclase, and with a lower affinity for the butyrophenones (e.g. haloperidol) and substituted benzamides (e.g. sulpiride); the D2 receptor was defined as the receptor that was not coupled to adenylate cyclase, but with a high affinity for the above-mentioned pharmacological agents. Shortly after, it was proved that the D2 receptor inhibits adenylate cyclase (De Camilli et al., 1979; Enjalbert and Bockaert, 1983).

The cloning of the gene and cDNA for the hamster β_2-adrenergic receptor in 1986 initiated and fueled the field of GPCRs (Dixon et al., 1986). The predicted topography of the cloned receptor was shown to resemble that of rhodopsin, a seven transmembrane-spanning receptor. The striking structural relationship between rhodopsin and the β_2-adrenergic receptor led to the hypothesis that all GPCRs might be seven transmembrane-spanning receptors. Based on suspected homology with already-cloned members of the family, more and more GPCRs were cloned.

The molecular cloning of the dopamine receptors identified 5 receptor subtypes. A D_2 receptor cDNA was first isolated in 1988 (Bunzow et al., 1988). The D_2 receptor exists as two alternatively spliced isoforms differing in the insertion of a stretch of 29 amino acids in the third intracellular loop (D_{2S} and D_{2L}) (Fig. 1-5). Following the cloning of the D_2 receptor, the other members were cloned quickly owing to their homology to other dopamine or monoamine

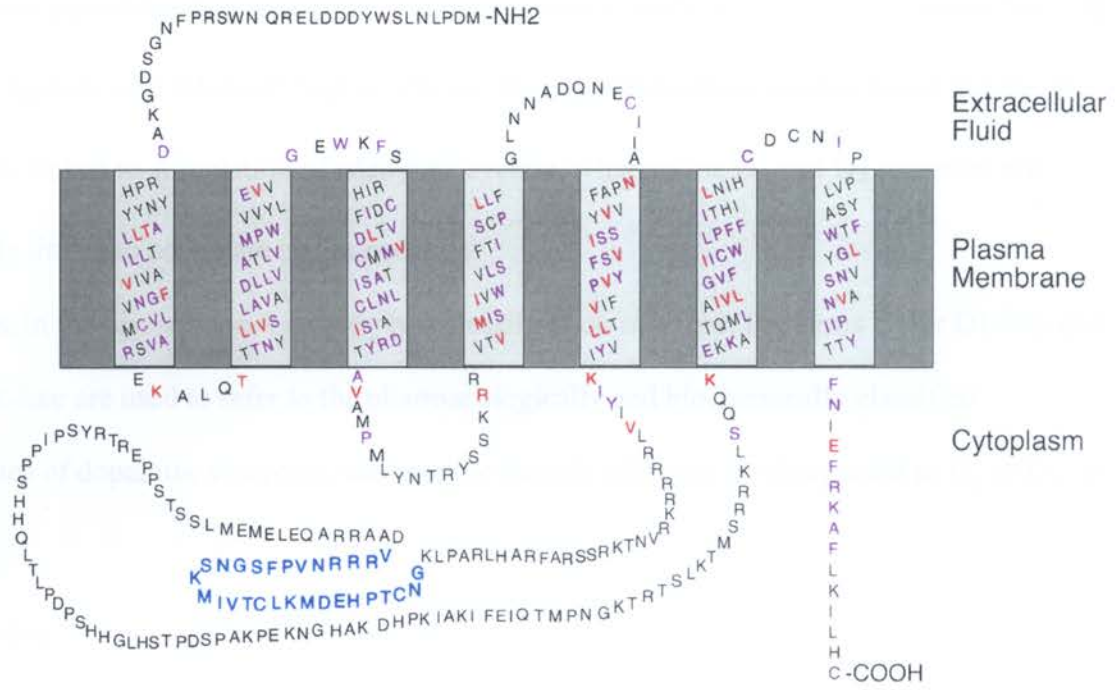

Fig. 1-5 The seven transmembrane α-helix structure of the human D_2 dopamine receptor. In purple are amino acids identical in the aligned D_1 receptor sequence. In red are residues not identical but conservative between the D_2 and D_1 receptors. Only residues in and proximal to the transmembrane domains are compared. The two receptors share ~45% amino acid identity in the transmembrane domains and the homology increases to over 60% when conservative residues are included. The alternatively spliced region containing 29 amino acids in the D_{2L} receptor is highlighted in blue. Compared to the D_2 receptor, the D_1 receptor is characterized by a longer carboxyl-terminus and by a shorter third intracellular loop.

receptors (Dearry et al., 1990; Monsma, Jr. et al., 1990; O'Dowd et al., 1990; Sokoloff et al., 1990; Sunahara et al., 1990; Zhou et al., 1990; Sunahara et al., 1991; Van Tol et al., 1991). Subsequent studies revealed that all dopamine receptor subtypes fit into one of the two originally classified receptor categories. First, structurally, the D_1 and D_5 receptors share a very high amino acid sequence homology in their transmembrane helices (TMs). This is also true among D_2, D_3, and D_4 receptors. Second, pharmacological studies demonstrated that the D_5 receptor exhibits

the classical ligand-binding specificity of the D1 receptor, and the D_3 and D_4 receptors bind D2-selective ligands with relatively higher affinity. Finally, biochemical studies found that the D_5 receptor is linked to stimulation of adenylate cyclase, whereas the D_3 and D_4 receptors are negatively linked to adenylate cyclase activity.

Thus, in the dissertation, I employ two classification schemes: the terms D1 or D1-like and D2 or D2-like are used to refer to the pharmacologically and biochemically classified subfamilies of dopamine receptors, whereas the genetic subtypes are designated as D_1 or D_5; D_2, D_3, or D_4.

Distribution

The dopamine receptors are widely distributed throughout the brain, pituitary, retina, cardiovascular system, and kidney. Because specific ligands have not been developed for each receptor subtype, *in situ* hybridization has been extensively used to study the distribution of dopamine receptor mRNAs. Caution should be taken when interpreting the data because: first, mRNA abundance does not necessarily reflect the level of the encoded protein or the number of ligand binding sites. For example, the mismatch of mRNA level to protein distribution may reflect differential protein and mRNA stability and mRNA translation rates; second, in neurons, proteins are synthesized in the cell body and are subsequently delivered to distal projections. *In situ* hybridization only detects cell body mRNA levels, and the results do not reflect receptor distribution within dendrites and axons. Accordingly, the key to fully elucidate the distribution patterns of each of the dopamine receptor subtypes is the development of ligands or antipeptide antibodies with high levels of subtype-selectivity.

The D_1 receptor is the most abundant dopamine receptor and is expressed at the highest level among all dopamine receptors. D_1 mRNA is highly expressed in the striatum, the nucleus

accumbens, and the olfactory tubercle (Dearry et al., 1990; Monsma, Jr. et al., 1990; Meador-Woodruff et al., 1991; Mansour et al., 1992). In addition, mRNA has been detected in the rostral neocortex, amygdala, and retina. However, in some areas where the D_1 receptor protein is highly expressed such as the substantia nigra pars reticulata and the globus pallidus (Le Moine et al., 1991; Gerfen, 1992), no mRNA has been detected, most likely reflecting that the D_1 receptor protein in these regions is localized on projections originating from other brain regions (Dearry et al., 1990; Fremeau, Jr. et al., 1991; Weiner et al., 1991).

Compared with the D_1 receptor, the D_5 receptor is poorly expressed in the brain. According to the original reports, little or no mRNA was detected in the dorsal striatum, nucleus accumbens, and olfactory tubercle; instead, a limited distribution was found in the hippocampus, the lateral mamillary nuceus, the parafascicular nucleus of the thalamus, and the anterior pretectal nuclei (Tiberi et al., 1991; Meador-Woodruff et al., 1992). Upon further examination, a low level of the D_5 receptor mRNA has been found throughout the striatum and cortex in human and monkey (Huntley et al., 1992; Rappaport et al., 1993)

The D_2 receptor is found mainly in the striatum (in medium- and large-sized neurons), the olfactory tubercle, and the nucleus accumbens, where it is expressed by GABAergic neurons coexpressing enkephalins or neurotensin (Le Moine et al., 1990; Bouthenet et al., 1991; Le Moine and Bloch, 1995). D_2 receptor mRNA and protein are also present in globus pallidus, substantia nigra pars compacta, ventral tegmental area, amygdala, cerebral cortex, and the pituitary (Le Moine and Bloch, 1991; Surmeier et al., 1992; Rappaport et al., 1993; Lester et al., 1993; Chronwall et al., 1994).

The D_3 receptor has a specific distribution to limbic areas such as the ventral striatal complex comprised of the nucleus accumbens, olfactory tubercle, ventral pallidum, ventral

tegemental area, and islands of Calleja (Sokoloff et al., 1990). Compared to the D_2 receptor, its distribution is more restricted, with poor expression in the dorsal striatum and no D_3 receptor mRNA within the pituitary gland. However, later studies suggested D_3 mRNA is co-distributed with D_2 mRNA in the medium-sized cells of the striatum (Surmeier et al., 1992).

D_4 receptor mRNA is most abundant in the frontal cortex, amygdala, hippocampus, hypothalamus, and mesencephalon, with low levels of expression in the basal ganglia (Van Tol et al., 1991; O'Malley et al., 1992). D_4 mRNA is also expressed in the retina (Cohen et al., 1992).

Dopamine receptors also exist in the periphery. It has been reported that all the cloned dopamine receptors are present in the kidney (Sokoloff et al., 1990; Nash et al., 1993; Yamaguchi et al., 1993; Gao et al., 1994; Matsumoto et al., 1995) and that the D_4 receptors are present in the heart (O'Malley et al., 1992). However, little is known about the molecular nature of dopamine receptors in blood vessels (both D1- and D2-like) (Goldberg et al., 1978), in postganglionic sympathetic nerve terminals (D2-like) (Lyon et al., 1987; Pupilli et al., 1994), and in the adrenal cortex (both D1 and D2-like) (Missale et al., 1989), so their distribution and classification in these regions are largely based on pharmacological data.

Signaling Pathways

Studies of dopamine receptor-mediated signaling events have been carried out intensively either in native tissues or in heterologous systems. Native tissues, brain tissues in particular, usually express multiple dopamine receptor subtypes, which makes it difficult to deduce subtype-specific properties because of the lack of ligands selective for each receptor subtype. Heterologous expression systems make it possible to work with a single subtype of receptors; however, these systems are mostly fibroblast in nature whereas endogenous dopamine receptors are expressed primarily in neuronal cells. Thus, each cell line may contain different cellular

components from others and neurons/cells *in vivo*, leading to conflicting results. Furthermore, dopamine receptors regulate multiple effectors within the same cell, and it has been observed that a subset of signaling events mediated by these receptors can be selectively weakened or enhanced by intracellular regulators (Liu et al., 1992; Di Marzo et al., 1993). In other cases, one receptor subtype might modulate a response through multiple signaling mechanisms (Ganz et al., 1990; Neve et al., 1992; Chio et al., 1994). In addition, another exciting possibility is that some agonists may induce an agonist-specific conformation of the receptor which favors only a certain type of G protein interaction, thus activating only a subset of signaling events (Gurwitz et al., 1994). Bear these in mind when reading through the following summary of dopamine receptor-mediated signaling events (A-D, second messengers; the rest, other "effectors" by broader means) (Fig. 1-3).

A. Adenylate Cyclase. D1-like dopamine receptors couple to the stimulatory G-proteins $G_{s/olf}$, stimulate adenylate cyclase, and cause cAMP accumulation upon agonist stimulation. In contrast, D2-like receptors couple to the inhibitory G proteins $G_{i/o}$ and inhibit adenylate cyclase, with D_3 showing only a weak effect.

B. Calcium Channels. Generally, D1-like receptors mediate an increase of inward calcium currents whereas D2-like receptors mediate an inhibition.

C. Potassium Channels. The role of D1-like receptors is controversial in this issue. D2-like receptors increase outward potassium currents, leading to cell hyperpolarization.

D. Arachidonic Acid. D2-like receptors cause an increase whereas D1-like receptors cause an inhibition of calcium-evoked release of arachidonic acid.

E. Na^+/H^+ Exchangers. D2-like receptors activate Na^+/H^+ exchangers (extracellular acidification/Na^+ absorption). In contrast, D1-like receptors inhibit them.

F. Na^+-K^+-ATPase. Stimulation of D1-like receptors evokes an inhibition of it. The role of D2-like receptors is not clear.

G. Phospholipase C. D1-like receptors activate phospholipase C which in turn stimulates phosphatidylinositol hydrolysis. Pituitary D_2 receptors decrease phospholipase C activity, leading to an inhibition of phosphatidylinositol metabolism.

H. Mitogenesis and cell differentiation: D2-like receptors promote through a variety of mechanisms including activation of mitogen-activated protein kinases (MAP kinases).

Function

A. Motor function. The major dopamine pathway involved in motor activity is the nigrostriatal pathway. Forward locomotion is determined by synergistic interaction between D1 and D2 receptors (Breese et al., 1987; Dreher and Jackson, 1989). Administration of dopamine receptor agonists to laboratory animals elicits hyperactivity and repetitive, stereotyped behavior such as sniffing, rearing, licking, and gnawing.

B. Reward and motivation. Administration of drugs of abuse such as opiates induces an increase of dopamine release in the mesolimbic areas, whereas withdrawal of these drugs is followed by a reduction in basal dopamine transmission *in vivo*. These results imply that mesolimbocortical dopamine is implicated in reward and reinforcement mechanisms (Ramsey and Van Ree, 1992; Di Chiara, 1995).

C. Integration of sensory and motor pathways. Dopamine acts as a neuromodulator of the inducible patterns of activity within the sensory-motor circuit (Dasari and Cooper, 2004).

D. Learning and memory. It has been reported that activation of both D1-like and D2-like receptors improves working memory tasks (Sawaguchi and Goldman-Rakic, 1991; White and Viaud, 1991), although some inconsistencies are present in the literature.

E. Regulation of gene expression. Activation of dopamine receptors results in modulation of both neuropeptide and immediate early gene expression. The D1-like receptors mediate the stimulatory effects of dopamine on substance P and dynorphin expression, whereas the D2-like receptors mediate the inhibition of preproenkephalin A (precursor of enkephalin) expression (Le Moine et al., 1990; Le Moine et al., 1991; Gerfen, 1992). However, it is likely that simultaneous stimulation of D1-like and D2-like receptors produces synergistic effects on both immediate early gene and peptide expression (LaHoste et al., 1993; Keefe and Gerfen, 1995).

F. Endocrine regulation. In the pituitary, D_2 receptors mediate the tonic inhibitory control of hypothalamic dopamine on prolactin and α-melanocyte-stimulating hormone secretion (Ben Jonathan, 1985; Stack and Surprenant, 1991).

G. Roles in the periphery include modulation of cardiovascular function, norepinephrine release, hormone secretion, vascular tone, renal function, and gastrointestinal motility. For example, activation of postjunctional D1-like receptors produces direct vasodilation, and activation of prejunctional D2-like receptors inhibits norepinephrine release, thus indirectly inducing vasodilation and decrease of cardiac contractility (Goldberg et al., 1978).

Because of the lack of highly selective ligands for each dopamine receptor subtypes, gene knockout studies have been instrumental in clarifying the physiological functions of dopamine receptors (Glickstein and Schmauss, 2001; Holmes et al., 2004). For D_1 receptor knockout mice, in general, they appear normal, but show growth retardation and low survival rate after weaning. Behavioral tests implicate the importance of D_1 receptor in maintaining spontaneous motor behaviors and its involvement in reward-related behaviors. In addition, these mice exhibit

deficits in prefrontal cortex-mediated behaviors including working memory. However, some but not all hippocampus-mediated behavioral tasks appear abnormal in D_1 receptor knockout mice. D_2 receptor knockout mice are viable but exhibit disorders of pituitary hormone synthesis and secretion, adrenal hypertrophy, and hypertension. A number of studies have reported decreased locomotor activity and delayed initiation of movements in D_2 receptor knockout mice, and the role for the receptor in gait, posture and motor coordination has also been suggested. Reward-related responses to drugs of abuse are reduced in D_2 receptor knockout mice. Moreover, these mice fail to show amphetamine-induced sensorimotor gating deficits. Interestingly, data from these studies suggest that the two isoforms of the D_2 receptor, D_{2S} and D_{2L}, mediate discrete functions of the D_2 receptor, with D_{2L} responsible for most postsynaptic effects while D_{2S} act as a presynaptic autoreceptor controlling dopamine release. For the other three subtypes that are expressed at much lower levels in the brain, gene knockout studies suggest that the D_3 receptor may play a prominent role in mediating reward-related behaviors, that the D_4 receptor may generally inhibit (or stimulate) dopaminergic neurotransmission in certain brain regions, and that the D_5 receptor may modulate hippocampus-mediated cognition. As a final note, data from gene knockout studies should be interpreted with caution as differences in genetic background and variations in testing procedures have produced variable results across laboratories and the potential compensatory changes during development may alter the normal function of a dopamine receptor subtype.

THE LIGAND BINDING POCKET OF DOPAMINE RECEPTORS

Following the molecular cloning of dopamine receptors, a great deal of work has accumulated regarding the structure and function of these receptors. However, the lack of subtype-selective ligands hinders the accurate mapping of receptor subtype distribution, the characterization of their physiological and pharmacological roles, and the linkage of the pathophysiological state of individual receptor subtypes to certain disorders. In other words, a better understanding of the structure of the receptors and of the identity of critical residues and subdomains that participate in ligand binding specificity would provide a foundation that will help develop highly subtype-selective ligands and ultimately lead to the development of pharmacological tools and clinically useful drugs.

Methods to Identify Binding Site Residues

Affinity labeling

A photoactivatable group is attached to an agonist or antagonist ligand that can bind to the receptor in a reversible manner, and then bound ligand can be rendered reactive by exposure to UV light. This approach is straightforward but the problems are the lack of affinity reagents and that not all amino acid residues can be labeled.

Site-directed mutagenesis

Site-directed mutagenesis has been proven to be a powerful approach for identifying the ligand binding site residues of dopamine receptors. With *in vitro* mutagenesis, a residue suspected to be involved in ligand binding is mutated to a given residue with different size, charge, or polarity. If such a mutation causes loss of binding, this implies a direct contribution of the residue to binding. This approach has also been used in determining residues contributing to pharmacological specificity, such as in the cases of D_2 vs. D_4 and D_1 vs. D_2 receptors (Simpson et al., 1999; Lan et al., 2006). For two receptors that differ in ligand binding

capability/specificity, aligned non-conserved residues exposed in the ligand binding site are swapped, on the assumption that a mutant receptor will exhibit loss of binding of its own selective ligands and/or gain of binding of ligands selective for the other receptor. However, caution should be taken when interpreting data that show loss of function since mutations can also cause non-specific distortion of receptor structure.

The Substituted-Cysteine Accessibility Method

This approach has been designed to identify residues that form the water-accessible ligand binding pocket of the D_2 receptor. Consecutive residues extending from the extracellular surface of the receptor into the transmembrane domains are mutated to cysteine, one at a time, and the mutant receptors are expressed in a heterologous cell system. Subsequently, charged, sulfhydryl-specific, methanethiosulfonate (MTS) derivatives are used to irreversibly inhibit radioligand binding to the receptors. The sulfhydryl of the engineered cysteine should readily react with MTS reagents if the residue is accessible from the aqueous phase, and should display an inhibitory effect on ligand binding if the residue faces the ligand binding pocket. Then it can be deduced whether the corresponding wild-type residue is exposed in the binding pocket.

The Primary Binding Site

The primary binding pocket in catecholamine receptors includes residues in TM3, TM5, and TM6 (Dixon et al., 1988; Strader et al., 1989; Cho et al., 1995; Javitch et al., 1998). In particular, the conserved Asp3.32 (See chapter 2, *Materials and Methods, Numbering of residues*) is thought to make a direct contact with the protonated nitrogen of all catecholamine ligands via an electrostatic interaction. Moreover, a cluster of three Ser residues in TM5 form hydrogen-bonds with catechol hydroxyls. These interactions exist between Ser5.42 and Ser5.46 and the meta- and para-hydroxyl substituents of catecholamine agonists, and antagonists also interact with Ser5.42.

In the cases of the β_2-adrenergic receptor and dopamine D_1 and D_2 receptors, an additional interaction of Ser5.43 with the meta-hydroxyl has been reported (Strader et al., 1989; Cox et al., 1992; Pollock et al., 1992; Wilcox et al., 2000). In addition, a cluster of aromatic residues in TM6, including Trp6.48, Phe6.51 and Phe6.52, interact with an aromatic ring of catecholamine ligands.

Structural Determinants of Pharmacological Specificity

Although dopamine binds to D_1 and D_2 receptors with similar affinity, numerous synthetic compounds preferentially bind to one receptor or the other. For example, dopamine receptor agonists bromocriptine, 6,7-ADTN, and 7-OH-DPAT and antagonists spiperone and sulpiride are D_2-selective, and other compounds such as the agonist fenoldopam and the antagonist *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390) bind to the D_1 receptor with higher affinity (Neve and Neve, 1997). Among residues lining the ligand binding pocket, those conserved between D_1 and D_2 receptors are not likely to account for differential pharmacological properties. Pharmacological specificity is more likely to reside in those residues that are non-conserved between D_1 and D_2 receptors (Fig. 1-5).

In the D_2 receptor homology models constructed by our collaborator, Dr. Martha Teeter, the binding site is located in the extracellular half of the transmembrane domains, consisting of a water-accessible primary binding pocket (see above *The primary binding site*) and an additional hydrophobic ancillary binding pocket. The ancillary pocket is composed of a cluster of aromatic and nonpolar residues between TM2, TM3, and TM7 on the extracellular side of the primary binding pocket, such as at position 3.28 (Trp in D_1 and D_5, Phe in D_2 and D_3, and Leu in D_4) and position 7.43 (Trp in D1-like and Tyr in D2-like) (Teeter et al., 1994; Neve et al., 2003). One possibility is that these ancillary pocket residues contribute to pharmacological selectivity by

stabilizing the binding of drugs with aromatic or nonpolar groups oriented toward the ancillary pocket. An aromatic microdomain located within TM2, TM3, and TM7 contributes to selectivity between dopamine D_2 and D_4 receptors (Simpson et al., 1999). Several residues in this microdomain are also components of the ancillary pocket, which lends further support to our speculation.

In addition, findings from my dissertation work implied that a considerable portion of D_1/D_2 pharmacological selectivity is caused by non-conserved residues that do not interact directly with ligands, but that change the shape of the primary and ancillary binding pockets and thus alter ligand interactions with conserved residues. For example, mutation of Ala/Val5.39 caused reciprocal changes in affinity of the receptors for D1 and D2-selective ligands, reflecting possibly altered packing of the interface of helices 5 and 6 and the altered distance between critical conserved contact residues, thus contributing to pharmacological selectivity indirectly (Lan et al., 2006).

The second extracellular loop (EL2) of rhodopsin-like GPCRs had not received much attention until the 2.8Å crystal structure of rhodopsin was resolved (Palczewski et al., 2000). According to its crystal structure, the EL2 of bovine rhodopsin dives down into the TMs and contacts retinal. Emerging evidence demonstrated that the overall structures of rhodopsin and biogenic amine receptors are very similar and that EL2s of these receptors are very likely to be part of the ligand binding pocket and/or to play a role in the determination of ligand selectivity. For example, antagonist binding profiles of α_{1a}- and α_{1b}-adrenoceptors are interchangeable when three residues in EL2 are exchanged (Zhao et al., 1996), and one residue difference in EL2 determines much of the difference between the affinity of canine and human 5-HT_{1D} receptors for the antagonist ketanserin (Wurch and Pauwels, 2000). In the D_2 receptor, five EL2 residues

were found to be exposed in the ligand binding pocket (Shi and Javitch, 2004). Findings from my dissertation work support the notion that EL2 in dopamine receptors participates in forming the ligand binding pocket, but the contribution of EL2 to pharmacological specificity of D_1/D_2 receptors needs further examination.

Receptor Homology Modeling and Ligand Docking

GPCR homology models and ligand docking experiments have been useful in rationalizing experimental results and formulating hypotheses for further testing. Together with receptor mutagenesis studies and drug structure-activity analysis, knowledge of the three-dimensional structure of the receptor and ligand-receptor interaction facilitates development of improved therapeutic agents having both high binding affinity and selectivity for the receptor. Homology modeling refers to the prediction of the structure of a protein from the known structure of a related protein. The predicted structural homology of the biogenic amine receptors and rhodopsin, along with the 2.8Å high-resolution crystal structure of rhodopsin, lay the foundation for constructing these receptor models (Dixon et al., 1986; Palczewski et al., 2000). In brief, the sequences of biogenic amine receptors are aligned with rhodopsin, and then in the computer, amino acids from these receptors are substituted for the side chains of rhodopsin in the crystal structure. Finally, the protein interior is repacked with the new side chains and refined by energy minimization or molecular dynamics. In the dopamine receptor models of Dr. Martha Teeter, no energy minimization is performed nor is molecular dynamics run because these procedures distort the models, however, local geometry optimization, proline template replacements, and side chain rotation are used to refine the models, and close contacts are eliminated manually (Teeter et al., 1994; Lan et al., 2006).

Next, a ligand is docked into the binding site within the receptor, which allows one to bring together the ligand and the receptor in the computer and to study the ligand-receptor interaction within the binding pocket. Usually, ligand conformation is derived either from its crystal structure or from ab initio calculations. In our studies, the positively-charged nitrogen in a dopaminergic ligand interacts with the conserved, negatively-charged Asp3.32, and the conserved contact residues in TM5, Ser5.42 and Ser5.46, bind to polar atoms on the ligand (the O, N, -OH groups, or halogens Cl or F). Drugs with relatively rigid structures are particularly useful for rationalizing experimental data in ligand-docking studies, because the binding of a rigid drug has more constraints than the binding of a drug such as dopamine that can adopt a variety of low-energy conformations. For example, on the basis of mutagenesis data, more rigid spiperone and less rigid haloperidol were docked into the D_2 receptor model, which elaborated ligand structure-activity relationships and provided new insights into the three-dimensional structure of the receptor (Lan et al., 2006).

ARRESTINS

The Arrestin Family

GRK-mediated phosphorylation alone cannot account for the full inactivation of most GPCRs. Full inactivation requires an additional component, arrestin. The first identified arrestin protein was rod arrestin, which was found to bind to light-activated rhodopsin and quench the acitivity of cGMP phophodiesterase (Pfister et al., 1985). In 1987, it was proposed that an analog of rod arrestin is required for efficient desensitization of β_2-adrenergic receptor (Benovic et al., 1987). So far, four arrestin family members have been identified. Based on sequence homology, function, and tissue distribution, they can be divided into two groups: visual arrestins and non-

visual arrestins. Visual arrestins include rod arrestin (also known as arrestin-1 or visual arrestin.) and cone arrestin (also known as arrestin-4). Non-visual arrestins (β-arrestins) include β-arrestin1 and β-arrestin2, also known as arrestin-2 and arrestin-3, respectively. Both arrestin-1 and arrestin-4 are localized primarily to the retina, being highly enriched in the photosensor rods and cones, respectively. Non-visual arrestins are ubiquitously expressed outside the retina, but are more abundant in neuronal tissues and in the spleen, and are highly concentrated at neuronal synapses along with GRKs. Thus, these proteins are ideally localized to modulate neuronal function. Alternative splice variants have been identified for arrestin-1, -2 and -3. However, for non-visual arrestin splice variants, there are no reported differences in functional activity.

Biological Roles: Desensitization, Resensitization, and Signaling

Desensitization

The loss of responsiveness of GPCRs following prolonged or repeated activation is called desensitization. The mechanisms of desensitization include uncoupling of the receptor from heterotrimeric G protein, internalization of cell surface receptors to intracellular compartments, and downregulation of receptors due to reduced receptor mRNA and protein synthesis, as well as both the lysosomal and plasma membrane degradation of receptors. In this section, the focus is on rapid homologous desensitization, that is, the uncoupling of the receptor from G protein-mediated signaling pathways following agonist occupancy of the receptor. This process involves GRKs and arrestins. GRKs phosphorylate only agonist-occupied receptors and this phosphorylation promotes the binding of arrestins to the receptor. Arrestin binding physically uncouples the receptor from G proteins and also targets the receptor for internalization (see below). Two points need to be clarified. First, GRK-mediated phosphorylation itself does not alter receptor responsiveness greatly, but rather targets the activated receptors for uncoupling.

Second, arrestins preferentially bind to agonist-activated and GRK-phosphorylated GPCRs, however, substantial binding to both agonist-activated but non-phosphorylated receptors and phosphorylated non-activated receptors has also been observed (Gurevich et al., 1995).

Internalization and Resensitization

An important aspect of GPCR regulation is the internalization of agonist-activated receptors into the intracellular membrane compartments of the cell. Non-visual arrestins were found to participate in initiating the internalization of many GPCRs, including dopamine receptors. It is now generally believed that GRK-mediated GPCR phosphorylation and binding of arrestins to the receptor promote agonist-induced internalization (Ferguson, 2001). The extent of arrestin involvement varies, depending on the receptor, agonist, and cell type. In particular, variations in endogenous patterns of GRK and arrestin expression significantly contribute to the extent of GPCR internalization. For instance, in different cell lines, the levels of GRK and arrestin expression determine the maximal extent of β_2-adrenergic receptor internalization (Menard et al., 1997).

Non-visual arrestins contain two motifs within the C-terminal tail of the molecule that allow them to function as adapter proteins that link the GPCR to components of the clathrin-dependent internalization machinery, namely the clathrin heavy chain and the β_2-adaptin subunit of the heterotetromeric AP-2 adaptor complex (Goodman, Jr. et al., 1997; Laporte et al., 1999; Laporte et al., 2000). So, arrestins bind to GRK-phosphorylated receptor and recruit clathrin and AP-2, leading to the co-localization of the receptor and arrestins in punctuated vesicles at the cell surface. Soon afterwards, the receptor, either alone or together with arrestin, internalizes to acidic endosomes, where it is either dephosphorylated and recycled to the cell surface, or degraded in lysosomes. Although many receptors internalize through clathrin-coated vesicles,

there are additional pathways of GPCR internalization, including caveolae, which are independent of both clathrin and arrestin (Raposo et al., 1989; Couet et al., 1997). In addition, some receptors such as β_3- and α_{2a}-adrenergic receptors, and rhodopsin, do not internalize in response to agonist stimulation (Palczewski et al., 1989; Jockers et al., 1996; Pierce et al., 2000). Visual arrestin does not contain the clathrin- and β_2-adaptin-binding motifs that are conserved between non-visual arrestins, thus it cannot link rhodopsin to the internalization machinery. As for the other two receptors, they do not serve as substrates for GRKs and interacting partners for arrestins, which may also be true for the dopamine D_3 receptor (Kim et al., 2001).

Receptor internalization is required for resensitization of many GPCRs. Presumably, in the endosome, agonist is released from receptor, followed by the loss of the active receptor conformation. This conformational change results in the release of arrestin and the exposure of receptor-attached phosphates. Subsequently, the receptor is dephosphorylated by a GPCR-specific phosphatase and regains responsiveness to agonist stimulation (Gurevich and Gurevich, 2006). The resensitized receptor then can be recycled back to the plasma membrane.

Signaling

Recent evidence indicates that non-visual arrestins contribute to the assembly of signaling protein complexes (Luttrell et al., 1999; Barlic et al., 2000; DeFea et al., 2000). They play an essential role in the recruitment of Src kinases in GPCR signaling, including c-Src, Hck and c-Fgr, thus coupling the termination of receptor-G protein coupling with the initiation of alternative signaling in which the desensitized receptor and arrestins act as scaffolds. This alternative signaling is physiologically relevant. For example, arrestin-mediated Hck activation and redistribution was shown to be required for the Hck-dependent exocytosis of granules in neutrophils (Barlic et al., 2000). In addition, non-visual arrestins act as scaffolds for the

activation and targeting of MAP kinases. In response to the activation of protease-activated receptor 2, non-visual arrestins interact directly with both Raf-1 (a MAP kinase kinase kinase) and extracellular signal-regulated kinase (ERK, a MAP kinase), forming a receptor-arrestin-ERK complex in endosomal vesicles (DeFea et al., 2000). As a consequence, ERK is activated, but the formation of the complex in vesicles prevents the translocation of activated ERK to the nucleus and ERK activity is limited to cytosolic targets. Similar mechanisms have been proposed for regulation of ERK and JNK MAP kinase cascades by the AT_{1a} angiotensin receptor (Luttrell et al., 2001).

GPCR Determinants for Arrestin Binding

GPCRs form a large superfamily of cell-surface receptors (>1000 members in human) that respond to an enormous array of stimuli. In contrast, only four members of the arrestin gene family have been discovered. Visual arrestin shows strong selectivity to its cognate receptor, rhodopsin (Gurevich et al., 1995), whereas non-visual arrestins have the ability to recognize and bind to a large number of GPCRs.

The molecular mechanisms of arrestin-GPCR interaction are best understood for visual arrestin and rhodopsin (Gurevich and Gurevich, 2006) (Fig. 1-6). Arrestin is divided into the N domain and C domain. According to this model, in the basal state, the conformation of arrestin is maintained by three groups of intramolecular interactions: hydrophobic interaction between the interfaces of N and C domains, ionic interactions among charged residues within the polar core, and a three-element interaction among β-strand I, α helix I and the carboxyl terminus. Upon agonist (light) stimulation, the conformation of receptor changes as the receptor is activated and subsequently phosphorylated by GRK1 (also termed rhodopsin kinase), followed by multiple interactions with arrestin. Arrestin binds via its activation sensor to receptor intracellular regions

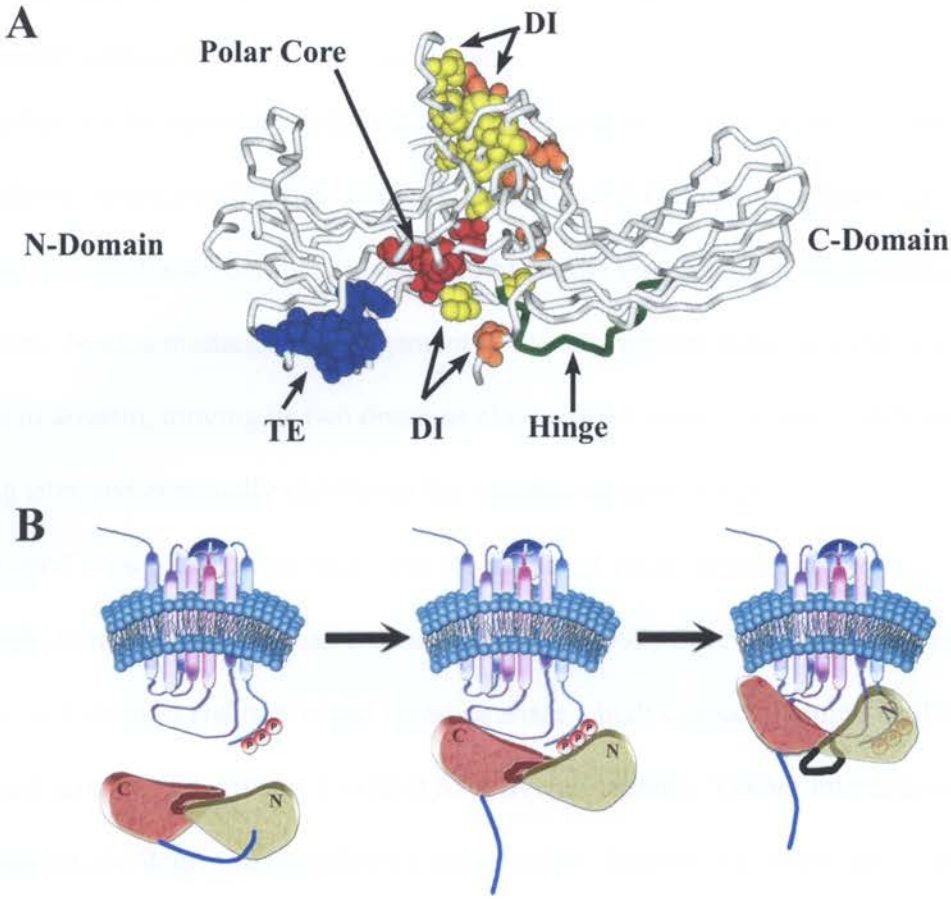

Fig. 1-6 The molecular mechanisms of arrestin-GPCR interaction. A. In its basal state, arrestin is an elongated two-domain molecule supported by three groups of intra-molecular interactions. Side chains of participating residues are shown in CPK: (1) DI, hydrophobic residues participating in the extensive interaction between the bodies of the two domains are shown in lighter (N-domain residues) or darker (C-domain residues) pattern; (2) polar core; (3) TE (three-element interaction), an interaction between β-strand I and α-helix I in the N-domain, and the arrestin C-tail, which folds back from the C-domain. The inter-domain hinge is also highlighted. B. Model of arrestin-receptor interaction. Arrestin binds via its activation sensor to receptor elements that change conformation upon activation (phosphorylation-independent interaction) and via the phosphate sensor to receptor-attached phosphates (phosphorylation-dependent interaction). Simultaneous engagement of both sensors promotes arrestin transition into the active state with concomitant engagement of additional binding sites, stabilizing the arrestin-receptor complex. Note that arrestin in panel B is shown with its N-domain on the right-handed side and that the

arrestin C-tail is highlighted in blue and is released prior to the transition of arrestin into the active state. (adapted from Gurevich and Gurevich, 2006 with modifications)

that change conformation upon activation; this binding event most likely interferes with the hydrophobic interaction between the interfaces of N and C domains. Meanwhile, receptor-attached phosphates sequentially invade and break the three-element interaction and then the polar core. Next, simultaneous engagement of the two sensors induces a global conformational change in arrestin, moving its two domains close to each other, exposing additional receptor binding sites and eventually stabilizing the receptor-arrestin complex.

Several lines of evidence imply that the model of visual arrestin-rhodopsin interaction may also apply to non-visual arrestins (Attramadal et al., 1992; Gurevich and Gurevich, 2006). First of all, visual arrestin and non-visual arrestins share a high degree of amino acid homology. In the rat, visual arrestin and arrestin-3 exhibit 65% overall identity. Taking into account conservative substitutions, the degree of homology is even higher. Second, the crystal structures of visual arrestin and arrestin-2 show extensive similarity. Third, biochemical studies using purified GPCRs, together with mutagenesis and structural studies, demonstrate that the polar core and the three-element interaction exist in all arrestins. Finally, data from these studies imply that activation-sensing mechnism also plays a role in the interaction between non-visual arrestin and GPCRs.

Emerging evidence supports the opinion that when arrestin binds to a receptor, extensive multi-element contact between the two partners takes place, which possibly determines the orientation of arrestin relative to the receptor in the receptor-arrestin complex and the receptor-specific conformation of arrestin (Gurevich and Gurevich, 2006). Two points need to be emphasized. First, the flexibility of the arrestin molecule ensures its best fit to the receptor upon binding, and the receptor subtype specificity resides in inherent structural properties of the

receptor. Second, structurally different receptor-arrestin complexes yield diverse functional consequences, such as different patterns of receptor internalization, resensitization, and degradation. Receptor elements in arrestin binding can be roughly divided into phosphorylation sites (or negatively-charged phosphate mimics) and non-phosphorylation sites. It has become clear that relevant phosphorylation sites and non-phosphorylated binding sites in different receptors could be localized almost anywhere on the intracellular surface of the receptor. For example, in many receptors phosphorylation sites relevant for arrestin binding are in the C-terminal tail or in the IC3, but in some cases they are also found in IC1 or IC2 (Gurevich and Gurevich, 2006); for non-phosphorylation sites, several residues in IC1 and IC2 of rhodopsin have been proved to be directly involved in the interaction with visual arrestin (Gray-Keller et al., 1997; Raman et al., 1999; Raman et al., 2003), whereas for many GPCRs with much larger IC3s and/or C-terminal tails, the direct arrestin binding sites have been localized to these receptor segments (Wu et al., 1997; Gelber et al., 1999; DeGraff et al., 2002). In addition, arrestin-3 was found to bind to the IC2 of the dopamine D_2 receptor, although to a lesser extent than to IC3 (Macey et al., 2004). Most of these data were derived from experiments using synthetic or overexpressed and purified (e.g., GST fusion proteins) peptides representing intracellular receptor fragments. As complementary approaches, arrestin translocation assays and receptor internalization assays have been used extensively to explore arrestin-binding receptor determinants (Gurevich and Gurevich, 2006). In these assays, mutant and chimeric receptors are expressed in a heterologous cellular system and subsequently, upon agonist stimulation, arrestin translocation to the plasma membrane and receptor sequestration from cell surface are examined. Data derived from these approaches are often consistent with those from *in vitro* assays. The

drawback is that these assays themselves often cannot distinguish between phosphorylation-dependent and non-phosphorylation-dependent interactions.

Because of the very large ICs and/or CTs of many GPCRs and the qualitative nature of most *in vitro* assays, precisely localizing receptor determinants for arrestin binding is a challenging task. In my dissertation work, a standard curve was generated using background optical density (i.e., no arrestin) and 3-5 concentrations of purified arrestin-2 or arrestin-3, from which the amount of bound arrestin-2 or arrestin-3 was calculated. The method developed to quantify arrestin makes it possible to compare binding capability of mutant and chimeric receptor peptides and to narrow down the specific binding sites. Taking advantage of this quantitative method, I was able to identify one residue in IC2 and a stretch of 4 residues in IC3 critical for the interaction of the D_2 receptor and arrestin-3.

SPECIFIC AIMS

Specific Aim 1: **To identify critical amino acids that determine ligand binding specificity between D_1 and D_2 dopamine receptors.** The lack of highly selective ligands for individual dopamine receptor subtypes greatly hinders the understanding of physiological functions of each subtype and the development of new pharmacotherapies. D_1 and D_2 receptors are widely distributed and most similar to the pharmacologically defined D1 and D2 receptors, respectively. Potential residues involved in D_1/D_2 pharmacological specificity are most likely to be exposed in the ligand binding pocket but not conserved between these two receptor subtypes. In addition, certain residues that are not exposed in the pocket but proximal to primary binding sites were also included in this investigation because they might indirectly affect the conformation of ligand binding pocket. These selected residues were switched between the D_1 and D_2 receptors and the

receptors were stably expressed in HEK 293 cells, followed by radioligand binding to determine the affinities of the mutant receptors for D_1- and D_2-selective antagonists. Finally, experimental results were rationalized by receptor homology modeling and ligand docking.

Specific Aim 2: **To test the hypothesis that the IC2 and IC3 of dopamine D_2 and D_3 receptors differentially bind arrestins.** The differential regulation of D_2 and D_3 receptors by arrestins is attributed to the different composition of IC2 and IC3 of the two receptors because swapping these loops reverses (presumably) arrestin-dependent receptor trafficking (Kim et al., 2001). Little is known concerning the direct interactions between these two receptors and arrestins. As an initial step, the IC2- and IC3-GST fusions were constructed, expressed, and purified and then were examined for their interactions with purified arrestin-2 and -3 using a GST pull-down assay.

Specific Aim 3: **To further identify residues/regions within IC2 that contribute to the different arrestin-3 binding profiles for dopamine D_2 and D_3 receptors and to identify residues/regions within IC3 that are important for high affinity arrestin-3 binding.** Initial studies demonstrated robust binding of arrestin-3 to IC3 and binding preference for D_2-IC2 over D_3-IC2. To identify residues important for the difference between D_2-IC2 and D_3-IC2, chimeric and reciprocal substitution mutants of GST-IC2 fusions were investigated for their direct interactions with purified arrestin-3. To identify residues important for binding of arrestin-3 within IC3, truncation and substitution mutants of GST-IC3 fusions were investigated for their direct interactions with purified arrestin-3. All these studies were performed using a GST pull-down assay.

Specific Aim 4: **To characterize the potential arrestin binding sites of the dopamine D_2 receptor in HEK 293 cells.** Arrestin-3 and D_2 receptor mutants were overexpressed in HEK 293

cells. These studies examined, first, the trafficking of D_2 receptor mutants from the cell surface to cytoplasm following dopamine stimulation, using radiolabeled antagonists as tools; second, the translocation of arrestin-3 to the plasma membrane following dopamine stimulation using Western blotting; and finally, the ligand binding properties and G-protein coupling of D_2 receptor mutants using radioligand binding assays (including antagonist saturation binding and agonist competition binding assays). These studies were designed to investigate whether the changes in receptor internalization and arrestin translocation caused by mutations in D_2 were attributed directly to disrupted/attenuated arrestin binding or due to non-specific effects such as altered G-protein coupling.

II. STRUCTURAL DETERMINANTS OF PHARMACOLOGICAL SPECIFICITY BETWEEN D_1 AND D_2 DOPAMINE RECEPTORS

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ABSTRACT

To test the hypothesis that pharmacological differentiation between D_1 and D_2 dopamine receptors results from interactions of selective ligands with non-conserved residues lining the binding pocket, we mutated amino acid residues in the D_2 receptor to the corresponding aligned residues in the D_1 receptor and *vice versa*, and expressed the receptors in human embryonic kidney 293 cells. Determinations of the affinity of the 14 mutant D_2 receptors and 11 mutant D_1 receptors for D_1- and D_2-selective antagonists, and rhodopsin-based homology models of the two receptors, identified two residues whose direct interactions with certain ligands probably contribute to ligand selectivity. The D_1 receptor mutant $W99^{3.28}F$ showed dramatically increased affinity for several D_2-selective antagonists, particularly spiperone (225-fold), whereas the D_2 receptor mutant $Y417^{7.43}W$ had greatly decreased affinity for benzamide ligands such as raclopride (200-fold) and sulpiride (125-fold). The binding of the D_1-selective ligand SCH23390 was unaffected, indicating that SCH23390 makes little contact with these ancillary pocket residues. Mutation of $A/V^{5.39}$ caused modest but consistent and reciprocal changes in affinity of the receptors for D_1 and D_2-selective ligands, perhaps reflecting altered packing of the interface of helices 5 and 6. We also obtained some evidence that residues in the second extracellular loop contribute to ligand binding. We conclude that additional determinants of D_1/D_2 receptor-selective binding either are located in that loop or are in the transmembrane helices but, like residue 5.39, indirectly influence the interactions of selective ligands with conserved residues by altering the shape of the primary and ancillary binding pockets.

INTRODUCTION

Dopamine modulates diverse biological functions, including movement, endocrine function, and memory formation, through activation of five distinct dopamine receptor subtypes that belong to the G protein-coupled receptor (GPCR) superfamily and are grouped into two subfamilies, D_1-like dopamine receptors and D_2-like dopamine receptors, based on their structure, pharmacology and transduction pathways. The D_1 and D_2 receptors are the most abundant dopamine receptor subtypes and are most similar to the classical, pharmacologically defined D1 and D2 receptors (Kebabian and Calne, 1979). The D_1 receptor has a long carboxyl terminus and a short third intracellular loop, couples to the adenylate cyclase stimulatory G proteins $G\alpha_{s/olf}$, and stimulates cyclic AMP accumulation. In contrast, the D_2 receptor has a short carboxyl terminus and a long third intracellular loop, couples to the pertussis toxin-sensitive G proteins $G\alpha_{i/o}$, inhibits cyclic AMP accumulation, and also modulates a variety of $G\beta\gamma$-regulated effectors such as calcium and potassium ion channels, mitogen-activated protein kinases, and phospholipases (Neve et al., 2004).

D_1 and D_2 receptor-selective agonists and antagonists are current or potential therapeutic drugs for treatment of schizophrenia, Parkinson's disease, and other neuropsychiatric disorders (Sidhu et al., 2003). Although there are numerous drugs that are highly selective for the D_2 receptor over the D_1 receptor, the chemical diversity of D_1 receptor-selective drugs is lower, and there is little information on the structural features of the two receptors that contribute to D_1/D_2 pharmacological selectivity. The primary binding pocket in catecholamine receptors includes residues in transmembrane helix (TM) 3, TM5, and TM6; in particular, Asp3.32 and a cluster of 3 Ser residues in TM5 interact with the protonated nitrogen and catechol hydroxyls, respectively,

in catecholamine ligands (Strader et al., 1989). In D_2 receptor homology models, we have identified an ancillary binding pocket composed of a cluster of aromatic and nonpolar residues between transmembrane helices 2, 3, and 7 on the extracellular side of the primary binding pocket (Teeter et al., 1994; Neve et al., 2003). We have speculated that these ancillary pocket residues stabilize the binding of drugs with aromatic or non-polar groups oriented towards the ancillary pocket, and that ancillary pocket residues contribute to pharmacological selectivity. For example, several ancillary pocket residues are part of an aromatic microdomain that is important for selectivity between dopamine D_2 and D_4 receptors (Simpson et al., 1999).

We now describe the pharmacological characterization of D_1 and D_2 receptor mutants in which one or more residues were mutated to the corresponding residue(s) in the other receptor subtype. The mutation effects on ligand affinity were rationalized by ligand docking in rhodopsin-based homology models of the D_1 and D_2 receptors. Mutations of residues at three positions in the receptor transmembrane helices, including two ancillary pocket residues, changed receptor affinity for some ligands in a manner consistent with the hypothesis that the residues contribute to pharmacological specificity: position 3.28 (Trp99 in the D_1 receptor), position 7.43 (Tyr417 in the D_2 receptor), and position 5.39 (Ala195 and Val190 in the D_1 and D_2 receptors, respectively). Receptor modeling and ligand docking studies suggest that Trp99 and Tyr417 interact directly with some ligands, but that position 5.39 contributes to pharmacological selectivity indirectly by determining the distance between other binding site residues. Our data also provide some indication that residues in the second extracellular loop (EL2) contribute to D_1/D_2 selectivity.

MATERIALS AND METHODS

Materials. [^{3}H]Spiperone (107 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ), and [^{3}H]SCH23390 (86 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Serum was purchased from Hyclone Laboratories (Logan, UT). (+)-Butaclamol, SCH23390, S-(-)-raclopride, domperidone, haloperidol, spiperone, S-(-)-sulpiride, and most other drugs and reagents, including culture medium, were purchased from Sigma-Aldrich (St. Louis, MO). Piquindone, tropapride, and YM09151-2 were obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program.

Numbering of Residues. Residues are numbered according to their positions in the rat D_{2L} receptor sequence (Monsma, Jr. et al., 1989) or in the rhesus macaque D_1 receptor sequence (Machida et al., 1992). To simplify the identification of corresponding residues in D_1 and D_2 receptors, we also use an index system in which each residue has a number that denotes the transmembrane helix (TM) in which it lies and its location relative to the most conserved residue in that helix (Ballesteros and Weinstein, 1995). The most conserved residue within each helix is assigned the number 50; *e.g.,* the most highly conserved residue in TM3 of the D_2 receptor, Arg132, has the index number 3.50 and is designated Arg132$^{3.50}$. One residue towards the N-terminus from Arg132 is Asp131$^{3.49}$, and one residue towards the C-terminus is Tyr133$^{3.51}$. The position and index numbers of TM residues mutated in this study are provided in Table 1.

Production of Cell Lines. Mutants of the rat D_{2L} receptor and the rhesus macaque D_1 receptor were constructed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Double mutants were obtained through one or two cycles of mutagenesis, whereas triple/quadruple mutants were achieved through two or three cycles. Wild-type and mutant receptors in pcDNA3.1 were transfected into human embryonic kidney 293 cells with

Lipofectamine (Invitrogen, Carlsbad, CA), and clonal cell lines stably expressing the receptors were isolated after selection with G418 (800 μg/ml). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 5% iron-supplemented calf bovine serum, 5% fetal bovine serum, and 600μg/ml G418 at 37°C and 10% CO_2.

Radioligand Binding Assays. Cells were lysed in ice-cold hypotonic buffer (1 mM Na^+HEPES, pH 7.4, 2 mM EDTA) for 15 min, scraped from the plate, and centrifuged at 17,000*g* for 20 min. The resulting crude membrane fraction was resuspended with a Brinkmann Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 6 for 8 to 10 s in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 0.9% NaCl). Membrane proteins (40-100 μg) were incubated in duplicate for 45 min at 37°C, in the case of D_2 receptor, in a total reaction volume of 1 ml with [^{3}H]spiperone at concentrations ranging from 0.01-0.4 nM for saturation binding or ~0.1 nM with the appropriate concentration of the competing drug for competition binding. For characterization of wildtype and mutant D_1 receptors, incubations were carried out in 0.5 ml final volume containing [^{3}H]SCH23390 at concentrations ranging from 0.1-3.0 nM for saturation binding or ~1.0 nM with the appropriate concentration of the competing drug for competition binding. (+)-Butaclamol (2 μM) was used to define nonspecific binding. Data for saturation and competition binding were analyzed by nonlinear regression using the computer program Prism (GraphPad, San Diego, CA) to determine K_d and IC_{50} values. Apparent affinity (K_i) values were calculated from the IC_{50} values by the method of Cheng and Prusoff (1973). In all assays, the free concentration of radioligand was calculated as the concentration added minus the concentration specifically bound.

Receptor Homology Modeling and Ligand Docking.* Based on known homology of

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rhodopsin and dopamine receptors, the sequences of the dopamine D_1 and D_2 receptors were aligned with rhodopsin. The alignments agreed with those found in the G protein-coupled receptor database (www.gpcr.org). Modeling procedures were similar to those previously used to model the sodium site in the D_2 receptor (Neve et al., 2001). Briefly, amino acids for the respective receptor were substituted for the side chains of rhodopsin in the crystal structure (1l9h; Okada et al., 2002) and geometry around Pro substitutions adjusted using a Pro template (Teeter et al., 1994). Improvement of poor contacts by rotamer change and repacking of helices (primarily TM5 and TM6) was accomplished manually using the program Chain (Sack, 1988). Only transmembrane helices were modeled for this study since binding and specificity sites are substantially located in these regions. No energy minimization was used but close contacts were eliminated manually. This modeling procedure has accurately predicted Na^+ binding residues, as confirmed by mutagenesis (Teeter et al., 1994; Neve et al., 2001). Our modeling approach relies heavily on the experimentally determined X-ray structure of rhodopsin (Teeter et al., 1994; Neve et al., 2003).

Ligands were docked into the binding site using previously identified polar groups on the protein as attachment points (Strader et al., 1989): the conserved negatively-charged residue $Asp^{3.32}$ which binds to the positively-charged nitrogen in the aminergic ligands, and the Ser residues 5.42 and 5.46 which interact with polar atoms on the ligand (the O, N, -OH groups or halogens Cl or F). Ligand conformations were either from crystal structures of the ligands or from *ab initio* calculations. The crystal structures of piquindone (Olson et al., 1981), spiperone (Liang et al., 1998), and haloperidol (Reed and Schaefer, 1973) were described previously. For tropapride, 8 conformations were generated from the degrees of freedom and subjected to *ab initio* quantum mechanical calculations using the basis sets 3-21G* and 6-31G* in the program

Spartan (Wavefunction, Inc., Irvine, CA), producing minimized conformations of approximately equal energy. One conformation matched three of the available crystal structures and fit well in our D_2 receptor model (Teeter et al., 2001). The structure of SCH23390 was based on energy minimization and analysis of conformationally constrained analogues (Pettersson et al., 1990).

Once ligands were docked, interactions in the ancillary pocket could be assessed, as described below. Aromatic and aliphatic groups that could bind in the hydrophobic ancillary pocket have varying degrees of rigidity relative to the docked portion of the structure, ranging from spiperone as most rigid to haloperidol as least rigid.

Our model derived from rhodopsin is expected to be the inactive state structure of a GPCR since the rhodopsin crystal structure is in the ground state (*i.e.*, bound to 11-*cis*-retinal). Although the D_2 receptor residue Ser194$^{5.43}$ has also been identified from mutagenesis as important for binding of agonists (Cox et al., 1992), it cannot readily interact directly with the ligand in our ground state model of the dopamine receptors, and may be utilized for the activated state of the receptor.

RESULTS AND DISCUSSION

Mutations Based on Solvent Accessibility and Non-conservation. Seven mutant D_2 receptors were constructed based on the criteria of Javitch and colleagues (Simpson et al., 1999) for identifying amino acid residues that potentially contribute to receptor subtype selectivity: the residues must be exposed in the binding pocket and residue side chain properties should not be conserved between D_1 and D_2 receptors. One residue, Phe110$^{3.28}$, is in TM3 and is predicted to be in the ancillary binding pocket (Teeter et al., 1994; Neve et al., 2003), and a second residue is in TM6 (His394$^{6.55}$). Five residues are in TM7, with three of them (Tyr409$^{7.35}$, Thr413$^{7.39}$, and Tyr417$^{7.43}$) predicted to be in the ancillary binding pocket (Table 2-1). Each residue was mutated to the corresponding residue in the D_1 receptor, Mutant receptors were stably expressed in human embryonic kidney 293 cells, and drug affinity was determined by saturation analysis of the binding of the D_2-like receptor radioligand [^{3}H]spiperone and competition analysis of the binding of seven additional D_2-selective antagonists and the D_1-selective antagonist SCH23390 (Table 2-2; Fig. 2-1).

D_2-Y417W had substantially decreased affinity for most D_2-selective antagonists, consistent with data from other receptors implicating residue 7.43 in ligand binding (Roth et al., 1997; Mialet et al., 2000; Matsui et al., 1995; Cavalli et al., 1996). Substituted benzamides (sulpiride, raclopride, tropapride, and YM-09151-02) were particularly sensitive to this mutation, with their binding reduced 60- to 200-fold. Each of the other mutations caused a modest reduction in affinity for one or more D_2-selective antagonists. None of the mutants had markedly increased affinity for SCH23390, in contrast to what would be expected if they contributed to the D_1 receptor selectivity of this ligand. The lack of effect of mutation of the residues in TM7 on the

binding of SCH23390 is inconsistent with our prior analysis of chimeric D_1/D_2 receptors, which identified this region as being particularly important for the selective binding of SCH23390 and several other benzazepine ligands (Kozell et al., 1994), but our model supports the conclusion that SCH23390 does not contact these ancillary pocket residues (see below).

The sensitivity of ligands to the D_2-Y417W mutation can be explained by the larger size of the Trp residue and its different orientation in the ancillary pocket (Fig. 2-2). For the benzamides, the orientation of the benzyl and ethyl substituents on the five-membered pyridyl ring with the charged nitrogen is key (Fig. 2-1). These all extend toward the cytoplasmic side of the ancillary pocket where they contact residue D_2-417. When Tyr417 is mutated to the bulkier Trp, the affinity of these ligands is decreased.

We created D_1 receptor mutants that were reciprocals of four of the D_2 receptor mutants (Table 2-1). Mutations that contribute to subtype selectivity would be expected to decrease the affinity of SCH23390 and increase the affinity of D_2 receptor ligands. Consistent with this expectation, each mutation caused a modest but statistically significant reduction in affinity for [^{3}H]SCH23390 as determined by saturation analysis (Table 2-2). In contrast, three of the mutations caused little gain of affinity for D_2-selective ligands; D_1-N292$^{6.55}$H, D_1-V317$^{7.39}$T, and D_1-W321$^{7.43}$Y, reciprocals of D_2 receptor mutations that generally decreased the affinity of D_2 ligands, had unchanged or slightly decreased affinity for D_2 ligands except for an almost 4-fold increase in the affinity of D_1-W321Y for piquindone. The mutant D_1-W99F, however, had a 225-fold increase in apparent affinity for spiperone, with smaller increases of 45-, 24-, 3.1-, and 2.7-fold for domperidone, YM-0915-02, tropapride, and haloperidol, respectively. This was surprising because the reciprocal mutation F110$^{3.28}$W had little effect on the affinity of the D_2

receptor for ligands. Fig. 2-3 depicts the dramatic leftward shift in the spiperone competition binding curve (toward D_2 wildtype) observed for D_1-W99F.

The increased affinity for D_2 antagonists that results from the D_1-W99F mutation could be due to both the altered size of this residue at the ancillary pocket opening and the orientation of the aromatic group on the ring with the protonated nitrogen of the ligand. The 225-fold increase in spiperone binding affinity likely comes from the smaller Phe side chain that opens the ancillary pocket in the D_1 receptor. When residue 3.28 is Trp, the pocket is effectively closed (Fig. 2-4A). The mutant Phe residue also has a favorable stacking interaction with the nonpolar N1-phenyl ring that is relatively rigidly held in spiperone (Fig. 2-4B).

Haloperidol matches spiperone in structure except for the more flexible chloro-phenyl substituent *para* to the nitrogen in the pyrrole ring (Fig. 2-1). That the flexible chloro-phenyl substituent can rotate away from Trp99 in native D1 as well as its less optimal stacking with Phe99 in the mutant receptor (Fig. 2-4C) make the improvement in affinity of D_1-W99F for haloperidol relatively smaller than the considerable binding improvement for spiperone. Interestingly, differences between the interactions of haloperidol and spiperone with this residue (Trp99) can account for the entire difference in D_1/D_2 selectivity for spiperone (0.05 nM K_d and 400 nM K_i at D_2 and D_1, respectively, in the experiments in which the wildtype receptors were analyzed together with the mutants D_1-W99F and D_2-F110W; almost 8000-fold selective) and haloperidol (0.8 nM and 68 nM K_i at D_2 and D_1, respectively; 85-fold selective); both ligands are approximately 35-fold selective for the D_2 receptor over D_1-W99F (spiperone and haloperidol K_i for mutant receptor of 1.9 nM and 25 nM, respectively).

Whereas spiperone has a relatively rigidly held phenyl ring, the corresponding substituent on domperidone and YM-09151-02 is free to rotate on the central ring. In domperidone the

substituent is *meta* to the nitrogen in the central piperidine ring, rather than *para* as in spiperone, and in YM-09151-02 the substituent is bound to the charged nitrogen of the pyridyl ring (Fig. 2-1). The similarity in substituent for domperidone and spiperone despite large differences in the effect of the W99F mutation suggests that the orientation of the substituent may be the more important factor in the relative effect of Trp99 on receptor affinity for the two ligands. This argument also applies to YM-09151-02, where the orientation of the phenyl substituent is less favorable for stacking with the mutant Phe99. For D_2-selective ligands whose binding affinity is only slightly elevated or unaffected by the W99F mutation, geometry and flexibility both come into play. The ethyl or benzyl substituents of sulpiride, raclopride, and tropapride are relatively flexible and point towards residue 7.43 rather than residue 3.28 (see above and Fig. 2-2), so that the removal of $Trp99^{3.28}$ enhances their binding weakly or not at all.

Residue 3.28 also contributes modestly to ligand selectivity between D_2 and D_4 receptors, since D_2-F110L has slightly decreased affinity for [^{3}H]spiperone and 5-fold enhanced affinity for the D_4-selective ligand CPPMA (Simpson et al., 1999), and mutation of residues $Leu^{3.28}$ and $Met^{3.29}$ in the D_4 receptor to the corresponding D_2 receptor residues decreases the affinity of many D_4-selective ligands (Kortagere et al., 2004). Thus, the aromaticity and shape of the side chain at this position affects the receptor subtype selectivity of ligands to an extent that depends on the geometry, flexibility, and stacking potential of ligand substituents that are oriented towards outer TM3 and the opening of the ancillary binding pocket.

Although mutations of the two ancillary pocket residues 3.28 and 7.43 have effects that suggest a contribution to the D_2 receptor-selectivity of ligands, none of the mutations substantially changed receptor affinity for the D_1-selective ligand SCH23390 (Table 2-2). D_2-selective antagonists such as spiperone are longer than SCH23390 (Fig. 2-1). Although the

distance from polar halide or –OH to the protonated nitrogen is comparable, the D_2-selective ligands have relatively rigid groups that extend beyond the protonated nitrogen and are parallel to the rest of the molecule, reaching into the ancillary pocket which leads from the primary binding pocket perpendicular to the helix axes. SCH23390, however, contains a phenyl ring perpendicular to the rest of the ligand extending from the ring containing the protonated nitrogen (Pettersson et al., 1990). SCH23390 docked in the D2 receptor model has few interactions in the ancillary pocket because its 1-phenyl substituent extends toward the extracellular surface of the receptor, parallel to the helix axes.

Lack of Reciprocal Effects. The absence of a D_1/D_2 reciprocal effect for the mutations at 3.28 and 7.43 is puzzling. Why did mutation of residue 3.28 enhance binding of some D_2-selective ligands to the D_1 receptor without decreasing their binding to the D_2 receptor, and why did mutation of residue 7.43 decrease binding of some D_2-selective ligands to the D_2 receptor without enhancing their binding to the D_1 receptor? Our D_2 receptor model depicting tight packing of hydrophobic residues in the ancillary pocket (Neve et al., 2003) suggested the hypothesis that the absence of a D_1/D_2 reciprocal effect for mutations at positions 3.28 and 7.43 reflected the context in which the point mutation was made. For example, perhaps changing between Phe and Trp at position 3.28 affects the binding of spiperone only in a receptor (*e.g.*, the D_1 receptor) that also has the bulkier Trp at position 7.43.

To test this hypothesis, we combined the two mutations in the double-mutant receptors D_1-W99F/W321Y and D_2-F110W/Y417W. We also created the triple mutants D_1-W99F/V317T/W321Y and D_2-F110W/T413V/Y417W because Thr413$^{7.39}$ is located within the ancillary binding pocket together with residues 3.28 and 7.43 in our D_2 receptor model (Neve et

al., 2003), and its mutation from Thr to Val modestly reduced D_2 receptor affinity for many D_2-selective ligands (Table 2-2). We predicted that combining the mutations would have additive or synergistic effects on ligand affinity. In contrast to our prediction, the D_1 double and triple mutants had lower affinity for spiperone (*i.e.*, were less D_2-like) than the single mutant W99F (Table 2-3). Furthermore, the extra mutations caused no further increase in affinity for YM-09151-02 or piquindone over that observed for D_1-W99F or D_1-W321Y, respectively (Tables 2-2 and 2-3). On the other hand, the 10-fold decrease in affinity of D_1-W99F/W321Y and D_1-W99F/V317T/W321Y for SCH23390 was greater than the decrease resulting from single mutations of any of the residues, and the D_2-F110W/Y417W double mutant had decreased affinity for [^{3}H]spiperone and tropapride that was roughly equivalent to the additive effects of the two single mutants. Adding the third mutation to the D_2 receptor (D_2-F110W/T413V/Y417W) had little or no additional effect. Overall, these results provided only slight support for our hypothesis that residues at positions 3.28, 7.43, and 7.39 have additive or synergistic effects on the affinity of subtype-selective ligands.

Do D_1 and D_2 Receptors Have the Same Binding Pockets? An alternative hypothesis for the lack of reciprocal effects of the mutations on the binding of D_2-selective ligands is that the specificity/binding sites may not be identical for D_1 and D_2 receptors. While the central hydrogen bonding and electrostatic interactions in the binding site (Ser residues on TM5 and Asp on TM3 – see Fig. 2-2) are conserved between the two receptors, the selectivity (ancillary) pockets may be quite different. As noted above, D_2-selective ligands have relatively rigid groups extending beyond the protonated nitrogen that are parallel to the rest of the molecule, and to the membrane plane, and that reach into the ancillary binding pocket, whereas SCH23390

contains a phenyl ring perpendicular to the rest of the molecule and the membrane plane and parallel to the helix axes. Furthermore, as discussed below, the position of TM5 relative to TM6 appears to differ in the D_1 and D_2 receptors. In the D_1 receptor, this would move the fluorine-substituted ring on spiperone that binds to the Ser residues closer to TM6 and move the rigid N1-phenyl ring closer to the mutated residue in the ancillary pocket ($W99F^{3.28}$). According to our model, on the other hand, reducing the size of the side chain at position 7.43 from Trp to Tyr in D_1-W321Y does not enhance the binding of substituted benzamides because these D_2-selective ligands, with the exception of piquindone, are prevented from reaching 7.43 by the bulky $Trp^{3.28}$ (Fig. 2-2 and Fig. 2-4A). Although one would predict that opening up the ancillary pocket by removing Trp99 in the double mutant D_1-W99F/W321Y would cause benzamide ligands to bind with more D_2-like affinity, we speculate that replacing the two Trp residues with smaller aromatic residues may destabilize helix packing and the ancillary pocket.

Why is piquindone, with modestly enhanced binding to D_1-W321Y (Table 2-2), an exception to this rule? Structure/activity relations for this Na^+-dependent ligand and its derivatives (Teeter and DuRand, 1996) support its binding in a small cleft, adjacent to the ancillary pocket, that stretches in an intracellular direction from $Asp114^{3.32}$ in TM3 towards Na^+-binding pocket residues including $Asp80^{2.50}$ (Neve et al., 2001). In the D_1 receptor, this puts piquindone in Van der Waals contact with Trp321 at the intracellular end of the ancillary pocket, and mutation to Tyr opens up this pocket. Thus, binding of piquindone is enhanced not by the W99F mutation at the mouth of the ancillary pocket, but rather by the W321Y mutation. Finally, the lack of a gain of affinity for D_2-selective ligands with the mutant D_1-W321Y could also be explained by assuming that Tyr417 in the D_2 receptor does <u>not</u> interact directly with benzamide

ligands, and that the loss of affinity for these ligands is an indirect consequence of a mutation-induced perturbation of helix packing.

Mutations Based on Proximity to Primary Binding Residues. Residues that are one helix turn away from key ligand-contacting residues are frequently important for pharmacological specificity (Shi and Javitch, 2002), with a good example being residue 3.28, which is one turn away from the TM3 $Asp^{3.32}$ residue that is the primary contact residue for biogenic amine ligands (Shi and Javitch, 2002). We therefore made the double mutant D_1-Y194F/A195V. $Tyr194^{5.38}$ and $Ala195^{5.39}$ are approximately one helix turn away from two serine residues that are important for agonist binding to dopamine receptors (Cox et al., 1992; Neve et al., 2003), and are part of a stretch of 11 contiguous residues in TM5 that, in the D_2 receptor, are exposed to the water-accessible binding pocket as indicated by their high or moderate reactivity with water-soluble cysteine-modifying reagents (Javitch et al., 1995). The Ala/Val substitution at position 5.39 is quite conservative. The Tyr/Phe substitution at position 5.38 is less conservative, but seemed unlikely to be a major determinant of D_1/D_2 subtype selectivity because the D_4 receptor has the Tyr residue that is shared by all of the D_1-like receptors at this position, instead of the Phe shared by the other D_2-like receptors. Nevertheless, the D_1-Y194F/A195V double mutant showed strong evidence for the presence of selectivity determinants at this locus, with 4- to 12-fold enhanced affinities for the four D_2-selective antagonists tested, and 14-fold decreased affinity for [^{3}H]SCH23390 (Table 2-3).

To explore this region further, we tested the two single mutants D_1-Y194F and D_1-A195V, as well as the reciprocal mutants D_2-F189Y/V190A, D_2-F189Y, and D_2-V190A. We observed that the affinity of the mutant D_1-A195V for [^{3}H]SCH23390 was decreased 11-fold, while the affinity of D_1-Y194F for [^{3}H]SCH 23390 was decreased only 2-fold (Table 2-3); competition

analysis further showed that the mutant D_1-A195V had increased affinity for the D_2-selective antagonists tropapride and piquindone. Thus, most of the effect of the double mutation on the binding of these subtype-selective ligands could be explained by mutation of Ala195. The reciprocal D_2 mutants had changes in affinity that were smaller than those observed for the D_1 mutants, but in the direction consistent with the hypothesis that the residue at position 5.39 contributes to D_1/D_2 selectivity (Table 2-3).

Interestingly, changing the residue at position 5.39 in the α_{1b}–adrenoceptor from Ala to Val, its corresponding residue in the α_{1a}–adrenoceptor, confers on the receptor a more α_{1a}–like pharmacological profile (Perez et al., 1998). This effect was additive with the effect of a mutation from Leu to Met at position 6.55, although for the dopamine receptors we found only a modest effect of mutating D_2-His394$^{6.55}$ or D_1-Asn292$^{6.55}$ (Table 2-2).

How does the relatively conservative Ala/Val substitution, in the amino-terminal part of TM5, reciprocally affect the binding of ligands that differentiate between D_1 and D_2 receptors? In our D_1 and D_2 receptor models, residue 5.39 packs against residue 6.59 on the extracellular side of TM6 (Fig. 2-5). In the D_2 receptor, these residues are relatively large (Val packs against Ile) compared to the D_1 receptor where Ala contacts Pro. Thus, the helices at the extracellular TM5/TM6 interface of the D_1 receptor are closer than the corresponding residues are in the D_2 receptor. In the model, the Cβ-Cβ distance between residues 5.39 and 6.59 is less than 4 Å for D_1 and more than 5 Å for D_2.

Residues in TM5 and TM6 make important contributions to the ligand-binding site. In particular, the TM5 Ser residues at one end of the ligand-binding pocket likely interact directly with ligand and create a polar environment for ligands. Hydrophobic Trp and Phe residues in

TM6 cradle the ligand-binding site on one side. The shorter distance in the D_1 receptor at positions 5.39 and 6.59 results in the Ser residues being closer to TM6 in D_1 receptor (9 Å from $Ser^{5.42}$ Cβ to $Phe^{6.52}$ Cβ) than in the D_2 receptor (10 Å). This brings SCH23390 closer to the aromatic residues on TM6. When Ala in the D_1 receptor is mutated to the Val (as in D_2), the tight packing with Pro causes an increase in the TM5-TM6 distance and movement of a ligand away from the aromatic residues in TM6, enhancing the binding of D_2 ligands, which have a wider profile in the binding pocket, while decreasing affinity for SCH23390.

An interesting aspect of the effect of position 5.39 on D_1/D_2 selectivity is that docking ligands in our receptor models provided no indication of a direct interaction with this residue, one to two helix turns above the primary binding pocket residues in TM5 and TM6. Instead of interacting directly with ligands, position 5.39 appears to affect ligand binding by altering the relative positions of other primary binding pocket residues that are conserved between D_1 and D_2 receptors.

Mutations in the Second Extracellular Loop. The second extracellular loop (EL2) of rhodopsin-family GPCRs has been suggested to play a role in pharmacological specificity (Shi and Javitch, 2002). This is consistent with the structure of EL2 in rhodopsin, where the ligand is covalently attached to the receptor and does not dissociate; EL2 is inserted into the binding pocket in such a way that several residues, surrounding a Cys residue that forms a highly conserved disulfide bond with a Cys residue in TM3, contact retinal (Palczewski et al., 2000). The pharmacological profiles of subtypes of α-adrenoceptors (Zhao et al., 1996), 5-HT receptors (Wurch and Pauwels, 2000), and adenosine receptors (Kim et al., 1996; Olah et al., 1994), are also influenced by residues in EL2. For example, switching three consecutive residues that follow the conserved cysteine in EL2 between α_{1a}- and α_{1b}-adrenoceptors is sufficient to switch

the subtype selectivity of some antagonists (Zhao et al., 1996), and much of the difference between the affinity of canine and human 5-HT_{1D} receptors for ketanserin can be attributed to the presence of a Gln or Leu residue immediately following the conserved Cys in EL2 (Wurch and Pauwels, 2000). Shi and Javitch (2004) identified 5 residues in EL2 of the D_2 receptor that line the binding-site crevice, as determined by the substituted cysteine accessibility method, including 2 residues (Ile184 and Asn186, +2 and +4 relative to the conserved Cys182) that are protected from cysteine-modifying reagents by antagonist binding. Ile184 is shared by D_2 and D_3 receptors, with a conservative Leu substitution in the D_4 receptor. Asn186 is also conserved in D_2 and D_3 receptors, but the D_4 receptor has an Asp residue at that position. To test the hypothesis that these residues contribute to D_1/D_2 receptor pharmacological selectivity, we mutated three (EL2.3) or four (EL2.4) consecutive residues immediately following the conserved Cys in EL2 in the D_1 and D_2 receptors to the corresponding residues in the other subtype. D_2-EL2.3 and D_2-EL2.4 both had substantially decreased affinity for the D_2 receptor antagonist tropapride and modestly decreased affinity for [^{3}H]spiperone, but both mutants also had modestly decreased affinity for SCH23390 (Table 2-3). Similarly, D_1-EL2.4 had substantially decreased affinity for [^{3}H]SCH23390, but unchanged or slightly decreased affinity for the D_2 receptor-selective antagonists (Table 2-3). The loss of affinity observed for some ligands provides some support for the hypothesis that this region of EL2 in dopamine receptors contributes to forming the ligand-binding pocket, but the lack of any gain-of-function (*i.e.*, increased affinity, which is the most rigorous criterion for identifying receptor determinants of pharmacological selectivity) weakens the hypothesis that EL2 contributes to D_1/D_2 receptor selectivity. These results should, however, be interpreted with caution. Residues at positions –1

and –5 relative to the conserved Cys were also identified as lining the binding-site crevice of the D_2 receptor, but were not tested in these studies because the presence of a ligand did not protect them from cysteine-modifying reagents (Shi and Javitch, 2004). Furthermore, the EL2 is considerably longer in the D_1 receptor than in the D_2 receptor, and possibly arrayed very differently in the two receptors in a way that cannot be mimicked by simply exchanging three or four residues. We have not modeled the loops because of their considerable difference and our philosophy to be initially conservative in modeling large differences from rhodopsin.

Dopamine Receptor Ligand Specificity Regions. The specificity regions identified in this study appear to be quite distinct for the D_1 and D_2 receptors (Fig. 2-6). The D_2 receptor contains a specificity pocket consisting of aromatic groups that can increase ligand binding affinity. Protein aromatic groups are well-suited to packing with ligand aromatic groups because their rotation can accommodate different geometries in the ligand. Also, rotation of the protein aromatic group can permit a more closely packed pocket in the absence of the ligand than can other side chains. According to our models, this pocket is not accessible in the D_1 receptor unless it is opened up by mutation of $Trp99^{3.28}$.

In the case of the D_1 receptor, it is the packing of TM5 and TM6 and the size of the primary binding pocket in the vicinity of the aromatic rings of SCH23390 that contribute to D_1 receptor-selective binding. Although we have not modeled the loops for the receptor because of the lack of structural information, the 1-phenyl of SCH23390 is oriented towards and possibly interacts with EL2, so that residues there could influence specificity. Our exploration of residues near the conserved Cys in EL2 has not yet identified such residues.

Summary and Conclusions. To identify structural determinants of D_1/D_2 receptor pharmacological specificity, we mutated residues based on several criteria. Some residues were

selected because they are accessible in the binding site crevice and differ non-conservatively between D_1 and D_2 receptors while being shared within the D_1-like and D_2-like subclasses. Others were selected based on their proximity to primary binding pocket residues. Still other residues were selected to test the hypothesis that a region of EL2 immediately C-terminal to a conserved Cys residue contributes to pharmacological specificity for these receptors.

We identified 2 residues in TM3 and TM7 that appear to contribute to the selectivity of certain D_2 receptor-selective ligands by making direct contact with ligand substituents: residues 3.28 and 7.43. In the D_1 receptor, the mutation $W99F^{3.28}$ enhanced the affinity of ligands that are sufficiently long and inflexible to interact negatively with the bulkier Trp residue, particularly if the ligand geometry permitted a stacking interaction with the Phe residue; this Trp residue accounted for all of the difference in selectivity between the structurally related compounds spiperone and haloperidol. In the D_2 receptor, our model suggests that the mutation $Y417^{7.43}W$ greatly decreased the affinity of ligands such as benzamides because of the the larger size and differing orientation of Trp. In addition, a Val/Ala switch at position 5.39 had reciprocal effects on the binding of D_1- and D_2-selective antagonists, consistent with a role for this residue in pharmacological selectivity. Our D_1 and D_2 receptor models suggest that changes at this position alter the size of the binding pocket by modulating the distance between the extracellular ends of TM5 and TM6. Finally, we obtained modest support for the hypothesis that residues following the conserved Cys in EL2 contribute to pharmacological specificity.

Overall, we have observed that structural determinants of D_1/D_2 receptor-selective binding vary among different classes of dopamine ligands and even within a group of structurally similar ligands. We have mutated most residues that are believed to be exposed to the binding site crevice, that differ between D_1 and D_2 receptors, and that are conserved within the D_1-like and

D_2-like subclasses, and conclude that residues contributing to pharmacological specificity are not in the same location on the two receptors (Fig. 2-6). Furthermore, the residues that have been identified as contributing to pharmacological specificity can account for only a fraction of the difference between D_1 and D_2 receptors. We hypothesize that additional significant determinants of D_1/D_2 receptor-selective binding either are in EL2 or, like Ala/Val$^{5.39}$, affect the overall shape of the primary and ancillary binding pockets rather than interacting directly with ligands.

Footnotes

This work was supported by the VA Merit Review and Career Scientist programs. M.M.T. and C.J.D. thank UC Davis Department of Chemistry for research facilities (M.M.T.) and the Department of Psychiatry for support (M.M.T. and C.J.D.), while on leave from Boston College (M.M.T.).

TABLE 2-1 Numbering of residues mutated in this study. Numbering is provided for both receptors at positions that were mutated in either receptor. For TM residues the index number is that of Ballesteros and Weinstein (Ballesteros and Weinstein, 1995), and for EL2 residues the index denotes the position of the residue relative to the conserved Cys in that loop. * residues predicted to be in the ancillary binding pocket according to our D_2 receptor homology model (Neve et al., 2003).

D_2 Residue	D_1 Residue	Index Number
Phe110*	Trp99	3.28
Ile183	Asp187	+1
Ile184	Ser188	+2
Ala185	Ser189	+3
Asn186	Leu190	+4
Phe189	Tyr194	5.38
Val190	Ala195	5.39
His394	Asn292	6.55
Leu408	Thr312	7.34
Tyr409*	Phe313	7.35
Ser410	Asp314	7.36
Thr413*	Val317	7.39
Tyr417*	Trp321	7.43

TABLE 2-2 Pharmacological characterization of single-residue mutants of the D_1 and D_2 receptors. Affinity values (expressed as mean pK_d or $pK_i \pm$ S.E.) are shown for each ligand at the indicated wildtype or mutant receptor. The number below the affinity value in each cell is the ratio of the K_d or K_i of the wildtype receptor to that of the mutant (mean ± S.E.); a number greater than 1 indicates that the mutation enhanced the affinity of the receptor for a given ligand, while a number less than 1 indicates a loss of affinity. Affinity values for spiperone at wildtype and mutant D_2 receptors and for SCH23390 at wildtype and mutant D_1 receptors are the pK_d determined by saturation analysis of radioligand binding. The pK values for wildtype receptors (D_2-WT and D_1-WT) are the means of all experiments for a given drug, whereas the fold-change for a particular mutant and the statistical significance of the difference in pK values were calculated from only the experiments in which that mutant and the wildtype receptor were tested together. The number of experiments used to determine the fold change is in parentheses. An *asterisk* denotes a pK value that was significantly different from wildtype ($P < 0.05$). Mutation-induced affinity changes that are greater than 10-fold are indicated by bold font.

Receptor	*Drug affinity*								
	SCH23390	Spiperone	Domperidone	Haloperidol	Piquindone	Raclopride	Sulpiride	Tropapride	YM-09151-02
D_2-WT	5.73 ± 0.26	10.31 ± 0.07	9.23 ± 0.08	8.85 ± 0.13	8.25 ± 0.10	8.57 ± 0.10	8.45 ± 0.07	10.46 ± 0.10	10.23 ± 0.16
	1 (7)	1 (18)	1 (6)	1 (6)	1 (7)	1 (6)	1 (9)	1 (9)	1 (6)
D_2-F110W	6.26 ± 0.04	10.04 ± 0.05	8.59 ± 0.04	8.88 ± 0.08	8.25 ± 0.01	8.35 ± 0.13	8.12 ± 0.02	9.77 ± 0.05	9.66 ± 0.19
	1.5 ± 0.1 (4)*	0.6 ± 0.1 (4)*	0.3 ± 0.02 (3)*	0.7 ± 0.1 (3)	1.6 ± 0.4 (4)	0.9 ± 0.1 (3)	0.7 ± 0.03 (4)*	0.4 ± 0.1 (5)*	0.5 ± 0.04 (3)
D_2-H394N	6.58 ± 0.05	10.01 ± 0.10	8.94 ± 0.03	8.20 ± 0.17	7.59 ± 0.03	8.04 ± 0.12	**7.05 ± 0.11**	10.55 ± 0.09	10.28 ± 0.13
	1.7 ± 0.1 (3)*	0.2 ± 0.03 (5)*	0.3 ± 0.01 (3)*	0.4 ± 0.1 (3)	0.1 ± 0.01 (3)*	0.2 ± 0.02 (3)*	**0.04 ± 0.01 (5)***	0.7 ± 0.1 (4)	0.6 ± 0.1 (3)
D_2-L408T	6.26 ± 0.06	10.65 ± 0.05	9.34 ± 0.05	8.73 ± 0.21	8.26 ± 0.06	8.53 ± 0.13	8.44 ± 0.05	10.62 ± 0.08	10.52 ± 0.18
	0.8 ± 0.1 (3)	0.9 ± 0.1 (5)	0.9 ± 0.04 (3)	1.3 ± 0.2 (3)	0.6 ± 0.1 (3)*	0.6 ± 0.1 (3)	0.8 ± 0.1 (5)	0.8 ± 0.1 (4)	1.0 ± 0.3 (3)
D_2-Y409F	6.36 ± 0.07	10.92 ± 0.08	9.44 ± 0.05	8.52 ± 0.08	7.76 ± 0.05	8.01 ± 0.12	8.57 ± 0.08	10.81 ± 0.12	11.00 ± 0.00
	1.0 ± 0.2 (3)	1.7 ± 0.2 (5)	1.1 ± 0.1 (3)	0.8 ± 0.1 (3)	0.2 ± 0.01 (3)*	0.2 ± 0.02 (3)*	1.0 ± 0.2 (5)	1.3 ± 0.3 (4)	3.0 ± 0.4 (3)*
D_2-S410D	6.30 ± 0.04	10.22 ± 0.08	9.05 ± 0.03	8.38 ± 0.16	8.26 ± 0.03	8.64 ± 0.11	8.54 ± 0.13	10.62 ± 0.08	10.54 ± 0.09
	0.9 ± 0.1 (3)	0.3 ± 0.1 (4)*	0.4 ± 0.02 (3)*	0.6 ± 0.1 (3)	0.6 ± 0.03 (3)*	0.8 ± 0.04 (3)	1.0 ± 0.3 (4)	0.8 ± 0.1 (4)	1.0 ± 0.03 (3)
D_2-T413V	5.47 ± 0.28	10.01 ± 0.03	8.51 ± 0.03	8.50 ± 0.07	8.25 ± 0.06	8.28 ± 0.13	7.92 ± 0.08	9.38 ± 0.10	9.51 ± 0.09
	1.1 ± 0.3 (3)	0.5 ± 0.03 (4)*	0.3 ± 0.02 (3)*	0.3 ± 0.1 (3)*	1.6 ± 0.4 (4)	0.8 ± 0.1 (3)	0.4 ± 0.1 (4)*	0.2 ± 0.04 (5)*	0.4 ± 0.1 (3)
D_2-Y417W	5.24 ± 0.64	**9.29 ± 0.04**	8.54 ± 0.03	8.64 ± 0.13	7.27 ± 0.02	**6.10 ± 0.13**	**6.21 ± 0.02**	**8.32 ± 0.12**	**8.00 ± 0.10**
	1.5 ± 0.4 (4)	**0.1 ± 0.01 (4)***	0.3 ± 0.02 (3)*	0.4 ± 0.1 (3)	0.2 ± 0.04 (4)*	**0.005 ± 0.000 (3)***	**0.008 ± 0.001 (4)***	**0.016 ± 0.004 (4)***	**0.014 ± 0.004 (3)***
D_1-WT	9.26 ± 0.04	6.36 ± 0.03	5.83 ± 0.02	7.17 ± 0.01	5.70 ± 0.01	3.82 ± 0.15	4.77 ± 0.08	6.08 ± 0.02	6.09 ± 0.02
	1 (5)	1 (4)	1 (3)	1 (3)	1 (3)	1 (3)	1 (3)	1 (3)	1 (3)
D_1-W99F	9.12 ± 0.02	**8.71 ± 0.02**	**7.48 ± 0.03**	7.60 ± 0.01	5.44 ± 0.04	3.83 ± 0.23	4.21 ± 0.12	6.57 ± 0.03	**7.47 ± 0.03**
	0.7 ± 0.1 (5)*	**225 ± 14 (4)***	**45 ± 4.6 (3)***	2.7 ± 0.03 (3)*	0.5 ± 0.04 (3)*	1.0 ± 0.3 (3)	0.3 ± 0.03 (3)*	3.1 ± 0.1 (3)*	**24 ± 1.2 (3)***
D_1-N292H	8.58 ± 0.03	6.13 ± 0.12	5.64 ± 0.02	6.75 ± 0.04	5.22 ± 0.01	3.13 ± 0.11	4.41 ± 0.07	5.56 ± 0.01	5.86 ± 0.05
	0.2 ± 0.01 (5)*	0.7 ± 0.2 (4)	0.7 ± 0.1 (3)*	0.4 ± 0.04 (3)*	0.3 ± 0.01 (3)*	0.2 ± 0.01 (3)*	0.4 ± 0.04 (3)*	0.3 ± 0.01 (3)*	0.6 ± 0.03 (3)*
D_1-V317T	8.93 ± 0.10	5.88 ± 0.03	5.57 ± 0.05	6.91 ± 0.02	5.76 ± 0.04	3.81 ± 0.12	5.11 ± 0.13	**4.78 ± 0.19**	5.69 ± 0.03
	0.5 ± 0.1 (5)*	0.3 ± 0.02 (4)*	0.6 ± 0.04 (3)*	0.6 ± 0.03 (3)*	1.2 ± 0.1 (3)	1.0 ± 0.04 (3)	2.2 ± 0.5 (3)	**0.05 ± 0.02 (3)***	0.4 ± 0.01 (3)*
D_1-W321Y	8.81 ± 0.03	6.09 ± 0.02	5.81 ± 0.03	6.62 ± 0.04	6.30 ± 0.03	3.56 ± 0.14	4.73 ± 0.05	5.49 ± 0.09	6.02 ± 0.01
	0.4 ± 0.04 (5)*	0.5 ± 0.04 (4)*	0.9 ± 0.04 (3)	0.3 ± 0.02 (3)*	3.9 ± 0.1 (3)*	0.6 ± 0.1 (3)	0.9 ± 0.1 (3)	0.3 ±0.04 (3)*	0.9 ± 0.1 (3)

TABLE 2-3 Pharmacological characterization of multiple-residue mutants of the D_1 and D_2 receptors.

Affinity values (expressed as mean pK_d or $pK_i \pm$ S.E.) are shown for each ligand at the indicated wildtype or mutant receptor. The number below the affinity value is the ratio of the K_d or K_i of the wildtype receptor to that of the mutant (mean ± S.E.); a number greater than 1 indicates that the mutation enhanced the affinity of the receptor for the ligand, while a number less than 1 indicates a loss of affinity. Single-residue mutations to determine the basis for the effects of the 5.38/5.39 double mutant are also included in this table. Affinity values for spiperone at wildtype and mutant D_2 receptors and for SCH23390 at wildtype and mutant D_1 receptors are the pK_d determined by saturation analysis of radioligand binding. The pK values for wildtype receptors (D_2-WT and D_1-WT) are the means of all experiments for a given drug, whereas the fold-change for a particular mutant and the statistical significance of the difference in pK values were calculated from only the experiments in which that mutant and the wildtype receptor were tested together. The number of experiments used to determine the fold change is in parentheses. An *asterisk* denotes a pK value that was significantly different from wildtype ($P < 0.05$). Mutation-induced affinity changes that were greater than 10-fold are indicated by bold font. ND, not determined.

Receptor	*Drug Affinity*				
	SCH23390	Spiperone	Piquindone	Tropapride	YM-09151-02
D_1-WT	9.18 ± 0.03 1 (21)	6.33 ± 0.04 1 (9)	5.57 ± 0.07 1 (6)	5.60 ± 0.1 1 (6)	6.07 ± 0.05 1 (3)
D_1-W99F/W321Y	**8.23 ± 0.04** **0.1 ± 0.01 (3)***	**7.59 ± 0.05** **22 ± 1 (3)***	6.13 ± 0.03 2.5 ± 0.2 (3)*	6.22 ± 0.09 8.8 ± 1.9 (3)*	**7.39 ± 0.05** **20.7 ± 0.3 (3)***
D_1-W99F/V317T/W321Y	**8.21 ± 0.06** **0.1 ± 0.02 (5)***	7.07 ± 0.02 5.5 ± 0.2 (3)*	6.02 ± 0.03 2.0 ± 0.1 (3)*	6.09 ± 0.10 6.5 ± 1.4 (3)*	**7.48 ± 0.03** **25.7 ± 2.4 (3)***
D_1-Y194F/A195V	**7.90 ± 0.03** **0.07 ± 0.01**	7.00 ± 0.02 3.7 ± 0.4 (3)*	**6.84 ± 0.00** **12.5 ± 0.6 (3)***	6.27 ± 0.06 9.4 ± 1.5 (3)*	6.93 ± 0.09 7.2 ± 0.6 (3)*
D_1-Y194F	8.91 ± 0.08 0.5 ± 0.1 (4)*	ND	5.25 ± 0.12 0.7 ± 0.2 (3)	5.59 ± 0.00 0.6 ± 0.1 (3)	ND
D_1-A195V	**8.17 ± 0.05** **0.09 ± 0.03**	ND	6.07 ± 0.07 4.6 ± 0.5 (3)*	6.46 ± 0.02 4.4 ± 1.1 (3)*	ND
D_1-EL2.3 DSS/IIA	9.28 ± 0.10 1.3 ± 0.3 (4)	6.15 ± 0.10 0.7 ± 0.2 (3)	5.52 ± 0.03 0.6 ± 0.03 (3)*	5.08 ± 0.08 0.5 ± 0.1 (3)	5.77 ± 0.04 0.5 ± 0.1 (3)*
D_1-EL2.4 DSSL/IIAN	**7.77 ± 0.03** **0.05 ± 0.004**	5.87 ± 0.08 0.3 ± 0.1 (3)*	5.41 ± 0.06 0.5 ± 0.1 (3)*	4.97 ± 0.19 0.6 ± 0.2 (3)	5.78 ± 0.14 0.5 ± 0.1 (3)
D_2-WT	6.06 ± 0.02 1 (6)	10.04 ± 0.03 1 (12)	ND	9.95 ± 0.05 1 (6)	ND
D_2-F110W/Y417W	6.35 ± 0.03 1.9 ± 0.3 (3)*	**8.52 ± 0.10** **0.04 ± 0.01 (3)***	ND	**7.61 ± 0.49** **0.004 ± 0.001**	ND
D_2-F110W/T413V/Y417W	6.15 ± 0.07 1.2 ± 0.2 (3)	**8.89 ± 0.09** **0.09 ± 0.02 (3)***	ND	**7.53 ± 0.04** **0.003± 0.0005**	ND
D_2-F189Y/V190V	6.46 ± 0.06 2.7 ± 0.3 (3)*	9.56 ± 0.06 0.3 ± 0.04 (7)*	ND	9.71 ± 0.09 0.7 ± 0.1 (3)	ND
D_2-F189Y	5.91 ± 0.03 0.8 ± 0.1 (3)	9.66 ± 0.04 0.4 ± 0.1 (7)*	ND	9.78 ± 0.05 0.8 ± 0.1 (3)	ND
D_2-V190A	6.31 ± 0.06 1.9 ± 0.2 (3)*	9.47 ± 0.08 0.3 ± 0.04 (7)*	ND	9.54 ± 0.07 0.5 ± 0.1 (3)*	ND
D_2-EL2.3 IIA/DSS	5.52 ± 0.07 0.3 ± 0.03 (3)*	9.89 ± 0.03 0.7 ± 0.1 (5)*	ND	**8.32 ± 0.03** **0.02 ± 0.005 (3)***	ND
D_2-EL2.4 IIAN/DSSL	5.46 ± 0.02 0.2 ± 0.02 (3)*	9.33 ± 0.07 0.2 ± 0.04 (5)*	ND	**7.51 ± 0.46** **0.004 ± 0.001**	ND

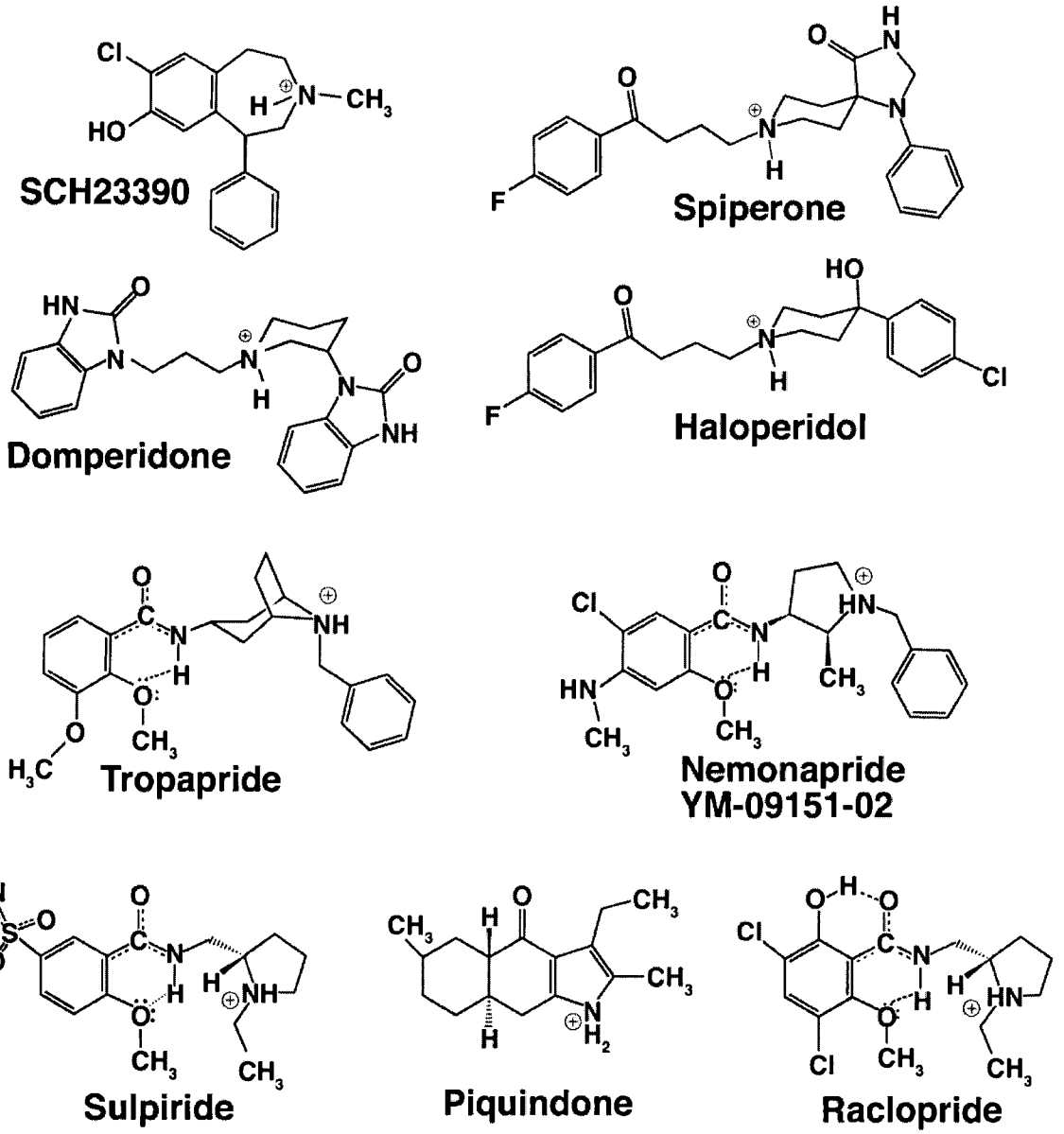

Fig. 2-1 Structures of ligands used in this study.

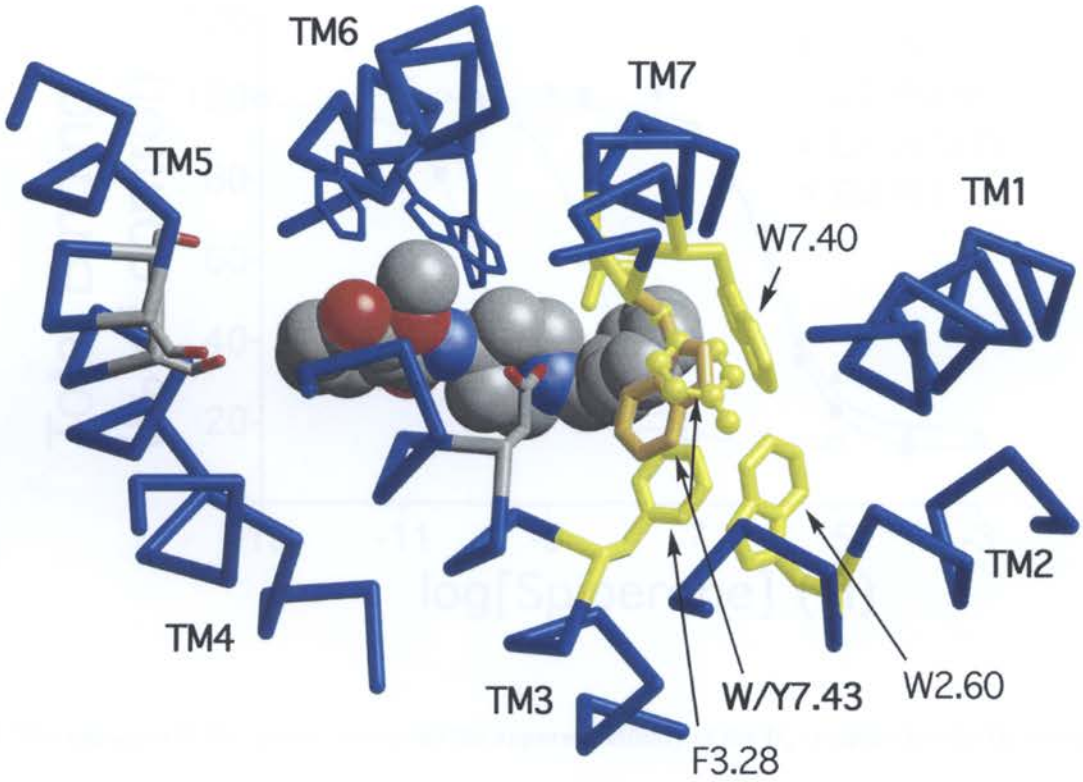

Fig. 2-2 Tropapride binding in the D_2 receptor is decreased by the point mutation of Y417W. The view is from the intracellular side of the primary and ancillary binding pockets. In *yellow* are important residues in the ancillary binding pocket: 2.60, 3.28, 7.40, 7.43. Residues are numbered according to the index of Ballesteros and Weinstein (1995). The differing orientations relative to the ligand of Trp and Tyr at position 7.43 are shown. The D_2 residue Tyr417 (*yellow, ball and stick*) extends across the top of the pocket whereas the D_1 residue Trp (*gold*) extends into the pocket overlapping the benzyl group of tropapride and decreases this ligand's binding to the mutated receptor. Primary binding pocket residues Asp114$^{3.32}$ and Ser193$^{5.42}$ and Ser197$^{5.46}$ are also depicted with oxygen in red and carbon in gray. TM6 aromatic residues in deep blue (Trp358$^{6.48}$, Phe361$^{6.51}$, and Phe362$^{6.52}$) line the binding pocket. The backbone is drawn from Cα to Cα of the respective helices. Tropapride, shown as spacefilling, has colors as above plus nitrogen in blue (work of Dr. Martha Teeter, UC Davis).

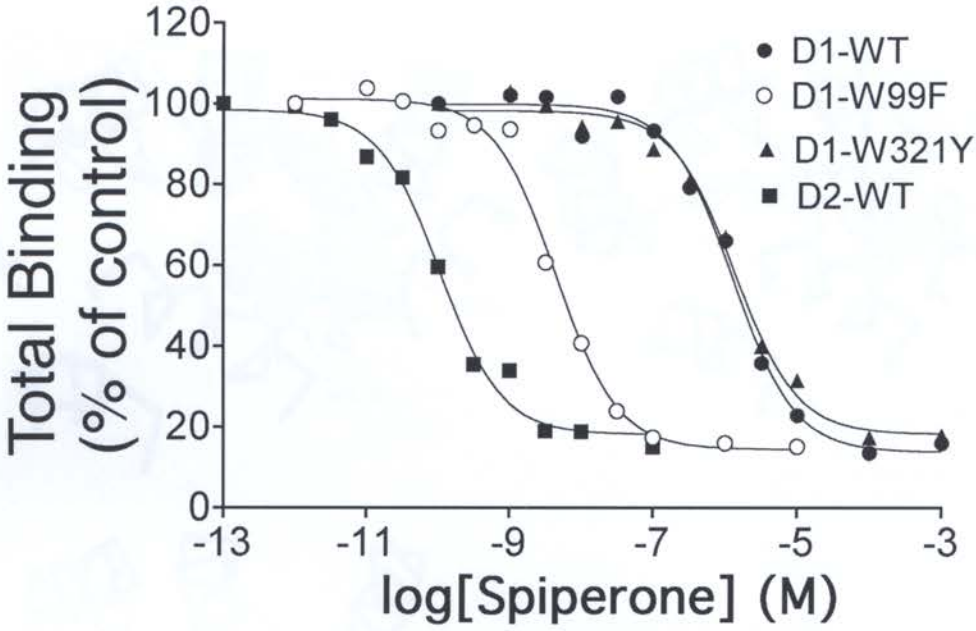

Fig. 2-3 The mutation W99F greatly enhanced the apparent affinity of the D_1 receptor for the D_2 antagonist spiperone. Data are shown from one of three or more independent experiments in which inhibition of the binding of radioligand to the indicated receptor (wildtype D_1 and D_2, and the D_1 receptor mutants $W99^{3.28}F$ and $W321^{7.43}Y$) was determined. Data are plotted as a percentage of the total binding in the absence of spiperone versus the logarithm of the concentration of spiperone. The radioligand was [^{3}H]SCH 23390 for the D_1 wildtype and mutant receptors and [^{3}H]spiperone for the D_2 receptor.

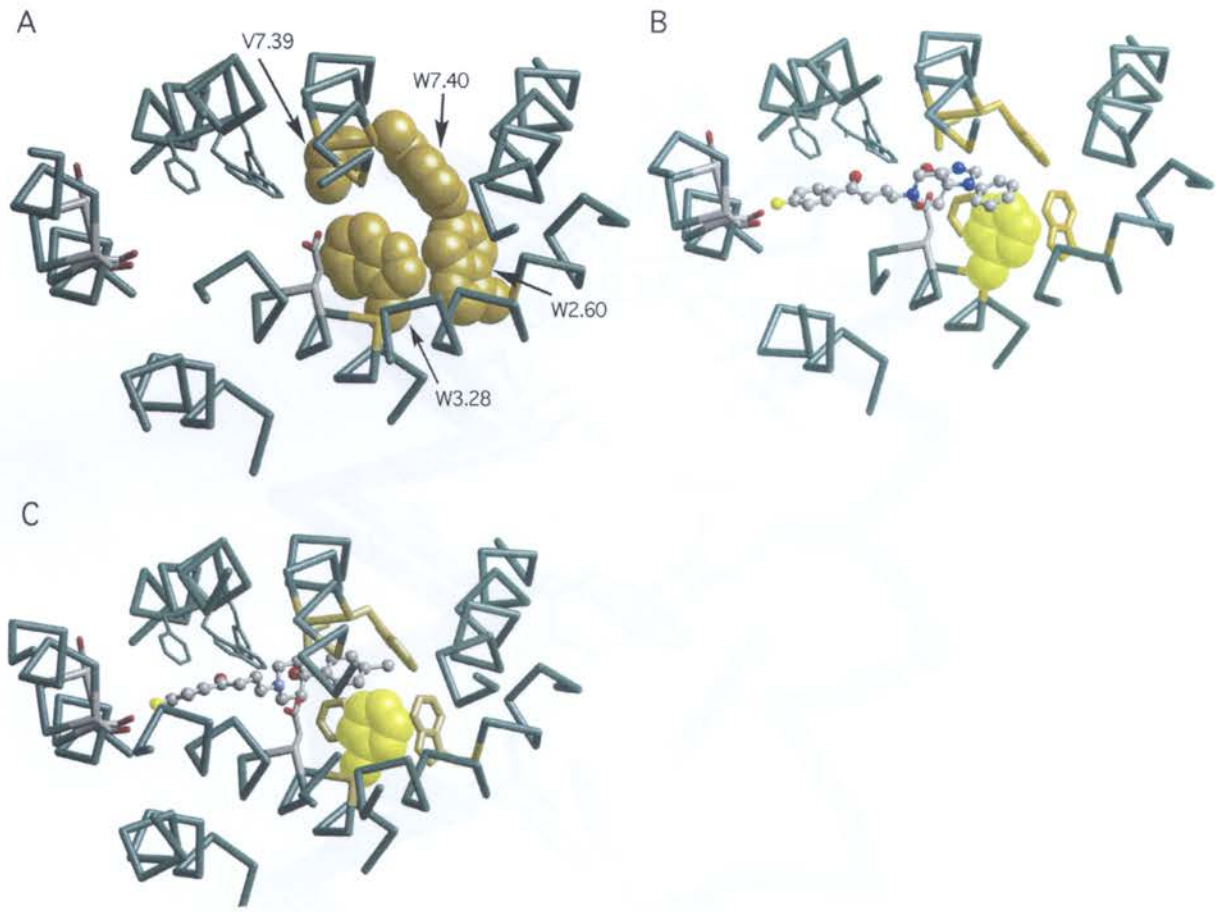

Fig. 2-4 W99F mutation opens the ancillary pocket for binding of D_2 receptor-selective ligands. View of the D_1 receptor from the intracellular side shows the ancillary pocket residues (*gold, space filling*). Side chains of conserved aromatic residues on TM6 involved in binding are shown in *green*. The key contact residues in the binding site (Asp110$^{3.32}$ and Ser residues on TM5) have oxygen color red and carbon gray. **A,** Ancillary pocket of D_1 receptor is constricted by Trp99$^{3.28}$. **B and C,** Depiction of spiperone (*panel B*) or haloperidol (*panel C*) docked into the D_1 receptor with both the D_2 residue Phe (*yellow, space filling*) and wildtype D_1 residue Trp99 (*gold*) shown at position 3.28. Trp99 is partially obscured behind Phe$^{3.28}$. The phenyl ring of spiperone overlaps with Trp99 but is well stacked with Phe. In contrast, the chlorophenyl substituent in haloperidol is able to move away from Trp99 and is edge-to-edge with Phe at that position (work of Dr. Martha Teeter, UC Davis).

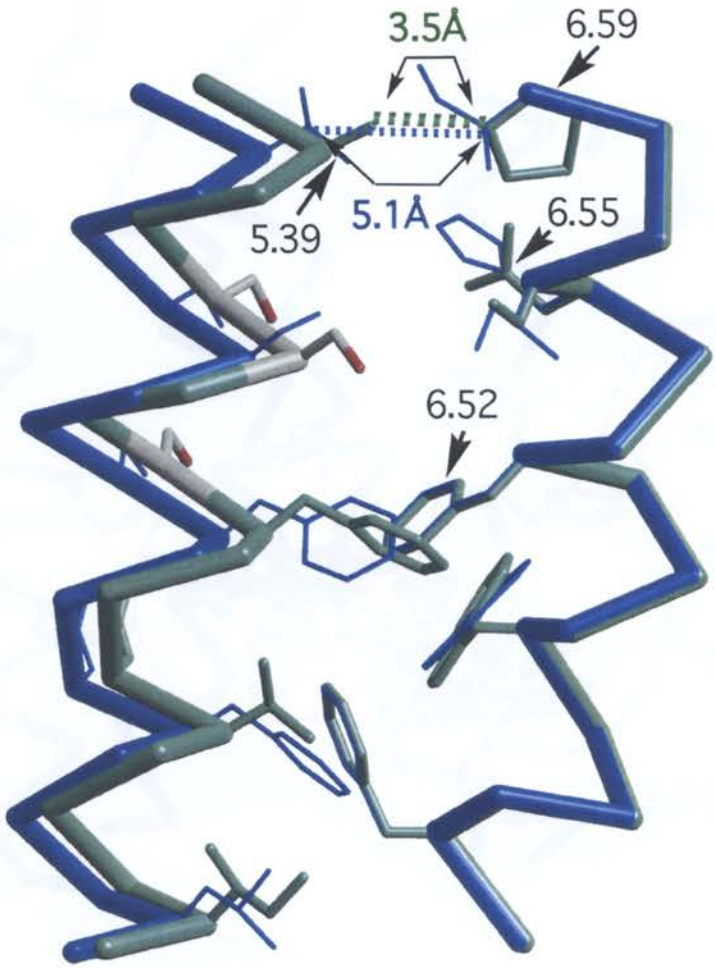

Fig. 2-5 Difference between TM5 to TM6 helix contacts at the Val/Ala mutation site (residue 5.39) contributes to D_1/D_2 receptor binding differences. Residues 5.39 and 6.59 towards the extracellular face of the membrane (top of figure) pack more closely for D_1 (*green*) than for D_2 (*blue*). In the D_1 receptor, the Cβ-Cβ distance for residues 5.39 and 6.59 (Ala and Pro) is relatively close (3.5 Å, large green dots). In the D_2 receptor, the Cβ-Cβ distance for residues 5.39 and 6.59 is longer (~5 Å, fine blue dots) because the Val to Ile contact residues are larger. The positions of two TM6 residues discussed in the text are also indicated (work of Dr. Martha Teeter, UC Davis).

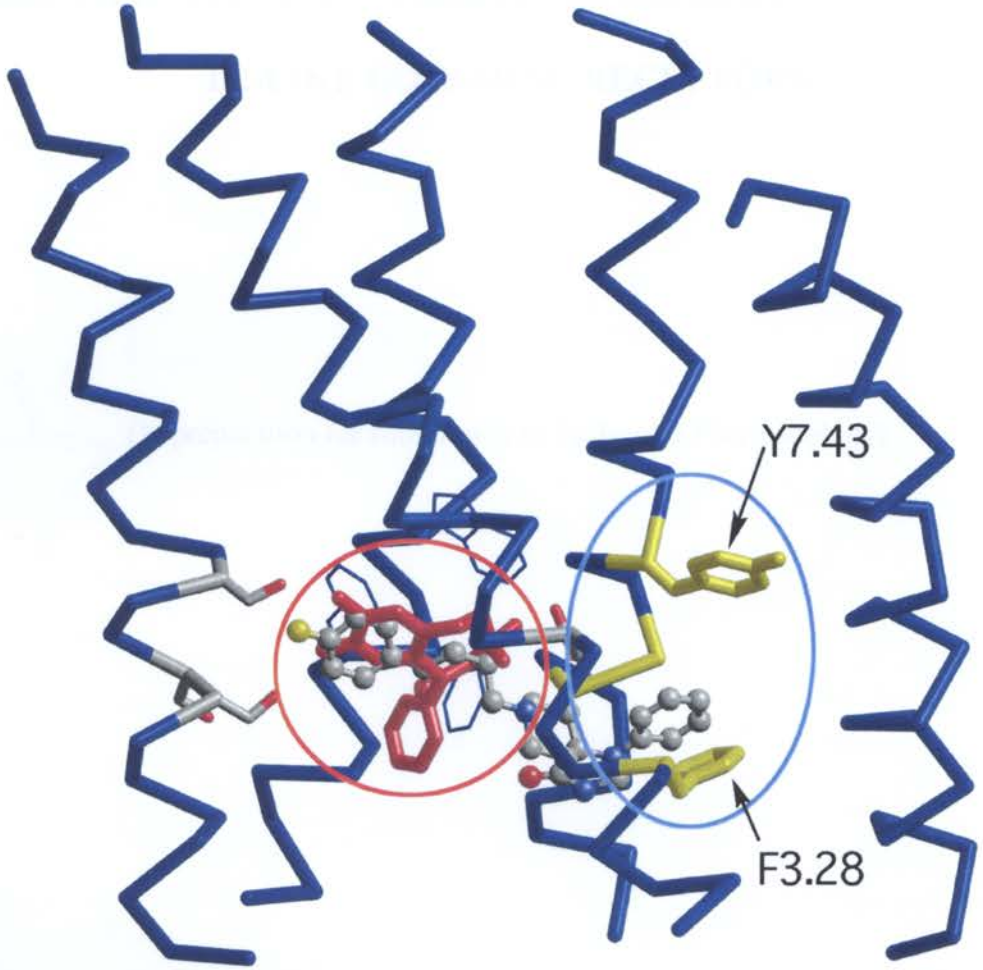

Fig. 2-6 Regions that contribute to selective high-affinity binding to D_1 and D_2 receptors are non-overlapping. D_2 receptor model is depicted with the extracellular face of the membrane at the bottom. For the D_2 receptor, with spiperone docked in the binding pocket, ancillary pocket residues (*yellow* with blue circle) appear most important. For the D_1 receptor, regions of the primary binding pocket in contact with the benzazepine rings of SCH23390 (red circle) appear to contribute most to specificity (work of Dr. Martha Teeter, UC Davis).

III. IDENTIFICATION OF ARRESTIN-BINDING DETERMINANTS ON D2-LIKE DOPAMINE RECEPTORS

(In preparation for submission to Molecular Pharmacology)

ABSTRACT

Non-visual arrestins (arrestin-2 and -3) serve as adaptors linking agonist-activated G protein-coupled receptors (GPCRs) to the endocytotic machinery and as scaffolds for other signaling proteins. It is likely that all GPCRs bind arrestins, but the molecular determinants of binding are still being elucidated. The dopamine D_2 and D_3 receptors have similar structures and signaling mechanisms, but distinct characteristics of interaction with arrestins. Initially, we investigated the molecular mechanism underlying *in vitro* interaction between the two receptors and arrestins. The ability of purified arrestins to bind to glutathione S-transferase (GST) fusion proteins containing different intracellular segments of the receptors, i.e., the second and third intracellular loops (IC2 and IC3), was assessed. Arrestin-3 bound to IC3 and IC2 of both receptors, although IC3 had higher affinity than IC2. Furthermore, IC2 of the D_2 receptor bound arrestin-3 with an affinity higher than that of the D_3 receptor; D_2 residue K149 was particularly important for the preferential binding of arrestin to D_2-IC2. Mutagenesis of the GST-IC3 fusion proteins identified an important determinant of the binding of arrestin3 in the N-terminal region of IC3. Mutation of a stretch of 4 amino acids (IYIV212-215) in this region of the full-length D_2 receptor disrupted receptor-mediated arrestin-3 translocation to the membrane and agonist-induced internalization of the receptor in human embryonic kidney (HEK) 293 cells, without affecting ligand binding or G-protein coupling. These results imply that the differential effects of D_2 and D_3 receptor activation on translocation of arrestin-3 to the cell membrane and the differential modes of receptor internalization are at least in part due to the different binding affinities of D_2- and D_3-IC2 for arrestin-3, and that the sequence IYIV212-215 at the N-terminus of IC3 of the D_2 receptor is required to form a binding site for arrestin.

INTRODUCTION

The non-visual arrestins arrestin-2 and -3 (also termed β-arrestin-1 and -2) are cytosolic proteins involved in homologous desensitization and resensitization of GPCRs, and serve as adaptors to link GPCRs to the endocytotic machinery. In addition, they also redirect GPCRs to alternative G protein-independent signaling pathways (Pierce and Lefkowitz, 2001). Although most or all GPCRs bind arrestins, and phosphorylated serine and threonine residues often comprise part of the arrestin binding site, little information is available concerning common features of receptors that cause them to be recognized by arrestin.

Three D2-like receptors, D_2, D_3, and D_4, have been identified. D2-like receptors are the major targets of antipsychotic drugs. When activated, they couple to the $G\alpha_{i/o}$ family of G proteins, leading to the regulation of adenylate cyclases, potassium channels, calcium channels, and other effectors (Neve et al., 2004). The D2-like receptors are characterized by a long IC3 and a short carboxyl-terminus. Despite the close sequence homology between D_2 and D_3 receptors, agonist-induced receptor phosphorylation, arrestin translocation to the plasma membrane, and receptor internalization for the two subtypes differ dramatically, differences that can be attributed primarily to IC2 and IC3 of the two receptors (Kim et al., 2001).

Numerous studies have examined the role of IC2, IC3, and the carboxyl-terminus of GPCRs in receptor internalization. In the classical model of G protein-coupled receptor kinase- (GRK-) and arrestin-mediated intracellular trafficking of GPCRs, GRK phosphorylates residues in the intracellular segments of receptors, recruiting arrestin to bind to the phosphorylated residues and to other residues whose accessibility is regulated by receptor phosphorylation and by the activation state of the receptor (Lee et al., 2000; Pierce and Lefkowitz, 2001; Kim et al., 2004; Gurevich and Gurevich, 2006). One way to quantify the contribution of non-phosphorylated

residues to the binding of arrestin is to use peptides representing intracellular receptor domains, either measuring their ability to inhibit arrestin binding to receptors or measuring direct binding of arrestin to the receptor fragments. Such studies have demonstrated that arrestin binds to multiple unphosphorylated intracellular domains of GPCRs (Wu et al., 1997; Gelber et al., 1999; Cen et al., 2001; DeGraff et al., 2002; Macey et al., 2004; Macey et al., 2005)

To identify non-phosphorylated arrestin-binding sites of D_2 and D_3 receptors, and the potential contribution of those sites to the preferential binding of arrestins to D_2 over D_3 receptors, we generated GST fusion proteins of two intracellular loops of the receptors and used them in direct binding assays with purified arrestins. We now report that arrestin-3 bound more avidly than arrestin-2 to intracellular domains of both receptors, and more avidly to IC3 than to IC2. Moreover, although arrestin-3 bound with similar affinity to D_2-IC3 and D_3-IC3, arrestin-3 bound preferentially to D_2-IC2 over D_3-IC2. The preferential binding to D_2-IC2 was in large part due to K149 in D_2-IC2; the D_3 receptor has a cysteine residue at this position. Binding of arrestin-3 to D_2-IC2 required 4-5 residues at the N-terminus of the loop. Simultaneous substitution of alanine for four of these residues in the full-length D_2 receptor abolished receptor-mediated recruitment of arrestin-3 to the membrane and agonist-induced receptor internalization in HEK 293 cells, without altering high- or low-affinity agonist binding to the receptor.

MATERIALS AND METHODS

Materials. [^{3}H]spiperone (83 Ci/mmol) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and [^{3}H]sulpiride (77.7 Ci/mmol) from PerkinElmer Life and Analytical Sciences (Boston, MA). Serum was purchased from Hyclone Laboratories (Logan, UT). Dopamine, (+)-Butaclamol, haloperidol, and most reagents, including culture medium, were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies used include: mouse anti-rat arrestin-2 (1/300 dilution; A47520 from BD Transduction Laboratories, Lexington, KY), mouse anti-human arrestin-3 (1/400 and 1/100 dilutions; sc-13140 from Santa Cruz Biotechnology, Santa Cruz, CA), and secondary antibody horseradish peroxidase conjugated goat anti-mouse IgG (1/10,000 dilution; 31430, from Pierce Biotechnology, Rockford, IL). Arrestin-3-pCMV5 was a generous gift from Dr. Marc Caron.

Generation of GST Fusion Proteins and D_2 Receptor Constructs. For construction of the GST fusion proteins the IC2 of the rat dopamine D_{2L} receptor (D_2-IC2), amino acids 130-154, IC2 of the rat dopamine D_3 receptor (D_3-IC2), amino acids 125-151, IC3 of the rat dopamine D_{2L} receptor (D_2-IC3), amino acids 211-371, and IC3 of the rat dopamine D_3 receptor (D_3-IC3), amino acids 210-372, were PCR-amplified, subcloned into *Spe*I-*Xho*I sites in pET-41a(+) (Novagen, Madison, Wisconsin), transformed into NovaBlue competent cells, sequenced, and subsequently transformed into Rosetta 2(DE3) competent cells (Novagen, Madison, Wisconsin). For purification of GST fusion proteins, Rosetta 2(DE3) cells were grown in 2X YTK medium containing kanamycin (50 ug/ml) at 37° C to $A_{600} = 0.8$ and induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 hr at 32° C. Cells were pelleted, resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, 0.5 mg/ml lysozyme, pH 8.0) containing Complete protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany), and incubated for 20 min with gentle rotation at

room temperature. The homogenates were clarified by centrifugation, and supernatants were applied to microcentrifuge tubes containing Glutathione Sepharose 4B beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and purified as described by the manufacturer (GE Healthcare). To quantify the amounts of fusion proteins, SDS sample loading buffer was applied to beads bound with purified proteins, samples were separated by SDS-PAGE and the gel was stained with Gel Code Blue (Pierce, Rockford, IL). BSA was used as a standard.

The IC2 chimeras, D_2-IC3 substitution mutants, and the A4 mutant (myc-D_2-IYIV212-215A4) were constructed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) through one or more mutagenesis steps, with GST-D_2-IC2, GST-D_2-IC3, or the myc-D_2 receptor as a template. The IC3 truncation mutants were generated using a QuikChange method modified to introduce large truncations (Makarova et al., 2000), using GST-D_2-IC3 or GST-D_3-IC3 plasmid as template. The myc-D_2 receptor (referred to herein as wildtype D_2 to differentiate it from the D_2-A4 mutant) was constructed by cloning a rat D_{2L} receptor cDNA into *Sfi*I-*Xho*I sites in the pCMV-Myc vector (BD Biosciences Clontech, Mountain View, CA).

Purified Arrestin Binding to GST Fusion Proteins. GST fusion proteins bound to glutathione Sepharose 4B beads were incubated with purified bovine arrestin-2 or arrestin-3 in arrestin binding buffer (25 mM Tris.HCl, 150 mM NaCl, pH 7.2, Complete protease inhibitor tablet, 0.1% Triton X-100) for 30 min at room temperature. Incubation mixtures were washed four times in wash buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.2, 0.1% Triton X-100), and the proteins were released with SDS sample loading buffer. Proteins were separated by SDS-PAGE, transferred to PVDF membranes that were then blocked with 5% nonfat-dry milk in Tris-buffered saline (TBS), and detected by immunoblotting using anti-arrestin-2 (1:300 dilution in TBS) or anti-arrestin-3 (1:400 dilution in TBS) antibody, with horseradish peroxidase-

conjugated goat anti-mouse IgG (1:10,000 dilution in TBS) as secondary antibody. Visualization of the secondary antibody was performed using the SuperSignal West Pico Chemiluminescence kit (Pierce, Rockford, IL) and quantified by IP Lab (Scanalytics, Fairfax, VA). The amount of bound arrestin-2 or arrestin-3 was calculated from linear regression of a standard curve generated using background optical density (i.e., no arrestin) and 3-6 concentrations of arrestin-2 or arrestin-3 varying between 0.25 and 20 ng. Saturation analysis of the binding of arrestin was carried out by incubating various concentrations of arrestin-2 or -3 with a fixed concentration of GST alone (150 or 175 ng), GST-IC2 (200 ng) or GST-IC3 (300 ng) for 30 min at room temperature. The resulting concentration-response curves were analyzed by nonlinear regression using Prism 3.0 (Graphpad Software) and statistical comparisons of the curves were made using two-way ANOVA followed by Bonferroni post test analysis. In experiments where only one concentration of arrestin was used, statistical significance was evaluated using a paired *t* test.

Internalization Assay. Internalization was measured using the intact cell [^{3}H]sulpiride binding assay described by Itokowa et al. (Itokawa et al., 1996). HEK 293 cells grown to 80% confluency were co-transfected with 30 ng D_2 wildtype or 10 μg D_2-A4 mutant receptor DNA (the A4 mutant was expressed on the membrane at a much lower density so higher amount of DNA was used to achieve similar expression level) and 3 μg arrestin-3-pCMV5 using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Cells were split into 2 plates after 12 hr, and 2 days later rinsed once with pre-warmed, calcium- and magnesium-free phosphate-buffered saline (CMF-PBS; 138 mM NaCl, 4.1 mM KCl, 5.1 mM sodium phosphate, 5 mM potassium phosphate, and 0.2% glucose, pH 7.4), and preincubated for 15 min with pre-warmed, CO_2-saturated serum-free Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.4, at 37° C. Cells were stimulated with 10 μM dopamine in the same HEPES-buffered medium at

37° C for 20 min. Stimulation was terminated by quickly cooling the plates on ice and washing the cells three times with ice-cold CMF-PBS, after which cells were gently scraped from the plate in 2 ml of ice-cold CMF-PBS assay buffer (CMF-PBS containing 2 mM EDTA and 0.001% bovine serum albumin). Cells were gently mixed, added to assay tubes in a final volume of 250 μl with [^{3}H]sulpiride (5 nM final concentration), and incubated at 4° C for 150 min in the absence and presence of unlabeled haloperidol (10 μM final concentration). The assay was terminated by filtration through Whatman GF/C filters presoaked with 0.05% polyethylenimine using a 96-well Tomtec cell harvester (Orange, CT) and ice-cold wash buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl). Filters were allowed to dry, and BetaPlate scintillation fluid (50 μl) was added to each sample. Radioactivity on the filters was determined using a Wallac 1205 BetaPlate scintillation counter (Gaithersburg, MD). To confirm the expression of arrestin-3, the remaining cells were pelleted and resuspended with ice-cold lysis buffer (20 mM HEPES, 20 mM NaCl, 5 mM EDTA, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and Complete protease inhibitor tablet). The suspensions were gently rocked on an orbital shaker at 4° C for 60 min and then were centrifuged at 100,000*g* for 30 min at 4° C. The supernatant was saved and immunoblotting of overexpressed arrestin-3 was performed as described below.

Arrestin-3 Translocation. Transient expression of wildtype D_2 or D_2-A4 mutant receptor with arrestin-3 and dopamine stimulation of cells were performed as described above, except that each plate was split into 3 plates after transfection (one plate for the intact cell [^{3}H]sulpiride binding to detect the levels of receptor expression, performed as described above but without dopamine treatment). Stimulation was terminated by quickly cooling the plates on ice and washing the cells once with ice-cold CMF-PBS. Cells were lysed with 1 ml ice-cold lysis buffer (20 mM HEPES, 20 mM NaCl, 5 mM EDTA, and Complete protease inhibitor tablet), scraped,

collected, homogenized with a glass-Teflon homogenizer, and sonicated for 8-10 seconds. Samples were centrifuged at 1,000*g* for 10 min at 4° C. Supernatants were transferred to new centrifuge tubes and centrifuged at 100,000*g* for 30 min at 4° C. Supernatants were collected; pellets were rinsed carefully with ice-cold CMF-PBS and then resuspended with 100 µl CMF-PBS. The abundance of arrestin-3 in both pellet and supernatant fractions was quantified by immunoblotting as described above, except with a 1:100 dilution of the anti-arrestin-3 antibody and the addition of 0.1% Tween 20 and 5% dry milk to the incubations with primary and secondary antibodies.

Radioligand Binding Assays. Cells expressing wildtype D_2 receptor or the D_2-A4 mutant were lysed in ice-cold hypotonic buffer (1 mM Na^+HEPES, pH 7.4, 2 mM EDTA) for 15 min, scraped from the plate, and centrifuged at 17,000*g* for 20 min. The resulting crude membrane fraction was resuspended with a Brinkmann Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 6 for 8 to 10 s in TBS for saturation assays of the binding of [^{3}H]spiperone, or resuspended in preincubation buffer (50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol), preincubated for 30 min at 37° C, centrifuged at 17,000*g* for 10 min, and resuspended again in Tris assay buffer (50 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.001% BSA, 0.002% ascorbic acid) for competition binding studies in which dopamine displacement of the binding of [^{3}H]spiperone was assessed. Membranes (40-100 µg protein) were incubated in duplicate in a total reaction volume of 1 ml with [^{3}H]spiperone at concentrations ranging from 0.01-0.6 nM for saturation binding or ~0.1 nM with the appropriate concentration of the competing drug dopamine for competition binding. (+)-Butaclamol (2 µM final) was used to define nonspecific binding. Reactions were incubated at 37° C for 45 min and terminated by filtration as described above. Data for saturation and

competition binding were analyzed by nonlinear regression using the computer program Prism 3.0 (GraphPad, San Diego, CA) to determine K_d and IC_{50} values. Apparent affinity (K_i) values were calculated from the IC_{50} values by the method of Cheng and Prusoff (Cheng and Prusoff, 1973). In all assays, the free concentration of radioligand was calculated as the concentration added minus the concentration specifically bound.

RESULTS

Robust Binding of Arrestin-3 to IC3 and Binding Preference for D_2-IC2 over D_3-IC2. Four receptor fragment fusion proteins, GST-D_2-IC3, GST-D_3-IC3, GST-D_2-IC2, and GST-D_3-IC2, were constructed and the binding of arrestin determined using an *in vitro* GST pull-down assay. To identify conditions for equilibrium binding, the rate of association of arrestin-3 with GST-D_2-IC3 was determined. The half-time for binding was approximately 2 minutes and the binding approached equilibrium within 15 minutes. All other GST binding assays were carried out for 30 minutes. Arrestin-3 bound avidly to both GST-D_2-IC3 and GST-D_3-IC3, showing no apparent difference between the two IC3 fusion proteins (Fig. 3-1). Arrestin-3 also bound to IC2, although with a lower affinity than to IC3 (Fig. 3-2, Table 3-1). Maximal binding of arrestin-3 was also greater for GST-D_2-IC3 (10.1 ± 0.3 ng) than for GST-D_2-IC2 (5.5 ± 0.7 ng) (Table 3-1). Interestingly, arrestin-3 bound much more avidly to IC2 from the D_2 receptor than to IC2 from the D_3 receptor (Fig 3-2). Arrestin-2 bound weakly to all of the fusion proteins (Fig. 3-1 and Fig. 3-2).

Identification of Arrestin-3 Binding Sites within IC2 of the D_2 Receptor. To identify residues that contribute to the stronger binding of arrestin-3 to GST-D_2-IC2 than to GST-D_3-IC2 and that might contribute to preferential binding of arrestins to the full-length D_2 receptor than to the D_3 receptor, we constructed chimeric proteins combining portions of IC2 from the D_2 and D_3 receptors and carried out additional binding assays using a single concentration of arrestin-3 (300 ng). A chimera in which the amino-terminal half of D_2-IC2 was replaced with that portion of D_3-IC2 (D_2-IC2/NT-D_3; Fig. 3-3A) bound arrestin as avidly as GST-D_2-IC2 (Fig. 3-3B). In contrast, replacing the carboxyl-terminal half of D_2-IC2 with that portion of D_3-IC2 (D_2-IC2/CT-D_3)

dramatically reduced the amount of arrestin binding to about the level of GST-D_3-IC2 (Fig 3-3A, 3-3B). Thus, the stronger binding of arrestin to GST-D_2-IC2 than to GST-D_3-IC2 can be attributed to residues in the carboxyl-terminal half of IC2.

Additional mutants were constructed to define key binding residues within the C-terminal half of D_2-IC2 (Fig. 3-3A). The 7 most C-terminal residues in IC2 are shared between the D_2 and D_3 receptors except for K149 and T153 in the D_2 receptor, which are C147 and A151 in the D_3 receptor. The D_2 double mutant D2-IC2-K149C/T153A showed lower arrestin binding that was indistinguishable from arrestin binding to GST-D_3-IC2, and its reciprocal D_3 mutant D_3-IC2-C147K/A151T gained binding of arrestin to a level between GST-D_2-IC2 and GST-D_3-IC2 (Fig. 3-3C). Analysis of the single-residue mutants in this region indicated that K149 of the D_2 receptor is a possible contact residue for the binding of arrestin to GST-D_2-IC2, because binding of arrestin to D_2-IC2-K149C was similar to the double mutant D_2-IC2-K149C/T153A and to D_3-IC2, and binding to D_3-IC2-C147K was similar to the double D_3-IC2 mutant D_3-IC2-C147K/A151T. On the other hand, single-residue substitutions at the D_2-T153 and D_3-A151 position had little effect on the binding of arrestin-3 (Fig. 3-3D).

Effects of mutations within the first 4-6 residues of the C-terminal half of IC2, where there is no amino acid identity between D_2 and D_3 receptor, could not be explained solely by whether residues were from the D_2-IC2 or the D_3-IC2. For example, D_3-IC2 is two residues longer than D_2-IC2, and inserting two residues from D_3-IC2 (G143 and Q144) into the corresponding position in D_2-IC2 (D_2-IC2+GQ) dramatically reduced arrestin binding, but the reciprocal D_3-IC2 mutant in which G143 and Q144 were deleted (D_3-IC2-GQ) did not gain arrestin binding (Fig. 3-3C). Furthermore additional D_2-to-D_3 mutations in D_2-IC2+GQ, changing N143 to Q

and T144 to H (D_2-IC2M4), restored arrestin binding to a level equal to or higher than wildtype D_2-IC2, while the further addition of D_2-to-D_3 mutations R145G and Y146T (D_2-IC2M6) virtually abolished arrestin binding. Similarly, although whether the N-terminal half of IC2 is from D_2 or D_3 had no influence on arrestin binding in the context of wildtype C-terminal half of IC2 (D_2-IC2 vs. D_2-IC2/NT-D_3; Figure 3-3B), the mutants D_2-IC2M6 and D_3-IC2-C147K/A151T have identical C-terminal halves but had very different arrestin-binding properties (Fig. 3-3C). Surprisingly, in the context of this chimeric C-terminal half of IC2, more arrestin binding was observed when the N-terminal half of IC2 was derived from the D_3 receptor (D_3-IC2-C147K/A151T) than from the D_2 receptor (D_2-IC2M6). It seems that for residues 143-146 of the D_2 receptor and 139-144 of the D_3 receptor, the overall context in which individual residues are displayed is critical for determining their effect on the binding of arrestin.

Identification of Arrestin-3 Binding Sites within IC3 of the D_2 and D_3 Receptors. The higher affinity of IC3 than IC2 for arrestin-3 (Table 3-1) suggests that IC3 plays a more important role than IC2 in arrestin-3 binding to both D_2 and D_3 receptors. Intracellular residues in proximity to the transmembrane domains 5 and 6 are critical for many GPCR-cytosolic protein interactions, so we made truncation/deletion mutants to identify regions of arrestin-3 binding to IC3, focusing on residues close to those transmembrane domains. GST pulldown assays revealed that deletion of the first 20 residues of D_2-IC3 (D_2-IC3ΔNT20) dramatically decreased its arrestin-binding capability; when the last 20 amino acids were deleted (D_2-IC3ΔCT20), the binding capability also decreased, though to a lesser extent; when both the first and last 20 residues were deleted (D_2-IC3Δ(NT20+CT20)), the binding was totally disrupted (Fig 3-4). In contrast, deletion of ~120 residues comprising all of IC3 except the 20 residues at each terminus

(D_2-IC3ΔMID) had little effect on the binding of arrestin (Fig 3-4). For the D_3 receptor, the arrestin binding patterns of the truncation/deletion mutants were the same as for the D_2 receptor (Fig 3-4C).

Having localized arrestin-3 binding to the first and last 20 residues of IC3, with the N-terminal segment playing a more important role, we constructed additional N-terminal truncation mutants of D_2-IC3 in which the first 5 or 10 or the second 10 amino acids were deleted (Fig 3-5A). In the GST pull-down assay, deletion of either the first 5 (D_2-IC3ΔNT5) or 10 (D_2-IC3ΔNT10) residues decreased arrestin binding by about 60%, whereas deletion of the second 10 residues (D_2-IC3ΔNT10-2) had little effect (Fig 3-5B). We also made smaller C-terminal truncation/deletion mutants, deleting the last 5 or 10 or the penultimate 10 residues, but none of these deletions caused a significant reduction in the binding of arrestin (data not shown). This is consistent with the lesser role of the C-terminus suggested by the 20-residue truncation, and suggests that any contiguous 10 residues in the C-terminus are sufficient for the smaller contribution of the C-terminus to the binding of arrestin.

Because most of the effect of deleting the N-terminal 20 residues of D_2-IC3 could be attributed to the first 5 residues (D_2-IC3ΔNT5), we made additional mutations within this segment (Fig. 3-6A). Deletion of the first 3 residues (D_2-IC3ΔNT3) had no effect on the binding of arrestin-3 (Fig 3-6B). On the other hand, deletion of D_2-IC3 residues 2-5 (IYIV212-215 in the D_2 receptor, D_2-IC3ΔIYIV) was as deleterious to the binding of arrestin as deletion of the first 5 residues (D_2-IC3ΔNT5) (Fig 3-6B).

Within the cytosolic domains of many integral membrane proteins, the motif YXXϕ (where Y is tyrosine, X is any amino acid, and ϕ is an amino acid with a bulky hydrophobic group)

mediates endocytosis and other intracellular trafficking events (Boll et al., 1996; Collins et al., 2002; Vogt et al., 2005). A copy of this motif, YIVL213-216, is at positions 3-6 of D_2-IC3, and a deletion mutant of residues 2-6 that removes the entire motif (D_2-IC3ΔIYIVL) caused a 90% loss of arrestin binding that was greater than the loss of binding to D_2-IC3ΔIYIV (Fig. 3-6A and 3-6B) and similar to the 83% loss of binding caused by the N-terminal 20-residue truncation (D_2-IC3ΔNT20; Fig. 3-5). Finally, we mutated IYIV212-215 or IYIVL212-216 to alanines, creating the substitution mutants D_2-IC3IYIV212-215A4 and D_2-IC3IYIVL212-216A5 that correspond to the deletion mutants D_2-IC3ΔIYIV and D_2-IC3ΔIYIVL (Fig. 3-6A). The substitution mutations were as effective or more effective than their corresponding deletion mutations at decreasing the binding of arrestin (Fig. 3-6C).

The A4 Mutation Had no Effect on D_2 Receptor Affinity for Ligands. The effect of the A4 (IYIV212-215A4) mutation in the context of the full-length D_2 receptor was evaluated by analysis of radioligand binding to membranes prepared from HEK 293 cells transiently expressing wildtype D_2 and D_2-A4 mutant receptors. Saturation analysis of the binding of the D_2-selective antagonist radioligand, [^{3}H]spiperone, yielded K_d values of 83 ± 13 pM and 86 ± 4 pM for D_2 and D_2-A4 receptors, respectively (Table 3-2, Fig 3-7A). Competition binding analysis of the ability of dopamine to decrease the binding of [^{3}H]spiperone indicated that high- and low-affinity binding of dopamine to the A4 mutant was indistinguishable from binding to the wildtype D_2 receptor (Table 3-2; Fig 3-7B), suggesting that the A4 mutation did not alter D_2 receptor affinity for ligands or coupling to G proteins.

The A4 Mutation Abolished Arrestin-3 Translocation and Receptor Internalization. The interaction of D_2 and D_2-A4 receptors with arrestin was evaluated by quantifying agonist-

induced translocation of arrestin to the cell membrane and agonist-induced receptor internalization in HEK 293 cells transiently co-expressing wildtype or mutant D_2 receptor and arrestin-3. The amount of arrestin-3 in the membrane fraction of cells transfected with the wildtype D_2 receptor was doubled from 1.0 ± 0.2 ng of arrestin-3 in untreated cells to 2.1 ± 0.3 ng after treatment with 10 μM dopamine for 20 min (Fig 3-8A). In contrast, in cells coexpressing D_2-A4 and arrestin-3, the levels of arrestin-3 in the membrane preparations were equal in vehicle- and dopamine-treated cells (1.2 ± 0.1 vs. 1.1 ± 0.1, respectively), indicating that the A4 mutation prevented dopamine-induced arrestin-3 translocation to the plasma membrane (Fig 3-8A).

D_2 receptor internalization was assessed by quantifying the agonist-induced loss of cell surface binding of the hydrophilic ligand [^{3}H]sulpiride in an intact cell binding assay. In cells transiently expressing the wildtype D_2 receptor and arrestin-3, treatment with 10 μM dopamine for 20 min decreased binding of [^{3}H]sulpiride by 31 ± 2% (Fig. 3-8B). Consistent with the lack of arrestin-3 translocation mediated by the A4 mutant and the role of arrestin binding in D_2 receptor internalization (Macey et al., 2004), the A4 mutation abolished dopamine-induced receptor internalization. Together, these data suggest that the motif IYIV212-215 at the N-terminus of the D_2 receptor IC3 is required for a functional interaction between the receptor and arrestin-3.

DISCUSSION

Studies of receptor-mediated translocation of GFP-tagged arrestin to the cell membrane have identified IC2 and IC3 as being particularly important for the interaction between D2-like receptors and arrestin and for the preferential association of arrestin with the D_2 over the D_3 receptor (Kim et al., 2001). In the present study, we constructed receptor fragment-GST fusion proteins as "bait" to identify arrestin-binding subdomains. We determined that IC2 and IC3 from both D_2 and D_3 receptors bound arrestin-3 more avidly than arrestin-2 and that arrestin binding to IC3 was stronger than to IC2. Our data also indicated that D_2-IC2 bound arrestin more strongly than D_3-IC2, but that D_2-IC3 and D_3-IC3 bound arrestin with similar affinities. Four residues at the N-terminus of D_2-IC3 were critical for arrestin binding, and alanine mutations of those four residues abolished D_2 receptor-mediated translocation of arrestin and receptor internalization, without altering receptor coupling to G proteins.

Accumulating evidence suggests that multiple mechanisms contribute to GPCR-arrestin interactions. Phosphorylation of receptor intracellular domains by GRKs may promote arrestin binding due to electrostatic interactions between negatively charged phosphates on the receptor and positively charged arrestin residues that serve as a phosphorylation sensor (Gurevich and Gurevich, 2006). These electrostatic interactions may also induce a conformational change in arrestin that enhances its binding to the receptor. Phosphorylation of the receptor may also contribute indirectly to arrestin binding by initiating conformational changes of the intracellular domains that expose binding sites for arrestin (Kim et al., 2004; Liu et al., 2004). Receptor activation is also accompanied by conformational changes, exposing receptor sites that interact with a theoretical activation sensor in arrestin (Gurevich and Gurevich, 2006). Because the GST fusion proteins are not phosphorylated, this method does not identify receptor phosphorylation

sites that bind arrestin, but can identify only determinants of binding that are revealed by activation- or phosphorylation-dependent conformational changes of the receptor intracellular domains; the determinants are presumably occluded in the inactive and/or unphosphorylated full-length receptor but exposed when the receptor fragments are expressed as GST fusion proteins, free from restrictions imparted by other domains of the intact receptor. Thus, this *in vitro* binding assay may identify both phosphorylation-independent and some phosphorylation-dependent determinants of the interaction between receptor and arrestin.

Arrestin-binding domains/residues vary among GPCRs, based on results from binding studies with purified arrestins along with recombinant or synthetic peptides representing receptor intracellular fragments. Both arrestin-2 and arrestin-3 bind to IC3 of the 5-HT_{2A} receptor (Gelber et al., 1999) and the δ-opioid receptor (Cen et al., 2001), and to the C-terminal domain of δ- and κ-opioid receptors, with certain serine/threonine residues in these receptor regions being important for binding (Cen et al., 2001). Binding of arrestin-2 to the M_3-muscarinic receptor requires both N- and C-terminal regions of the IC3 of the M_3-muscarinic receptor subdomains, whereas for arrestin-3 the C-terminal region of the IC3 is sufficient for binding (Wu et al., 1997). Attempts to define more precisely non-phosphorylated residues that are sites of arrestin binding have identified the highly conserved DRY sequence at the N-terminus of IC2 (Hüttenrauch et al., 2002), an aspartate residue in IC3 of the luteinizing hormone/choriogonadotropin receptor that is thought to mimic a phosphorylated residue (Mükherjee et al., 2002), and BXXBB motifs present in the N- and C-terminal portions of IC3 of the α_2-adrenoceptors (DeGraff et al., 2002). Our studies on basic residues in similar locations in the D_2 receptor suggest that they are not involved in arrestin-3 binding (see Chapter IV).

Our GST pull-down studies demonstrated the ability of D2-like receptor IC2 and IC3 to bind arrestin, and also that arrestin bound with higher affinity to GST-D_2-IC3 (K_d = 72 nM) than to GST-D_2-IC2 (242 nM). These values are lower than the micromolar affinities derived from surface plasmon resonance studies (Cen et al., 2001; Liu et al., 2004). Although arrestin bound with equal affinity to IC3 from D_2 or D_3 receptors, we observed that there was a marked difference in the binding of arrestin to IC2 from the two receptors, with binding to GST-D_2-IC2 being much stronger than to GST-D_3-IC2. Preferential arrestin binding to D_2-IC2 could contribute to the stronger interactions between the D_2 receptor and arrestin in intact cells. Additional studies to identify the determinants of selective arrestin binding to D_2-IC2 revealed that residues in the C-terminal half of IC2 account for the preference of arrestin-3 for D_2-IC2 over D_3-IC2, with K149 playing a particularly important role, although additional unidentified residues and/or the overall conformation of the loop also seem to be involved in selective arrestin binding.

Studies with full-length receptors have used direct binding of arrestin, arrestin translocation, and receptor internalization as assays to explore receptor determinants of arrestin binding (Gurevich and Gurevich, 2006), although with these assays it is sometimes difficult to distinguish between phosphorylation-dependent and -independent determinants of binding. These approaches revealed that receptor elements involved in arrestin binding can be localized almost anywhere on the intracellular surface of the receptor, including in the C-terminal tail (Qian et al., 2001; Charest and Bouvier, 2003; Liang et al., 2003; Barthet et al., 2005), IC3 (Lee et al., 2000; Kim et al., 2001; DeGraff et al., 2002; Wang and Limbird, 2002; Namkung and Sibley, 2004), IC2 (Raman et al., 1999; Bennett et al., 2000; Kim et al., 2001), and IC1 (Raman et al., 1999; Kishi et al., 2002).

For the D2-like receptors, where both IC2 and IC3 participate in the stronger interaction of arrestin with the D_2 receptor than the D_3 receptor, preferential phosphorylation of the D_2 receptor contributes to selective binding of arrestin (Kim et al., 2001). Our results suggest that phosphorylation-independent features also contribute to selective binding, or that at least part of the effect of receptor phosphorylation is to alter the accessibility of other receptor subdomains, including perhaps IC2. A recent report on the IC2s of rhodopsin-family GPCRs implicates the amino half of IC2 in the association between receptor and arrestin, in particular a conserved proline residue 6 positions after the conserved DRY motif (Marion et al., 2006). Our data from GST pull-down assays support a direct contribution of IC2 to the binding of arrestin; on the other hand, the stronger interaction of arrestin with D_2-IC2 than with D_3-IC2 was owing to non-conserved sequences in the carboxyl-terminal half of IC2.

The predominant pathway for GPCR endocytosis/internalization involves arrestin-dependent recruitment into clathrin-coated vesicles (Ferguson, 2001). A number of motifs have been proposed to be required for receptor endocytosis, including NP($X_{2,3}$)Y, DRYXXV/IXXPL, BXXBB, and a dileucine motif, all located within or close to the transmembrane proximal domains of IC2, IC3, and the carboxyl-terminus of GPCRs (Barak et al., 1994; Moro et al., 1994; Arora et al., 1995; Gabilondo et al., 1997; DeGraff et al., 2002). Interestingly, a membrane-proximal YXXϕ motif, where ϕ is any bulky hydrophobic residue, was reported to interact with μ2 subunit of the AP2 complex and to be important for clathrin-mediated endocytosis of many integral membrane proteins (Ohno et al., 1995; Boll et al., 1996; Owen and Evans, 1998; Collins et al., 2002; Royle et al., 2005; Vogt et al., 2005). In our study, via GST pull-down assays, we identified a 5-residue site at the N-terminus of D_2-IC3 that incorporates the YXXϕ motif and that is required for high-affinity binding of arrestin, IYIVL. Mutation of four of these residues to

alanine in the full-length receptor prevented both D_2 receptor-mediated translocation of arrestin to the membrane and agonist-induced receptor internalization.

When identifying receptor determinants for arrestin binding and receptor internalization, it is important to consider that the membrane proximal regions of receptor intracellular segments serve as binding sites for many receptor-interacting proteins, including G proteins, making it difficult to establish that effects of a mutation are due to loss of binding of one particular protein. In our studies, we were able to use the GST pull-down assay to identify a short stretch of amino acid residues that were critical for arrestin binding but whose mutation to alanine did not alter high-affinity binding of dopamine to the D_2 receptor, suggesting that coupling of the mutant receptor to G proteins was not affected by the mutation. Although binding of arrestin and G proteins to GPCRs is presumably mutually exclusive, which is the basis for arrestin-induced desensitization, our data indicate that the receptor structural determinants of binding for arrestin and G proteins can be distinguished.

TABLE 3-1 Saturation analysis of binding of arrestin-3 to receptor fragments. K_d values were calculated based on the molecular weight of arrestin-3 (~54 kDa). B_{max} values represent the amount of arrestin-3 bound to 300 ng of GST-IC3 (~44 kDa) or 200 ng of GST-IC2 (~30 kDa). Each value represents the mean ± S.E. of 3 or more independent experiments. Although for GST-D_3-IC2 the highest concentration of arrestin used was lower than the calculated K_d, the similarity between B_{max} values for GST-D_2-IC2 and GST-D_3-IC2 suggests that the K_d estimate for GST-D_3-IC2 is also reliable.

Fusion Protein	Arrestin-3	
	K_d (*nM*)	B_{max} (*ng*)
GST-D_2-IC3	72 ± 14	10.1 ± 0.3
GST-D_3-IC3	79 ± 14	10.2 ± 0.3
GST-D_2-IC2	242 ± 66	5.5 ± 0.7
GST-D_3-IC2	1100	5.0

TABLE 3-2 Binding characteristics of the wildtype and A4 mutant dopamine D_2 receptors. Each value represents the mean ± S.E. of 3 or more independent experiments.

Receptor	K_d [^{3}H]Spiperone (*pM*)	$K_i$high for dopamine (*nM*)	$K_i$low for dopamine (*μM*)	Receptor in high affinity state (%)
D_2 wildtype	83 ± 13	9.0 ± 2.9	4.3 ± 0.5	22.1 ± 3.5
D_2-A4	86 ± 4	11.2 ± 3.2	3.6 ± 0.7	22.7 ± 3.4

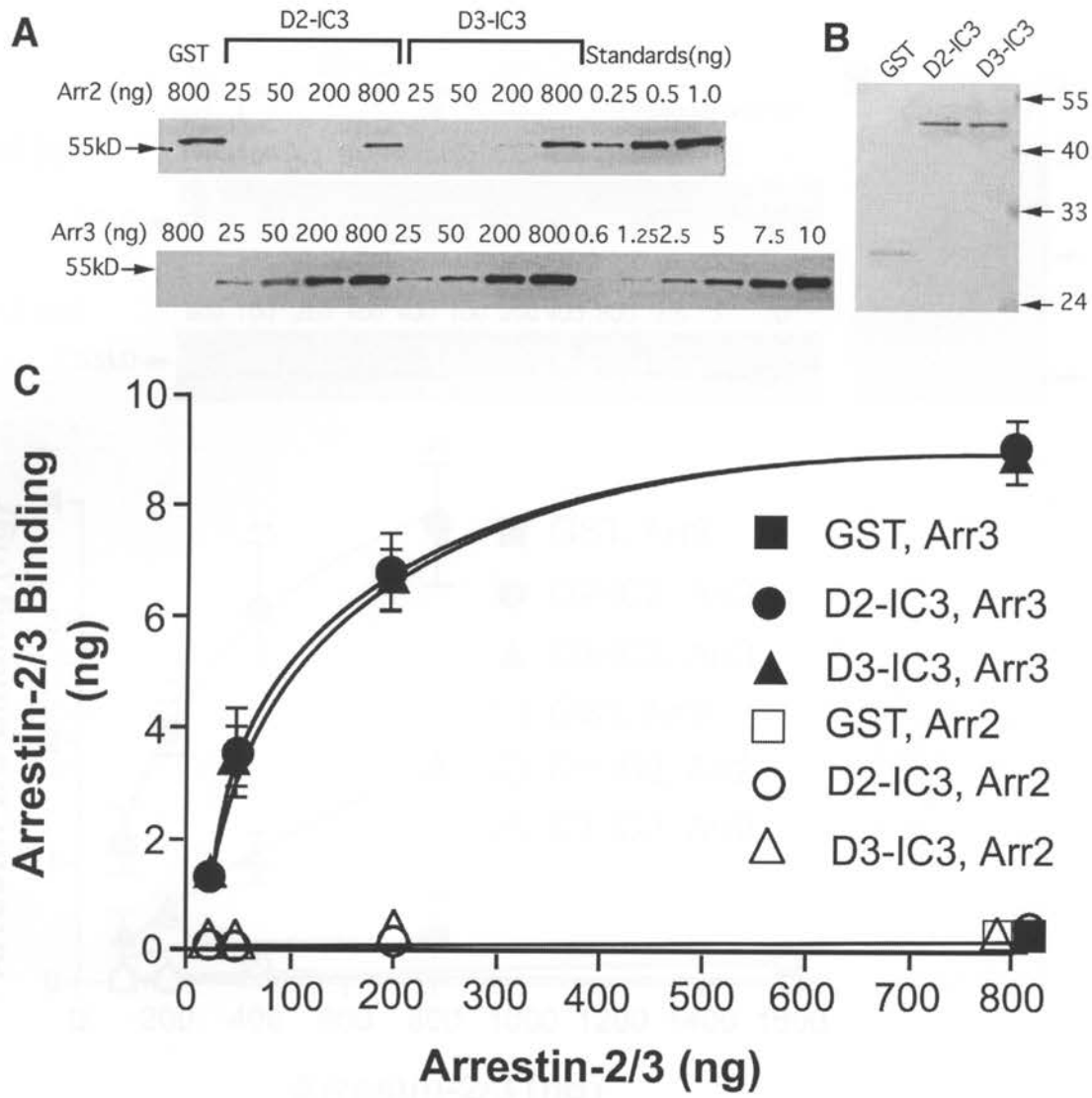

Fig. 3-1 Binding of arrestins to GST-D_2-IC3 and GST-D_3-IC3 fusion proteins. GST alone (GST, 150 ng) or receptor third intracellular loop GST fusion proteins (GST-D_2-IC3 and GST-D_3-IC3, 300 ng) were incubated with the indicated amount of arrestin-2 or arrestin-3. The amount of arrestin that co-eluted with GST or the GST fusion proteins was determined by immunoblotting with anti-arrestin antibodies. Results were quantified using standard curves constructed with known amounts of arrestin-2 and arrestin-3. A. Immunoblots are shown from an experiment representative of 4 independent experiments. Arrestin standards are shown on the right of each blot. B. The protein-stained gel demonstrates that equal amounts of GST-D_2-IC3 and GST-D_3-IC3 were included in the reactions. C. Mean ± SE are shown for the binding of arrestins to IC3 fusion proteins. The amount of arrestin bound is plotted against the amount of arrestin included in the pull-down assay.

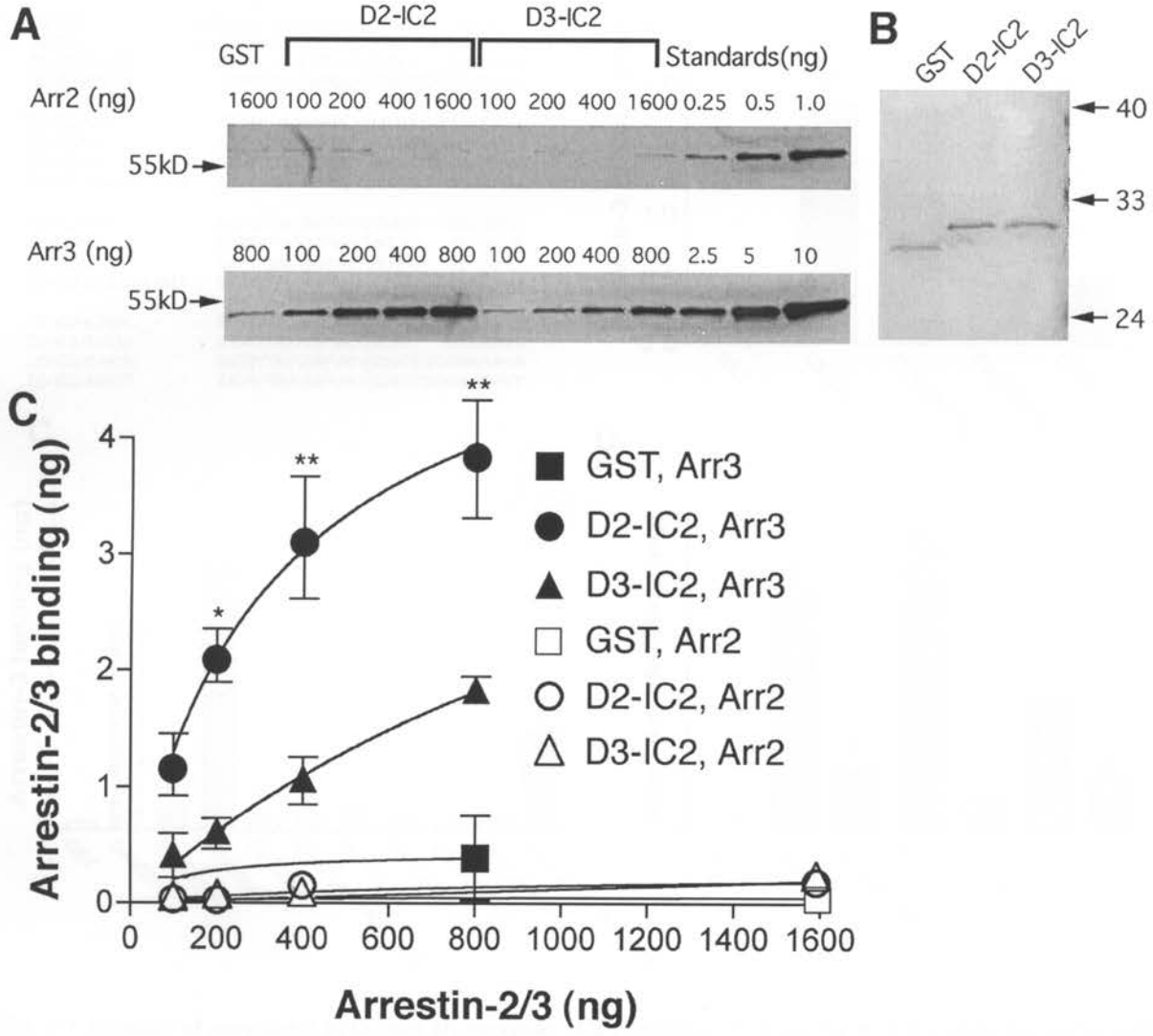

Fig. 3-2 Binding of arrestins to GST-D_2-IC2 and GST-D_3-IC2 fusion proteins. GST alone (GST, 175 ng) or receptor second intracellular loop GST fusion proteins (GST-D_2-IC2 and GST-D_3-IC2, 200 ng) were incubated with the indicated amount of arrestin-2 or arrestin-3. The amount of arrestin that co-eluted with GST or the GST fusion proteins was determined by immunoblotting with anti-arrestin antibodies. Results were quantified using standard curves constructed with known amounts of arrestin-2 and arrestin-3. A. Immunoblots are shown from an experiment representative of 3 independent experiments. Arrestin standards are shown on the right of each blot. B. The protein-stained gel demonstrates that equal amounts of GST-D_2-IC2 and GST-D_3-IC2 were included in the reactions. C. Mean ± SE are shown for the binding of arrestins to IC3 fusion proteins. The amount of arrestin bound is plotted against the amount of arrestin included in the pull-down assay. * $P < 0.05$, ** $P < 0.01$ compared to D_3-IC2 by Bonferroni post test.

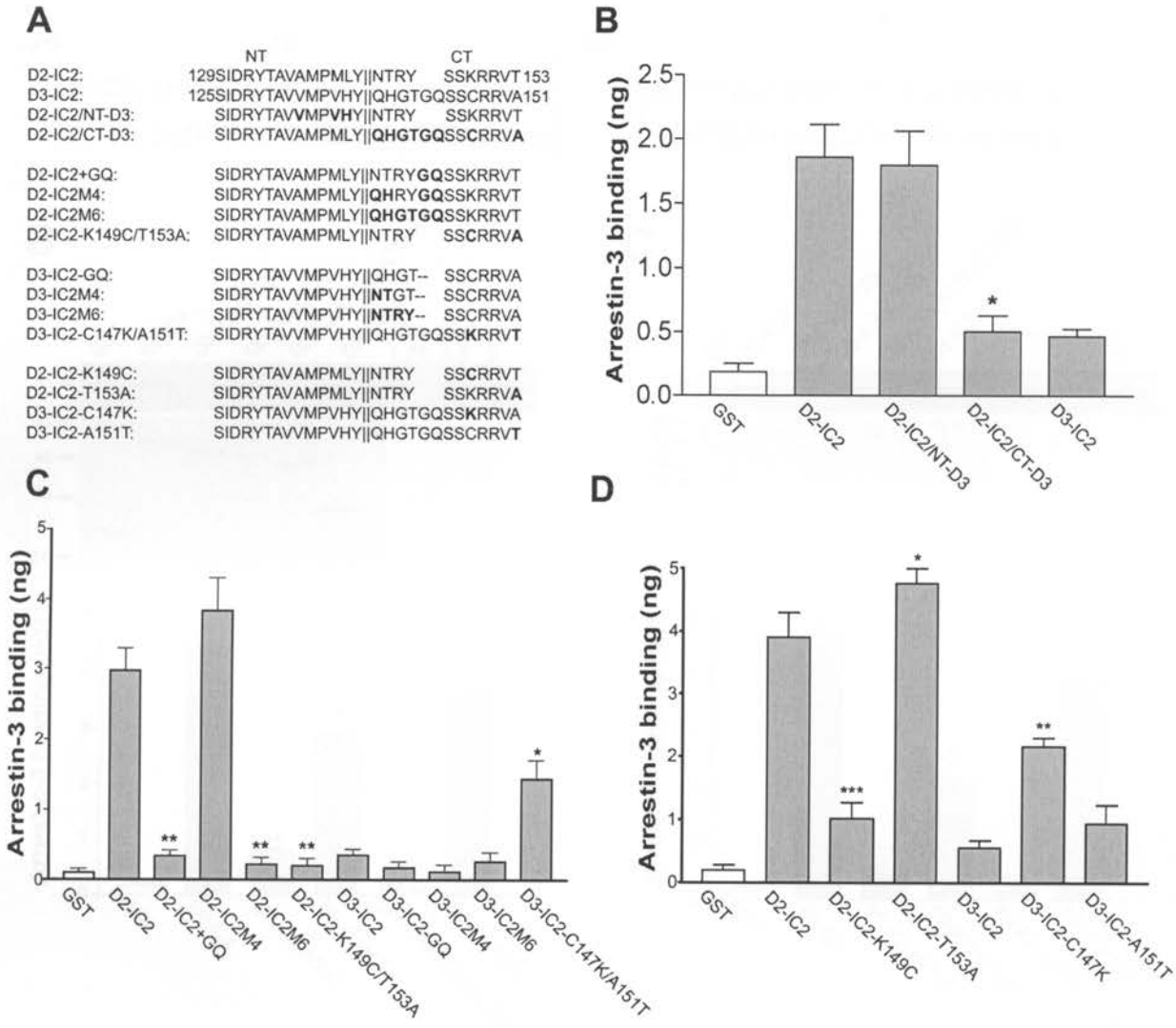

Fig. 3-3 Binding of arrestin-3 to D_2 and D_3 receptor IC2 chimeras. A. Alignment of IC2 from the rat D_2 and D_3 dopamine receptors, listing the name of each construct on the left and its sequence on the right, with mutated residues in bold. Each IC2 is divided into amino-terminal and carboxyl-terminal halves (NT and CT). The position in the full-length receptor of the first and last residues of each IC2 is indicated for the wildtype sequences. B-D. Purified GST or GST fusion proteins (400 ng in B, 500 ng in C, and 250 ng in D) were incubated with 300 ng of purified arrestin-3, and the amount of arrestin bound was determined by quantitative immunoblotting. The results shown are the mean ± SE from 4-6 independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the respective wildtype fusion protein by paired *t* test).

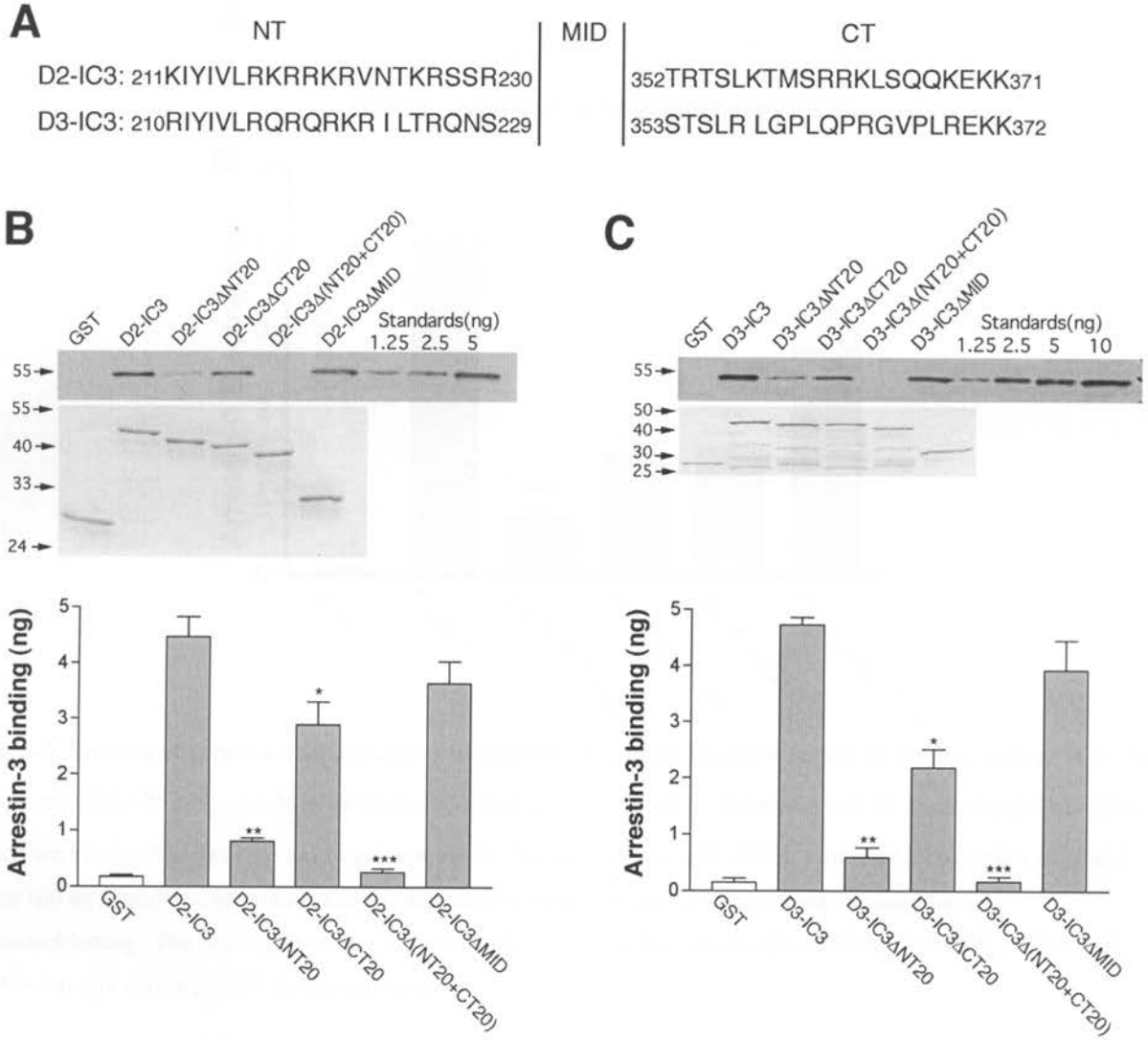

Fig. 3-4 Binding of arrestin-3 to D_2 and D_3 receptor IC3 truncation mutants. A. Alignment of IC3 from the rat D_2 and D_3 dopamine receptors. Each IC3 is divided into NT (20 residues), MID (121 residues not shown for D_2 and 123 residues for D_3) and CT (20 residues). B and C. Purified GST or GST fusion proteins (ranging from 500 to 800 ng to keep the molar concentration constant) were incubated with 100 ng of purified arrestin-3, and the amount of arrestin bound was determined by quantitative immunoblotting. *Upper panels*, representative arrestin immunoblots are shown. The amount of bound arrestin was quantified using a standard curve constructed with known amounts of arrestin as shown on the right of each blot. *Middle panels*, protein-stained gels used to verify the size of the truncation mutants and to quantify the amount of each fusion protein are shown. *Lower panels*, the results shown are the mean ± SE from 4 independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the respective wildtype fusion protein by paired *t* test).

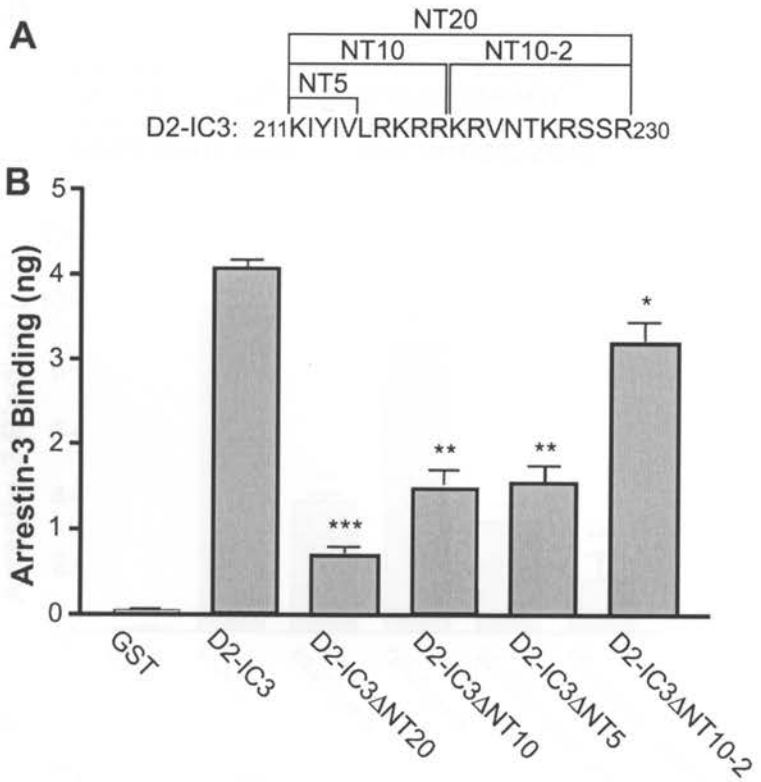

Fig. 3-5 Binding of arrestin-3 to truncation mutants of the amino-terminal region of the D_2 receptor IC3. A. Sequence of the 20 amino acids in the amino-terminal region of D_2-IC3. The first 5 and 10 or the second 10 amino acids were deleted to form the indicated mutants. B. Purified GST or GST fusion proteins (400ng) were incubated with 100 ng of purified arrestin-3, and the amount of arrestin bound was determined by quantitative immunoblotting. The results shown are the mean ± SE from 4 independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus D_2-IC3 by paired t test).

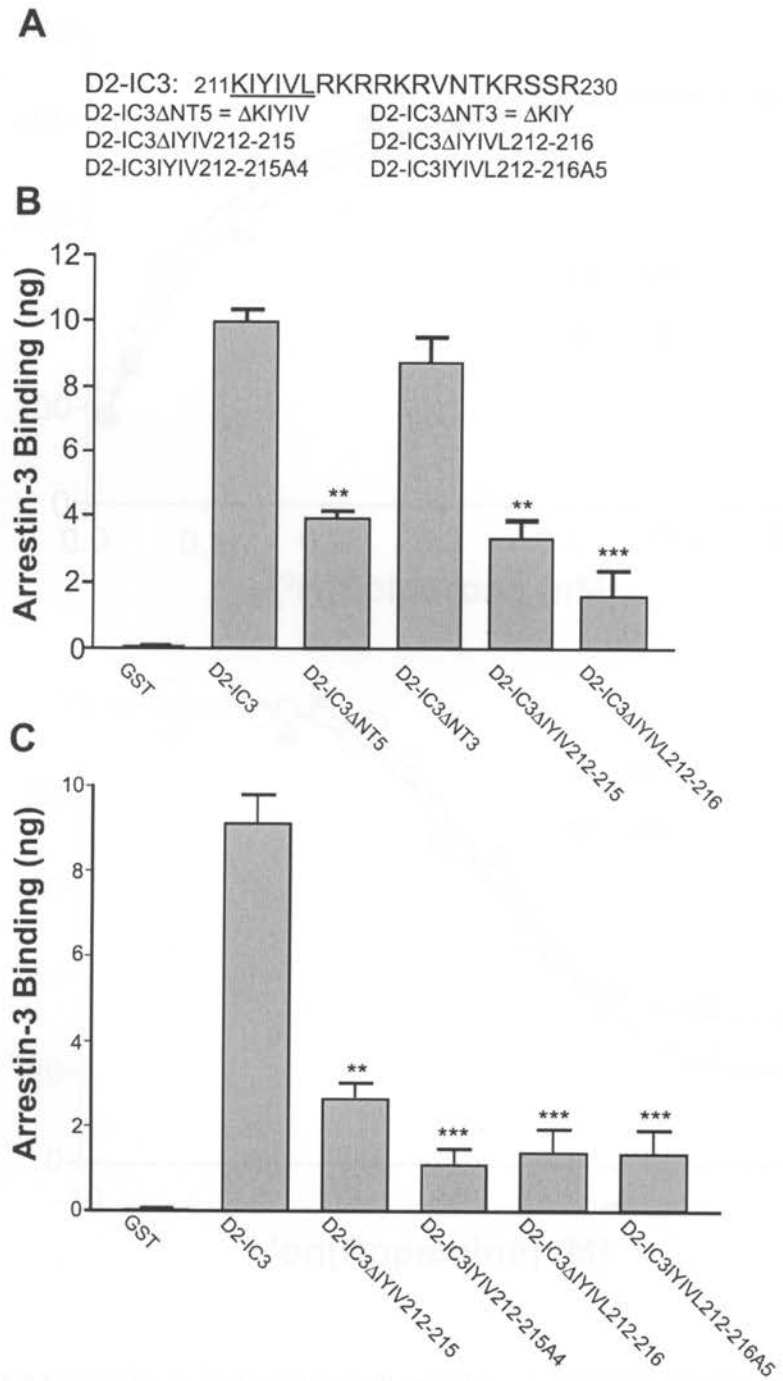

Fig. 3-6 Binding of arrestin-3 to 3-, 4-, and 5-residue deletion mutants and 4- and 5-residue substitution mutants of the amino-terminal region of D_2-IC3. A. Sequence of amino acids in the amino-terminal region of D_2-IC3, along with the name of each construct where Δ denotes the residues that were deleted. B and C. Purified GST or GST fusion proteins (600 ng) were incubated with 100 ng of purified arrestin-3, and the amount of arrestin bound was determined by quantitative immunoblotting. Results shown are the mean ± SE from 4 (B) or 5 (C) independent experiments. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus D_2-IC3 by paired t test).

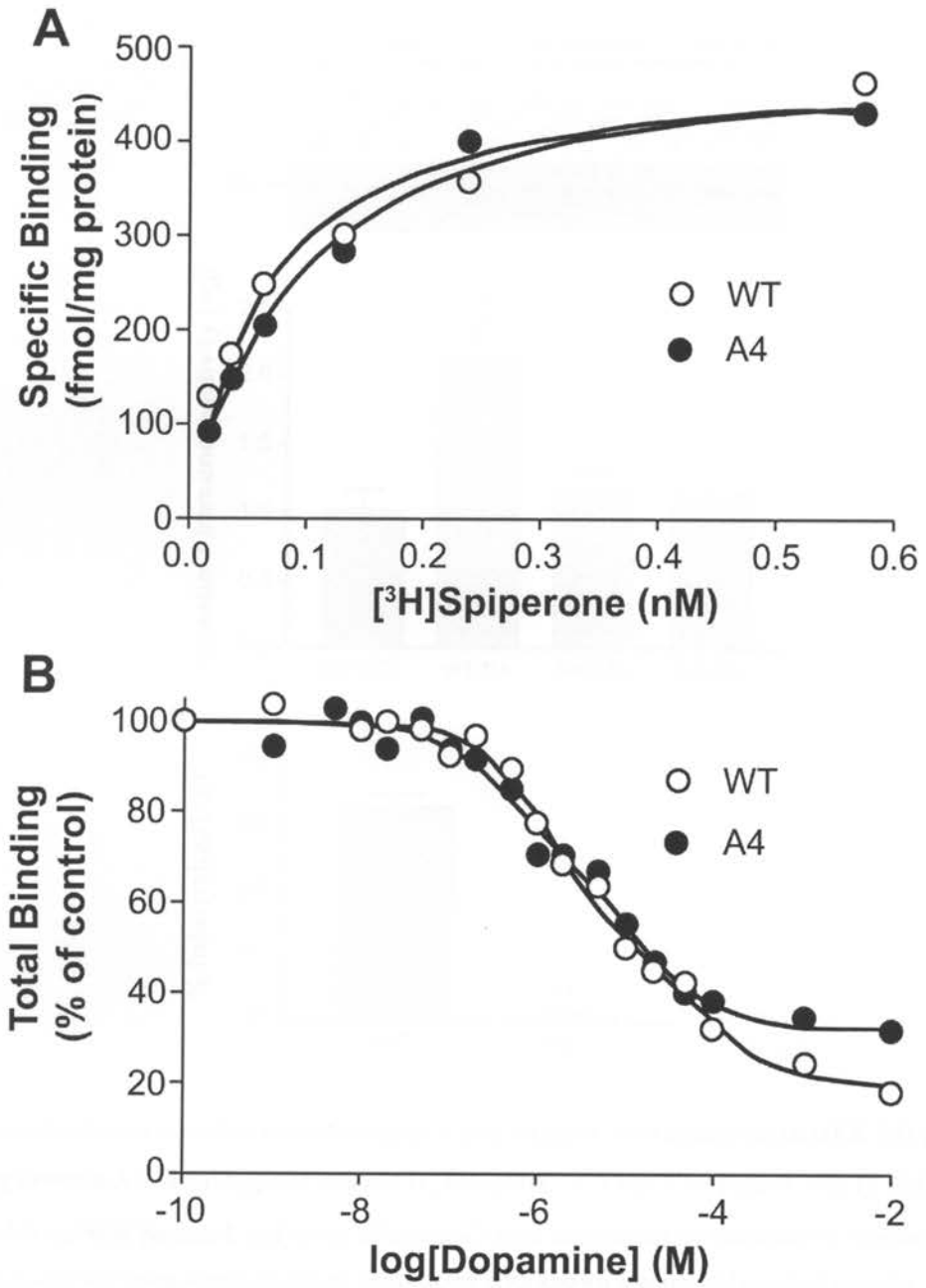

Fig. 3-7 Ligand binding properties of the D_2-A4 mutant receptor. A. Saturation analysis of the binding of the D_2 receptor antagonist [3H]spiperone to membrane preparations from HEK 293 cells expressing wildtype D_2 or D_2-A4 receptors. Data are plotted as specific binding (fmol/mg protein) versus the free ligand concentration of radioligand. B. Inhibition of the binding of [^{3}H]spiperone by dopamine. Data are plotted as a percentage of the total binding in the absence of dopamine versus the logarithm of the concentration of dopamine. In both A and B, the experiment shown is representative of three or more independent experiments.

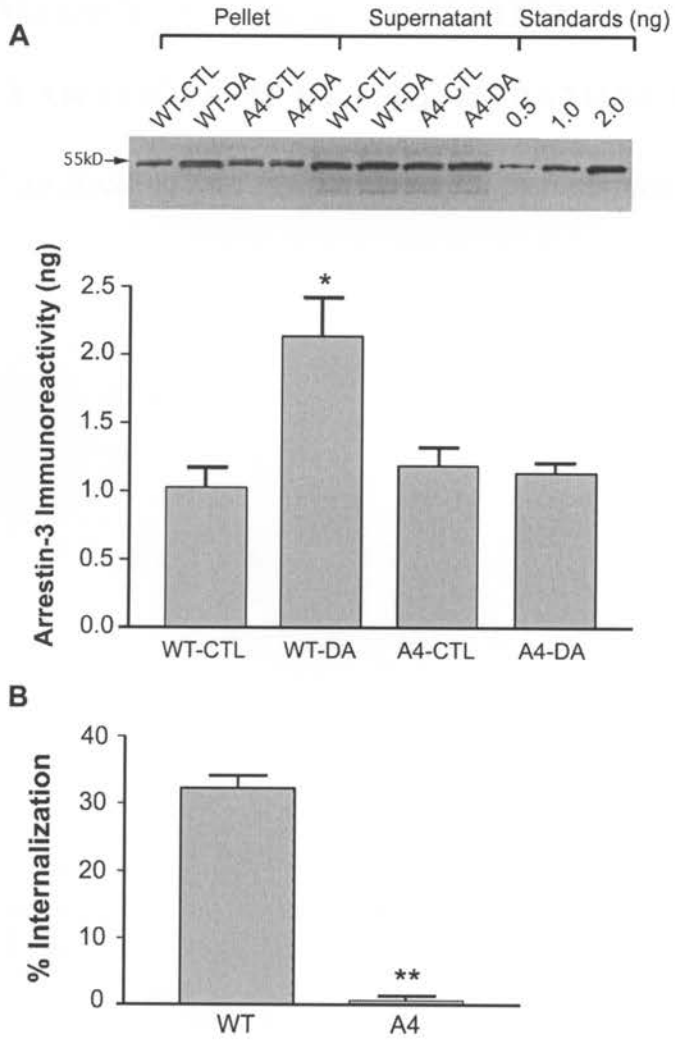

Fig. 3-8 Agonist-induced translocation of arrestin-3 and receptor internalization in HEK 293 cells coexpressing arrestin-3 and wildtype or mutant D_2 receptors. A. Cells were treated with 10 μM dopamine for 20 min, membranes were prepared, and levels of arrestin-3 were determined by quantitative immunoblotting. Results were quantified using standard curves constructed with known amounts of purified arrestin-3. The expression of cell surface receptor for the A4 mutant is similar to or higher than that for D_2 wildtype, as determined by intact cell [^{3}H]sulpiride binding(*data not shown*). *Upper panel*, an immunoblot is shown from an experiment representative of 4 independent experiments. Supernatants were loaded to show similar expression levels of arrestin-3. *Lower panel*, Results from all four experiments are shown as the mean ± SE. B. Cells were treated with 10 μM dopamine for 20 min, and were subjected to the intact cell [^{3}H]sulpiride binding assay. Results are expressed as the percentage by which the binding of [^{3}H]sulpiride to cell surface receptors decreased after agonist stimulation, and are shown as the mean ± SE from 4 independent experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus wildtype by paired *t* test). The expression levels of arrestin-3 were similar in cells transfected with wildtype or mutant receptors (data not shown).

IV. BINDING OF NON-VISUAL ARRESTINS TO THE THIRD INTRACELLULAR LOOPS OF D2-LIKE DOPAMINE RECEPTORS:

Effects of GRK2 on Binding and Role of Arrestin in Receptor Internalization

ABSTRACT

Arrestin binding determinants in the dopamine D_2 and D_3 receptors were further investigated, with a focus on the third intracellular loop (IC3) of the receptors and arrestin-3. Deletions of each of two stretches of basic residues located in the amino- and carboxyl-terminal regions of D_2-IC3 (RKRRKR217-222 and KEKK368-371) and deletions of 5- and 10-residue within the C-terminal region of D_2-IC3 had no/little effect on arrestin-3 binding, as demonstrated by the GST pull-down assay. In contrast to the GST pull-down of purified arrestin-3 (see Chapter III), only the IC3 of the D_3 receptor interacted with arrestin-3 in rat striatal homogenate. The effects of GRK2 on the interaction between the receptors and arrestins and the roles of arrestin-3 in the internalization of the D_2 receptors were also evaluated. IC3 from both receptors appeared to be poor substrates for GRK2 *in vitro*, and GRK2 did not affect binding of purified arrestin to GST-IC3 fusion proteins. In contrast, in HEK 293 cells GRK2 co-transfection increased arrestin-3 translocation to the plasma membrane in the basal state. Agonist-induced internalization of the D_2 receptor increased with the co-transfection of arrestin-3 in HEK 293 cells.

INTRODUCTION

During the course of the arrestin project, I performed additional experiments as controls, to support the primary data, or to address related questions, experiments that because of limited space were not included in Chapter III, which is being prepared for submission to a journal. Data from these experiments are put together in this chapter. The questions I address in this chapter include:

1. What is the rate of association of arrestin-3 with GST-D_2-IC3 (GST fusion protein containing the third intracellular loop of the D_2 receptor)? In other words, how long does it take for arrestin-3 binding to reach equilibrium?
2. Stretches of basic residues are located in the amino- and carboxyl-terminal regions of D_2-IC3. Do these residues play a role in arrestin-3 binding?
3. The last 20 residues of D_2-IC3 were demonstrated to play a role in arrestin-3 binding. Does any contiguous shorter amino sequence within the last 20 residues account for the contribution of the C-terminus of D_2-IC3 to the binding of arrestin?
4. Are GST fusion proteins (GST-D_2-IC2, GST-D_2-IC3, GST-D_3-IC2 and GST-D_3-IC3) capable of interacting with arrestins in rat striatal homogenate?
5. Can GRK2 phosphorylate GST-D_2-IC3 and GST-D_3-IC3 fusions *in vitro* and does it affect arrestin binding to these fusions?
6. Does coexpressing GRK2 with arrestin-3 and D_2 receptor affect arrestin-3 translocation to the plasma membrane?
7. In HEK 293 cells, is agonist-induced internalization of the D_2 receptor arrestin-dependent?

8. As a complementary approach to explore agonist-induced receptor internalization of wildtype or mutant D_2 receptors, the intact cell [^{3}H]spiperone binding assay was applied. Are results from the [^{3}H]spiperone binding assay consistent with those from the [^{3}H]sulpiride assay?
9. In the intact cell [^{3}H]sulpiride binding assay, does the loss of binding of [^{3}H]sulpiride reflect only receptor internalization? Is it partly due to persistent binding of agonist?

MATERIALS AND METHODS

Materials. S-(-)-sulpiride and spiperone were purchased from Sigma-Aldrich (St. Louis, MO). GRK2-pBC12BI and purified GRK2 were generous gifts from Dr. Jeffrey Benovic. See Chapter III for others.

GST Fusion Proteins. See Chapter III for description of the creation and purification of fusion proteins GST-D_2-IC3, GST-D_3-IC3, GST-D_2-IC2 and GST-D_3-IC2. The IC3 truncation mutants were generated as described in Chapter III.

Purified Arrestin Binding to GST Fusion Proteins. The binding of arrestin-3 to deletion mutants of GST-D_2-IC3 was carried out as described in Chapter III. The time course of arrestin-3 binding was determined by incubating 600 ng of GST-D_2-IC3 with 100 ng of purified arrestin-3 for periods of up to 60 min at room temperature. The resulting data were analyzed by nonlinear regression using the computer program Prism 3.0 (GraphPad, San Diego, CA) to determine the half time for binding.

Tissue GST Pull-down. Striata were dissected from Fisher rats and homogenized in solubilization buffer (50 mM Tris-HCl, pH 7.4, 0.05 mM EDTA, 10 mM CHAPS, 1 mM DTT, and Complete protease inhibitor tablet) with five strokes of a glass-Teflon Dounce homogenizer. Samples were centrifuged at 40,000*g* for 30 min, and the protein concentration in the resulting supernatant was determined by the Bradford assay using Coomassie Plus protein assay reagent (Pierce, Rockford, IL). Glutathione Sepharose 4B beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) containing similar molar amounts of GST alone, GST-D_2-IC2, GST-D_3-IC2 (~200 ng each), GST-D_2-IC3 and GST- D_3-IC3 (~400 ng each) were incubated with striatal homogenate (1 mg protein) for 2 hr at room temperature. The beads were washed twice with TBS containing 300 μM phenylmethylsulfonyl fluoride (PMSF) and 0.1% Triton X-100, and the

proteins were released with SDS sample loading buffer. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. For immunoblotting of arrestin-2, membranes were blocked with 5% dry milk in TBS, and incubated sequentially with anti-arrestin-2 antibody (1:300 dilution in TBS) as primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,500 dilution in TBS containing 5% dry milk) as secondary antibody. For immunoblotting of arrestin-3, membranes were incubated sequentially with anti-arrestin-3 antibody (1:100 dilution in TBS containing 0.1% Tween 20 and 5% dry milk) as primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,500 dilution in TBS containing 0.1% Tween 20 and 5% dry milk) as secondary antibody. The blots were developed using the SuperSignal West Pico Chemiluminescence kit (Pierce, Rockford, IL).

***In vitro* Phosphorylation Assay.** Glutathione Sepharose 4B beads containing similar molar amounts of GST alone (~700 ng), GST-D_2-IC3 and GST-D_3-IC3 (~1 μg each) were incubated at 30 °C for 1 hr in the absence and presence of 0.5 μg GRK2 (~150 nM) in 25 mM Tris-HCl, pH 7.2, 2 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, Complete protease inhibitor tablet, 0.5 mM ATP, and ~10 μCi of [γ-^{33}P]ATP in a final volume of 15 μl. Reactions were stopped by the addition of SDS sample loading buffer and incubation at 60 °C for 15 min. Proteins were separated by SDS-PAGE. The gel was sealed in a sample bag, placed in exposure cassette under storage phosphor screen, and the resulting autoradiograph of ^{33}P-labeled proteins was captured with Typhoon 9410 (GE Healthcare, Little Chalfont, Buckinghamshire, UK)

Purified Arrestin Binding to GST Fusion Proteins Pre-incubated with GRK2. Pre-incubation of purified fusion proteins in the absence and presence of GRK2 was performed as described above except that no [γ-^{33}P]ATP was included. Then, GST alone (400 ng), GST-D_2-

IC3 (600 ng), or GST-D_3-IC3 (600 ng) was incubated with arrestin-2 or arrestin-3, followed by immunoblotting as described in Chapter III.

Arrestin-3 Translocation. HEK 293 cells grown to 80% confluency were co-transfected with 3 μg D_2 receptor cDNA and 3 μg arrestin-3-pCMV5 in the absence or presence of 3 μg GRK2-pBC12BI using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Cells were split into 4 plates after 12 hr, 2 days later rinsed once with pre-warmed calcium- and magnesium-free phosphate-buffered saline (CMF-PBS; 138 mM NaCl, 4.1 mM KCl, 5.1 mM sodium phosphate, 5 mM potassium phosphate, and 0.2% glucose, pH 7.4), and preincubated for 15 min with pre-warmed, CO_2-saturated serum-free Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.4, at 37° C. Cells were stimulated with 10 μM 7-OH-DPAT in the same HEPES-buffered medium at 37° C for periods of up to 120 min. Stimulation was terminated by quickly cooling the plates on ice and washing the cells once with ice-cold CMF-PBS. Cells were lysed with 1 ml ice-cold lysis buffer (20 mM HEPES, 20 mM NaCl, 5 mM EDTA, and Complete protease inhibitor tablet), scraped, collected, homogenized with a glass-Teflon homogenizer, and sonicated for 8-10 seconds. Samples were centrifuged at 1,000*g* for 10 min at 4° C. Supernatants were transferred to new centrifuge tubes and centrifuged at 100,000*g* for 30 min at 4° C. Supernatants were collected; pellets were rinsed carefully with ice-cold CMF-PBS and then resuspended with 100 μl CMF-PBS. The abundance of arrestin-3 in both pellet and supernatant fractions was quantified by immunoblotting as described in Chapter III.

Internalization Assays. Two different strategies were used to evaluate D_2 receptor internalization. The intact cell [^{3}H]sulpiride binding assay is described in Chapter III. The second assay, the intact cell [^{3}H]spiperone binding assay, was described by Kim et al. (2001). This assay was performed as described for the first assay except that cells were added to assay

tubes in a final volume of 1 ml with [^{3}H]spiperone (final concentration, 0.3 nM), and incubated at 14 °C for three hr in the absence and presence of unlabeled spiperone (final concentration, 10 μM) or sulpiride (final concentration, 3 μM). To test arrestin-3 regulation of receptor internalization, HEK 293 cells were transfected with 30 ng pCMV-myc-D_2 receptor (referred to herein as wildtype D_2 to differentiate it from the D_2 mutants) with or without 3 μg arrestin-3-pCMV5. For the [^{3}H]sulpiride assay, to exclude the possibility of residual binding of agonist after washing step, dopamine treatment was carried out at 4°C as a control.

RESULTS AND DISCUSSION

The Time Course of Arrestin-3 Binding to GST- D_2-IC3. To explore conditions for equilibrium binding of arrestin to GST fusion proteins, GST-D_2-IC3 was incubated with purified arrestin-3 for periods up to 60 min. The binding reaction progressed quickly, having a half time of less than 2 min and reaching equilibrium within 15 min (Fig. 4-1). I chose an incubation time of 30 min for all other GST binding assays.

Basic Residues in D_2-IC3 Had no Effect on Binding of Purified Arrestin-3. Several basic residues in the N- and C-terminal portions of IC3 of the α_{2b}-adrenergic receptor have been found to play a critical role in the binding of purified arrestin-3 to that receptor (DeGraff et al., 2002). For both D_2 and D_3 receptors, arrestin-3 binding was primarily localized to the first and last 20 residues of IC3 (See Chapter III). Two stretches of basic residues, **RKRRKR**217-222 and **KEKK**368-371, are present in these regions in the D_2 receptors (Fig. 4-2) and are essentially conserved between D_2 and D_3 receptors. Unexpectedly, mutagenesis studies revealed that deletion of these two sequences in GST-D_2-IC3 did not affect binding of arrestin, indicating that these basic residues are NOT important for arrestin binding *in vitro* (Fig. 4-2). Although both D_2 and α_{2b}-adrenergic receptors belong to the catecholamine receptor subfamily and both of them couple to $G\alpha_{i/o}$ family of G proteins, arrestin-binding determinants could be quite different. Mutation of those basic residues in α_{2b}-adrenergic receptor severely impairs agonist-induced receptor internalization, consistent with the involvement of arrestin in this process (DeGraff et al., 2002). Interestingly, some of these basic residues in the α_2-adrenergic receptor are part of a G-protein binding and activation domain (Okamoto et al., 1992; Wade et al., 1999), suggesting a competition between G protein and arrestin for receptor binding. For the D_2 receptor, the

sequence IYIV212-215 at the N-terminus of D_2-IC3 was critical for the binding of arrestin but not G proteins (see Chapter III). Our data imply that the structural determinants of arrestin and G protein binding can be distinguished.

Identification of Arrestin-3 Binding Sites within the C-terminal Region of D_2-IC3. The last 20 residues of D_2-IC3 were implicated in binding of arrestin-3 (see Chapter III). We constructed additional C-terminal truncation mutants of D_2-IC3 in which the last 5 or 10 or the penultimate 10 amino acids were deleted (Fig. 4-3). In the GST pull-down assay, none of these smaller deletions caused a significant reduction in arrestin binding (Fig. 4-3). This is consistent with the less important role of the C-terminus suggested by the modest effect of the 20-residue truncation on the binding of arrestin to D_2-IC3 (Fig. 3-4).

Interaction of IC3 of the D_3 Receptor with Arrestin-3 in Rat Striatal Homogenate. Having determined that arrestin-3 bound more avidly than arrestin-2 to IC2 and IC3 from both D_2 and D_3 receptors and that binding of arrestin to IC3 is stronger than to IC2 (see Chapter III), we further investigated the association of these receptor intracellular loops with endogenous arrestins. GST-D_2-IC2, GST-D_3-IC2, GST-D_2-IC3, and GST-D_3-IC3 fusion proteins immobilized on glutathione-Sepharose beads were incubated with rat striatal homogenate, and arrestin binding was quantified by immunoblotting. The signals from the tissue sample demonstrated the abundance of both arrestins. Consistent with purified arrestin-2 binding, none of these fusion proteins interacted with arrestin-2 (Fig 4-4). Despite the robust binding of purified arrestin-3 to IC3 from both D_2 and D_3 receptors, only IC3 from the D_3 receptor showed a direct association with endogenous arrestin-3 (Fig 4-4). This is also inconsistent with prior determinations of agonist-induced translocation of arrestin-3 to the plasma membrane and receptor internalization, for both of which the magnitude was much greater for the D_2 receptor

than for the D_3 receptor in a heterologous system (Kim et al., 2001). The discrepancy might be caused by different experimental conditions such as use and choice of detergents. Furthermore, other D_2-IC3 binding proteins present in striatal homogenate might compete for binding and affect the results. However, we cannot rule out the possibility that in the striatum, in the basal state, arrestin-3 might actually have a preference for the less abundant D_3 receptor over the more abundant D_2 receptor.

IC3s from D_2 and D_3 Receptors Were Poor Substrates for GRK2 *in vitro*. For many GPCRs, GRK-catalyzed receptor phosphorylation plays a critical role in arrestin-binding and receptor internalization. Activation of the D_2 receptor causes GRK-mediated receptor phosphorylation, whereas in the case of the D_3 receptor, the phosphorylation level is much lower, and phosphorylation correlates with arrestin translocation and receptor internalization (Kim et al., 2001). The difference is attributed to distinct sequences in IC2 and IC3 of the two receptors. To investigate phosphorylation-dependent arrestin binding, purified GST-D_2-IC3 and GST-D_3-IC3 were incubated with purified GRK2 in the presence of [γ-^{33}P]ATP, followed by storage phosphor screen autoradiography. Very weak phosphorylation of D_2-IC3 was observed, whereas for D_3-IC3 phosphorylation was hardly detectable (Fig. 4-5A). This was in agreement with the notion that the D_2 receptor is a better substrate for GRK2 than the D_3 receptor (Kim et al., 2001). G$\beta\gamma$ subunits and phosphatidyl inositol are activators of GRK2 (Ferguson, 2001). However, addition of 70 nm G$\beta\gamma$ subunits and 300 μM phosphatidyl inositol did not improve phosphorylation of these GST fusions (data not shown).

Arrestin binding to GST-D_2-IC3 and GST-D_3-IC3 was further tested following GRK2 pre-treatment, which showed that GRK2 had no effect on arrestin binding (Fig. 4-6), most likely due

to the low stoichiometry of phosphate incorporation under these experimental conditions. This is not surprising because, typically, GRKs interact with multiple domains on a receptor and are activated by the receptor; in contrast, receptor fragment peptides tend to be poor substrates for GRKs because of poor binding resulting from the loss of some binding sites for GRKs and ensuing low enzyme activity (Pao and Benovic, 2005). Thus, investigation of phosphorylation sites on the D_2 receptor may still rely on intact cell phosphorylation assays and mass spectrum analysis.

GRK2 Increased Arrestin-3 Translocation in the Basal State. In an effort to optimize conditions for arrestin-3 translocation to the plasma membrane in response to agonist-induced activation of the wildtype D_2 receptor in HEK 293 cells, GRK2 was co-expressed with receptor and arrestin. Without GRK2 co-expression, arrestin translocation increased upon stimulation with 7-OH-DPAT for 5 min and reached maximal level by 20 min. In contrast, with concomitant GRK2 overexpression, membrane-associated arrestin reached maximal level in the basal state, and agonist treatment for periods of up to 120 min did not cause any further effect (Fig. 4-7). Although GRK2 co-expression did not improve the arrestin translocation assay itself (so all other translocation assays were performed without its co-transfection), these findings implied that GRK2 had the capability to phosphorylate some GPCR(s) in the basal state, resulting in an increase in arrestin translocation to the plasma membrane.

D_2 Receptor Internalization was Modulated by Arrestin. Agonist-induced internalization of the D_2 receptor greatly decreases upon siRNA-induced depletion of arrestins in NS20Y cells (Macey et al., 2004). Compared to NS20Y cells, endogenous arrestin is less abundant in HEK 293 cells. Is agonist-induced internalization of the D_2 receptor arrestin-3-regulated in HEK 293 cells? To address the question, HEK 293 cells transiently expressing the wildtype D_2 receptor in

the absence and presence of overexpressed arrestin-3 were subjected to both [^{3}H]sulpiride and [^{3}H]spiperone internalization assays (see Chapter V for comparison of the two assays). In the [^{3}H]sulpiride assay, with the overexpression of arrestin, agonist treatment caused an internalization of 32 ± 2%, compared to 13 ± 5% without the overexpression of arrestin. Similarly, in the [^{3}H]spiperone assay, with the overexpression of arrestin, the internalization level was 9 ± 0.5%, in sharp contrast to 0 ± 1% when there was no arrestin overexpression. Variations in magnitude between these two internalization assays originated from different calculation methods (in the [^{3}H]sulpiride assay the percentage is calculated from a denominator of the extracellular receptors whereas in the [^{3}H]spiperone assay the percentage is calculated from a denominator of the total receptors) and more importantly, a difference in accuracy of these assays. These results confirmed that arrestin regulated the internalization of D_2 receptor caused by agonist activation. Thus, all other internalization assays were performed with the overexpression of comparable levels of arrestin-3 in HEK 293 cells expressing wildtype D_2 or mutant D_2-A4 receptors, as confirmed by immunoblotting (Fig. 4-8B).

During the course of internalization assays, we found that overexpression of arrestin-3 increased D_2 receptor expression and transport of receptors to the cell surface. For example, in the presence of arrestin-3 co-expression, total receptor was 9,800 cpm (counts per min), extracellular receptor was 6,600 cpm and the percentage of extracellular receptor was 67%, in contrast to 3,400 cpm, 1,000 cpm and 29%, respectively, in the absence of arrestin-3 co-expression (total receptor is specific binding defined by [^{3}H]spiperone binding assay and extracellular receptor is specific binding defined by [^{3}H]sulpiride binding assay). The mechanism of this phenomenon is totally unknown. One explanation is that the arrestin DNA simply enhances the efficiency of transfection. Alternatively, arrestin-3 might function as a

chaperone by binding to and stabilizing a specific conformation of the receptor during protein synthesis, thus facilitating folding and proper targeting of receptors.

The A4 Mutation Abolished Receptor Internalization. As a complementary approach to confirm the results reported in the previous chapter, the intact cell [^{3}H]spiperone binding assay was employed to assess D_2 receptor internalization. In HEK 293 cells transiently expressing the wildtype D_2 receptor and arrestin-3, treatment with 10 μM dopamine for 20 min increased the intracellular receptors by 36% (from 25 ± 1% to 34 ± 0.4%, intracellular receptors expressed as a percentage of total receptors, Fig 4-8A). In contrast, the A4 mutation abolished dopamine-induced receptor internalization. These results were consistent with those from the intact cell [^{3}H]sulpiride binding assay. The difference in the basal level of intracellular receptors between wildtype D_2 and D_2-A4 receptors (25 ± 1% vs. 31 ± 3%) was most likely a reflection of different receptor expression levels because the difference was minimized when receptor expression levels were equal (data not shown).

No Persistent Binding of Agonist in the Intact Cell [^{3}H]Sulpiride Assay. In the intact cell [^{3}H]sulpiride binding assay, the hydrophilic ligand [^{3}H]sulpiride binds solely to the extracellular receptors. Potentially, residual agonist binding would greatly affect final results by occupying the ligand binding site and thus decreasing [^{3}H]sulpiride binding. To rule out this possibility, agonist treatment was carried out at 4 °C, a temperature at which receptor internalization is blocked, but agonist still binds the receptors. At 37 °C, stimulation of wildtype D_2 receptor with dopamine produced a receptor internalization level of 26%, but at 4 °C no significant receptor internalization was observed (0.1%). We concluded that the washing procedure after agonist treatment was sufficient to remove agonist.

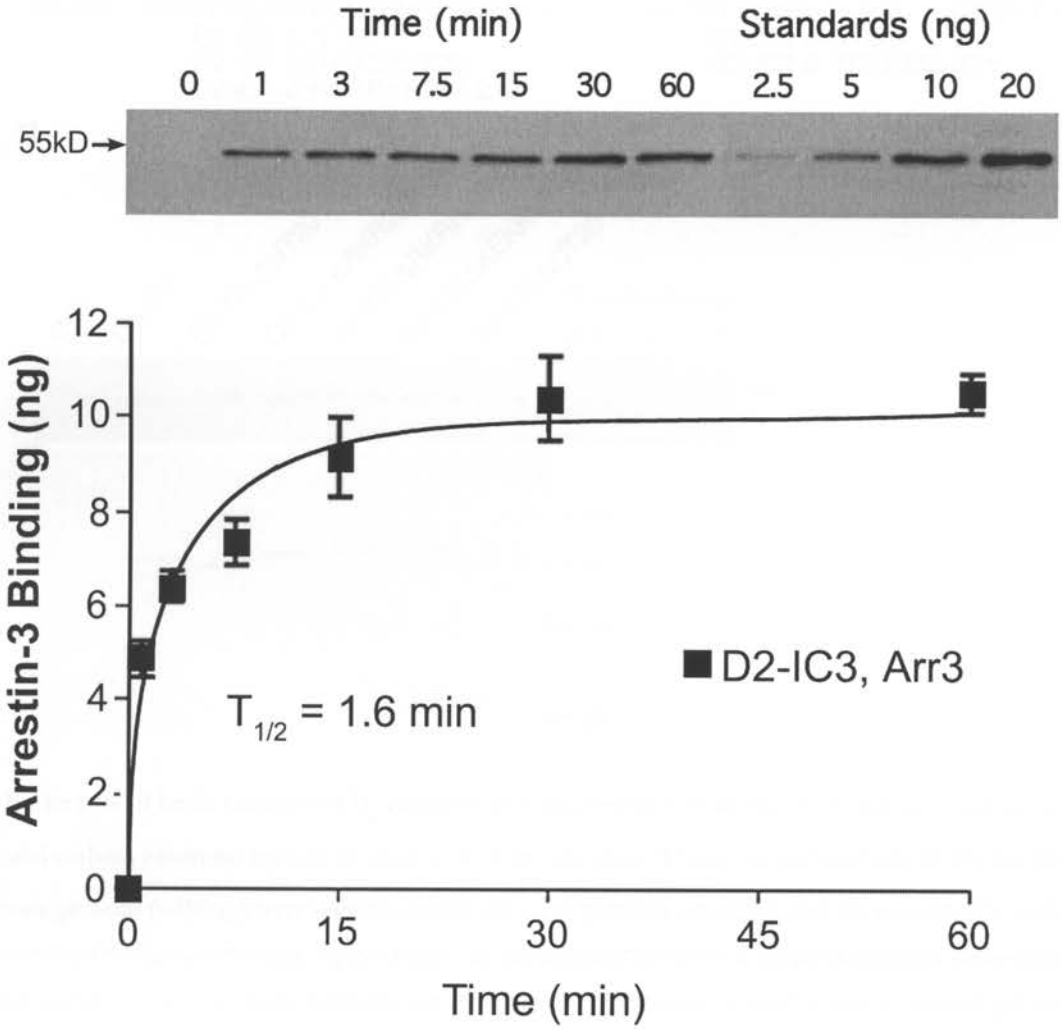

Fig. 4-1 The time course of binding of arrestin-3 to GST-D_2-IC3 fusion protein. GST-D_2-IC3 (600ng) was incubated with 100 ng of purified arrestin-3 for periods up to 60 min. The amount of arrestin that co-eluted with the GST fusion protein was determined by immunoblotting with anti-arrestin-3 antibody. Results were quantified using standard curves constructed with known amounts of arrestin-3. *Upper panel*, an immunoblot is shown from an experiment representative of 3 independent experiments, in which the zero time point indicates that no arrestin was included in the reaction. Arrestin standards are shown on the right. *Lower panel*, the results are displayed as the mean ± SE. The amount of arrestin bound is plotted against incubation time.

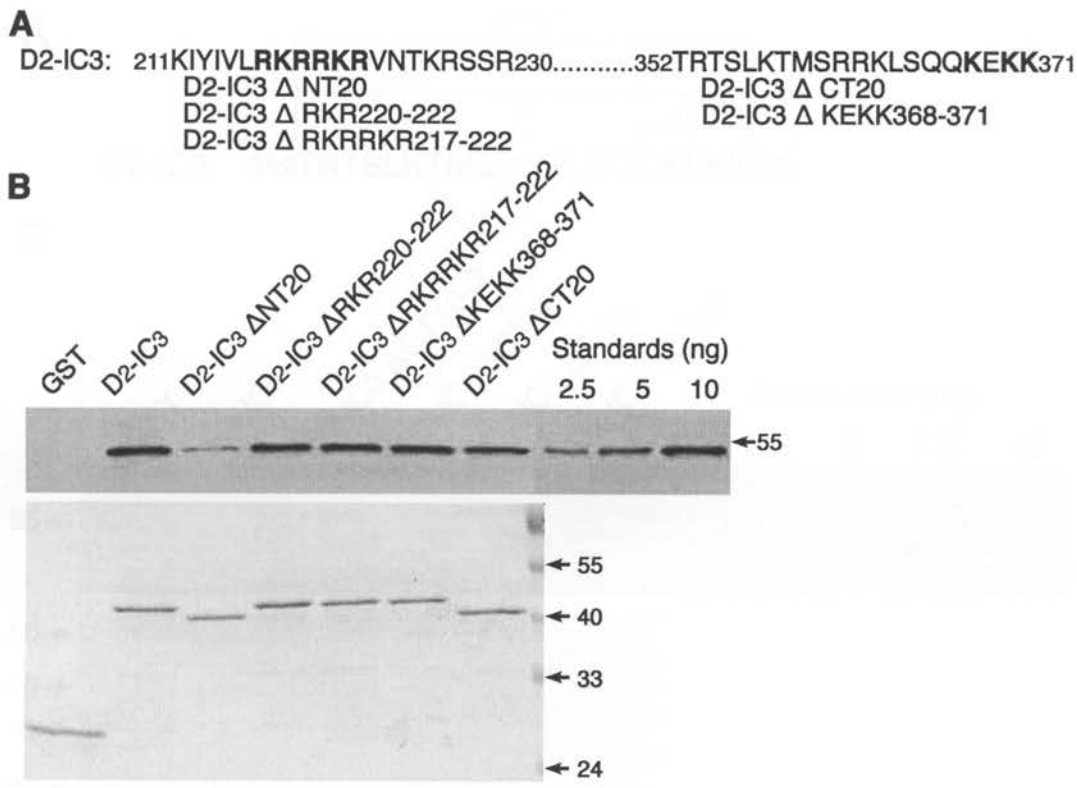

Fig. 4-2 The role of basic residues of D_2 receptor IC3 in arrestin-3 binding. A. Sequence of amino acids in the amino- and carboxyl-terminal regions of D_2-IC3, with the stretches of basic residues in bold. B. Purified GST or GST fusion proteins (~400ng) were incubated with 100 ng of purified arrestin-3, and the amount of arrestin bound was determined by immunoblotting. *Upper panel*, an immunoblot is shown from an experiment representative of 4 independent experiments. Arrestin standards are shown on the right. *Lower panel*, a protein-stained gel that was used to verify the size of the truncation mutants and to quantify the amount of each fusion protein is shown.

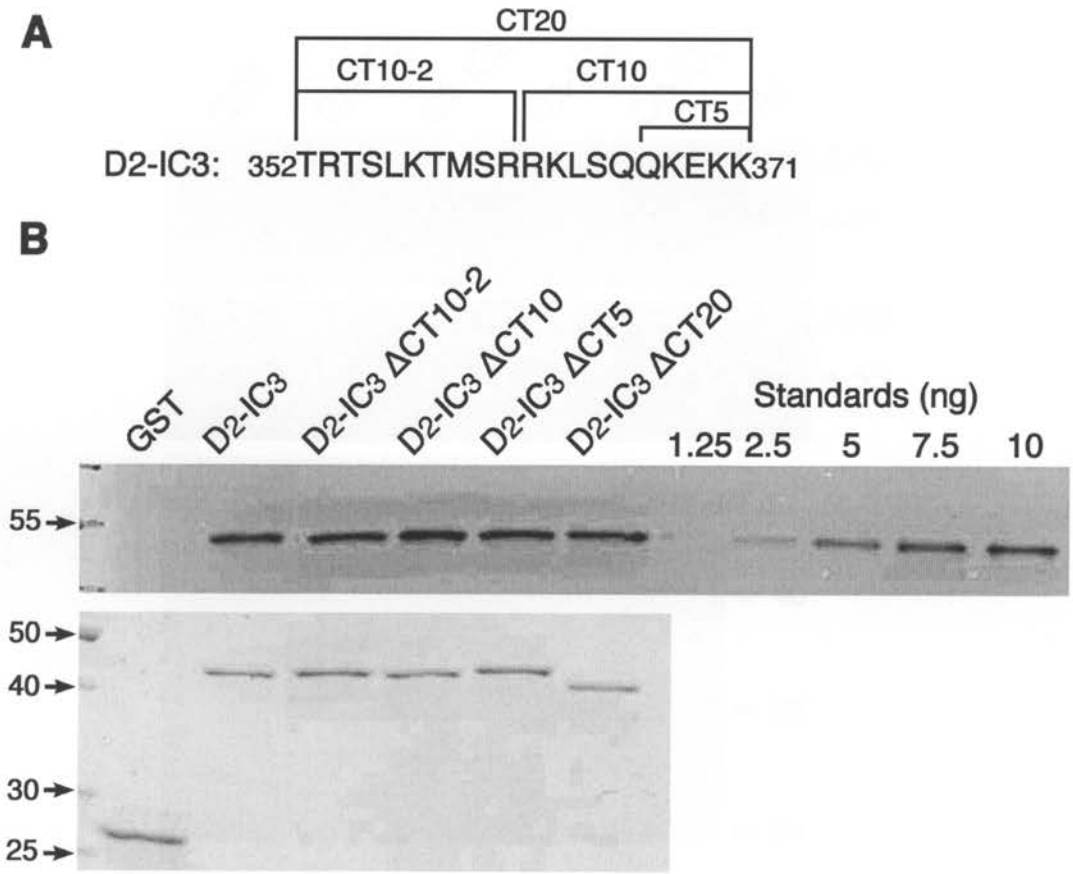

Fig. 4-3 Binding of arrestin-3 to truncation mutants of the carboxyl-terminal region of the D_2 receptor IC3. A. Sequence of the 20 amino acids in the carboxyl-terminal region of D_2-IC3. The last 5, 10, or 20 residues or the penultimate 10 residues were deleted to form the indicated mutants. B. Purified GST or GST fusion proteins (400ng) were incubated with 100 ng of purified arrestin-3, and the amount of arrestin bound was determined by immunoblotting. *Upper panel*, an immunoblot is shown from an experiment representative of 4 independent experiments. Arrestin standards are shown on the right. *Lower panel*, a protein-stained gel that was used to verify the size of the truncation mutants and to quantify the amount of each fusion protein is shown.

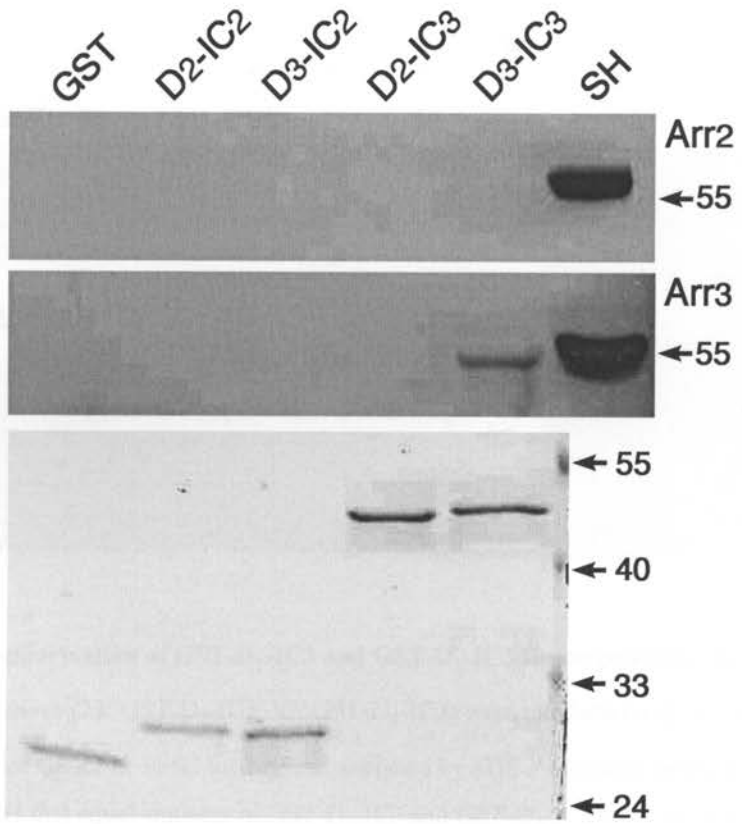

Fig. 4-4 Interaction of D_2 and D_3 receptor intracellular loops with arrestins in preparations of rat striatum. GST alone (GST, 200 ng), receptor second intracellular loop GST fusion proteins (GST-D_2-IC2 and GST-D_3-IC2, 200 ng) or receptor third intracellular loop GST fusion proteins (GST-D_2-IC3 and GST-D_3-IC3, 400 ng) were *incubated with striatal homogenate (1 mg protein), and the amount of arrestin bound was determined by* immunoblotting. *Upper and middle panels*, immunoblots are shown from an experiment representative of 4 independent experiments. Aliquots (10 μg of protein) of the striatal homogenate (SH) were run in two lanes to demonstrate the presence of both arrestins in the striatal homogenate. *Lower panel*, a protein-stained gel that was used to verify the size of the truncation mutants and to quantify the amount of each fusion protein is shown.

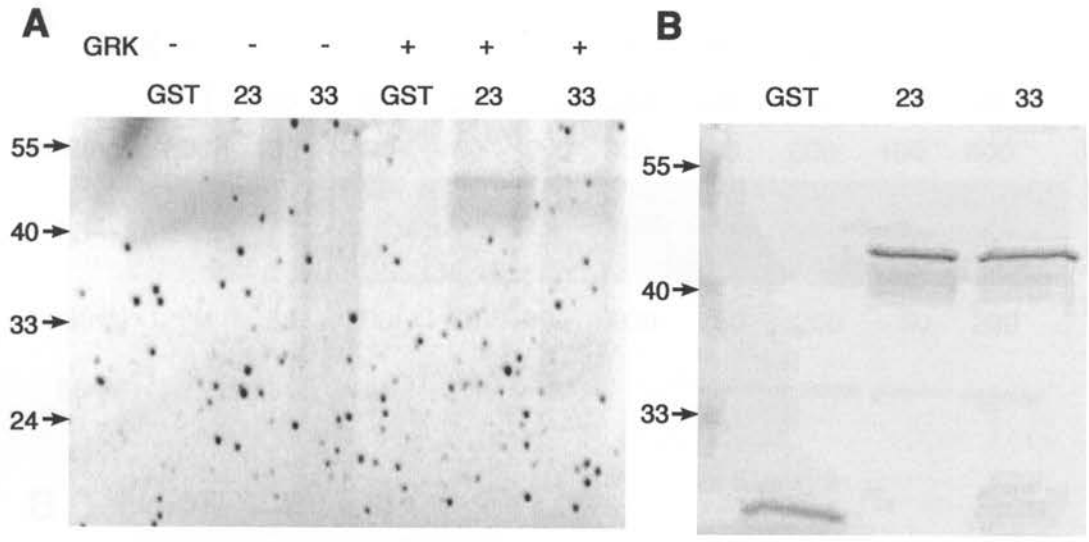

Fig. 4-5 *In vitro* phosphorylation of GST-D_2-IC3 and GST-D_3-IC3 fusion proteins. A. Either 700 ng of GST alone or 1 μg of IC3 fusions (23: GST-D_2-IC3; 33: GST-D_3-IC3) were incubated with [γ-^{33}P]ATP in the absence (control) and presence of GRK2 at 30 °C for 1 hr and analyzed by SDS-PAGE and autoradiography. B. The protein-stained gel demonstrates that equal amounts of GST-D_2-IC3 and GST-D_3-IC3 were included in the reactions.

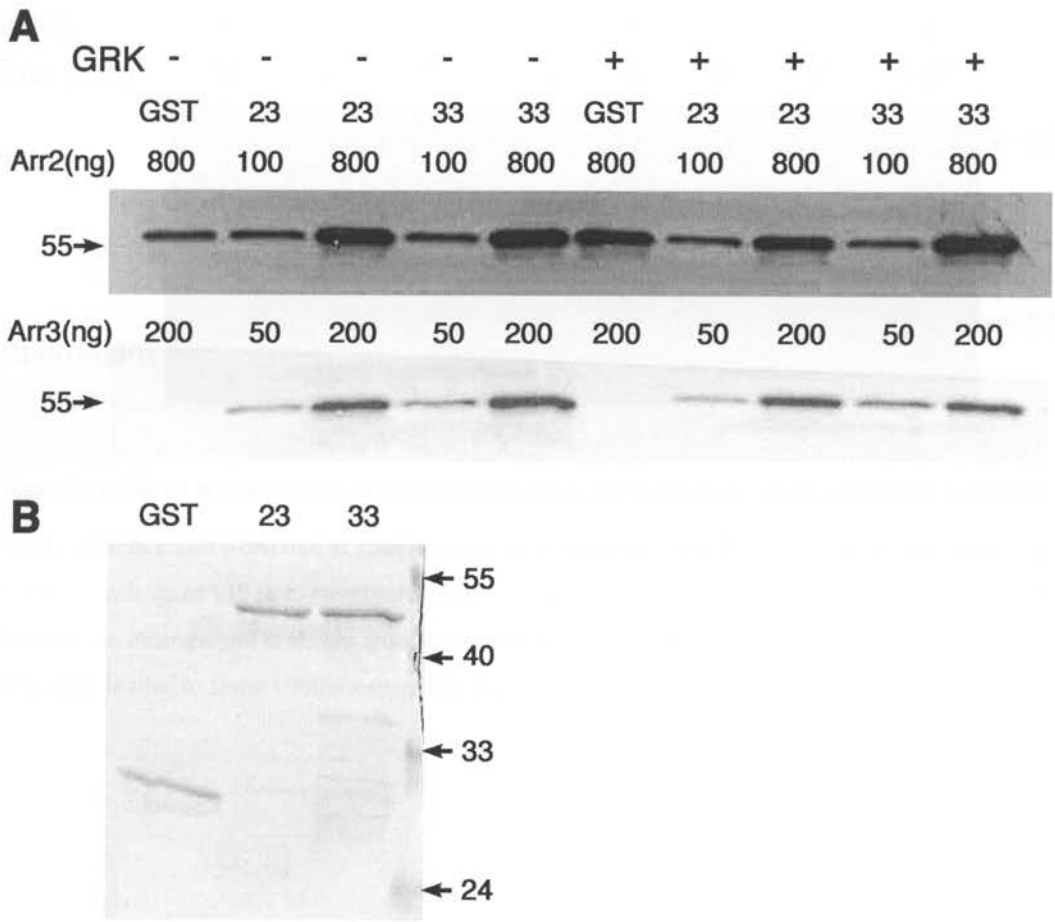

Fig. 4-6 Effect of GRK2 on arrestin binding to GST-D_2-IC3 and GST-D_3-IC3 fusion proteins. *In vitro* phosphorylation of fusion proteins by GRK2 were performed as in Fig 4-5 except that no [γ-^{33}P]ATP was included. GST alone (GST, 400 ng) or receptor third intracellular loop GST fusion proteins (23: GST-D_2-IC3 and 33: GST-D_3-IC3, 600 ng) were incubated with the indicated amounts of arrestin-2 or arrestin-3 for 30 min at room temperature. The amount of arrestin that co-eluted with GST or the GST fusion proteins was determined by immunoblotting with anti-arrestin antibodies. A. Immunoblots are shown from an experiment representative of 3 independent experiments. B. The protein-stained gel demonstrates that equal amounts of GST-D_2-IC3 and GST-D_3-IC3 were included in the reactions.

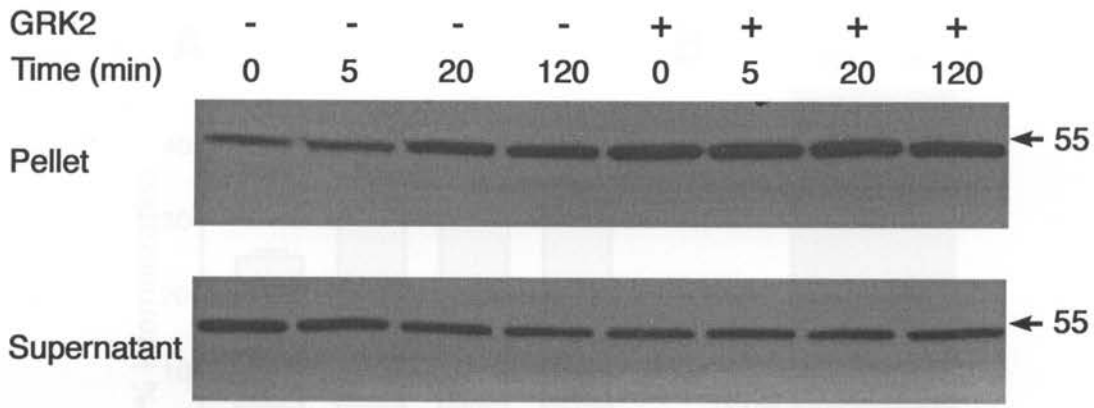

Fig. 4-7 Agonist-induced translocation of arrestin-3 in HEK 293 cells expressing arrestin-3 and wildtype D_2 receptor in the absence and presence of concomitant expression of GRK2. Cells were treated with 10 μM 7-OH-DPAT for periods up to 120 min, membranes were prepared, and levels of arrestin-3 were determined by immunoblotting. An immunoblot is shown from an experiment representative of 2 independent experiments. Supernatants were loaded to show similar expression levels of arrestin-3.

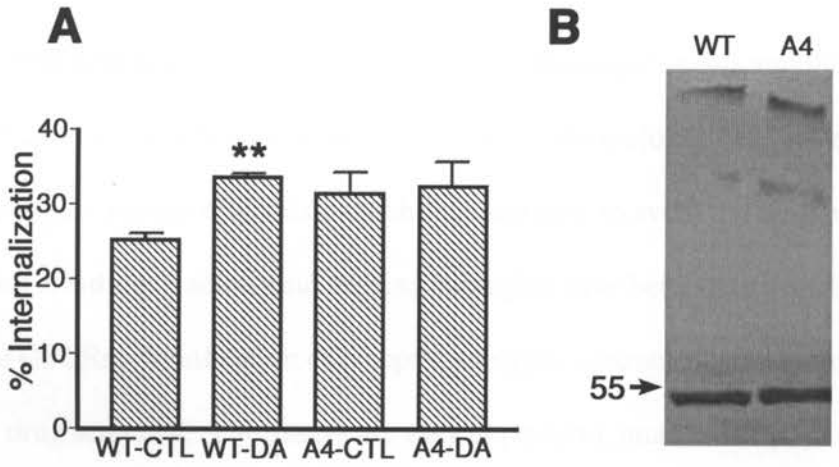

Fig. 4-8 Agonist-induced receptor internalization in HEK 293 cells overexpressing arrestin-3 and wildtype D_2 or mutant D_2-A4 receptors. A. Cells were treated with 10 μM dopamine for 20 min, and were subjected to the intact cell [^{3}H]spiperone binding assay. Results displayed are intracellular receptors expressed as percentages of total receptors in the basal state (control) and after dopamine stimulation, and are shown as the mean ± SE from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the respective control by paired t test. B. The expression levels of arrestin-3 were similar in cells expressed with wildtype or mutant receptors, as determined by Western blotting. Arrestin levels were measured to confirm arrestin-3 expression for both [^{3}H]spiperone and [^{3}H]sulpiride assays.

V. METHODOLOGICAL CONCERNS

There are some potential concerns when performing the experiments and interpreting data. In this section, I address the advantages and limitations of the major techniques employed in the dissertation, how these factors affect data reliability, and how to avoid the drawbacks effectively.

Radioligand Binding. Radioligand binding strategies have been used broadly in direct identification of GPCRs, classification of receptor subtypes, characterization of receptor regulation, and drug screening. The assays are easy to perform, quantitative, and easy to analyze (can be analyzed by curve fitting with nonlinear regression using commercially available software such as Prism). Below are several considerations when performing antagonist saturation and competition binding experiments:

(1) Receptor density: antagonist affinity is not generally influenced by the receptor density, but I try to use cell lines that express receptors at densities between 500 and 1000 fmol/mg of protein in case characterization of agonist binding or function will be carried out, because of the possibility that overexpression of receptors might lead to non-physiological interactions with G proteins.

(2) Ligand depletion: when performing radioligand binding assays, I make sure that only a small fraction of radioligand binds (ideally <10%), therefore the free concentration is close to the concentration added, and the free concentration is similar for determination of total and nonspecific binding. Increasing the amount of radioligand by increasing the assay volume or decreasing the amount of tissue may be necessary to prevent ligand depletion.

(3) Non-specific effects of mutations: it is important to distinguish between mutation-induced effects on ligand-receptor interactions that are due to contributions of specific

receptor domains or amino acid residues, and effects that are simply due to nonspecific distortion of the structure of the mutant receptor. In particular, caution should be taken when interpreting data that show decreased binding affinity. For this reason I give more weight to results showing a gain of affinity, which is the most rigorous criterion for identifying residues critical for ligand binding selectivity.

(4) Mutation of certain residues could lead to loss of detectable ligand binding due either to the loss of a critical ligand contact residue or to impaired trafficking to the membrane because of the importance of some residues for maintaining receptor structure. Although my results showed that none of the mutations caused complete loss of detectable ligand binding, immunocytochemistry was employed occasionally to verify that the mutant receptor was transported to the cell membrane.

(5) Agonists *vs.* antagonists: why did I choose antagonists for the proposed research instead of agonists? First, compared to the agonists, the available antagonists display higher selectivity and/or higher affinity. Second, for agonists, there is often considerable variability arising from buffer conditions, and interpretation of data is complicated by the presence or absence of two affinity states related to G protein coupling.

(6) Species difference: in the studies, I used rat D_2 and rhesus macaque D_1 receptors. The transmembrane domains of both D_1 and D_2 receptors are virtually identical between rat and rhesus macaque, and the pharmacological profiles show no species difference within mammals, suggesting that consequences of mutations in these regions will be the same regardless of species. In contrast, for receptor loops, such as in the case of EL2, sequence divergence is relatively more apparent between rat and monkey. Fortunately, since the length of the loop and the localization of the conserved cysteine are consistent between

the species, it is likely that the conformation of EL2 is virtually identical in mammalian orthologs of D_1 and D_2 receptors.

GST Pull-down. Affinity chromatography of receptor fragment-GST fusion protein is useful for identifying and confirming a protein that associates directly with a given GPCR. Non-visual arrestins are believed to be dopamine receptor-interacting proteins, but evidence for direct association between these receptors and arrestins is rare. The purpose of my research on the interaction between D2-like receptors and arrestins was to confirm direct association, and more importantly, to identify receptor regions that bind arrestin and that contribute to the difference between the D_2 and D_3 receptors.

This biochemical method is technically simple and suitable for medium-scale screenings. Purification and immobilization of the receptor fragment is achieved by rapid and high-affinity binding between glutathione-S-transferase and its substrate glutathione, which is conjugated onto beads. Purified proteins as well as tissue homogenates or cell and bacterial lysates can all be applied. However, binding and washing conditions and the choice of detergent greatly affect experimental outcome, and fusion proteins may not have the conformation that is required for efficient binding to the full-length native protein. Complementary functional approaches such as intact cell internalization assays and arrestin translocation assays are usually required to confirm data derived from GST pull-down. In addition, in the course of this project, I found several phenomena that were worth noting:

(1) Selection of starting and ending points of receptor fragments. Adding or deleting a few residues at the N- and/or C-termini of the receptor fragment can greatly change the quality and quantity of the fusion protein.

(2) Selection of bacterial cell lines for transformation. Receptor fragments encoded by DNA containing codons rarely used in E. coli may be poorly expressed. To avoid this kind of problem, it is better to use a bacterial strain designed to enhance the expression of eukaryotic proteins that are encoded by DNA containing rare codons.

(3) Context-dependent effects. As in the cases of reciprocal mutants of D_2-IC2 and D_3-IC2, the overall conformation of the fusion protein and the accessibility of individual residues in the context is critical for determining their effect on the binding of arrestin (see Chapter III).

(4) Data from purified protein pull-down and tissue pull-down may not be consistent, as described and discussed in Chapter IV.

Functional Assays. To confirm the arrestin binding determinants in the D_2 receptor and to investigate the functional significance of these potential binding sites, five techniques were used in preliminary studies: confocal microscopy, biotinylation, arrestin translocation, [^{3}H]spiperone binding assay, and [^{3}H]sulpiride binding assay. Confocal microscopy of arrestin3-GFP translocation to the plasma membrane upon agonist activation of D_2 receptor was not pursued due to its qualitative nature. I also discontinued use of the biotinylation assay for receptor internalization because the presence of multiple diffuse bands of D_2 receptor immunoreactivity on immunoblots makes it difficult to do quantitative analysis. I focused on the other three approaches.

(1) Agonist-induced translocation of arrestin-3 in HEK 293 cells transiently expressing arrestin-3 and either wildtype or mutant D_2 receptors. After agonist stimulation, a membrane pellet was prepared and the abundance of arrestin in the membrane pellet was detected by immunoblotting. This assay is simple, and sharp bands of arrestin

immunoreactivity can be quantified with computer programs such as IP Lab ((Scanalytics, Fairfax, VA). The weakness was that the basal level of arrestin in the membrane was high and was only doubled by agonist stimulation, making it difficult to detect subtle changes caused by some D_2 receptor mutants. I tried prolonging the preincubation with serum-free medium to 2 hr and did not get improved results.

(2) Intact cell [^{3}H]spiperone binding assay and [^{3}H]sulpiride binding assay. Both spiperone and sulpiride are high-affinity D_2 antagonists. The receptor internalization assays share a highly quantitative nature and specific binding is readily determined (unlabeled antagonists are used to determine non-specific binding, see Chapters III and IV, *Materials and Methods*). The [^{3}H]spiperone assay provides more information. Total, intracellular, and extracellular receptor amounts before and after agonist stimulation can all be obtained. The [^{3}H]sulpiride assay only measures cell surface receptors, and the data derived from this assay are more straightforward. More importantly, according to my experience, this method is more accurate compared to the other method and the results are less affected by receptor expression level. However, caution should be taken when performing the [^{3}H]sulpiride assay since persistent binding of agonist could be mistaken for receptor internalization. Under my experimental conditions, treatment with agonist at 4°C caused no significant loss of receptor binding (see Chapter IV).

When performing these functional assays, it is important to keep the expression levels of receptor and arrestin consistent or at least comparable among cells expressing either wildtype or mutant receptors. The intact cell [^{3}H]sulpiride assay and the immunoblotting of arrestin-3 are used to monitor expression levels.

VI. DISCUSSION

OVERVIEW

Dopamine receptors are integral membrane proteins. Towards the extracellular side of the membrane-spanning domains, they contain a ligand binding pocket. On the intracellular side, they hold multiple binding domains for cytosolic proteins including arrestins. In this dissertation, I investigated the determinants of ligand binding specificity between D_1 and D_2 dopamine receptors and of arrestin binding in D_2 and D_3 dopamine receptors. In the past two decades, gene-cloning techniques have revolutionized the field of dopamine receptors, helping elucidate their structural and transductional properties. In contrast, two major issues remain open: first, selective ligands for some receptor subtypes are still lacking, making the localization of individual receptor subtypes difficult and hindering characterization of the physiological roles of each subtype; second, the physiological roles and pathological implications of dopamine receptor subtypes are ambiguous or are largely unknown.

The D_1 and D_2 receptors are widely distributed and most similar to the pharmacologically defined D1-like and D2-like receptors, respectively. Although the D_1 receptor is the most abundant dopamine receptor, few antagonists highly selective for the D1-like receptors have been developed, and there are no ligands that distinguish between the D_1 and D_5 receptor subtypes. The development of additional D_2-selective ligands is also urgent because most nominally "D_2-selective" ligands are actually D2-like-selective and cannot distinguish the D_2 receptor from D_3 and D_4 receptors. The first goal of my dissertation is to identify the amino acids that determine the properties and shape of the ligand binding pockets and thus contribute to ligand binding selectivity between D_1 and D_2 receptors. This subtle structural information will

help develop highly D_1- or D_2-selective drugs, and in the long run, lay the foundation for the discovery of agonists and antagonists with even greater selectivity for each of the dopamine receptor subtypes.

The D_2 and D_3 receptors share similar signaling pathways. It has been speculated that different regulatory properties of these two D2-like receptors may contribute to their distinct physiological roles, and arrestin may be one of the key regulators (Kim et al., 2001). Arrestins are involved in desensitization, internalization, and resensitization of GPCRs as well as the assembly of signaling protein complexes (Luttrell et al., 1999; Barlic et al., 2000; DeFea et al., 2000). In addition, my dissertation work revealed that co-expression of arrestin-3 with the D_2 receptor increased receptor expression level. Arrestin-3 might function as a chaperone for the D_2 receptor, promoting trafficking of receptor to the plasma membrane. In summary, first of all, arrestins have been demonstrated to contribute to the regulation of desensitization and internalization of dopamine receptors; secondly, their involvement in dopamine receptor signaling is very likely; and finally, the roles of arrestins remain to be expanded and characterized. Thus, a better understanding of the molecular mechanism underlying the interactions between D2-like receptors and arrestins may greatly contribute to the clarification of the physiological roles of these receptors and may identify novel targets for therapeutic intervention.

In the first half of this dissertation, I investigated D_1/D_2 receptor selectivity determinants. Three primary conclusions were reached: 1) Residues 3.28 (Trp99 in D_1 and Phe110 in D_2) and 7.43 (Trp321 in D_1 and Tyr417 in D_2) were identified as contributing to D_1/D_2 receptor selectivity, presumably by making direct contact with certain ligands; 2) The residue at position 5.39 (Ala195 in D_1 and Val190 in D_2) played a role in pharmacological selectivity by altering

the size/shape of the binding pocket rather than interacting directly with ligands; 3) EL2 was implicated in the formation of the ligand binding pocket. In the second half of this dissertation, I investigated the determinants of binding of arrestin in D2-like receptors. Major findings include: 1) Arrestin-3 bound more avidly than arrestin-2 to both D_2 and D_3 receptor intracellular domains, and more avidly to IC3 than to IC2; 2) Arrestin-3 bound preferentially to D_2-IC2 over D_3-IC2 and the preference could be partly attributed to Lys149 in the C-terminal half of D_2-IC2; 3) For both D_2 and D_3 receptors, arrestin-3 binding was primarily localized to the first and last 20 residues of IC3, with the N-terminal segment of IC3 playing a more important role; 4) Mutation of IYIV212-215 at the N-terminus of D_2-IC3 blocked D2 receptor interaction with arrestin-3 without affecting coupling to G proteins.

FUTURE ISSUES TO ADDRESS

In the following sections I address the issues raised by the results of the research that were not addressed in the discussion of each chapter. In each section I will briefly state the background behind each issue and then discuss its implications and propose an approach to further address it.

Involvement of the EL2 in D_1/D_2 Receptor Selectivity. The EL2 of rhodopsin-family GPCRs has been suggested to line the ligand binding pocket and to play a role in pharmacological specificity, particularly those residues that immediately follow the conserved cysteine in EL2 (Olah et al., 1994; Kim et al., 1996; Zhao et al., 1996; Palczewski et al., 2000; Wurch and Pauwels, 2000; Shi and Javitch, 2004). I switched three (EL2.3) or four (EL2.4) consecutive residues following the conserved cysteine in the D_1 and D_2 receptors. All mutants showed loss of affinity for antagonists selective for corresponding wildtype receptors, giving

some support for the hypothesis that this region is part of the ligand binding pocket (Table 2-3). However, no gain of function was observed for all these mutants, making the role of EL2 in D_1/D_2 receptor selectivity ambiguous.

	4.60 NT	CT 5.36
Rhodopsin	171PLVGWSRYIPEGMQCS	**C**(187)GIDYYTPHEETNNE201
D_2 Rat	170LLFGLNNTDQNE	**C**(182)I I A N P187
D_1 Monkey	158PVQLSWHKAKPTS PSDGNATSLAETIDN	**C**(186)DSSLSR192

Fig. 6-1 Aligment of EL2s of bovine rhodopsin, rat D_2 and monkey D_1 receptors. The conserved cysteine that forms a disulfide bond with the cysteine at the extracellular end of TM3 is shown in bold. Each EL2 is divided into NT and CT by the conserved cysteine.

As we can see from Fig 6-1, the portions of EL2 that are to the N- and C-terminal sides of the conserved Cys residue both differ markedly among rhodopsin and the D_1 and D_2 receptors in both length and sequence. In addition, the conserved Cys in rhodopsin is in the middle of the EL2, but towards the C-terminal end of the loop in both dopamine receptors. Due to the overall structural divergence, it is difficult to predict how the EL2s of D_1 and D_2 receptors are arrayed. This may also explain why exchanging 3 or 4 residues in the loop does not confer gain of affinity for any ligands. The answer could be that hypothetical ligand-interacting residues in one subtype are not positioned properly in the context of the different-length loop from the other receptor subtype. To test more rigorously the contribution of EL2 to D_1/D_2 receptor selectivity, I suggest making chimeric receptors in which the EL2 from one receptor subtype is replaced with that from the other. If a chimeric receptor with the intact EL2 replaced shows gain-of-affinity for antagonists of the other receptor subtype, successively smaller substitutions, beginning with replacement of the residues N-terminal to the conserved Cys, could be used to narrow down the putative determinants of receptor selectivity. In addition, according to unpublished receptor modeling work by our collaborator, Dr. Martha Teeter, the longer EL2 of the D_1 receptor may

come closer to the end of TM5, possibly interacting with the residue at position 5.39 (Ala in D_1 and Val in D_2). If the chimeric receptors provide evidence that EL2 is a structural determinant of receptor selectivity, an intriguing additional experiment would be to make chimeric receptors combining the Ala/Val mutation at 5.39 with the EL2 substitution to see if these manipulations produce additive effects.

Additional Determinants of D_1/D_2 Receptor Selectivity that Indirectly Affect Ligand Binding. I have mutated most residues that line the ligand binding pocket but are non-conserved between D_1 and D_2 receptors. Residues at positions 3.28 and 5.39 have been determined to be critical residues for D_1/D_2 receptor selectivity, as demonstrated by gain of affinity for antagonists selective for the other receptor subtype, and the residue at position 7.43 may also sterically inhibit the binding of certain subtype-selective ligands (Lan et al., 2006). However, none of these residues can fully account for the difference in ligand selectivity between D_1 and D_2 receptors. Even if the effects of the individual mutations were assumed to be additive, their combination would leave most of the pharmacological difference between D1 and D2 receptors unexplained. It is most likely that additional determinants of D_1/D_2 receptor selectivity exist, either in EL2 or in residues/regions that are not exposed in but proximal to the ligand binding pocket and indirectly influence the interactions of selective ligands with the conserved primary contact residues (see Chapter I), as in the case of residue 5.39. This residue packs against residue 6.59 (Fig 2-5). Mutation of Ala in the D_1 receptor to aligned D_2 residue Val, which is bigger than Ala, increases the distance between TM5 and TM6, resulting in different packing of these two helices and consequently changing the shape and/or size of the ligand binding pocket.

Based on previous data, it will be interesting to examine whether mutating the residue at position 6.59 in the D_1 receptor from the smaller Pro to the bigger Ile in the D_2 receptor

increases affinity for D_2-selective antagonists. Furthermore, if mutation at position 6.59 does have the effect that we expect, combining the 5.39 and 6.59 mutations is very likely to confer even more of a D_2-like binding profile on the D_1 receptor. Finally, to convert the D_1 receptor to an even more D_2-like binding profile, I suggest combining the 3.28, 5.39, and 6.59 mutations, making a triple mutant.

The distance between residues 5.39 and 6.59 affects the spacing between critical serine residues in TM5 (Ser5.42, Ser5.43, and Ser5.46) and critical aromatic residues in TM6 (Phe6.52, Phe6.51, and Trp6.48) (see Chapter I and Fig 2-5). Phe5.47 packs against Phe6.52 in both D_1 and D_2 receptors (Fig 2-5). I hypothesize that mutating Phe at position 5.47 in the D_1 receptor to the bigger Trp will increase the distance between TM5 and TM6, thus leading to the same effect as Ala/Val mutation at position 5.39, that is, gain of binding for D_2-selective antagonists and loss of binding for the D_1-selective antagonist SCH23390.

Does the D_2-A4 Mutation Have any Effect on MAP Kinase Activation? The four-residue mutation IYIV212-215A4 was found to disrupt binding of purified arrestin-3 to D_2-IC3 in the GST pull-down assay and to abolish arrestin translocation and receptor internalization in the context of full length receptor, but not to affect G protein coupling. The activation of MAP kinases such as extracellular signal-regulated kinases (ERKs) by GPCRs plays a role in DNA synthesis and mitogenesis (Dhanasekaran et al., 1998). The D_2 receptor-mediated activation of ERKs depends on transactivation of either the epidermal growth factor receptor or the platelet-derived growth factor receptor (Wang et al., 2005). Interestingly, the cellular internalization machinery is involved in both direct EGF stimulation of ERK1/2 activity and GPCR-mediated signaling to ERK1/2 (Maudsley et al., 2000). In addition, stimulation of some GPCRs results in the assembly of a protein complex in which arrestin acts as a scaffold protein, recruiting MAP

kinase cascades and agonist-occupied GPCR and facilitating GPCR-activated kinase activation (Luttrell et al., 1999; McDonald et al., 2000). Because the D_2-A4 mutation abolished receptor internalization and arrestin-3 translocation, one intriguing issue is whether this mutation inhibits ERK activation. To investigate this problem, I propose to measure ERK phosphorylation upon dopamine stimulation of cells expressing arrestin-3 and either wildtype or mutant D_2 receptors.

Do the D_2-K149C and D_3-C147K Mutations Affect Receptor Internalization and Arrestin Translocation? Data from GST pull-down of purified arrestin-3 suggested the importance of D_2 residue Lys149 in the preferential binding of arrestin to D_2-IC2 over D_3-IC2, strongly evidenced by the loss of arrestin binding to GST-D_2-IC2-K149C and the gain of binding to the reciprocal mutant GST-D_3-IC2-C147K. However, the importance of Lys149 has not been confirmed by functional assays in the context of the full-length receptor. I propose to use the [^{3}H]sulpiride binding assay first for the whole receptor mutant D_2-K149C, followed by the arrestin translocation assay as performed for D_2-A4 mutant. If this single mutant does decrease receptor internalization and/or arrestin translocation to the plasma membrane in response to dopamine stimulation, that would confirm the role of Lys149 as a critical contact residue for arrestin-3. This will be an exciting finding since it will identify a novel mechanism contributing to the differential regulation of the D_2 and D_3 receptors by arrestin-3 (Kim et al., 2001). In addition, it may also be worthwhile to test the reciprocal mutation C147K in the full-length D_3 receptor. Obviously, the gain of function of this D_3 mutant will provide further support.

Does the D_2-IC3ΔMID Mutation Affect Receptor Internalization and Arrestin Translocation? We have localized arrestin-3 binding sites within IC3 to the first and last 20 residues, with the N-terminal segment, in particular a stretch of residues, IYIV212-215, playing a

more important role (see Chapter III). In contrast, deletion of 121 residues comprising the middle segment of IC3 (D_2-IC3ΔMID) had little effect on the binding of arrestin (Fig 2-4). It is surprising considering the length of this segment. However, GST pull-down assay only assesses the role of non-phosphorylated arrestin binding sites and the middle segment contains Ser/Thr residues that are potentially phosphorylated by GRKs. Functional assays using the whole receptor mutant D_2-IC3ΔMID will provide additional support for previous data and rule out the possibility of any context-dependent effect. I hypothesize that data from receptor internalization and arrestin translocation assays will be consistent with GST pull-down data; that is, this mutation will have little effect on the ability of the receptor to undergo agonist-induced internalization or to induce the translocation of arrestin. If my hypothesis is correct, the story of arrestin binding determinants within the IC3 will be more complete and convincing.

CONCLUDING REMARKS

In this dissertation, I provided new insights into the ligand binding pocket of the D_1 and D_2 dopamine receptors and D_1/D_2 pharmacological selectivity, and the determinants of arrestin-binding in D2-like receptors. Future work is required to completely accomplish the goals of these projects, as I detail in this chapter. The experiments performed and proposed will greatly add to the current understanding of the structure and function relationship of dopamine receptors, and perhaps even more importantly, give implications for molecular GPCR research and for drug discovery within the pharmaceutical industry.

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