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# Dopamine D1 and D2 Receptor Interaction with Endogenous Arrestin2 and 3

by

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A DISSERTATION

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## ABBREVIATIONS

| | |
|---|---|
| cAMP | cyclic AMP; adenosine 3', 5'-cyclic monophosphate |
| D2-EGFP | D2 receptor with enhanced green fluorescent protein attached to the C terminus |
| DMEM | Dulbecco's modified Eagle's medium |
| EGFP | enhanced green fluorescent protein |
| ERK | extracellular-signal-related kinase |
| FRET | Fluorescence Resonance Energy Transfer |
| G protein | heterotrimeric GTP-binding protein; |
| GPCR | G protein-coupled receptor |
| GRK | G protein-coupled receptor kinase |
| GST | glutathione-S-transferase |
| HEK | human embryonic kidney cells |
| $K_D$ | equilibrium dissociation constant |
| MEM | minimal essential medium |
| NS20Y | mouse neuroblastoma cells |
| 7-OH DPAT | 7-hydroxy-2-(N,N-di-n-propylamino)tetraline |
| PTX | pertusis toxin |
| siRNA | small interfering RNA |
| SDS-PAGE | polyacrylamide gel electrophoresis |
| SKF-82958 | (+/-)6-chloro-7,8-dihydroxy-3-ally1- phenyl-2,3,4,5-tetrahydro-1H-3benzazepine hydrobromide |
| TBS | Tris-buffered saline |

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Finally, a special thank you to my family and close friends-NJ represent!

I dedicate this thesis to Wes, Wil, and the beagles!

**Abstract**

The goal of my dissertation research was to assess the interaction of the dopamine D1 and D2 receptors and endogenous arrestin2 and arrestin3. Dopamine receptors are 7-transmembrane domain G-protein coupled receptors (GPCR) and transduce signal produced by dopamine stimulation. Arrestins are cytosolic proteins that bind to the GPCR and cause desensitization of the receptor. Specifically, I wanted to determine if there was a difference between each receptor type and its interaction with either isoform of arrestin.

To elucidate the interaction between the dopamine D1 and D2 receptor and each form of arrestin I used various molecular and biochemical techniques. Neostriatal cultures were used to investigate the endogenous dopamine D1 and D2 receptor and endogenous arrestins. In addition, the interaction of the D2 receptor and endogenous arrestins was investigated in NS20Y neuroblastoma cells expressing an enhanced green fluorescent protein-tagged D2 receptor (D2-EGFP).

The colocalization of the dopamine D1 and D2 receptor with arrestin2 and arrestin3 was investigated using confocal microscopy, translocation studies, and co-immunoprecipitation. In neostriatal neurons, 2, 5, and 20 min agonist treatment increased the colocalization of the D1 receptor and arrestin3 immunoreactivity. There was no change in the colocalization of the D1 receptor and arrestin2. D1 receptor agonist treatment for 5 and 20 min caused translocation of arrestin3, but not arrestin2, to the membrane. The binding of arrestin3, but not arrestin2, to the D1 receptor was increased, as assessed by co-immunoprecipitation following agonist treatment for 5 and 20 min. In

NS20Y cells, D2 agonist stimulation enhanced the colocalization of D2-EGFP with endogenous arrestin2 and 3 at 2 hr. In neurons, 2-hr agonist treatment selectively increased the colocalization of the endogenous D2 receptor with arrestin2, but colocalization with arrestin3 was reduced. Translocation of arrestin2, but not arrestin3, to the membrane in neurons, and an increase in the interaction of the D2 receptor and arrestin2 as assessed with co-immunoprecipitation occurred upon agonist activation.

Internalization of the dopamine D1 and D2 receptors was characterized. Agonist treatment of neurons induced D1 receptor internalization (35-45%) that was maximal within 2-5 min. The internalization of the endogenous D2 receptor in neostriatal neurons was also maximal at 20 minutes. In D2-EGFP-NS20Y cells, agonist treatment induced D2 receptor internalization (36-46%) that was maximal within 20 min, but that was prevented by siRNA-induced depletion of arrestin2 and 3.

GST fusion protein constructs were made to the C-terminus, second loop, or third loop of the dopamine D1 receptor and the dopamine D2 receptor. The fusion proteins were incubated with striatal brain homogenate or purified arrestin2 or arrestin3. We hypothesized that the C-terminus of the dopamine D1 receptor would bind arrestin3. Endogenous arrestin2 and 3 in striatal homogenates bound to the C-terminus of the D1 receptor, with arrestin3 binding more strongly. The D1 C-terminus and, to a lesser extent, the third cytoplasmic loop, also bound purified arrestin2 and 3. We hypothesized that the third loop of the dopamine D2 receptor would bind arrestin2 and arrestin3. Arrestin2 and 3 in striatal homogenates bound to the third cytoplasmic loop of the D2 receptor, and purified arrestin2 and 3 bound to the second and third loops and C-terminus of the D2 receptor. Overall, these data indicate that the D1 receptor preferentially

interacts with arrestin3 in neostriatal neurons. In addition, there was a selective agonist-induced interaction between the D2 receptor and arrestin2 in neurons.

## 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, four chapters of original data, 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*.

Chapter 2 contains data, figures, and text as they appear in an original paper that has been published previously (Macey et al., 2004). Chapter 3 contains data, figures, and text as they appear in an original paper that has been accepted for publication (Macey et al., 2005). Chapter 4 and Chapter 5 represent data that were collected during the project.

### Papers representative of this work

Macey, Tara A., Gurevich, Vsevolod V., and Neve, Kim A., (2004) Preferential interaction between the dopamine D2 Receptor and arrestin2 in neostriatal neurons, *Molecular Pharmacology,* 66(6):1635-42.

Macey, Tara A., Liu, Yong, Gurevich, Vsevolod V., and Neve, Kim A. (2005) Dopamine D1 receptor interaction with arrestin3 in neostriatal neurons, accepted 11/20/04 to *J. Neurochemistry, in press*

### Abstracts representative of this work

Macey, Tara A. and Neve, Kim A. (2005) Dopamine Receptor Interactions with Arrestins in Neostriatal Neurons. WCBR.

Macey, Tara A., Buck, Dave C., and Neve, Kim A. (2004) Differences in the interaction of the dopamine D2 receptor and endogenous β-arrestin1 and 2 in primary striatal cultures and D2 NS20Y cells. Experimental Biology, Washington, DC.

Macey, Tara A. and Neve, Kim A. (2003) Interaction of the endogenous dopamine D2 receptor and endogenous β–arrestin1 and 2 in primary striatal cultures. Society for Neuroscience, New Orleans, LA.

Macey, Tara A. and Neve, Kim A. (2003) Interaction of the dopamine D2 receptor and endogenous β-arrestin 1 and 2. Experimental Biology, San Diego, CA.

## PROJECT INTRODUCTION

## DOPAMINE AS A NEUROTRANSMITTER

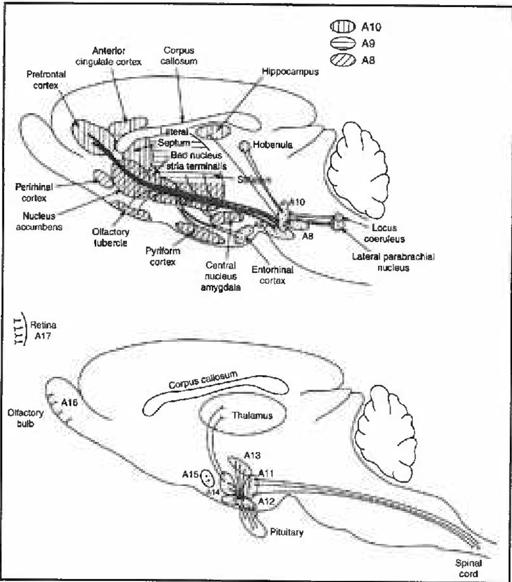
Dopamine activity is imperative to central nervous system (CNS) function. Dopamine dysfunction is responsible for various neurological and psychological disorders, including Parkinson's disease and schizophrenia. Dopamine binds to dopamine receptors, which are 7-transmembrane domain membrane-bound G-protein coupled receptors (GPCR). Dopamine receptors transduce signal produced by dopamine stimulation across the membrane and associate with additional signaling proteins, such as heterotrimeric GTP-binding protein (G protein), GPCR kinases (GRKs), and arrestins. GRKs bind the agonist-occupied GPCR and phosphorylate the receptor. Once the GPCR is phosphorylated, cytosolic proteins termed arrestins bind to the GPCR and cause dissociation of the cognate G protein, followed by desensitization of the GPCR. The association of dopamine receptors with various signaling proteins is important to the understanding of CNS function. The purpose of my dissertation is to investigate the trafficking of the dopamine D1 and D2 receptors and elucidate the interaction of each receptor type with both isoforms of endogenous arrestin. The dopamine D1 receptor interacts with arrestin3 in D1- and GRK-transfected mammalian cells. The dopamine D2 receptor has been characterized to interact with both forms of arrestin when both arrestin and GRKs are added to D2-transfected mammalian cell lines. The internalization of both receptor types has not been extensively studied. To further investigate the interaction of the dopamine D1 and D2 receptor with endogenous arrestins, agonist-induced colocalization, co-immunoprecipitation, and translocation of arrestins were determined in primary striatal cultures. To visualize the dopamine D2 receptor without the use of

antibody, the dopamine D2 receptor was fused at its C-terminus to the enhanced green fluorescent protein (D2-EGFP) and transfected into mouse neuroblastoma cells (D2-EGFP NS20Y). The enhanced green fluorescent tag emits green fluorescence when stimulated at 488 nm. The interaction of the D2-EGFP with endogenous arrestin was investigated using D2-EGFP NS20Y cells. When siRNA to both isoforms of arrestin were introduced, internalization of the dopamine D2 receptor in D2-EGFP cells was attenuated, suggesting the dopamine D2 receptor is dependent on arrestin for internalization. The C-terminus and the third intracellular loop of both dopamine receptor subtypes and the second intracellular loop of the dopamine D2 receptor were investigated for their possible interaction with endogenous arrestin and purified arrestin using GST fusion protein constructs. This introductory section includes background information on the neurotransmitter dopamine, and more specifically the signal transduction and regulation of the dopamine D1 and D2 receptors. In addition, the introduction will focus on the phenomenon of desensitization and the role of arrestin in the desensitization of the dopamine D1 and D2 receptors.

**History.** Dopamine is derived from the amino acid tyrosine and is grouped as a catecholamine with other neurotransmitters that are also derived from tyrosine, including norepinephrine and epinephrine. Dopamine plays a role in several physiological responses, including cognition, cardiovascular responses, endocrine function, and motor coordination. The ability for dopamine to act as a neurotransmitter in the brain was investigated in 1958 by Carlsson and others (Carlsson et al., 1958). Dysfunction of dopamine neurotransmission contributes to both psychiatric disorders such as schizophrenia, and neurological diseases such as Huntington's and Parkinson's disease.

In addition, dysregulation of the dopamine system has been implicated in the development of movement disorders, such as tardive dyskinesia that occurs with chronic neuroleptic treatment. Dopamine dysfunction is also involved in opiate and cocaine addiction. Norepinephrine is primarily localized to the hypothalamus and dopamine is highly localized within the nuclei of the basal ganglia. Dopamine, as a neurotransmitter, and its involvement in motor control was investigated by Ehringer and Hornykiewicz in 1960 (Ehringer and Hornykiewicz, 1960). Ehringer and Hornykiewicz determined that there was a decrease in dopamine specific to the basal ganglia of Parkinson's disease patients (Ehringer and Hornykiewicz, 1960).

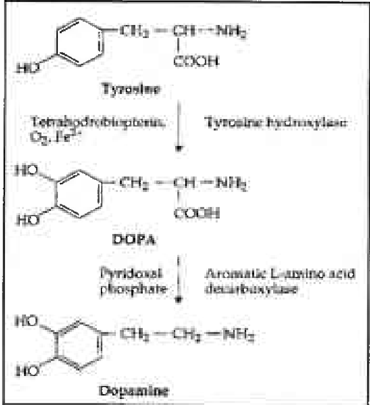
**Figure 1-1**




**Neuroanatomy.** The organization of the central dopamine-containing systems is complex and is depicted in Figure 1-1. There is 3 to 4 times the amount of dopamine cells in the brain compared to noradrenergic neurons, and several major dopamine-containing nuclei (Cooper et al., 2003). The dopamine systems are divided into 3 categories based on the lengths of the efferent dopamine fibers: ultrashort, intermediate, and long. The two ultrashort systems, also termed interneurons, make extremely localized connections. The interplexiform amacrine-like neurons (A17) link the outer and inner plexiform layers of the retina. The periglomerular dopamine cells in the olfactory bulb (A16) link the mitral cell dendrites in separated adjacent glomeruli. Next, the intermediate-length dopamine systems include the tuberohypophysial dopamine cells (A12, 14), the

incertohypothalamic neurons (A11, 13, 15), and the medullary periventricular group. The tuberoinfundibular system projects from the arcuate and periventricular nuclei and ends in the intermediate lobe of the pituitary or in the median eminence. The second intermediate-length system is comprised of incertohypothalamic neurons, which link the dorsal and posterior hypothalamus with the dorsal anterior hypothalamus and lateral septal nucei. The final intermediate-length dopamine system is the medullary periventicular group, which includes the dopamine cells in the perimeter of the dorsal motor nucleus of the vagus nerve, the nucleus tractus solitarius, and the cells within the tegmental radiation of the periaqueductal gray matter. The long system comprises the final part of the central dopamine-containing network. The long system includes the projections of the dopamine cells that link the ventral tegmental area (A8, A10) and the substantia nigra (A9) with three target areas, the neostriatum, the limbic cortex, and other limbic groups. The limbic cortex, including the medial prefrontal, cingulate, and entorhinal areas, are termed the mesocortical dopamine projections. The mesolimbic dopamine projections include other limbic structures such as the regions of the septum, olfactory tubercle, nucleus accumbens, amygdaloid complex, and the piriform cortex (Cooper et al., 2003).

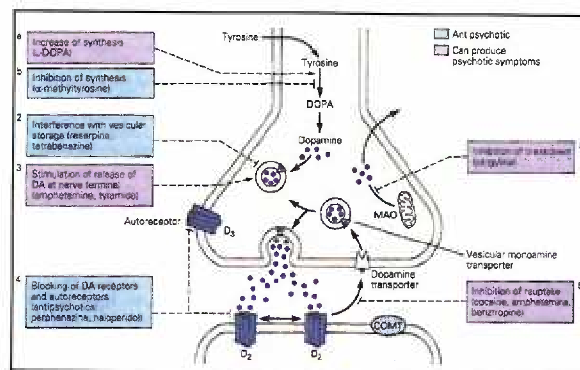
**Figure 1-2**




**Biosynthesis and modulation of neuronal activity.** Dopamine synthesis originates from the conversion the amino acid, L-tyrosine, to L-dihydroxyphenylalanine (DOPA), by the rate limiting enzyme tyrosine hydroxlase (Figure 1-2) (Cooper et al., 2003). Dopamine is produced when DOPA is metabolized by DOPA decarboxylase, or L-aromatic amino acid

decarboxylase. The synthesis of dopamine is modulated by several different sources, including the availability of substrate for dopamine synthesis, vesicle storage, dopamine receptors, and dopamine transporters (Figure 1-3). The availability of the amino acid tyrosine can affect dopamine synthesis. Tyrosine hydroxylase, the rate limiting enzyme of dopamine synthesis, requires a cofactor for its activation. This cofactor, tetrahydrobiopterin (BH4), is affected by competitive binding of dopamine or other catecholamines. Tyrosine hydroxylase is also modified by pharmacological agents, which inhibit its activity, including α-methyl-para-tyrosine. Tyrosine hydroxylase activity is enhanced by increases in neuronal activity or by increases in intracellular cAMP or $Ca^{2+}$ levels, which stimulate increases in phosphorylation. Dopamine synthesis is affected by vesicular storage of dopamine. For example when reserpine is applied, a long-lasting depletion of dopamine can occur, which prevents dopamine uptake and storage (Figure 1-3). In addition, when amphetamine is administered, the stimulation of dopamine release at the nerve terminal alters dopamine synthesis and the reuptake of dopamine is blocked (Figure 1-3). Dopamine synthesis is affected when dopamine receptors and autoreceptors are blocked by antipsychotics because receptor signaling via dopamine is prevented. Cocaine or amphetamine prevents the reuptake of dopamine via dopamine transporters (Figure 1-3).

**Figure 1-3**




After synthesis, dopamine is released into the synaptic cleft and can interact with autoreceptors and postsynaptic dopamine receptors (Figure 1-3). Autoreceptors are

localized on the cell bodies or axon terminals of dopamine neurons. Postsynaptic dopamine receptors are located on the axon terminals on non-dopaminergic cells, for example GABA-containing neurons. The stimulation of autoreceptors or postsynaptic dopamine receptors modulates the 2$^{nd}$ messenger signals to other neurotransmitters or channels. For example, the postsynaptic dopamine D1 receptor can stimulate adenylate cyclase activity in the cell, raising levels of intracellular cAMP, thereby activating protein kinase A (PKA). PKA acts to phosphorylate proteins within the cell. The stimulation of dopamine receptors varies from cell to cell based on populations of the receptors and the populations of proteins that the receptor may affect. The stimulation of dopamine receptors may increase or decrease the release of neurotransmitters. Dopamine receptors enhance or inhibit the ability of a cell to respond to a reduction in membrane potential, or depolarization, leading to the generation of an action potential.

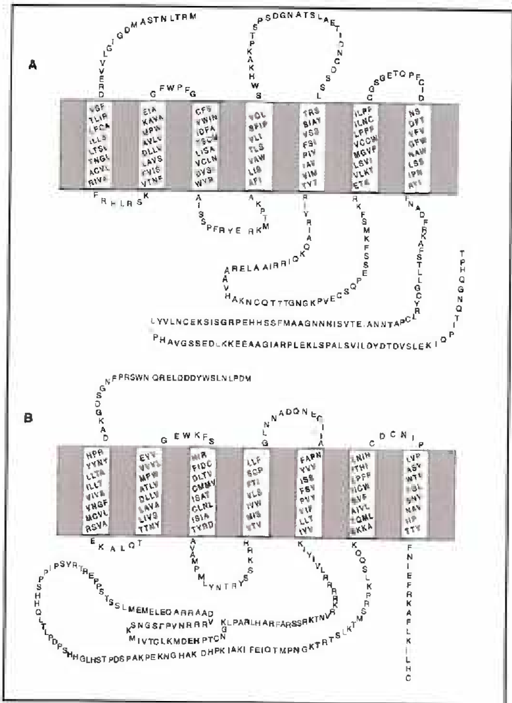
Dopamine transporters terminate the actions of dopamine. The plasma membrane dopamine transporter is a 619-amino acid protein with 12 membrane-spanning domains. The dopamine transporter recaptures dopamine soon after its release, and modulates its concentration in the synapse. The dopamine transporter is dependent on $Na^+$ and $Cl^-$. The energy for the dopamine transporter is provided via the $Na^+$ gradient generated by the $Na^+/K^-$ exchanging adenosine triphosphatase. The action of the dopamine transporter can be inhibited by the drugs benztropine, cocaine, and a series of GBR compounds.

The major metabolites of dopamine in the CNS are homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC). Dopamine is converted to DOPAC by intraneuronal monoamine oxidase (MAO) after reuptake by the nerve terminal. Released dopamine is also metabolized to HVA through the sequential action of catechol-O-

methyl-transferase (COMT) and MAO. Dopamine converted to 3-methoxytyramine (MT) via COMT and can be further metabolized by MAO to HVA in glial cells.

## MOLECULAR BIOLOGY OF DOPAMINE RECEPTORS

Figure 1-4



In the late 1970's, after more than one type of dopamine receptor was thought to exist in the brain based on biochemical and pharmacological evidence, two classes of dopamine receptors were proposed. Specific drugs that antagonized dopamine in the pituitary did not block dopamine activity in the neostriatum. The 2 families of dopamine receptors proposed were the dopamine D1-like and dopamine D2-like receptors. The dopamine D1 family of receptors is comprised of D1 (Fig 1-4, A) and D5 dopamine receptors. The dopamine D2 family of receptors includes the D2 (Fig 1-4, B), D3, and D4 dopamine receptors.

**The dopamine D1 receptor family.** The structure of the dopamine D1 receptor includes a long C-terminus and a shorter third intracellular loop, and both regions of the receptor include several residues that are involved in the desensitization of the receptor (Fig 1-4, A). Four groups isolated DNA encoding the D1 receptor (Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990). The mammalian D1 receptors are 446 residues and have a molecular weight of 49 kDa (Zhou et al., 1990). The human dopamine D1 receptor is located on chromosome 5, q35.1 (Grandy et al., 1990). The

highest abundance of the rat and human D1 receptor mRNA is located within the striatum, which includes the caudate, nucleus accumbens, and olfactory tubercle (Dearry et al., 1990; Monsma et al., 1990; Mansour et al., 1991).

The dopamine D1 receptor belongs to a subfamily of 7-transmembrane domain GPCRs that interact with the G protein $G\alpha_S$ to modulate several downstream effectors including stimulation of adenylate cyclase, stimulation of phosphatidylinositol (PI), and activation of L-type $Ca^{2+}$ channels via activation of protein kinase A (Neve and Neve, 1997). The intracellular third loop of the D1 receptor is responsible for coupling to $G\alpha s$ and the stimulation of adenylate cyclase (Kozell et al., 1994). Dopamine D1 receptor signaling in the striatum is mediated by the G protein $G_{olf}$ (Zhuang et al., 2000). When the dopamine D1 receptor is transfected into mammalian cell lines, the receptor binds the D1 antagonist SCH 23390 with high affinity (<1 nM) and binds the D1 antagonist (+)-butaclamol with nM affinity. The recombinant receptors exhibit high affinity for D1 agonists fenoldepam and SKF 38393 compared to D2-selective or nondopaminergic agonists. The dopamine D1 receptor has μM affinity for dopamine (Neve and Neve, 1997).

Desensitization of the dopamine D1 receptor has been described in a variety of cell lines and tissue preparations (Ng et al., 1994; Tiberi et al., 1996; Gardner et al., 2001; Lamey et al., 2002; Mason et al., 2002; Kim et al., 2004a). Both second messenger-dependent protein kinases, such as PKA, and GRK-mediated phosphorylation are responsible for the desensitization of the D1 receptor. Thr-136, within the second intracellular loop, and Thr-268, within the third cytoplasmic loop, of the dopamine D1 receptor are potential phosphorylation sites for PKA. The long C-terminal tail of the

dopamine D1 receptor contains multiple serine and threonine residues that are potentially phosphorylated by GRKs (Neve and Neve, 1997).

Mutagenesis studies within the dopamine D1 receptor have identified critical residues for receptor desensitization. Sites within the C-terminus and third loop responsible for the desensitization of the dopamine D1 receptor have been described (Lamey et al., 2002; Jackson et al., 2002; Mason et al., 2002; Kim et al., 2004a; Tiberi et al., 1996). Both Jackson *et al.* and Lamey *et al.* demonstrated that specific residues within the C-terminus of the receptor are responsible for phosphorylation-dependent desensitization of the D1 receptor (Lamey et al., 2002; Jackson et al., 2002). Tiberi *et al.* demonstrated GRK-mediated phosphorylation of the D1 receptor. When the dopamine D1 receptor is transfected in HEK293 cells and co-expressed with GRK2, GRK3, or GRK5 there is a significant increase in agonist-induced phosphorylation of the receptor on serine residues (Tiberi et al., 1996). In the third intracellular loop of the D1 receptor, Mason *et al.* found that residue T268 is phosphorylated in a PKA-dependent manner and the rate of the desensitization of the receptor or the sorting of the receptor to the perinuclear region of the cell is regulated by T268 (Mason et al., 2002).

The interaction of arrestins and the dopamine D1 receptor and the contribution of this interaction to receptor internalization have also been investigated, but only in studies using heterologous expression of arrestins and the D1 receptor in non-neuronal cells. Internalization is necessary for recycling of the receptor back to the membrane for resensitization and for signaling that occurs via the D1 receptor, such as ERK1/2 activation (Chen et al., 2004). Activation of the heterologously expressed dopamine D1 receptor causes GRK-dependent receptor phosphorylation, an increase of the

translocation of GFP-tagged arrestin3 compared to GFP-tagged arrestin2 to the cell membrane, and receptor internalization followed by the disassociation of arrestin at or near the membrane (Zhang et al., 1999; Oakley et al., 2000; Kim et al., 2004a).

The dopamine D5 receptor is also part of the dopamine D1 receptor family. The primary pharmacological difference between the dopamine D1 receptor and the dopamine D5 receptor is that the D5 receptor has an increased affinity for dopamine and other agonists. There are no selective ligands that have been developed to distinguish the dopamine D1 receptor from the dopamine D5 receptor. The dopamine D5 receptor mRNA is localized primarily to the hippocampus and the thalamus.

**The dopamine D2 receptor family.** In 1988, the dopamine D2 receptor was cloned, when a cDNA encoding a 415 amino acid protein was isolated from a rat brain cDNA library (Bunzow et al., 1988). The mRNA encoding the D2 receptor was localized in the hypothalamus and basal ganglia. The two forms of the receptor, D2L and D2S, are produced as RNA splice variants of one another. The rat and murine D2S and D2L receptors are 415 and 444 residues in length with a predicted molecular weight of 47 kDa and 50.9 kDa, respectively (Bunzow et al., 1988; Monsma et al., 1989; Montmayeur et al., 1991). The D2S and D2L sequences are identical with the exception of a 29 amino acid insert that begins at residue 242 of the D2L receptor. The human dopamine D2 receptor has been localized to chromosome 11q22-q23 (Grandy et al., 1989). The D2 receptor mRNA is abundant in the nervous system, and in the brain is localized to the caudate putamen, nucleus accumbens, olfactory tubercle, globus pallidus, substantia nigra, and ventral tegmental area (Le Moine et al., 1991; Surmeier et al., 1992; Lester et al., 1993; Rappaport et al., 1993).

The dopamine D2 receptor belongs to a subfamily of 7-transmembrane domain GPCRs that interact with the G proteins $G\alpha_i$ and $G\alpha_o$ to modulate several downstream effectors including inhibition of adenylate cyclase, activation of phospholipases, activation of K+ currents, and deactivation of $Ca^{2+}$ channels (Neve and Neve, 1997). The third cytoplasmic loop of the dopamine D2 receptor determines the selectivity in the coupling of the receptor to G proteins, similar to other GPCRs (Neve and Neve, 1997). It has been demonstrated that the second and third intracellular loop of the dopamine D2 receptor are involved with the inhibition of adenylate cyclase and both loops are necessary for minimal inhibition of adenylate cyclase (Kozell et al., 1994; Robinson and Caron, 1996). In addition, the third loop of the dopamine D2 receptor has been implicated in the activation of ERK (Takeuchi and Fukunaga, 2004).

Transfection of the cDNA into mammalian cell lines resulted in expression of a receptor with D2-like pharmacology. The D2 receptor exhibited high affinity for spiperone and sulpiride and mediated the inhibition of adenylate cyclase in response to agonist (Bunzow et al., 1988; Neve et al., 1989; Albert et al., 1990). The recombinant dopamine D2 receptor binds dopamine and quinpriole with μM affinity and has nM affinity for bromocriptine (Neve and Neve, 1997). The D2 receptor has the following rank order of potency: spiperone > (+)-butaclamol > haloperidol > sulpiride > SCH23390 = ketanserin > (-)-butaclamol (Bunzow et al., 1988). The D2L and D2S receptors have similar pharmacological profiles, however D2S does exhibit two- to threefold higher affinity for some substituted benzamide derivatives (Castro and Strange, 1993b). The D2S receptor inhibits adenylate cyclase more efficiently than D2L possibly

due to the ability for D2S to couple to G proteins with greater efficiency (Castro and Strange, 1993a).

The structure of the dopamine D2 receptor includes a long intracellular loop that contains multiple residues that are potentially involved in desensitization of the receptor and a short carboxyl-terminal tail (Neve and Neve, 1997). The potential PKA phosphorylation of the dopamine D2 receptor is located in the third cytoplasmic loop at Ser-364, the only residue that contains the optimal consensus sequence for phosophorylation by PKA (Neve and Neve, 1997). Other potential phosphorylation sites in the D2 receptor include Ser-147, -148 in the second cytoplasmic loop, and in the third cytoplasmic loop Ser-229, -296, and –354.

Desensitization and internalization of the dopamine D2 receptor have been described in a variety of cell lines and tissue preparations (Barton et al., 1991; Zhang et al., 1994; Boundy et al., 1995; Ng et al., 1997; Sibley and Neve, 1997; Vickery and von Zastrow, 1999; Kim et al., 2001; Kim et al., 2004b). The interaction of arrestins and the dopamine D2 receptor and the contribution of this interaction to receptor internalization have also been investigated, but chiefly in studies using heterologous expression of arrestins, GRKs, and the D2 receptor in non-neuronal cells. Activation of the heterologously expressed dopamine D2 receptor causes GRK-dependent receptor phosphorylation, translocation of GFP-tagged arrestin2 and 3 to the cell membrane, and receptor internalization that is enhanced by overexpression of GRKs or arrestins and prevented by overexpression of a dominant negative mutant of arrestin3 (Kim et al., 2001; Kim et al., 2004b).

The two other dopamine receptors in the D2-like family of receptors are the dopamine D3 and D4 receptors. The D3 and D4 receptors are similar to the D2 receptor based on pharmacological profiles, sequence homology to the D2 receptor (53% and 41%, respectively), and gene structure. Both D3 and D4 receptors have high affinity for D2 receptor ligands but both have their own pharmacological profiles. The dopamine D3 receptor has a higher affinity than the D2 receptor for several agonists, including dopamine, 7-OH DPAT, dihydrexidine, and quinpirole (Sokoloff et al., 1990; Sokoloff et al., 1992; Castro and Strange, 1993a; Watts et al., 1993; Freedman et al., 1994; Kula et al., 1994; MacKenzie et al., 1994). The dopamine D4 receptor has higher affinity for clozapine compared to the dopamine D2 receptor (Van Tol et al., 1991; Lawson et al., 1994). However, not all studies have confirmed the increased affinity of the dopamine D4 receptor for clozapine.

Kim and others have shown that the dopamine D3 receptor has a reduced ability to translocate arrestin to the membrane and it internalizes less than the D2 receptor (Kim et al., 2001). With agonist stimulation a larger fraction of D2 receptors desensitized and disappeared from the membrane compared to the D3 receptor. In addition, when the second and third cytoplasmic loops of the dopamine D2 receptor are replaced with the second and third loops from the dopamine D3 receptor, the ability of the D2 receptor to regulate translocation of arrestin and receptor sequestration is decreased (Kim et al., 2001).

## ARRESTINS

**History of arrestins.** Non-visual arrestin was first identified when the desensitization of the β-adrenergic receptor was investigated and suggested that a cofactor was necessary

for β-adrenergic receptor kinase (βARK)-mediated inhibition of receptor function (Lohse et al., 1990). The cofactor necessary for βARK-mediated inhibition was lost when βARK was completely purified. However, when βARK was partially purified and incubated with purified β-adrenergic receptor, the agonist-stimulated GTPase activity of $G_s$ was inhibited (Benovic et al., 1987). It was determined that desensitization of rhodopsin required the binding of rhodopsin arrestin, or visual arrestin (Wilden et al., 1986). When visual arrestin was added *in vitro* to purified β-adrenergic receptors and βARK, desensitization of the receptor was only partially restored, indicating there was another protein related to visual arrestin responsible for β-adrenergic receptor desensitization (Wilden et al., 1986). Two arrestin homologues were cloned, arrestin2 (β-arrestin1) and arrestin3 (β-arrestin2), and both desensitized the β-adrenergic receptor (Lohse et al., 1990; Attramadal et al., 1992). The deduced amino acid sequence of the putative arrestin protein was 47.1-kD in size, slightly larger than visual arrestin at 45.3 kD (Lohse et al., 1990). The partially purified arrestin inhibited the function of βARK-phosphorylated receptors by more than 75% and exhibited a 20- to 40-fold preference for βARK-phosphorylated receptors compared to unphosphorylated receptors (Lohse et al., 1990). Agonist occupancy of the β-adrenergic receptor transforms the receptor into a substrate for βARK, followed by the binding of arrestin to the βARK-phosphorylated β-adrenergic receptor. Binding of arrestin prevents the interaction of the receptor with $G_s$.

**Members of the arrestin family.** Arrestins are a family of cytosolic proteins of which two distinct members have been identified and divided into four subfamilies based on sequence homology and tissue distribution: visual arrestins comprised of rod and cone arrestin, and non-visual arrestins, or β-arrestins, comprised of arrestin2 (β-arrestin1) and

arrestin3 (β-arrestin2) (Ferguson et al., 1996). Arrestin2 (Lohse et al., 1990) and arrestin3 (Attramadal et al., 1992) share high homology with one another and visual arrestin. There are two variant forms of each type of arrestin. The variant forms of arrestin2 differ only by the insertion of 8 amino acids between amino acids 333 and 334, and the variant forms of arrestin3 are identical except for an 11 amino acid insert between amino acids 361 and 362 (Sterne-Marr et al., 1993). Immunohistochemical investigation of arrestin2 and arrestin3 in rat brain showed extensive, but differentially distributed, neuronal labeling of the two proteins (Attramadal et al., 1992). Arrestins are concentrated at synapses with GRKs, suggesting that they have the potential to regulate many GPCRs (Attramadal et al., 1992). The distribution of visual arrestins, is predominantly localized in photoreceptor cells of the retina but can also be found in other tissues including the pineal gland, brain, and leukocytes (Smith and Justice, 1994).

**Arrestin: GPCR desensitization.** Receptor desensitization is a phenomenon in which receptor responsiveness decreases after continued or repeated stimulation with an agonist. After termination of agonist stimulation, desensitization is followed by resensitization, the reinstatement of the ability to respond to ligands (Krupnick and Benovic, 1998). For GPCRs, trafficking of the receptor through various subcellular compartments is an important part of desensitization and resensitization. A model of GPCR desensitization, best characterized for the $\beta_2$-adrenergic receptor, has been developed in which the agonist-activated GPCR is phosphorylated by GRKs or second messenger-dependent kinases such as protein kinase A (Krupnick and Benovic, 1998). Phosphorylation by GRKs enhances the interaction of the GPCR with arrestins. Binding of arrestin causes rapid desensitization of the receptor by inhibiting receptor binding to G proteins, and also

targets the receptor to clathrin-coated pits for internalization and either degradation or resensitization (Pippig et al., 1995; Tsao et al., 2001).

**Arrestin: GPCR internalization.** GPCRs are sequestered or internalized when exposed to agonists, and become inaccessible to the extracellular ligand (Ferguson et al., 1996). Arrestin serves as an adapter molecule by binding to clathrin-coated pits and targeting the receptor for endocytosis (Sterne-Marr et al., 1993). Arrestin molecules defective in clathrin binding fail to support internalization of the β-adrenergic receptor (Lin et al., 1997). It is still unknown to what extent the two forms of arrestin play different roles in the desensitization or sequestration of GPCRs. Studies in fibroblast lines derived from mouse embryos (MEFs) lacking either one or both arrestins demonstrate that arrestin2 and arrestin3 exhibit functional specialization (Kohout et al., 2001). Desensitization of the β2-adrenergic receptor and angiotensin AT1a receptor was impaired in arrestin2 and arrestin3 knockout MEFs. However, sequestration of the β2-adrenergic receptor was reduced only in arrestin3 knockout and double-knockout MEFs, not in arrestin2 knockout MEFs, indicating that arrestin3 was required for internalization of the β2-adrenergic receptor (Kohout et al., 2001). From the above experiments, it was determined arrestin3 is 100-fold more potent in its ability to sequester the β2-adrenergic receptor compared to arrestin2. Angiotensin AT1a receptor sequestration is minimally affected in arrestin2 knockout MEFs, unaffected in arrestin3 knockout MEFs, and markedly impaired only in the double-knockout MEFs, indicating that either isoform of arrestin is sufficient for internalization of the angiotensin AT1a receptor (Kohout et al., 2001). In the experiment described above, there are no differences observed in the efficiency of arrestin2 and 3 to mediate angiotensin AT1a receptor sequestration (Kohout et al., 2001). This evidence

suggests that both isoforms of arrestin are capable of regulating the desensitization of GPCRs, but the specificity of one form compared to another may rely on both the specificity for the receptor and tissue-specific expression of the different forms of arrestin. There is some evidence that GPCRs can internalize independently of arrestin. It was determined that the muscarinic M2 ($M_2$ mAChR) receptor internalized independently from arrestin. A dominant negative arrestin prevented internalization of the of muscarinic receptor subtypes (M1, M3, and M4) however the internalization of the $M_2$ mAChR was not prevented (Vögler et al., 1998).

**Arrestin: GPCR resensitization.** Once the agonist is removed, desensitization is followed by resensitization, the reinstatement of the ability to respond to ligands (Lin et al., 1997). Preventing GPCR endocytosis by treatment with concanavalin A or through the use of mutant receptors defective in endocytosis demonstrated that sequestration of the receptor is usually required for resensitization (Ito et al., 1999). Overexpression of arrestins in COS-7 cells enhances the rate of β-adrenergic receptor resensitization, indicating that arrestin-dependent endocytosis plays a role in the process (Zhang et al., 1997). The stability of the receptor:arrestin complex determines the rate of resensitization of the GPCR. There are two groups of GPCRs, class A and class B, that have been characterized based on their rates of internalization and affinities for arrestin. Class A receptors are rapidly dephosphorylated and recycled back to the membrane, and have higher affinity for arrestin3 than for arrestin2 (Shenoy and Lefkowitz, 2003). Class B receptors remain associated with arrestin during internalization, which makes the recycling and dephosphorylation of the receptor occur more slowly, and have similar affinity for arrestin2 and arrestin3 (Shenoy and Lefkowitz, 2003). Residues within the

carboxyl-terminal tail appear to determine the characteristics of Class A or Class B GPCRs (Oakley et al., 2000). The dopamine D1 receptor has been classified as a Class A GPCR, whereas the neurokinin NK-1 and vasopression V2 receptors have characterized as Class B GPCRs (Oakley et al., 2000). There are exceptions, however, to this GPCR classification. For example the luteinizing hormone/choriogonadotropin receptor preferentially interacts with arrestin2, and the $M_2$ muscarinic receptor internalizes independent of arrestin compared to the $M_1$ and $M_3$ muscarinic receptor, which are both class A receptors (Claing et al., 2000; Mundell and Benovic, 2000; Wu et al., 1997).

**Arrestin: GPCR signaling.** The first evidence that arrestins may play a role in the signaling of GPCRs was obtained when it was demonstrated that arrestin2 formed a complex with the β-adrenergic receptor and the protein tyrosine kinase, Src (Luttrell et al., 1999). Src was co-immunoprecipitated with β-adrenergic receptor in an agonist- and arrestin-dependent manner, suggesting a direct interaction between arrestin and Src. Mutations within arrestin that prevent its interaction with Src inhibit β-adrenergic receptor stimulation of the ERK cascade. In addition, arrestins can act as agonist-regulated scaffolds for both the ERK cascade (Defea et al., 2000b; Defea et al., 2000a; Luttrell et al., 2001) and c-Jun N-terminal kinase type 3 (JNK3) cascade (McDonald et al., 2000). Arrestins, when acting as scaffolding proteins, facilitate protein-protein interactions, ensure signal specificity, and maintain the correct subcellular distribution for both the ERK and JNK3 cascades.

**Arrestins: in vivo.** The development of mice that lack arrestin2 or arrestin3 has facilitated the investigation of the physiological roles of arrestin *in vivo*. Knockout of both arrestins is lethal. Arrestin2 knockout mice are normal except for an increase in

cardiac ejection fraction, the amount of blood ejected from the left ventricle with each heartbeat with β-adrenergic receptor stimulation (Conner et al., 1997). Arrestin3 knockout animals are physiologically normal but fail to develop tolerance to the repeated administration of morphine (Bohn et al., 1999). Arrestin2 knockout animals treated with morphine displayed the same antinociceptive profiles as their wildtype littermates (Bohn et al., 1999). Arrestin3 knockout animals displayed enhanced and prolonged antinociception to morphine and heroin, but antinociception was attenuated with etorphine, fentayl, or methadone administration (Bohn et al., 2004). Overall, arrestin3 was required for desensization of the μ-opioid receptor by morphine or heroin. Arrestin2 was not required for desensitization of the μ-opioid receptor.

**Specific Aims**

The desensitization of the dopamine D1 and D2 receptor has been investigated, but studies are limited to the transfection of receptors, GRKs, and arrestins into mammalian cell lines. In my dissertation, the interaction of the different dopamine receptors with endogenous arrestin was investigated. Both arrestin2 and arrestin3 interaction with dopamine D1 and D2 receptors was investigated using GST fusion protein constructs, D2 transfected NS20Y cells, and primary striatal cultures.

Specific Aim 1: ***Test the hypothesis that the dopamine D1 and D2 receptors interact with endogenous arrestin2 and/or arrestin3.*** These studies examined the trafficking of the D1 receptor and endogenous arrestin in primary striatal cultures. The possible interaction of the D2 receptor and endogenous arrestin was investigated in D2-EGFP receptor-transfected NS20Y cells and in primary striatal neuronal cultures. Cells were treated with agonist, and interaction of the D1 or D2 receptor and arrestins was

investigated at various time points through confocal microscopy and immunoprecipitation studies.

Specific Aim 2: ***Characterize the internalization of the dopamine D1 and D2 receptor.*** These studies examined the trafficking of the endogenous dopamine D1 and D2 receptor using neostriatial neurons. In addition D2-EGFP receptor-transfected NS20Y cells were used to characterize the internalization rate of the dopamine D2 receptor. In D2-EGFP NS20Y cells, the expression of both arrestin2 and arrestin3 was prevented by siRNA-interference. When siRNA was transfected into the D2-EGFP NS20Y cells, the internalization of the dopamine D2 receptor was assessed.

Specific Aim 3: ***Test the hypothesis that the third cytoplasmic loop, the second cytoplasmic loop, and/or the C-terminus of the dopamine D1 and D2 receptor is responsible for interaction of the dopamine D1 and D2 receptor and arrestins.*** GST fusion proteins containing the C-terminus or the third loop of the dopamine D1 receptor, or the C-terminus, or the second or third cytoplasmic loops of the D2 receptor were generated, and the interaction of the fusion proteins with arrestin2 and arrestin3 in striatal brain homogenates and with purified arrestin2 and 3 was investigated.

# II. Preferential Interaction between the Dopamine D2 Receptor and Arrestin2 in Neostriatal Neurons

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**ABSTRACT**

Dopamine D2 receptor interactions with arrestins and arrestin-dependent internalization have been characterized using heterologously expressed D2 receptor and arrestins. The purpose of this study was to investigate D2 receptor interaction with endogenous arrestins. Arrestin2 and 3 in striatal homogenates bound to the third cytoplasmic loop of the D2 receptor, and purified arrestin2 and 3 bound to the second and third loops and C-terminus of the D2 receptor, in a GST pull-down assay. In NS20Y neuroblastoma cells expressing an enhanced green fluorescent protein-tagged D2 receptor (D2-EGFP), 2-hr D2 agonist stimulation enhanced the colocalization of D2-EGFP with endogenous arrestin2 and 3. These results suggest that the D2 receptor has the intrinsic ability to bind both non-visual arrestins. Agonist treatment of D2-EGFP NS20Y cells induced D2 receptor internalization (36-46%) that was maximal within 20 min, but that was prevented by siRNA-induced depletion of arrestin2 and 3. In neostriatal neurons, 2-hr agonist treatment selectively increased the colocalization of the endogenous D2 receptor with arrestin2, whereas receptor colocalization with arrestin3 was reduced. Agonist stimulation caused translocation of arrestin2, but not arrestin3, to the membrane in neurons, and selectively enhanced the co-immunoprecipitation of the D2 receptor and arrestin2. All three measures of receptor:arrestin interaction (colocalization, translocation, and coprecipitation) demonstrated selective agonist-induced interaction between the D2 receptor and arrestin2 in neurons.

## Introduction

Receptor desensitization is a phenomenon in which receptor responsiveness decreases after continued or repeated stimulation with an agonist. After termination of agonist stimulation, desensitization is followed by resensitization, the reinstatement of the ability to respond to ligands (Krupnick and Benovic, 1998). For G protein-coupled receptors (GPCRs), trafficking of the receptor through various subcellular compartments is an important part of desensitization and resensitization. A model of GPCR desensitization, best characterized for the $\beta_2$-adrenergic receptor, has been developed in which the agonist-activated GPCR is phosphorylated by GPCR kinases (GRKs) or second messenger-dependent kinases such as protein kinase A (Krupnick and Benovic, 1998). Phosphorylation by GRKs enhances the interaction of the GPCR with additional proteins, termed arrestins. Binding of arrestin causes rapid desensitization of the receptor by inhibiting receptor binding to G proteins, and also targets the receptor to clathrin-coated pits for internalization and either degradation or resensitization (Pippig et al., 1995; Tsao et al., 2001). Arrestin can also act as a scaffolding protein, promoting the stable association of signaling proteins with the receptor (Luttrell et al., 2001).

The rate of GPCR resensitization depends on the stability of the receptor:arrestin complex. Receptors that dissociate from arrestin near the cell membrane (called class A receptors) are rapidly dephosphorylated and recycled, whereas receptors that remain associated with arrestin during internalization (class B receptors) are dephosphorylated and recycled more slowly (Shenoy and Lefkowitz, 2003). Class A and B receptors can also be differentiated on the basis of their affinities for arrestins, with class A receptors, including the D1 dopamine receptor, having higher affinity for arrestin3 than for

arrestin2, whereas class B receptors have similar affinity for arrestin2 and arrestin3 (Oakley et al., 2000).

Desensitization and internalization of the dopamine D2 receptor have been described in a variety of cell lines and tissue preparations (Ng et al., 1997; Boundy et al., 1995; Barton et al., 1991; Zhang et al., 1994; Sibley and Neve, 1997; Kim et al., 2001; Vickery and von Zastrow, 1999; Kim et al., 2004b). The interaction of arrestins and the dopamine D2 receptor and the contribution of this interaction to receptor internalization have also been investigated, but chiefly in studies using heterologous expression of arrestins, GRKs, and the D2 receptor in non-neuronal cells. Activation of the heterologously expressed dopamine D2 receptor causes GRK-dependent receptor phosphorylation, translocation of GFP-tagged arrestin2 and 3 to the cell membrane, and receptor internalization that is enhanced by overexpression of GRKs or arrestins and prevented by overexpression of a dominant negative mutant of arrestin3 (Kim et al., 2001; Kim et al., 2004b).

We now report that agonist stimulation caused rapid internalization of the D2 receptor heterologously expressed in NS20Y neuroblastoma cells, and that depletion of endogenous arrestins prevented receptor internalization. In NS20Y cells, agonist-induced colocalization of the D2 receptor with both arrestin2 and arrestin3 suggests that the receptor interacts with both forms, as described for class B receptors, an interpretation supported by the direct binding of both forms of arrestin to the receptor second and third cytoplasmic loops and C-terminus. In neurons, however, the endogenous dopamine D2 receptor preferentially interacted with arrestin2, as indicated by selective agonist-induced

D2 receptor colocalization with, co-immunoprecipitation with, and translocation of arrestin2.

**Materials and Methods**

**Materials.** [$^3$H]Spiperone was purchased from Amersham Life Sciences (Arlington Heights, IL), and [$^3$H]sulpiride from New England Nuclear Life Science Products (Boston, MA). Serum was purchased from HyClone (Logan, UT). Other reagents, including culture media, 7-OH-DPAT, haloperidol, and (+)-butaclamol, were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies used include: rabbit anti-dopamine D2L/S (1/250 dilution, AB5084P from Chemicon, Temecula, CA), mouse anti-arrestin2 (1/300 dilution, A47520 from Transduction Laboratories, Lexington, KY), mouse anti-arrestin3 (1/250 dilution, sc-13140 from Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-GST (1/500 dilution, 27-4577-01 from Amersham Biosciences, Piscataway, NJ), monoclonal anti-GFP (1/300, Clontech, Palo Alto, CA), and monoclonal anti-GAPDH (1/50,000, MAB374 from Chemicon, Temecula, CA). Secondary antibodies for confocal microscopy were purchased from Molecular Probes (Eugene, OR), and secondary antibodies for immunoblot analysis from Santa Cruz Biotechnology (Santa Cruz, CA). The blocking reagent I-block was purchased from Tropix (Bedford, MA). Pregnant Sprague-Dawley rats at gestation day 13 were obtained from Harlan (Indianapolis, IN).

**Generation of GST Fusion Proteins.** For construction of the GST fusion protein the second cytoplasmic loop of the dopamine $D2_L$ receptor (D2-IC2), amino acids 119-154, the third cytoplasmic loop of the dopamine $D2_L$ receptor (D2-IC3), amino acids 212-369, and the C-terminus of the dopamine $D2_L$ receptor (D2-CT), amino acids 419-444 were

PCR-amplified, subcloned into *Bam*HI-*Sal*I sites in pGEX-4T-3 (Amersham Biosciences, Piscataway, NJ), and transformed into BL21 cells. Transformants were screened by induction with 50 $\mu$M IPTG and immunoblot analysis using an anti-GST antibody. For larger-scale purification, the GST fusion protein was grown in LB broth containing ampicillin (100 $\mu$g/mL) to $A_{600}$ = 0.5 and stimulated with 50 $\mu$M IPTG for 3 hr at room temperature. Bacteria were pelleted and washed with phosphate-buffered saline. Pellets were resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, 1 mg/ml lysozyme, pH 8.0) and incubated for 1 hr with gentle rotation at room temperature. The homogenates were clarified by centrifugation, and 600 µl of supernatant, typically ~1 mg, was applied to the MicroSpin GST Purification Module (Amersham Biosciences, Piscataway, NJ) containing Glutathione Sepharose 4B beads, and purified according to manufacturer's instructions. Eluates were separated by SDS-PAGE and the gel was stained with Gel Code Blue (Pierce, Rockford, IL) to determine the correct molecular weight of each fusion protein. In addition, a BCA protein assay was used to determine protein concentrations of the GST fusion proteins.

**GST Pulldown.** For GST pulldown experiments, striata were dissected from Sprague-Dawley rats and homogenized in GST solubilization buffer (50 mM Tris-HCl, pH 7.4, 0.05 mM EDTA, 10 mM CHAPS, and a Complete protease inhibitor tablet/50 ml) with 5 strokes of a glass-Teflon dounce homogenizer. Samples were centrifuged at 38,000 x *g* for 30 min and the protein concentration in the resulting supernatant was determined using the BCA Protein Assay kit. To obtain purified arrestins, plasmids were expressed in BL21 cells and arrestins purified using heparin-sepharose chromoatography, followed by Q-Sepharose chromatography (Han et al., 2001). Glutathione Sepharose 4B beads

containing equal amounts of D2-IC3 GST, D2-IC2 GST, D2-CT GST or GST without insert (~1 mg of protein for each), were incubated with 500 μg of striatal brain homogenate overnight at $4^{o}$ C with gentle rotation or with 25 ng of purified arrestin2 or arrestin3 at $4^{o}$ C for 2 hours. The beads were washed three times with 20 mM Tris-HCl, pH 6.9, containing 70 mM NaCl. Samples were eluted with elution buffer (50 mM Tris-HCl, 10 mM glutathione, pH 8.0) for 20 min at room temperature with gentle rotation. Bound proteins were analyzed by immunoblotting with anti-arrestin2 or anti-arrestin3 as described below. In experiments using purified arrestins, the amount of bound arrestin2 or 3 was calculated from a 4-point standard curve generated using background optical density (*i.e.*, no arrestin) and 3 concentrations of arrestin2 or 3 between 0.625 and 2.5 ng. The amount bound to GST alone was subtracted from the total amount bound to each fusion protein to arrive at a value for amount of arrestin2 or 3 specifically bound to D2-IC3, D2-IC2, or D2-CT.

**D2 Receptor-expressing NS20Y Cells.** The D2–EGFP receptor was constructed by cloning a rat $D2_L$ receptor cDNA into the pEGFP-N1 N-Terminal Protein Fusion Vector (Clontech, Palo Alto, CA). Three mutations were introduced into the wildtype $D_2$ receptor using the QuikChange mutagenesis kit (Stratagene,Cedar Creek, TX) to eliminate the stop codon from the $D_2$ receptor and add an *ApaI* restriction site for cloning into the EGFP vector in the proper reading frame: CTGCTGAGTCTG => CTGCTGGGCCCG. Wildtype $D2_L$ receptor (in pcDNA3.1) and D2-EGFP were stably expressed in mouse neuroblastoma NS20Y cells by calcium phosphate coprecipitation (Neve et al., 1991). After selection for resistance to G418 (600 μg/ml), pooled populations of D2-EGFP-expressing cells were isolated using a BD FACSVantage SE

flow cytometer (Becton Dickinson, San Jose, CA) with excitation at 488 nm. Cells were maintained at 37°C in a humidified atmosphere with 10% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum and 5% calf bovine serum, with 300 μg/ml of G418 Sulfate (Calbiochem Co, San Diego, CA). The EGFP tagged D2 receptor and the wildtype D2 receptor had similar affinity for [$^3$H]spiperone ($K_d$ = 49 ± 22 pM for D2-EGFP-NS20Y and 44 ± 4 pM for D2-NS20Y cells; data not shown). The integrity of the fusion protein was demonstrated by colocalization of D2 receptor immunoreactivity and EGFP autofluorescence in D2-EGFP-NS20Y cells (data not shown).

**Confocal Microscopy.** D2-EGFP NS20Y cells and neostriatal neurons grown on glass coverslips were treated for 5, 10, 60, or 120 min at 37°C with 7-OH DPAT (10 μM) or vehicle (1% ethanol). Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (58 mM $Na_2HPO_4$, 17 mM $NaH_2PO_4$, 68 mM NaCl, pH 7.4) for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, then blocked with 5% goat serum for 1 hr at room temperature. All cells were incubated with mouse anti-arrestin2 or anti-arrestin3, washed, and incubated for 1 hr with Alexa Fluor-Red-tagged goat anti-mouse IgG (1/400). Localization of the endogenous D2 receptor in neurons and of the wildtype D2 receptor in D2-NS20Y cells was done using rabbit anti-dopamine D2L/S, followed by incubation for 1 hr with Alexa Fluor-Green-tagged goat anti-rabbit IgG (1/400). Coverslips were washed, mounted onto a slide with Slowfade (Molecular Probes, Eugene, OR), and imaged with a Leica SP laser scanning confocal microscope. The extent of colocalization of arrestin immunoreactivity with either D2 receptor immunoreactivity or EGFP autofluorescence (pixels expressing both red and green

fluorescence) is expressed as a percentage of the total number of pixels expressing the green fluorescence of the dopamine D2 receptor. Colocalization was quantified for each image using IP Lab software (Fairfax, VA). Three independent experiments were done for each cell type and arrestin isoform, with an average of 20 cells per experiment analyzed for each time point.

**Receptor Sequestration.** Sequestration was measured using the intact cell [$^3$H]sulpiride binding assay described by Kim *et al.* (2001). D2-EGFP- and D2-expressing NS20Y cells were grown to 80% confluency. Cells were rinsed and preincubated with serum-free minimal essential medium (MEM) containing 10 mM $Na^+$-HEPES, pH 7.4, at 37°C. Cells were stimulated with 10 $\mu$M 7-OH DPAT for 0, 20, or 120 min as indicated. Stimulation was terminated by quickly cooling the plates on ice and washing three times with ice-cold serum-free MEM with 20 mM HEPES, pH 7.4. Cells were gently pipetted, collected, and incubated with 250 $\mu$l [$^3$H]sulpiride (final concentration, 2.2 nm) at 4°C for 150 minutes in the absence and presence of unlabeled competitive inhibitor (10 $\mu$m haloperidol). The assay was terminated by filtration (Whatman GF/C filters) using a 96-well Tomtec cell harvester. 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. Statistical comparisons were made using ANOVA followed by Dunnett's *post hoc* test.

**Biotinylation Sequestration Assay.** D2-EGFP NS20Y cells grown to 80% confluency on 10 cm tissue culture plates were treated with 7-OH DPAT (10 μM) or vehicle (1% ethanol) in DMEM for 20 min, after which the medium was decanted and the plates were placed on ice. The remaining cell surface proteins were then biotinylated with 0.5 mg/ml

of EZ™-Link NHS-SS-biotin (Pierce, Rockford, IL) and homogenized in solubilization buffer (25 mM Tris, 150 mM NaCl, 1% CHAPS, pH 7.4), including a Complete protease inhibitor tablet (1 tablet/50 ml, Boehringer-Mannheim, Mannheim, Germany) with a glass-Teflon homogenizer. Lysates were centrifuged at 16000 x *g* and supernatants containing equal amounts of total protein were incubated with ImmunoPure™ Immobilized strepavidin beads (Pierce, Rockford, Illinois) to capture biotinylated proteins. The protein concentration of each sample was determined using the BCA Assay kit (Pierce, Rockford, IL). After washing in extraction buffer, biotinylated proteins were eluted from strepavidin beads by heating at 60°C for 20 min in sample buffer, separated by SDS-PAGE, and immunoblotted using anti-GFP or anti-dopamine D2 receptor antibodies. A one-way ANOVA and Dunnett's *post hoc* comparison were used to analyze data.

**Transfection of siRNAs.** Chemically synthesized siRNAs with 19-nt duplex RNA and 2-nt 3' dTdT overhangs were purchased from (Invitrogen, Carlsbad, CA). The siRNA sequences targeting mouse arrestin2 (NM_177231) and arrestin3 (NM_145429) were 5'-AGCCUUCUGUGCUGAGAAC-3' and 5'-GGACCGGAAAGUGUUUGUG-3', respectively. Twenty-four hr before transfection D2-EGFP expressing NS20Y cells were plated in DMEM containing 10% fetal calf serum and were about 30% confluent the next day. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was added to MEM according to the manufacturer's instructions, while RNA mixtures at a final concentration of 25 nM were prepared in MEM. RNA mixtures were added dropwise to the Lipofectamine2000 mixture and incubated at room temperature for 30 min. After the incubation, the total mixture was added to cells in a 6-well tissue culture cluster, for immunoblotting, or a 10

cm plate, for the internalization assay. Some wells received Lipofectamine 2000 only as a negative control. Additional MEM was added to each well or plate 24 hr after the addition of the RNA mixtures. Twenty-four hr later, the siRNA/MEM mixture was replaced by DMEM containing 10% fetal calf serum. Cells were harvested on the third day following transfection for quantification of arrestin and GAPDH immunoreactivity or D2 receptor sequestration as described above.

**Neostriatal Neuronal Cultures.** The striatal region was dissected from 4 day-old Sprague-Dawley rats and incubated in MEM containing 20 U/ml papain for 2 hours at 37°C. The tissue was then triturated using fire-polished Pasteur pipettes in MEM supplemented with 10% fetal bovine serum, 0.45% glucose, 5 pg/ml insulin, 0.5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated on poly-D-lysine-treated glass coverslips at a density of 75,000 cells per coverslip. Neuronal medium containing 50% MEM, 39% Ham's F12 medium, 10% horse serum, 1% fetal bovine serum, 0.45% glucose, 5 pg/ml insulin, 0.1 mg/ml apotransferrin, 0.5 mM kynurenic acid, and 1 μg/ml glia-derived neurotrophic factor, was added one hour after initial plating. The medium was first conditioned with glial cells for 24 hours. Cells were grown in a humidified 5% $CO_2$ incubator at 37°C and used after 6-8 days in culture.

**Arrestin Translocation.** Striatal cultures were treated with 7-OH DPAT (10 μM) or vehicle for 20 or 120 min, then rinsed with calcium- and magnesium-free phosphate-buffered saline containing 25 mM EDTA. Cultures were scraped from plates and triturated, after which nuclei were pelleted by centrifugation at 500 x *g* for 10 min at 4°C. The supernatant was centrifuged at 100,000 x *g* for 30 minutes at 4°C. The pellets were resuspended in solubilization buffer (25 mM Tris, 150 mM NaCl with 1% CHAPS, pH

7.4), including a Complete protease inhibitor tablet and sonicated for 10 seconds. Protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL), and 50 μg of protein/sample was used for quantification of arrestin2 and 3 by immunoblotting.

**Immunoblots.** Proteins were separated by SDS-PAGE through a 10% polyacrylamide gel and transferred to polyvinyl membranes (Millipore, Bedford, MA). The membranes were blocked overnight with I-block (0.2% with 0.1% Tween 20 in Tris-buffered saline (TBS), pH 7.4) at 4°C, washed twice for 5 min, followed by two 10-min washes with TBS, and incubated with anti-arrestin2 or anti-arrestin3 antibody at room temperature for 2 hr or with anti-dopamine D2 receptor antibody overnight at 4°C. The PVDF membranes were again washed twice for 5 min, and twice for 10 min TBS, then incubated with secondary antibody at a dilution of 1:3000 (alkaline-phosphatase conjugated anti-mouse IgG or anti rabbit IgG, from Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 hr. Membranes immunoblotted with anti-GAPDH were first stripped with 0.2M NaOH for 15 min at room temperature, followed by 2 15-min washes with TBS, then incubated with I-block as described. Stripped blots were then incubated with anti-GAPDH antibody, washed as described, and incubated with alkaline-phosphatase conjugated anti-mouse IgG at a dilution of 1:25,000 as described. Immunodetection was accomplished using an ECF Western blotting kit (Amersham Biosciences, Piscataway, NJ). Proteins were visualized using the Typhoon phosphorimaging system and quantified with ImageQuaNT (Molecular Dynamics, Sunnyvale, CA). A one-way ANOVA and Dunnett's post hoc comparison was used to analyze data.

**Co-immunoprecipitation.** Striatal cultures were treated with 7-OH DPAT (10 μM) or vehicle for 20 or 120 min, rinsed with phosphate-buffered saline, and incubated at room temperature for 30 min with 2 mM disuccinimidyl suberate (Pierce, Rockford, IL) dissolved in phosphate-buffered saline containing a Complete protease inhibitor tablet. The crosslinking reaction was quenched with a final concentration of 10 mM Tris-HCl, pH 7.5, for 15 min at room temperature. Cultures were scraped and collected, and CHAPS was added at a final concentration of 1%. Lysates were incubated on ice for 1 hr, and then centrifuged at 17, 500 x g for 15 min at 4°C. Protein concentrations of the supernatants were determined using the BCA Protein Assay (Pierce, Rockford, IL). Cell lysate (500 μg of protein), Antibody Capture Affinity Ligand, and 4 μg of anti-dopamine D2 antibody were added to pre-washed Catch and Release beads and rotated overnight at 4°C. Beads were washed 3 times for 15 min and samples were eluted according to the manufacturer's instructions, heated at 60°C for 20 min in sample buffer, separated by SDS-PAGE, and immunoblotted using anti-arrestin2 or –arrestin3 antibodies. A one-way ANOVA and Dunnett's *post hoc* comparison were used to analyze data.

## Results

**Direct Interaction of the D2 Receptor with Arrestin2 and 3.** D2-IC2-GST, D2-IC3-GST, and D2-CT-GST fusion proteins were constructed to identify direct binding of arrestins to intracellular domains of the D2 receptor. The fusion proteins were immobilized on glutathione-Sepharose beads and incubated with rat striatal homogenates or purified arrestins. Arrestin2 and 3 immunoreactivity in the eluates was determined by immunblotting. Both arrestin2 and arrestin3 were detected in the eluates from the D2-IC3 incubated with striatal brain homogenates, suggesting a direct interaction of the third

loop of the D2 receptor with both isoforms of arrestin (Fig. 2.1A). There was little or no specific binding of arrestin2 or 3 to D2-IC2 and D2-CT, as indicated by the lack of arrestin immunoreactivity in eluates from both fusion proteins incubated with striatal homogenates (Fig. 2.1A). Similar experiments were carried out with purified arrestin2 and arrestin3 (Fig. 2.1B). D2-IC3 bound an average of 1.3 ± 0.3 ng and 1.6 ± 0.4 ng of purified arrestin2 and 3, respectively (N = 4). D2-CT also bound purified arrestin2 (0.5 ± 0.04 ng, N = 3) and arrestin3 (0.3 ± 0.1 ng, N = 3). The D2-IC2 bound purified arrestin2 (0.4 ± 0.1 ng, N = 3) and to a greater extent arrestin3 (1.2 ± 0.3 ng, N = 3).

**Agonist-induced Colocalization of the D2 Receptor and Endogenous Arrestin2 and 3 in NS20Y Cells.** Agonist-induced trafficking of the dopamine D2 receptor and arrestin was evaluated in D2-EGFP NS20Y cells. Cells were grown on glass coverslips and treated with 7-OH DPAT (10 μM) for 10, 60, or 120 min. Agonist-treated cells were compared to cells treated with vehicle to assess changes in the colocalization of D2-EGFP with arrestin2 or 3 immunoreactivity. Treatment with 7-OH DPAT for 120 min increased the colocalization of the receptor with arrestin2 and 3 compared to untreated cells ($p < 0.01$, N = 3; Fig.2.2), whereas treatments for only 10 or 60 min had no significant effect.

We also used D2-NS20Y cells to confirm that similar results were observed for wildtype D2 and D2-EGFP receptors. Treatment with 7-OH DPAT (10 μM) for 120 min increased the colocalization of the D2 receptor with arrestin2 and 3 from basal levels of 10 ± 2% and 9.5 ± 3%, respectively, to 46 ± 5 % and 55 ± 10% after treatment with the D2 receptor agonist ($p < 0.001$, N = 3).

**Agonist-induced Internalization of the D2 Receptor in NS20Y Cells.** Internalization of the D2 receptor was induced by treatment with the agonist 7-OH DPAT (10 μM). Internalization was quantified as the loss of binding of the hydrophilic ligand [$^3$H]sulpiride to intact NS20Y cells stably expressing D2-EGFP or the wildtype D2 receptor after agonist treatment for 20 or 120 min. Cells were treated with 7 OH-DPAT, washed, and incubated with [$^3$H]sulpiride (2 nM) at 4°C for 90 min. The maximal loss of [$^3$H]sulpiride binding was 36 ± 6% ($p < 0.001$, N = 3) for D2-EGFP and 45 ± 1% for wildtype D2 receptor after agonist treatment for 20 min ($p < 0.001$, N = 3; Fig. 2.3). No further reduction in [$^3$H]sulpiride binding was observed after treatment with agonist for 2 hr. Carrying out the 7 OH-DPAT treatment at 4° C caused a modest reduction in binding that was not statistically significant (data not shown), indicating that the loss of binding of [$^3$H]sulpiride reflected receptor internalization rather than persistent binding of the agonist. In addition, D2-EGFP-NS20Y cell surface proteins were biotinylated after a 20 min incubation with agonist. Treatment with 7-OH DPAT for 20 min reduced D2-EGFP receptor immunoreactivity on the cell membrane by 36 ± 8%, compared to untreated cells (N = 3, $p < 0.01$).

**Prevention of D2 Receptor Internalization in NS20Y Cells.** Treating D2-EGFP NS20Y cells with siRNAs directed against arrestin2 and 3 decreased the abundance of both arrestin isoforms to levels barely detectable by immunoblotting, without affecting GAPDH immunoreactivity (Fig. 2.4A). This siRNA-induced depletion of arrestins largely prevented agonist-induced sequestration of the D2 receptor. Thus, treatment with 10 μM 7-OH DPAT for 20 min decreased the binding of [$^3$H]sulpiride by 38 ± 11% ($p <$ 0.01, N = 3) in D2-EGFP cells, but decreased the binding of [$^3$H] sulpiride by only 5.5 ±

5.5% for D2-EGFP cells in which arrestins were depleted by transfection with arrestin2 and arrestin3 siRNAs (Fig. 2.4B).

**Agonist-induced Colocalization of Endogenous D2 Receptor and Arrestin2, but not Arrestin3, in Neostriatal Neurons.** Agonist-induced trafficking of the endogenous dopamine D2 receptor and arrestin2 and 3 in neostriatal cultures was investigated using cells that were treated with 7-OH DPAT, fixed with paraformaldehyde, and immunostained with D2 receptor and arrestin antibodies as described in *Materials and Methods*. Treatment with 7-OH DPAT (10 μM) increased the colocalization of arrestin2 and the endogenous D2 receptor from 20 $\pm$ 5% in untreated cells to 46 $\pm$ 7% after agonist treatment for 2 hr ($p < 0.05$, N = 3; Fig. 2.5). Shorter durations of treatment had no significant effect on colocalization with arrestin2. In contrast, the colocalization of the endogenous D2 receptor with arrestin3 decreased from 43 $\pm$ 10% to 13 $\pm$ 2%, 10 $\pm$ 4%, and 18 $\pm$ 6% after 7 OH-DPAT treatment for 5, 60, or 120 min, respectively (Fig. 2.5).

**Agonist-induced Translocation of Arrestin2 in Neostriatal Neurons.** To confirm that agonist-induced colocalization of D2 receptor and arrestin2 in neurons represents translocation of the adaptor protein to the membrane, the abundance of arrestin2 and 3 was determined in membranes prepared from neostriatal neurons treated with 7-OH DPAT (10 μM) for 20 or 120 min. The abundance of arrestin2 in the membrane was enhanced by 91 $\pm$ 31% ($p < 0.05$, N = 3) after treatment with agonist for 2 hr but not after 20 min (Fig. 2.6). There was no significant translocation of endogenous arrestin3 to the membrane at 20 or 120 min of 7-OH DPAT treatment.

**Agonist-induced Co-immunoprecipitation of the D2 Receptor and Arrestin2 in Neostriatal Cultures.** Cultures were treated with 7-OH-DPAT, crosslinked with

disuccinimidyl suberate, immunoprecipitated with anti-dopamine D2 antibody, and immunoblotted with anti-arrestin2 or arrestin3 antibody. Crosslinking of the D2 receptor and arrestin2 or 3 formed a complex that was detected at ~100 kDa. Agonist treatment of neurons for 20 or 120 min increased the coprecipitation of the dopamine D2 receptor and arrestin2 by 33 ± 11% ($p < 0.05$, N=3) and 36 ± 8% ($p < 0.05$, N=3), respectively (Fig. 2.7). There was no significant effect of agonist treatment on the coprecipitation of the dopamine D2 receptor and arrestin3.

**Discussion**

The mechanisms of desensitization and resensitization of the dopamine D2 receptor have not been thoroughly elucidated, although studies using heterologously expressed arrestin and/or GRKs in non-neuronal cells suggest an important role for those proteins (Kim et al., 2001; Ito et al., 1999; Kim et al., 2004b). Our aim was to investigate whether the dopamine D2 receptor differentiates between the two isoforms of endogenous non-visual arrestins.

For many GPCRs, the third intracellular loop is the main site of interaction with arrestin (Gelber et al., 1999; DeGraff et al., 2002; Krupnick et al., 1994; Wu et al., 1997; Mukherjee et al., 1999; Kim et al., 2001; Cen et al., 2001), although the first and second intracellular loops and the carboxy terminus also contribute to the binding of arrestin to some GPCRs (Cen et al., 2001; Raman et al., 1999; Bennett et al., 2000; Nakamura et al., 2000; Hüttenrauch et al., 2002). Using a GST pull-down assay, we determined that the third intracellular loop of the D2 receptor bound both arrestin2 and 3 in neostriatal homogenate and bound similar amounts of purified arrestin2 and 3, suggesting that the third loop of the D2 receptor has no inherent selectivity for either form of arrestin. Both

forms of purifed arrestin bound to the C-terminus and the second cytoplasmic loop of the D2 receptor, but less avidly than to D2-IC3, which may account for the lack of specific binding when using neostriatal homogenate as the source of arrestins. The C-terminus bound purified arrestin3 preferentially, but a lesser contribution of this receptor domain to arrestin binding might account for the lack of other evidence for preferential binding of arrestin3.

Arrestin binding is driven both by a conserved "phosphate sensor", involving salt bridges in the polar core of arrestin that are disrupted by the electrostatic interaction of phosphorylated residues in the GPCR with positively charged residues in arrestin, and by a theoretical "GPCR activation sensor" that interacts with residues exposed in the activated GPCR (Gurevich and Gurevich, 2004) . Thus, although binding of arrestin to GPCRs is enhanced by receptor phosphorylation, there are also phosphorylation-independent determinants of binding that are sufficient for binding to occur between isolated GPCR cytoplasmic domains and arrestin (Wu et al., 1997; DeGraff et al., 2002; Cen et al., 2001; Shiina et al., 2000). Arrestin is presumably binding to sites on the fusion proteins that are occluded in the inactive GPCR and made accessible by receptor activation or, in this case, by removing them from the context of the intact receptor.

To evaluate the effects of D2 receptor stimulation on endogenous arrestins in a cell system, we first used confocal microscopy to quantify their agonist-induced colocalization. In NS20Y cells stably expressing D2-EGFP, colocalization of the receptor with both arrestin2 and 3 was markedly enhanced by agonist treatment for 2 hr, but not by shorter treatments. Although colocalization is not proof of interaction, this is consistent with the well-established observation that agonist-activated GPCRs bind

arrestins, and consistent with the binding of both arrestin2 and 3 to D2-IC3, D2-IC2, and D2-CT. Similar results were observed using GFP-tagged arrestins co-expressed with $D2_L$ in CHO cells (Kim et al., 2004b). The apparent nonselectivity of the D2 receptor for the arrestin subtypes is typical of class B receptors such as the neurotensin NT1 and vasopressin V2 receptors (Oakley et al., 2000).

In neostriatal neurons, in contrast, agonist treatment for 2 hr selectively enhanced the colocalization of the endogenous D2 receptor with endogenous arrestin2, whereas colocalization with arrestin3 was rapidly decreased by 7-OH-DPAT, an efficacious dopamine D2-like receptor-selective agonist (Chio et al., 1994). Selective translocation of arrestin2 in neurons was confirmed by quantifying arrestin immunoreactivity in membranes prepared from agonist-treated neuronal cultures; the abundance of arrestin2, but not arrestin3, was enhanced after treatment for 2 hr, but not 20 min. In addition, there was a selective agonist-induced increase in the direct interaction of the endogenous D2 receptor and arrestin2 as assessed by co-immunoprecipitation of the two proteins. These data suggest a preferential agonist-induced interaction of the endogenous D2 receptor with endogenous arrestin2 in neostriatal neurons.

The difference in results between D2 receptor and arrestin colocalization in D2-EGFP NS20Y cells (no apparent selectivity) and in neostriatal neurons (selectivity for arrestin2) could have been due to the different cell types or due to the use of a receptor-EGFP fusion protein in NS20Y cells. To eliminate the possibility that the presence of EGFP inhibited a selective interaction with arrestin2, we evaluated the colocalization of a recombinant wildtype D2 receptor with arrestins in D2-NS20Y cells, and determined that agonist treatment enhanced the colocalization of D2 receptor immunoreactivity with both

arrestin2 and arrestin3. Taken together with the results of the GST pull-down assay, we propose that the D2 receptor can bind both arrestin2 and 3, but that an interaction with arrestin3 is prevented in neostriatal neurons. One possible mechanism for selective interaction with arrestin2 in neurons is separate compartmentalization of the D2 receptor and arrestin3. Another possibility is that arrestin2 is simply much more abundant in neostriatal tissue (Gurevich et al., 2002), although the ability to pull down similar amounts of arrestin2 and arrestin3 from neostriatal homogenates using D2-IC3-GST suggests that our results cannot be explained in this way. The high basal colocalization of arrestin3 and the D2 receptor in neurons may not reflect an interaction between the proteins, since any stimulus such as constitutive activity of other GPCRs that recruits arrestins to the cell membrane will alter the apparent colocalization of arrestins with all other membrane proteins.

We also evaluated the role of arrestins in D2 receptor internalization in NS20Y cells. Using direct binding of the hydrophilic ligand [$^3$H]sulpiride, we observed substantial internalization of the D2 receptor that was maximal after 20 min of treatment with 7-OH DPAT. A similar internalization time course was determined using a cell-surface protein biotinylation assay in both NS20Y cells and neostriatal neurons (T.A.M. and K.A.N., unpublished observations). These results are similar to prior work demonstrating agonist-induced internalization of the $D2_L$ receptor in CHO, HEK293, and Neuro2A neuroblastoma cells (Itokawa et al., 1996; Vickery and von Zastrow, 1999), although others have observed little or no internalization in the absence of overexpressed GRK or arrestin (Ito et al., 1999; Kim et al., 2001; Kim et al., 2004b). Suppression of the expression of arrestin2 and 3 by transfection with siRNAs greatly decreased the

internalization of the D2 receptor, indicating that D2 receptor internalization requires arrestin. This is consistent with prior work demonstrating that expression of a dominant negative arrestin mutant inhibits D2 receptor internalization (Kim et al., 2004b).

The time course of the trafficking of endogenous arrestins, however, was much slower than that determined using heterologously expressed arrestins (Shenoy and Lefkowitz, 2003). Translocation of endogenous arrestins to the membrane and agonist-induced colocalization of the D2 receptor and arrestins were not significantly increased for at least 60 min, and only co-precipitation of the D2 receptor and arrestin2 was significantly enhanced within 20 min of agonist treatment, when receptor internalization was maximal. According to the prevailing model of GPCR trafficking in which the binding of arrestin to agonist-activated, phosphorylated receptor mediates receptor desensitization and internalization (Krupnick and Benovic, 1998), agonist-induced translocation of arrestins should precede receptor internalization. One possibility is that constitutive interaction of arrestins with the D2 receptor that is suggested by figure 7 suffices to support receptor internalization. Another consideration is that D2 receptor colocalization with arrestin2 and 3 in NS20Y cells, receptor colocalization with arrestin2 in neurons, and translocation of arrestin2 to the membrane in neurons all tended to be increased after 5-20 min of agonist treatment, although none of these effects reached statistical significance. If endogenous arrestin is much more abundant than D2 receptor expression, rapid receptor-induced changes in the distribution of a fraction of endogenous arrestin might be difficult to detect on the background noise of high arrestin concentrations. In the coprecipitation assay, on the other hand, where rapid agonist-induced changes were observed, a high ratio of arrestin to receptor would not affect the

signal-to-noise ratio, because the only arrestin being measured is that which is bound to the receptor. Despite the seeming mismatch between the time courses of receptor internalization and some measures of arrestin translocation, the finding that siRNA-induced depletion of endogenous arrestins prevents receptor internalization provides strong support for the hypothesis that arrestin binding to the D2 receptor is required for receptor internalization.

The classification of GPCRs has been investigated chiefly using recombinant GPCR- and arrestin-overexpressing cell lines (Oakley et al., 2000; Shenoy and Lefkowitz, 2003), and other work suggests that not all rhodopsin-family GPCRs fit neatly within the A or B classification (Mukherjee et al., 2002; Tulipano et al., 2004). In this study we have determined that although the D2 receptor interacts with endogenous arrestin2 and 3 in NS20Y cells and *in vitro*, characteristic of a class B receptor, the neostriatal D2 receptor interacts selectively with endogenous arrestin2 upon agonist activation.

Figure 2.1

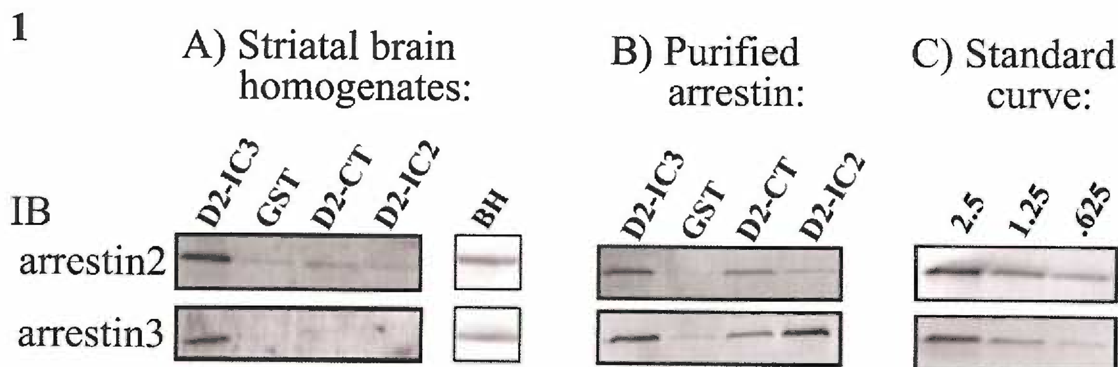


**Fig. 2.1.** Binding of arrestins to D2 receptor intracellular domains. Fusion proteins of GST and the D2 receptor third cytoplasmic loop (D2-IC3), second cytoplasmic loop (D2-IC2), or carboxy terminus (D2-CT), or GST alone (GST) were incubated with striatal homogenate **(A)** or purified arrestin2 or 3 **(B)** and purified as described in *Materials and Methods.* Eluates were immunoblotted with anti-arrestin2 or anti-arrestin3 antibody, as indicated, with the binding of arrestin resulting in a band at ~55 kDa. The figures shown are representative of 3-4 independent experiments. **A,** each preparation was incubated with striatal homogenate (500 μg of protein). Aliquots (20 μg protein) of the striatal homogenate (brain homogenate, BH) were run in two lanes to demonstrate the presence of both arrestin2 and 3 in the striatal homogenate. **B,** each preparation was incubated with 25 ng of purified arrestin2 or 3. **C,** the standard curve that was used to calculate arrestin binding in (B) is depicted. Each experiment with purified arrestins included a 4-point standard curve (background and 3 concentrations of arrestin2 or 3 as indicated, in ng).

Figure 2.2

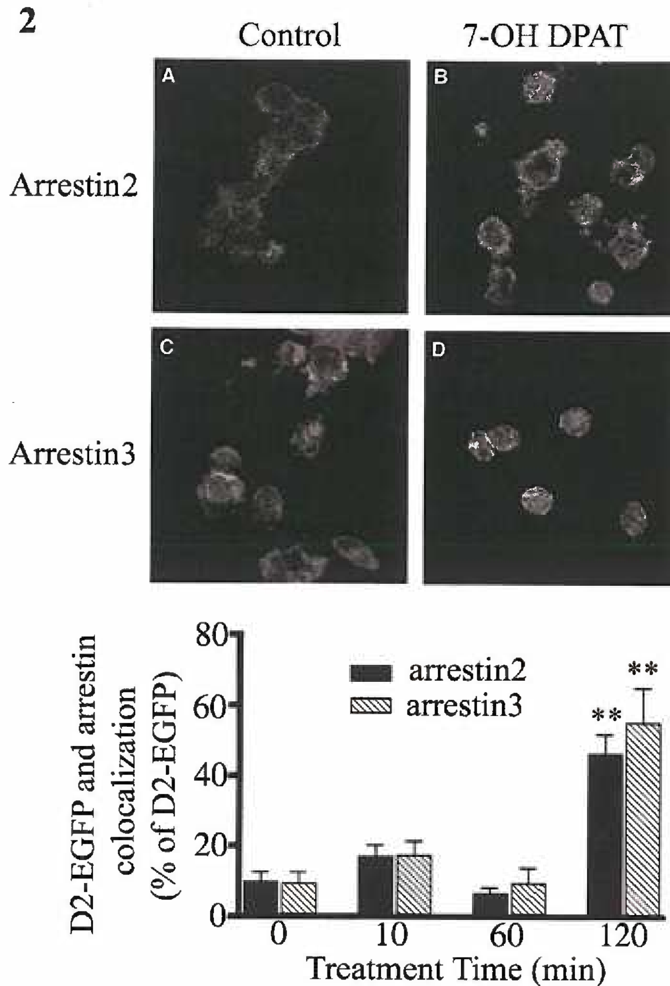


**Fig. 2.2.** Agonist-induced colocalization of D2-EGFP and endogenous arrestin2 and 3 in NS20Y cells. NS20Y cells expressing D2-EGFP were grown on coverslips for 24-48 hours and treated with 7-OH DPAT (10 μM) for 10, 60, or 120 min. Paraformaldehyde-fixed cells were incubated with mouse anti-arrestin2 or anti-arrestin3 antibody and imaged by confocal microscopy. Untreated cells were compared to treated cells at each time point to assess differences in colocalization of D2-EGFP and arrestin2 immunoreactivity. **Images,** Representative confocal fluorescence images of the colocalization of D2-EGFP and endogenous arrestins in NS20Y cells. Cells treated as described above were used to assess colocalization of D2-EGFP fluorescence and arrestin2 (Arr2, top row) or arrestin3 (Arr3, bottom row) immunoreactivity in vehicle-treated (left column, A and C) and agonist-treated cells (right column, B and D). Green and red fluorescence images were merged, and pixels containing only green or red fluoresence were subtracted to show only pixels containing both green and red fluorescence. **Graph,** the results shown are the mean ± S.E.M. for colocalization of D2-EGFP autofluorescence and arrestin2 or arrestin3 immunoreactivity, expressed as a percentage of total D2-EGFP fluorescence, in cells treated with vehicle (0) or agonist for the indicated time. Treatment with 7-OH DPAT significantly altered colocalization of D2 EGFP and arrestin2 and 3 ($p < 0.0001$ by one-way ANOVA) with a significant increase observed after treatment for 2 hr (**$p < 0.01$ by Dunnett's *post hoc* comparison, N=3).

Figure 2.3

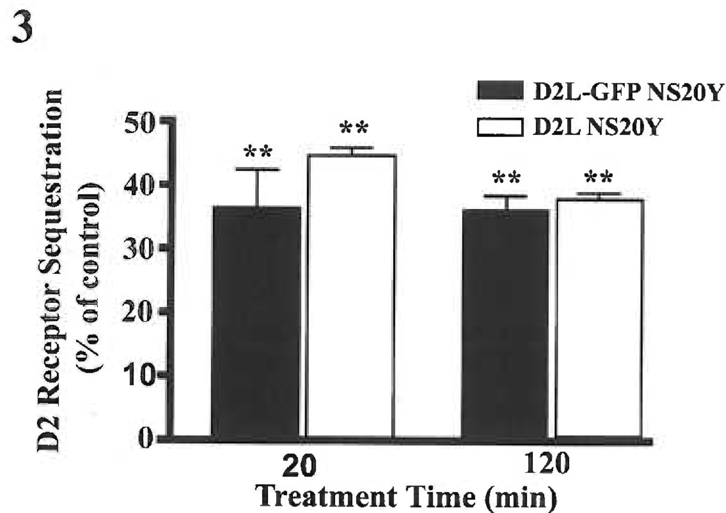


**Fig. 2.3.** Agonist-induced internalization of the D2 receptor in NS20Y cells assessed using [$^3$H]sulpiride. NS20Y cells expressing either wildtype D2 or D2-EGFP were treated with 7-OH DPAT (10 μM) for 20 or 120 min before quantifying internalization as loss of binding of [$^3$H]sulpiride, expressed as the percent reduction from the value in vehicle-treated cells. Treatment with 7-OH DPAT caused internalization of the dopamine D2 receptor ($p < 0.0001$ by one-way ANOVA). The four conditions shown were all significantly different from control (**$p < 0.01$ by Dunnett's *post hoc* comparison, N=3).

Figure 2.4

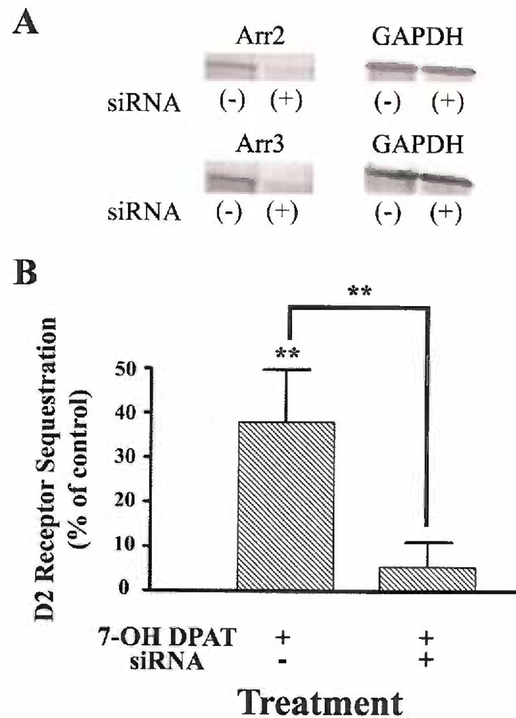


**Fig. 2.4.** Inhibition of D2 receptor internalization in NS20Y cells by siRNA-induced depletion of endogenous arrestins. **A,** Immunoreactivity of arrestin2 and arrestin3 in lysates (50 μg protein) was determined in cells transfected with siRNAs specific to arrestin2 and arrestin3 as described in *Materials and Methods.* To control for equal loading of protein, membranes were stripped, and immunoblotted with anti-GAPDH (right column). The amount of arrestin in each sample was calculated from arrestin2 and 3 standard curves similar to the representative curve depicted in Fig. 1C. **B,** NS20Y cells expressing D2-EGFP were transfected with siRNAs specific to arrestin2 and arrestin3 and treated with 7-OH DPAT (10 μM) for 20 min before quantifying internalization as loss of binding of $[^3H]$sulpiride, expressed as the percent reduction from the value in vehicle-treated cells (control). Treatment with 7-OH DPAT caused internalization of the dopamine D2 receptor that was significantly different from control and from siRNA-treated cells ($p < 0.01$ by Dunnett's t-test for both comparisons).

Figure 2.5 **5**

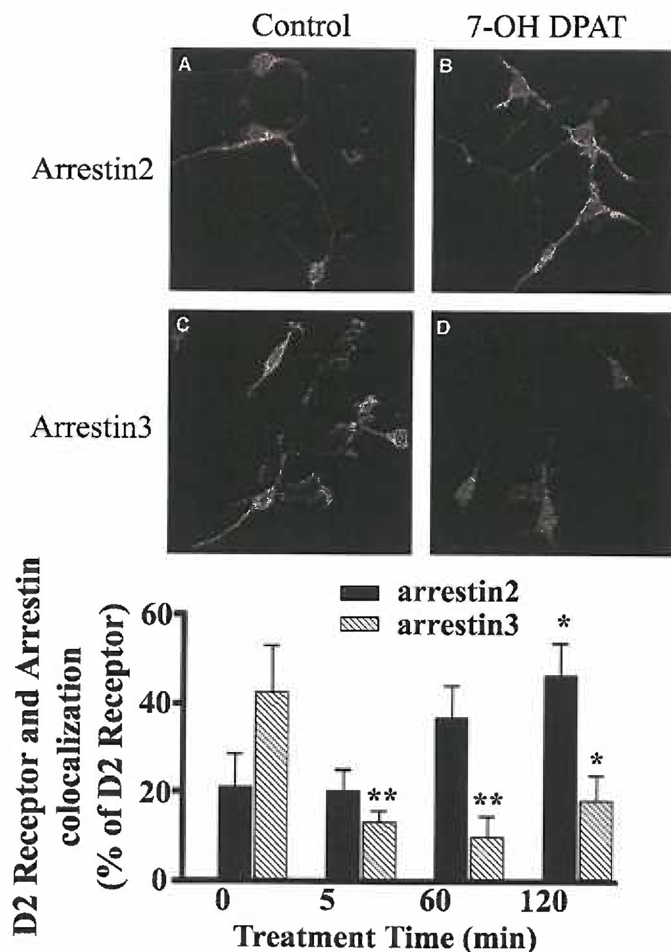


**Fig. 2.5.** Agonist-induced colocalization of the endogenous D2 receptor and endogenous arrestin2, but not arrestin3, in neostriatal neurons. Neuronal cultures prepared as described in *Materials and Methods* and treated as described in the legend to Figure 3 were used to quantify colocalization of D2 receptor and arrestin2 or arrestin 3 immunoreactivity. **Images**, Representative confocal fluorescence images are shown for the colocalization of D2 and arrestin immunoreactivity in neostriatal neuronal cultures. The representative experiment shown depicts colocalization of endogenous D2 receptor immunoreactivity and immunoreactivity for arrestin2 (Arr2, top row) or arrestin3 (Arr3, bottom row) in vehicle treated cells (left column, A and C) or agonist-treated cells (right column, B and D). Green and red fluorescence images were merged, and pixels containing only green or red fluoresence were subtracted to show only pixels containing both green and red fluorescence. **Graph,** The results shown are the mean ± S.E.M. for colocalization of D2 and arrestin2 or arrestin3 immunoreactivity, expressed as a percentage of total D2 receptor immunoreactivity, in cells treated with vehicle (0) or agonist for the indicated time. Treatment with 7-OH DPAT significantly altered colocalization of D2 receptor and arrestin2 immunoreactivity ($p < 0.01$ by one-way ANOVA) with a significant increase observed after treatment for 2 hr (*$p < 0.05$ by Dunnett's *post hoc* comparison, N= 3). Treatment with 7-OH DPAT significantly altered colocalization of D2 receptor and arrestin3 immunoreactivity ($p < 0.01$ by one-way ANOVA) with significant decreases observed after treatment for 5, 60, and 120 min (* $p < 0.05$, **$p < 0.01$ by Dunnett's *post hoc* comparison, N = 3).

Figure 2.6

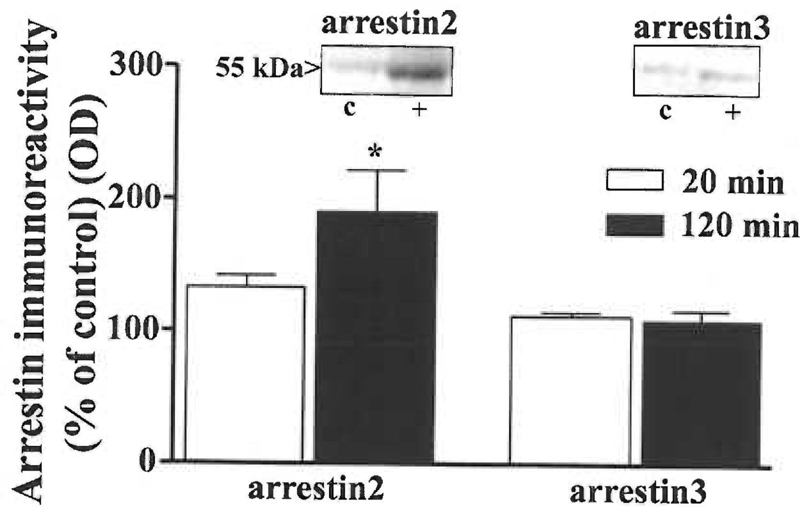


**Fig. 2.6**. Agonist-induced translocation of arrestin in neostriatal neurons. Neostriatal neurons were treated with 7-OH DPAT (10 μM) for 20 or 120 min, membranes were prepared, and levels of endogenous arrestin2 and arrestin3 were assessed using immunoblotting. Results are the mean ± S.E.M. from 3 experiments, expressed as a percentage of the band density in membranes from vehicle-treated cells. The inset depicts representative experiments in which cells were treated with vehicle (c) or agonist (+) for 120 min. (*$p < 0.05$ by Dunnett's *post hoc* comparison).

Figure 2.7

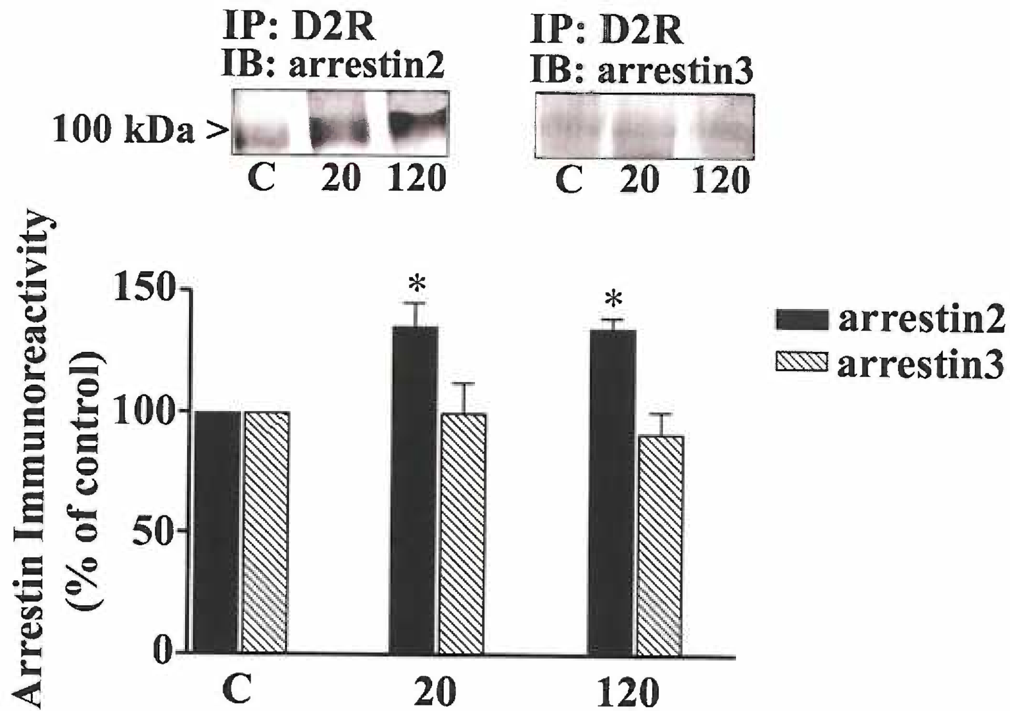


**Fig. 2.7.** Agonist-induced co-immunoprecipitation of the dopamine D2 receptor and arrestin2 and 3 in neostriatal cultures. Striatal neurons were treated with 7-OH DPAT or vehicle, crosslinked with disuccinimidyl suberate, and co-immunoprecipitated with anti-dopamine D2 antibody. Lysates were separated by SDS-PAGE and immunoblotted with anti-arrestin2 or 3 antibody. ***Gel,*** the picture depicts representative experiments in which cells were treated with vehicle (C) or 7-OH DPAT for 20 or 120 min. ***Graph***, Results are the mean $\pm$ S.E.M. from 3 experiments, expressed as a percentage of the band density in membranes from vehicle-treated cells (* $p < 0.05$ by Dunnett's *post hoc* comparison).

## III. Dopamine D1 Receptor Interaction with Arrestin3 in Neostriatal Neurons

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**Abstract**

Dopamine D1 receptor interactions with arrestins has been characterized using heterologously expressed D1 receptor and arrestins. The purpose of this study was to investigate the interaction of the endogenous D1 receptor with endogenous arrestin2 and arrestin3 in neostriatal neurons. Endogenous arrestin2 and 3 in striatal homogenates bound to the C-terminus of the D1 receptor in a GST pull-down assay, with arrestin3 binding more strongly. The D1 C-terminus and, to a lesser extent, the third cytoplasmic loop, also bound purified arrestin2 and 3. In neostriatal neurons, 2, 5, and 20 min agonist treatment increased the colocalization of the D1 receptor and arrestin3 immunoreactivity, without altering the colocalization of the D1 receptor and arrestin2. Further, agonist treatment for 5 and 20 min caused translocation of arrestin3, but not arrestin2, to the membrane. The binding of arrestin3, but not arrestin2, to the D1 receptor was increased, as assessed by co-immunoprecipitation following agonist treatment for 5 and 20 min. Agonist treatment of neurons induced D1 receptor internalization (35-45%) that was maximal within 2-5 min, a time course similar to that of the increase in colocalization of the D1 receptor with arrestin3. These data indicate that the D1 receptor preferentially interacts with arrestin3 in neostriatal neurons.

Key Words: D1 dopamine receptor, arrestin, receptor internalization, G protein-coupled receptor

Running Title: D1 receptor and arrestin3 in neostriatal neurons

**Introduction**

Arrestin plays a critical role in the densensitization of G protein-coupled receptors (GPCRs) by binding to GPCR kinase (GRK) -phosphorylated receptors, resulting in disassociation of the G protein from the receptor. Arrestin then targets the receptor to clathrin-coated pits for internalization and either degradation or recycling back to the membrane for resensitization (Pippig et al., 1995; Tsao et al., 2001). Arrestin can also promote the stable interaction of signaling proteins with the GPCR (Luttrell et al., 2001).

Based on their rates of resensitization and relative affinities for arrestin2 and 3, GPCRs have been classified into two groups, class A and class B. Residues within the carboxyl-terminal tail of the receptor appear to contribute to the stability of the receptor: arrestin complex, which determines the rate of resensitization of the GPCR (Oakley et al., 2000). Class A receptors dissociate from arrestin relatively fast and are rapidly dephosphorylated and recycled back to the membrane. Class A receptors also have higher affinity for arrestin3 than for arrestin2 (Shenoy and Lefkowitz, 2003). Class B receptors remain associated with arrestin during internalization, which makes the recycling and dephosphorylation of the receptor occur more slowly, and have similar affinity for arrestin2 and arrestin3 (Shenoy and Lefkowitz, 2003). The dopamine D1 receptor has been designated a class A GPCR, along with the $\beta_2$-adrenoceptor, whereas the neurokinin NK-1 and vasopression V2 receptors are examples of class B GPCRs (Oakley et al., 2000).

Desensitization and internalization of the dopamine D1 receptor have been described in a variety of cell lines and tissue preparations (Ng et al., 1994; Tiberi et al., 1996; Gardner et al., 2001; Lamey et al., 2002; Mason et al., 2002; Kim et al., 2004a).

Desensitization of the D1 receptor may depend on phosphorylation of the receptor by both GRK and second messenger-dependent protein kinases such as protein kinase A. The interaction of arrestin and the dopamine D1 receptor has been investigated, but only in studies using heterologous expression of arrestins and the D1 receptor in non-neuronal cells. Activation of the heterologously expressed dopamine D1 receptor causes translocation of GFP-tagged arrestin3 to the cell membrane, with arrestin3 showing greater translocation than arrestin2, and receptor internalization followed by the disassociation of arrestin at or near the membrane (Zhang et al., 1999; Oakley et al., 2000; Kim et al., 2004a).

We previously demonstrated that the dopamine D2 receptor in transfected mouse neuroblastoma (NS20Y) cells exhibits agonist-induced colocalization with endogenous arrestin2 and 3, but that in primary neostriatal neurons the endogenous dopamine D2 receptor preferentially interacts with arrestin2 (Macey et al., 2004). Thus, although the D2 receptor displayed some of the characteristics of a class B receptor when expressed in NS20Y cells, the endogenous dopamine D2 receptor could not be classified as class A or class B in neostriatal neurons. We now report that in a GST fusion protein assay, the C-terminus of the D1 receptor bound endogenous arrestin3 more avidly than arrestin2. The C-terminus of the D1 receptor bound purified arrestin2 and arrestin3 with similar avidity, as did the third cytoplasmic loop of the D1 receptor to a lesser extent. Agonist-induced rapid internalization and preferential colocalization of the D1 receptor with arrestin3 suggests that the endogenous D1 receptor could be described as a class A receptor. The endogenous dopamine D1 receptor selectively interacted with arrestin3 in neostriatal

neurons, as indicated by selective agonist-induced D1 receptor co-immunoprecipitation with arrestin3, and translocation of arrestin3 in response to the D1 agonist, SKF-82958.

## Experimental Procedures

### Materials

Serum was purchased from HyClone (Logan, UT). SKF-82958 and culture media were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies used include: rabbit anti-dopamine D1R (1/300, RDI-D1Rabrx, Research Diagnostics, Inc., Flanders, NJ), mouse anti-arrestin2 (1/300 dilution, A47520 from BD Transduction Laboratories, Lexington, KY), mouse anti-arrestin3 (1/250 dilution, sc-13140 from Santa Cruz Biotechnology, Santa Cruz, CA), and goat anti-GST (1/500 dilution, 27-4577-01 from Amersham Biosciences, Piscataway, NJ). Secondary antibodies for confocal microscopy were purchased from Molecular Probes (Eugene, OR), and secondary antibodies for immunoblot analysis from Santa Cruz Biotechnology (Santa Cruz, CA). The blocking reagent I-block was purchased from Tropix (Bedford, MA). Pregnant Sprague-Dawley rats at gestation day 13 were obtained from Harlan (Indianapolis, IN).

### Generation of the GST fusion proteins

For construction of the GST fusion proteins, the third cytoplasmic loop of the dopamine D1 receptor (D1-IC3), amino acids 216-268, and the C-terminus of the dopamine D1 receptor (D1-CT), amino acids 329-446, were PCR-amplified, subcloned into *Bam*HI-*Sal*I sites in pGEX-4T-3 (Amersham Biosciences, Piscataway, NJ), and transformed into BL21 cells. Yong Liu was responsible for construction of the D1-CT. Transformants were screened by induction with 50 $\mu$M isopropyl β-D-thiogalactoside and immunoblot analysis using an anti-GST antibody. For larger-scale purification, the GST fusion

protein was grown in LB broth containing ampicillin (100 $\mu$g/mL) to $A_{600} = 0.5$ and stimulated with 50 $\mu$M isopropyl β-D-thiogalactoside for 3 hr at room temperature. Bacteria were pelleted and washed with phosphate-buffered saline. Pellets were resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, 1 mg/ml lysozyme, pH 8.0) and incubated for 1 hr with gentle rotation at room temperature. The homogenates were clarified by centrifugation, and 600 µl of supernatant, typically ~1 mg, was applied to the MicroSpin GST Purification Module (Amersham Biosciences, Piscataway, NJ) containing glutathione Sepharose 4B beads, and purified according to manufacturer's instructions. Eluates were separated by SDS-PAGE and the gel was stained with Gel Code Blue (Pierce, Rockford, IL) to determine the correct molecular weight of each fusion protein. In addition, the BCA Protein Assay kit (Pierce) was used to determine protein concentrations of the GST fusion proteins.

**GST pulldown**

For GST pulldown experiments, striata were dissected from Sprague-Dawley rats and homogenized in GST solubilization buffer (50 mM Tris-HCl, pH 7.4, 0.05 mM EDTA, 10 mM CHAPS, and a Complete protease inhibitor tablet/50 ml) with 5 strokes of a glass-Teflon dounce homogenizer. Samples were centrifuged at 38,000 x *g* for 30 min and the protein concentration in the resulting supernatant was determined using the BCA Protein Assay kit. To obtain purified arrestins, plasmids were expressed in BL21 cells and arrestins purified using heparin-Sepharose chromatography, followed by Q-Sepharose chromatography (Han et al., 2001). Glutathione Sepharose 4B beads containing equal amounts of D1-IC3 GST, D1-CT GST or GST without insert, were incubated with 500 µg of striatal brain homogenate overnight at 4° C with gentle rotation or with 25 ng of purified arrestin2 or arrestin3 at 4° C for 2 hr. The beads were washed

three times with 20 mM Tris-HCl, pH 6.9, containing 70 mM NaCl. Samples were eluted with elution buffer (50 mM Tris-HCl, 10 mM glutathione, pH 8.0) for 20 min at room temperature with gentle rotation. Bound proteins were analyzed by immunoblotting with anti-arrestin2 or anti-arrestin3 as described below. In experiments using purified arrestins, the amount of bound arrestin2 or 3 was calculated from a 4-point standard curve generated using background optical density (*i.e.*, no arrestin) and 3 concentrations of arrestin2 or 3 between 0.625 and 2.5 ng. The amount bound to GST alone was subtracted from the total amount bound to D1-IC3-GST or D1-CT-GST to arrive at a value for amount of arrestin2 or 3 specifically bound to each receptor fragment.

**Neostriatal neuronal cultures**

The striatal region was dissected from 4 day-old Sprague-Dawley rats and incubated in minimum essential medium (MEM) containing 20 U/ml papain for 2 hr at 37°C. The tissue was then triturated using fire-polished Pasteur pipettes in MEM supplemented with 10% fetal bovine serum, 0.45% glucose, 5 pg/ml insulin, 0.5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated on poly-D-lysine-treated glass coverslips at a density of 75,000 cells per coverslip, for confocal microscopy, or in 10 cm tissue culture plates for coprecipitation or translocation analyses. An equal volume of neuronal medium containing 50% MEM, 39% Ham's F12 medium, 10% horse serum, 1% fetal bovine serum, 0.45% glucose, 5 pg/ml insulin, 0.1 mg/ml apotransferrin, 0.5 mM kynurenic acid, and 1 μg/ml glia-derived neurotrophic factor, was added to the coverslips one hr after initial plating. Tissue culture plates received 2 ml of the neostriatal cell suspension and an additional 5 ml of the neuronal medium at the time of plating. The neuronal medium was first conditioned with glial cells for 24 hr. Cells were grown in a humidified 5% $CO_2$ incubator at 37°C and used after 6-8 days in culture.

**Confocal microscopy**

Neostriatal neurons grown on glass coverslips were treated for 2, 5, 20, or 60 min at 37°C with SKF-82958 (50 μM) or vehicle (1% ethanol). Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (58 mM $Na_2HPO_4$, 17 mM $NaH_2PO_4$, 68 mM NaCl, pH 7.4) for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, then blocked with 5% goat serum for 1 hr at room temperature. All cells were incubated with mouse anti-arrestin2 or anti-arrestin3, washed, and incubated for 1 hr with Alexa Fluor-Red-tagged goat anti–mouse IgG (1/400). Localization of the endogenous D1 receptor in neurons was done using rabbit anti-dopamine D1 receptor, followed by incubation for 1 hr with Alexa Fluor-Green-tagged goat anti-rabbit IgG (1/400). Coverslips were washed, mounted onto a slide with Slowfade (Molecular Probes, Eugene, OR), and imaged with a Leica SP laser scanning confocal microscope. The extent of colocalization of arrestin immunoreactivity with D1 receptor immunoreactivity (pixels expressing both red and green fluorescence) is expressed as a percentage of the total number of pixels expressing the green fluorescence of the dopamine D1 receptor. Colocalization was quantified for each image using IP Lab software (Fairfax, VA). Three independent experiments were done for each arrestin isoform, with an average of 20 cells per experiment analyzed for each time point.

**Arrestin translocation**

Striatal cultures were treated with SKF-82958 (50 μM) or vehicle for 5 or 20 min, then rinsed with calcium- and magnesium-free phosphate-buffered saline containing 25 mM EDTA. Cultures were scraped from plates and triturated, after which nuclei were pelleted by centrifugation at 500 x *g* for 10 min at 4°C. The supernatant was centrifuged at 100,000 x *g* for 30 minutes at 4°C. The pellets were resuspended in solubilization

buffer (25 mM Tris, 150 mM NaCl with 1% CHAPS, pH 7.4), including a Complete protease inhibitor tablet and sonicated for 10 seconds. After determining protein concentrations using the BCA Protein Assay kit, 50 μg of protein/sample was used for quantification of arrestin2 and 3 by immunoblotting.

**Co-immunoprecipitation**

Striatal cultures were treated with SKF-82958 (50 μM) or vehicle for 5 or 20 min, rinsed with phosphate-buffered saline, incubated with solubilization buffer on ice for 1 hr, and then centrifuged at 17,500 x *g* for 15 min at 4°C. Protein concentrations of the supernatants were determined using the BCA Protein Assay kit. Cell lysate (500 μg of protein), Antibody Capture Affinity Ligand, and 4 μg of anti-dopamine D1 antibody were added to pre-washed Catch and Release beads and rotated overnight at 4°C. Beads were washed 3 times for 15 min and samples were eluted according to the manufacturer's instructions, then heated at 60°C for 20 min in sample buffer, separated by SDS-PAGE, and immunoblotted using anti-arrestin2 or –arrestin3 antibodies as described below.

**Biotinylation sequestration assay**

Neostriatal neurons were labeled for 2 min at 37°C with EZ™-Link NHS-SS-biotin (0.3 mg/ml) to biotinylate cell surface proteins prior to treatment with agonist. After washing in Tris-buffered saline and one rinse with 0.1 M glycine, neurons were incubated in neuronal medium containing vehicle or 50 μM SKF-82958 for 2, 5, 20, or 60 min. Agonist treatment was terminated by rapid cooling on ice. Biotinylated proteins remaining on the cell surface were stripped of biotin by the non-permanent reducing agent glutathione (150 mM glutathione, 150 mM NaCl, pH 8.75). Glutathione was subsequently neutralized by 50 mM iodoacetamide in phosphate-buffered saline, and the cells were homogenized in solubilization buffer as described above. After centrifugation

at 16,000 x *g*, supernatants containing equal amounts of total protein were incubated with ImmunoPure™ Immobilized strepavidin beads (Pierce, Rockford, Illinois) to capture biotinylated proteins. The protein concentration of each sample was determined using the BCA Protein Assay kit. After washing in extraction buffer, biotinylated proteins were eluted from strepavidin beads by heating at 60°C for 20 min in sample buffer, separated by SDS-PAGE, and immunoblotted using anti-dopamine D1 receptor antibodies as described below.

**Immunoblots**

Proteins were separated by SDS-PAGE through a 10% polyacrylamide gel and transferred to polyvinyl membranes (Millipore, Bedford, MA). The membranes were blocked overnight with I-block (0.2% with 0.1% Tween 20 in Tris-buffered saline, pH 7.4, at 4°C) washed twice for 5 min, followed by two 10-min washes with Tris-buffered saline, and incubated with anti-arrestin2 or anti-arrestin3 antibody at room temperature for 2 hr or with anti-dopamine D1 receptor antibody overnight at 4°C. The PVDF membranes were washed twice for 5 min, followed by two 10-min washes with Tris-buffered saline, then incubated with secondary antibody at a dilution of 1:3000 (alkaline-phosphatase conjugated anti-mouse IgG or anti rabbit IgG) at room temperature for 1 hr. Immunodetection was accomplished using an ECF Western blotting kit (Amersham Biosciences, Piscataway, NJ). Proteins were visualized using the Typhoon phosphorimaging system and quantified with ImageQuaNT (Amersham Biosciences). Statistical significance was determined using a one-way ANOVA and Dunnett's post hoc comparison.

## Results

### Direct interaction of the D1 receptor with arrestin2 and 3

D1-IC3-GST and D1-CT-GST fusion proteins were constructed to identify direct binding of endogenous arrestins to the third cytoplasmic loop and the C-terminus of the D1 receptor. The D1-IC3 and D1-CT fusion proteins were immobilized on glutathione-Sepharose beads and incubated with rat striatal homogenate or purified arrestins. In brain homogenates, arrestin2 and arrestin3 were detected in the eluates from D1-CT-GST at a level significantly greater than in the eluates from GST alone ($p<0.05$, Fig. 3.1a, graph). Binding of arrestin2 and –3 to D1-CT-GST was 48 ± 7% and 161 ± 18%, respectively, above binding to GST (control). Although arrestins were also present in eluates from D1-IC3-GST, the amount of immunoreactivity for arrestin2 (20 ± 17% above control) and arrestin3 (36 ± 12% above control) was not significantly different from that in eluates from GST alone. The binding of arrestin3 to the D1-CT fusion protein was significantly greater than to all other constructs in Fig. 3.1a ($p<0.01$).

The D1-IC3 and D1-CT fusion proteins both bound purified arrestin2 and arrestin3 (Fig. 3.1b). D1-CT-GST bound an average of 1.8 ± 0.4 ng and 1.6 ng ± 0.2 of purified arrestin2 and arrestin3, respectively, and D1-IC3-GST bound an average of 0.9 ± 0.04 ng and 0.8 ± 0.2 ng of purified arrestin2 and 3, respectively. All of these except the binding of arrestin3 to D1-IC3-GST were significantly different from binding to GST alone ($p<0.05$). Overall, D1-CT-GST bound significantly more purified arrestins than D1-IC3-GST ($p < 0.0001$, 2-way ANOVA), but there was no significant difference between arrestin2 and arrestin3.

**Agonist-induced colocalization of endogenous dopamine D1 receptor and arrestin3, but not arrestin2**

Agonist-induced trafficking of the endogenous dopamine D1 receptor and arrestin was evaluated in neostriatal neurons. Cells were grown on glass coverslips and treated with SKF-82958 (50 μM) for 2, 5, 20, or 60 min. Agonist-treated cells were compared to cells treated with vehicle to assess changes in the colocalization of D1 receptor and arrestin2 or 3 immunoreactivity. Treatment with SKF-82958 for 2, 5, or 20 min increased the colocalization of the receptor with arrestin3 compared to untreated cells, whereas treatment for 60 min had no significant effect. Treatment with SKF-82958 did not significantly change the colocalization of the D1 receptor and arrestin2 (Fig. 3.2).

**Agonist-induced translocation of arrestin3 in neostriatal neurons**

To confirm that agonist-induced colocalization of D1 receptor and arrestin3 in neurons represents translocation of the adaptor protein to the membrane, the abundance of arrestin2 and 3 was determined in membranes prepared from neostriatal neurons treated with SKF-82958 (50 μM) for 5 or 20 min. The abundance of arrestin3 in the membrane was enhanced by 75 ± 22% ($p < 0.05$, N = 3) after treatment with agonist for 20 min, with a nonsignificant increase of 30 ± 9% observed after 5 min (Fig. 3.3). There was no significant translocation of arrestin2 to the membrane after 5 or 20 min of treatment with SKF-82958.

**Agonist-induced co-immunoprecipitation of the D1 receptor and arrestin3**

Neuronal cultures were treated with SKF-82958, lysed, and solubilized, after which the co-immunoprecipitation of arrestin2 or 3 with the dopamine D1 receptor was determined.

Agonist treatment of neurons for 5 and 20 min increased the co-immunoprecipitation of the dopamine D1 receptor and arrestin3 by 69 ± 16% and 78 ± 15%, respectively (Fig. 3.4). There was no significant effect of agonist treatment on the co-immunoprecipitation of the dopamine D1 receptor and arrestin2.

**Agonist-induced internalization of the endogenous D1 receptor**

Neostriatal cultures were biotinylated and then treated with SKF-82958 (50 μM) for 0, 2, 5, 20, or 60 min to induce receptor internalization. After separation of biotinylated proteins by avidin gel matrix purification, the proportion of internalized D1 receptor was determined by immunoblot analysis. Treatment with SKF-82958 for 2, 5, or 60 min increased the proportion of internalized receptor compared to untreated cells (Fig. 3.5; 35 ± 3% increase at 2 min, 45 ± 6% at 5 min, and 16 ± 2% at 60 min).

**Discussion**

Investigations of the interactions of arrestins with the dopamine D1 receptor and their role in D1 receptor desensitization and resensitization have used heterologously expressed arrestin in non-neuronal cells (Oakley et al., 2000; Kim et al., 2004a). Our aim was to investigate whether the dopamine D1 receptor selectively interacted with one isoform of endogenous arrestin using D1-GST fusion proteins composed of different regions of the receptor, and endogenous D1 receptor and arrestins in rat neostriatal neuronal cultures.

For some GPCRs, the third intracellular loop is the main site of interaction with arrestin (Krupnick et al., 1994; Wu et al., 1997; Gelber et al., 1999; Mukherjee et al., 1999; Kim et al., 2001; DeGraff et al., 2002). For other GPCRs, the C-terminus is a

major site of arrestin binding (Raman et al., 1999; Nakamura et al., 2000; Bennett et al., 2001; Cen et al., 2001; Hüttenrauch et al., 2002). In our study, the third intracellular loop of the D1 receptor was not significantly more effective than GST at binding either form of endogenous arrestin in the complex mixture of proteins and other molecules present in the striatal homogenate, but the D1 C-terminus pulled down arrestin2 and, most robustly, arrestin3. Studies using purified arrestins indicated that both arrestin subtypes bound to D1-CT or D1-IC3 with similar affinity, but that binding to D1-IC3 was less strong than to D1-CT. The difference between tissue preparations (purified arrestins vs. neostriatal homogenate) could reflect lower concentrations of arrestins in the latter preparation, the presence of other receptor-interacting proteins that compete with the binding of arrestins, particularly arrestin2, to D1-IC3 and D1-CT, or even the presence of a protein that specifically binds arrestin2 or otherwise prevents its binding to the D1 receptor. Overall, however, the more robust binding of arrestin3 than arrestin2 to the C-terminus of the D1 receptor when the fusion protein was incubated with striatal homogenate is consistent with a preferential interaction of the receptor with arrestin3, although it is puzzling that this selectivity is not reflected in the binding of purified arrestins as has been described for the $\alpha_{2A}$-and $\alpha_{2C}$-adrenoceptor (DeGraff et al., 2002). Furthermore, based on the hypothesis that arrestin binding depends on both a phosphorylation sensor and a receptor activation sensor (Gurevich and Gurevich, 2004), these results suggest that D1-CT is of greater importance than D1-IC3 for the phosphorylation-independent binding of arrestins to the D1 receptor. This seems to contradict previous work demonstrating that the D1 C-terminus is not required for phosphorylation-independent agonist-induced recruitment of arrestin3 to the cell membrane (Kim et al., 2004a); it may be that in the context of the

intact D1 receptor, IC3 and the C-terminus serve redundant roles with respect to binding of arrestin.

For further evaluation of the ability of the D1 receptor to preferentially interact with one form of arrestin in a cell system, we used confocal microscopy to assess the colocalization of the endogenous D1 receptor with both endogenous arrestin2 and arrestin3 in neostriatal neurons. Treatment for 2, 5, and 20 min with SKF-82958, an efficacious dopamine D1-like receptor-selective agonist (Martin-Negrier et al., 2000), enhanced the colocalization of the D1 receptor with arrestin3, and not arrestin2. Translocation of arrestin3 to the membrane in neurons was confirmed by quantification of arrestin immunoreactivity in membranes prepared from agonist-treated neuronal cultures; the abundance in the membrane of arrestin3, but not arrestin2, was enhanced by D1 receptor activation. In addition, agonist treatment increased the direct interaction of the endogenous D1 receptor and arrestin3 as assessed by co-immunoprecipitation of the two proteins. These data are consistent with other studies that demonstrate a preferential interaction between the D1 receptor and arrestin3, characteristic of class A GPCRs, but this study is the first to characterize the interactions of the endogenous proteins in a neuronal system.

As previously described for the D1 receptor and other class A GPCRs, the D1 receptor was rapidly internalized and probably disassociated from arrestin near the membrane, as suggested by a decrease in receptor-arrestin colocalization at 60 min and by inspection of the confocal images. These results are consistent with prior work demonstrating agonist-induced internalization of the D1 receptor in heterologous expression systems (Oakley et al., 2000; Gardner et al., 2001; Mason et al., 2002).

In contrast to the D2 receptor, for which agonist-induced internalization seemed to occur more rapidly than receptor-arrestin colocalization or arrestin translocation to the membrane, agonist-induced colocalization of the D1 receptor and arrestin3 was rapid and consistent with a model in which the binding of arrestin to agonist-activated, phosphorylated receptor mediates receptor desensitization and internalization (Krupnick and Benovic, 1998). Translocation of arrestin3 to the membrane also tended to be increased within 5 min of agonist stimulation, although the increase was not statistically significant until the measurement at 20 min, and agonist treatment increased the co-immunoprecipitation of the D1 receptor and arrestin3 within 5 min.

The classification of GPCRs has been investigated chiefly using recombinant GPCR- and arrestin-overexpressing cell lines (Oakley et al., 2000; Shenoy and Lefkowitz, 2003), and other work suggests that not all rhodopsin-family GPCRs fit neatly within the A or B classification (Mukherjee et al., 2002; Tulipano et al., 2004; Macey et al., 2004). In this study we have determined that the D1 receptor interacts with arrestin3 in neostriatal cultures, and that the rapid time course of the interaction is consistent with arrestin-mediated receptor internalization; preferential interaction with arrestin3 and rapid receptor internalization and disassociation from arrestin are both characteristics of a class A receptor.

Figure 3.1

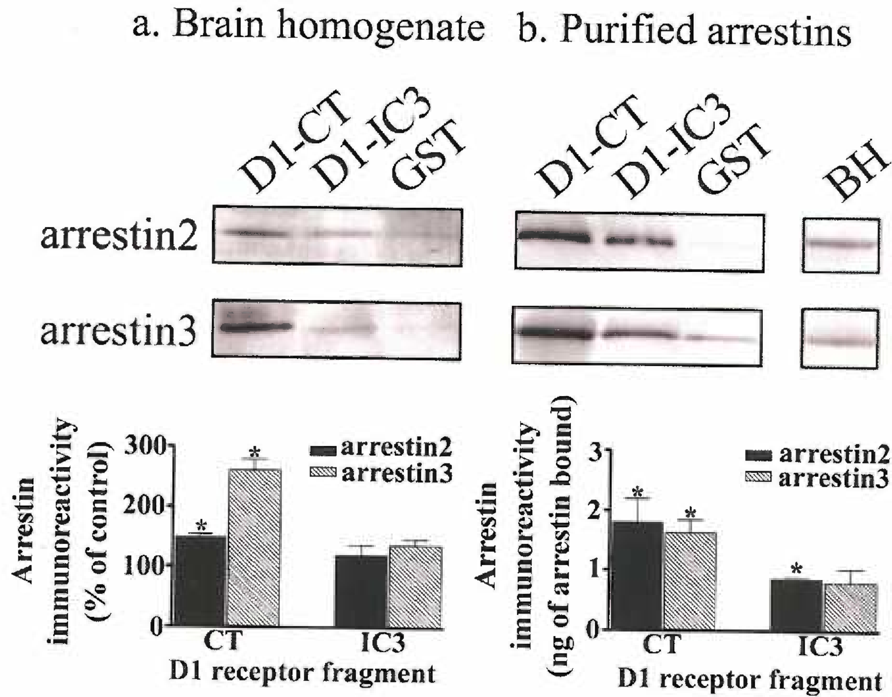


**Fig. 3.1** Direct interaction of the third intracellular loop and C-terminus of the dopamine D1 receptor with arrestin2 and arrestin3. Fusion proteins of GST and the third cytoplasmic loop of the D1 receptor (D1-IC3), the D1 C-terminus (D1-CT), or GST alone (GST) were incubated with (a) 500 µg of striatal homogenate, or (b) 25 ng of purified arrestin2 or arrestin3, purified, and immunoblotted with anti-arrestin2 or anti-arrestin3 antibody, as indicated. Aliquots (20 µg protein) of the striatal homogenate (brain homogenate, BH) were run in two lanes to demonstrate the presence of both arrestin2 and 3 in the striatal homogenate. The upper panels depict experiments representative of 3 independent experiments. The lower panels show the means $\pm$ SE from all 3 experiments, expressed as a percentage of the band density of eluates for GST alone (*lower left panel*) or as ng of arrestin specifically bound (*lower right panel*). (*$p$ <0.05 compared to control (GST alone) by Student's $t$-test analysis).

Figure 3.2

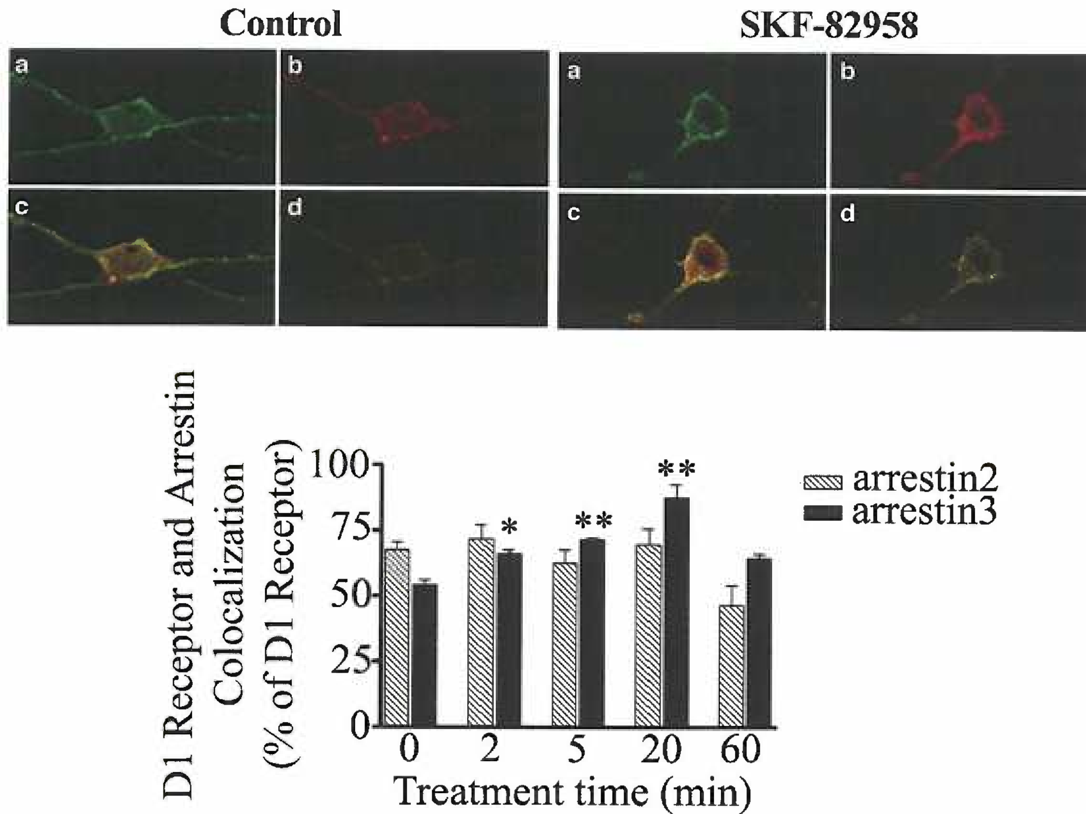


**Fig. 3.2** Agonist-induced colocalization of the endogenous D1 receptor and endogenous arrestin3, but not arrestin2, in neostriatal neurons. Neuronal cultures prepared as described in Experimental Prodecures were used to quantify colocalization of D1 receptor and arrestin2 or arrestin 3 immunoreactivity. ***Upper panels***, Confocal fluorescence images are shown for colocalization (c) of anti-arrestin3 (b) with anti-D1 receptor (a) in neuronal cultures treated with vehicle (*left panel*) or with SKF-82958 for 5 min (right panel). Merged and subtracted images (d) show only pixels containing both green and red fluorescence. ***Lower panel***, The results shown are the means ± SE for colocalization of D1 receptor immunoreactivity and arrestin2 or 3 immunoreactivity, expressed as a percentage of total D1 receptor immunoreactivity, in cells treated with vehicle (0) or agonist for the indicated time. Treatment with SKF-82958 significantly altered colocalization of D1 receptor and arrestin3 immunoreactivity ($p < 0.0001$ by one-way ANOVA) with a significant increase observed after treatment for 2, 5, and 20 min (*$p$ <0.05, **$p$ <0.01 by Dunnett's *post hoc* comparison, N = 3).

Figure 3.3

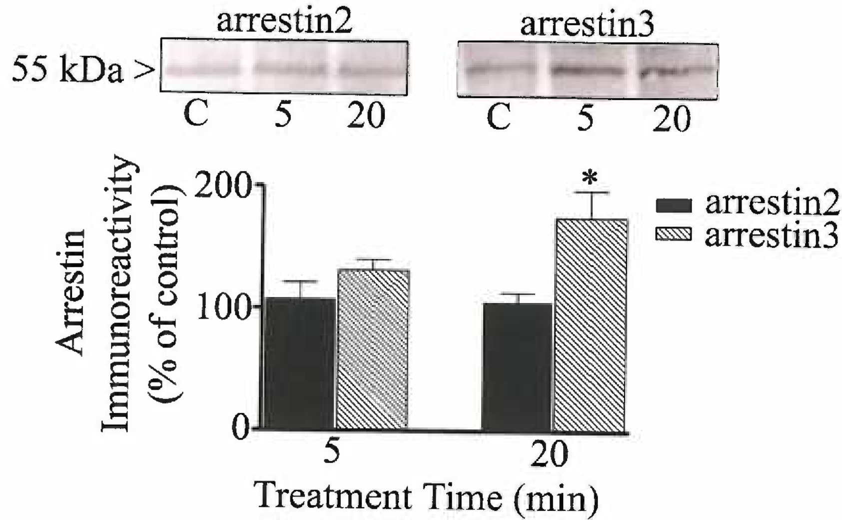


**Fig. 3.3** Agonist-induced translocation of arrestin in neostriatal neurons. Neostriatal neurons were treated with SKF-82958 (50 μM) for 5 or 20 min, membranes were prepared, and levels of endogenous arrestin2 and arrestin3 were assessed using immunoblotting. Results are the means ± SE from 3 experiments, expressed as a percentage of the band density in membranes from vehicle-treated cells (*$p < 0.05$ by Dunnett's *post hoc* comparison). A representative immunoblot is shown in which cells were treated with vehicle (C) or agonist for 5 and 20 min (*upper panel*).

Figure 3.4

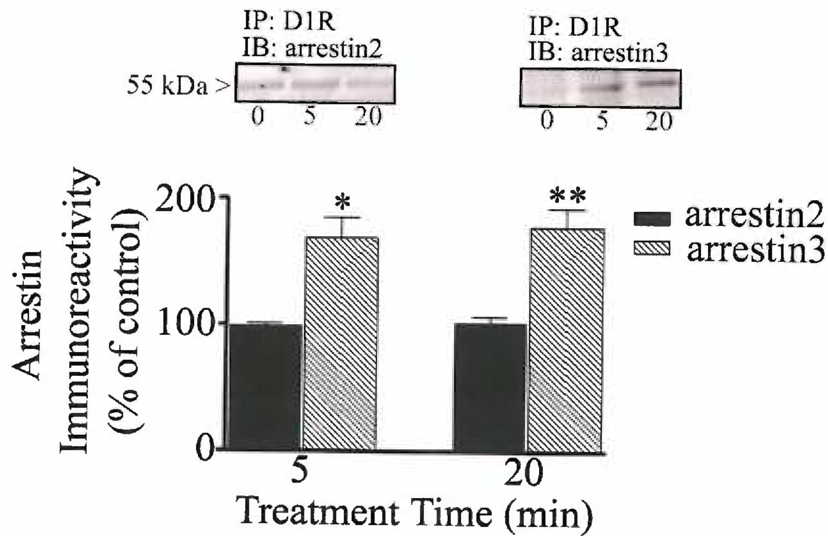


**Fig. 3.4** Agonist-induced co-immunoprecipitation of the dopamine D1 receptor and arrestin2 and 3 in neostriatal cultures. Striatal neurons were treated with vehicle or with SKF-82958 (50 μM) for 5 or 20 min. The D1 receptor was immunoprecipitated, after which proteins in the pellet were separated by SDS-PAGE and immunoblotted with anti-arrestin2 or 3 antibody. The upper panel depicts representative experiments in which cells were treated with vehicle (0) or SKF-82958 for 5 or 20 min. The lower panel shows the means ± SE from 3 experiments, expressed as a percentage of the band density in membranes from vehicle-treated cells (*$p < 0.05$, **$p < 0.01$, by Dunnett's *post hoc* comparison).

Figure 3.4

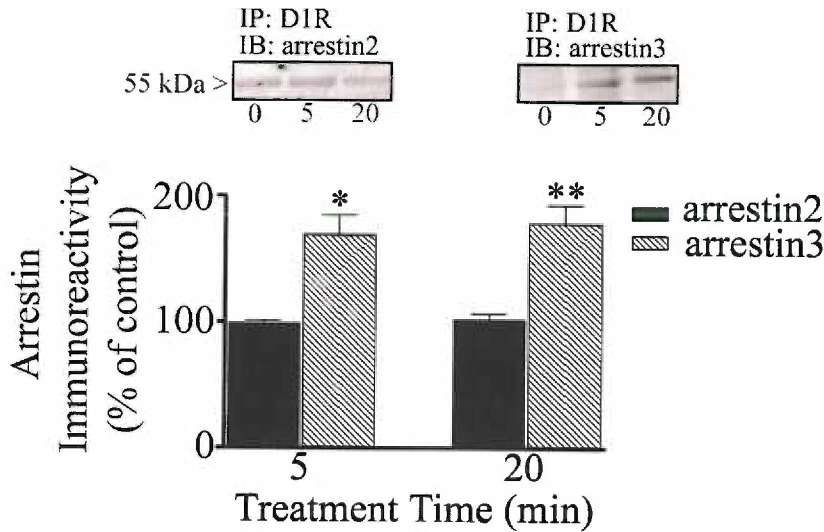


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Figure 3.5

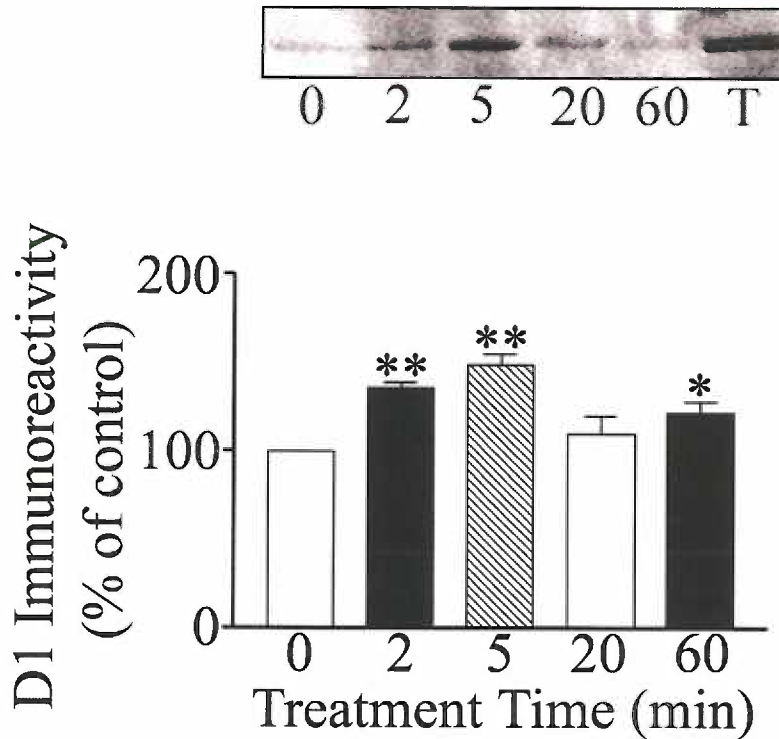



**Fig. 3.5** Agonist-induced internalization of the D1 receptor in neostriatal neurons. Striatal cultures were biotinylated, treated with SKF-82958 (50 μM), reduced by glutathione, avidin purified, separated by SDS-PAGE, and immunoblotted with anti-dopamine D1 receptor antibody as described in Experimental Procedures. (a) Representative immunoblot from striatal cultures treated with vehicle (0) or SKF-82958 for 2, 5, 20, or 60 minutes. Also shown is the total biotinylated product (T). (b) The means ± SE of the band densities from vehicle- or agonist-treated cells are shown, expressed as a percentage of the optical density in vehicle-treated cells. (* $p < 0.05$, ** $p < 0.01$ compared to control, Dunnett's *post hoc* comparison, N = 3).

# IV. Dopamine D2 Receptor Internalization in Neostriatal Neurons

## ABSTRACT

Dopamine D2 receptor internalization has been characterized in heterologously expressed D2 receptor-transfected mammalian cells with exogenous addition of GRKs and arrestins. The purpose of this study was to investigate internalization of endogenous dopamine D2 receptors in neurons. Neurons were treated at 5, 20, and 120 min with the selective D2 agonist, 7-OH DPAT. Agonist treatment of neostriatal neurons induced D2 receptor internalization that was maximal within 20 min. When neurons were pretreated with pertusis toxin (PTX), an inhibitor of $G_{i/o}$ signaling, and treated with agonist for 20 min, internalization of the receptor was attenuated. These data demonstrate the time course of the internalization of the endogenous dopamine D2 receptor in primary striatal cultures.

## Introduction

When the responsiveness of a receptor decreases after continued or repeated stimulation with an agonist, the phenomenon is known as desensitization. Resensitization, the reinstatement of the ability for the receptor to respond to ligands, follows desensitization (Krupnick and Benovic, 1998). Trafficking of GPCRs through various subcellular compartments is an important part of desensitization and resensitization. The $\beta_2$-adrenergic receptor has been used to extensively model GPCR desensitization. Once the GPCR has been activated by agonist, the GPCR is phosphorylated by GRKs or second messenger-dependent kinases such as protein kinase A (Krupnick and Benovic, 1998). Arrestins bind to the phosophorylated GPCRs, preventing receptor coupling to G proteins. The GPCR can be targeted to clathrin-coated pits for internalization and either degradation or resensitization (Pippig et al., 1995; Tsao et al., 2001). Arrestin can also promote the stable association of signaling proteins with the receptor (Luttrell et al., 2001).

The stability of the receptor:arrestin complex determines the rate of GPCR resensitization. Class A GPCRs dissociate from arrestin near the cell membrane, are rapidly dephosphorylated and recycled, and have been characterized to internalize within seconds to minutes. Arrestin3 preferentially interacts with class A GPCRs, such as the dopamine D1 receptor and the $\beta_2$-adrenergic receptor. In contrast, class B GPCRs remain associated with arrestin during internalization and are dephosphorylated and recycled more slowly (Shenoy and Lefkowitz, 2003). Internalization of class B receptors occurs more slowly compared to class A receptors. Class B receptors, such as the vasopression V2 receptors, have similar affinity for arrestin2 and arrestin3 (Oakley et al., 2000).

Both desensitization and internalization of the dopamine D2 receptor have been described in a variety of cell lines and tissue preparations (Ng et al., 1997; Boundy et al., 1995; Barton et al., 1991; Zhang et al., 1994; Sibley and Neve, 1997; Kim et al., 2001; Vickery and von Zastrow, 1999; Kim et al., 2004b). The interaction of arrestins and the dopamine D2 receptor and the contribution of this interaction to receptor internalization have also been investigated, but have been limited to studies using heterologous expression of arrestins, GRKs, and the D2 receptor in non-neuronal cells. We recently were able to demonstrate that internalization of the D2 receptor was attenuated when arrestin2 and arrestin3 was knocked down by siRNA interference in D2-transfected mouse neuroblastoma cells (Macey et al., 2004). In this study D2 internalization was assessed without the addition of exogenous GRK or arrestins into the system (Macey et al., 2004). Our data agree with a study that used a dominant negative mutant of arrestin3 to prevent D2 internalization (Kim et al., 2001).

We now report that agonist stimulation caused internalization of the endogenous D2 receptor in neostriatal neurons that was maximal at 20 minutes. Based on our data, the internalization rate of the dopamine D2 receptor can be described as class B receptor. The D2 receptor exhibited slow internalization, compared to a class A receptor that has been characterized to internalize within minutes.

## Materials and Methods

**Materials.** Serum was purchased from HyClone (Logan, UT). Other reagents, including culture media, 7-OH-DPAT, and PTX, were purchased from Sigma Chemical Co. (St. Louis, MO). The antibody used was the rabbit anti-dopamine D2L/S (1/250 dilution, AB5084P from Chemicon, Temecula, CA). The secondary antibody for immunoblot

analysis was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The blocking reagent I-block was purchased from Tropix (Bedford, MA). Pregnant Sprague-Dawley rats at gestation day 13 were obtained from Harlan (Indianapolis, IN).

**Neostriatal Neuronal Cultures.** The striatal region was dissected from 4 day-old Sprague-Dawley rats and incubated in minimum essential medium (MEM) containing 20 U/ml papain for 2 hours at 37°C. The tissue was then triturated using fire-polished Pasteur pipettes in MEM supplemented with 10% fetal bovine serum, 0.45% glucose, 5 pg/ml insulin, 0.5 mM glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. Cells were plated on poly-D-lysine-treated 10 cm plates. Neuronal medium containing 50% MEM, 39% Ham's F12 medium, 10% horse serum, 1% fetal bovine serum, 0.45% glucose, 5 pg/ml insulin, 0.1 mg/ml apotransferrin, 0.5 mM kynurenic acid, and 1 $\mu$g/ml glia-derived neurotrophic factor, was added one hour after initial plating. The medium was first conditioned with glial cells for 24 hours. Tissue culture plates received 2 ml of the neostriatal cell suspension and an additional 5 ml of the neuronal medium at the time of plating. Cells were grown in a humidified 5% $CO_2$ incubator at 37°C and used after 6-8 days in culture.

**Biotinylation Sequestration Assays.** Neostriatal neurons were labeled for 2 min at 37°C with EZ™-Link NHS-SS-biotin (0.3 mg/ml) to biotinylate cell surface proteins prior to treatment with agonist. After washing in Tris-buffered saline and one rinse with 0.1 M glycine, neurons were incubated in neuronal medium containing vehicle or 10 µM 7-OH DPAT for 5, 20, or 120 min. In some experiments, cells were first pre-treated with PTX (50 ng/ml) overnight. Agonist treatment was terminated by rapid cooling on ice. Biotinylated proteins remaining on the cell surface were stripped of biotin by the non-

permanent reducing agent glutathione (150 mM glutathione, 150 mM NaCl, pH 8.75). Glutathione was subsequently neutralized by 50 mM iodoacteamide in phosphate buffered saline, and the cells were homogenized in solubilization buffer as described above. Cells were centrifuged at 16000 x g, and supernatants containing equal amounts of total protein were incubated with ImmunoPure™ Immobilized strepavidin beads (Pierce, Rockford, Illinois) to capture biotinylated proteins. The protein concentration of each sample was determined using the BCA Assay kit (Pierce, Rockford, IL). After washing in extraction buffer, biotinylated proteins were eluted from strepavidin beads by heating at 60°C for 20 min in sample buffer, separated by SDS-PAGE, and immunoblotted using anti-dopamine D2 receptor antibodies. A one-way ANOVA and Dunnett's *post hoc* comparison were used to analyze data.

**Immunoblots.** Proteins were separated by SDS-PAGE through a 10% polyacrylamide gel and transferred to polyvinyl membranes (Millipore, Bedford, MA). The membranes were blocked overnight with I-block (0.2% with 0.1% Tween 20 in Tris-buffered saline (TBS), pH 7.4) at 4°C, washed twice for 5 min, followed by 2 10-min washes with TBS, and incubated with anti-dopamine D2 receptor antibody overnight at 4°C. The PVDF membranes were washed twice for 5 min, followed by 2 10-min washes with TBS, then incubated with secondary antibody at a dilution of 1:3000 (alkaline-phosphatase conjugated anti-rabbit IgG, from Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 hr. Immunodetection was accomplished using an ECF Western blotting kit (Amersham Biosciences, Piscataway, NJ). Proteins were visualized using the Typhoon phosphorimaging system and quantified with ImageQuaNT (Molecular

Dynamics, Sunnyvale, CA). A one-way ANOVA and Dunnett's post hoc comparison were used to analyze data.

## Results

**Agonist-Induced Internalization of the Endogenous D2 Receptor in Neostriatal Neurons.** Neostriatal cultures were biotinylated and then treated with 7-OH DPAT (10 μM) for 0, 5, 20, or 120 min to induce receptor internalization. After separation of biotinylated proteins by avidin gel matrix purification, the proportion of internalized D2 receptor was determined by immunoblot analysis. Treatment with 7-OH DPAT for 20 min increased the proportion of internalized receptor compared to untreated cells (Fig. 4.1; 59 ± 9% increase, $p < 0.01$, n = 3). D2 receptor internalization was prevented by pretreatment with PTX, demonstrating that internalization requires D2 receptor interaction with the pertussis toxin-sensitive G proteins $G\alpha_{i/o}$.

## Discussion

We used biotinylation to quantify D2 receptor internalization induced by 7-OH DPAT, an efficacious dopamine D2-like receptor-selective agonist (Chio et al., 1994). Using neostriatal neurons, we observed substantial internalization of the D2 receptor within 20 min of agonist treatment. This work agrees closely with other work we have done using D2-transfected NS20Y cells with no exogenous addition of arrestins or GRKs. We used both direct binding of the hydrophilic ligand [$^3$H]sulpiride and a different version of the biotinylation assay in D2-transfected NS20Y cells, and found that internalization of the dopamine D2 receptor was maximal at 20 minutes (Macey et al., 2004). These results are similar to prior work demonstrating agonist-induced

internalization of the $D2_L$ receptor in CHO, HEK293, and Neuro2A neuroblastoma cells (Itokawa et al., 1996; Vickery and von Zastrow, 1999), although others have observed little or no internalization in the absence of overexpressed GRK or arrestin (Ito et al., 1999; Kim et al., 2001; Kim et al., 2004b). Internalization of the biotinylated receptor was maximal at 20 min. Agonist-induced D2 receptor internalization was attenuated by pretreatment with PTX, demonstrating a requirement for D2 receptor interaction with the PTX-sensitive G proteins $G\alpha_{i/o}$.

Figure 4.1

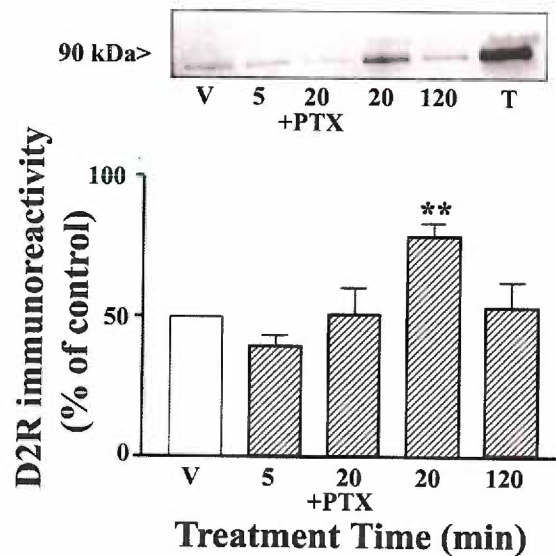



**Fig. 4.1.** Agonist-induced internalization of the D2 receptor in neostriatal neurons. Striatal cultures were biotinylated, treated with 7-OH DPAT (10 μM), reduced by glutathione, avidin purified, separated by SDS-PAGE, and immunoblotted with anti-dopamine D2 receptor antibody as described in Methods. *A*, Representative immunoblot from striatal cultures treated with vehicle (V) or 7-OH DPAT (10 μM) for 5 (5') and 20 (20') minutes, or pretreated with pertussis toxin (50 ng/ml) prior to treatment with 7-OH DPAT for 20 min (20' + PTX). Also shown is the total biotinylated product (total). ***B***, The mean ± S.E.M. of the band densities from vehicle- or agonist-treated cells is shown, expressed as a percentage of the optical density in vehicle-treated cells. (** $p < 0.01$ compared to control, Dunnett's *post hoc* comparison, N = 3).

# V. The interaction of the dopamine D1 and D2 receptors with constitutively active arrestin mutants

**Abstract**

Dopamine D1 and D2 receptor interactions with purified arrestins have been characterized using receptor fragments expressed as GST fusion proteins. The purpose of this study was to further investigate D1 and D2 receptor interaction with constitutively active arrestins. The C-terminus and the third intracellular loop of the dopamine D1 receptor (D1-CT and D1-IC3) and the third intracellular loop of the D2 receptor (D2-IC3) were generated as GST fusion proteins. In previous studies, I demonstrated that the D1-CT, D1-IC3, and D2-IC3 bound wildtype arrestin2 and arrestin3. The first constitutively active arrestin, arrestin2 (3A), consisted of 3 residue changes, I386A, V387A, and F388A in arrestin2. The binding of the D1-CT to arrestin2 (3A) was significant compared to GST alone. The D1-IC3 and the D2-IC3 to did not significantly bind arrestin2 (3A) compared to GST alone. The second constitutively active arrestin mutation in arrestin2 was based on the residue Arg-169, arrestin2 (R169E). Both the D1-CT and D1-IC3 bound arrestin2 (R169E) significantly, but the D2-IC3 did not significantly bind the mutant arrestin, compared to GST alone. The third mutation, arrestin3 (1-392), involves the truncation of the C-terminus of arrestin3, residues 393-409. The D1-IC3 bound arrestin3 (1-392) significantly, but the D1-CT or D2-IC3 did not bind the mutant arrestin3 significantly, compared to GST alone. Interestingly, in our study, the D1-IC3 significantly bound arrestin3 (1-392).

## Introduction

Arrestin plays a critical role in the desensitization of GPCRs by binding to GRK-phosphorylated receptors, resulting in dissociation of the G protein from the receptor. Arrestin then targets the receptor to clathrin-coated pits for internalization and either degradation or recycling back to the membrane for resensitization (Pippig et al., 1995; Tsao et al., 2001). The stability of the arrestin:GPCR complex determines whether the GPCR interacts with additional signaling proteins (Luttrell et al., 2001). The model of the rhodopsin-visual arrestin system proposed by Gurevich and others is the basis for the model of GPCR and arrestin association. The binding is driven both by a conserved "phosphate sensor" and an "activation sensor" (Gurevich and Gurevich, 2004).

The phosphate sensor of arrestin involves salt bridges in the polar core of arrestin. The key salt bridge in the polar core of visual arrestin is between Arg-175 and Asp-296. In arrestin2, the homologous residues are Arg-169 and Asp-290 (Hirsch et al., 1999). Phosphate binding neutralizes the charge on Arg-169 and destabilizes the polar core, allowing the arrestin to bind to the activated receptor (Kovoor et al., 1999). A charge reversal of Arg-169 in arrestin2 yields a phosphorylation-independent arrestin that binds with high affinity to the activated unphosphorylated receptor (Kovoor et al., 1999). The GPCR activation sensor of arrestin interacts with residues exposed in the activated GPCR (Gurevich and Gurevich, 2004).

There were three constitutively active arrestins used in this study that all were made in arrestin2 or arrestin3 based on robust phosphorylation-independent binding found in visual arrestin (Celver et al., 2002). The three mutant arrestins demonstrate the typical phenotype of a phosphorylation-independent mutant. The first mutant, arrestin2 (3A),

consisted of 3 residue changes, I386A, V387A, and F388A, which have been identified in visual arrestin to yield robust phosphorylation-independent binding (Celver et al., 2002). Arrestin2 (3A) binds to the unphosphorylated but activated $\beta_2$-adrenoreceptor, and phosphorylation does not increase the binding of the mutant to the receptor. The second arrestin2 mutant investigated was based on the residue Arg-169, arrestin2 (R169E). Gray and others demonstrated that this charge reversal in arrestin2 caused the arrestin-insensitive $5HT_{2A}$ receptor to become phosphorylation-dependent, and when the mutant was coexpressed with the receptor, led to the recruitment of arrestin2 (R169E) to the membrane in transfected HEK-293 cells (Gray et al., 2003). The third arrestin mutant involved the truncation of the C-terminus of arrestin3, arrestin3 (1-392). The mutation removes the Arg-393, homologous to Arg-382 in visual arrestin, which yielded a mutant with reduced arrestin selectivity.

In this study, the difference in the binding of each of the GST fusion proteins, D1-CT, D1-IC3, and D2-IC3, was assessed using the arrestin mutants, arrestin2 (3A), arrestin2 (R169E), and arrestin3 (1-392), and compared to the each construct's ability to bind wildtype arrestin2 and arrestin3. In our previous studies, the fusion proteins bound to the wildtype arrestins independent of phosphorylation. It was expected that the constructs would bind the mutant arrestins. The D1-IC3 bound wildtype arrestin2 and arrestin3 to a lesser extent compared to the D1-CT and D2-IC3.

## Material and Methods

### Materials

Antibodies used include: mouse anti-arrestin2 (1/300 dilution, A47520 from BD Transduction Laboratories, Lexington, KY), mouse anti-arrestin3 (1/250 dilution, sc-13140 from Santa Cruz Biotechnology, Santa Cruz, CA), and goat anti-GST (1/500 dilution, 27-4577-01 from Amersham Biosciences, Piscataway, NJ). Secondary antibodies for immunoblot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The blocking reagent I-block was purchased from Tropix (Bedford, MA).

### Generation of the GST fusion proteins

For construction of the GST fusion proteins, the third cytoplasmic loop of the dopamine D1 receptor (D1-IC3), amino acids 216-268, and the C-terminus of the dopamine D1 receptor (D1-CT), amino acids 329-446, and the third cytoplasmic loop of the dopamine $D2_L$ receptor (D2-IC3), amino acids 212-369 were PCR-amplified, subcloned into *Bam*HI-*Sal*I sites in pGEX-4T-3 (Amersham Biosciences, Piscataway, NJ), and transformed into BL21 cells. The D1-CT was subcloned by Yong Liu. Transformants were screened by induction with 50 $\mu$M isopropyl β-D-thiogalactoside and immunoblot analysis using an anti-GST antibody. For larger-scale purification, the GST fusion protein was grown in LB broth containing ampicillin (100 $\mu$g/mL) to $A_{600} = 0.5$ and stimulated with 50 $\mu$M isopropyl β-D-thiogalactoside for 3 hr at room temperature. Bacteria were pelleted and washed with phosphate-buffered saline. Pellets were resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, 1 mg/ml lysozyme, pH 8.0) and incubated for 1 hr with gentle rotation at room temperature. The homogenates were

clarified by centrifugation, and 600 μl of supernatant, typically ~1 mg, was applied to the MicroSpin GST Purification Module (Amersham Biosciences, Piscataway, NJ) containing glutathione Sepharose 4B beads, and purified according to manufacturer's instructions. Eluates were separated by SDS-PAGE and the gel was stained with Gel Code Blue (Pierce, Rockford, IL) to determine the correct molecular weight of each fusion protein. In addition, the BCA Protein Assay kit (Pierce) was used to determine protein concentrations of the GST fusion proteins.

**GST pulldown**

For GST pulldown experiments, purified arrestins were obtained when plasmids were expressed in BL21 cells and arrestins purified using heparin-Sepharose chromatography, followed by Q-Sepharose chromatography (Han et al., 2001). Purified arrestins were supplied by Vsevold Gurevich. Glutathione Sepharose 4B beads containing equal amounts of D1-IC3 GST, D1-CT GST, D2-IC3 GST, or GST without insert, were incubated with 25 ng of purified mutant arrestin2 or mutant arrestin3 at 4° C for 2 hr. The beads were washed three times with 20 mM Tris-HCl, pH 6.9, containing 70 mM NaCl. Samples were eluted with elution buffer (50 mM Tris-HCl, 10 mM glutathione, pH 8.0) for 20 min at room temperature with gentle rotation. Bound proteins were analyzed by immunoblotting with anti-arrestin2 or anti-arrestin3 as described below. The amount of bound arrestin2 or 3 was calculated from a 4-point standard curve generated using background optical density (*i.e.*, no arrestin) and 3 concentrations of arrestin2 or 3 between 0.625 and 2.5 ng. The amount bound to GST alone was subtracted from the total amount bound to D1-IC3-GST, D1-CT-GST, or D2-IC3 GST to arrive at a value for amount of arrestin2 or 3 specifically bound to each receptor fragment.

**Immunoblots**

Proteins were separated by SDS-PAGE through a 10% polyacrylamide gel and transferred to polyvinyl membranes (Millipore, Bedford, MA). The membranes were blocked overnight with I-block (0.2% with 0.1% Tween 20 in Tris-buffered saline, pH 7.4, at 4°C) washed twice for 5 min, followed by two 10-min washes with Tris-buffered saline, and incubated with anti-arrestin2 or anti-arrestin3 antibody at room temperature for 2 hr at RT. The PVDF membranes were washed twice for 5 min, followed by two 10-min washes with Tris-buffered saline, then incubated with secondary antibody at a dilution of 1:3000 (alkaline-phosphatase conjugated anti-mouse IgG) at room temperature for 1 hr. Immunodetection was accomplished using an ECF Western blotting kit (Amersham Biosciences, Piscataway, NJ). Proteins were visualized using the Typhoon phosphorimaging system and quantified with ImageQuaNT (Amersham Biosciences). Statistical significance was determined using a one-way ANOVA and Dunnett's post hoc comparison.

**Results**

For the first arrestin mutant, the D1-CT bound 0.6 ng of arrestin2 (3A), and there was no dectable binding of the mutant to the D2-IC3 or the D1-IC3. The binding of the D1-CT was significant compared to GST alone ($p<0.05$, Figure 5.1). The D1-IC3 and the D2-IC3 did not bind significantly to arrestin2 (3A) compared to GST alone. The same fusion protein constructs did bind wildtype arrestin2 in a phosophorylation-independent manner (Macey et al., 2004).

The D1-CT bound 1.1 ng of arrestin2 (R169E), the D1-IC3 bound 0.54 ng of arrestin2 (R169E), and the D2-IC3 bound 0.14 ng of arrestin2 (R169E). The D1-CT and

D1-IC3 bound arrestin2 (R169E) significantly compared to GST alone ($p<0.05$, Figure 5.1).

The D1-IC3 bound 0.82 ng of arrestin3 (1-392) significantly compared to GST alone ($p<0.01$, Figure 5.2). The D1-CT and D2-IC3 did not bind the truncated arrestin3 mutant.

**Discussion**

Mutant arrestins, that were constructed to be constitutively active, were incubated with the D1-CT, D2-IC3, and D2-CT GST fusion proteins. D1-CT significantly bound to arrestin2 (3A) and there was no detectable binding of the D2-IC3 or the D1-IC3 to the mutant. The same fusion protein constructs did bind wildtype arrestin2 in a phosphorylation-independent manner (see chapters II and III). It would be expected that the D1-IC3 would have bound arrestin2 (3A) with more affinity than wildtype arrestin2 because arrestin2 (3A) demonstrates the typical phenotype of a phosphorylation-independent mutant.

Both the D1-CT and D1-IC3 bound arrestin2 (R169E) significantly compared to GST alone. The D2-IC3 did not bind the mutant arrestin significantly compared to GST alone. The D1-CT, D1-IC3, and D2-IC3 bound wildtype arrestin2 in a phosphorylation-independent manner (see chapters II and III). For the D1-CT and D1-IC3 the mutation did not affect the binding of either construct to arrestin (R169E). The D1-CT bound more than twice the amount of the mutant arrestin compared to the D1-IC3, similar to the amount bound to wildtype arrestin2. It was surprising that the D2-IC3 did not bind arrestin2 (R169E) with greater affinity, because the D2-IC3 did not require activation or phosphorylation to bind to wildtype arrestin2. The mutation of arrestin2 (R169E)

affected the binding of the mutant arrestin to the D2-IC3. The region of the third intracellular loop contained in the construct may not interact with arrestin2 (R169E) or may not be accessible to the mutant arrestin. Again, wildtype arrestin2 significantly bound the D2-IC3 compared to GST alone, indicating the mutation has an effect on its interaction with the D2-IC3 (Macey et al., 2004).

The third mutation, arrestin3 (1-392) involves the truncation of the C-terminus of arrestin3, residues 393-409. Arrestin3 (1-392) generates a mutant arrestin with reduced selectivity. The D1-IC3 significantly bound arrestin3 (1-392) compared to GST alone. Both the D1-CT and D2-IC3 did not bind the truncated arrestin3 mutant significantly compared to GST alone. The D1-IC3 did not significantly bind wildtype arrestin3 (Macey et al., 2005). There is a possibility that the selectivity of arrestin3 was removed and allowed the D1-IC3 to bind to the mutant. At the same time selectivity was removed from arrestin3, critical residues required for binding of the D1-CT and D2-IC3 were removed or accessibility of the mutant arrestin was altered compared to wildtype arrestin3, and therefore both fusion proteins did not bind the mutated arrestin.

Previously it was demonstrated that the D1-CT, D1-IC3, and D2-IC3 bound to the purified wildtype arrestins in the absence of phosphorylation (Macey et al., 2004; Macey et al., 2005). The mutant arrestins investigated in this study did not have the same binding profile to the constructs as expected. It was expected that the constructs would bind the constitutively active arrestins with greater affinity compared to the wildtype arrestins, because there is constitutive phosphorylation that would increase the binding of each construct to arrestin. However, the D2-IC3 did not bind two of the mutant arrestins significantly compared to GST alone. If additional regions were included in the construct

or were eliminated from the D2-IC3 a difference in the binding of the GST fusion protein to the different mutant arrestins could be obsereved. Again, because the D2-IC3 did not bind the mutant arrestin significantly does not indicate that the third intracellular loop is incapable of binding the mutant arrestins, only that the region contained within the specified construct does not bind to the mutant arrestins, indicating that additional binding sites may be required. Differences between the wildtype arrestins and mutant arrestins include the possibility that conformational changes or structure changes in the mutant arrestins prevented, or in the case of the D1-IC3 allowed, the binding of the different constructs. To further elucidate the differences in binding of the different regions of the dopamine D1 and D2 receptor to the mutant arrestins, more constructs containing different regions of the receptors could be made or the intact receptors could be incubated with each mutant arrestin.

Figure 5.1

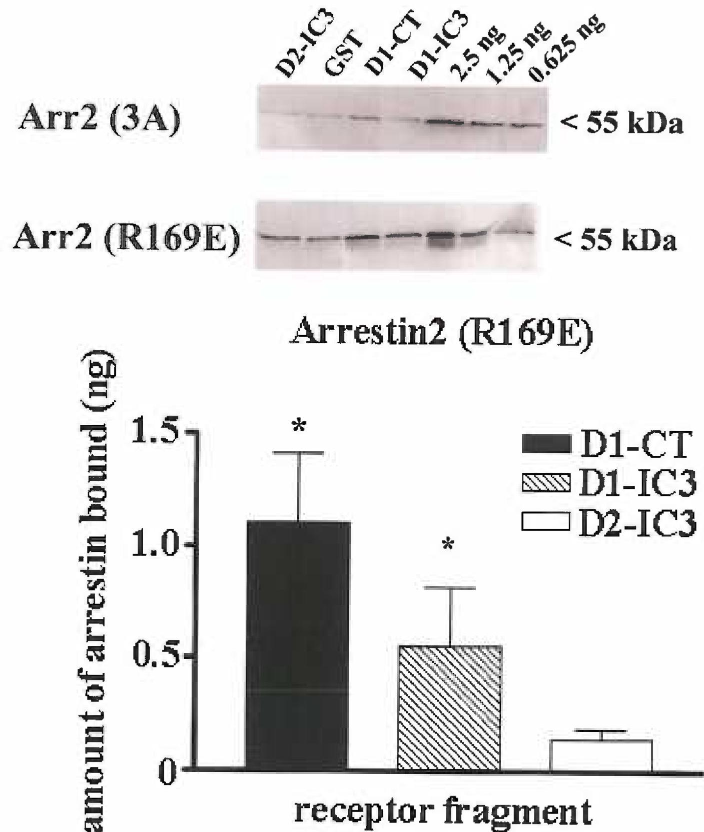



**Fig. 5.1.** Direct interaction of the third intracellular loop and C-terminus of the dopamine D1 receptor with constitutively active mutant arrestin2 (3A) and and arrestin2 (R169E). Fusion proteins of GST and the third cytoplasmic loop of the D1 receptor (D1-IC3), the D1 C-terminus (D1-CT), or GST alone (GST) were incubated with 25 ng of purified arrestin2 (3A) or arrestin2 (R169E), purified, and immunoblotted with anti-arrestin2 antibody, as indicated. The upper panels depict experiments representative of 3 independent experiments. The lower panels show the means ± SE from all 3 experiments for arrestin2 (R169E), as ng of arrestin specifically bound (*lower right panel*). (*$p$ <0.05 compared to control (GST alone) by Dunnett's *post hoc* comparison)

Figure 5.2

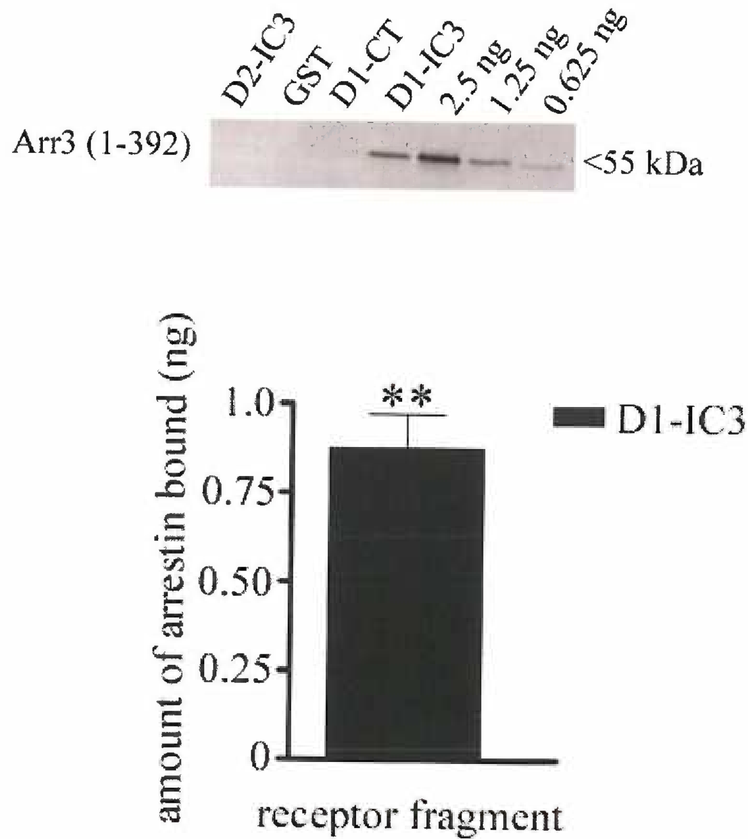



**Fig. 5.2.** Direct interaction of the third intracellular loop and C-terminus of the dopamine D1 receptor with arrestin3 (1-392). Fusion proteins of GST and the third cytoplasmic loop of the D1 receptor (D1-IC3), the D1 C-terminus (D1-CT), or GST alone (GST) were incubated with 25 ng of purified arrestin3 (1-392), purified, and immunoblotted with anti-arrestin3 antibody, as indicated. The upper panels depict experiments representative of 3 independent experiments. The lower panels show the means $\pm$ SE from all 3 experiments, as ng of arrestin specifically bound (*lower right panel*). (**$p$ <0.01 compared to control (GST alone) by Dunnett's *post hoc* comparison).

## Discussion

In this dissertation, I investigated the interaction of the dopamine D1 and D2 receptors with endogenous arrestin2 and arrestin3. I used various molecular and biochemical techniques to elucidate the interaction of the dopamine receptors and arrestins including GST fusion proteins, primary neuronal cultures, and a dopamine D2-receptor transfected cell line. In addition, the internalization of the D1 and D2 receptor was characterized. In a D2-receptor transfected cell line, the internalization of the dopamine D2 receptor was determined to be arrestin-dependent.

The dopamine D1 receptor, a class A receptor, internalized quickly and preferentially interacted with endogenous arrestin3. In the case of the dopamine D2 receptor, in D2-EGFP NS20Y cells, the D2-EGFP interacted with both endogenous arrestin2 and arrestin3 and exhibited slower arrestin-dependent internalization. In our investigation of the endogenous dopamine D2 receptor, we observed the same pattern of slow internalization but preferential interaction with arrestin2 compared to arrestin3.

### ***Specific Aim 1: Interaction of the dopamine D1 and D2 receptors with endogenous arrestins.***

Following agonist stimulation, the endogenous dopamine D1 receptor preferentially interacted with arrestin3, as determined using confocal microscopy, co-immunoprecipitation studies, and translocation studies. In a heterologously D2 receptor expressing cell line, agonist stimulation increased the colocalization of the dopamine D2 receptor and arrestin2 and arrestin3. In primary neostriatal cultures, the dopamine D2

receptor selectively interacted with arrestin2 following agonist stimulation, as determined by the use of confocal microscopy, co-immunoprecipitation, and translocation studies.

Confocal microscopy has become a useful tool to investigate protein-protein interactions in mammalian cells. The confocal microscope allows the visualization of fluorescent tags. The advantage of a confocal microscope is its confocal pinhole, which allows the microscope to reject out-of-focus fluorescent light, one of the problems of regular fluorescence microscopy. With regular fluorescence microscopy the sample is completely illuminated by the excitation light, so the entire sample is fluorescing at the same time, which contributes to a background haze in the resulting image. The image from a confocal microscope is taken from a thin optical section of a sample. A three-dimensional image can be constructed from multiple scans of thin sections through the same sample. Confocal microscopy can be used for live cell imaging and to analyze subcellular functions, such as pH gradients, membrane potentials, and intracellular changes in ion concentrations such as calcium, sodium, magnesium, zinc and potassium using fluorescent dyes.

In this study, I used NS20Y cells transfected with the dopamine D2 receptor that was tagged with EGFP. The D2-EGFP eliminated the need for the use of the primary antibody for the dopamine D2 receptor. In primary cultures, anti-dopamine D1 and D2 receptor antibodies that were conjugated to AlexaFluor488, a label that when excited at 488 nm emits green fluorescence, recognized both the endogenous dopamine D1 and D2 receptor. For both arrestin2 and arrestin3, primary antibodies were conjugated to AlexaFluor560, which emits red fluorescence when excited at 568 nm. After treatment with the selective agonist for both dopamine receptors, cells or neurons were fixed, and

incubated with the dopamine D1 or D2 antibody and antibody to either form of arrestin. Colocalization was measured using IP Lab software (Fairfax, VA).

Confocal microscopy also detects the location of proteins. Both the dopamine D1 and D2 receptor are membrane bound receptors and localized to the membrane. Arrestin is expressed ubiquitously throughout the cell and in response to agonist translocates to the membrane, to colocalize with the receptor. For example, agonist treatment enhanced the colocalization of arrestin3 and the dopamine D1 receptor, and arrestin2 and the dopamine D2 receptor, in neurons. Agonist treatment enhanced the colocalization of both isoforms of arrestin and the D2-EGFP in D2-EGFP-expressing NS20Y cells. The translocation of arrestins was confirmed when selective agonists for the dopamine D1 and D2 receptor enhanced abundance of arrestin3 and arrestin2 at the membrane, respectively.

There are some limitations to the use of confocal microscopy. One of the most prevalent issues is the specificity of the primary or secondary antibody. Appropriate controls must be run with confocal microscopy experiments. If possible, a tissue not containing the protein of interest should be treated, fixed, and incubated with both primary and secondary antibody. In our study we were able to use cerebellar neurons to account for any non-specific labeling of the dopamine D1 and D2 receptor, because the cerebellum does not contain these receptors. For arrestin antibody specificity we were limited to controlling for secondary antibody background because we did not have access to arrestin knockout animals or mouse embryonic fibroblasts (MEFs) lacking arrestins. Secondary antibody labeling is controlled for when cells are incubated with blocking buffer and only secondary antibody for any background that may exist.

We determined for the D2-EGFP NS20Y cells that agonist treatment enhanced the colocalization of the receptor and both isoforms of arrestin. There are several possible reasons why the D2-EGFP receptor interacted with both forms of arrestin compared to the endogenous D2 receptor, which exhibited a preference for arrestin2. The first possible explanation of why the D2-EGFP interacted with both isoforms of arrestin is that D2-EGFP NS20Y cells were overexpressing the dopamine D2 receptor, while striatal primary cultures were expressing endogenous dopamine D2 receptors. With an overexpressed cell system there is the disadvantage that when the protein of interest is overexpressed, non-physiological protein-protein interactions may occur. There is the possibility that in D2-EGFP NS20Y cells the overexpression of the dopamine D2 receptor allows the receptor to colocalize with arrestin3. However, the D2-EGFP was not highly overexpressed. An additional explanation for differences in the interaction of the D2-EGFP and both forms of arrestin includes the use of the EGFP tag. The size of the EGFP tag is large, and the tag may interfere with the trafficking of the receptor, or the tag may alter the ability of the signaling of the receptor. In our studies, the functional integrity of the fusion protein was assessed using [$^3$H]spiperone binding and inhibition of adenylate cyclase. D2-EGFP was localized to the membrane, as assessed by D2 antibody studies. Tagging of the receptor with EGFP did not affect the signaling, localization, or function of the receptor.

We did confocal microscopy experiments to confirm the EGFP tag did not affect the increase in colocalization that was observed with both isoforms of arrestin and the D2-EGFP. We used an untagged D2-transfected NS20Y cell line and observed a significant increase in colocalization of the D2 receptor with both arrestin2 and arrestin3

at 120 minutes. When internalization of the D2-EGFP NS20Y cells was characterized, NS20Y cells expressing an untagged D2 receptor were run in parallel. There were no differences between the tagged and untagged receptor in its colocalization with arrestin or in the internalization patterns. An additional confound of the EGFP tag is that the tag may cause the tagged protein to be localized within the perinuclear region (Mason et al., 2002). We did see some expression of the D2-EGFP tag in the perinuclear region of the transfected NS20Y cells but it was not evident in every NS20Y cell expressing the D2-EGFP. The EGFP tag most likely did not contribute to the non-selective increase in colocalization of the D2-EGFP and both isoforms of arrestin that was observed.

Agonist-induced interaction of each receptor with arrestins was further determined using co-immunoprecipitation studies in primary striatal cultures. The agonist induced co-immunoprecipitation of the dopamine D1 receptor and arrestin3 was increased 5 and 20 minutes, a time course similar to confocal and translocation studies. There was agonist-induced co-immunoprecipitation and colocalization, respectively, of the dopamine D2 receptor and arrestin2 at 20 and 120 minutes. The time course of the interaction of the dopamine D2 receptor and arrestin2 was not as straightforward, compared to the dopamine D1 receptor and arrestin3. Confocal and translocation studies suggest an increase in colocalization of the endogenous dopamine D2 receptor and arrestin2 at 120 minutes, not 20 minutes. However, the internalization of the dopamine D2 receptor is maximal at 20 minutes, which is supported by the co-immunoprecipitation data. There is a possibility in the confocal microscopy studies that a high ratio of arrestin to receptor increased the signal-to-noise ratio and prevented the detection of the increase in colocalization of the receptor and arrestin at 20 minutes. It should be noted that there

was increased colocalization of the endogenous dopamine D2 receptor and arrestin in neostriatal cultures at 20 minutes, although it was not significant. Overall, the co-immunoprecipitation study of both dopamine receptor subtypes with each form of arrestin was valuable in elucidating the differences in selectivity of each dopamine receptor with each isoform of arrestin and the time course of this interaction. Other methods to assess real time physical interactions of proteins include Fluorescence Resonance Energy Transfer (FRET) and real-time confocal microscopy. FRET measurements provide information on the molecular distances, environments, and interactions of proteins. Proteins are labelled with two fluorophores with the emission spectrum of the donor overlapping the absorption spectrum of the acceptor. Non-radiative energy can then be transferred from the excited donor molecule to the acceptor molecule, which then emits fluorescent light (Leica Microsystems).

Additional experiments could have been done to further elucidate the interaction of the dopamine D1 and D2 receptors with arrestin2 and arrestin3. Because other dopamine D1 and D2 agonists are available, additional experiments include treatments of cells or neurons with other agonists to each receptor type. Outcomes include the possibility that a specific agonist could change the time course of the increase in colocalization observed with the agonists used in these studies or change the preference of the receptor from one form of arrestin to the other. For example, when μ-opioid receptor-transfected HEK 293 cells were treated with morphine or heroin there was minimal translocation of arrestin3 (Bohn et al., 2004). Other μ-opioid receptor agonists including methadone, etophine, or fentanyl when administered to the same μ-opioid receptor-transfected HEK 293 cells demonstrated a robust translocation of arrestin3 to the

membrane (Bohn et al., 2004). Again, to further elucidate the interaction of the dopamine receptors and arrestin2 or arrestin3, additional experiments include antagonist treatment of each receptor type, added at the same time as receptor agonist. It would be expected that the addition of antagonist would prevent or decrease the increase in colocalization of the receptor and arrestin. Finally, additional time points could have been included. If cells or neurons were treated for three to four hours there could have been a further increase from basal levels in transfected cells or cultures that we were not able to observe within our defined time course.

Overall, our data suggest that the endogenous dopamine D1 and D2 receptors exhibited a preference for arrestin3 or arrestin2, respectively, with agonist stimulation. The D2 receptor in transfected cells exhibited increased colocalization with both forms of arrestin following agonist treatment.

***Specific Aim 2: Characterize the internalization of the dopamine D1 and D2 receptor.***

Internalization of the dopamine D1 and D2 receptor has been characterized using heterologously expressed receptors in mammalian cell lines and the addition of exogenous proteins involved with desensitization of the receptor. In this study the maximal internalization rate for both the endogenous dopamine D1 and D2 receptors in primary neostriatal cultures was determined. In addition, using D2-EGFP NS20Y cells, internalization of the D2-EGFP was assessed. Using siRNA interference, the expression of arrestin2 and 3 was suppressed in D2-EGFP NS20Y cells and internalization of the D2-EGFP was assessed.

Previous studies that have investigated the internalization of the dopamine D1 receptor have not provided a full time course of agonist treatment to determine the time

course of the internalization of the receptor. In this study, we characterized the internalization of the dopamine D1 receptor with the D1 receptor agonist SKF-82958. Membrane-bound receptors were biotinylated and treated with agonist for 2, 5, 20, or 60 minutes. Internalization of the receptor was significant at 2, 5, and 60 minutes following agonist treatment. Quick internalization of the dopamine D1 receptor was expected based on its classification as a class A GPCR. Again, class A GPCRs internalize within seconds to minutes and selectively interact with arrestin3. The time course of the internalization of the dopamine D1 receptor was consistent with the interaction of the receptor and arrestin described in our confocal microscopy studies, co-immunoprecipitation studies, and translocation studies.

The internalization of the dopamine D2 receptor has been characterized, but similar to the dopamine D1 receptor, cells transfected with the dopamine D2 receptor, arrestins, and GRKs have been used. In this study the internalization of the D2 receptor was assessed using D2-EGFP NS20Y cells, D2-NS20Y cells, and primary neostriatal cultures. In D2-transfected cells two approaches to characterize dopamine D2 receptor internalization were used, and in neostriatal cultures we used the same version of the biotinylation assay used to characterize dopamine D1 receptor internalization. Cells and neurons were treated with the D2 agonist, 7-OH DPAT. Agonist treatment caused substantial internalization of the D2 receptor within 20 minutes for both D2-transfected cell lines and for the endogenous dopamine D2 receptor in neostriatal neurons. In neurons, agonist-induced D2 receptor internalization was attenuated by pretreatment with pertussis toxin, demonstrating a requirement for D2 receptor interaction with the pertussis

toxin-sensitive G proteins $G\alpha_{i/o}$. In addition, there is the possibility that the arrestin could not bind and prevented the internalization of the receptor.

The time course of the internalization of the dopamine D2 receptor was consistent with our co-immunoprecipitation data of the D2 receptor and arrestin2 in neurons. An agonist-induced interaction of the receptor and arrestin was increased within 20 minutes. In our confocal microscopy studies we did observe an increase of the D2 receptor and arrestin2. There was agonist-induced colocalization and co-immunoprecipitation of the D2 receptor and arrestin2 and an agonist-induced increase of arrestin2 at the membrane. However, there was some increase in colocalization of the dopamine D2 receptor and arrestin2 in neurons at 20 minutes in the microscopy studies, though it was not significant compared to basal levels. In translocation studies, there was some arrestin2 in the membrane preparations from neurons treated at 20 minutes, but not significant compared to basal levels of arrestin2 at the membrane. Co-immunoprecipitation of the dopamine D2 receptor and arrestin2 measured only arrestin bound to the dopamine D2 receptor and these data may provide the most consistent results as far as the time course of the interaction of arrestin2 and the dopamine D2 receptor at 20 and 120 minutes. Together, the internalization and co-immunoprecipitation data suggest that arrestin may be necessary for internalization of the dopamine D2 receptor.

Agonist-induced internalization of the $D2_L$ receptor described in CHO, HEK293, and Neuro2A neuroblastoma cells agrees with our data for agonist-induced internalization of the dopamine D2 receptor in terms of the time-course (Itokawa et al., 1996; Vickery and von Zastrow, 1999). Other studies have consistently used a treatment time of 30 minutes to observe D2 receptor internalization, similar to our treatment time of

20 minutes. Overexpression of arrestin can enhance D2 receptor internalization (Kim et al., 2001; Kim et al., 2004b). The dominant negative arrestin, V53D, binds with high affinity to clathrin cages but is unable to interact with GPCRs and suppresses internalization of the receptor (Ferguson et al., 1996). Transfection of V53D in D2 receptor-transfected HEK 293 cells reduced the sequestration of dopamine D2 receptor (Kim et al., 2004b). The dependence on arrestin for internalization of the D2 receptor was also described in COS-7 cells not overexpressing exogenous arrestin (Kim et al., 2004b). Taken together, these data suggest that the dopamine D2 receptor sequesters into clathrin-coated vesicles in an arrestin- and dynamin-dependent manner. However, the dominant negative arrestin used in the described study suggests that the dopamine D2 receptor interacts with clathrin because D2 receptor sequestration is reduced when V53D is introduced. In a conflicting study also using D2 receptor transfected HEK 293 cells, evidence was found to support a dynamin-independent mechanism of internalization of the D2 receptor, not involving clathrin-coated vesicles (Vickery and von Zastrow, 1999). Due to this conflicting evidence in the literature, to further elucidate the mechanism of internalization for the D2 receptor, I suppressed arrestin2 and arrestin3 interactions with the D2 receptor using a different approach.

We used siRNA interference to suppress expression levels of arrestin2 and arrestin3, based on our confocal results that the D2-EGFP receptor colocalized with both arrestin2 and 3, to further characterize the possible arrestin-dependent internalization of the dopamine D2 receptor. The sequence used for the design of the siRNA sequences was based on arrestin2 and 3 sequence by Ahn and others (Ahn et al., 2003). The mouse and human sequences were aligned and the mouse sequence siRNA duplex was designed and

constructed for transfection into D2-EGFP NS20Y cells. Cells were transfected, treated with agonist for 20 minutes, and labeled with the hydrophilic ligand [$^3$H]sulpiride. When arrestin2 and arrestin3 were suppressed in D2-EGFP siRNA-transfected NS20Y cells, internalization of the D2-EGFP was prevented compared to non-transfected D2-EGFP NS20Y cells. Attenuation of the internalization of the receptor suggests that internalization of the D2-EGFP is dependent on either or both arrestin2 and arrestin3.

Additional investigation of the internalization of the dopamine D1 and D2 receptor could include the suppression of arrestin2 and/or arrestin3 in neostriatal neurons. However, tranfection of siRNA duplexes into primary neuronal cultures presents a challenge. Advances continue in the area of siRNA delivery, but transfection reagents have limited efficiency in neurons. Transfection products that have been developed often base their transfection efficiencies on hippocampal neurons, which are more durable then striatal neurons. Lipofectamine 2000 (Invitrogen), used to transfect the D2-EGFP NS20Y cells in this study, has only about 30% transfection efficiency in neurons. We also tried an amine- and-lipid based transfection reagent from Ambion, and siIMPORTER (Upstate). Our final attempt for transfection of the siRNA duplex into neurons was a product from Amaxa that uses a proprietary transfection reagent to resuspend freshly prepared neurons, and then the neurons are inserted into an electroporator. The electroporator is designed specifically for the delivery of siRNA duplexes into neurons. Our best results have been using the products from Amaxa, but the integrity of the neurons was compromised, as suggested by our inability to observe agonist-induced internalization of the endogenous D2 receptor in sham-transfected neurons. Most measures of receptor internalization require that the plasma membrane be

intact; it is possible that the transfection reagent caused persistent changes to the integrity of the membrane. Furthermore, the expression of both arrestins was only partially blocked by the siRNA treatment. An additional approach to transfect siRNA into neurons involves viral packaging, such as herpes simplex virus or adenovirus, or using a vector-based system for transfection with similar reagents described above.

Overall, the characterization of the internalization of the dopamine D1 and D2 receptor has provided additional data to elucidate the time course of the interaction of the receptor and arrestin. For the dopamine D1 receptor, the receptor internalized within minutes following agonist treatment, characteristic of a class A GPCR. In the case of the dopamine D2 receptor, in D2-EGFP transfected NS20Y cells and in neurons, there was significant agonist-induced internalization observed at 20 minutes, slow internalization as characterized for class B GPCRs. When arrestin2 and arrestin3 expression was suppressed in D2-EGFP transfected NS20Y cells, agonist-induced internalization was attenuated, suggesting that internalization of the dopamine D2 receptor is dependent on arrestin.

***Specific Aim 3: Interaction of the dopamine D1 and D2 receptor with arrestin using GST fusion proteins.***

To elucidate which specific region of the dopamine D1 and D2 receptor bound to arrestin, GST fusion proteins were constructed. For our study, we constructed five GST fusion proteins, which included: the C-terminus of the dopamine D1 receptor (D1-CT), the third intracellular loop of the dopamine D1 receptor (D1-IC3), the C-terminus of the dopamine D2 receptor (D2-CT), the third intracellular loop of the dopamine D2 receptor (D2-IC3), and the second intracellular loop of the dopamine D2 receptor (D2-IC2).

These regions of the dopamine D1 and D2 receptors were chosen based on previous data in the literature that suggested the third loop, second loop, and C-terminus of GPCRs were involved in desensization of the receptors, as discussed in the Introduction. The GST fusion proteins were incubated with striatal brain homogenates or purified arrestin2 or arrestin3. Incubation with striatal brain homogenates allowed the fusion protein to interact with endogenous arrestins, and most likely required a higher affinity of arrestin for the receptor due to the mixture of proteins in the striatal homogenate. When purified arrestins were incubated with fusion proteins, a lower affinity was most likely sufficient because the purity of the arrestin protein was near 100%.

Subcloning into the glutathione-S-transferase (GST) vector allows the protein of interest to be expressed, purified, and detected by immunoblotting. Once the protein of interest is subcloned into the GST vector, protein-protein interactions can be determined. Compared to other protein purification systems, advantages of using the GST system include that the GST fusion protein is produced in bacterial cultures (*E.coli)*, the GST system is inducible, there is a possibility for a high-level expression of the genes or gene fragments, the GST fusion proteins are easily purified using glutathione sepharose 4B beads, and fusion proteins can be detected using immunoblotting with the anti-GST antibody. Disadvantages of the GST system include issues such as, the purification of fusion proteins can be difficult if inclusion bodies are present, nonspecific binding of the GST tag can occur and must be appropriately controlled for, and the possibility of premature termination of translation.

In most GPCRs, receptor phosphorylation is required for GPCR interaction with arrestin, and the necessity for phosphorylation -dependence or phosphorylation-

independence of the GST fusion protein constructs should be addressed. Resolved crystal structures of arrestin explain arrestin selectivity for the phosphorylated active form of the receptor. There is one model of GPCR-arrestin interaction that suggests arrestin has two sensor sites, one site recognizes the phosphorylated receptor and a second site that recognizes the activated receptor. The activation sensor and/or additional sites that mobilize arrestin binding, not the phosphate-binding elements, determine receptor preference (Vishnivetskiy et al., 2004). Phosphate sensor function is highly conserved among the arrestin family. If the sensors involved in the recognition of phosphorylation sites are swapped, there is no consequence on receptor specificity.

There is a concern that phosphorylation of the constructs is necessary for the binding to arrestin to occur, but a number of studies have investigated receptor-arrestin interactions using unphosphorylated GPCRs. Ferguson et al. found that $\beta_2$ adrenergic receptors can internalize in the absence of GRK-mediated phosphorylation when arrestin2 and arrestin3 were expressed at high levels (Ferguson et al., 1996). In addition, Shiina et al. demonstrated the binding of *in vitro* translated arrestin to the carboxyl tail of the intact β2 adrenergic receptor that was not phosphorylated by GRKs, suggesting a phosphorylation-independent internalization of the β2 adrenergic receptor (Shiina et al., 2000). Wu et al. demonstrated the interaction of arrestins with the third intracellular loop GST fusion proteins for the $M_2$-muscurinic receptor, the $M_3$-muscrinic receptor, and the $\alpha_{2A/D}$-adrenergic receptor in the absence of peptide phosphorylation (Wu et al., 1997). Based on these data, phosphorylation of the dopamine D1-CT or D2-IC3 should not be required to demonstrate its interaction with arrestin2 or arrestin3. In our experiments, when the different regions of each receptor were taken out of the context of the whole

receptor, specific regions of each receptor type interacted with arrestin in a phosphorylation-independent manner. Fusion protein constructs were incubated with both a purified preparation of arrestin and striatal brain homogenates, and the interaction of the fusion proteins and arrestins was detected in both preparations.

When the D1-CT and the D2-IC3 were incubated with striatal brain homogenates, arrestin2 and arrestin3 were detected in the eluates. When the interaction of the D1-CT with arrestins was further analyzed, it was determined that the D1-CT preferentially interacted with arrestin3 compared to arrestin2. There was no difference in the interaction of arrestin2 and arrestin3 and the D2-IC3. It was not surprising that the D1-CT and the D2-IC3 were the main regions that exhibited high affinity binding to endogenous arrestin, because both are involved in the desensitization of the receptors (Kim et al., 2001; Lamey et al., 2002; Jackson et al., 2002). The D1-IC3 bound to endogenous arrestin, but less than what was observed for the D1-CT. The other constructs, which include the D2-CT and D2-IC2, did not bind endogenous arrestin.

The D1-CT bound purified arrestin2 and arrestin3, $1.8 \pm 0.4$ ng and $1.6$ ng $\pm 0.2$ ng, respectively. The D1-IC3 bound arrestin2 and arrestin3 to a lesser extent than the D1-CT, $0.9 \pm 0.04$ ng and $0.8 \pm 0.2$ ng, respectively. The two constructs were able to bind the purified arrestins without phosphorylation. The interaction of both constructs of the D1 receptor with arrestin did not follow a typical class A receptor, there was not a preference for arrestin3 compared to arrestin2. However, these data do suggest that the C-terminus of the D1 receptor is possibly the main site of arrestin binding, as found in the striatal brain homogenate data. All of these except the binding of arrestin3 to D1-IC3-GST were significantly different from binding to GST alone. Overall, D1-CT-GST

bound significantly more purified arrestins than D1-IC3-GST, but there was no significant difference between arrestin2 and arrestin3.

The D2-IC3 bound purified arrestin2 and arrestin3, 1.3 ± 0.3 ng and 1.6 ± 0.4 ng, respectively. The D2-CT also bound purified arrestin2 (0.5 ± 0.04 ng) and arrestin3 (0.3 ± 0.1 ng). The D2-IC2 bound purified arrestin2 (0.4 ± 0.1 ng) and to a greater extent arrestin3 (1.2 ± 0.3 ng). The three constructs were able to bind the purified arrestins without phosphorylation. The interaction of the D2-IC3 with both arrestin2 and arrestin3 is typical of a class B receptor. These data do suggest that the intracellular third loop of the D2 receptor is possibly the main site of arrestin binding, as found in the striatal brain homogenate data. It was interesting that the second intracellular loop bound arrestin3 more avidely than arrestin2. There is a possibility the D2-IC2 mediates selectivity of the dopamine D2 receptor for arrestin3, however this does not agree with our data investigating D2 receptor interaction with arrestins in primary neostriatal cultures or D2-EGFP NS20Y cells.

There could be several reasons why there was a difference in results between preparations of arrestin, purified arrestins versus striatal homogenates, when incubated with the GST fusion proteins. Again, only the D1-CT and D2-IC3 bound endogenous arrestin when incubated with striatal homogenates. Striatal homogenates could have other receptor-interacting proteins that compete with the binding of arrestins. Other interacting proteins may include additional trafficking proteins, such as clathrin, or mediators of downstream signaling, including ERK1/2 or other proteins that directly bind the C-terminus or the third intracellular loop of either the D1 or D2 receptor. If proteins competing with arrestin are binding within similar regions of the receptor, for example

the third loop of the receptor, or at the same sites, differences in how the receptor binds the specific isoform of arrestin could be affected (Smith et al., 1999). As a future experiment, it would be interesting to incubate the different GST fusion protein constructs with both purified arrestin2 and arrestin3 simultaneously to determine whether a similar phenomenon occurs. For example, if arrestin2 and arrestin3 are incubated with the D1-CT, the C-terminus of the D1 receptor may preferentially bind to arrestin3.

Mutant arrestins that were constructed to be constitutively active, or phosphorylation-independent, were also incubated with the D1-CT, D2-IC3, and D2-CT fusion proteins. The overall hypothesis was that each constitutively active mutant would bind the D1-CT, D1-IC3, and D2-IC3, and perhaps more avidly than wildtype arrestins because the arrestin mutation might mimic the effect of phosphorylation of the receptor. Three constitutively active arrestins used in the study were arrestin2 (3A), arrestin2 (R169E), and arrestin3 (1-392).

The first constitutively active arrestin, arrestin2 (3A), consisted of 3 amino acid residue changes, I386A, V387A, and F388A (Celver et al., 2002). The D1-CT bound arrestin2 (3A) significantly compared to GST alone. The D2-IC3 and the D1-IC3 did not bind arrestin2 (3A). See Table 1 for exact values of arrestin (ng) bound.

The second constitutively activating mutation altered residue Arg-169, arrestin2 (R169E). The D1-CT bound the highest amount of arrestin2 (R169E), followed by the D1-IC3, and the D2-IC3 bound the least amount of arrestin2 (R169E). The D1-CT bound arrestin2 (R169E) significantly compared to GST alone. The D1-CT bound more than twice the amount of the mutant arrestin R169E compared to the D1-IC3. The D2-IC3 did

not significantly bind arrestin2 (R169E) compared to GST alone. See Table 1 for exact values of arrestin (ng) bound.

It would be interesting to incubate the D2-IC3 GST construct with the wildtype arrestin2 and mutant arrestin2 at the same time to determine if the binding of wildtype arrestin2 is altered. Antibodies specific to the mutated arrestin could be generated to detect if mutant arrestin was binding more or less compared to the wildtype arrestin. In addition, if both the wildtype and mutant arrestins were incubated with the D2-IC3 simultaneously, the detection of arrestin2 in the presence of the mutant arrestin could be compared to determine if there was a competitive effect of the mutant arrestin.

The third mutation, arrestin3 (1-392), involves the truncation of the C-terminus of arrestin3, residues 393-409. Arrestin3 (1-392) significantly bound to the D1-IC3 compared to GST alone. Arrestin3 (1-392) did not bind to the D1-CT or D2-IC3. See Table 1 for exact values of arrestin (ng) bound.

Most interesting was that arrestin3 (1-392) significantly bound to the D1-IC3, which did not significantly bind wildtype arrestin3 compared to GST alone. One possible interpretation of these data is that this mutant rescued the ability of the D1-IC3 to bind arrestin3. An additional interesting result was that arrestin2 (R169E) did not bind the D2-IC3. Arrestin2 (R169E) may not be accessible to the third loop of the D2 receptor or this residue may not be required to bind to the receptor as observed for wildtype arrestin2 and arrestin3. In additional experiments it would be interesting to incubate the D2-IC2 and the D2-CT with arrestin2 (R169E).

**Table 1**

| GST Fusion Protein | Arrestin2 | Arrestin3 | Arrestin2 (3A) | Arrestin2 (R169E) | Arrestin3 (1-392) |
|---|---|---|---|---|---|
| D1-CT | 1.8 ng | 1.6 ng | 0.6 ng | 1.1 ng | no binding |
| D1-IC3 | 0.9 ng | 0.8 ng | no binding | 0.54 ng | 0.82 ng |
| D2-IC3 | 1.3 ng | 1.6 ng | no binding | 0.14 ng | no binding |
| D2-CT | 0.5 ng | 0.3 ng | n/a | n/a | n/a |
| D2-IC2 | 0.4 ng | 1.2 ng | n/a | n/a | n/a |

Selectivity of one isoform of arrestin versus another is reflected in GST fusion protein construct data collected from striatal brain homogenates. For example the D1-CT preferentially interacts with arrestin3. The D2-IC2 did not bind arrestin2, only arrestin3, suggesting that the second loop of the dopamine D2 receptor could contain selectivity for arrestin3 compared to other regions within the receptor. To further elucidate the selectivity of one arrestin, different GST constructs could be constructed that contained more narrow regions. With additional GST constructs that contain more narrow regions in which arrestins bind, the selectivity of using purified arrestin2 or arrestin3 and their interaction with the dopamine D1 or D2 receptor could be defined.

There were differences between results that we collected on the interaction of the dopamine D1 and D2 receptors and arrestin using GST fusion proteins compared to the data we generated using other techniques, including confocal microscopy, co-immunoprecipitation, and translocation assays. In neostriatal cultures, there was a preferential interaction of the dopamine D1 receptor for arrestin3. The D1-CT showed a preferential interaction for arrestin3 in striatal brain homogenates, but the D1-CT and D1-

IC3 bound both purified forms of arrestin. The D2 receptor in cultures selectively bound arrestin2, unlike the transfected D2 receptor, which bound both forms of arrestin. The D2-IC3 bound both forms of arrestin in brain homogenates and when incubated with purified arrestins, however the D2-IC2 selectively bound purified arrestin3. The discrepancy in results between the two experimental approaches may include differences in how the receptors were or were not phosphorylated in the different assays, the possibility of receptor dimerization with endogenous or transfected receptors, or differences in the stoichemetry of arrestin:receptor complexes between assays.

There are additional experiments that would be interesting to run using the GST fusion proteins. The phosphorylation state of the dopamine D1 and D2 receptor was different between assays. In the GST fusion protein assays there was no phosphorylation of the receptor fragments, but in assays that investigated the whole receptor, the receptor was most likely phosphorylated following agonist stimulation. It would be interesting to phosphorylate the GST fusion protein constructs using mutagenesis to make the receptor constitutively phosphorylated. The constructs once phosphorylated could be incubated with purified arrestins or striatal brain homogenates as described. It would be expected that all of the constructs could bind endogenous arrestin if phosphorylated, because all include potential phosphorylation sites that may alter the affinity of the receptor for arrestins. There could also be an increase in the amount of purified arrestin bound to constructs that interact with the arrestin phosphorylation sensor. An additional approach to elucidating the specific regions or more specific residues of each receptor that are involved in arrestin interaction involves mutagenesis within the whole receptor. Serine and threonine residues, residues identified in other GPCRs capable of binding arrestin,

could be altered and incubated with purified arrestins or striatal brain homogenates. If a specific residue, or the combination of several residues, in a specific region or regions of the receptor changes the binding properties of the receptor to arrestin, the identified residues could be responsible for the interaction of the receptor and arrestin. Binding of the different regions of the receptor to arrestin was not surprising because phosphorylation and/or activation of the whole receptor exposes the binding sites of the GPCR, allowing arrestin to bind. This phenomenon has been described for the dopamine D1 receptor (Kim et al., 2004a).

The stoichiometry of arrestin and GPCRs remains to be elucidated. Gurevich and others believe there is a relationship of one arrestin to one GPCR, or two arrestins to one receptor dimer (Gurevich and Gurevich, 2004). The stoichiometry of the receptor:arrestin complex could influence the results of our study. In the GST fusion protein study, we are investigating a specific region of the receptor and its interaction with arrestin, both in its purified form and in its endogenous form. In the GST protein assay we can assume one fusion protein is interacting with one arrestin or there is the possibility that one arrestin is binding two fusion proteins. The fusion proteins are small and have a limited number of arrestin binding sites. There is also the possibility that the structure of the fusion protein does not allow arrestin to bind. We cannot assume that, because there is no or minimal binding of arrestin to a specfic GST construct, the region contained within the construct does not interact with arrestin.

As suggested above, there is a possibility that *in vivo* receptor dimerization could occur and thereby influence the interaction of the receptor with arrestin. Terrillon and others found that the vasopressin V1a and V2 receptors form a heterodimer, though both

receptor subtypes have distinct mechanisms of agonist-promoted trafficking with arrestin when transfected in HEK 293 cells (Terrillon et al., 2004). The V1a receptor rapidly dissociates from arrestin and quickly recycles back to the plasma membrane. The V2 receptor remains associated with arrestin in endosomes, leading to intracellular accumulation. When a nonselective agonist was used for its activation, the heterodimer acted like the V2 receptor and stably associated with arrestin. When the heterodimer was treated with a V1a receptor agonist, the heterodimer rapidly dissociated from arrestin (Terrillon et al., 2004). These data suggest that the acivation of the specific receptor within the heterodimer regulates the endocytotic processing of the GPCRs. Though this study uses an overexpressed cell line, it provides insight into the possibility of other GPCRs to potentially form heterodimers. If a GPCR is classified as a class A or a class B receptor, its classification could change depending on its ability to form a heterodimer with another GPCR.

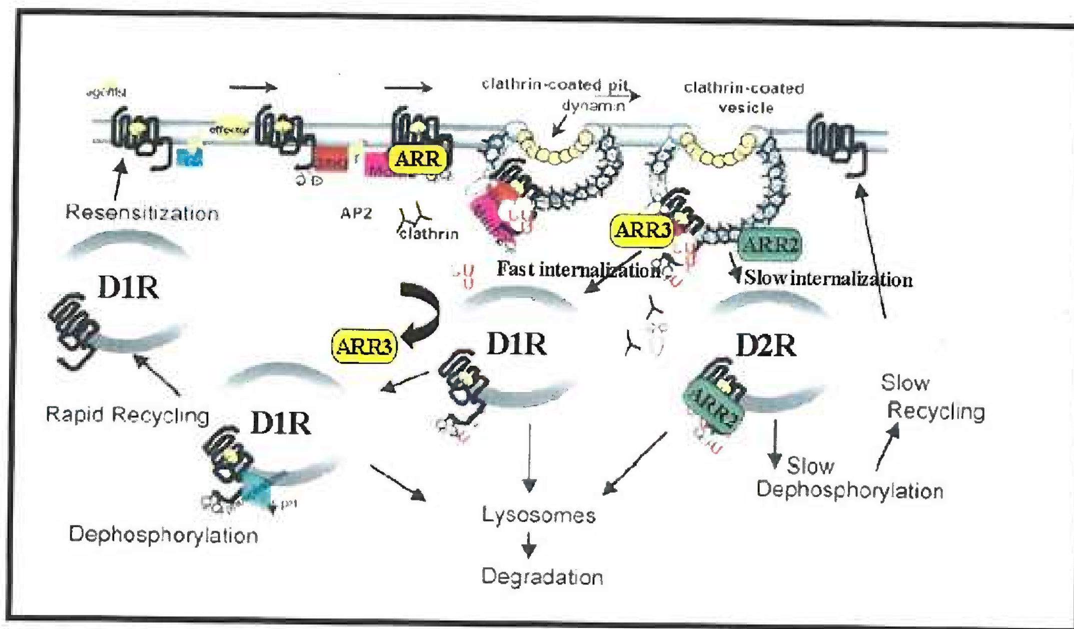
Overall, the use of GST fusion proteins to elucidate which regions of the D1 and D2 receptor bound arrestin2 and arrestin3 provided valuable data on each receptor's ability to interact with arrestin, both in purified and endogenous preparations. For the dopamine D1 receptor, the results suggest that the D1-CT is more important than the D1-IC3 for phosphorylation-independent binding of arrestins to the receptor. In the case of the dopamine D2 receptor, the D2-IC3 is more important than either the D2-CT or D2-IC2 in the phosophorylation-independent interaction of arrestin with the receptor. Our data followed the hypothesis that arrestin binding depends on both a phosphorylation sensor and a receptor activation sensor (Gurevich and Gurevich, 2004). For the dopamine D1 receptor, it can be speculated that the phosphorylation sensor may

recognize sites in the D1-IC3, while the activation sensor may bind chiefly to D1-CT and less strongly to D1-IC3. For the dopamine D2 receptor, it can be speculated that the phosphorylation sensor may recognize sites in the D2-CT and D2-IC2, while the activation sensor may bind mainly to D2-IC3 and less to the D2-CT and D2-IC2.

***Summary of conclusions.***

In this dissertation, the interaction of the dopamine D1 and D2 receptor with endogenous arrestin2 and arrestin3 was elucidated. The dopamine D1 receptor selectively interacted with arrestin3 upon agonist stimulation (Figure 6-1), and the C-terminus of the D1 receptor bound to endogenous arrestin3 in striatal brain homogenates. The D1-CT and D1-IC3 bound to both forms of purified arrestin. The dopamine D1 receptor internalized within minutes with agonist stimulation (Figure 6-1). The dopamine D2 receptor in neostriatal cultures preferentially interacted with arrestin2 with agonist treatment (Figure 6-1). However, in a D2-transfected cell line, the D2 receptor interacted with both arrestin2 and arrestin3 upon agonist stimulation, which was also observed for the D2-IC3 when incubated with striatal brain homogenates and both forms of purified arrestins. The dopamine D2 receptor required arrestin for its internalization, which was slow compared to the dopamine D1 receptor and was maximal at 20 minutes (Figure 6-1).

**Figure 6-1. Interaction of the dopamine D1 receptor (D1R) and the dopamine D2 receptor (D2R) with arrestin2 (ARR2) and arrestin3 (ARR3) following agonist stimulation.** As described the D1R preferentially interacts with arrestin3 and the D2R interacts with arrestin2 in primary neostriatal neurons. Our data suggests that the D1R interacts with arrestin 3 transiently, which causes rapid dissociation of the D1R and arrestin3. Internalization of the D1R is rapid. Our data suggests the D2R and arrestin2 remain stably associated following agonist treatment and the internalization of the D2R is slow.




adapted from Shenoy and Lefkowitz, 2003

Reference List


Ahn S, Nelson CD, Garrison TR, Miller WE and Lefkowitz RJ (2003) Desensitization, internalization, and signaling functions of β-arrestins demonstrated by RNA interference. *Proc Natl Acad Sci USA* **100**:1740-1744.

Albert PR, Neve KA, Bunzow JR and Civelli O (1990) Coupling of a cloned rat dopamine-$D_2$ receptor to inhibition of adenylate cyclase and prolactin secretion. *J Biol Chem* **265**:2098-2104.

Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG and Lefkowitz RJ (1992) β-arrestin2, a novel member of the arrestin/β-arrestin gene family. *J Biol Chem* **267**:17882-17890.

Barton AC, Black LE and Sibley DR (1991) Agonist-induced desensitization of $D_2$ dopamine receptors in human Y-79 retinoblastomal cells. *Mol Pharmacol* **39**:650-658.

Bennett TA, Foutz TD, Gurevich VV, Sklar LA and Prossnitz ER (2001) Partial phosphorylation of the *N*-formyl peptide receptor inhibits G protein association independent of arrestin binding. *J Biol Chem* **276**:49195-49203.

Bennett TA, Maestas DC and Prossnitz ER (2000) Arrestin binding to the G protein-coupled *N*-formyl peptide receptor is regulated by the conserved "DRY" sequence. *J Biol Chem* **275**:24590-24594.

Benovic JL, Kuhn H, Weyand I, Codina J, Caron MG and Lefkowitz RJ (1987) Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc Natl Acad Sci U S A* **84**:8879-8882.

Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG and Barak LS (2004) Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Mol Pharmacol* **66**:106-112.

Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG and Lin FT (1999) Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* **286**:2495-2498.

Boundy VA, Pacheco MA, Guan W and Molinoff PB (1995) Agonists and antagonists differentially regulate the high affinity state of the $D2_L$ receptor in human embryonic kidney 293 cells. *Mol Pharmacol* **48**:956-964.

Bunzow JR, Van Tol HHM, Grandy DK, Albert P, Salon J, Christie M, Machida CA, Neve KA and Civelli O (1988) Cloning and expression of a rat $D_2$ dopamine receptor cDNA. *Nature* **336**:783-787.

Carlsson A, Lindqvist M, Magnusson T and Waldeck B (1958) On the presence of 3-hydroxytyramine in brain. *Science* **15**:471.

Castro SW and Strange PG (1993a) Coupling of $D_2$ and $D_3$ dopamine receptors to G-proteins. *FEBS Lett* **315**:223-226.

Castro SW and Strange PG (1993b) Differences in the ligand binding properties of the short and long versions of the D2 dopamine receptor. *J Neurochem* **60**:372-375.

Celver J, Vishnivetskiy SA, Chavkin C and Gurevich VV (2002) Conservation of the phosphate-sensitive elements in the arrestin family of proteins. *J Biol Chem* **277**:9043-9048.

Cen B, Xiong Y, Ma L and Pei G (2001) Direct and differential interaction of β-arrestins with the intracellular domains of different opioid receptors. *Mol Pharmacol* **59**:758-764.

Chen J, Rusnak M, Luedtke RR and Sidhu A (2004) D1 dopamine receptor mediates dopamine-induced cytotoxicity via the ERK signal cascade. *J Biol Chem* **279**:39317-39330.

Chio CL, Lajiness ME and Huff RM (1994) Activation of heterologously expressed D3 dopamine receptors: comparison with D2 dopamine receptors. *Mol Pharmacol* **45**:51-60.

Claing A, Perry SJ, Achiriloaie M, Walker JKL, Albanesi JP, Lefkowitz RJ and Premont RT (2000) Multiple endocytic pathways of C protein-coupled receptors delineated by GIT1 sensitivity. *Proc Natl Acad Sci USA* **97**:1119-1124.

Conner DA, Mathier MA, Mortensen RM, Christe M, Vatner SF, Seidman CE and Seidman JG (1997) beta-Arrestin1 knockout mice appear normal but demonstrate altered cardiac responses to beta-adrenergic stimulation. *Circ Res* **81**:1021-1026.

Cooper JR, Bloom F E and Roth B L (2003) in *The Biochemical Basis of Neuropharmacology* pp 225-270, Oxford University Press.

Dearry A, Gingrich JA, Falardeau P, Fremeau RT, Bates MD and Caron MG (1990) Molecular cloning and expression of the gene for a human $D_1$ dopamine receptor. *Nature* **347**:72-75.

Defea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O and Bunnett NW (2000a) The proliferative and antiapoptotic effects of substance P are facilitated by formation of a β-arrestin-dependent scaffolding complex. *Proc Natl Acad Sci USA* **97**:11086-11091.

Defea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD and Bunnett NW (2000b) β-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* **148**:1267-1281.

DeGraff JL, Gurevich VV and Benovic JL (2002) The third intracellular loop of $\alpha_2$-adrenergic receptors determines subtype specificity of arrestin interaction. *J Biol Chem* **277**:43247-43252.

Ehringer H and Hornykiewicz O (1960) Verteilung von Noradrenalin und Dopamin (3-Hydroxytyramin) im Gehirn des Menschen und ihr Verhalten bei Erkrankungen des extrapyramidalen Systems. *Klin Wochenschr* **38**:1236-1239.

Ferguson SSG, Downey WE, III, Colapietro AM, Barak LS, Ménard L and Caron MG (1996) Role of β-arrestin in mediating agonist-promoted G protein- coupled receptor internalization. *Science* **271**:363-366.

Freedman SB, Patel S, Marwood R, Emms F, Seabrook GR, Knowles MR and McAllister G (1994) Expression and pharmacological characterization of the human $D_3$ dopamine receptor. *J Pharmacol Exp Ther* **268**:417-426.

Gardner B, Liu ZF, Jiang D and Sibley DR (2001) The role of phosphorylation/dephosphorylation in agonist-induced desensitization of $D_1$ dopamine receptor function: evidence for a novel pathway for receptor dephosphorylation. *Mol Pharmacol* **59**:310-321.

Gelber EI, Kroeze WK, Willins DL, Gray JA, Sinar CA, Hyde EG, Gurevich V, Benovic J and Roth BL (1999) Structure and function of the third intracellular loop of the 5-hydroxytryptamine$_{2A}$ receptor: The third intracellular loop is α-helical and binds purified arrestins. *J Neurochem* **72**:2206-2214.

Grandy DK, Litt M, Allen L, Bunzow JR, Marchionni M, Makam H, Reed L, Magenis RE and Civelli O (1989) The human dopamine D2 receptor gene is located on chromosome 11 at q22-q23 and identifies a TaqI RFLP. *Am J Hum Genet* **45**:778-785.

Grandy DK, Zhou Q-Y, Allen L, Litt R, Magenis RE, Civelli O and Litt M (1990) A human $D_1$ dopamine receptor gene is located on chromosome 5 at q35.1 and identifies an EcoRI RFLP. *Am J Hum Genet* **47**:828-834.

Gray JA, Bhatnagar A, Gurevich VV and Roth BL (2003) The interaction of a constitutively active arrestin with the arrestin-insensitive 5-HT$_{2A}$ receptor induces agonist-independent internalization. *Mol Pharmacol* **63**:961-972.

Gurevich EV, Benovic JL and Gurevich VV (2002) Arrestin2 and arrestin3 are differentially expressed in the rat brain during postnatal development. *Neuroscience* **109**:421-436.

Gurevich VV and Gurevich EV (2004) The molecular acrobatics of arrestin activation. *TIPS* **25**:105-111.

Han M, Gurevich VV, Vishnivetskiy SA, Sigler PB and Schubert C (2001) Crystal structure of β-arrestin at 1.9 Å: Possible mechanism of receptor binding and membrane translocation. *Struct Fold Des* **9**:869-880.

Hirsch JA, Schubert C, Gurevich VV and Sigler PB (1999) The 2.8 A crystal structure of visual arrestin: a model for arrestin's regulation. *Cell* **97**:257-269.

Hüttenrauch F, Nitzki A, Lin FT, Höning S and Oppermann M (2002) β-arrestin binding to CC chemokine receptor 5 requires multiple C-terminal receptor phosphorylation sites and involves a conserved Asp-Arg-Tyr sequence motif. *J Biol Chem* **277**:30769-30777.

Ito K, Haga T, Lameh J and Sadée W (1999) Sequestration of dopamine D2 receptors depends on coexpression of G-protein-coupled receptor kinases 2 or 5. *Eur J Biochem* **260**:112-119.

Itokawa M, Toru M, Ito K, Tsuga H, Kameyama K, Haga T, Arinami T and Hamaguchi H (1996) Sequestration of the short and long isoforms of dopamine $D_2$ receptors expressed in Chinese hamster ovary cells. *Mol Pharmacol* **49**:560-566.

Jackson A, Iwasiow RM, Chaar ZY, Nantel MF and Tiberi M (2002) Homologous regulation of the heptahelical D1A receptor responsiveness: specific cytoplasmic tail regions mediate dopamine-induced phosphorylation, desensitization and endocytosis. *J Neurochem* **82**:683-697.

Kim KM, Valenzano KJ, Robinson SR, Yao WD, Barak LS and Caron MG (2001) Differential regulation of the dopamine $D_2$ and $D_3$ receptors by G protein-coupled receptor kinases and β-arrestins. *J Biol Chem* **276**:37409-37414.

Kim OJ, Gardner BR, Williams DB, Marinec PS, Cabrera DM, Peters JD, Mak CC, Kim KM and Sibley DR (2004a) The role of phosphorylation in D1 dopamine receptor desensitization: evidence for a novel mechanism of arrestin association. *J Biol Chem* **279**:7999-8010.

Kim SJ, Kim MY, Lee EJ, Ahn YS and Baik JH (2004b) Distinct regulation of internalization and mitogen-activated protein kinase activation by two isoforms of the dopamine D2 receptor. *Mol Endocrinol* **18**:640-652.

Kohout TA, Lin FT, Perry SJ, Conner DA and Lefkowitz RJ (2001) β-arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Natl Acad Sci USA* **98**:1601-1606.

Kovoor A, Celver J, Abdryashitov RI, Chavkin C and Gurevich VV (1999) Targeted construction of phosphorylation-independent beta-arrestin mutants with constitutive activity in cells. *J Biol Chem* **274**:6831-6834.

Kozell LB, Machida CA, Neve RL and Neve KA (1994) Chimeric D1/D2 dopamine receptors: distinct determinants of selective efficacy, potency, and signal transduction. *J Biol Chem* **269**:30299-30306.

Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38**:289-319.

Krupnick JG, Gurevich VV, Schepers T, Hamm HE and Benovic JL (1994) Arrestin-rhodopsin interaction. Multi-site binding delineated by peptide inhibition. *J Biol Chem* **269**:3226-3232.

Kula NS, Baldessarini RJ, Kebabian JW and Neumeyer JL (1994) *S*-(+)-aporphines are not selective for human $D_3$ dopamine receptors. *Cell Mol Neurobiol* **14**:185-192.

Lamey M, Thompson M, Varghese G, Chi H, Sawzdargo M, George SR and O'Dowd BF (2002) Distinct residues in the carboxyl tail mediate agonist-induced desensitization and internalization of the human dopamine $D_1$ receptor. *J Biol Chem* **277**:9415-9421.

Lawson CF, Mortimore RA, Schlachter SK and Smith MW (1994) Pharmacology of a human dopamine $D_4$ receptor expressed in HEK293 cells. *Methods Find Exp Clin Pharmacol* **16**:303-307.

Le Moine C, Normand E and Bloch B (1991) Phenotypical characterization of the rat striatal neurons expressing the $D_1$ dopamine receptor gene. *Proc Natl Acad Sci USA* **88**:4205-4209.

Lester J, Fink S, Aronin N and Difiglia M (1993) Colocalization of $D_1$ and $D_2$ dopamine receptor mRNAs in striatal neurons. *Brain Res* **621**:106-110.

Lin FT, Krueger KM, Kendall HE, Daaka Y, Fredericks ZL, Pitcher JA and Lefkowitz RJ (1997) Clathrin-mediated endocytosis of the β-adrenergic receptor is regulated by phosphorylation/dephosphorylation of β-arrestin1. *J Biol Chem* **272**:31051-31057.

Lohse MJ, Benovic JL, Caron MG and Lefkowitz RJ (1990) Multiple pathways of rapid $\beta_2$-adrenergic receptor desensitization: delineation with specific inhibitors. *J Biol Chem* **265**:3202-3209.

Luttrell LM, Ferguson SSG, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin FT, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ (1999) β-arrestin-dependent formation of $\beta_2$ adrenergic receptor Src protein kinase complexes. *Science* **283**:655-661.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL and Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds. *Proc Natl Acad Sci USA* **98**:2449-2454.

Macey TA, Gurevich VV and Neve KA (2004) Preferential interaction between the dopamine D2 receptor and arrestin2 in neostriatal neurons. *Mol Pharmacol* **66**.

MacKenzie RG, VanLeeuwen D, Pugsley TA, Shih Y-H, Demattos S, Tang L, Todd RD and O'Malley KL (1994) Characterization of the human dopamine $D_3$ receptor expressed in transfected cell lines. *Eur J Pharmacol Mol Pharmacol* **266**:79-85.

Mansour A, Meador-Woodruff JH, Zhou QY, Civelli O, Akil H and Watson SJ (1991) A comparison of D1 receptor binding and mRNA in rat brain using receptor autoradiographic and in situ hybridization techniques. *Neuroscience* **45**:359-371.

Martin-Negrier ML, Charron G and Bloch B (2000) Agonist stimulation provokes dendritic and axonal dopamine $D_1$ receptor redistribution in primary cultures of striatal neurons. *Neuroscience* **99**:257-266.

Mason JN, Kozell LB and Neve KA (2002) Regulation of dopamine $D_1$ receptor trafficking by protein kinase A-dependent phosphorylation. *Mol Pharmacol* **61**:806-816.

McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ and Lefkowitz RJ (2000) β-Arrestin 2: A receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**:1574-1577.

Monsma FJ, Mahan LC, McVittie LD, Gerfen CR and Sibley DR (1990) Molecular cloning and expression of a $D_1$ dopamine receptor linked to adenylyl cyclase activation. *Proc Natl Acad Sci USA* **87**:6723-6727.

Monsma FJ, McVittie LD, Gerfen CR, Mahan LC and Sibley DR (1989) Multiple $D_2$ dopamine receptors produced by alternative RNA splicing. *Nature* **342**:926-929.

Montmayeur JP, Bausero P, Amlaiky N, Maroteaux L, Hen R and Borrelli E (1991) Differential expression of the mouse $D_2$ dopamine receptor isoforms. *FEBS Lett* **278**:239-243.

Mukherjee S, Gurevich VV, Preninger A, Hamm HE, Bader MF, Fazleabas AT, Birnbaumer L and Hunzicker-Dunn M (2002) Aspartic acid 564 in the third cytoplasmic loop of the luteinizing hormone/choriogonadotropin receptor is crucial for phosphorylation-independent interaction with arrestin2. *J Biol Chem* **277**:17916-17927.

Mukherjee S, Palczewski K, Gurevich VV and Hunzicker-Dunn M (1999) beta-arrestin-dependent desensitization of luteinizing hormone/choriogonadotropin receptor is prevented by a synthetic peptide corresponding to the third intracellular loop of the receptor. *J Biol Chem* **274**:12984-12989.

Mundell SJ and Benovic JL (2000) Selective regulation of endogenous G protein-coupled receptors by arrestins in HEK293 cells. *J Biol Chem* **275**:12900-12908.

Nakamura K, Liu XB and Ascoli M (2000) Seven non-contiguous intracellular residues of the lutropin/choriogonadotropin receptor dictate the rate of agonist-induced internalization and its sensitivity to non-visual arrestins. *J Biol Chem* **275**:241-247.

Neve KA, Cox BA, Henningsen RA, Spanoyannis A and Neve RL (1991) Pivotal role for aspartate-80 in the regulation of D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol Pharmacol* **39**:733-739.

Neve KA, Henningsen RA, Bunzow JR and Civelli O (1989) Functional characterization of a rat dopamine D-2 receptor cDNA expressed in a mammalian cell line. *Mol Pharmacol* **36**:446-451.

Neve KA and Neve R L (1997) Molecular biology of dopamine receptors, in *The Dopamine Receptors* (Neve KA and Neve RL eds) pp 27-76, Humana Press, Totawa, NJ.

Ng GY, Mouillac B, George SR, Caron M, Dennis M, Bouvier M and O'Dowd BF (1994) Desensitization, phosphorylation and palmitoylation of the human dopamine $D_1$ receptor. *Eur J Pharmacol Mol Pharmacol* **267**:7-19.

Ng GY, Varghese G, Chung HT, Trogadis J, Seeman P, O'Dowd BF and George SR (1997) Resistance of the dopamine $D2_L$ receptor to desensitization accompanies the up-regulation of receptors on to the surface of Sf9 cells. *Endo* **138**:4199-4206.

Oakley RH, Laporte SK, Holt JA, Caron MG and Barak LS (2000) Differential affinities of visual arrestin, βarrestin1, and βarrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem* **275**:17201-17210.

Pippig S, Andexinger S and Lohse MJ (1995) Sequestration and recycling of $\beta_2$-adrenergic receptors permit receptor resensitization. *Mol Pharmacol* **47**:666-676.

Raman D, Osawa S and Weiss ER (1999) Binding of arrestin to cytoplasmic loop mutants of bovine rhodopsin. *Biochemistry* **38**:5117-5123.

Rappaport MS, Sealfon SC, Prikhozhan A, Huntley GW and Morrison JH (1993) Heterogeneous distribution of D1, D2 and D5 receptor mRNAs in monkey striatum. *Brain Res* **616**:242-250.

Robinson SW and Caron MG (1996) Chimeric $D_2/D_3$ dopamine receptors efficiently inhibit adenylyl cyclase in HEK 293 cells. *J Neurochem* **67**:212-219.

Shenoy SK and Lefkowitz RJ (2003) Trafficking patterns of β-arrestin and G protein-coupled receptors determined by the kinetics of β-arrestin deubiquitination. *J Biol Chem* **278**:14498-14506.

Shiina T, Kawasaki A, Nagao T and Kurose H (2000) Interaction with β-arrestin determines the difference in internalization behavior between $\beta_1$- and $\beta_2$-adrenergic receptors. *J Biol Chem* **275**:29082-29090.

Sibley DR and Neve K A (1997) Regulation of dopamine receptor function and expression, in *The Dopamine Receptors* (Neve KA and Neve RL eds) pp 383-424, Humana Press, Totowa, NJ.

Smith AD and Justice JB (1994) The effect of inhibition of synthesis, release, metabolism and uptake on the microdialysis extraction fraction of dopamine. *J Neurosci Methods* **54**:75-82.

Smith FD, Oxford GS and Milgram SL (1999) Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *J Biol Chem* **274**:19894-19900.

Sokoloff P, Andrieux M, Besançon R, Pilon C, Martres M-P, Giros B and Schwartz J-C (1992) Pharmacology of human dopamine $D_3$ receptor expressed in a mammalian cell line: comparison with $D_2$ receptor. *Eur J Pharmacol-Molec Pharm* **225**:331-337.

Sokoloff P, Giros B, Martres M-P, Bouthenet M-L and Schwartz J-C (1990) Molecular cloning and characterization of a novel dopamine receptor ($D_3$) as a target for neuroleptics. *Nature* **347**:146-151.

Sterne-Marr R, Gurevich VV, Goldsmith P, Bodine RC, Sanders C, Donoso LA and Benovic JL (1993) Polypeptide variants of beta-arrestin and arrestin3. *J Biol Chem* **268**:15640-15648.

Sunahara RK, Niznik HB, Weiner DM, Stormann TM, Brann MR, Kennedy JL, Gelernter JE, Rozmahel R, Yang Y, Israel Y, Seeman P and O'Dowd BF (1990) Human dopamine $D_1$ receptor encoded by an intronless gene on chromosome 5. *Nature* **347**:80-83.

Surmeier DJ, Eberwine J, Wilson CJ, Cao Y, Stefani A and Kitai ST (1992) Dopamine receptor subtypes colocalize in rat striatonigral neurons. *Proc Natl Acad Sci USA* **89**:10178-10182.

Takeuchi Y and Fukunaga K (2004) Dopamine D2 receptor activates extracellular signal-regulated kinase through the specific region in the third cytoplasmic loop. *J Neurochem* **89**:1498-1507.

Terrillon S, Barberis C and Bouvier M (2004) Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with beta-arrestin and their trafficking patterns. *Proc Natl Acad Sci U S A* **101**:1548-1553.

Tiberi M, Nash SR, Bertrand L, Lefkowitz RJ and Caron MG (1996) Differential regulation of dopamine D1A receptor responsiveness by various G protein-coupled receptor kinases. *J Biol Chem* **271**:3771-3778.

Tsao P, Cao T and von Zastrow M (2001) Role of endocytosis in mediating downregulation of G-protein-coupled receptors. *Trends Pharmacol Sci* **22**:91-96.

Tulipano G, Stumm R, Pfeiffer M, Kreienkamp HJ, Hollt V and Schulz S (2004) Differential beta -arrestin trafficking and endosomal sorting of somatostatin receptor subtypes. *J Biol Chem* 279:21374-21382.

Van Tol HHM, Bunzow JR, Guan H-C, Sunahara RK, Seeman P, Niznik HB and Civelli O (1991) Cloning of the gene for a human dopamine $D_4$ receptor with high affinity for the antipsychotic clozapine. *Nature* **350**:610-614.

Vickery RG and von Zastrow M (1999) Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. *J Cell Biol* **144**:31-43.

Vishnivetskiy SA, Hosey MM, Benovic JL and Gurevich VV (2004) Mapping the arrestin-receptor interface. Structural elements responsible for receptor specificity of arrestin proteins. *J Biol Chem* **279**:1262-1268.

Vögler O, Bogatkewitsch GS, Wriske C, Krummenerl P, Jakobs KH and Van Koppen CJ (1998) Receptor subtype-specific regulation of muscarinic acetylcholine receptor sequestration by dynamin: distinct sequestration of m2 receptors. *J Biol Chem* **273**:12155-12160.

Watts VJ, Lawler CP, Knoerzer T, Mayleben MA, Neve KA, Nichols DE and Mailman RB (1993) Hexahydrobenzo[a]phenanthridines: Novel dopamine $D_3$ receptor ligands. *Eur J Pharmacol* **239**:271-273.

Wilden U, Hall SW and Kuhn H (1986) Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc Natl Acad Sci U S A* **83**:1174-1178.

Wu GY, Krupnick JG, Benovic JL and Lanier SM (1997) Interaction of arrestins with intracellular domains of muscarinic and $\alpha_2$-adrenergic receptors. *J Biol Chem* **272**:17836-17842.

Zhang J, Barak LS, Anborgh PH, Laporte SA, Caron MG and Ferguson SSG (1999) Cellular trafficking of G protein-coupled receptor/β-arrestin endocytic complexes. *J Biol Chem* **274**:10999-11006.

Zhang J, Barak LS, Winkler KE, Caron MG and Ferguson SS (1997) A central role for β-arrestins and clathrin-coated vesicle-mediated endocytosis in $\beta_2$-adrenergic receptor resensitization - Differential regulation of receptor resensitization in two distinct cell types. *J Biol Chem* **272**:27005-27014.

Zhang L-J, Lachowicz JE and Sibley DR (1994) The $D_{2S}$ and $D_{2L}$ dopamine receptor isoforms are differentially regulated in Chinese hamster ovary cells. *Mol Pharmacol* **45**:878-889.

Zhou Q-Y, Grandy DK, Thambi L, Kushner JA, Van Tol HHM, Cone R, Pribnow D, Salon J, Bunzow JR and Civelli O (1990) Cloning and expression of human and rat $D_1$ dopamine receptors. *Nature* **347**:76-80.

Zhuang X, Belluscio L and Hen R (2000) $G_{olf\alpha}$ mediates dopamine $D_1$ receptor signaling. *J Neurosci* **20**:RC91(1-5).