The Dopamine D1 Receptor: Regulation of Signaling and Trafficking

by

John Nicholas Mason

A DISSERTATION

Presented to the Department of Physiology and Pharmacology

and Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements of the degree of

Doctor of Philosophy

February 2002

School of Medicine
Oregon Health & Science University

CERTIFICATION OF APPROVAL

This is to certify that the Ph.D. thesis of

John Nicholas Mason

has been approved

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Member

Associate Dean for Graduate Studies

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

cAMP	cyclic AMP;adenosine 3',5'-cyclic monophosphate
Chloro-APB	6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3 benzazepine; SKF 82958
EC50	concentration that produces 50% of maximal effect
EEA1	early endosomal antigen one
EE	early endosome
EGFP	enhanced green fluorescent protein
FYVE	first letters of (Fablp, YOTB, Vaclp, and EEA1)
GPCR	G protein-coupled receptor
H-89	{N-[2-((p-bromocinnamyl)amino)ethyl] -5-isoquinolinesulfonamide,HCl}
K_D	equilibrium dissociation constant
K_H	dissociation constant for high affinity state
K_i	apparent equilibrium
mTF	mouse transferring
7-OH DPAT	7-hydroxy-2-(N,N-di-n-propylamino)tetraline
PAGE	Polyacrylamide gel electrophoresis
PKA	cyclic AMP-dependent protein kinase
PVDF	polyvinylidene difluoride
Rab11	GTPase rab11
SE	sorting endosome
SCH 23390	7-chloro-8-dihydroxy-1-phenyl-2,3,4,5,-tetrahydro-1Hbenzazepine

ACKNOWLEDGMENTS

I wish to thank my mentor Kim Neve who made this dissertation possible and for his insight and advice that will prove invaluable in the years to come. I would also like to thank Aaron Janowsky and Amy Eshleman for creating an atmosphere within the lab that encouraged the exchange of opinions and ideas.

ABSTRACT

The goal of my dissertation research was to determine the effect that potential cyclic AMP-dependent protein kinase (PKA) phosphorylation sites of the dopamine D1 receptor have on agonist-stimulated phosphorylation, desensitization and internalization of the receptor.

To facilitate purification and imaging of the D1 receptor, I attached a polyhistidine tag to the amino terminus and the enhanced green fluorescent protein (EGFP) to the carboxy terminus (D1-EGFP). I found the properties of the D1-EGFP receptor to be similar to the non-tagged D1 receptor in terms of affinity for agonists and antagonists ligands, coupling to G proteins, cyclic AMP-dependent responsiveness, and cellular redistribution.

When I substituted either the potential PKA phosphorylation site Thr268 or Ser380 with Ala, I measured no effect on desensitization of the D1-EGFP receptor when stably expressed in NS20Y neuroblastoma cells. When I pretreated the wildtype D1-EGFP with the PKA inhibitor H89 there was also no effect on desensitization.

Stimulation by dopamine caused an increase in the incorporation of phosphate by wildtype and the S380A mutant D1-EGFP receptor. However, the T268A mutant showed no increase in phosphorylation. Pretreatment of the wildtype D1-EGFP expressing cells with PKA inhibitor H89 prevented an increase in agonist-induced phosphorylation to an extent similar to that caused by mutation of Thr268.

Using real-time fluorescent microscopy I demonstrated that mutation of Thr268 attenuates the rate of dopamine-induced fluorescence accumulation of the D1-EGFP

receptor in the perinuclear region of NS20Y neuroblastoma cells. I confirmed that this attenuated increase in accumulated fluorescence was due to a difference in the intracellular sorting of the T268A mutant receptor and not from an inability of the T268A mutant to be internalized. I demonstrated that this effect could be replicated in the wildtype by pretreatment with H89.

Based on these results I then hypothesized that this decrease in fluorescent accumulation in the perinuclear region was due to the absence of Thr268 phosphorylation that could affect the sorting of the D1-EGFP between recycling compartments of the long recycling pathway. To test this hypothesis I used fluorescent microscopy to assess for differences between the wildtype and T268A mutant receptors in their colocalization with endosomal markers EEA1, mTF, and Rab11 of the long recycling pathway.

Colocalization results indicated that T268A promotes constitutive accumulation of D1-EGFP in mTF and Rab11 compartments and expedites the movement of the D1-EGFP through the long recycling pathway in response to dopamine. My results also demonstrated that in response to agonist, T268A causes the formation of enlarged early endosomes colocalized with D1-EGFP immediately adjacent to the plasma membrane.

In this dissertation I conclude that the phosphorylation of Thr268 of the dopamine D1-EGFP receptor by PKA regulates the sorting of the receptor to compartments of the long recycling pathway that does not involve desensitization of the receptor.

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, two chapters of original data, and a discussion and conclusions chapter. Each data chapter includes an abstract, introduction, materials and methods, results, and discussion and conclusion section. All data and results reported in this dissertation are my own work with the exception of figure 2-6. References are listed separately in the order in which they first appear, and follow the format of *Neuroscience.*

Papers representative of this work

Mason J.N. and Neve, K.A., Trafficking of a Thr268-Ala dopamine D1 receptor mutant. *Mol Pharm: (to be submitted).*

Mason J.N., Laura B. Kozell, and Neve, K.A., Regulation of dopamine D1 receptor trafficking by protein kinase A-dependent phosphorylation. *Mol Pharm: (in press, 2002).*

Abstracts

Neve, K.A., Macey, T.A., Mason, J.N. Agonist induced trafficking of EGFP-tagged dopamine receptors. *Abstract No. 30: The 34th Annual Winter Conference on Brain Research 2001.*

Mason, J.N., Hauge, X., and Neve K.A. Agonist-induced trafficking of DA D1-EGFP receptors lacking putative protein kinase A phsphorylation sites. *Abstract 532.13, Part II: Society for Neuroscience 2000.*

Mason, J.N., and Neve, K.N. Agonist-Regulated phoshorylation and trafficking of the dopamine D1 receptor. *Abstract 389.12, Vol. 13, 1: Exper. Biology 1999.*

Mason, J.N., Weins, B.W., Neve, K.A. Agonist-regulated phoshorylation and trafficking of the dopamine D1 receptor. *Abstract: 584.2, Part II: Society for Neuroscience 1999.*

I. INTRODUCTION

Dopamine is an important neurotransmitter that affects many central nervous system (CNS) functions. It has been implicated in psychiatric and neurological disorders such as Parkinson's disease, schizophrenia, and Huntington's disease. Dopamine acts by binding to and activating integral membrane proteins, dopamine receptors, which then transduce the signal across the cell membrane. These signals lead to downstream effects that include phosphorylation of the receptor. An understanding of how agonist-induced phosphorylation affects the function of the dopamine receptor is important for understanding the role of dopamine in the CNS. The goal of my dissertation was to examine how agonist-induced phosphorylation alters dopamine receptor function. Specifically, I wanted to elucidate the effect of cyclic AMP-dependent protein kinase phosphorylation of the dopamine D1 receptor on adenylate cyclase responsiveness, phosphorylation, and trafficking. Therefore, potential cAMP- dependent phosphorylation sites of the dopamine D1 receptor were removed and their effect on desensitization and trafficking assessed.

This introductory section contains background information on dopamine as a neurotransmitter, but its primary purpose is to characterize and distinguish the D1 dopamine receptor from other dopamine receptors, and then to discuss the evidence for a role for agonist-induced phosphorylation in regulation of D1 receptor responsiveness and trafficking. Finally, I will also discuss the use of different fluorescent probes as tools for tracking subcellular redistribution of the dopamine D1 receptor.

DOPAMINE AS A NEUROTRANSMITTER

Historical

Dopamine (3,4-dihydrophenylethylamine) is the major catecholamine in the CNS. Dopamine neurotransmission is involved in numerous physiological processes, including motor control, cardiovascular homeostasis, endocrine function, and cognition. Disease states that have been linked to dysfunction of dopamine neurotransmission include Huntington's chorea, Parkinson's disease, schizophrenia and affective disorders, and endocrine disturbances. Not until the mid 1950s was dopamine hypothesized to be a neurotransmitter [20]. Before this time dopamine was considered to be exclusively an intermediate in the biosynthesis of the catecholamines norepinephrine and epinephrine. Norepinephrine is concentrated in the hypothalamus, with small quantities found throughout other brain regions, whereas dopamine is concentrated in nuclei within the basal ganglia. This marked difference in regional distribution of dopamine and norepinephrine led investigators to propose a biological role for dopamine independent of its function as a precursor for norepinephrine biosynthesis [20].

The first behavioral evidence for dopamine as a neurotransmitter came from the akinetic reversal effect of L-DOPA administration on rabbits depleted of catecholamine stores after treatment by reserpine. Further analysis revealed that the behavioral action of L-DOPA closely correlated with the accumulation of dopamine in the brain [20]. This and subsequent studies demonstrating that the majority of dopamine is confined to the basal ganglia of the brain, led to the hypothesis that dopamine might be involved in motor control and that decreased striatal dopamine could be the cause of extrapyramidal symptoms of Parkinson's disease. Subsequent studies confirmed that dopamine is

released and taken back up by nerve terminals, and its occurrence in neural pathways was demonstrated using histochemical fluorescence methods [34,50].

Dopamine pathways in the CNS

The mapping studies of Dahlstrom and Fuxe in the mid 1960s, based on fluorescent staining, showed that dopaminergic neurons are organized in three main systems [28]. Accounting for about 75% of the dopamine in the brain is the nigrostriatal pathway (Fig. 1-1). Cell bodies of this pathway lie in the substantia nigra, and project to the neostriatum (caudate and putamen in human brain). The second important system is comprised of the mesolimbic and mesocortical pathways which project from the ventral tegmental area (VTA) to the olfactory tubercle, nucleus accumbens, amygdala, and regions of the frontal cortex (prefrontal, cingulate, and entorhinal cortex).

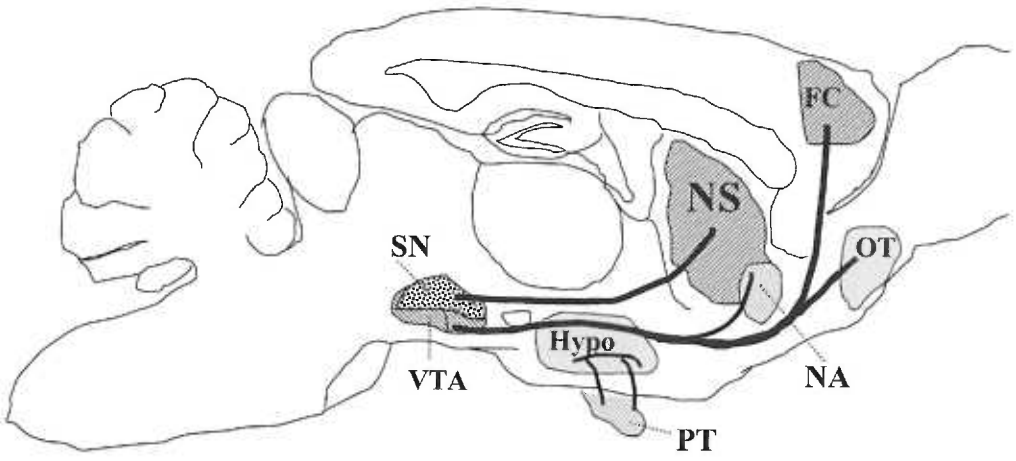

Figure 1-1 Major dopamine pathways in the rat.
Major dopaminergic neurons project from the substantia nigra (SN) to the neostriatum (NS) and from the ventral tegmental area (VTA) to the nucleus accumbens (NA), olfactory tubercule (OT), and frontal cortex (FC).

The tuberoinfundibular pathway is a group of short projections running from the arcuate nucleus of the hypothalamus to the median eminence and pituitary gland, regulating release of pituitary hormones. Finally, other dopaminergic pathways include the incertohypothalamic dopamine pathway, which links the dorsal and posterior hypothalamus with the dorsal anterior hypothalamus and lateral septal nuclei, and the medullary periventricular dopamine-containing cells, which are located near the vagus nerve, the nucleus tractus solitarius, and the cells scattered in the tegmental radiation of the periaqueductal grey matter. There are also dopaminergic interneurons in the olfactory bulb and in the retina.

Dopamine biosynthesis

Dopamine synthesis begins with the amino acid precursor L-tyrosine, which must be transported across the blood brain barrier into the dopamine neuron. Once in the neuron, L-tyrosine is converted to L-dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (Fig 1-2). This is the rate limiting step, as DOPA is very quickly converted to dopamine by L-aromatic amino acid decarboxylase (DOPA decarboxylase).

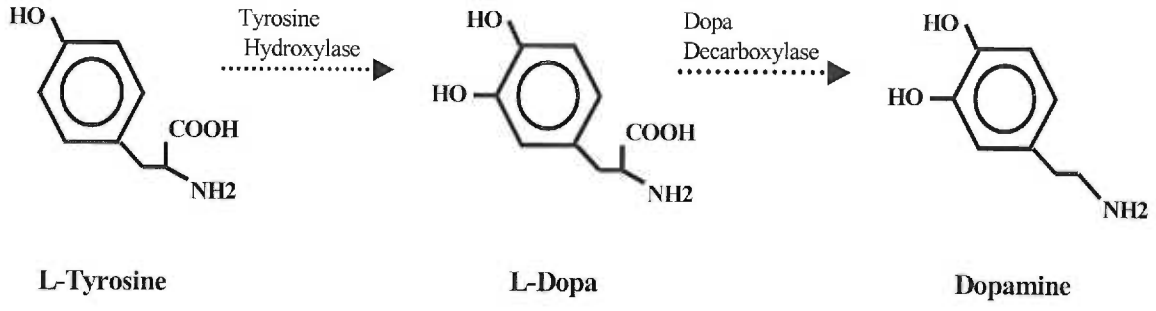

Figure 1-2 Dopamine (3, 4-dihydroxyphenylethylamine) is synthesized from L-tyrosine.

Endogenous mechanisms for regulating the rate of dopamine synthesis in dopaminergic neurons primarily involve modulation of tyrosine hydroxylase activity. Dopamine acts as an end product inhibitor of tyrosine hydroxylase (TH) by competing with a tetrahydrobiopterin (BH4) cofactor for a binding site on the enzyme [2]. Phosphorylation of TH results in its conversion to an activated form with greater affinity for BH4 cofactor and reduced affinity for dopamine [68]. Presynaptic dopamine autoreceptors activated by dopamine released from the nerve terminal modulate the rate of tyrosine hydroxylation by inhibition of TH phosphorylation via reduction of adenylate cyclase activity. The synthesis of dopamine also depends on the rate of impulse flow in the nigrostriatal pathway. High impulse flow increases the rate of tyrosine hydroxylation through kinetic activation of TH that increases its affinity for BH4 and decreases its affinity for the end product inhibitor dopamine [2].

The release of dopamine from the nerve terminal is calcium dependent. Invasion of the terminal by an action potential results in the release of calcium from intracellular stores, the subsequent fusion of dopamine-containing vesicles to the plasma membrane, and the release of dopamine into the synaptic cleft. After its release into the synaptic cleft, dopamine can interact with presynaptic (autoreceptors) and postsynaptic dopamine receptors (Fig. 1-3). Postsynaptic dopamine receptors may be on the axon terminals or dendrites of nondopaminergic cells, whereas autoreceptors may be located on the cell bodies or the axon terminals of dopamine-containing neurons. Dopamine is largely recaptured from the synaptic cleft by a high affinity neurotransmitter transporter, through which reuptake depends upon Na^+ and Cl^- gradients and ATP. A number of drugs inhibit high affinity uptake, including cocaine, nomifensine, and benztropine.

After reuptake by the nerve terminal, dopamine is converted to dihydroyphenylacetic acid (DOPAC) by monoamine oxidase (MAO) or packaged into vesicles for re-release. Dopamine not taken back into the nerve terminal is converted to homovanillic acid (HVA) by catechol-O-methyltransferase (COMT) and MAO [39,61].

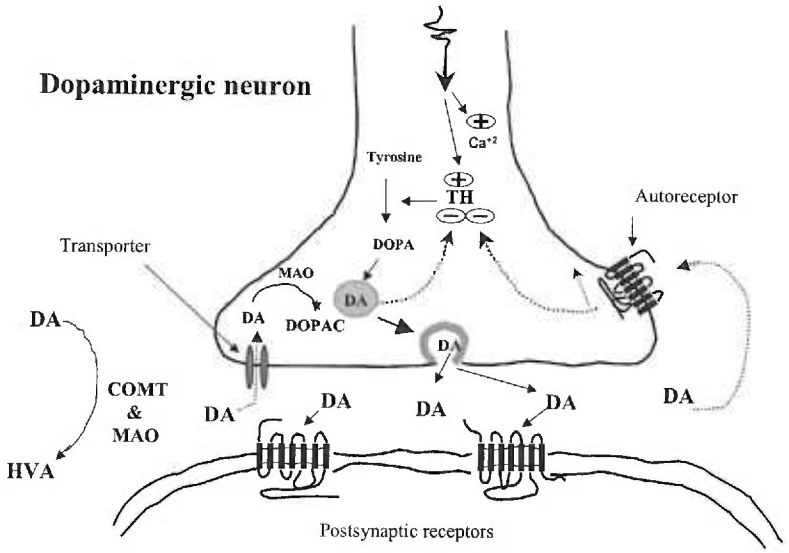

Figure 1-3 Model of a dopaminergic nerve terminal.
Diagram illustrates the life cycle of dopamine and the mechanisms that modulate dopamine synthesis, release, and storage. Invasion of the terminal by a nerve impulse results in the Ca2+-dependent release of dopamine. This release process is attenuated by release-modulating autoreceptors. The conversion of tyrosine to L-DOPA by tyrosine hydroxylase is the rate limiting step in dopamine biosynthesis. Newly synthesized dopamine may be sequestered into storage vesicles for subsequent release. The plasma membrane dopamine transporter recycles dopamine released into the synaptic cleft. The vesicular amine transporter (VAT) transports cytoplasmic dopamine into storage vesicles, decreasing cytoplasmic concentrations of dopamine and preventing metabolism by monoamine oxidase (MAO)

Dopamine receptors

The first neurochemical evidence for the existence of dopamine receptors came in 1972, when it was demonstrated that dopamine stimulates adenylate cyclase activity [17,56]. Pharmacological and biochemical studies by Cools and Van Rossum et al. [26] provided evidence for the existence of multiple binding sites for dopamine. Spano and Govoni et al. [104] proposed the existence of two populations of dopamine receptors, one positively

coupled to adenylate cyclase and the other independent of this effector. In 1979 Kebabian and Calne [55] suggested the classification system of D1 and D2 receptors (Table 1-1).

Table 1-1 Signaling pathways of D1 and D2 like receptors

D1-like	D2-like
Adenylate cyclase activation	Inhibition of adenylate cyclase
Phosphoinositide (PI) hydrolysis	Inhibition of Ca++ and enhancement of K+ conductances
	Modulation of PI turnover

In 1988, a cDNA encoding a 415 amino acid protein was isolated from a rat brain cDNA library [19]. Hydrophobicity plots suggested that the protein had seven membrane spanning domains, a characteristic of G protein-coupled receptors. Transfection of the cDNA into mammalian cell lines resulted in the expression of a receptor with D2-like pharmacology that mediated inhibition of adenylate cyclase in response to dopamine.

In 1990 several groups nearly simultaneously cloned full length cDNAs and genes of the dopamine D1 receptor [31,41,80,121]. The identity of the D1 receptor cDNAs/genes was confirmed on the basis that transfection of any of the cloned cDNAs or genes into mammalian cells produced a receptor that was pharmacologically indistinguishable from the D1 class of receptors proposed by Kababian and Calne [55].

Gene cloning studies in the early 1990s split the D2 receptor type into multiple subgroups. The second D2-like receptor to be cloned, D2L, is generated by alternative splicing of the same gene that encodes the D2S receptor, the first D2 receptor to be

cloned [42]. The D2L and D2S receptor sequences are identical with the exception of a 29 amino acid insert in the third cytoplasmic loop of D2L. Based on pharmacological profiles, sequence homology with the D2 receptor (53% and 41%, respectively), and gene structure, two other dopamine receptors (D3 and D4) fit into the D2-subtype class [103,110].

At approximately the same time as the initial cloning of the D4 receptor, there was the cloning of a novel D1-like receptor named D5 or D1B [43,52]. The D5 receptor gene product was identified as a D1-like receptor based on its sequence and pharmacological profile. The sequence of the D5 receptor is more closely related to that of the D1 receptor than to other dopamine or catecholamine receptors.

MOLECULAR BIOLOGY OF DOPAMINE RECEPTORS

Gene and protein structure

All the mammalian D1 receptors are 446-amino acid proteins, with a predicted molecular weight of ~49,000 [121]. The mammalian D1 receptors have two potential sites for N-linked glycosylation: Asn-5 at the amino terminus, and Asn175 in the third extracellular domain (Fig 1-4). Potential sites for phoshorylation by cyclic AMP-dependent kinase (PKA) are Thr136 in the second cytoplasmic loop, within the sequence R-K-X-T, Thr268, at the C-terminal end of the third cytoplasmic loop, within the less commonly phosphorylated sequence R-X-T, and Ser380 (R-X-S). The human dopamine D1 receptor gene resides on the long arm of chromosome 5, at q35.1 [44]. The human D5 receptor is a 477-amino acid protein with ~60% amino acid identity with the D1 receptor, increasing to ~80% in the transmembrane regions. D1-like receptors have multiple serine and threonine residues in the long cytoplasmic tail in close proximity to acidic residues,

making them potential sites for phosphorylation by the G-protein coupled receptor kinase family. Finally, D1 receptors do not have introns within their coding regions [106].

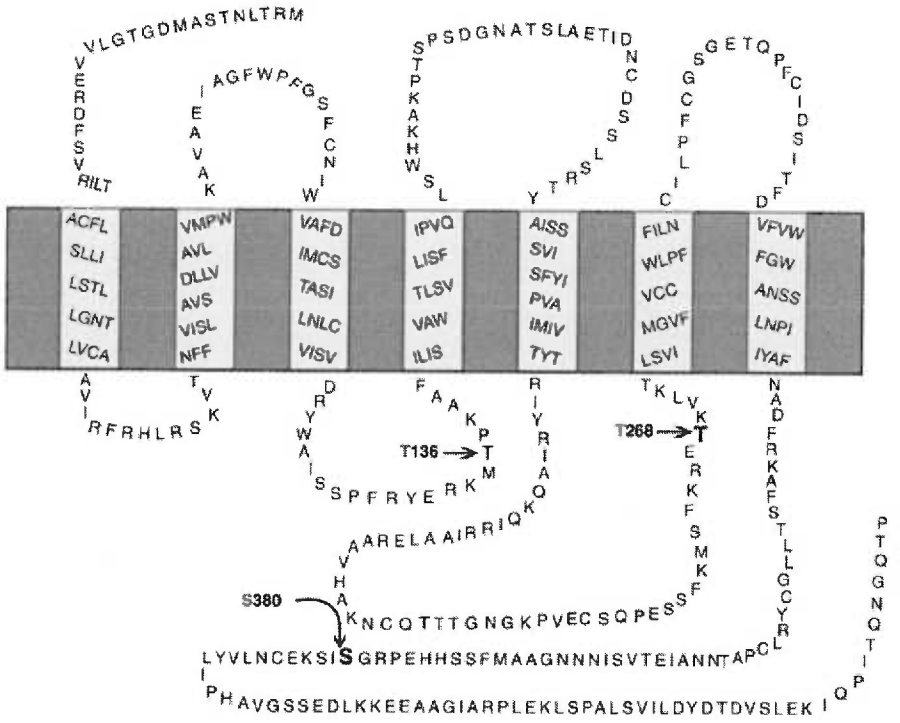

Figure 1-4 Map showing predicted structure of dopamine D1 receptor.
Residues that are potential phosphorylation sites for PKA are Thr136, Thr268, and Ser380, indicated by **bold** lettering.

All D2-like receptors contain several introns within their coding region, allowing for alternative splicing of a single gene, and resulting in multiple gene products such as D2L and D2S [19,29]. The human D2S and D2L receptors are 414 and 443 amino acids long, respectively. The human D3 receptor is 400 amino acid residues long. Many allelic variants of the human D4 receptor gene have been identified that differ in the number and order of copies of a direct, imperfect 48 base pair repeat in the third cytoplasmic loop. This 48 base pair repeat gives rise to human D4 receptors that vary in amino acid sequence and length. The shortest human D4 receptor variant, D4.2, has 387 amino acids, whereas the most common human D4 variant, D4.4, has 419 amino acids.

The D4 has relatively low homology of ~40% to the other receptors in its subclass, the D2 and D3 receptors.

Table 1-2 Distinguishing molecular characteristics of dopamine receptors

	D1-like		D2-like		
	D1	*D5*	*D2*	*D3*	*D4*
Chromosomal localization	5q 35.1	4p 15.1-16.1	11q 22-23	3q 13.3	11p 15.5
Introns in coding region	No	No	Yes	Yes	Yes
Amino acids (human)	446	477	414 (S) 443 (L)	400	387
mRNA size	3.8 kb	3.0 kb	2.5 kb	8.3 kb	5.3 kb

Pharmacology

The dopamine D1-like receptors are distinguished from the D2-like receptors on the basis of drug potency and their ability to activate adenylate cyclase. Selective D1-like (D1 and D5) receptor agonists are fenoldepam and SKF 38393. At present, no ligands have been developed that can distinguish between D1 and D5 receptors. The major pharmacological characteristic that distinguishes D1 and D5 receptors is the increased affinity of the D5 receptor for dopamine, SKF 38393, and other agonists.

Selective ligands have been developed that can differentiate D2-like receptors. The agonists quinpirole and 7-OH DPAT have a higher affinity for D3 receptors than for other D2-like subtypes. Bromocriptine has a higher affinity for D2 than for D3 and D4 (see Table 1-3). For antagonists, raclopride distinguishes D4 from D2 and D3 subtypes because is has lower affinity for D4, and clozapine has a slightly higher affinity for D4 than for D2 and D3.

Table 1-3 Receptor pharmacology

	D1-like		D2-like		
	D1	*D5*	*$D2_{S\&L}$*	*D3*	*D4*
Agonists	SKF-38393 Dihydrexidine ABT-431	SKF-38393	Bromocriptine Quinpirole	7-OH-DPAT Quinpirole	?
Antagonists	SCH-23390	SCH-23390	Spiperone Sulpiride	(+)UH232 PNU-99194A	Clozapine

AGONIST-INDUCED RESPONSES

Signaling

The D2-like dopamine receptors couple to the G-proteins Gi or Go to inhibit the activity of adenylate cyclase. Although both D2 receptor splice variants D2S and D2L couple to G proteins, there appear to be differences in the G-protein that each receptor couples to [73]. Another difference between the splice variants is the enhanced potency of agonists and greater inhibition of adenylate cyclase activity by the D2S splice variant in some cell lines [13,29].

The D3 receptor inhibits adenylate cyclase activity, although this inhibition is very weak compared to inhibition via the D2 receptor [23,29]. The D2-like dopamine receptors have also been shown to modulate arachidonic acid release, Na^+/H^+ exchange, K^+ currents, and Ca^{++} currents [8,33,69]. The D4 receptor can couple to most of the same pathways as the D2 receptor, although like the D2 and D3 receptors, this appears to be somewhat dependent on the cell type in which it is expressed.

Desensitization

Persistent agonist occupancy of various G-protein coupled receptors can lead to a reversible loss of responsiveness [10], called desensitization. Receptor phosphorylation, internalization, and down regulation can contribute to desensitization [36]. The contribution of each of these events to desensitization is believed to be receptor and cell type dependent [36].

Dopamine D1 receptor desensitization has been demonstrated in a variety of cells that express the dopamine D1 receptor endogenously, such as NS20Y neuroblastoma, D384 astrocytoma-derived, and opossum kidney cells [5,10,12,85]. For the dopamine D2 receptor Barton et al. [9] demonstrated that agonist treatment of retinoblastoma Y-79 cells endogenously expressing the D2 receptor, decreased the ability of the D2 receptor to mediate inhibition of adenylate cyclase activity. Nesby et al. [83] demonstrated that in response to morphine administration, D2 receptors in rat striatum show a profound decrease in the apparent affinity for the agonist quinpirole.

Phosphorylation

Agonist–stimulated phosphorylation of G-protein coupled receptors (GPCRs) is required for desensitization and internalization of numerous GPCRs (β-adrenergic receptor, μ-opioid, histamine H2, etc.) [88]. However, agonist-induced phosphorylation has a minimal effect on desensitization and/or internalization for GPCRs such as vasopressin V2, parathyroid hormone, angiotensin I, and M3 muscarinic [18,72]. Agonist induced phosphorylation of D1 and D2-like dopamine receptors occurs in a variety of cell lines. In Sf9 cells that over-express the dopamine D2L receptor, agonist stimulation increased phosphate incorporation by 2 fold [86]. For the D1 dopamine receptor, Ng et al. [85,87]

demonstrated that Sf9 cells over-expressing the dopamine D1 receptor undergo phosphorylation following a 15 minute exposure to 10 μM dopamine, and that the incorporation of phosphate was increased 1.7 fold over basal. After whole cell ^{32}P labeling and subsequent treatment with 10 μM dopamine for 15 minutes, a 50% increase in dopamine D1 receptor phosphorylation in HEK293 cells overexpressing the D1 receptor was demonstrated by Tiberi et al. [107]. Phosphorylation of the dopamine D1 receptor was increased 2 to 3 fold over basal after a 10 minute exposure to dopamine when over-expressed in HEK 293 cells that were metabolically labeled with ^{32}P [40]. For many GPCRs, phosphorylation plays an important role in regulating arrestin binding, adenylate cyclase responsiveness, and sequestration. However, there also are a number of GPCRs for which agonist-induced receptor phosphorylation has little or no effect on sequestration or desensitization. For example, mutations that abolished or greatly reduced agonist-induced incorporation of phosphate by parathyroid hormone, vasopressin V2, and the α_1 adrenergic receptors had no measurable effect on either desensitization or internalization [72,90,96].

Trafficking

Ariano et al. [4] examined immunofluorescent staining of dopamine D1 receptor protein expression in non-permeabilized Chinese Hamster Ovary (CHO) cells using antisera directed against the third cytoplasmic loop of the protein. After agonist exposure for as brief as 2 minutes, profound loss of immunofluorescence staining occurred. This agonist-induced loss was blocked by the antagonist (+)-butaclamol. Vickery et al. [112]

demonstrated that treatment by agonist for 5 minutes resulted in a 60% increase in D1 receptor internalization and 40% in D2 internalization.

Dynamin associates with clathrin coated vesicles and caveolae to promote their scission from the plasma membrane [51]. Over-expression of a dominant negative mutant of dynamin blocked D1 but not D2 internalization[112]. Thus, D1 and D2 receptors are selectively endocytosed by distinct mechanisms.

DESENSITIZATION AND NEUROLOGICAL DISEASE

Parkinson's Disease

Degenerative changes in the dopaminergic system are the main neuropathological features of Parkinson's Disease (PD). Standard therapy for treatment of PD is L-dopa, which may promote a refractory "off" period side effect. Apomorphine, a nonselective agonist at D1 and D2 dopamine receptors, is effective at reversing refractory L-dopa induced "off" periods [21], but the response to apomorphine is reduced after repeated administration. This loss of response may be due to dopamine receptor phosphorylation and desensitization [71]. Beneficial responses are obtained from the dopamine D1 receptor agonist A-77636 in animal models of PD. However, A-77636 is of limited potential for PD therapy because it induces rapid tolerance *in vivo*. This tolerance is thought to occur because A-77636 dissociates slowly from the D1 receptor causing prolonged receptor desensitization [67]. Clarification of the molecular bases for desensitization of the dopamine receptor may lead to a better understanding of the regulation of dopamine receptor responsiveness, and to more effective treatment of PD [71].

Schizophrenia

Antipsychotic drugs, such as chloropromazine and related phenothiazine neuroleptics, that are effective in the treatment of schizophrenia also block D2-like receptors. It was this correlation between alleviating the symptoms of schizophrenia and blockade of D2 receptors that suggested an excess of dopaminergic transmission may underlie some aspect(s) of the pathogenesis of schizophrenia. Studies by Lidow et al. [66] demonstrated in primates that dopamine D1 receptor sites in prefrontal cortex are down regulated by chronic treatment with D2 antagonists. The authors speculate that down regulation of the dopamine D1 receptor may be an obligatory consequence of D2 receptor pharmacological antagonism. On the other hand, Okubo et al. [89] used positron emission tomography (PET) to reveal reduced radioligand binding to the dopamine D1 receptor in the prefrontal cortex of schizophrenics. They observed that this reduction was related to severity of negative symptoms of schizophrenia. The reduced radioligand binding may be a result of desensitization from excess dopaminergic transmission. They suggest that dysfunction of the dopamine D1 receptor signaling in the prefrontal cortex may contribute to the negative symptoms and cognitive deficits seen in schizophrenia.

TRAFFICKING

GPCR Pathways

GPCRs such as the β adrenergic and μ opioid receptors traffic by three recognized pathways [36], (Fig. 1-5). These pathways are; 1) rapid recycling, 2) slow recycling, and 3) degradation [37]. The internalization of GPCRs is typically by a clathrin-dependent mechanism. However, more recently it has been shown that blocking clathrin-dependent internalization of GPCRs may cause internalization by other mechanisms [22]. Thus, the particular pathway through which a GPCR traffics may depend on the mechanism by which it is internalized.

Internalization

A well established first step is the delivery of receptors from the plasma membrane via clathrin-coated vesicles (CCV) into early endosomes. Coat propagation and fission of CCV from the plasma membrane regulated by a series of protein factors and lipids, including the small GTPase Rab5. Rab5 mediates the heterotypic tethering and fusion of CCV with early endosomes as well as the homotypic fusion between early endosomes.

Endosomal sorting

The Rab family is part of the Ras superfamily of small GTPases. The Rab GTPase switches between GDP- and GTP-bound conformations. Conversion from GDP- to the GTP-bound form is caused by nucleotide exchange, catalyzed by a GDP/GTP exchange factor (GEF). Conversion from the GTP-to the GDP-bound form

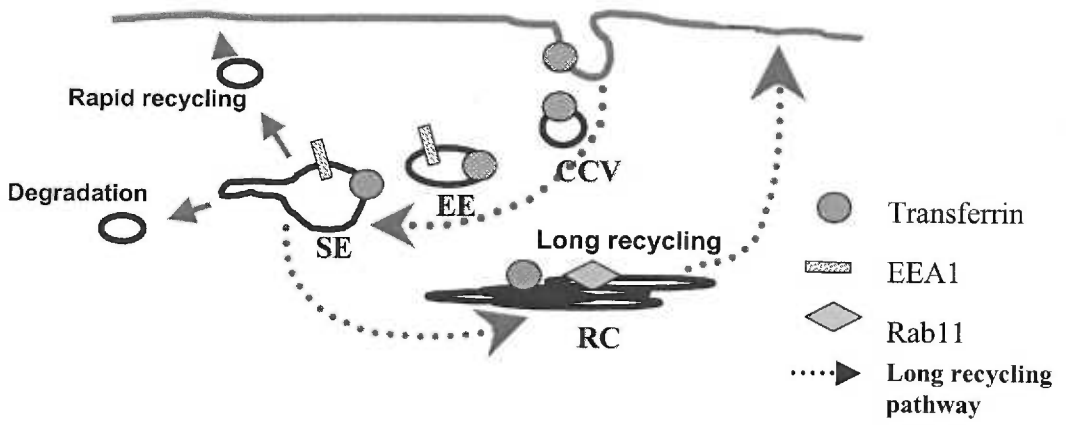

Figure 1-5: **Major trafficking pathways and endosomal markers.**
Diagram depicts 3 major pathways (rapid recycling, long recycling, and degradation) through which GPCRs are thought to traffic. Also shown are endosomes identified by transferrin and anti-EEA1and Rab11. SE= sorting endosome, EE=early endosome, CCV=clathrin coated vesicle, RC=Recycling compartment.

occurs by GTP hydrolysis, facilitated by a GTPase-activating protein (GAP). The GTP-bound form interacts with effector molecules [105].

Rab5 is symmetrically distributed between CCV and early endosomes [78]. Several Rab effectors are required for homotypic endosomal fusion, including early endosomal antigen 1 (EEA1) and PtdIns 3 kinase (PI3K).

One of the products of PI3K, Ptdlns 3-phosphate (PI3P), plays a major role in endocytic trafficking [101]. PI3P is highly enriched on early endosomes and in the internal vesicles of multivesicular endosomes. Proteins that contain a FYVE zinc finger domain, such as EEA1, are effectors of PI3P [117]. The restricted localization of PI3P to endosomes suggests that most FYVE domain proteins regulate endosomal function.

EEA1 is selective to early endosomes [78]. Recruitment of EEA1 to early endosomes requires both PI3P and Rab5, although in the presence of high levels of PI3P, Rab5 is not required. Low levels of Rab11 are associated with EEA1 endosomes and with

pericentriole recycling endosomes [108]. The hydrolysis of GTP on Rab11 is required for the direct delivery of transferrin from the pericentriole recycling compartment to the cell surface, but not from sorting endosomes [98]. Rab11 is localized to late Golgi and post-Golgi membranes, as well as to specialized recycling membranes, suggesting a role for Rab11 in late Golgi and post-Golgi transport [108]. These reports suggest that the precise function of Rab11 is uncertain.

The transferrin intracellular trafficking pathway has been extensively studied. After binding with iron, transferrin is internalized by clathrin coated pits into CCV, fused with sorting endosomes containing EEA1, and then to recycling endosomes before returning to the surface [82].

Green Fluorescent Protein

The gene encoding the green fluorescent protein (GFP) comes from the jellyfish *Aequoria victoria*. The gene was first isolated in 1992 and has rapidly become a popular reporter molecule for monitoring protein expression and localization. GFP is an autofluorescent protein of 238 amino acids of which 11 beta-strands form a hollow cylinder through which a cyclic tripeptide chromophore is threaded post-translationally (Fig 1-6).

GFP absorbs blue light at 395 nm, with a smaller peak at 475 nm, and emits green light at 508 nm. The existence of two excitation peaks complicates the use of wildtype GFP for routine fluorescence observations. Mutation of Ser65 to Thr (S65T) completely suppresses the 395-nm peak while amplifying the 475-nm excitation peak ~6 fold and shifting it to 489 nm. Mutation of Phe64 to Leu (F64L) improves folding at 37°C. Thus,

these two highly advantageous substitutions have been incorporated into a variant of GFP named Enhanced Green Fluorescent Protein (EGFP). Expression of functional GPCR-EGFP fusion proteins has been demonstrated for the β-adrenergic, GnRH, cholecystokinin (CCK), and muscarinic receptors. These receptors, when tethered to the EGFP, recapitulate the characteristics of the wild-type form. The intrinsically fluorescent properties of EGFP eliminate problems of substrate availability to the fluorescent marker, and EGFP fluorescence *in vivo* allows real-time imaging of the tagged receptor. Potential disadvantages of GPCR-EGFP chimeras are that expression in cells often produces labeling of protein biosynthetic compartments, such as the endoplasmic reticulum and Golgi, and that production of a GPCR-EGFP chimera might result in altered properties of the receptor.

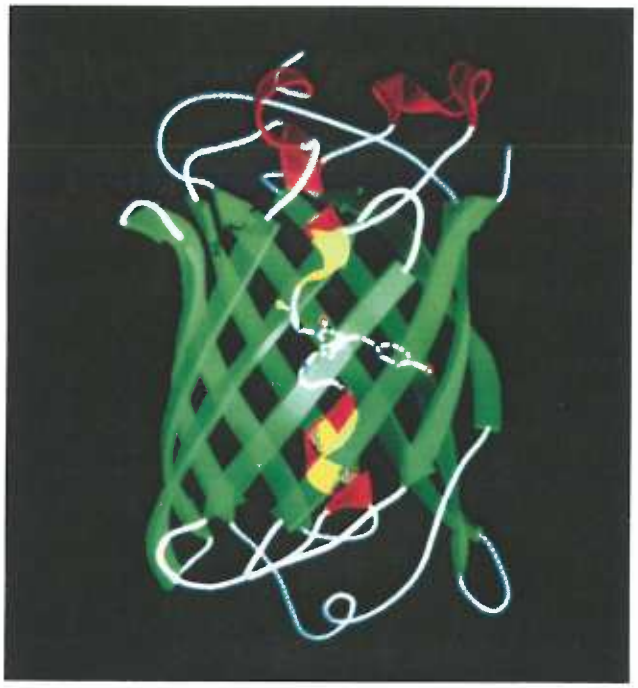

Fig. 1-6. Green Fluorescent Protein. Ribbon diagram of the wildtype GFP structure. The alpha-helices are shown in black and the beta-pleated sheets are shown in green. The chromophore is shown as a ball-and-stick[16].

SPECIFIC AIMS OF DISSERTATION

Specific aim #1

Both G protein-coupled receptor kinases (GRKs) and cyclic AMP-dependent protein kinase (PKA) are thought to contribute to agonist-induced phosphorylation of the dopamine D1 receptor. Over-expression of either GRK-2, -3, or -5 in dopamine D1 receptor-expressing HEK293 cells enhances phosphorylation of the dopamine D1 receptor [107]. Evidence for the role of PKA in agonist-induced phosphorylation is controversial. Based on reports by Ng et al. [87;85] that correlate phosphorylation with desensitization, and reports by Bates and Jiang [11,12,54] that demonstrate that agonist-stimulation of the dopamine D1 receptor reduces adenylate cyclase responsiveness, I hypothesized that phosphorylation of potential PKA sites of the receptor would alter agonist-induced receptor desensitization, phosphorylation, and trafficking. When the work that is the subject of this dissertation was begun, there was no direct evidence to link the contribution of D1 receptor GRK or PKA consensus site phosphorylation with agonist-induced phosphorylation of the D1 receptor. To determine the contribution of GRK and PKA potential phosphorylation sites in agonist-induced phosphorylation of the D1 receptor, each potential phosphorylation site must be individually removed. The impact of the mutation on dopamine-induced receptor phosphorylation is then assessed. The consensus sequences most frequently recognized by PKA have been identified, whereas those for GRKs have not been clearly identified [57,58]. Therefore, in this dissertation I chose to examine only the contribution of PKA to agonist-induced receptor phosphorylation. I also assessed the role of each PKA consensus site in regulating receptor responsiveness and trafficking.

To assess changes in phosphorylation of the receptor in response to the removal of individual PKA consensus sites, I placed a polyhistidine tag on the N-terminus of the receptor to separate the dopamine receptor from other phosphorylated proteins in the cell. To study the redistribution of the receptor in living cells, I placed green fluorescent protein on the C-terminus, to allow me to visually track and assess the movement of the receptor throughout the cell in real-time.

The possible effects of these modifications on antagonist/agonist affinity and adenylate cyclase response were assessed by radioligand binding and adenylate cyclase assays, respectively. Results indicated little difference between the tagged receptor and untagged receptors. Thus, I concluded that the tagged receptor recapitulated the characteristics of the wildtype receptor and was a useful tool to evaluate the effects of individual PKA site mutations on receptor phosphorylation, responsiveness, and trafficking, in response to agonists.

Specific aim #2

In the initial part of this dissertation, I identified a difference between the wildtype D1-EGFP and the mutant T268A D1-EGFP receptor in their accumulation in the perinuclear region of the cell in response to agonist. Agonist stimulation of the D1-EGFP receptor, but not T268A, led to an increase in the ratio of the perinuclear to plasma membrane fluorescence. We hypothesized that Thr268 acts as a sorting signal in the long recycling pathway, and that mutating it to alanine altered a sorting step in the pathway. Thus, the second aim of this study was to determine if the pathways through which the two receptors traffic differ. To further our understanding of how the

intracellular trafficking pathways of this mutant (T268A) might differ from the wildtype D1-EGFP receptor, we compared the colocalization of the mutant and wildtype receptors with the endosomal markers EEA1 (early endosomal antigen 1), Rab11 (GTPase Rab11), and TF (transferrin). We also used fluorescent recovery after photobleaching (FRAP) to look for possible differences between the receptors in how they tether to the plasma membrane.

II. REGULATION OF DOPAMINE D1 RECEPTOR TRAFFICKING BY PROTEIN KINASE A-DEPENDENT PHOSPHORYLATION

As published in
Molecular Pharmacology (Mar, 2002)

ABSTRACT

The aim of this study was to use pharmacological inhibition of protein kinase A and mutation of potential protein kinase A phosphorylation sites to determine the role of protein kinase A-catalyzed phosphorylation of the dopamine D_1 receptor in agonist-stimulated desensitization and internalization of the receptor. To facilitate purification and imaging of the D_1 receptor, we attached a polyhistidine tag to the amino terminus and enhanced green fluorescent protein to the carboxy terminus of the receptor (D_1-EGFP). D_1-EGFP was similar to the untagged D_1 receptor in terms of affinity for agonist and antagonist ligands, coupling to G proteins, and stimulation of cyclic AMP accumulation. D_1-EGFP and two mutants in which either Thr268 or Ser380 was replaced with Ala were stably expressed in NS20Y neuroblastoma cells. Pretreatment with the protein kinase A inhibitor H-89 or substitution of Ala for Thr268 reduced agonist-stimulated phosphorylation of the receptor and resulted in diminished trafficking of the receptor to the perinuclear region of the cell. Substitution of Ala for Thr268 had no effect, however, on agonist-induced receptor sequestration or desensitization of cyclic AMP accumulation. Substitution of Ala for Ser380 had no effect on D_1 receptor phosphorylation, sequestration, desensitization, or trafficking to the perinuclear region. We conclude that protein kinase A-dependent phosphorylation of the D_1 receptor on Thr268 regulates a late step in the sorting of the receptor to the perinuclear region of the cell, but that phosphorylation of Thr268 is not required for receptor sequestration or maximal desensitization of cyclic AMP accumulation.

INTRODUCTION

The dopamine D1 receptor belongs to the superfamily of heptahelical receptors that modulate the activity of effectors such as adenylate cyclase by activiation of specific heterotrimeric GTP-binding proteins (G-proteins). For many G protein coupled receptors (GPCR), phosphorylation by protein kinases such as protein kinase A (PKA) or G protein-coupled receptor kinases (GRK) is an early step in agonist-induced desensitization, the diminished responsiveness that occurs after continuous or repeated exposure of the receptors to agonist [49]. The mechanisms of desensitization and resensitization have been perhaps most thoroughly characterized for the β-adrenergic receptor, a $G\alpha_s$-coupled receptor that is structurally and functionally homologous to the D1 receptor. Occupation of the β-adrenergic receptor by agonists stabilizes a conformation of the receptor that increases its phosphorylation by GRK, which in turn increases the affinity of the receptor for β-arrestin [62]. The coupling of the receptor to heterotrimeric G proteins is inhibited by binding of β-arrestin which is likely to be the immediate cause of rapid desensitization. β-Arrestin also acts as an adaptor protein to facilitate receptor endocytosis [62]. Once internalized the receptor is either resensitized by dephosphorylation and then recycled back to the cell surface [91], or directed to an intracellular compartment for degradation [109].

Although this model closely describes the regulation of some GPCRs, the regulation of other receptors differs significantly in ways that may be both cell- and receptor-dependent [60]. For example, GPCR desensitization and internalization can occur despite greatly reduced or undetectable agonist-induced phosphorylation of the receptor [72,90,96], and dephosphorylation/resensitization can occur without internalization [40].

Desensitization of the dopamine D1 receptor has been demonstrated in a variety of tissue preparations, including brain slices and retina, primary neuronal culture, neuroblastoma, astrocytoma, or kidney cell lines expressing an endogenous D1 receptor, and cell lines expressing a recombinant D1 receptor [10,54,76,111]. The responsiveness of the D1 receptor is probably regulated by PKA- and GRK-catalyzed phosphorylation of the receptor, since dopamine-induced desensitization is temporally associated with or preceded by receptor phosphorylation [40,85], enhanced by activation of PKA or overexpression of GRK [107], and reduced by inhibitors of PKA or GRK. Furthermore, desensitization of the D1 receptor is blunted in cells deficient in PKA [111], and mutation of a potential site of PKA phosphorylation of the D1 receptor, Thr268, reduces the rate of agonist-induced desensitization [54]. Ser380 has also been proposed to be a site of phosphorylation by PKA, because a peptide comprised of D1 receptor amino acid residues 372-442 is phosphorylated by PKA on Ser380 [119].

In this study we assessed the role of two potential sites of phosphorylation by PKA, Thr268 and Ser380, in agonist-induced phosphorylation, desensitization, and internalization of the dopamine D_1 receptor. We created a D_1 receptor (D_1-EGFP) with enhanced green fluorescent protein at the carboxy terminus and a hexa-histidine tag at the amino terminus. Wildtype D_1-EGFP and mutants in which an alanine residue was substituted for Thr268 (T268A) or Ser380 (S380A) were stably expressed in NS20Y neuroblastoma cells. We now report that mutation of Thr268, but not Ser380, prevented dopamine-induced phosphorylation of D_1-EGFP and redistribution of the receptor to the perinuclear region of NS20Y cells, without altering maximal desensitization of cyclic

AMP accumulation or sequestration of the receptor away from the extracellular surface of the membrane.

MATERIALS AND METHODS

Construction of tagged and mutant receptors. We attached a polyhistidine tag to the N-terminus of the rhesus macaque D_1 receptor [70] using the polymerase chain reaction. The entire amplified portion of the gene was sequenced to confirm the absence of random mutations and cloned into pcDNA3. To place the enhanced green fluorescent protein (EGFP) to the C-terminus, we used the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) to introduce a *Bam*HI site at Pro445 of the histidine-tagged D_1 receptor, which resulted in the loss of the terminal Thr residue (position 446), and the substitution of Asn for His444. A *Hind*III - *Bam*HI fragment containing the histidine-tagged D_1 receptor was cloned into pEGFP-N1 (Clontech, Palo Alto, CA), creating a histidine-tagged D_1 receptor with EGFP tethered by a 6 residue linker at the C-terminus to form the construct D_1-EGFP (Fig. 2-1). Mutants of the D_1-EGFP construct were made in which two potential sites of phosphorylation by PKA, Thr268 and Ser380, were changed to alanine, yielding the mutants T268A and S380A (Fig. 2-1). Untagged alanine substitution mutants of Thr268 and Ser380 for expression in C_6 glioma cells were constructed separately using the trans-polymerase chain reaction (PCR) method described previously [84], except using *Pfu* thermostable DNA polymerase instead of *Taq*. The T268A mutant was cloned into pcDNA1, whereas the S380A mutant was cloned into pcDNA3. The PCR-amplified portion of each mutant was sequenced to confirm the presence of the desired mutation and to identify random errors introduced by PCR. The untagged T268A had two additional mutations that changed Ile34 → to Val in the first transmembrane domain and Gln436 → His close to the C terminus.

Creation and Maintenance of Cell Lines. Both tagged and untagged wildtype and mutant receptors were stably expressed in NS20Y neuroblastoma cells by calcium phosphate co-precipitation, and after selection for G418–resistance, pooled populations of D_1-EGFP-, T268A- and S380A-expressing cells were isolated using a BD FACSVantage SE flow cytometry system (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, Sigma Chemical Co, St. Louis, MO) supplemented with 5% fetal bovine serum and 5% calf bovine serum, with 600 μg/ml of G418 Sulfate (Calbiochem Co, San Diego, CA). Clonal C_6 cell lines expressing untagged wildtype and mutant D_1 receptors were created by co-transfection with pBabe-Puro [81] and selection with puromycin (wildtype D_1 and T268A, 2 μg/ml) or by transfection (in pcDNA3) and selection with G418 (S380A, 600 μg/ml), exactly as described previously [27]. C_6 cells expressing wildtype and mutant D_1 receptors were maintained in DMEM supplemented with 2% fetal bovine serum, 3% iron-supplemented calf bovine serum, penicillin (50 μg/ml), streptomycin (50 μg/ml), and either puromycin or G418.

[^{3}H]SCH 23390 Saturation Binding. Confluent cells in 10 cm-dia. tissue culture plates were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na^+-HEPES, pH 7.4, 2 mM EDTA). After swelling for 10-15 min, the cells were scraped off the plate and centrifuged at 24,000 x *g* for 20 min. The crude membrane fraction was resuspended in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 155 mM NaCl) with a Brinkmann Polytron homogenizer at setting 6 for 10 sec. Aliquots of the membrane preparation (~30

μg of protein) were added to duplicate assay tubes containing 50 mM Tris-HCl, pH 7.4, 155 mM NaCl, 0.001% bovine serum albumin, and 6 concentrations of [^{3}H]SCH23390 (75.5 Ci/mmol; NEN Life Science Products, Boston, MA) ranging from 0.05 to 2.0 nM in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of (+)-butaclamol (2 μM). Incubations were carried out at 30°C for 60 min and terminated by filtration using a 96-well Tomtec Mach II cell harvester (Orange, CT). Filters (BetaPlate filtermat A) were dried, and 50 μl of BetaPlate scintillation cocktail was added to each sample. Radioactivity on the filters was determined using a LKB/Wallac BetaPlate 1205 scintillation counter (Gaithersburg, MD).

Agonist Binding. The crude membrane fraction was resuspended in competition binding assay buffer (20 mM HEPES, pH 7.5, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol), incubated at 37°C for 15 to 20 min, re-centrifuged, and resuspended a second time in assay buffer. An aliquot of the membrane preparation was added to duplicate assay tubes containing assay buffer with 0.025% ascorbic acid, 0.001% bovine serum albumin, [^{3}H]SCH 23390 (~0.8 nM) and 24 concentrations of dopamine ranging from 1×10^{-12} - 1×10^{-3} M. Incubations were carried out and filtered as detailed above.

Cyclic AMP accumulation assay. NS20Y cells stably expressing wildtype or mutant D_1 receptors were plated out in 12-well tissue culture clusters and used to assess desensitization 36-48 hr later, when they were at a density of ~150,000 cells/well, whereas C_6 cells stably expressing untagged wildtype and mutant D_1 receptors were plated in 48-well clusters at a density of 100,000 to 150,000 cells/well and used 3-4 days later. Cells were pre-incubated for 10 min with 200 μL assay buffer (Earle's Balanced Salt Solution containing 0.02% ascorbic acid, 2% bovine calf serum, and 500 μM

isobutylmethylxanthine). The cells were then placed on ice and drugs added to triplicate wells as indicated. For measurement of dopamine-stimulated cyclic AMP accumulation in C_6 cells, 1 μM propranolol was added to prevent potential stimulation of endogenous β–adrenergic receptors by dopamine. After incubation for 15 min at 37°C, the assay buffer was decanted and cells were lysed with 3% trichloroacetic acid (100 μl/well). The cyclic AMP in each well was quantified using a competitive binding assay modified as described previously [114]. Ten μl of cell lysate was incubated in duplicate tubes containing 1.5 nM [^{3}H]cyclic AMP and crude adrenal extract (~100 μg protein) in 500 μl of cyclic AMP binding buffer (100 mM Tris/HCl, pH, 7.4, 100 mM NaCl, 5 mM EDTA) for 2 hr on ice, then harvested by filtration as described for radioligand binding assays, except using filtermat B. Unknown samples were plotted against a standard curve (0.1 to 100 pmol) to calculate the amount of cyclic AMP.

Metabolic Labeling. NS20Y neuroblastoma cells stably expressing the wildtype or mutant D_1-EGFP receptors were plated in 6-well tissue culture clusters and used 36-48 hr later, when they were at a density of ~320,000 cells/well. The following day plates were rinsed twice with 1 ml phosphate-free DMEM, then incubated for 1 hr at 37°C with 200 μCi/ml [^{32}P]PO_4 in 1 ml phosphate-free DMEM containing 25 mM Na-HEPES, pH 7.4, and 0.02% ascorbic acid. Cells were then treated with dopamine (25 μM) or vehicle for 5 or 20 min, followed by cell lysis and affinity purification of D_1-EGFP.

Affinity Purification. The medium was aspirated off and replaced with ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaF, 10 mM disodium pyrophosphate, and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cells were then loaded into 50 ml centrifuge tubes and centrifuged at 500 rpm for 5 min at 4°C. The

supernatant was decanted, and cells were resuspended in lysis buffer before centrifugation at 40,000 x *g* at 4°C for 20 min. The supernatant was removed and the pellet resuspended in 3 ml of ice-cold solubilization buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1% n-dodecylmaltose, 10 mM NaF, 10 mM disodium pyrophosphate, and 1 tablet protease inhibitor cocktail/50 ml). The resuspended pellet was sonicated on ice for 20 sec, and the contents were transferred to a 15 ml conical centrifuge tube and placed on a tilt shaker at 4°C for 1 hr. Following solubilization, the solution was centrifuged at 40,000 x *g* at 4°C to remove non-solubilized material. The His-tagged receptor was then purified using nickel-charged Chelating Sepharose Fast Flow beads (Pharmacia Biotech AB, Uppsala Sweden) as described [59]. The eluate (1 ml) from this procedure was collected in a 1.7 ml microfuge tube and 10 µl of 1 M phosphate buffer (pH 4.5) added for a final concentration of 10 mM. Next, 20 µl of Protein G Sepharose 4 Fast Flow (Pharmacia Biotech AB) and 1 µg of monoclonal anti-autofluorescent protein (Quantum Biotechnologies Inc., Montreal, Canada) was added. After incubating for 2 hr at 4°C on a tilt rocker, the tube was centrifuged at 2000 x *g* and the supernatant removed by aspiration. The pellet was resuspended in 1 ml radioimmunoprecipitation assay (RIPA) buffer (10mM Tris, 150mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate) and centrifuged at 2000 x *g*; this step was repeated 3 times. The washed pellet was resuspended in 30 µl Laemmli sample buffer and incubated at 65°C for 20 minutes to dissociate the D_1-EGFP receptor from the protein G sepharose. After centrifugation at 2000 x *g* for 5 minutes, the supernatant protein was separated by SDS PAGE and transferred to a PVDF membrane. The D_1-EGFP receptor was detected by immunoblotting as described below using monoclonal anti-GFP (Clontech, Palo Alto,

CA). Membranes were scanned using a Storm 840 Imaging System (Molecular Dynamics; Sunnyvale CA), then juxtaposed to Kodak X-OMAT film for 24-48 hours. The film was developed, and the resulting autoradiograph was digitally captured with a Hewlett Packard Scan Jet LP. The bands showing D_1-EGFP immunoreactivity were quantified by densitometry using ImageQuant software (Molecular Dynamics; Sunnyvale CA).

Immunoblotting. Proteins were separated by SDS-PAGE through a 10% polyacrylamide gel and transferred to PVDF membranes (Costar Corning, Cambridge, MA). The membranes were blocked overnight with 5% nonfat dry milk, washed with Tris-buffered saline, and incubated with the indicated primary antibody for 2 hr. The PVDF membranes were washed, incubated with secondary antibody (fluorescein-linked anti-mouse IgG, Amersham Life Science Ltd., Buckinghamshire, England), and immunodetection was accomplished using an ECF Western blotting kit (Amersham). In some cases membranes were stripped and re-blotted using a western blot recycling kit (Chemicon International, Temecula, CA) and a monoclonal antibody recognizing phosphothreonine (Santa Cruz Biotechnology, Santa Cruz, CA).

Fluorescence Microscopy. Sterile coverslips (Fisherbrand™ #1) were placed in 12 well tissue culture dishes and seeded with cells stably expressing D_1-EGFP. Imaging of live cells was performed the next day using either a Leica TCS SP scanning confocal microscope or a Nikon TE200 inverted fluorescent microscope with a CH350L camera. Images captured by the Nikon microscope were deconvolved using software by API Delta Vision (Applied Precision Co., Issaquah, WA). Cells were maintained at 37°C using a Delta T Stage Adapter (Bioptechs Co., Butler, PA) for the Nikon TE200

microscope and an RC 26 open bath imaging chamber on a PH-1 heater platform (Warner Instruments, Hamden, CT) for the Leica TCS SP. Image quantification was done using NIH Image V. 1.62B.

Biotinylation of membrane proteins. Cells grown to 80% confluency on 10 cm tissue culture plates were treated with dopamine (25 μM) or vehicle (0.02% ascorbic acid) 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 biotinylation and separated from nonbiotinylated proteins by ImmunoPure™ Immobilized strepavidin (Pierce, Rockford, IL) as described [75]. Protein was eluted from the strepavidin beads with 30 μl of Laemmli sample buffer and constant mixing for 20 min before separation by SDS PAGE and transfer to PVDF membranes for western blotting with monoclonal anti-GFP (Clontech, Palo Alto, CA).. D_1-EGFP immunoreactivity was quantified by densitometry using ImageQuant software (Molecular Dynamics; Sunnyvale CA).

Data analysis. Saturation isotherms, radioligand binding inhibition curves, and dose-response curves for cyclic AMP accumulation were analyzed by nonlinear regression using Prism 3.0 (GraphPad Software, San Diego, CA). K_I, K_D, and EC_{50} values are geometric means from three or more experiments followed by the limits defined by the asymmetric standard error of the mean. For displacement of radioligand binding by dopamine, curves were analyzed assuming the presence of one and two classes of binding sites. The assumption of two classes of binding sites was accepted when $p < 0.05$ for the improvement of the fit, determined using an F test. Statistical comparisons between cells expressing mutant and wildtype receptors or between treated and untreated cells were made using the Student's *t*-test.

RESULTS

Expression of D_1-EGFP Receptors in NS20Y Cells. We constructed a tagged dopamine D_1 receptor (D_1-EGFP) with a polyhistidine tag attached to the amino terminus and enhanced green fluorescent protein fused to the carboxy terminus of the receptor (Fig. 2-1). To eliminate two potential sites of phosphorylation by PKA, alanine substitution mutants of D_1-EGFP were made for Thr268 (T268A) and Ser380 (S380A). The untagged D_1 receptor as well as tagged wildtype and mutant receptors were stably expressed in NS20Y mouse neuroblastoma cells.

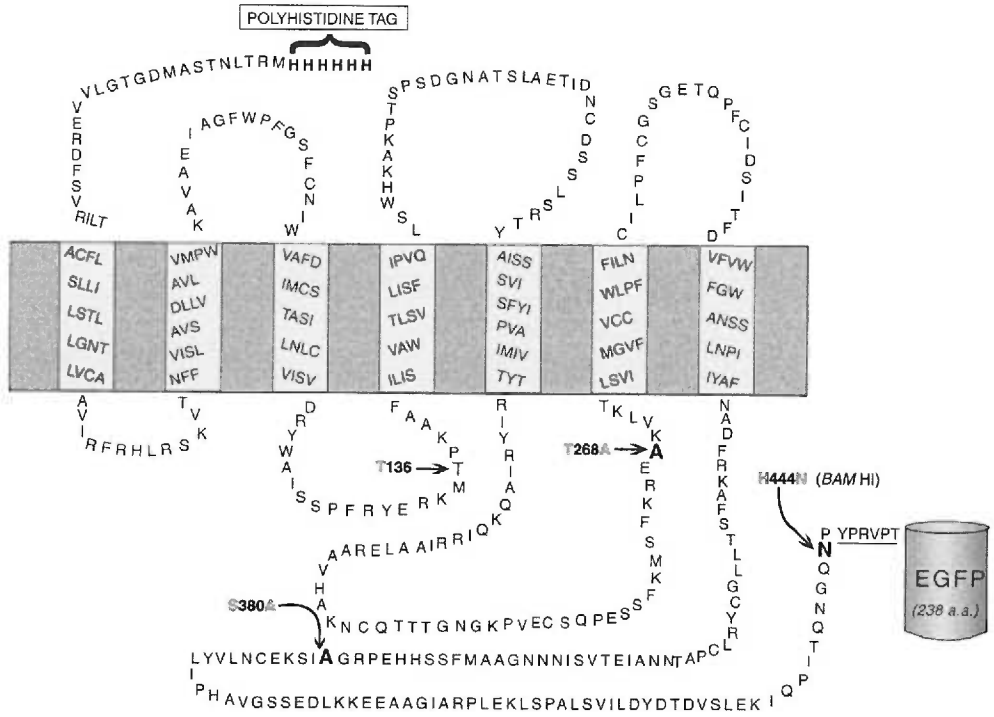

Fig. 2-1. Schematic diagram of the EGFP-tagged dopamine D_1 receptor.

Residues of the receptor that were mutated to Ala to eliminate potential sites of phosphorylation are shown, as well as the mutation His444→Asn which was the result of introducing a BamHI site for in-frame cloning of the receptor into pEGFP-N1. The six residues that link the D1 receptor and EGFP are indicated in the figure. A hexa-histidine tag was added to the N-terminus of the receptor.

Table 2-1. Binding properties and density of D_1 receptors stably expressed in NS20Y neuroblastoma cells

Receptor	[^{3}H]SCH 23390		Dopamine		
	K_D (nM)	B_{max} (pmol/mg)	K_H (nM)	K_L (nM)	%K_H
D1-EGFP	0.34 (0.28-0.42)	2.20 ± 0.30	11 (9-16)	1320 (890-1950)	37 ± 9
T268A	0.29 (0.24-0.36)	1.75 ± 0.25	8 (4-17)	1040 (1000-1100)	34 ± 8
S380A	0.56 (0.53-0.59)	2.36 ± 0.39	3 (2-5)	1470 (1170-1850)	24 ± 10
D1-EGFP (L)	0.33 (0.30-0.35)	0.50 ± 0.20	11 (6-22)	1070 (400-3020)	29 ± 7
Wt-D1	0.35 (0.28-0.43)	0.75 ± 0.23	9 (4-21)	1010 (740-1400)	36 ± 8

Cell membranes were prepared, and saturation and competition binding assays performed as described in *Materials and Methods*. Two populations of cells expressing D_1-EGFP were selected, a high receptor-density population (D1-EGFP) that was used in subsequent studies of phosphorylation, internalization, and trafficking of the D_1 receptor, and a low receptor-density population (D1-EGFP (L)) that was used in cyclic AMP accumulation experiments for comparison with the untagged D_1 receptor (Wt-D1). K_D and B_{max} values for the binding of [^{3}H] SCH23390 were determined by saturation analysis. Dopamine affinity values were calculated from competition binding curves analyzed in terms of high (K_H) and low (K_L) affinity classes of binding sites. All affinity values are the geometric mean of 12 ([^{3}H]SCH 23390) or 3-6 (dopamine) independent experiments followed by the limits defined by the asymmetric standard error of the mean.

For wildtype or mutant D_1-EGFP receptors, FACS enriched cell populations were selected that expressed each receptor at approximately 2 pmol/mg of membrane protein (Table 2-1). Because the cell line selected for untagged D_1 receptor expressed that receptor at a lower density (0.75 pmol/mg protein) a second population of cells expressing D_1-EGFP at a lower density was also used for comparison. Mutant and wildtype receptors had similar affinities for the antagonist radioligand [^{3}H]SCH 23390 (K_D = 0.3 - 0.6 nM; Table 2-1). Similarly, curves for inhibition of the binding of [^{3}H]SCH 23390 by the agonist dopamine were best fit by assuming the presence of two classes of binding sites for each wildtype and mutant receptor, and the proportions of sites with high affinity for dopamine (24-37%) were similar among the receptors, as were the K_H and K_L values for dopamine. Thus, the antagonist and agonist binding properties of the D_1 receptor were not altered by tagging the receptor or by mutation of Thr268 or Ser380.

To investigate further the functional properties of the tagged and mutant receptors, stimulation of cyclic AMP accumulation by dopamine and the agonist 6-chloro-PB (SKF 81297) was assessed. Maximal stimulation of cyclic AMP accumulation was similar in cells expressing wildtype D_1, D_1-EGFP, T268A, or S380A receptors (Table 2-2). In contrast, the mean EC_{50} values for the tagged wildtype and mutant receptors were lower than the value for the untagged wildtype receptor. This was particularly evident for dopamine, which was 3.5- to 7-fold more potent at the EGFP-tagged receptors than at the untagged D_1 receptor. The difference was not due to the higher level of expression of most of the EGFP-tagged receptors compared to the untagged

receptor, since the difference was also observed for a cell line expressing D_1-EGFP at a lower density (Table 2-2). Thus, neither His- and EGFP-tagging nor mutation of

Table 2-2. D_1 receptor agonist stimulated cyclic AMP accumulation

	Dopamine		Cl-PB	
Receptor	EC_{50} (nM)	V_{max}	EC_{50} (nM)	V_{max}
D1-EGFP	6 (1-26)	90 ± 25	9 (6-14)	60 ± 34
T268A	5 (2-14)	74 ± 20	27 (21-36)	51 ± 23
S380A	4 (3-5)	100 ± 22	13 (10-18)	66 ± 4
D1-EGFP (L)	14 (9-21)	39 ± 12	26 (13-54)	51 ± 11
Wt-D1	62 (23-170)	60 ± 30	77 (62-95)	63 ± 8

Cyclic AMP accumulation was assessed in NS20Y cells stably expressing the indicated receptor construct. Potency (EC_{50}) and maximal stimulation (V_{max}, expressed as pmol of cyclic AMP/well) were calculated from nonlinear regression analysis of dose response curves for the indicated agonist. The EC_{50} values are geometric means from 3-5 independent experiments followed by the limits defined by the asymmetric standard error of the mean. Two populations of cells expressing D1-EGFP were selected, a high receptor-density population (D1-EGFP) that was used in subsequent studies of phosphorylation, internalization, and trafficking of the D1 receptor, and a low receptor-density population (D1-EGFP (L)) that was used for comparison with agonist-stimulated cyclic AMP acccumulation by the untagged D1 receptor (Wt-D1).

Thr268 or Ser380 greatly altered the ability of the D_1 receptor to maximally stimulate adenylate cyclase. Tagging the receptor caused a modest left-ward shift in the potency of agonists that was similar for wildtype and mutant receptors.

Agonist-Induced Phosphorylation. To quantify agonist-induced incorporation of phosphate into the D_1-EGFP receptor, the receptor was purified by sequential nickel

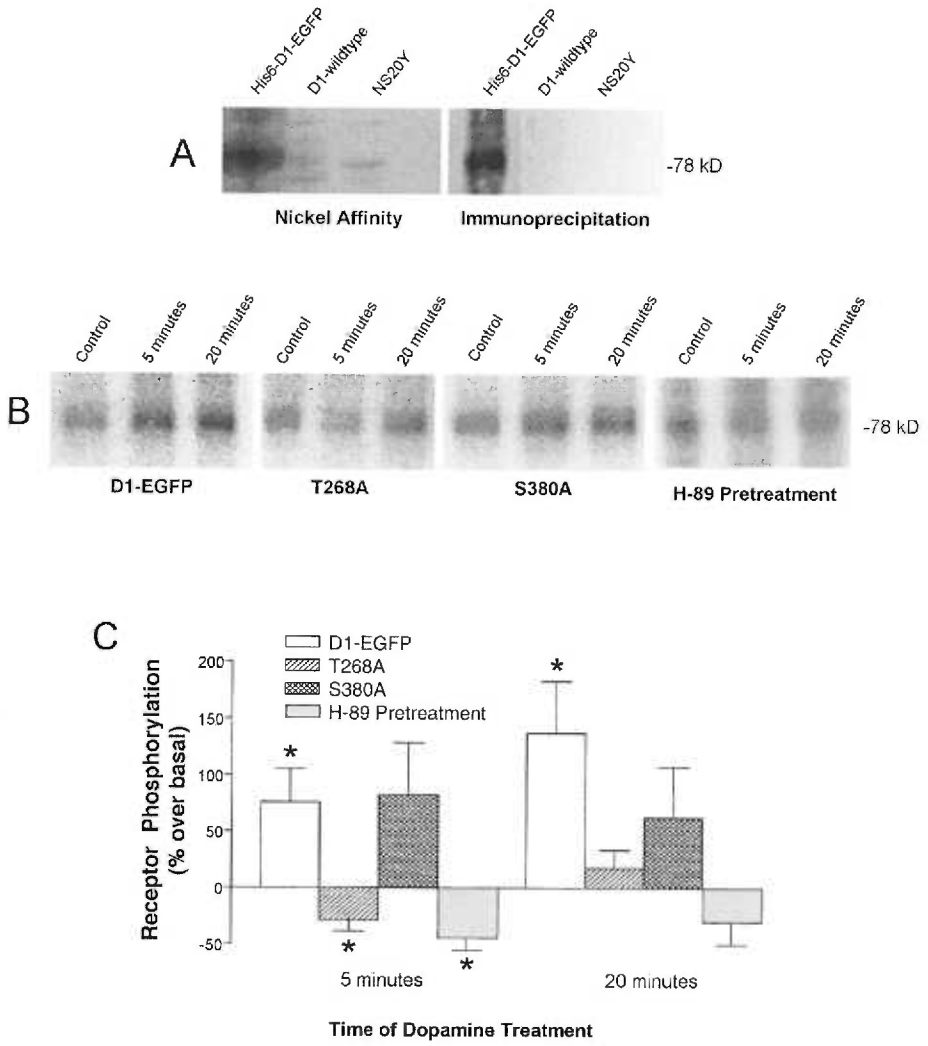

Fig. 2-2. Agonist-induced phosphorylation of D_1-EGFP and mutant receptors expressed in NS20Y neuroblastoma cells.

A, Immunoblot of preparations from untransfected NS20Y cells (NS20Y) or cells expressing D1-EGFP (His6-D1-EGFP) or the untagged D1 receptor (D1-wildtype). Receptors were purified by either nickel affinity resin alone (Nickel Affinity) or by nickel affinity resin followed by immunoprecipitation (Immunoprecipitation) with an anti-GFP monoclonal antibody (IgG2a). Following SDS-PAGE and transfer to PVDF membranes, D1-EGFP receptor protein was detected using a different anti-GFP monoclonal antibody (IgG1k). ***B****, NS20Y cells were labeled with [^{32}P]H_3PO_4, then treated with dopamine (25 μM) for 5 or 20 min. In some experiments, cells were treated with the PKA inhibitor H-89 (5 nM, 30 min) prior to dopamine stimulation. D1-EGFP, T268A, and S380A receptors were purified by sequential nickel affinity chromatography and immunoprecipitation prior to transfer of SDS-PAGE separated proteins to PVDF membranes and analysis by autoradiography.* ***C****, Autoradiographs such as the representative image in panel B were quantified as described in Materials and Methods. Band optical density was determined, and data for dopamine-treated cells were expressed as the percentage increase over the density for vehicle-treated cells in the same experiment. Results are the mean ± S.E. of 10-12 independent experiments. *$p < 0.05$ using a paired Student's t-test.*

affinity chromatography and immunoprecipitation from NS20Y cells metabolically labeled with ^{32}P. As shown in the western blot in Fig. 2-2A, the sequential purification procedure produced a cleaner receptor preparation than did nickel affinity chromatography alone. The incorporation of ^{32}P into the D_1-EGFP receptor was increased following treatment with dopamine (25 μM) for 5 min (76 ± 30%) or 20 min (137 ± 46%) compared to untreated cells (Fig. 2-2B and 2-2C). Dopamine treatment also increased the incorporation of ^{32}P into S380A, although neither the effect at 5 min (p = 0.2) nor the effect at 20 min (P = 0.08) was statistically significant. Collapsing the data for S380A across time resulted in a 73 ± 31% increase in dopamine-stimulated phosphorylation of the mutant ($p < 0.05$, N = 24). In contrast, dopamine treatment caused a slight reduction in the phosphorylation of T268A after 5 min. Reprobing a subset of the filters with anti-phosphothreonine demonstrated that 20 min treatment with dopamine increased phosphothreonine immunoreactivity in D_1-EGFP by 41 ± 15%, whereas the same treatment caused a nonsignificant decrease in phosphothreonine immunoreactivity in T268A (Fig. 2-3).

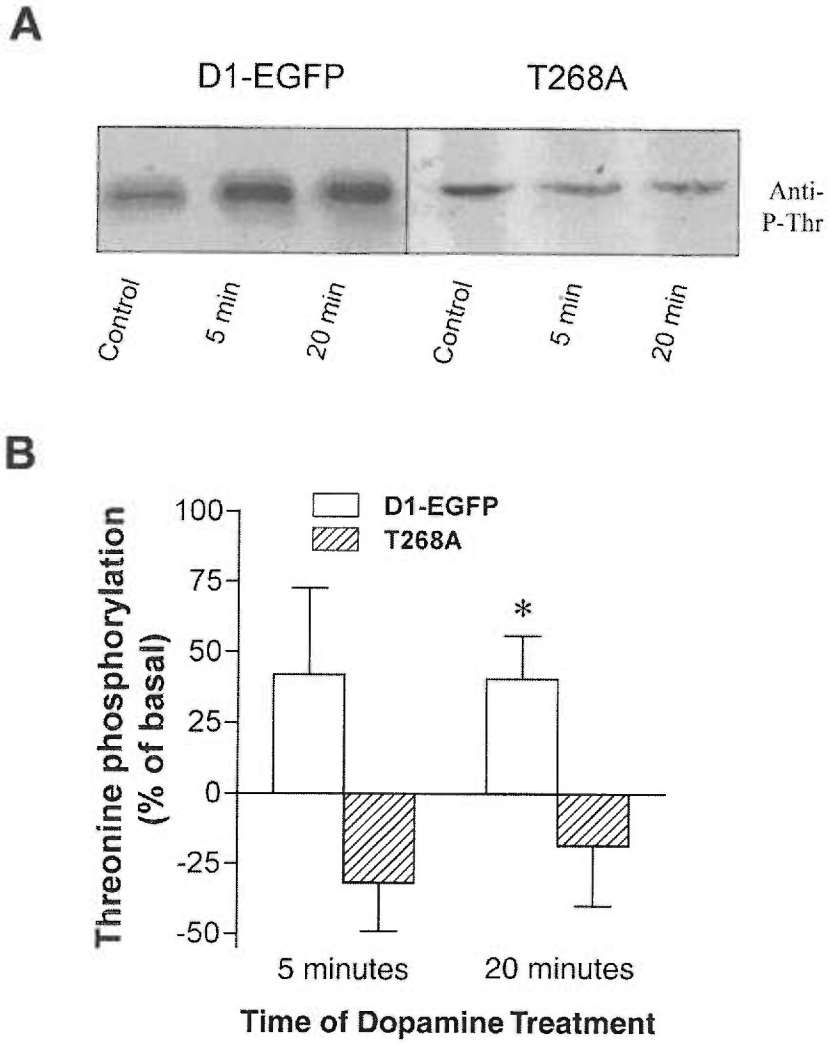

Fig. 2-3. Agonist-induced regulation of phosphothreonine immunoreactivity in D_1-EGFP and T268A receptors.

PVDF membranes used for the analysis of incorporation of ^{32}P depicted in Fig. 2-2B and C were stripped and re-probed with anti-phosphothreonine. ***A****, Representative blot of membranes from cells expressing D1-EGFP or T268A treated with vehicle or with dopamine (25 μM) for 5 or 20 min.* ***B****, Images such as that depicted in panel Awere quantified as described in Materials and Methods. Band optical density was determined, and data for dopamine-treated cells were expressed as the percentage increase over the density for vehicle-treated cells in the same experiment. Specificity of the anti-phosphothreonine antibody was demonstrated by co-incubation with phosphothreonine, which prevented detection of specific bands on the immunoblot (data not shown). Results are the mean ± S.E. of 3 independent experiments. *$p < 0.05$ using a paired Student's t-test.*

To address whether dephosphorylation might be occurring at the plasma membrane, we pretreated cells with 0.45 M sucrose to block receptor internalization. Sucrose pretreatment reduced basal receptor phosphorylation and also prevented dopamine-stimulated incorporation of phosphate (Fig. 2-4).

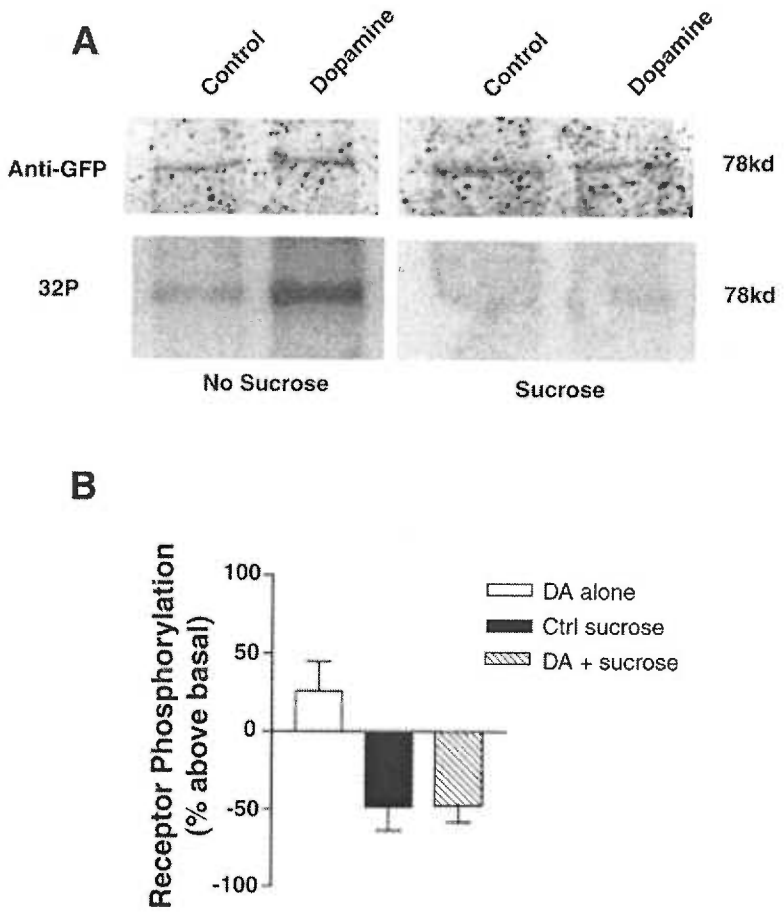

Fig. 2-4. Effect of sucrose on agonist-induced phosphorylation of D1-EGFP receptors.
NS20Y cells were labeled with [^{32}P]H_3PO_4, as described in Materials and Methods. Cells were pretreated with or without 0.45 M sucrose for 1 hour, with dopamine (25 μM) added for the final 5 min. ***A****, A representative immunoblot of D1-EGFP purified as described in the legend to Fig. 2-2 (upper panel), and the corresponding autoradiograph (lower panel).* ***B****, The optical density of bands in the autoradiographs corresponding to D1-EGFP immunoreactivity was quantified as described in Materials and Methods. Results are the mean ± S.E. of 3 independent experiments.*

To determine if the agonist-induced phosphorylation of the receptor was mediated by PKA, metabolically ^{32}P-labeled cells were pretreated with the PKA inhibitor H-89 (5 nM) for 30 min, followed by 5- or 20-min treatment with dopamine (25 μM). Pretreatment with H-89 prevented agonist-induced phosphorylation of D_1-EGFP (Fig. 2-2B and 2-2C).

Characterization of Agonist-Induced Desensitization. Desensitization of the D1 receptor was induced by pretreatment of NS20Y cells with dopamine (25 μM) prior to measuring cyclic AMP accumulation. The responsiveness of either the untagged (D1) or tagged (D1-EGFP) receptor decreased rapidly, so that maximal cyclic AMP accumulation was decreased by 40-50% within 5 min, with no further decrease observed following pretreatment for up to 60 min (Fig. 2-5A; Table 2-3). Thus, tagging the D1 receptor with His6 and EGFP did not alter acute desensitization of the receptor.

Because the T268A mutant was resistant to dopamine-induced phosphorylation, we determined if acute desensitization of the mutant was altered. The magnitude of desensitization of T268A was similar to that of D1-EGFP (Table 2-3; Fig. 2-5B), suggesting that PKA-catalyzed phosphorylation of the D1 receptor on Thr268 is not required for maximal acute desensitization. The responsiveness of S380A was also decreased by ~50% within 5 min of dopamine pretreatment (Table 2-3; Fig. 2-5B).

Mutation of Thr268 has been reported to decrease the rate of desensitization of the D_1 receptor expressed in C_6 glioma cells [54], so it is possible that phosphorylation on this residue regulates desensitization in a cell type-dependent manner. In the present studies, C_6 cells expressing untagged D_1, T268A, or S380A receptors were treated for

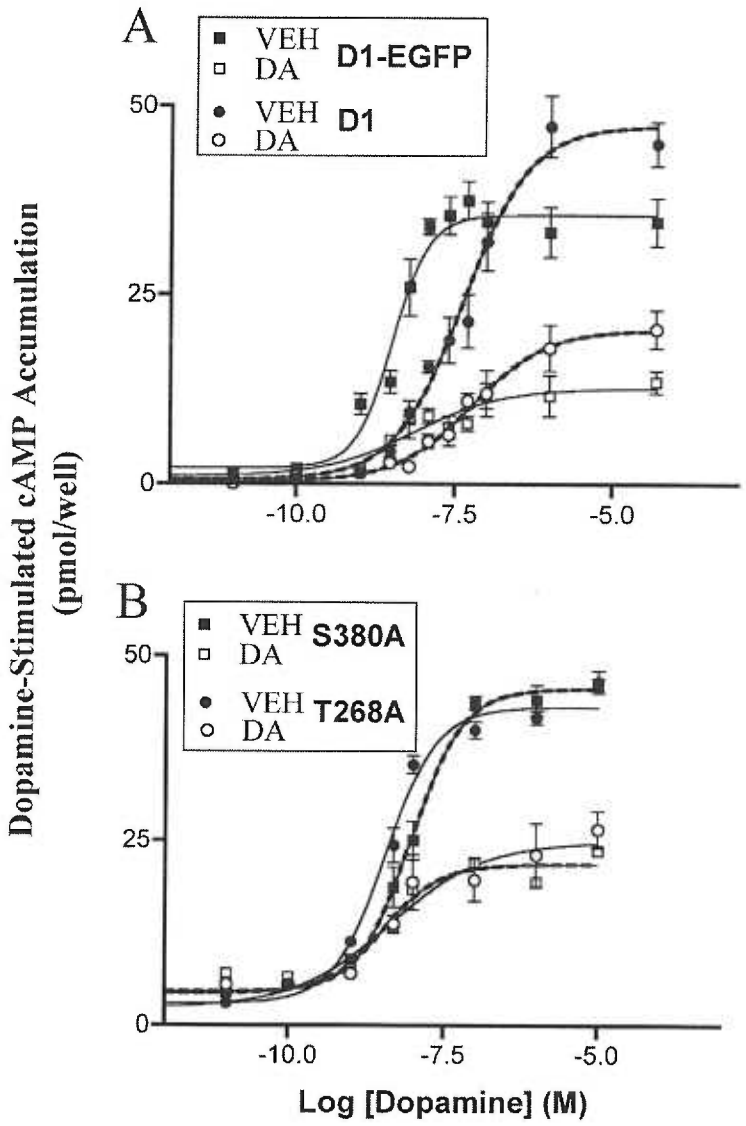

Fig. 2-5. Desensitization of dopamine-stimulated cyclic AMP accumulation by D_1 receptors stably expressed in NS20Y cells.

Cells expressing (A) the untagged D1 receptor or D1-EGFP, or (B) T268A or S380A were pretreated with dopamine (DA, 25 μM) or vehicle (VEH) for 5 min, followed by washing and quantification of dopamine-stimulated cyclic AMP accumulation. The curves shown are the mean ± S.E. of 3 independent experiments.

Table 2-3. Desensitization of D_1 receptor-stimulated cyclic AMP accumulation in NS20Y cells

Time (min)	Receptor				
	Wild-type	D1-EGFP(L)	D1-EGFP(H)	T268A	S380A
5	-43 ± 18	-48 ± 10	-52 ± 11	-57 ± 7	-54 ± 5
60	-54 ± 4	-43 ± 3	-50 ± 10	-63 ± 9	-60 ± 6

NS20Y neuroblastoma cells expressing the indicated receptor were pre-incubated with vehicle or dopamine (25 μM) for the indicated time. Cells were extensively washed with DMEM on ice, and then dopamine stimulated cyclic AMP production was assessed as described in *Materials and Methods*. Data are expressed as the percentage reduction in maximal cyclic AMP accumulation compared to untreated cells. Each value is the mean ± S.E. of 3-6 independent experiments.

one, two, or four hr with 10 nM, 100 nM, or 1 μM dopamine prior to measuring dopamine-stimulated cyclic AMP accumulation. Pretreatment of C6-D_1 cells with dopamine caused a time- and concentration-dependent decrease in subsequent maximal stimulation of cyclic AMP accumulation by dopamine, with the greatest decrease of ~60% being evident after treatment with 1 μM dopamine for 2-4 hr (Fig. 2-6A). As observed for the tagged mutant receptors expressed in NS20Y cells, the time- and dose-dependence of desensitization of the untagged T268A and S380A receptors stably expressed in C_6 cells were similar to that of the D_1 receptor (Fig. 2-6B and 2-6C).

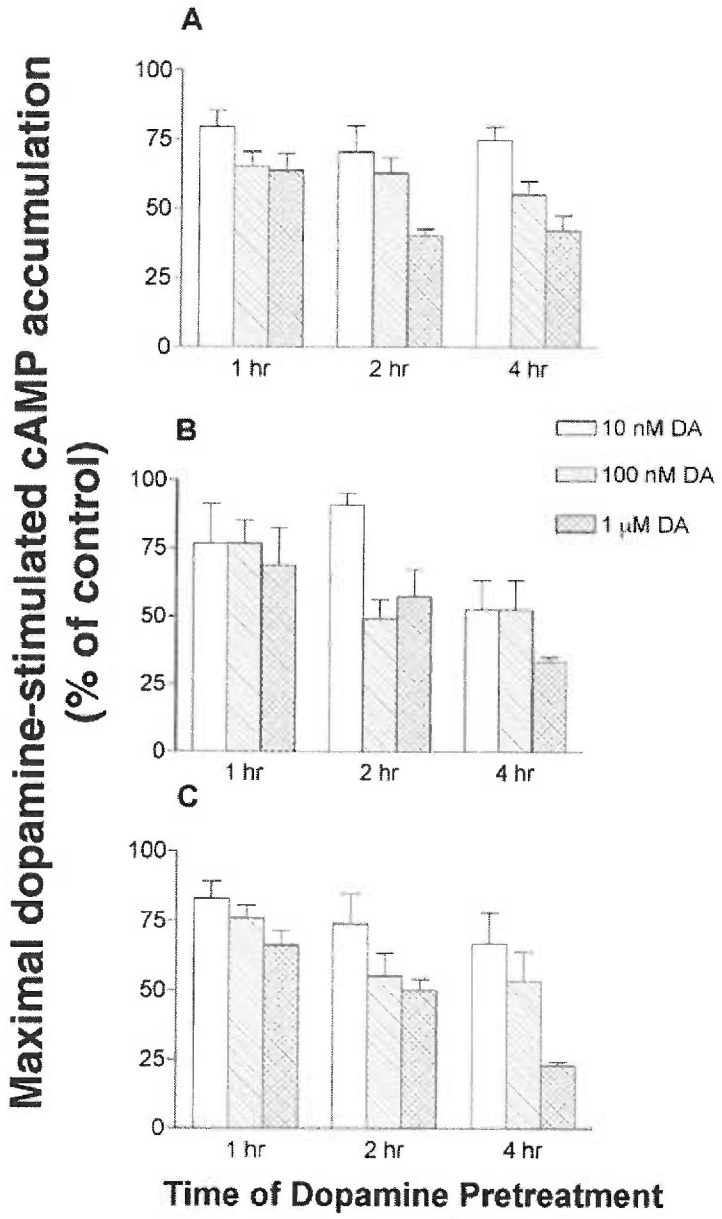

Fig. 2-6. Desensitization of maximal cyclic AMP accumulation by untagged D_1 receptors stably expressed in C_6 glioma cells.

Cells expressing **(A)** the D1 receptor, **(B)** T268A, or **(C)** S380A were treated with the indicated concentration of dopamine for 1, 2, or 4 hr prior to determination of dopamine-stimulated cyclic AMP accumulation as described in Materials and Methods. Values for maximal stimulation were determined by nonlinear regression of dose-response curves, and are expressed as the percentage of maximal stimulation in untreated cells. Results shown are the mean ± S.E. of 5-8 independent experiments.

Intracellular Trafficking. NS20Y cells expressing either D_1-EGFP or T268A were used to evaluate agonist-induced trafficking of the D_1 receptor. Cells were grown on coverslips that were placed in a heated (37°C) chamber on the microscope stage. The addition of dopamine (25 μM) to the medium markedly increased the intensity of fluorescence in the perinuclear region of cells expressing D_1-EGFP (Fig. 2-7A). The perinuclear and plasma membrane fluorescence was measured at the same coordinates of images captured every 2 min for 20 min, and the ratio of the two values was calculated for each time point. The ratio of perinuclear/membrane fluorescence increased linearly for up to 20 min of dopamine treatment of cells expressing D_1-EGFP, at which time the ratio had more than doubled (Fig. 2-7C). In contrast, although intracellular fluorescence increased in dopamine-treated cells expressing T268A (Fig. 2-7B), the accumulation of fluorescence in the perinuclear region was not observed, and the ratio of perinuclear fluorescence to fluorescence in the plasma membrane was only slightly enhanced compared to untreated cells (Fig. 2-7C).

If the altered trafficking of T268A reflects a lack of phosphorylation by PKA, then inhibition of PKA should similarly alter the trafficking of wildtype D_1-EGFP. Cells expressing wildtype D_1-EGFP were pretreated with the PKA inhibitor H-89 (5 nM) for 30 min, followed by the addition of 25 μM dopamine. Inhibition of PKA prevented the dopamine-induced increase in perinuclear fluorescence (Fig. 2-7C).

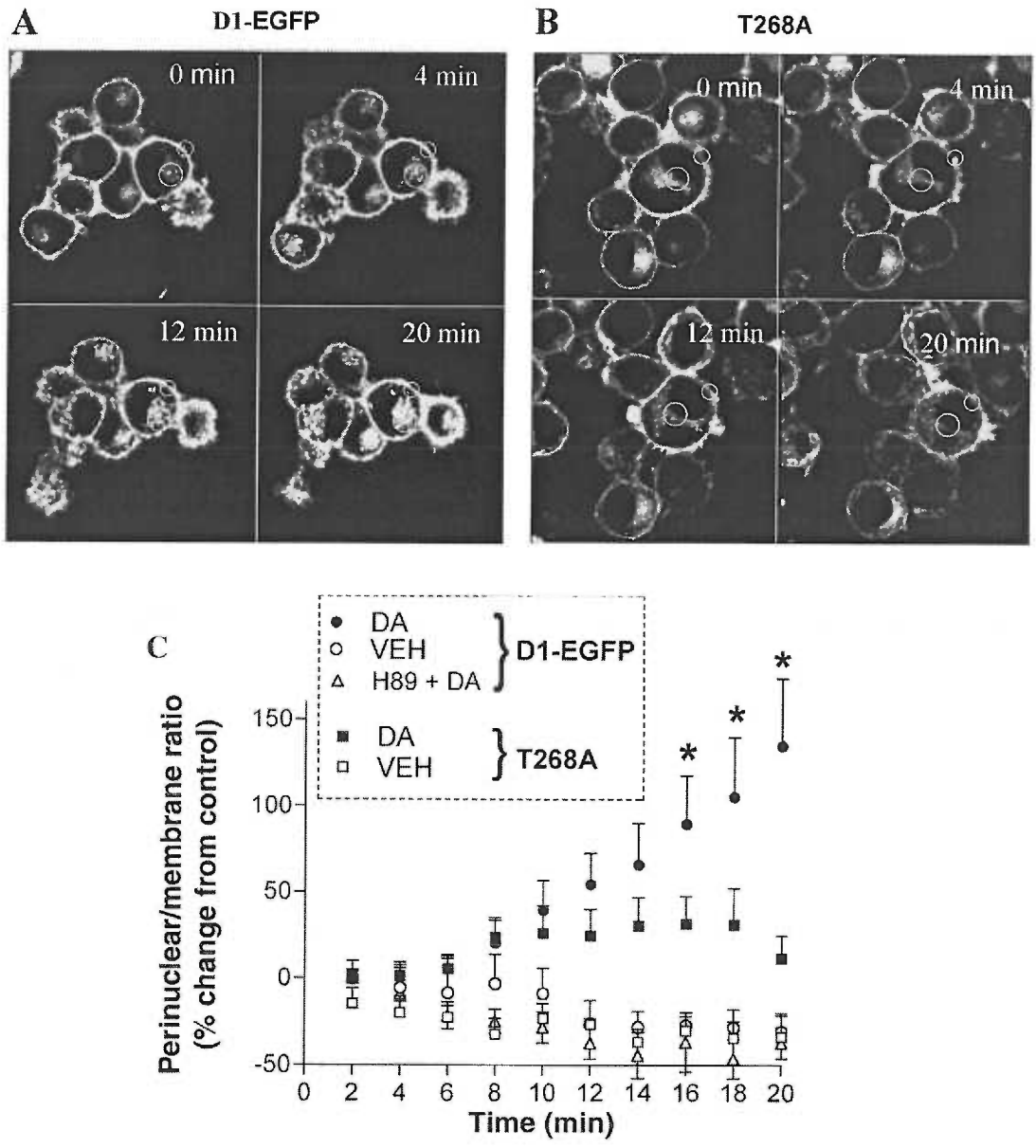

Fig. 2-7. Agonist-induced trafficking of D_1-EGFP receptors in NS20Y cells.

NS20Y neuroblastoma cells expressing ***(A)*** *D1-EGFP or* ***(B)*** *T268A were treated with dopamine (25 μM) at 37°. In some experiments, D1-EGFP cells were pretreated for 30 min with H-89 (5 nM) prior to incubation with dopamine. Cells were scanned at 2-min intervals; the time after the initiation of dopamine treatment is indicated in each frame. The fluorescence intensity within circles placed on the plasma membrane and on the region of perinuclear fluorescence was quantified and a ratio for each time point calculated as shown in* ***(C)****. The area and position of the circles for a given cell were held constant through consecutive scans of that cell.* ***C,*** *The ratio of the fluorescence intensity in the perinuclear region to that in the membrane was calculated and expressed as the percentage change from the ratio at time zero. Cells were treated with 25 μM dopamine (DA) or vehicle for the indicated time. Results are the mean ± S.E. of one cell from each of 19 independent experiments. * $p < 0.05$ compared to T268A cells treated with dopamine, Student's t-test.*

Agonist-induced Receptor Internalization. Biotinylation of cell surface proteins was used to quantify agonist-induced internalization of D_1 receptors. NS20Y neuroblastoma cells expressing either wildtype D_1-EGFP or T268A receptors were incubated with or without dopamine (25 μM), then placed on ice for biotinylation of the remaining surface receptors. Membranes were solubilized, and biotinylated proteins isolated by avidin gel matrix purification. The abundance of surface receptors before and after dopamine treatment was quantified by western blot using an antibody directed against green fluorescent protein (Fig. 2-8A).

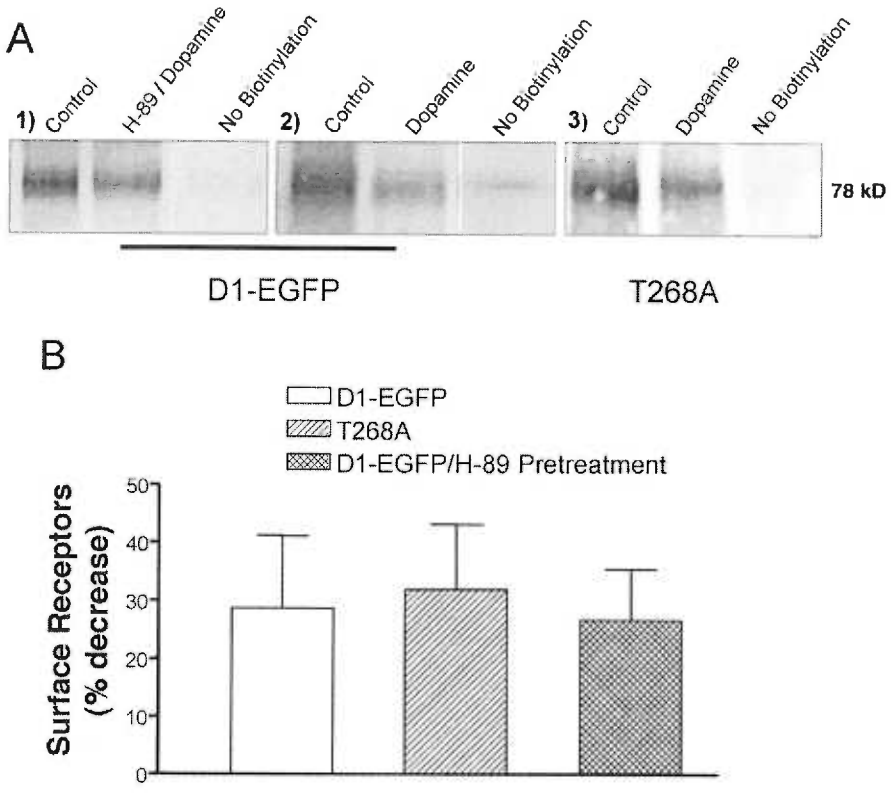

Fig. 2-8. Agonist-induced receptor sequestration in NS20Y cells.
Biotinylation, avidin purification, SDS-PAGE separation, and immunoblotting with anti-GFP were carried out as described in *Materials and Methods*. **A**, Representative immunoblots from **(1)** cells expressing wildtype D1-EGFP that were pretreated with H-89 (5 nM) for 30 min prior to treatment with dopamine (25 μM) for 20 min, or **(2-3)** cells expressing wildtype D1-EGFP or T268A treated with dopamine (25 μM) for 20 min. **B**, Band density was quantified and expressed as the percentage decrease in optical density compared to cells not treated with dopamine. The results shown are the mean ± S.E. from 4-6 experiments.

Treatment with dopamine for 20 min reduced the abundance of D_1-EGFP receptors on the surface of the membrane by 29 ± 11% compared to cells not treated with dopamine (Fig. 2-8B). Similarly, dopamine decreased the abundance of T268A on the surface of the cell membrane by 32 ± 12%, indicating that phosphorylation on Thr268 is not necessary for rapid receptor internalization.

To determine if PKA regulates rapid internalization of the D_1 receptor, dopamine-induced internalization was assessed after 30 min pretreatment with H-89 (5 nM). Under these conditions, dopamine treatment reduced the abundance of D_1-EGFP surface receptors by 27 ± 9%, indicating that phosphorylation by PKA is not a requirement for internalization.

The lack of effect of the T268A mutation on desensitization of cyclic AMP accumulation and on receptor internalization suggested that phosphorylation by PKA regulates a late step in the endocytotic trafficking of the D_1 receptor to the perinuclear region, but that agonist-induced trafficking close to the cell membrane is not regulated by PKA-dependent phosphorylation of Thr268. The agonist-induced increase in T268A-containing endocytic vesicles shown in Fig. 2-9B suggested a similar conclusion. To evaluate this more closely, we tracked the appearance and disappearance of EGFP-containing vesicles in NS20Y cells expressing D_1-EGFP or T268A. Dopamine treatment of either wildtype (Fig. 2-9A) or mutant (Fig. 2-9B) receptors caused the formation of vesicles that remained close to the plasma membrane and appeared to re-merge with the membrane within several minutes of vesicle formation.

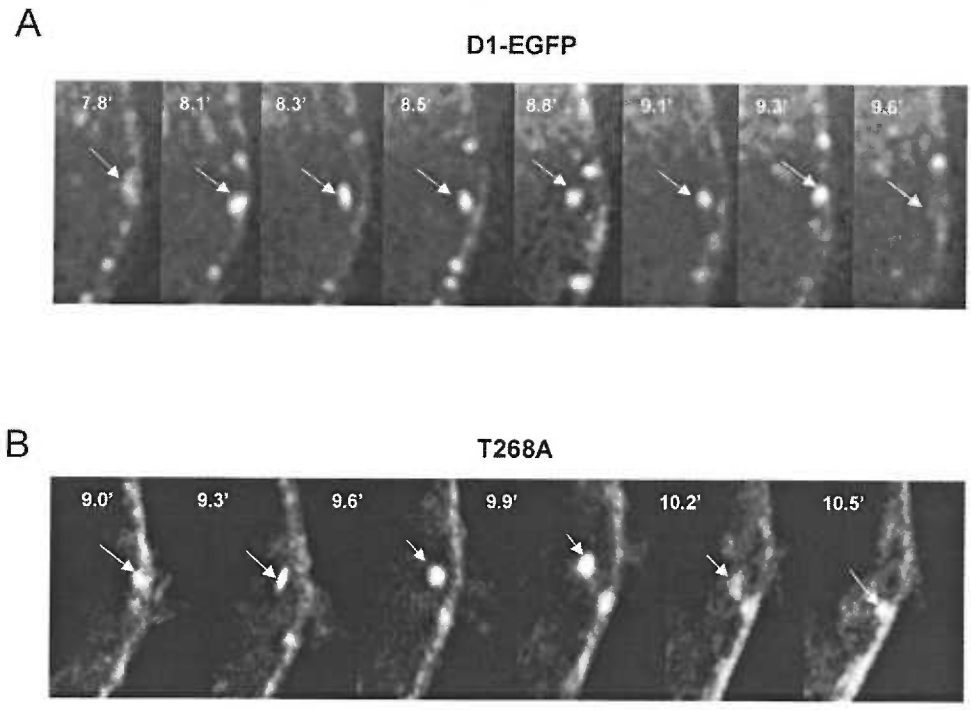

Fig. 2-9. Periplasmic recycling of endocytic vesicles containing the D1-EGFP receptor.
NS20Y neuroblastoma cells expressing D1-EGFP or T268A were treated with dopamine (25 μM) at 37°C, and images were collected approximately every 15 sec. In each panel, the arrow indicates a fluorescent vesicle that formed at the cell membrane, was internalized, then re-merged with the cell membrane. The time after the initiation of dopamine treatment is indicated in each frame. **A,** Cells expressing D1-EGFP. **B,** Cells expressing the mutant T268A.

DISCUSSION

The mechanisms of desensitization and resensitization of the dopamine D_1 receptor are not well understood. Although the high homology of the D_1 receptor with the β_2-adrenergic receptor might indicate that similar mechanisms regulate the responsiveness of the receptors, even subtypes of β_2-adrenergic receptors differ substantially in this respect [100]. For β-adrenergic receptors, a distinction has been made between desensitization that is homologous (resulting from stimulation of the same receptor) or heterologous (resulting from stimulation of a different receptor subtype, or non-receptor mediated activation of a second messenger) [46]. A mechanism for heterologous desensitization is phosphorylation of the receptor by PKA [25,48], whereas homologous desensitization of the β_2-adrenergic receptor involves phosphorylation of the agonist-occupied receptor by both GRK [14] and PKA [79,92]. Heterologous desensitization of the β_2-adrenergic receptor is mediated by PKA-dependent phosphorylation of Ser262, located in the carboxyl terminal portion of the third cytoplasmic loop, a position similar to that of Thr268 in the D_1 receptor [25,48,118]. In contrast, the PKA-dependent component of homologous (agonist-dependent) desensitization of the β_2-adrenergic receptor involves phosphorylation of the PKA site Ser345,346 in the cytoplasmic tail of the receptor [79]. For the D_1 receptor, too, PKA and GRK may both contribute to homologous desensitization [122]. The aim of this study was to determine the role of PKA-catalyzed phosphorylation of the D_1 receptor in agonist-stimulated receptor desensitization and internalization.

To facilitate purification and imaging of the receptor, we attached a polyhistidine tag to the amino terminus and EGFP to the carboxy terminus of the D_1 receptor. We chose NS20Y neuroblastoma cells for stable expression or the receptors because the line has some characteristics of striatal neurons [1], suggesting that it may be more physiologically relevant than non-neuronal cells for the characterization of a neuronal receptor such as the D_1 receptor. Although NS20Y cells have been reported to express endogenous dopamine D_1 receptors at low levels [10], in our hands both D_1 receptors and dopamine-stimulated cyclic AMP accumulation (data not shown) were barely detectable in untransfected cells.

The utility of any tagged molecule depends on the degree to which the tagged protein retains the functional characteristics of the wildtype protein. The polyhistidine- and EGFP-tagged D_1 receptor was similar to the untagged D_1 receptor in several respects. First, the receptors had similar affinities for the antagonist [^{3}H]SCH23390 and for the agonist dopamine. Second, the receptors had similar abilities to couple to G proteins, as indicated by the proportion of receptors with high affinity for dopamine in competition binding assays. Third, the subcellular localization of D_1-EGFP in NS20Y cells was comparable to that determined in other cell types using immunocytochemistry [4,85,87,112]. Finally, both receptors mediated agonist-stimulated cyclic AMP accumulation to a similar extent, although dopamine, in particular, was more potent at D_1-EGFP than at the untagged D_1 receptor.

Agonist-induced phosphorylation of the dopamine D_1 receptor has been demonstrated in Sf9 [86], HEK293 [107], and C_6 [40] cells. We report here that dopamine-

induced phosphorylation of D_1-EGFP expression in NS20Y neuroblastoma cells occurred within 5 min, and that dopamine treatment increased the phosphorylation of the receptor approximately 2-fold. The increased incorporation of phosphate was accompanied by increased phosphothreonine immunoreactivity. The primate D_1 receptor has three potential site of PKA-catalyzed phosphorylation in cytoplasmic domains. Although the predicted rank order of preference of the sites for phosphorylation by PKA is Thr136>Thr268=Ser 380 [58], we mutated the two residues suggested by previous work to be involved in the desensitization of the D_1 receptor (Thr268 [54]), or to be a site of PKA-catalyzed phosphorylation (Ser380 [119]). Whereas mutation of Ser380 had no effect on dopamine-stimulated phosphorylation of the receptor, mutation of Thr268 prevented the dopamine-induced incorporation of phosphate. These data strongly suggest that occupancy of the D_1 receptor by dopamine stimulates phosphorylation of the receptor at Thr268. Pretreatment with the PKA inhibitor H-89 abolished dopamine-induced receptor phosphorylation, supporting the hypothesis that phosphorylation was due to activation of PKA. Dopamine treatment tended to decrease the phosphorylation of T268A and of the D_1 receptor in the presence of H-89, particularly after short (5 min) dopamine treatments. It is possible that dopamine treatment enhances phosphatase activity, an effect that would be detectable only when dopamine-induced phosphorylation is prevented.

Although mutation of Thr262 prevented PKA-dependent phosphorylation of the D_1 receptor, the same mutation had no detectable effect on desensitization of dopamine-stimulated cyclic AMP accumulation in either NS20Y or C_6 cells, suggesting that desensitization in either cell line does not depend on PKA-dependent phosphorylation of this residue. The lack of effect of the PKA inhibitor H-89 on desensitization might

suggest that PKA does not play any role in acute desensitization, but this result should be interpreted with caution because of the considerable evidence implicating PKA in homologous desensitization of the D_1 receptor [40,54,111]. In particular, previous work has demonstrated that mutation of Thr268 reduces the rate more than the extent of desensitization of the D_1 receptor [54]. It may be significant that D_1 receptor desensitization in C6 cells occurred more slowly and to a lesser maximal extent in the present work with this cell line [54,65], which could explain the discrepancy in the observed role of Thr268 in receptor desensitization. Furthermore, since desensitization in NS20Y cells was a maximal at the first time point, it is possible that prevention of PKA-dependent phosphorylation caused a reduction in the rate of desensitization that could not be detected by this experimental design. Species differences may also be important, since the rat D_1 receptor has several Ser/Thr residues in the C-terminus and a potential PKA phosphorylation site at Ser229 that are not shared by the primate D_1 receptor.

It is surprising that inhibition of receptor phosphorylation by PKA, either by treatment with H-89 or by mutation of Thr268, prevented detectable agonist-induced phosphorylation of the D_1 receptor without preventing receptor desensitization. Work with the D_1 receptor [40,107], and other closely related receptors suggests, on the one hand, that GRK-mediated phosphorylation would be detectable even when PKA is inhibited and, on the other hand, that the rate and extent of homologous desensitization would be greatly reduced in the complete absence of agonist-induced phosphorylation. One possibility is that the D_1 receptor is not phosphorylated by GRKs in our line of NS20Y cells. An alternative explanation for these results is that the GRK-phosphorylated D_1

receptor is rapidly dephosphorylated in NS20Y cells, so that our inability to detect agonist-induced phosphorylation when PKA was inhibited was a false negative result. This hypothesis is consistent with the observation that dephosphorylation of the D_1 receptor can occur in the plasma membrane, in the absence of receptor internalization [40], and also with preliminary data indicating that sucrose-induced inhibition of receptor internalization greatly decreases basal and dopamine-induced phosphorylation of the D_1 receptor, and that phosphorylation of T268A or of the D_1 receptor in the presence of H-89 is enhanced by dopamine if cells are pretreated with the phosphatase inhibitor calyculin (unpublished observations). An alternative explanation is that phosphorylation by PKA is a prerequisite for phosphorylation by GRK, so that preventing the former also prevents the latter. This could be similar to the PKA-induced enhancement of β_2-adrenergic receptor phosphorylation by GRK [79], but would also suggest that agonist-induced phosphorylation is not necessary for D_1 receptor desensitization, as has been suggested for several other GPCR subtypes [72,90,96]. Finally as noted above, the particular characteristics of desensitization observed in the present studies might have precluded detection of an effect of PKA inhibition on D_1 receptor desensitization.

In agreement with the previous studies of the D_1 receptor [4,87,112], agonist treatment caused a rapid accumulation of D_1-EGFP in the cytoplasm of NS20Y cells. In cells expressing the wildtype receptor, the accumulation was particularly evident in a perinuclear region that may represent an accumulation of recycling vesicles. Comparing agonist-induced cellular redistribution of the wildtype D_1-EGFP to that of the mutant T268A receptor, we found there to be significantly less accumulation of T268A in the

perinuclear region. Consistent with the hypothesis that the effect of mutation of Thr268 was due to the loss of a PKA phosphorylation site, treatment of cells expressing wildtype D_1-EGFP with H-89 also prevented the accumulation of fluorescence in the perinuclear region.

Vesicles that form at the plasma membrane rapidly (<1 min) fuse to early endosomes, which are comprised of sorting endosomes and the recycling compartment [82]. After initial fusion with sorting endosomes, internalized receptors are thought to be sorted to the recycling compartment or to late endosomes, from which they are recycled to the membrane or shuttled to lysosomes, respectively. Depending on the cell type, the recycling compartment may be concentrated in the juxtanuclear region or dispersed throughout the cell, whereas late endosomes are mainly perinuclear and close to the trans-Golgi network. Agonist-induced trafficking of D_1-EGFP to the perinuclear region could represent accumulation in the recycling compartment or sorting to late endosomes, suggesting that PKA-dependent phosphorylation of the D_1 receptor regulates trafficking into one of these compartments. The perinuclear accumulation of fluorescence showed little colocalization with fluorescent markers for lysosomes or the Golgi apparatus (data not shown), indicating that D_1-EGFP was not accumulating in either of those compartments.

Although trafficking of the D_1 receptor to the juxtanuclear region was inhibited by mutation of Thr268, the T268A mutant desensitized normally and appeared to accumulate intracellularly during treatment with dopamine. We used two approaches to evaluate internalization and recycling of the D_1-EGFP receptor. To quantify receptor

internalization, cell surface receptors were biotinylated after dopamine treatment. Under these conditions, a decreased amount of biotinylated receptor is presumed to represent receptor that has been internalized by dopamine treatment. We found that dopamine treatment reduced the surface density of receptors by a similar amount in cells expressing D_1-EGFP or T268A. In the second approach, we used high-resolution fluorescence microscopy to confirm that, in cells expressing wildtype or T268A mutant receptor, EGFP-containing vesicles form and recycle to the membrane during treatment with dopamine. Thus, as suggested for the cholecystokinin receptor [95], the D_1-EGFP receptor may be resensitized through rapid dephosphorylation in a vesicular compartment adjacent to the plasma membrane. On the other hand, since inhibition of endocytosis of the D_1 receptor in C_6 glioma cells does not prevent receptor dephosphorylation [40], endocytosis may not be required for resensitization.

The signals that regulate the passage of internalized receptor to different endosomal compartments are not known, although receptor phosphorylation sites may contribute to this sorting. Phosphorylation of the epidermal growth factor receptor by protein kinase C shunts the receptor from the late endosome/lysosome pathway into a recycling pathway [6]. NMDA-induced internalization of the AMPA receptor is associated with dephosphorylation of a PKA site on the AMPA receptor GluR1 subunit, and inhibition of PKA reduces the rate of reinsertion of the receptor into the cell membrane[32]. Our data demonstrate that inhibiting PKA-catalyzed phosphorylation of the D_1-EGFP receptor pharmacologically or by mutation of Thr268 prevented agonist-induced trafficking of the receptor to a perinuclear region, without altering receptor internalization or desensitization. The results are consistent with a model in which phosphorylation of

Thr268 of the D_1 receptor by PKA regulates a late step in the sorting of the receptors to the recycling compartment or to late endosomes.

II. TRAFFICKING OF A Thr268-Ala DOPAMINE D1 RECEPTOR MUTANT

(In preparation for submission to Molecular Pharmacology)

ABSTRACT

We have previously demonstrated that removal of the potential cyclic AMP-dependent kinase phosphorylation site Thr268 of the dopamine D_1-EGFP receptor attenuates agonist-induced accumulation of the receptor in the perinuclear region when expressed in NS20Y neuroblastoma cell. We hypothesized that this difference between the T268A mutant and the wildtype D_1-EGFP receptor might be due to a difference in a sorting step in the long recycling pathway. To further investigate which in compartment(s) this might be occurring, we labeled endosomal compartments of the cell to assess for differences in colocalization between wildtype and mutant T268A D_1-EGFP before and after treatment with dopamine. The T268A mutant constitutively accumulated to a greater extent in transferrin and Rab11-labeled compartments than did the wildtype receptor. Upon treating with agonist, the T268A mutant began to move through compartments of the transferrin and Rab11 pathway sooner than the wildtype receptor. When this difference is considered, there was no difference in their patterns of accumulation in and dispersal from these compartments. In addition dopamine treatment induced an increase in the mean area of colocalization events of EEA1 with the mutant T268A, but not wildtype D_1-EGFP, immediately adjacent to the plasma membrane. In conclusion, we hypothesize that in the absence of agonist, the T268A mutant is preferentially trafficked to transferrin and Rab11-labeled compartments in the cell and that this results in T268A moving through the recycling pathway in advance of the wildtype receptor.

INTRODUCTION

Stimulation of GPCRs promotes their redistribution on the surface of the cell and their subsequent internalization by clathrin-dependent or -independent mechanisms [22,24]. Receptor internalization serves as a primary mechanism to attenuate signal transduction and to promote functional resensitization by delivering phosphorylated (desensitized) receptors to an endosome-associate phosphatase [35].

The fate of the internalized receptor is dependent on the pathway that it enters. Generally, GPCRs are believed to sort into three distinct trafficking pathways; 1) rapid recycling, 2) long recycling, and 3) degradation [37] (Fig. 1). Although the mechanisms of sorting among these pathways are poorly understood, there is evidence that serine/threonine phosphorylation affects how receptors are sorted [6,32,115]. Agonist stimulation of the dopamine D_1 receptor causes a redistribution of the receptor, forming clusters in the plasma membrane that appear punctate when visualized by fluorescence microscopy [113]. Within minutes of agonist stimulation, the D_1 receptor is internalized in a dynamin-dependent manner through a clathrin-mediated pathway [112], eventually accumulating in the perinuclear region of NS20Y neuroblastoma cells.

We have previously shown that removal of a cyclic-AMP dependent protein kinase (PKA) phosphorylation site at Thr268 of the dopamine D_1 receptor attenuates the rate of sorting of the receptor to the perinuclear region, implicating PKA in intracellular trafficking of the D_1 receptor. To further our understanding of how the intracellular trafficking pathways of this mutant (T268A) might differ from the wildtype D_1-EGFP receptor, we have compared the colocalization of the mutant and wildtype receptors with

the endosomal markers EEA1 (early endosomal antigen 1), Rab11 (GTPase Rab11), and TF (transferrin).

EEA1 is a protein unique to the cytoplasmic surface of endosomes involved in initial trafficking of proteins after endocytosis [78]. Endosomes that have EEA1 on their surface are referred to as early endosomes. Immediately following sequestration, GPCR protoendosomes fuse with early endosomes. In this process the head group of phosphatidylinositol-3 phosphate (PI3P), located on the surface of the protoendosome, binds to the hydrophobic loop in the FYVE domain of the EEA1 protein [77,101].

Rab11-labeled endosomes are located predominately in the long recycling pathway. Rab11 is a member of the Ras superfamily of small GTPases [105]. Rab11 associates with pericentriole recycling endosomes as well as late Golgi and post-Golgi membranes. The hydrolysis of GTP by Rab11 is required for delivery of transferrin to the cell surface from the pericentriole recycling compartment, but not from sorting endosomes [94].

The TF receptor traffics predominately through the long recycling pathway [45,99]. In addition to labeling pericentriole recycling endosomes, TF is located in early endosomes. Thus, in this study TF was used as a marker to assess colocalization of wildtype and mutant D_1-EGFP receptors in all compartments of the long recycling pathway.

Here we report that the wildtype and mutant receptors traffic through TF and Rab11-labeled compartments at similar rates, except that the wildtype receptor is delayed by about 4 minutes. We present evidence to suggest that this phase shift is caused by greater constitutive accumulation of the T268A mutant receptor in the TF and Rab11

compartments, perhaps due to differences between the tethering of the wildtype and mutant receptors at the plasma membrane. In addition we show that D_1-EGFP receptor-containing early endosomes in mutant but not wildtype receptor-expressing cells fuse to form enlarged endosomes in the peri-plasma membrane region in response to D_1 receptor stimulation.

MATERIALS AND METHODS

Construction and expression of tagged wildtype and mutant receptors. We created EGFP-tagged forms of the dopamine D_1 receptor (D1-EGFP) and a Thr268 → Ala mutant (T268A) by cloning the rhesus macaque D_1 receptor gene[70] into pEGFP-N1 (Clontech, Palo Alto, CA) and mutation using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) as described previously [74]. D_1-EGFP and T268A were stably expressed in NS20Y neuroblastoma cells by calcium phosphate co-precipitation. After selection for G418–resistance, pooled populations of D_1-EGFP- or T268A-expressing cells were isolated using a BD FACSVantage SE flow cytometry system (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, Sigma Chemical Co, St. Louis, MO) supplemented with 5% fetal bovine serum, 5% calf bovine serum, and 600 μg/ml of G418 sulfate (Calbiochem Co, San Diego, CA).

Immunofluorescent labeling.: NS20Y neuroblastoma cells expressing either wildtype or mutant receptors were seeded at 10,000 cells/cm^2 on coverslips (Fisherbrand™ #1) in 12-well clusters containing DMEM. After 48 hr, the cells were treated with 25 μM

dopamine for 0-20 min. The medium was removed and cells fixed in 4% paraformaldehyde for 20 min. The paraformaldehyde was decanted and cells were rinsed 2 x 5 min with TBS (Tris buffered saline) before permeabilizing cells with 1% Triton X-100 for five min. After blocking with 5% goat serum, cells were rinsed with TBS prior to overnight incubation with either 1:200 anti-Rab11 (Transduction laboratories, San Diego, CA) or anti–EEA1 (Transduction laboratories, San Diego, CA), followed by incubation with with 1:500 anti-mouse IgG conjugated to Alex-Red^{TM} 594 (Molecular Probes, Inc.) and treatment with Antifade^{TM} (Molecular Probes, Inc.). Coverslips were placed on a glass slide, sealed with nail polish, and stored in the dark at 4^0C until imaged.

Conjugation of Alexa Red^{TM} to TF. Mouse apo-transferrin (2 mg; Sigma Chemical Co, St. Louis, MO) was incubated in 100 µl of 5 mM Na-bicarbonate on ice for 30 min prior to the addition of 15 µl of $FeCl_2$ (0.15 M) and incubation on ice for 60 min. The solution was dialyzed in phosphate-buffered saline overnight at 4^0C using Spectra/Por^{TM} membrane tubing (6-8,000 MW cut-off; Spectrum Medical Industries, Inc., Los Angeles, CA). The contents of the tube were removed and the 465/280 OD ratio determined. Iron-rich TF was then conjugated to Alexa Fluorophore 594^{TM} using the Alexa Fluor 594 Protein Labeling Kit (Molecular Probes, Inc., Eugene, OR).

Cell labeling by TF. NS20Y neuroblastoma cells grown on coverslips in 12-well clusters were treated for 4 hr with fresh DMEM containing 5 µg/ml of Alexa-Red-conjugated TF to label endosomal compartments throughout the TF pathway. Cells were treated with 25 µM dopamine for 0-20 min, fixed with 4% paraformaldehyde, and prepared for imaging as described above.

Confocal laser-scanning microscopy and processing: Three cells from each treatment condition labeled with one of the 3 endosomal markers were selected at random. Six consecutive sections 200 nm apart, bracketing the center of the cell, were scanned alternating between 488 and 568 nm using a Leica SP confocal laser scanning microscope. System settings were held constant for all imaging. Images were digitally captured and then deconvolved and analyzed using Power HazeBuster^{TM} imaging software (VayTek, Inc., Fairfield, IA). All image analysis to determine colocalization was carried out using software by Power HazeBuster^{TM} . The colocalization of D_1-EGFP with each endosomal marker was determined by first identifying the coordinates of each fluorescent pixel captured at 488 nm (green or EGFP) and 568 nm (red or endosomal marker). Green and red pixels with the same coordinates were identified as being dually labeled (i.e., D_1-EGFP and the endosomal marker were colocalized). By summing the area of contiguous dually labeled pixels we derived the area of each colocalized event. The areas of each of these events were then summed and divided by the total number of events to yield the mean area of colocalization. The extent of colocalization was then determined from the ratio of the colocalization area to the total area of one of the fluorophores in a cell.

The total fluorescence intensity for a given fluorophore was determined by summing the intensities of each pixel of the cell. To obtain the total fluorescence intensity for colocalization we first summed the intensity of EGFP fluorescence in all dually labeled pixels, and expressed this value as a percentage of the total fluorescence intensity for EGFP in the cell.

FRAP (Fluorescence Recovery after Photobleaching). Sterile coverslips (Fisherbrand™ #1) were placed in 12 well tissue culture dishes and seeded with cells stable expressing D_1-EGFP. Photobleaching and imaging of live cells was performed after 48 hours using a Leica TCS SP scanning confocal microscope. Cells were maintained at 37°C using an RC 26 open bath imaging chamber on a PH-1 heater platform (Warner Instruments, Hamden, CT) for the Leica TCS SP. Sections 2 μm in length of the plasma membrane were exposed to an Argon 488 nm laser at 50% maximum for 30 seconds. Images were then captured immediately after bleaching, and at 8, and 16 minutes after bleaching. Data were analyzed using IPLab™ image processing software (Scanalytics, Inc., Fairfax, VA).

RESULTS

Constitutive internalization of wildtype and mutant D1 receptors.

In NS20Y cells expressing D_1-EGFP, receptor autofluorescence was observed not only on the cell membrane, but also in punctate accumulations throughout the cytoplasm (Fig. 3-1A). The distribution of the D_1 receptor mutant T268A was similar, except that in a percentage of cells (~40%), receptor accumulation was most abundant in the perinuclear region (Fig. 3-1B).

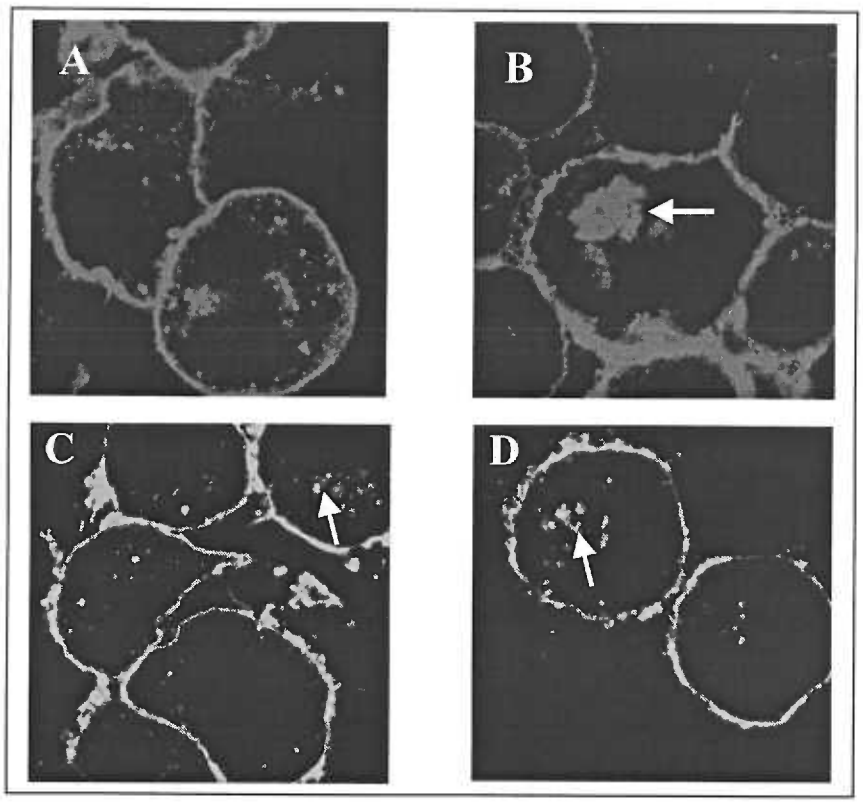

Fig. 3-1. Constitutive internalization of D1-EGFP.
NS20Y neuroblastoma cells expressing wildtype (A & C), and mutant (B & D) D1-EGFP receptors were treated with cycloheximide (10μg/ml for 8 hr). ***A & B****, before cycloheximide treatment some cells displayed high levels of mutant D1-EGFP in the perinuclear region (arrow in panel B).* ***C & D****, following treatment with cycloheximide, wildtype and mutant D1-EGFP receptors are present intracellularly (arrows) and on the cell membrane.*

To determine if the intracellular localization of D_1-EGFP and T268A might at least in part be due to constitutive internalization of receptors, we treated cells with cycloheximide for 8 hr to eliminate intracellular fluorescence resulting from D_1-EGFP receptor synthesis. Following treatment, D_1-EGFP fluorescence was still present within cells, suggesting that constitutive internalization of wildtype and mutant D_1-EGFP receptors was occurring (Fig. 3-1, C-D).

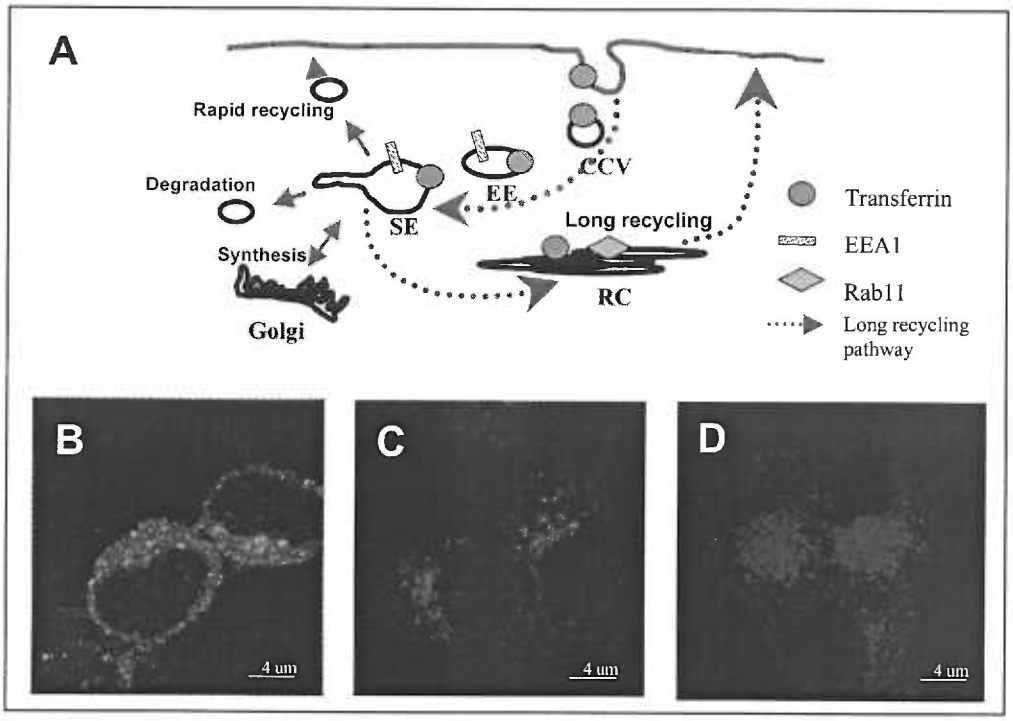

Fig. 3-2. Major trafficking pathways and endosomal markers.
A, Diagram depicts 4 major pathways (rapid recycling, long recycling, degradation, and synthesis) through which GPCRs are thought to traffic. Also shown are endosomes identified by transferrin, EEA1, and Rab11. SE= sorting endosome, EE=early endosome, CCV=clathrin coated vesicle, RC=recycling compartment. Representative images acquired with a laser scanning confocal microscope are shown for NS20Y neuroblastoma cells ***B****, after 4 hr incubation with 5μg/ml of mTF conjugated to the fluorophore Alexa-Red™,* ***C****, labeled with anti-EEA1, and* ***D****, labeled with anti-Rab11.*

Labeling of endosomal compartments with mTF, anti-EEA1, and anti-Rab11.

To track the movement of D1-EGFP receptors through the long recycling pathway we used three endosomal markers: 1) mouse transferrin (mTF) conjugated to Alexa Red to detect endosomes throughout the pathway, 2) anti-EEA1 (early endosome antigen 1) to detect EEA1 on the cytoplasmic surface of early endosomes and sorting endosomes, and 3) anti-Rab11 to detect the GTPase Rab11 on the membranes of endosomes in the perinuclear recycling compartment (Fig. 3-2A). EEA1 immunoreactivity was present throughout the cytosol of NS20Y cells (Fig. 3-2B). Incubation with mTF for 4 hr produced labeling that was most abundant in the perinuclear region (Fig. 3-2C). Labeling of Rab11 was also restricted mostly to the perinuclear region (Fig. 3-2D).

Constitutive localization of D_1-EGFP and T268A in endosomal compartments.

In untreated cells, the area of D_1-EGFP colocalization with either Rab11- or mTF-labeled endosomes, expressed as a percentage of the total area of endosomal labeling (mTF or Rab11; Fig. 3-3A) or EGFP fluorescence (Fig. 3-3B), was greater for the mutant than for the wildtype D_1-EGFP receptor. As indicated above, the distribution of intracellular EGFP was distinctly bimodal in cells expressing T268A, with approximately 40% of the cells having abundant fluorescence in the perinuclear region and the remainder having very little. This bimodal distribution was also reflected in the colocalization of EGFP with either mTF or Rab11, so that some T268A-expressing cells exhibited no colocalization with either marker, whereas for others the extent of colocalization was high. In cells in which colocalization with endosomal markers was detected (that is, was greater than zero), the enhanced colocalization of T268A compared to wildtype D_1-EGFP

with mTF was statistically significant when expressed as a percentage of the total area of mTF labeling, and the enhanced colocalization of T268A with Rabll was statistically significant when expressed as a percentage of the total area of EGFP fluorescence. The other two comparisons suggesting enhanced colocalization of T268A with these two endosomal markers just missed statistical significance (Fig. 3-3).

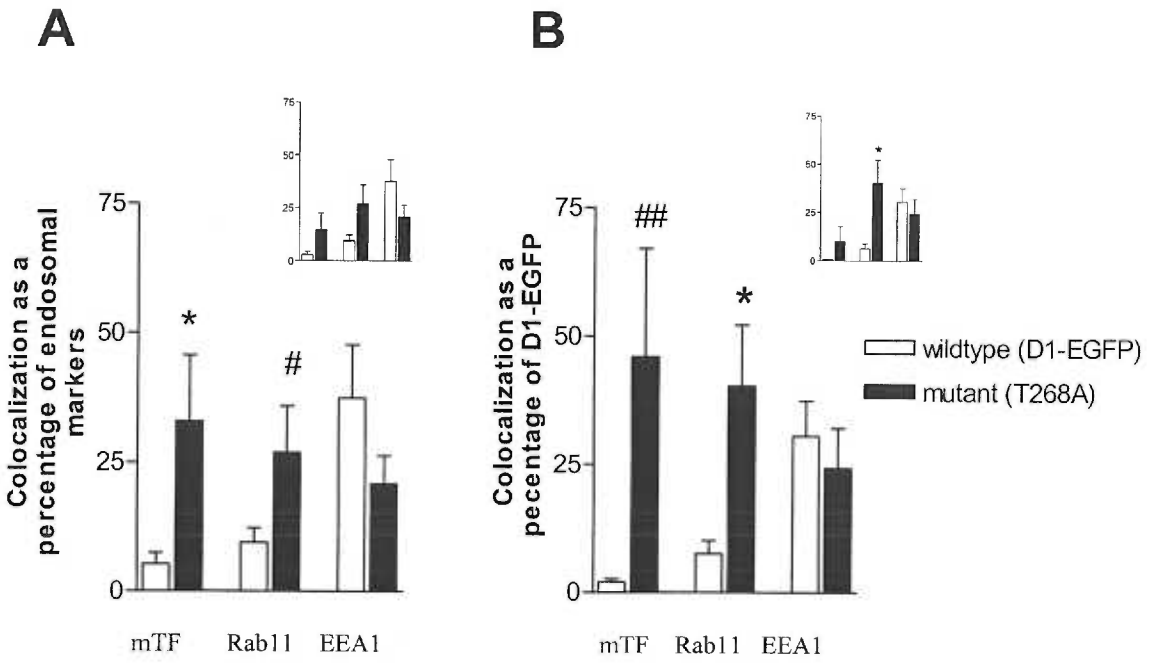

Fig. 3-3. Constitutive colocalization of wildtype and mutant D1-EGFP with mTF, Rab11, and EEA1. *A, Wildtype or mutant D1-EGFP colocalization with mTF, Rab11, or EEA1 as a percentage of the total area of TF- or Rab11-labeled endosomes.* ***B****, Wildtype or mutant D1-EGFP colocalization with mTF, Rab11, or EEA1 as a percentage of the total area of EGFP-labeled endosomes.* ***A and B insets****, Results from all cells analyzed, including those in which no colocalization was detected. # p= 0.085, ## p=0.067, * p<0.05 using Student's t-test. N = 3-9.*

Results from all cells analyzed, including those with no colocalization, show a similar trend except that only the enhanced colocalization of T268A with Rab11 as a percentage of Rab11 labeling was statistically significant (Fig. 3-3, insets). Similar results were

obtained when colocalization was calculated using total fluorescence intensities (the sum of the fluorescence intensities in all pixels containing emissions of a given color; compare wildtype and T268A at 0 min in Fig. 3-5A and B, and in 3-6A and B). Thus, T268A was constitutively sorted into these endosomal compartments to a greater extent than was D_1-EGFP. In contrast, there was little difference between the magnitude of constitutive colocalization of D_1-EGFP or T268A with EEA1 (Fig. 3-3).

Dopamine induced trafficking of D_1-EGFP through endosomal compartments.

To determine if treatment with dopamine altered the colocalization of receptor fluorescence with distinct endosomal compartments and if D_1-EGFP and T268A receptors differed in their response to dopamine, we compared colocalization as a percentage of the area of EEA1-, mTF-, or Rab11-labeled endosomes every 4 min for 20 min of treatment with 25 μM dopamine. Dopamine treatment caused a rapid increase in the area of colocalization of EGFP and EEA1 that was maximal by 4 min, after which there was no further change in receptor colocalization with EEA1 (Fig. 3-4A). There was no significant difference between D_1-EGFP and T268A in the rate or magnitude of dopamine-induced colocalization with EEA1. For either mTF or Rab11, however, treatment with dopamine differentially affected the area of colocalization of D_1-EGFP and T268A with the endosomal marker ($p<0.01$ for the interaction between duration of treatment and receptor subtype on the area of colocalization, 2-way ANOVA). Although

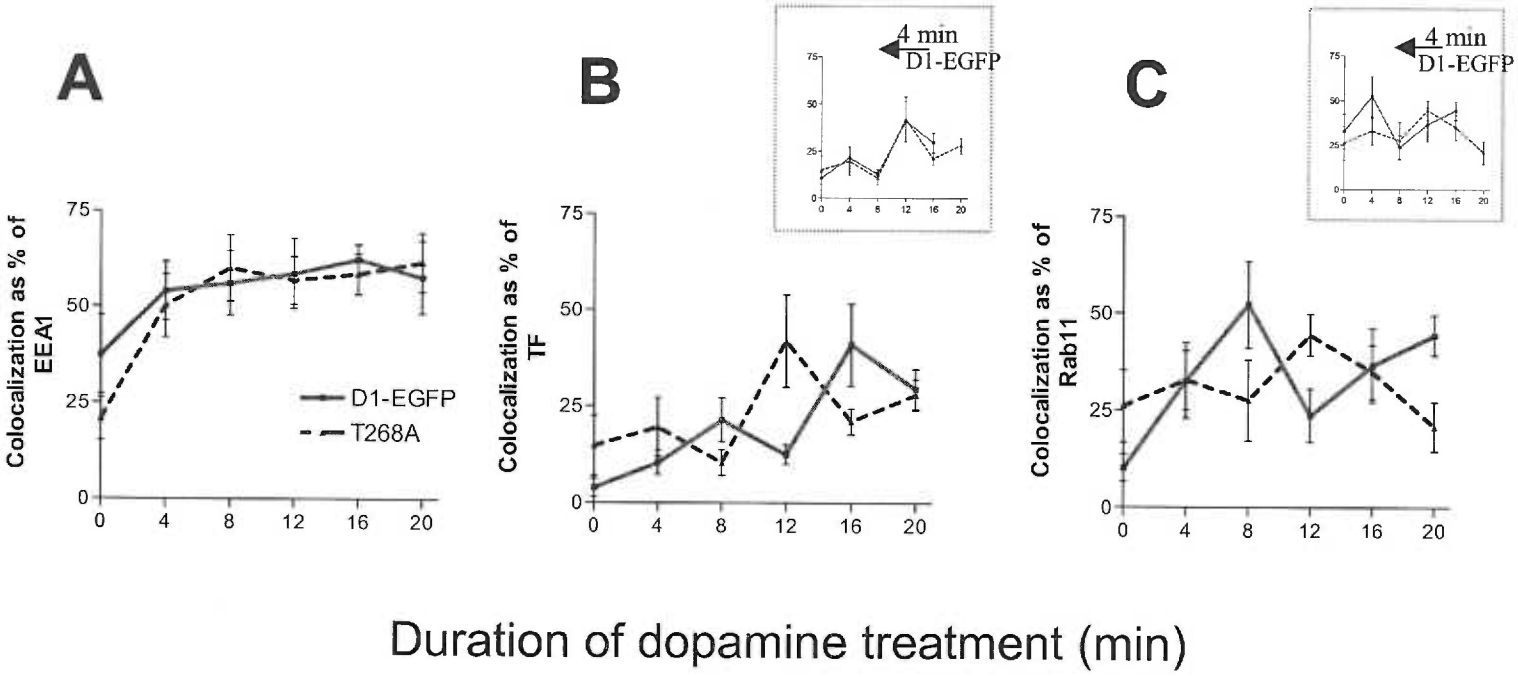

Fig. 3-4. Dopamine induced colocalization of D1-EGFP with endosomal markers.
All results are expressed as the percentage of the total area labeled by the respective endosomal marker. ***A****, Colocalization of EGFP and EEA1.* ***B****, Colocalization of EGFP and mTF.* ***C****, Colocalization of EGFP and Rab11. Insets, Wildtype shifted to left by 4 min. N = 9.*

the receptors passed through mTF- and Rab11-containing compartments at similar rates, the wildtype receptor lagged at the initial phase by 4 min (Fig. 3-4B and 3-4C). When the lag time was taken into consideration, there was no difference in the trafficking of the mutant and wildtype dopamine D_1 receptors (mTF, p=0.98; Rab11, p=0.65; for the interaction between time and receptor subtype; Fig. 3-4B and 3-4C, insets).

When we calculated the percentage of the total cellular EGFP fluorescence intensity that was colocalized with mTF or Rab11, we found that stimulation by dopamine caused a significant increase over time in the colocalization of mutant D_1-EGFP and Rab11 ($p<0.01$ by one-way ANOVA; $p<0.05$ by linear trend analysis), but not in the colocalization of wildtype D1-EGFP and Rab11 (Fig. 3-5). There was a tendency towards increased colocalization of wildtype D_1-EGFP and mTF over time that was not observed with the mutant (Fig. 3-6).

Dopamine-induced trafficking of D1-EGFP through EEA1-labeled compartments.

Although dopamine initially increased the area of colocalization with EEA1, it caused no further increase in the area of colocalization after four minutes. When we analyzed colocalization events throughout the cytosol separately from those in or immediately adjacent to the plasma membrane, we determined that dopamine treatment caused a gradual increase in the mean area of EGFP:EEA1 colocalization events in the cytosol for both D1-EGFP and T268A ($p < 0.05$, linear trend analysis; Fig. 3-7 and 3-8A). In the region near the plasma membrane, dopamine treatment increased the mean area of EGFP:EEA1 colocalization events for up to 16 minutes in cells expressing T268A ($p<0.05$, linear trend analysis), but had no significant effect on colocalization of wildtype D1-EGFP and EEA1 in this region of the cell (Fig. 3-7 and 3-8B).

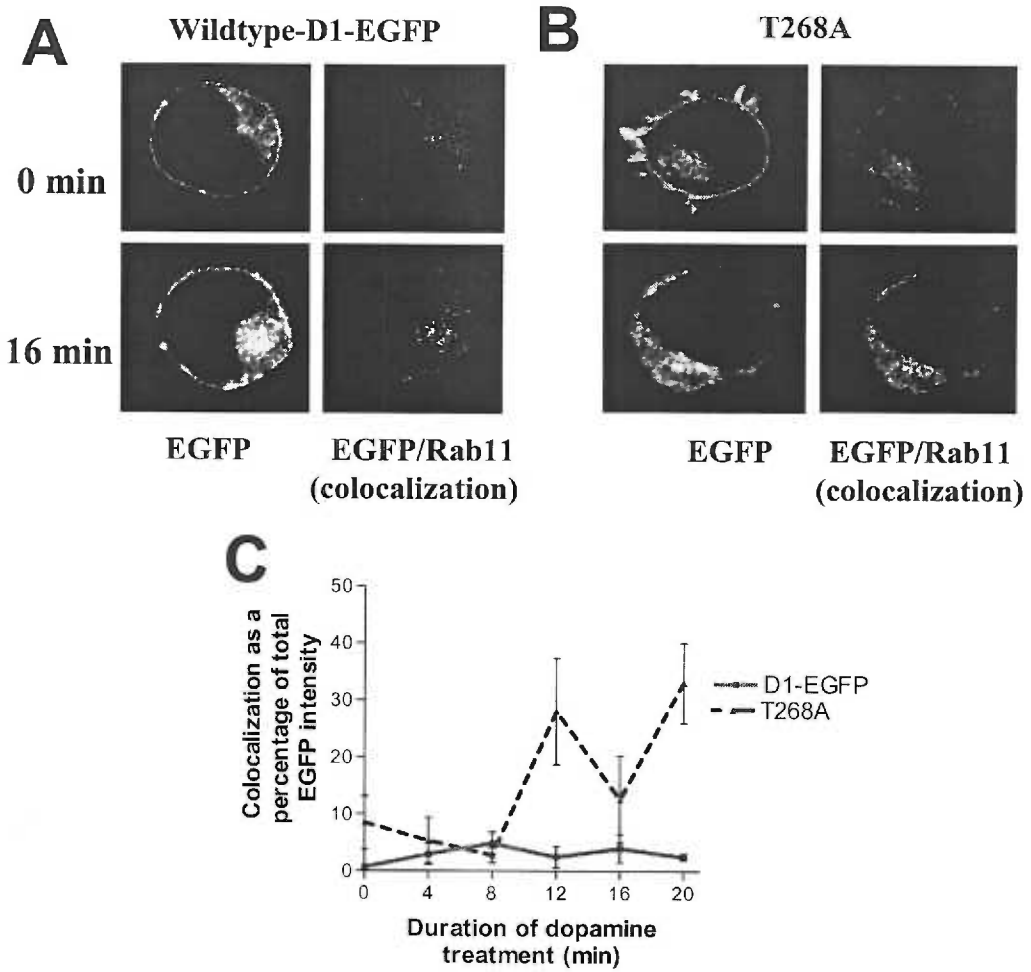

Fig. 3-5. D1-EGFP colocalization with Rab11. ***A & B****, Representative images are shown for total EGFP fluorescence (left) and colocalization of EGFP and Rab11 (right) in cells expressing (A) wildtype and (B) T268A D1-EGFP receptors 0 and 16 min after treatment with dopamine (25 μM).* ***C****, The green fluorescence intensity was determined in dually labeled pixels from 9 randomly selected cells at each time point after the initiation of dopamine treatment and expressed as a percentage of the total EGFP fluorescence intensity in each cell.*

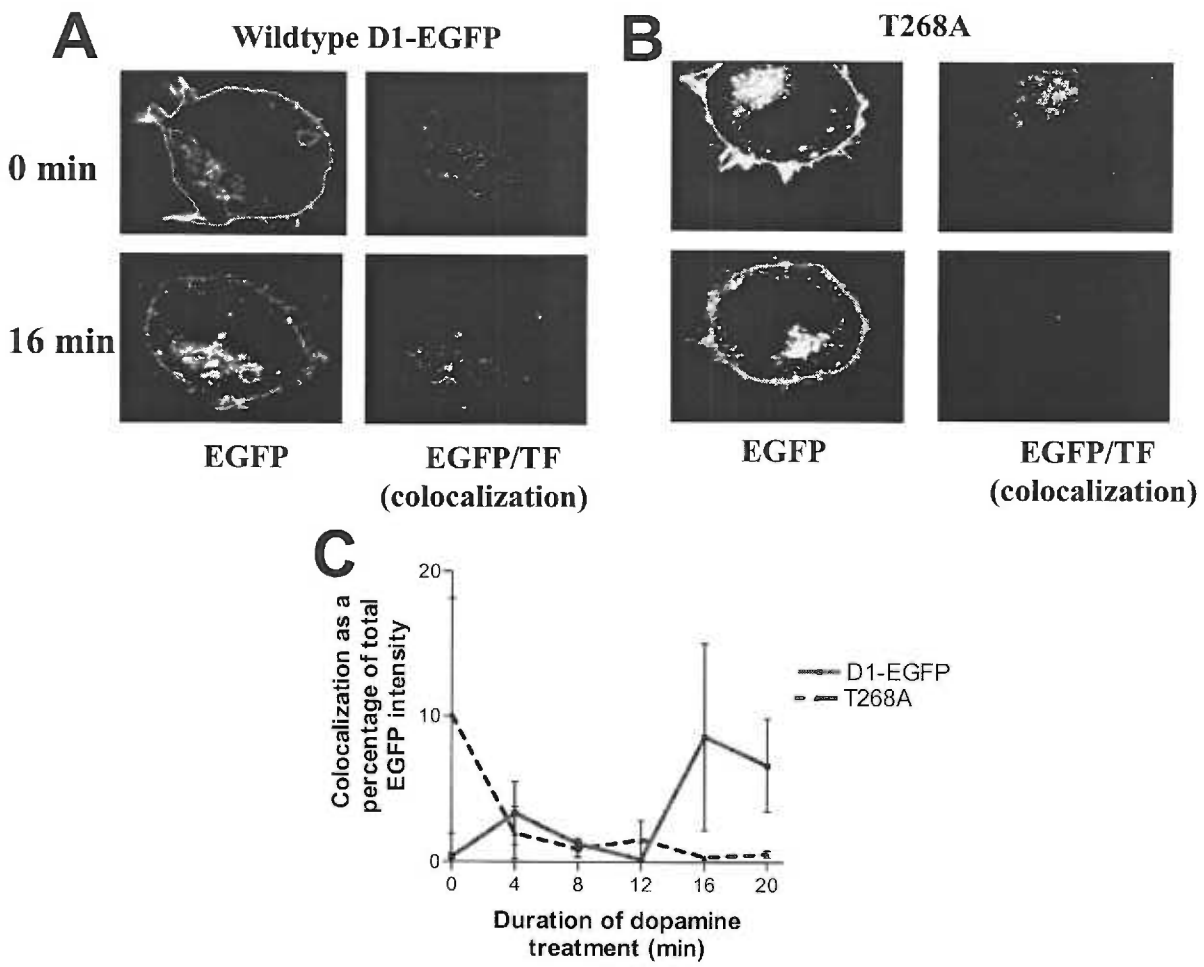

Fig. 3-6. D1-EGFP colocalization with mTF. ***A & B****, Representative images are shown for total EGFP fluorescence (left) and colocalization of EGFP and mTF (right) in cells expressing (A) wildtype and (B) mutant D1-EGFP receptors 0 and 16 min after treatment with dopamine (25 μM).* ***C****, The green fluorescence intensity was determined in dually labeled pixels from 9 randomly selected cells at each time point after the initiation of dopamine treatment and expressed as a percentage of total EGFP fluorescence intensity in each cell.*

.

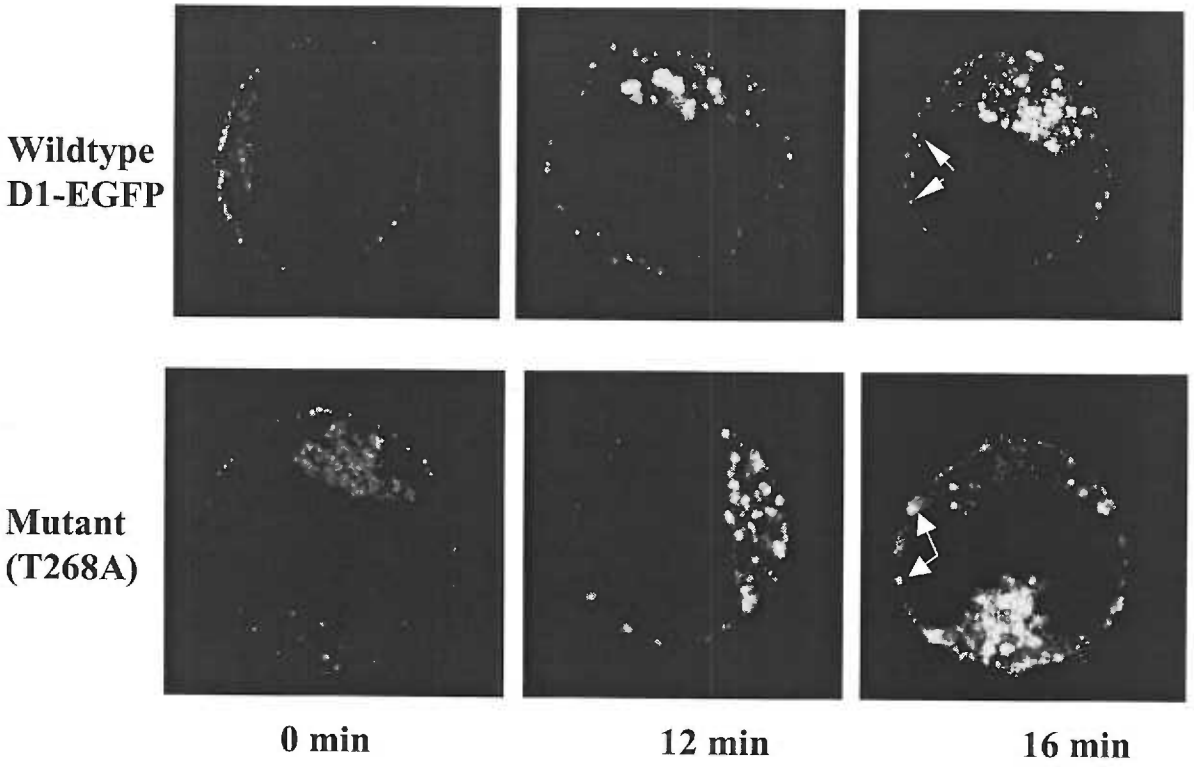

Fig. 3-7. D1-EGFP/EEA1 colocalization after dopamine treatment.
Images from representative cells expressing wildtype D1-EGFP (top row) or T268(bottom row) are shown for cells treated with dopamine (25 μM) for the indicated time. Pixels containing only green or red fluorescence were subtracted from the images, leaving only dually labeled pixels. Arrows indicate colocalization events near the plasma membrane that were increased in size in cells expressing T268A, but not in cells expressing wildtype D1-EGFP. Comparison of untreated and treated cells illustrates the dopamine-induced increase in the total area of colocalization and in the mean area of colocalization events in the cytosol of cells expressing either receptor.

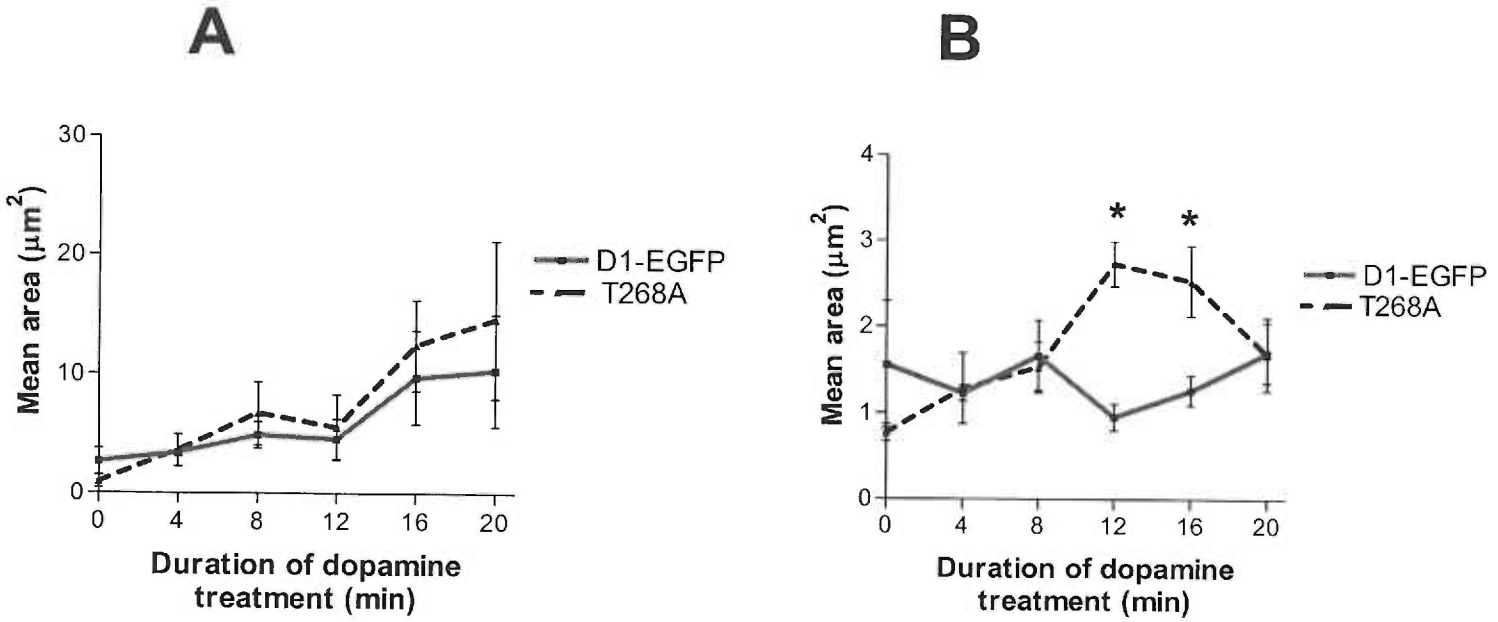

Fig. 3-8. The effect of dopamine on the mean area of D1-EGFP/EEA1 colocalization events. *The mean area of colocalization events (that is, sets of contiguous dually labeled pixels) was calculated in randomly selected cells such as those in figure 7.* ***A****, The mean area of D1-EGFP/EEA1 colocalization events in the cytosol significantly increased in cells expressing either wildtype D1-EGFP or T268A (N = 9),* ***B,*** *The mean area of D1-EGFP/EEA1 colocalization events in or immediately adjacent to the cell membrane increased significantly for T268A , (N = 9) but not for wildtype D1-EGFP. * $p < 0.05$, Dunnetts t-test.*

Comparison of plasma membrane FRAP between wildtype and mutant receptors

To determine if the mobility of D_1-EGFP at the plasma membrane was different from that of T268A, we photobleached the membrane of cells expressing D_1-EGFP or T268A for 30 sec (Fig. 3-9), and then assessed the FRAP. The FRAP, as a percentage of initial plasma membrane fluorescence, had a similar rate of recovery for the wildtype and mutant receptor expressing cells. However, the FRAP, as a percentage of plasma membrane fluorescence after photobleaching, indicates that wildtype D_1-EGFP receptor-expressing cells recover more rapidly than do mutant-expressing cells (Fig. 3-10).

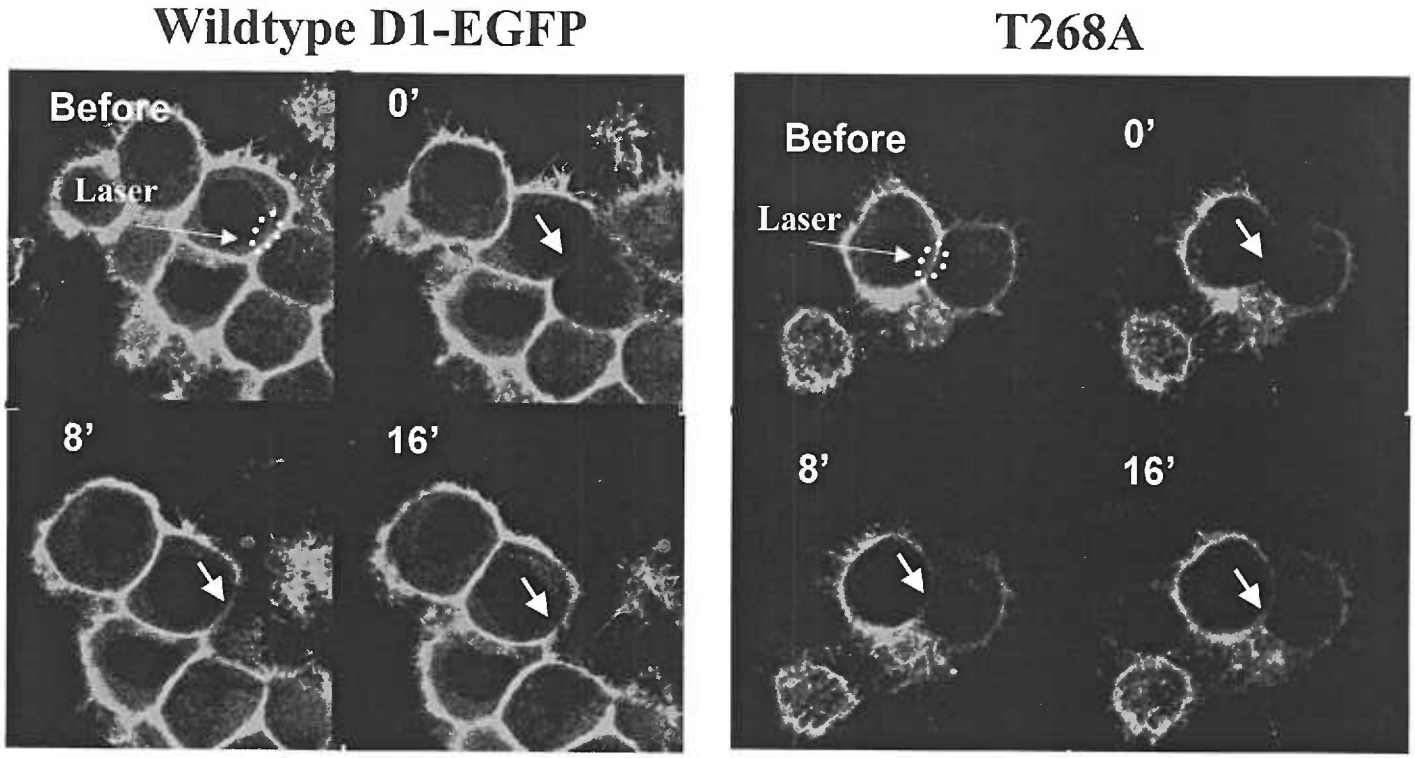

Fig. 3-9: Fluorescent recovery after photobleaching (FRAP). *Live NS20Y neuroblastoma cells expressing either wildtype D1-EGFP or T268A mutant D1-EGFP receptors. Arrows in each panel indicate area of plasma membrane before exposure to the 488 laser and number of minutes after exposure.*

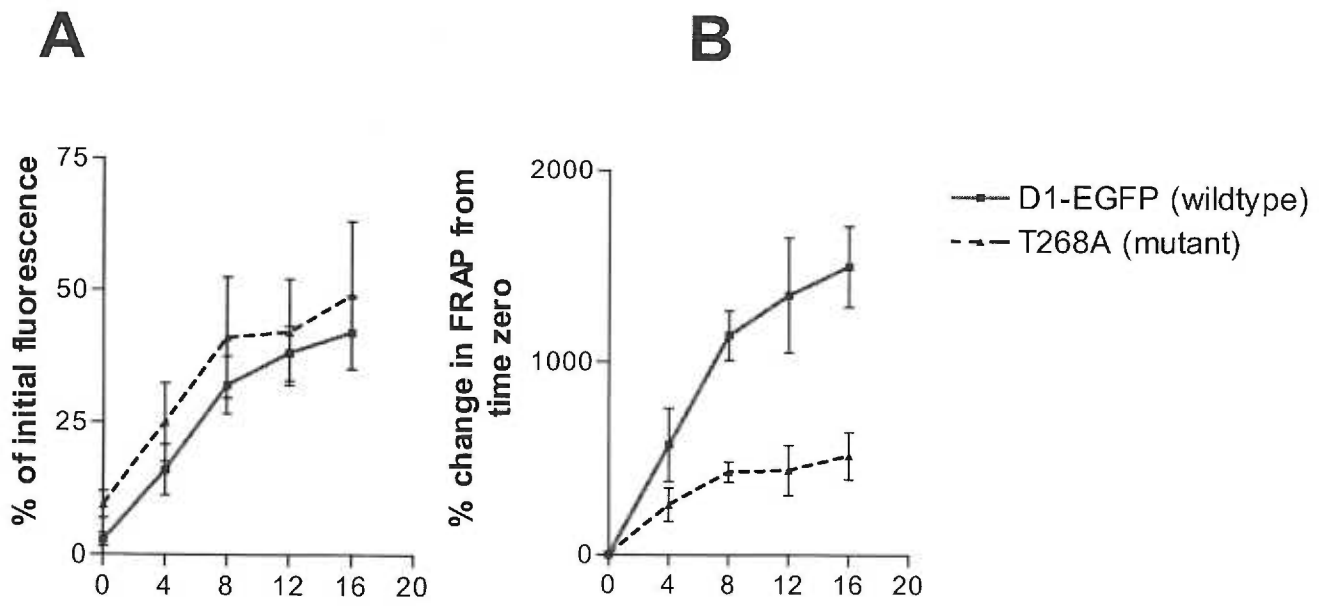

Fig. 3-10: Fluorescent recovery after photobleaching (FRAP) of plasma membrane. **A**, FRAP as percentage of initial fluorescence, and **B**, FRAP as percent recovery after photobleaching.

DISCUSSION

We previously demonstrated that mutation of Thr268, a site of PKA-dependent phosphorylation on the dopamine D_1 receptor, attenuates the rate of dopamine-induced accumulaton of the EGFP-tagged receptor in the perinuclear region of NS20Y neuroblastoma cells [74]. In the present study we investigated the hypothesis that this represents decreased trafficking of the mutant D_1 receptor to a recycling compartment due to an alteration in a PKA-dependent sorting step in the long recycling pathway. A PKA-dependent sorting step might participate in either constitutive or agonist-induced receptor trafficking.

We observed intracellular accumulations of fluorescence in some NS20Y cells expressing the EGFP-tagged receptors, accumulations that were particularly abundant in a population of the T268A-expressing cells. The occurrence of intracellular receptors in cells not treated with a D_1 receptor agonist could result from either newly synthesized or constitutively internalized receptors. The persistence of the intracellular fluorescence following prolonged treatment with cycloheximide to eliminate receptor synthesis suggests that constitutive internalization of wildtype and mutant D_1-EGFP receptors was occurring.

To determine if there was a difference in the trafficking of wildtype and mutant receptors through the long recycling pathway we evaluated colocalization of D_1-EGFP with the endosomal markers EEA1, mTF, and Rab11. EAA1 immunoreactivity was present on the plasma membrane and throughout the cytosol, whereas the endosomal markers mTF and anti-Rab11 produced labeling that was restricted mostly to the perinuclear region. The area of colocalization of EGFP with either Rab11- or mTF-

labeled endosomes, expressed as a percentage of the total area of either endosomal (mTF or Rab11) or EGFP labeling, was greater for the mutant than the wildtype receptor. We obtained similar results when we compared mutant and wildtype D_1-EGFP colocalization with endosomal markers by calculating the ratio of the summed fluorescence intensity of dually labeled and total green pixels. From these results we conclude that the T268A mutant was constitutively sorted into both the mTF- and Rab11-labeled compartments to a greater extent than the wildtype D_1-EGFP receptor. In contrast, the constitutive colocalization of wildtype D_1-EGFP with EEA1 was similar to that of T268A.

Following treatment with dopamine, the area of colocalization of D_1-EGFP and T268A with either mTF or Rab11 was differentially affected. The rate of movement of the receptors in and out of the labeled compartments was similar, except that the wildtype receptor appeared to lag behind T268A by ~4 min. When this lag time for rate of movement was taken into consideration, the data revealed a pattern of accumulation and dispersal that was similar for the wildtype and mutant receptors.

Internalized receptors that use the same pathway may not necessarily be concentrated within different compartments of the pathway to the same extent. As an additional measure of the accumulation of wildtype and mutant D_1-EGFP receptors in mTF- and Rab11-labeled compartments, we determined the fluorescence intensity of D_1-EGFP colocalized with Rab11 or mTF as a percentage of the total D_1-EGFP fluorescence intensity in the cell. Dopamine stimulation caused a time-dependent increase in the fluorescence intensity of T268A in Rab11-containing compartments that was not observed for wildtype D_1-EGFP. On the other hand, dopamine treatment tended to

increase the abundance of the wildtype receptor, but not T268A, in mTF-labeled compartments.

The constitutive accumulation of the mutant D_1-EGFP receptor within mTF and Rab11 compartments and its expeditious movement through the pathway in comparison to the wildtype D_1-EGFP receptor suggest that the mutant receptor may be sorted preferentially into mTF- and Rab11-containing compartments in the absence of dopamine stimulation. Our hypothesis is that when activated by dopamine the mutant D_1-EGFP begins to cycle immediately through mTF- and Rab11-containing compartments of the long recycling pathway, whereas the wildtype D_1-EGFP must first accumulate in this pathway. Our data also indicate that dopamine treatment causes a more gradual accumulation of T268A, but not wildtype D_1-EGFP, in Rab11-containing compartments. Since Rab11 is associated with the trafficking of endosomes to the surface [98], this difference between receptors may contribute to the attenuated dopamine-induced accumulation of the mutant D_1-EGFP receptor in the perinuclear region [74] by facilitating the movement of mutant D_1-EGFP receptors back to the membrane.

We hypothesized that a difference between the sorting of wildtype and mutant D1-EGFP receptors early in the long recycling pathway might play a role in determining the eventual fate of the receptors during dopamine-induced trafficking. To address this we compared the colocalization of wildtype and mutant D_1-EGFP with EEA1-labeled vesicles, which are thought to be early endosomes and sorting endosomes. Dopamine treatment caused two distinct changes in the colocalization of D_1-EGFP with EEA1. Dopamine initially increased the area of colocalization of both D_1-EGFP and T268A with

EEA1, presumably representing agonist-induced internalization of the receptors into CCVs that rapidly fuse with EEA1-labeled early endosomes. Dopamine treatment also caused more gradual changes in the size of the endosomes in which D_1-EGFP and EEA1 were colocalized. The mean area of the dually labeled endosomes increased in the cytosol of cells expressing either mutant or wildtype D_1-EGFP, whereas the mean area of dually labeled endosomes in and immediately adjacent to the plasma membrane was increased by dopamine in cells expressing T268A, but not wildtype D_1-EGFP. Since we measured no change in the mean size of EEA1-labeled vesicles in either T268A or wildtype expressing cells (data not shown), we hypothesis that T268A goes into vesicles that undergo a fate (homotypic fusion) that is different from the fate of the vesicles that the wildtype receptor goes into.

Recent studies have identified several proteins that may link the endocytic machinery of receptors with actin filament dynamics [97]. Thus, we hypothesized that wildtype and mutant D_1-EGFP receptors might differ in the proteins used as links between components of the endocytic and actin cytoskeletal machines that promote internalization. Since the fluidity of the plasma membrane allows for mobility of receptors, we photobleached membranes of cells expressing wildtype or mutant receptors and compared their FRAP for possible mobility differences.

The rate of FRAP as a percentage of initial plasma membrane fluorescence was similar between wildtype and mutant D_1-EGFP expressing cells. However, the FRAP as a percentage of plasma membrane fluorescence after bleaching indicated a more rapid rate of recovery for the wildtype receptor. This apparent difference in the mobility of the receptors coupled with a difference in the mean area of colocalization with EEA1

immediately adjacent to the plasma membrane, suggests a difference between wildtype and T268A in endocytic mechanisms.

Evidence for phosphorylation as a mechanism for regulating the sorting of receptors between compartments of different trafficking pathways is accumulating. Phosphorylation of the epidermal growth factor receptor by protein kinase C shunts the receptor from the late endosome/lysosome pathway into a recycling pathway [6]. NMDA-induced internalization of the AMPA receptor is associated with dephosphorylation of a PKA site on the AMPA receptor GluR1 subunit, and inhibition of PKA reduces the rate of reinsertion of the receptor into the cell membrane [32]. Our data are consistent with a model in which phosphorylation of Thr268 of the D_1 receptor by PKA regulates the sorting of the receptors to a compartment of the long recycling pathway.

IV. DISCUSSION

OVERVIEW

The goal of this dissertation was to determine the effect that the potential cyclic AMP-dependent protein kinase (PKA) sites of the dopamine D_1 receptor have on agonist-induced receptor phosphorylation, adenylate cyclase responsiveness, and trafficking. In the relatively few years since the cloning of the first G-protein coupled receptors (GPCRs), it has become apparent that the functional activity of GPCRs extends far beyond the traditional model of 'receptor→ G protein→ effector'. GPCR activity represents a coordinated balance between molecular mechanisms governing receptor signaling, desensitization, and resensitization. The exposure of a GPCR to its agonist typically causes a rapid loss in its responsiveness. This acute process, termed desensitization, is thought to be a result of the receptor uncoupling from its heterotrimeric G-protein complex and/or sequestration from the cell surface.

With some GPCRs, such as the β_2 adrenergic receptor (β2AR), agonist-induced phosphorylation is thought to be the mechanism that triggers desensitization and internalization. Agonist stimulation of the dopamine D_1 receptor increases the phosphorylation of the receptor [85,87,107]. Agonist-induced incorporation of phosphate by the dopamine D_1 receptor is correlated with desensitization and internalization [40,85,87]. Whether agonist-induced incorporation of phosphate by the dopamine D_1 receptor contributes to desensitization and/or internalization is not clear. A direct approach to assess the effect of agonist-induced phosphorylation on the dopamine D_1 receptor is to

mutate each potential phosphorylation site to determine the impact of that site on phosphorylation, desensitization, and trafficking of the receptor.

It is generally accepted that both second messenger dependent protein kinases [PKA and protein kinase C], and G protein-coupled receptor kinases (GRKs), phosphorylate serine and threonine residues within the intracellular loops and carboxyl-terminal tail domains of GPCRs [62]. The consensus sequences most frequently recognized by PKA have been identified, whereas the consensus sequences recognized by GRKs remain controversial [58]. Therefore, in this dissertation I chose to examine only the contribution of PKA consensus sites to agonist-induced receptor phosphorylation, adenylate cyclase response, and trafficking of the dopamine D1 receptor.

FUTURE ISSUES TO ADDRESS

In this dissertation three primary conclusions were reached; 1) PKA phosphorylation has no effect on desensitization, 2) Thr268 effects agonist-induced phosphorylation, and 3) Thr268 effects a sorting step(s) in the long recycling pathway. In the following sections I address the issues raised by the results of this study which were not addressed in the discussion of each chapter. In each section I will briefly state the background behind each issue and then discuss its implications and propose an approach to further address it.

EGFP alone enhances dopamine potency. Before I could use the dopamine D_1 receptor with the polyhistidine tag fused to the amino-terminus and the EGFP tethered to the C-terminus (from here on to be referred to as D_1-EGFP), I had to consider the possibility that the N- and C-terminal tags might alter the D_1 receptor function.

Therefore, experiments comparing the D1-EGFP and the wildtype D_1 receptor were conducted. These experiments demonstrated that although the tags had no effect on the affinity of the receptor for antagonists or agonists, the tags did cause a 3.5-7 fold shift in agonist potency for inhibition of adenylate cyclase.

Further investigation indicated that this shift in potency was due to the attachment of the EGFP and not the 6XHis, since tagging the D_1 with only the 6XHis had no effect (data not shown). Also, when a myc/His tag (epitope from myc oncogene/6XHis) was attached to the carboxy terminus there was no effect on potency (data not shown). Based on these data I conclude that attaching the EGFP alone to the D_1 receptor was responsible for enhancing the potency of dopamine.

How the EGFP interacts with the D_1 receptor to increase the potency of dopamine is not clear. To determine if this interaction is only in response to dopamine, I suggest that the adenylate cyclase responsiveness to other agonists be evaluated. If other agonists produced different responses, then the effect of attaching EGFP to the D_1 receptor may be related to how dopamine binds to the receptor to alter its interaction with G-proteins. However, if there is no difference between agonists in their effects on adenylate cyclase responsiveness, then I suggest co-transfecting with both D_1-EGFP and untagged D_1 receptor. This would provide insight into whether the enhanced adenylate cyclase responsiveness might be related to the effect of the EGFP on receptor dimerization.

Ser136-Ala prevents D_1 plasma membrane insertion. The potential PKA phosphorylation sites of the primate dopamine D_1 receptor are Thr136 of the second cytoplasmic loop, Thr268 of the third cytoplasmic loop, and Ser380 of the cytoplasmic

tail. In this dissertation I mutated each of these potential PKA sites, and all except T136A were found to traffic normally to the plasma membrane. Why the T136A mutant did not traffic to the plasma membrane was not resolved in this study. One possibility is that the interaction between the T136A mutation and the attached EGFP altered a sorting signal on the D_1 receptor preventing its targeting to the plasma membrane and causing it to be shunted to a degradation pathway. To test this hypothesis I suggest immunoprecipitating the mutant T136A D_1-EGFP using anti-EGFP, and then separating the receptor on a SDS gel for detection by Western blot using a D_1 receptor antibody. Thus, if the size of the construct had been altered by degradation, I would be able to detect bands smaller than the 78 kd D_1-EGFP.

Membrane or cytosolic dephosphorylation. The generally accepted trafficking route of GPCRs following agonist-induced phosphorylation is internalization and subsequent fusion with endosomes in which the pH is lowered, the ligand is released, and the receptor is dephosphorylated by Ser/Thr phosphatases within these endosomes. This event is then followed by recycling of the receptor back to the surface where it can be reactivated by dopamine. In this study, however, the results questioned this paradigm.

Other studies in which the D_1 receptor has been examined indicated that maximal phosphorylation of the D_1 receptor occurs within 1 minute after exposure to agonist [40,120]. Using NS20Y neuroblastoma cells that expressed the T268A mutant D_1-EGFP receptor, I measured no increase in receptor phosphorylation over basal after 5 minutes of stimulation by dopamine. Because agonist stimulation did not induce internalization of the T268A mutant D_1-EGFP receptor until after 4 minutes (determined by real-time

fluorescent microscopy), I concluded that internalization of the D_1-EGFP was independent of agonist-induced phosphorylation and hypothesized that dephosphorylation might instead be occurring at the plasma membrane.

To further test the hypothesized that dephosphorylation of the T268A mutant D_1-EGFP occurs at the plasma membrane, I metabolically labeled NS20Y cells expressing wildtype D_1-EGFP with ^{32}P, and then treated the cells with hypertonic sucrose to block agonist-induced internalization through clathrin coated vesicles. Results indicated that after pretreatment with sucrose, both control and dopamine-stimulated D_1-EGFP receptors were phosphorylated less than receptors of cells not pretreated with sucrose. Therefore, I concluded that dephosphorylation of the D_1-EGFP receptor may occur at the plasma membrane. However, this conclusion assumes that the receptor is only internalized by a mechanism that uses clathrin.

To address the hypothesis that the clathrin pathway may not be the only mechanism for internalization and that dephosphorylation occurs only after internalization via a clathrin-independent pathway, I propose that following hypertonic sucrose pretreatment, sucrose density gradient centrifugation be used to separate out membrane and cytosolic fractions to determine whether membrane bound receptors are dephosphorylated more than cytosolic receptors following agonist treatment. Since hypertonic sucrose would block internalization by clathrin coated pits, the decrease in phosphorylation by surface receptors should be greater than in the cytosol.

Thr268 and regulation of phosphatases. Protein kinase C promotes phosphorylation of the serotonin transporter by causing the release of a Ser/Thr protein phosphatase [3]. Thus, one possible explanation for the decrease in agonist-induced phosphorylation of the

T268A mutant is that Thr268 regulates the association of a protein phosphatase with the D_1 receptor and that phosphorylation of Thr268 promotes the dissociation of the phosphatase from the receptor. When Thr268 is mutated, PKA cannot phosphorylate the receptor at this site, and the receptor-associated phosphatase continues to dephosphorylate the receptor. This model could also explain the decrease in agonist-induced phosphorylation of wildtype D_1-EGFP following pretreatment with the PKA inhibitor H89.

To further address this, I suggest mutating Thr268 to glutamic acid. Since glutamic acid mimics the acidic PO_4 substituent of a phosphorylated residue, this would determine whether Thr268 regulates the association of a D_1 phosphatase with the receptor. I would test this by pretreating T268E with a phosphatase inhibitor followed by an agonist, which should cause no change in receptor phosphorylation since it would mimic the hypothesized effect of T268E which is to prevent association of the receptor phosphatase.

Controversy over T268A and attenuated desensitization. When I mutated either Thr268 or Ser380 there was no effect on agonist-induced desensitization of the D_1-EGFP receptor. Agonist-induced desensitization of either the T268A or S380A mutant D_1-EGFP was maximal at 5 minutes, the same as the wildtype D_1-EGFP . Furthermore, pretreatment with the PKA inhibitor H89 had no measurable affect on desensitization of the wildtype D1-EGFP. In conclusion, I suggested that agonist-induced phosphorylation of potential PKA sites of the D1-EGFP receptor does not regulate desensitization.

It was recently demonstrated by Jiang et al. [54] that expression of the T268A D_1 mutant in C6 glioma cells causes an attenuation in the rate of agonist-induced desensitization of the receptor. When we expressed the T268A mutant D_1 non-tagged receptor in C6 glioma cells, there was no difference in the rate of agonist-induced desensitization between mutant and wildtype receptors. When I expressed the T268A mutant D1-EGFP in NS20Y neuroblastoma cells, I also measured no attenuation in the rate or extent of agonist-induced desensitization. Since I saw no effect of Thr268 on desensitization in either the C6 or NS20Y cells, I suggest that the discrepancy between studies may occur because of a difference between the C6 glioma cell lines used between the studies.

At present there is only one report demonstrating that mutating Thr268 to alanine attenuates agonist-induced desensitization. Therefore, I suggest obtaining the untransfected cell line from the investigator who has reported this result and transfecting these cells with the mutant T268A D_1-EGFP receptor for desensitization studies. The results from these studies would resolve whether the absence of attenuated desensitization in my study was because of a subtle difference in cell lines.

GRK phosphorylation and arrestin. The notion that agonist-stimulated phosphorylation of GPCRs is a prerequisite for internalization stems from studies of the β_2AR where inhibition of GRK phosphorylation sites in the cytoplasmic tail attenuates internalization [64]. In the β_2AR, phosphorylation by GRK promotes the binding of β-arrestin, which leads to association of the β_2AR/β-arrestin complex with an adapter protein that then binds to clathrin and triggers internalization.

Phosphorylation by GRK has been proposed as a prerequisite for the binding of arrestin-like proteins to GPCRs [37,38]. Although there are reports that indicate arrestins are recruited in an agonist-dependent manner to the plasma membrane of D_1 receptor expressing cells, there are no reports to indicate direct association of arrestins with the D_1 receptor [120]. In this study I demonstrated that mutation of Thr268 in the D_1-EGFP receptor greatly attenuated agonist-induced phosphorylation, but had no effect on internalization. Based on these results I suggest that the T268A mutant D_1-EGFP may not be phosphorylated by GRKs, and therefore does not associate with an arrestin-like protein in response agonists. Thus, I conclude that binding of an arrestin-like protein to the D_1-EGFP is not a prerequisite for its internalization.

On the other hand, the lack of a measurable increase in agonist-induced phosphorylation of the T268A mutant does not rule out the potential contribution of agonist-induced phosphorylation in promoting internalization of the receptor. Agonist-stimulation may initiate interactions between phosphatases and kinases that result in phosphorylation remodeling of critical and non critical internalization residues. Thus, depending on which sites are phosphorylated, remodeling may result in no effect on the net change in receptor phosphorylation. Therefore, another possibility is that T268A may prevent increased agonist-induced receptor phosphorylation without preventing phosphorylation of sites critical for internalization.

D_1 internalization and endocytic proteins. The signals that determine which compartments a receptor traffics through are not known. Internalization of GPCRs is typically thought to occur by a clathrin-mediated mechanism. However, evidence

indicates that inhibiting internalization through clathrin-coated vesicles causes rapid stimulation of other internalization mechanisms (caveolae, macropinosome, rafts, etc) [30] [24,63], perhaps as a compensatory response. It has been hypothesized that some of these mechanisms are shared between pathways [22].

The GTPase dynamin is required for endocytosis of the D_1 receptor [112]. A number of proteins have been identified as potential links between dynamin and the endocytic and actin cytoskeletal machines [53,116]. Phosphorylation of the D_1-EGFP receptor may regulate the association of the receptor with endocytic proteins. A difference between the wildtype and the T268A mutant D_1-EGFP in association with endocytic proteins may account for the different trafficking fates of the receptors.

Whether dynamin is a requirement for constitutive internalization and if so whether potential PKA phosphorylation sites play a role in the dynamin-dependent pathway is not clear. To address this, I propose expressing the K44E dominant negative dynamin mutant in cells that express the wildtype or mutant D_1-EGFP receptors. Trafficking results obtained from studies using these cells would provide insight into the role of the dynamin-dependent pathway in the regulation of constitutive and agonist-induced internalization.

Confirmation of D_1-EGFP fluorescence

In this study purification of the D_1-EGFP receptor by first using nickel affinity chromatography to separate out D_1-EGFP receptors with the 6XHis on the N-terminus, and then by immunoprecipitation using an antibody to the EGFP on the C-terminus revealed a single 78 KD band when detected on Western Blots using anti-EGFP. Thus,

these experiments indicated no degradation of the receptor was occurring. However, the question remains as to whether the EGFP alone could be contributing to the fluorescence, since by purifying first with the 6XHis all D_1-EGFP receptors detected by anti-EGFP would be full length.

To resolve this issue I suggest immunoprecipitating the D_1-EGFP using only anti-EGFP. By running the protein obtained from this purification procedure on an SDS gel, transferring to a membrane, and then detecting with anti-EGFP, it will be possible to determine if EGFP alone is present in the cell.

Desensitization and striatal neurotransmission. The dopamine D_1 receptor is most abundant in the striatum where dopaminergic neurons release dopamine. In this region 90-95% of neurons are GABAergic. GABAergic neurons in the striatum are the major targets for dopamine released from dopaminergic neurons of the substantia nigra [102]. Dopamine stimulation of the D_1 receptor in this region can promote depolarization-evoked release of GABA from GABAergic neurons [47]. Thus, agonist-induced desensitization, uncoupling, and internalization of the dopamine D_1 receptor may modulate neurotransmission by effecting release of GABA in the neostriatum.

In this study results indicated that agonist stimulation of the dopamine D_1 receptor, when expressed in NS20Y mouse neuroblastoma cells, caused rapid desensitization of the receptor and was not affected by mutation of individual potential PKA sites, or by pharmacological inhibition with the PKA inhibitor H89. Based on these results I speculate that when dopamine is released into the striatum via the substantia nigra it leads to rapid desensitization of the dopamine D_1 receptor by a mechanism other

than phosphorylation of Thr268 or Ser380. To further investigate whether potential PKA sites play a role in dopamine-induced D1 desensitization in the striatum I would develop of a mouse line deficient in each of the potential PKA sites, and then assess for changes in GABA release from GABAergic neurons.

Aside from PKA phosphorylation, another potential mechanism that may contribute to desensitization of the dopamine D_1 receptor and affect neurotransmission is phosphorylation by GRKs. A determination of the contribution of these kinases to phosphorylation of the D_1 receptor would help resolve the role that phosphorylation plays in desenzitization of the D_1 receptor.

Specific GRK inhibitors that are cell permeable were not available at the time of this study. Therefore, in this study it was not possible to pharmacologically block potential GRK phosphorylation of the D_1 receptor. An alternative approach to determine the effect of GRK phosphorylation on desensitization would be to overexpress dominant negative GRK subtypes in cells expressing D_1 receptors deficient in potential PKA sites. These dominant negative GRK subtypes would attenuate GRK phosphorylation and thereby allow evaluation of the role of receptor phosphorylation by GRKs on desensitization of the D_1 receptor.

In this study I examined the role of potential PKA phosphorylation sites of the D_1 receptor on internalization after overexpression in NS20Y neuroblastoma cells and found no effect. To address the role of these sites in both whole animal and primary culture, I propose developing a mouse line that expresses the D_1 receptor that is deficient in potential PKA sites and that is fused to green fluorescent protein. This approach would

allow the use of electrophysiological methods to study the effects of PKA mutations on neurotransmission while also assessing how these changes relate to internalization in the striatum and other regions of the brain that express the D_1 receptor.

Novel intracellular signaling: To control promiscuous phosphorylation and inappropriate cross talk between receptor signaling pathways within the cell, some receptors bind specialized proteins that possess binding sites for kinases. Binding of kinases to these specialized proteins restricts random phosphorylation within the cell. An example of a receptor that uses specialized proteins to regulate kinase activity is the β_2AR [93]. When phosphorylated by GPCR-kinases, binding of the protein β-arrestin occurs. In addition to promoting internalization and uncoupling, β-arrestin also serves as a "scaffolding protein" for the binding of kinases such as MAP kinase kinase, c-Jun amino-terminal kinases-3, and apotosis signal-regulating kinase 1. In order for these kinases to become active they must first be internalized with the receptor [93].

At present there have been no reports of a direct association of the dopamine D1 receptor with an arrestin-like protein. However, agonist treatment of cells that express both the D_1 receptor and β_2AR does stimulate recruitment of β-arrestin to the cell membrane. Thus, it is possibile that the D_1 receptor binds to an arrestin-like protein that could serve as a scaffold for other protein kinases.

In this study mutation of Thr268 had no effect on desensitization or internalization of the D_1 receptor, but, the mutation did alter total phosphorylation of the receptor. Although this suggests that phosphorylation of the D_1 receptor is not required for internalization, the study did not address whether reduced phosphorylation could have an affect on attachment of scaffolding-like proteins to the receptor. Thus, I propose that

when the Thr268 site is phosphorylated it may bind a scaffolding protein that in turn binds proteins that are involved in signaling. In the absence of Thr268 the normal intracellular signaling cascades might not be triggered and the receptor would then enter into a different set of trafficking compartments. Such a relationship between the D_1 receptor and a scaffolding protein would likely depend on both the cell and brain region in which the receptor was expressed.

This hypothesis in which intracellular signaling proteins associate with a scaffolding-like protein that normally associates with the D_1 receptor except when Thr268 is not phosphorylated, could be investigated further by co-immunoprecipitation of both wildtype and the mutant Thr268 followed by separation on a non-reducing polyacrylamide gel to prevent separation of receptor/signaling protein complexes. Then once transferred to a membrane, the membrane could be probed for suspected signalling proteins.

Another approach with which to examine intracellular signaling effects of the T268A mutant would be to measure cell-dependent or region-dependent signaling by looking for possible changes in effects on potassium and L-type calcium channels. Although this could be performed in a stable cell line that expresses either the T268A or wildtype D_1 receptor, an *in vivo* approach would be more informative. Thus, access to a mouse line that expresses the T268A would provide an opportunity to obtain primary cultures for studying the effects of changes in intracellular modification or signaling.

Mechanistic role of Thr268. The mechanistic role of Thr268 in altering phosphorylation and trafficking of the D_1-EGFP receptor was not resolved in this study. To effectively arrive at a plausible mechanism to account for the effect of Thr268, I propose conducting

a series of systematic experiments. To first determine whether the mechanism through which the Thr268 mutation produces its effect depends on phosphorylation, I propose that Thr268 be substituted with the residue glutamic acid which would mimic phosphorylation. If agonist-induced phosphorylation of the Thr268 site is a requirement for directing the D_1 receptor to specific endosomal compartments in the long recycling pathway, then substituting the Thr268 site with glutamic acid should result in no change in the trafficking of wildtype D_1 receptor. However, if a difference in trafficking occurred following substitution by glutamic acid, I would conclude that the difference in signals regulating trafficking resulted because of a conformational change rather than the absence of Thr268 phosphorylation.

Once it has been determined whether constitutive internalization of Thr268 is a prerequisite for normal trafficking of Thr268, it should be possible to speculate with more certainty about the potential interactions between Thr268 and other proteins in the cell that regulate sorting of the receptor between compartments of the long recycling pathway.

In the long recycling pathway the initial point at which trafficking of the mutant receptor diverges from the wildtype could provide clues about the mechanism through which Thr268 acts. My results indicate that in response to agonist the mean area of colocalization of the Thr268 mutant and EEA1 increased immediately adjacent to the membrane, whereas there was no change in the mean area of colocalization between EEA1 and the wildtype. This early departure in the T268A and wildtype pathways is reason to hypothesize that a difference in endocytic mechanisms, as well as sorting between compartments, may be the initial step at which T268A and the wildtype diverge.

Because it is not known which endocytic mechanism(s) (clathrin, caveolae, rafts, etc.) contribute to internalization of the D_1 receptor, and because each of these mechanisms might respond differently to the mutation T268A, I propose; 1) a series of experiments to determine whether previously defined internalization motifs of other GPCRs play a role in internalization of the D_1 receptor be conducted, and 2) a series of experiments to determine the impact of Thr268 on identified D_1 receptor internalization motifs that effect wildtype D_1 internalization.

The dopamine D_1 receptor has two motifs that have been identified as important in the internalization of other GPCRs. At residue 328, in the seventh transmembrane region, and at residue 345, in the cytoplasmic tail, is an NPXXY and an LL motif, respectively. Evidence from previous studies indicates that these are recognition sites for the adaptor protein AP2. For GPCRs, the NPXXY motif (X represents any amino acid) in the seventh transmembrane domain has been assumed to be an internalization sequence. The tyrosine residue in the NPXXY is essential for internalization of the β_2AR [7] and neurokinin 1 receptor [15]. NPXXY is believed to interact with the adaptor protein such as AP2 of the membrane clathrin lattice, a process that leads to efficient endocytosis via clathrin-coated pits. Mutagenesis of the NPXXY tyrosine residue to alanine in the D_1 receptor would determine if the motif plays a role in internalization and if so how it might be involved in regulating other functional responses. Thus, this approach would lead to a more complete understanding of the mechanisms associated with internalization as well as possible endocytic proteins that may interact with the cytoskeleton to promote internalization of the D_1 receptor.

LITERATURE CITED

1. Amano T., Richelson E., and Nirenberg M. (1972) Neurotransmitter synthesis by neuroblastoma clones (neuroblast differentiation-cell culture-choline acetyltransferase- acetylcholinesterase-tyrosine hydroxylase-axons-dendrites). *Proc. Natl. Acad. Sci. U. S. A* 69, 258-263.

2. Anastasiadis P.Z., Kuhn D.M., Blitz J., Imerman B.A., Louie M.C., and Levine R.A. (1996) Regulation of tyrosine hydroxylase and tetrahydrobiopterin biosynthetic enzymes in PC12 cells by NGF, EGF and IFN-gamma. *Brain Res.* 713, 125-133.

3. Apparsundaram S., Galli A., DeFelice L.J., Hartzell H.C., and Blakely R.D. (1998) Acute regulation of norepinephrine transport: I. protein kinase C- linked muscarinic receptors influence transport capacity and transporter density in SK-N-SH cells. *J. Pharmacol. Exp. Ther.* 287, 733-743.

4. Ariano M.A., Sortwell C.E., Ray M., Altemus K.L., Sibley D.R., and Levine M.S. (1997) Agonist-induced morphologic decrease in cellular D1A dopamine receptor staining. *Synapse* 27, 313-321.

5. Balmforth A.J., Yasunari K., Vaughan P.F., and Ball S.G. (1988) Characterization of dopamine and beta-adrenergic receptors linked to cyclic AMP formation in intact cells of the clone D384 derived from a human astrocytoma. *J. Neurochem.* 51, 1510-1515.

6. Bao J., Alroy I., Waterman H., Schejter E.D., Brodie C., Gruenberg J., and Yarden Y. (2000) Threonine phosphorylation diverts internalized epidermal growth factor receptors from a degradative pathway to the recycling endosome. *J. Biol. Chem.* 275, 26178-26186.

7. Barak L.S., Tiberi M., Freedman N.J., Kwatra M.M., Lefkowitz R.J., and Caron M.G. (1994) A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta 2-adrenergic receptor sequestration. *J. Biol. Chem.* 269, 2790-2795.

8. Barber D.L. (1991) Mechanisms of receptor-mediated regulation of Na-H exchange. *Cell Signal.* 3, 387-397.

9. Barton A.C., Black L.E., and Sibley D.R. (1991) Agonist-induced desensitization of D2 dopamine receptors in human Y-79 retinoblastoma cells. *Mol. Pharmacol.* 39, 650-658.

10. Barton A.C. and Sibley D.R. (1990) Agonist-induced desensitization of D1-dopamine receptors linked to adenylyl cyclase activity in cultured NS20Y neuroblastoma cells. *Mol. Pharmacol.* 38, 531-541.

11. Bates M.D., Caron M.G., and Raymond J.R. (1991) Desensitization of DA1 dopamine receptors coupled to adenylyl cyclase in opossum kidney cells. *Am. J. Physiol* 260, F937-F945.

12. Bates M.D., Olsen C.L., Becker B.N., Albers F.J., Middleton J.P., Mulheron J.G., Jin S.L., Conti M., and Raymond J.R. (1993) Elevation of cAMP is required for down-regulation, but not agonist- induced desensitization, of endogenous dopamine D1 receptors in opossum kidney cells. Studies in cells that stably express a rat cAMP phosphodiesterase (rPDE3) cDNA. *J. Biol. Chem.* 268, 14757-14763.

13. Bates M.D., Senogles S.E., Bunzow J.R., Liggett S.B., Civelli O., and Caron M.G. (1991) Regulation of responsiveness at D2 dopamine receptors by receptor desensitization and adenylyl cyclase sensitization. *Mol. Pharmacol.* 39, 55-63.

14. Benovic J.L., Strasser R.H., Caron M.G., and Lefkowitz R.J. (1986) Beta-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc. Natl. Acad. Sci. U. S. A* 83, 2797-2801.

15. Bohm S.K., Grady E.F., and Bunnett N.W. (1997) Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem. J.* 322 (Pt 1), 1-18.

16. Brejc K., Sixma T.K., Kitts P.A., Kain S.R., Tsien R.Y., Ormo M., and Remington S.J. (1997) Structural basis for dual excitation and photoisomerization of the Aequorea victoria green fluorescent protein. *Proc. Natl. Acad. Sci. U. S. A* 94, 2306-2311.

17. Brown J.H. and Makman M.H. (1972) Stimulation by dopamine of adenylate cyclase in retinal homogenates and of adenosine-3':5'-cyclic monophosphate formation in intact retina. *Proc. Natl. Acad. Sci. U. S. A* 69, 539-543.

18. Bundey R.A. and Nahorski S.R. (2001) Homologous and heterologous uncoupling of muscarinic M(3) and alpha(1B) adrenoceptors to Galpha(q/11) in SH-SY5Y human neuroblastoma cells. *Br. J. Pharmacol.* 134, 257-264.

19. Bunzow J.R., Van Tol H.H., Grandy D.K., Albert P., Salon J., Christie M., Machida C.A., Neve K.A., and Civelli O. (1988) Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature* 336, 783-787.

20. Carlsson A. (2001) A paradigm shift in brain research. *Science* 294, 1021-1024.

21. Carlsson M. and Carlsson A. (1990) Interactions between glutamatergic and monoaminergic systems within the basal ganglia--implications for schizophrenia and Parkinson's disease. *Trends Neurosci.* 13, 272-276.

22. Cavalli V., Corti M., and Gruenberg J. (2001) Endocytosis and signaling cascades: a close encounter. *FEBS Lett.* 498, 190-196.

23. Chio C.L., Lajiness M.E., and Huff R.M. (1994) Activation of heterologously expressed D3 dopamine receptors: comparison with D2 dopamine receptors. *Mol. Pharmacol.* 45, 51-60.

24. Claing A., Perry S.J., Achiriloaie M., Walker J.K., Albanesi J.P., Lefkowitz R.J., and Premont R.T. (2000) Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity. *Proc. Natl. Acad. Sci. U. S. A* 97, 1119-1124.

25. Clark R.B., Friedman J., Dixon R.A., and Strader C.D. (1989) Identification of a specific site required for rapid heterologous desensitization of the beta-adrenergic receptor by cAMP-dependent protein kinase. *Mol. Pharmacol.* 36, 343-348.

26. Cools A.R. and van Rossum J.M. (1976) Instrastriatal administration of monoamines: behavioral effects. *Pharmacol. Ther. [B]* 2, 129-136.

27. Cox B.A., Rosser M.P., Kozlowski M.R., Duwe K.M., Neve R.L., and Neve K.A. (1995) Regulation and functional characterization of a rat recombinant dopamine D3 receptor. *Synapse* 21, 1-9.

28. Dahlstrom A. and Fuxe K. (1964) Localization of monoamines in the lower brain stem. *Experientia* 20, 398-399.

29. Dal Toso R., Sommer B., Ewert M., Herb A., Pritchett D.B., Bach A., Shivers B.D., and Seeburg P.H. (1989) The dopamine D2 receptor: two molecular forms generated by alternative splicing. *EMBO J.* 8, 4025-4034.

30. Damke H., Baba T., van der Bliek A.M., and Schmid S.L. (1995) Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. *J. Cell Biol.* 131, 69-80.

31. Dearry A., Gingrich J.A., Falardeau P., Fremeau R.T., Jr., Bates M.D., and Caron M.G. (1990) Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature* 347, 72-76.

32. Ehlers M.D. (2002) Reinsertion or degradation of AMPA receptors determined by activity- dependent endocytic sorting. *Neuron* 28, 511-525.

33. Einhorn L.C., Gregerson K.A., and Oxford G.S. (1991) D2 dopamine receptor activation of potassium channels in identified rat lactotrophs: whole-cell and single-channel recording. *J. Neurosci.* 11, 3727-3737.

34. Falck B., Hillarp N.A., Thieme G., and Torp A. (1982) Fluorescence of catechol amines and related compounds condensed with formaldehyde. *Brain Res. Bull.* 9, xi-xv.

35. Ferguson S.S. (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* 53, 1-24.

36. Ferguson S.S., Barak L.S., Zhang J., and Caron M.G. (1996) G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. *Can. J. Physiol Pharmacol.* 74, 1095-1110.

37. Ferguson S.S., Downey W.E., III, Colapietro A.M., Barak L.S., Menard L., and Caron M.G. (1996) Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* 271, 363-366.

38. Ferguson S.S., Menard L., Barak L.S., Koch W.J., Colapietro A.M., and Caron M.G. (1995) Role of phosphorylation in agonist-promoted beta 2-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by beta ARK1. *J. Biol. Chem.* 270, 24782-24789.

39. Finberg J.P., Pacak K., Goldstein D.S., and Kopin I.J. (1994) Modification of cerebral cortical noradrenaline release by chronic inhibition of MAO-A. *J. Neural Transm. Suppl* 41, 123-125.

40. Gardner B., Liu Z.F., Jiang D., and Sibley D.R. (2001) The role of phosphorylation/dephosphorylation in agonist-induced desensitization of D1 dopamine receptor function: evidence for a novel pathway for receptor dephosphorylation. *Mol. Pharmacol.* 59, 310-321.

41. Gingrich J.A., Dearry A., Falardeau P., Fremeau R.T., Jr., Bates M.D., and Caron M.G. (1991) Molecular characterization of G-protein coupled receptors: isolation and cloning of a D1 dopamine receptor. *J. Recept. Res.* 11, 521-534.

42. Giros B., Sokoloff P., Martres M.P., Riou J.F., Emorine L.J., and Schwartz J.C. (1989) Alternative splicing directs the expression of two D2 dopamine receptor isoforms. *Nature* 342, 923-926.

43. Grandy D.K., Zhang Y.A., Bouvier C., Zhou Q.Y., Johnson R.A., Allen L., Buck K., Bunzow J.R., Salon J., and Civelli O. (1991) Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes. *Proc. Natl. Acad. Sci. U. S. A* 88, 9175-9179.

44. Grandy D.K., Zhou Q.Y., Allen L., Litt R., Magenis R.E., Civelli O., and Litt M. (1990) A human D1 dopamine receptor gene is located on chromosome 5 at q35.1 and identifies an EcoRI RFLP. *Am. J. Hum. Genet.* 47, 828-834.

45. Hao M., Lin S.X., Karylowski O.J., Wustner D., McGraw T.E., and Maxfield F.R. (2001) Vesicular and non-vesicular sterol transport in living cells: the endocytic recycling compartment is a major sterol storage organelle. *J. Biol. Chem.*

46. Harden T.K. (1983) Agonist-induced desensitization of the beta-adrenergic receptor-linked adenylate cyclase. *Pharmacol. Rev.* 35, 5-32.

47. Harsing L.G., Jr. and Zigmond M.J. (1997) Influence of dopamine on GABA release in striatum: evidence for D1-D2 interactions and non-synaptic influences. *Neuroscience* 77, 419-429.

48. Hausdorff W.P., Bouvier M., O'Dowd B.F., Irons G.P., Caron M.G., and Lefkowitz R.J. (1989) Phosphorylation sites on two domains of the beta 2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J. Biol. Chem.* 264, 12657-12665.

49. Hausdorff W.P., Caron M.G., and Lefkowitz R.J. (1990) Turning off the signal: desensitization of beta-adrenergic receptor function. *FASEB J.* 4, 2881-2889.

50. Hillarp N.A., Fuxe K., and Dahlstrom A. (1966) Demonstration and mapping of central neurons containing dopamine, noradrenaline, and 5-hydroxytryptamine and their reactions to psychopharmaca. *Pharmacol. Rev.* 18, 727-741.

51. Innamorati G., Le Gouill C., Balamotis M., and Birnbaumer M. (2001) The long and the short cycle. Alternative intracellular routes for trafficking of G-protein-coupled receptors. *J. Biol. Chem.* 276, 13096-13103.

52. Jarvie K.R., Tiberi M., Silvia C., Gingrich J.A., and Caron M.G. (1993) Molecular cloning, stable expression and desensitization of the human dopamine D1b/D5 receptor. *J. Recept. Res.* 13, 573-590.

53. Jeng R.L. and Welch M.D. (2001) Cytoskeleton: Actin and endocyto. *Curr. Biol.* 11, R691-R694.

54. Jiang D. and Sibley D.R. (1999) Regulation of D(1) dopamine receptors with mutations of protein kinase phosphorylation sites: attenuation of the rate of agonist-induced desensitization. *Mol. Pharmacol.* 56, 675-683.

55. Kebabian J.W. and Calne D.B. (1979) Multiple receptors for dopamine. *Nature* 277, 93-96.

56. Kebabian J.W., Kuo J.F., and Greengard P. (1972) Determination of relative levels of cyclic AMP in tissues or cells prelabeled with radioactive adenine. *Adv. Cyclic. Nucleotide. Res.* 2, 131-137.

57. Kennelly P.J. (1994) Identification of sites of serine and threonine phosphorylation via site-directed mutagenesis--site transformation versus site elimination. *Anal. Biochem.* 219, 384-386.

58. Kennelly P.J. and Krebs E.G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* 266, 15555-15558.

59. Kobilka B.K. (1995) Amino and carboxyl terminal modifications to facilitate the production and purification of a G protein-coupled receptor. *Anal. Biochem.* 231, 269-271.

60. Koenig J.A. and Edwardson J.M. (1997) Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol. Sci.* 18, 276-287.

61. Kopin I.J. (1994) Monoamine oxidase and catecholamine metabolism. *J. Neural Transm. Suppl* 41, 57-67.

62. Krupnick J.G. and Benovic J.L. (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol.* 38, 289-319.

63. Lamaze C. and Schmid S.L. (1995) Recruitment of epidermal growth factor receptors into coated pits requires their activated tyrosine kinase. *J. Cell Biol.* 129, 47-54.

64. Lefkowitz R.J. (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J. Biol. Chem.* 273, 18677-18680.

65. Lewis M.M., Watts V.J., Lawler C.P., Nichols D.E., and Mailman R.B. (1998) Homologous desensitization of the D1A dopamine receptor: efficacy in causing desensitization dissociates from both receptor occupancy and functional potency. *J. Pharmacol. Exp. Ther.* 286, 345-353.

66. Lidow M.S. and Goldman-Rakic P.S. (1994) A common action of clozapine, haloperidol, and remoxipride on D1- and D2-dopaminergic receptors in the primate cerebral cortex. *Proc. Natl. Acad. Sci. U. S. A* 91, 4353-4356.

67. Lin C.W., Bianchi B.R., Miller T.R., Stashko M.A., Wang S.S., Curzon P., Bednarz L., Asin K.E., and Britton D.R. (1996) Persistent activation of the dopamine D1 receptor contributes to prolonged receptor desensitization: studies with A-77636. *J. Pharmacol. Exp. Ther.* 276, 1022-1029.

68. Lindgren N., Xu Z.Q., Herrera-Marschitz M., Haycock J., Hokfelt T., and Fisone G. (2001) Dopamine D(2) receptors regulate tyrosine hydroxylase activity and phosphorylation at Ser40 in rat striatum. *Eur. J. Neurosci.* 13, 773-780.

69. Lledo P.M., Legendre P., Israel J.M., and Vincent J.D. (1990) Dopamine inhibits two characterized voltage-dependent calcium currents in identified rat lactotroph cells. *Endocrinology* 127, 990-1001.

70. Machida C.A., Searles R.P., Nipper V., Brown J.A., Kozell L.B., and Neve K.A. (1992) Molecular cloning and expression of the rhesus macaque D1 dopamine receptor gene. *Mol. Pharmacol.* 41, 652-659.

71. Maggio R., Barbier P., and Corsini G.U. (1995) Apomorphine continuous stimulation in Parkinson's disease: receptor desensitization as a possible mechanism of reduced motor response. *J. Neural Transm. Suppl* 45, 133-136.

72. Malecz N., Bambino T., Bencsik M., and Nissenson R.A. (1998) Identification of phosphorylation sites in the G protein-coupled receptor for parathyroid hormone. Receptor phosphorylation is not required for agonist-induced internalization. *Mol. Endocrinol.* 12, 1846-1856.

73. Malek D., Munch G., and Palm D. (1993) Two sites in the third inner loop of the dopamine D2 receptor are involved in functional G protein-mediated coupling to adenylate cyclase. *FEBS Lett.* 325, 215-219.

74. Mason J.N., Kozell L.B., and Neve K.A. Regulation of dopamine D1 receptor trafficking by protein kinase A-dependent phsophorylation. *Mol.Pharmacol.* 2002 (in press).

75. Melikian H.E. and Buckley K.M. (1999) Membrane trafficking regulates the activity of the human dopamine transporter. *J. Neurosci.* 19, 7699-7710.

76. Memo M., Lovenberg W., and Hanbauer I. (1982) Agonist-induced subsensitivity of adenylate cyclase coupled with a dopamine receptor in slices from rat corpus striatum. *Proc. Natl. Acad. Sci. U. S. A* 79, 4456-4460.

77. Mills I.G., Jones A.T., and Clague M.J. (1999) Regulation of endosome fusion. *Mol. Membr. Biol.* 16, 73-79.

78. Mills I.G., Urbe S., and Clague M.J. (2001) Relationships between EEA1 binding partners and their role in endosome fusion. *J. Cell Sci.* 114, 1959-1965.

79. Moffett S., Rousseau G., Lagace M., and Bouvier M. (2001) The palmitoylation state of the beta(2)-adrenergic receptor regulates the synergistic action of cyclic AMP-dependent protein kinase and beta- adrenergic receptor kinase involved in its phosphorylation and desensitization. *J. Neurochem.* 76, 269-279.

80. Monsma F.J., Jr., Mahan L.C., McVittie L.D., Gerfen C.R., and Sibley D.R. (1990) Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. *Proc. Natl. Acad. Sci. U. S. A* 87, 6723-6727.

81. Morgenstern J.P. and Land H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18, 3587-3596.

82. Mukherjee S., Ghosh R.N., and Maxfield F.R. (1997) Endocytosis. *Physiol Rev.* 77, 759-803.

83. Nestby P., Tjon G.H., Visser D.T., Drukarch B., Leysen J.E., Mulder A.H., and Schoffelmeer A.N. (1995) Intermittent morphine treatment causes long-term desensitization of functional dopamine D2 receptors in rat striatum. *Eur. J. Pharmacol.* 294, 771-777.

84. Neve K.A., Cox B.A., Henningsen R.A., Spanoyannis A., and Neve R.L. (1991) Pivotal role for aspartate-80 in the regulation of dopamine D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol. Pharmacol.* 39, 733-739.

85. Ng G.Y., Mouillac B., George S.R., Caron M., Dennis M., Bouvier M., and O'Dowd B.F. (1994) Desensitization, phosphorylation and palmitoylation of the human dopamine D1 receptor. *Eur. J. Pharmacol.* 267, 7-19.

86. Ng G.Y., O'Dowd B.F., Caron M., Dennis M., Brann M.R., and George S.R. (1994) Phosphorylation and palmitoylation of the human D2L dopamine receptor in Sf9 cells. *J. Neurochem.* 63, 1589-1595.

87. Ng G.Y., Trogadis J., Stevens J., Bouvier M., O'Dowd B.F., and George S.R. (1995) Agonist-induced desensitization of dopamine D1 receptor-stimulated adenylyl cyclase activity is temporally and biochemically separated from D1 receptor internalization. *Proc. Natl. Acad. Sci. U. S. A* 92, 10157-10161.

88. Oakley R.H., Laporte S.A., Holt J.A., Barak L.S., and Caron M.G. (1999) Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J. Biol. Chem.* 274, 32248-32257.

89. Okubo Y., Suhara T., Sudo Y., and Toru M. (1997) Possible role of dopamine D1 receptors in schizophrenia. *Mol. Psychiatry* 2, 291-292.

90. Olivares-Reyes J.A., Smith R.D., Hunyady L., Shah B.H., and Catt K.J. (2001) Agonist-induced signaling, desensitization and internalization of a phosphorylation-deficient AT{sub1}A angiotensin receptor. *J. Biol. Chem.*

91. Pippig S., Andexinger S., and Lohse M.J. (1995) Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* 47, 666-676.

92. Post S.R., Aguila-Buhain O., and Insel P.A. (1996) A key role for protein kinase A in homologous desensitization of the beta 2-adrenergic receptor pathway in S49 lymphoma cells. *J. Biol. Chem.* 271, 895-900.

93. Pouyssegur J. (2000) Signal transduction. An arresting start for MAPK. *Science* 290, 1515-1518.

94. Ren M., Xu G., Zeng J., Lemos-Chiarandini C., Adesnik M., and Sabatini D.D. (1998) Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. *Proc. Natl. Acad. Sci. U. S. A* 95, 6187-6192.

95. Roettger B.F., Rentsch R.U., Pinon D., Holicky E., Hadac E., Larkin J.M., and Miller L.J. (1995) Dual pathways of internalization of the cholecystokinin receptor. *J. Cell Biol.* 128, 1029-1041.

96. Sadeghi H.M., Innamorati G., Esqueda E., and Birnbaumer M. (1998) Processing and ligand-induced modifications of the V2 vasopressin receptor. *Adv. Exp. Med. Biol.* 449, 339-346.

97. Schafer D.A. (2002) Coupling actin dynamics and membrane dynamics during endocytosis. *Curr. Opin. Cell Biol.* 14, 76-81.

98. Schlierf B., Fey G.H., Hauber J., Hocke G.M., and Rosorius O. (2000) Rab11b is essential for recycling of transferrin to the plasma membrane. *Exp. Cell Res.* 259, 257-265.

99. Sheff D.R., Daro E.A., Hull M., and Mellman I. (1999) The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. *J. Cell Biol.* 145, 123-139.

100. Shiina T., Kawasaki A., Nagao T., and Kurose H. (2000) Interaction with beta-arrestin determines the difference in internalization behavor between b. *J. Biol. Chem.* 275, 29082-29090.

101. Simonsen A., Wurmser A.E., Emr S.D., and Stenmark H. (2001) The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* 13, 485-492.

102. Smith A.D. and Bolam J.P. (1990) The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. *Trends Neurosci.* 13, 259-265.

103. Sokoloff P., Giros B., Martres M.P., Bouthenet M.L., and Schwartz J.C. (1990) Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature* 347, 146-151.

104. Spano P.F., Govoni S., and Trabucchi M. (1978) Studies on the pharmacological properties of dopamine receptors in various areas of the central nervous system. *Adv. Biochem. Psychopharmacol.* 19, 155-165.

105. Stenmark H. and Olkkonen V.M. (2001) The Rab GTPase family. *Genome Biol.* 2, REVIEWS3007.

106. Tiberi M., Jarvie K.R., Silvia C., Falardeau P., Gingrich J.A., Godinot N., Bertrand L., Yang-Feng T.L., Fremeau R.T., Jr., and Caron M.G. (1991) Cloning,

molecular characterization, and chromosomal assignment of a gene encoding a second D1 dopamine receptor subtype: differential expression pattern in rat brain compared with the D1A receptor. *Proc. Natl. Acad. Sci. U. S. A* 88, 7491-7495.

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

108. Trischler M., Stoorvogel W., and Ullrich O. (1999) Biochemical analysis of distinct. *J. Cell Sci.* 112 (Pt 24), 4773-4783.

109. 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.

110. Van Tol H.H., Bunzow J.R., Guan H.C., Sunahara R.K., Seeman P., Niznik H.B., and Civelli O. (1991) Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature* 350, 610-614.

111. Ventura A.L. and Sibley D.R. (2000) Altered regulation of the D(1) dopamine receptor in mutant Chinese hamster ovary cells deficient in cyclic AMP-dependent protein kinase activity. *J. Pharmacol. Exp. Ther.* 293, 426-434.

112. Vickery R.G. 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.

113. von Zastrow M. (2001) Role of endocytosis in signalling and regulation of G-protein-coupled receptors. *Biochem. Soc. Trans.* 29, 500-504.

114. Watts V.J. and Neve K.A. (1996) Sensitization of endogenous and recombinant adenylate cyclase by activation of D2 dopamine receptors. *Mol. Pharmacol.* 50, 966-976.

115. Whistler J.L., Tsao P., and von Zastrow M. (2001) A phosphorylation-regulated brake mechanism controls the initial endocytosis of opioid receptors but is not required for post-endocytic sorting to lysosomes. *J. Biol. Chem.* 276, 34331-34338.

116. Whistler J.L. and von Zastrow M. (1999) Dissociation of functional roles of dynamin in receptor-mediated endocytosis and mitogenic signal transduction. *J. Biol. Chem.* 274, 24575-24578.

117. Wurmser A.E., Gary J.D., and Emr S.D. (1999) Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. *J. Biol. Chem.* 274, 9129-9132.

118. Yuan N., Friedman J., Whaley B.S., and Clark R.B. (1994) cAMP-dependent protein kinase and protein kinase C consensus site mutations of the beta-adrenergic receptor. Effect on desensitization and stimulation of adenylylcyclase. *J. Biol. Chem.* 269, 23032-23038.

119. Zamanillo D., Casanova E., Alonso-Llamazares A., Ovalle S., Chinchetru M.A., and Calvo P. (1995) Identification of a cyclic adenosine 3',5'-monophosphate-dependent protein kinase phosphorylation site in the carboxy terminal tail of human D1 dopamine receptor. *Neurosci. Lett.* 188, 183-186.

120. Zhang J., Barak L.S., Anborgh P.H., Laporte S.A., Caron M.G., and Ferguson S.S. (1999) Cellular trafficking of G protein-coupled receptor/beta-arrestin endocytic complexes. *J. Biol. Chem.* 274, 10999-11006.

121. Zhou Q.Y., Grandy D.K., Thambi L., Kushner J.A., Van Tol H.H., Cone R., Pribnow D., Salon J., Bunzow J.R., and Civelli O. (1990) Cloning and expression of human and rat D1 dopamine receptors. *Nature* 347, 76-80.

122. Zhou X.M., Curran P., Baumgold J., and Fishman P.H. (1994) Modulation of adenylylcyclase by protein kinase C in human neurotumor SK-N-MC cells: evidence that the alpha isozyme mediates both potentiation and desensitization. *J. Neurochem.* 63, 1361-1370.