**Title**
Regulation of Dopamine Signaling by $D_1$ Receptor Membrane Trafficking



**Author**
Kotowski, Sarah Jane

**Publication Date**
2009

Peer reviewed|Thesis/dissertation

Regulation of Dopamine Signaling by D1 Receptor Membrane Trafficking

by

Sarah Kotowski

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

[redacted]

# Acknowledgements

This body of work would not have been possible without the continued support of a number of people. Thank you to my thesis committee members Antonello Bonci, Robert Edwards and Roger Nicoll. Also, thanks to my neuroscience classmates and the rest of the UCSF community. Thank you to my mentor Mark von Zastrow for giving me the resources to grow as a scientist and as a person. Thanks to all of the current and former members of the von Zastrow lab for keeping me laughing and sane and for all of your help over the years. In particular, thanks to Rani Dhavan and Guillermo Yudowski for your guidance, advice and cheerleading when I needed it most. Special thanks to Stanford Chen, Stacy Henry, Peter Hein, Ben Lauffer, Joy Yu, Michael Tanowitz, Paul Temkin and Vu Dang.

Thank you to my closest friends and colleagues Kathy Nagel, David Schoppik, Joel Greenwood, James Hislop and Dawn Thompson. I am certain that I would not have survived this Ph.D. without your commiseration, love and support. Thanks to Kate Madsen Cole, Kelly Knaus, Jean Kim, Annmarie (Bean) Behan, and to Riptide. You keep me grounded. Thank you to Randy Madsen, I will never forget all that you gave me and I wish you could be here to share this.

Thank you to my parents, Richard and Jane Kotowski, for instilling me with the values and determination that I needed to make it this far. Thanks to my big brothers, Douglas and Matthew Kotowski and to my sister-in-law, Laura Kotowski. Finally, thank you to

Heather Moore for all of the patience, kindness and love you have shown me over these last few years. We did it.

Experimental data and conclusions presented in this thesis were primarily generated by Sarah J. Kotowski, in the laboratory of Mark von Zastrow. We would like to acknowledge F. Woodward Hopf and Taban Seif in the laboratory of Antonello Bonci for their contribution of electrophysiology data presented and discussed in figure 7 of chapter 3. Some microscopy data presented in this study were generated at the Nikon Imaging Center at the University of California, San Francisco.

# Abstract

**Regulation of dopamine signaling by $D_1$ receptor membrane trafficking.**

Dopamine is a major catecholamine neurotransmitter in the central nervous system (CNS). Dopaminergic signaling is a critical component of a number of complex physiological functions including movement, learning and memory, attention and goal-directed behaviors. The cellular actions of dopamine are mediated by a family of G protein-coupled receptors (GPCRs), the dopamine receptors. The $D_1$ receptor is the major excitatory transducer of dopaminergic signaling within the brain. In this study, we examine the contribution of $D_1$ receptor membrane trafficking to the regulation of dopaminergic signaling in a HEK 293 model system, as well as in cortical and striatal neurons known to natively express $D_1$ receptors. We find that $D_1$ receptor membrane trafficking does not play a significant role in determining cellular sensitivity to dopamine after prolonged (30 minutes to 1 hour) agonist incubation. However, when we examine $D_1$ receptor trafficking and signaling with much greater temporal resolution, we find that rapid endocytosis is essential for neuronal dopamine signaling. Further, the kinetics of this regulation approaches those of transient increases in extracellular dopamine observed within the intact brain. This body of work presents a novel, and previously unanticipated role for endocytosis in the regulation of $D_1$ receptor-mediated dopaminergic signaling in neurons.

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# Chapter 1: Introduction

## 1.1 A brief history of dopamine and dopamine receptors

Cells must be able to both sense and respond to changing conditions in their environment in order to sustain life. The evolution of cellular signaling likely dates back over 3.5 billion years with the appearance of the first unicellular organisms in the fossil record. As organisms have become more and more complex and the need to respond to a greater number of environmental cues has increased, cellular signaling has likewise increased in complexity. Multicellular organisms rely on specific and efficient signal transduction of extracellular molecules in order to promote cell-to-cell communication and maintain overall homeostasis. Dopamine is a catecholamine compound thought to act as a signaling molecule in all metazoans and the dopamine receptors are members of a family of G protein-coupled receptors (GPCRs) known to mediate the cellular actions of dopamine.

The function of dopamine as a modulatory neurotransmitter was first discovered in the late 1950s by Arvid Carlsson and colleagues. Prior to then, dopamine was thought to act primarily as an intermediate in the synthesis of norepinepherine and epinepherine. Carlsson demonstrated that intravenous injection of the dopamine precursor L-dihydroxyphenylalanine (L-DOPA) could reverse marked reserpine-induced tranquilization in animal models and also produce increased levels 3-hydroxytyramine (dopamine) in brain regions that did not show a corresponding elevation in norepinepherine or epinephrine (Carlsson, Lindqvist et al. 1957; Carlsson, Lindqvist et al. 1957; Carlsson 1959). Interestingly, dopamine's role as a neurotransmitter was initially met with overwhelming resistance, as the prevailing belief in the field was that synaptic

transmission in the CNS was electrical and not chemical. It was not until the 1970s that biochemical studies started to add credence to the characterization of dopamine as its own unique signaling molecule in the brain. Evidence of a mammalian, dopamine-sensitive, adenylate cyclase was first demonstrated in sympathetic ganglia of the peripheral nervous system and later confirmed in homogenized tissue of the caudate nucleus (Kebabian and Greengard 1971; Kebabian, Petzold et al. 1972).

The concept of a "dopamine receptor" was also introduced by Carlsson and colleagues in the 1960s, long before the evolution of molecular biology (Carlsson and Lindqvist 1963). A receptor was thought to be some component of the tissue could both recognize and respond to very small quantities of an endogenous substance or drugs that imitate or inhibit this substance. The finding that dopamine could exert differing effects on adenylyl cyclase activity, depending on the tissue assayed, led to the theory that there were two classes of dopamine receptors (Kebabian and Calne 1979). This theory is still widely held today. The first mammalian dopamine receptor, the $D_2$ receptor, was cloned in the late 1980s based on its sequence homology to another GPCR, the $\beta_2$-adrenergic receptor (Bunzow, Van Tol et al. 1988; Grandy, Marchionni et al. 1989). The $D_2$ receptor is the prototypical member of the $D_2$ receptor-like subclass that includes $D_2$, $D_3$ and $D_4$ receptors. $D_2$-like receptors couple to $G_i/G_o$ G-proteins and inhibit adenylyl cyclase. Shortly after the cloning of the $D_2$ receptor, the mammalian $D_1$ dopamine receptor was cloned (Monsma, Mahan et al. 1990; Sunahara, Niznik et al. 1990; Zhou, Grandy et al. 1990). The $D_1$ receptor also represents the prototype of a subclass. $D_1$-like receptors include $D_1$ and $D_5$ receptors (sometimes referred to as $D_{1A}$ and $D_{1B}$,

respectively), which couple to $G_s/G_{olf}$ proteins and stimulate adenylyl cyclase. This body of work investigates membrane trafficking of the $D_1$ dopamine receptor and identifies an important role for $D_1$ receptor trafficking with respect to dopaminergic signaling.

## 1.2 Dopamine: anatomy, physiology and CNS pathologies

Dopamine is synthesized by tyrosine hydroxylase-mediated hydroxylation of the amino acid L-Tyrosine and subsequent decarboxylation of L-DOPA by aromatic L-amino acid decarboxylase (DOPA-decarboxylase). These steps are carried out peripherally in the cells of the adrenal medulla and centrally in dopaminergic neurons. In the periphery, dopamine primarily acts on the sympathetic nervous system to increase heart rate and blood pressure. Within the CNS, midbrain dopaminergic neurons have cell bodies in substantia nigra pars compacta, ventral tegmental area and the retrorubral field (Saper 2000). These cells send out projections that provide dopaminergic input into the striatum (nigrostriatal pathway), frontotemporal cortex (mesocortical pathway) and the limbic system including the central nucleus of the amygdala and the lateral septum (mesolimbic pathway). Midbrain dopamine pathways are thought to be important for such complex physiological functions as movement, motivation, reward, memory, emotion and reason. Dopaminergic cell bodies are also found within the olfactory bulb, retina and hypothalamus where they send projections to the lower brain stem and spinal cord to where they regulate sympathetic preganglionic neurons. Dopaminergic neurons originating in the hypothalamus are also thought to function in the neuroendocrine system

by regulating prolactin release. As $D_1$ receptors are particularly enriched within the striatum and prefrontal cortex, and a number of fascinating behaviors can be attributed to dopaminergic transmission within these brain regions, the following study focuses on membrane trafficking and regulation of signaling by striatal and cortical $D_1$ dopamine receptors.

$D_1$ receptors are thought to be the major excitatory mediators of dopaminergic transmission in the CNS (Missale, Nash et al. 1998; Neve, Seamans et al. 2005). Anatomical studies in both primates and rodents have revealed substantial $D_1$ receptor immunoreactivity in the basal ganglia as well as pyramidal neurons of the cortex and hippocampus (Bergson, Mrzljak et al. 1995; Hersch, Ciliax et al. 1995; Yung, Bolam et al. 1995). $D_1$ receptors are most highly expressed on the plasma membrane of dendritic spines and shafts, as well as the cell bodies of gamma-aminobutyric (GABA)-ergic medium spiny neurons (MSNs) within the striatum. This is consistent with the primary function of $D_1$ receptors as postsynaptic mediators of dopaminergic transmission. However, $D_1$ receptor staining has also been identified on 25-29% of axonal membranes within the nucleus accumbens core and shell, suggesting that $D_1$ receptors might also play a role in presynaptic modulation of neurotransmission within specific brain regions (Dumartin, Doudnikoff et al. 2007).

Much of what is known about the anatomy and physiology of dopaminergic systems has come from our understanding of disease and therapeutic interventions that alter dopaminergic transmission. $D_1$ receptor signaling can contribute to both the pathology

and the treatment to a number of human diseases. As mentioned previously, the role of dopamine as a neurotransmitter was in part discovered by the therapeutic utility of the dopamine precursor L-DOPA in ameliorating the negative symptoms of Parkinson's disease (Carlsson 2001). The pathology of Parkinson's disease is thought to arise primarily from the death of dopaminergic neurons in the substantia nigra and the corresponding loss of input to the basal ganglia and the cortex. This hypodopaminergic state leads to a decline of both motor and cognitive functions, which is at least partially improved by dopamine receptor agonists. As a simplified model, activation of $D_1$ receptor signaling in direct pathway of the basal ganglia-thalamocortical circuit is thought to increase motor output in Parkinson's patients (DeLong 2000), though unfortunately it is also thought to contribute to the dyskinesias seen as a side-effect of long term L-DOPA therapy. Interestingly, a recent study has shown recruitment of $D_1$ receptors to the plasma membrane of striatal neurons in parkinsonian patients, suggesting that $D_1$ receptor trafficking may play a specific role in this disease (Guigoni, Doudnikoff et al. 2007).

Abnormal $D_1$ receptor signaling is also thought to be a component of a number of neuropsychiatric disorders including schizophrenia, attention-deficit hyperactivity disorder (ADHD) and Tourette's syndrome. Although the dopaminergic hypothesis of schizophrenia suggests that positive symptoms (delusions, hallucinations, paranoia, hostility) are associated with increased activity of $D_2$-like receptors in the mesolimbic pathway, there is growing evidence for the involvement of $D_1$ receptor signaling in negative symptoms (withdrawal, blunted affect, decreased planning and working

memory). Positron emission tomography (PET) imaging studies have revealed decreased radio-ligand binding of $D_1$ receptors in the prefrontal cortex of drug-naïve schizophrenics (Okubo, Suhara et al. 1997). This reduction was strongly correlated with the severity of negative symptoms. Surprisingly, another imaging study reported that high $D_1$ receptor density in the medial prefrontal cortex is associated with increasing genetic risk for schizophrenia (Hirvonen, van Erp et al. 2006). Effective therapies for ADHD, such as methylphenidate and amphetamine, are known to increase dopamine within the brain. The proposed role of $D_1$ receptor signaling in ADHD is thought to involve modulation of activity of neurons in the prefrontal cortex (Brennan and Arnsten 2008). Though it is still poorly understood how this modulation improves attention and working memory, one model is that moderate $D_1$ receptor-mediated signaling leads to the opening of cAMP-sensitive cyclic nucleotide-gated (HCN) channels on the spines of neurons in the prefrontal cortex and suppresses irrelevant input (noise). Current hypotheses of Tourette's syndrome suggest that the involuntary, stereotyped behaviors that produce verbal and motor tics are mediated by hyperactivity in the corticostriatalthalomocortical (CSTC) loop (Campbell, McGrath et al. 1999). Excessive activation of $D_1$ receptor expressing neurons in the direct pathway of the CTSC loop is thought to contribute to this pathology.

Finally, $D_1$ receptor mediated signaling is thought to play an important role in drug addiction and dependence. It is well known that psychostimulants, such as cocaine and amphetamine cause a massive increase in neural dopamine through their actions at monoamine transporters (Torres, Gainetdinov et al. 2003). Cocaine acts as a non-

selective inhibitor of the dopamine transporter (DAT), which leads to increased extracellular accumulation of dopamine. Amphetamine-like drugs are taken up by DAT and cause release of dopamine from synaptic vesicles and a reversal of the direction of DAT transport, leading to massive release of dopamine into the extracellular space. Interestingly, dopaminergic signaling seems to play a role in more than just psychostimulant addiction. All addictive drugs are thought to elevate synaptic levels of dopamine in the nucleus accumbens (Hyman, Malenka et al. 2006). This has prompted countless studies investigating how dopamine's actions are involved in addiction. The finding that dopamine is central to both reward-related learning and motivated behaviors involved in obtaining rewards (Berridge and Robinson 1998; Schultz 2006) has led to the hypothesis that dopaminergic modulation of learning and memory is central to addiction. Not surprisingly, $D_1$ receptor signaling is thought to contribute to long-term potentiation (LTP) in a number of the same regions implicated in addiction (Hyman, Malenka et al. 2006).

## 1.3 Regulation of dopaminergic transmission: release and reuptake

A number of regulatory mechanisms act in concert to control both the concentration and the residence time of dopamine within the synapse and thereby limit the number of dopamine receptors that can become activated. The amount of dopamine that is released into the extracellular space is primarily controlled by the activity of dopaminergic neurons. These neurons exhibit two distinct modes of firing, tonic and phasic, which

produce different profiles of dopamine release. Dopaminergic neurons fire in a pacemaker-like fashion, with a frequency of approximately 5Hz (Grace and Bunney 1984). This tonic firing is thought to arise from two key membrane currents, a spontaneous slow depolarization current and an afterhyperpolarization current mediated by calcium-activated potassium conductance ($I_{K(Ca)}$). Tonic firing is thought to generate baseline levels of extracellular dopamine within the striatum of about 10-20 nM (Goto, Otani et al. 2007). Dopaminergic neurons are also capable of burst firing at much higher frequencies (Grace and Bunney 1984; Hyland, Reynolds et al. 2002). This phasic firing is dependent upon glutamatergic inputs to dopaminergic neurons and is thought to generate transient increases in extra-synaptic dopamine concentrations on the order of micro- to millimolar levels (Heien and Wightman 2006; Goto, Otani et al. 2007). Interestingly, the pharmacological properties of the $D_1$ receptor suggest that it is particularly important for transducing signals in response to the high extracellular concentrations of dopamine characteristic of phasic release (Richfield, Penney et al. 1989).

Midbrain dopaminergic neurons form en passant symmetrical synapses on both the spines and shafts of striatal medium spiny neurons (MSNs) (Freund, Powell et al. 1984; Groves, Linder et al. 1994). As the majority of $D_1$ receptors on MSNs receiving this input are not directly adjacent to dopaminergic terminals (Bergson, Mrzljak et al. 1995; Caille, Dumartin et al. 1996), the amount of dopamine available to activate these receptors is thought to be limited by diffusion and the mechanisms that clear dopamine from the extracellular space. This spatially non-selective model of dopaminergic transmission is

commonly referred to as volume transmission. Whether or not it is an accurate model for dopaminergic transmission in the CNS is subject to considerable debate (Zoli, Torri et al. 1998; Arbuthnott and Wickens 2006; Rice and Cragg 2008). The primary means by which dopamine is cleared from the extracellular space is via uptake of dopamine transporters (DAT) on presynaptic terminals. DAT is a twelve transmembrane-spanning symporter that requires the binding of two sodium ions and one chloride ion to move dopamine across the plasma membrane (Torres, Gainetdinov et al. 2003). Dopamine taken up by DAT is subject to storage in synaptic vesicles by vesicular monoamine transporters (VMATs) or degradation by the enzyme monoamine oxidase (MAO). Dopamine can also be metabolized by the enzyme catechol-o-methyltransferase (COMT), which is found primarily in postsynaptic neurons and glial cells (Karhunen, Tilgmann et al. 1995). Finally, the actions of dopamine on $D_1$ receptors can be limited by $D_2$ or $D_3$ autoreceptors on axonal membranes of presynaptic neurons. Although the primary function of $D_2/D_3$ autoreceptors is to inhibit exocytic release of dopamine, they have also been shown to play a role in the uptake of dopamine within the striatum and nucleus accumbens (Feuerstein 2008).

## 1.4 $D_1$ receptor-mediated signaling

$D_1$ dopamine receptors are activated by the endogenous ligand dopamine as well as a number of synthetic compounds. Upon agonist binding, $D_1$ receptors undergo a conformational change that increases their affinity for heterotrimeric G-proteins $G_s$ or $G_{olf}$, depending on brain region. Activated $G\alpha_s$ and $G\alpha_{olf}$ bind to adenylyl cyclase in the

plasma membrane, which catalyzes the conversion of ATP to cyclic AMP (cAMP). cAMP can then bind to the regulatory subunits cAMP-dependent protein kinase (PKA), causing dissociation of activated subunits and promoting this enzymes catalytic activity. Nearly all of the cellular effects of dopamine signaling via $D_1$ receptors can be attributed to activation of PKA and phosphorylation of its downstream effectors. PKA phosphorylation can modulate several proteins within the CNS including ion channels, ionotropic receptors, enzymes and transcription factors. $D_1$ receptor activation has been shown to stimulate NMDA and AMPA receptor currents, both inhibit and stimulate $GABA_A$ receptor currents, decrease N and P/Q-type $Ca^{2+}$ channel conductance, increase L-type $Ca^{2+}$ conductance, inhibit both voltage gated and G protein-mediated inwardly rectifying $K^+$ (GIRK) channels and either increase or decrease $Na^+$ channel conductance depending on cell type and/or brain region in which it is measured (Greengard 2001; Neve, Seamans et al. 2005). In the striatum, $D_1$ receptor activation leads to PKA-dependent phosphorylation of the dopamine and cAMP regulated phosphoprotein, 32 kD (DARPP-32). Activated DARPP-32 can also contribute to the regulation of these receptors and channels via inhibition of protein phosphotase 1 (PP1). $D_1$ receptor stimulation has also been shown to affect expression of genes with cAMP response elements by PKA-mediated phosphorylation of cyclic AMP response element-binding protein (CREB). Given the myriad of downstream effects that can result from $D_1$ receptor activation, it is not surprising that the signaling of this receptor is highly regulated.

## 1.5 Regulation of $D_1$ receptor signaling: a potential role for membrane trafficking

Cellular responsiveness to dopamine receptor agonists is thought to depend on both the accessibility of receptor to ligand and receptor efficiency for stimulating downstream second-messengers. Shortly after agonist activation, several GPCRs can be phosphorylated by G protein-coupled receptor kinases (GRKs) or second messenger kinases, leading to decreased efficiency of receptor-mediated signaling (Ferguson 2001; Gainetdinov, Premont et al. 2004). This decrease in receptor-mediated signaling efficiency is commonly referred to as desensitization and often involves recruitment of arrestin proteins to activated, phosphorylated, GPCRs. Arrestin binding is thought to physically uncouple receptors from their G proteins and thereby contribute to desensitization. Arrestin 1 was first discovered in the visual system and a non-visual arrestin (βarrestin-1 or arrestin 2) was later identified as an important component of GRK2-mediated desensitization of the $\beta_2$ adrenergic receptor (Pfister, Chabre et al. 1985; Benovic, Kuhn et al. 1987; Lohse, Benovic et al. 1990).

Agonist-induced phosphorylation and subsequent desensitization of the $D_1$ dopamine receptors is well characterized. This was first demonstrated in Sf9 insect cells (Ng, Mouillac et al. 1994). $D_1$ receptors undergo agonist-dependent phosphorylation by GRK2, GRK3 and GRK5 when co-expressed in 293 cells (Tiberi, Nash et al. 1996). Co-expression of $D_1$ receptors and GRK2, GRK3 or GRK5 leads to a decrease in agonist-stimulated cAMP accumulation when compared to $D_1$ receptors that are expressed alone. Amino acid residue Thr360 represents a major target of GRK2-mediated phosphorylation

in the $D_1$ receptor, as alanine substitution of this residue results in significant inhibition of agonist-induced desensitization in Chinese Hamster Ovary (CHO) cells (Lamey, Thompson et al. 2002). It has been postulated that $D_1$ receptors undergo hierarchical phosphorylation by GRKs, as phosphorylation of key carboxyl terminal tail residues followed by phosphorylation of some combination of Ser256, Ser258 and Ser259 seems to be necessary for desensitization and arrestin recruitment in HEK 293 cells (Kim, Gardner et al. 2004). The second messenger kinase PKA also phosphorylates $D_1$ receptors in an agonist-dependent manner. $D_1$ receptors undergo far less desensitization with respect to cAMP accumulation in mutant CHO lines that express reduced levels of PKAI or PKAII, compared to $D_1$ receptors in parental CHO lines (Ventura and Sibley 2000). In C6 glioma cells, mutation of four PKA consensus phosphorylation sites at Thr135, Ser229, Thr268 and Ser380 of the $D_1$ receptor resulted in far less agonist-induced desensitization with respect to cAMP accumulation than wild type receptors (Jiang and Sibley 1999). Further investigation identified Thr268 as the residue most important for mediating this desensitization. Interestingly, other studies have reported that PKA inhibition has no effect on agonist-stimulated $D_1$ receptor phosphorylation seen after 10 minutes of dopamine treatment (Gardner, Liu et al. 2001) and that alanine substitution at Thr268 does not alter $D_1$ receptor-mediated cAMP accumulation in NS20Y neuroblastoma cells (Mason, Kozell et al. 2002). These studies suggest that the mechanistic details of agonist-induced phosphorylation and desensitization of $D_1$ receptors can differ depending on both cell type and the temporal parameters of a given experiment.

Following agonist-dependent phosphorylation, arrestin recruitment is additionally thought to mediate important interactions that lead to receptor endocytosis (Ferguson 2001; Gainetdinov, Premont et al. 2004). Arrestins can bind to the clathrin adaptor protein AP-2, as well as clathrin itself, to promote clathrin-mediated endocytosis of activated $\beta_2$-adrenergic receptors (Goodman, Krupnick et al. 1996). This model likely applies to the $D_1$ dopamine receptor as well. Agonist stimulation of $D_1$ receptors has been shown to preferentially recruit arrestin 3 (βarrestin-2) to the plasma membrane of $D_1$ receptor expressing HEK 293 cells (Oakley, Laporte et al. 2000), though there is evidence that activated $D_1$ receptors can also efficiently recruit arrestin 2 (βarrestin-1) to the plasma membrane (Kim, Gardner et al. 2004). Consistent with what has been demonstrated for the $\beta_2$-adrenergic receptor, $D_1$ receptors undergo clathrin-mediated endocytosis after agonist activation in HEK 293 cells (Vickery and von Zastrow 1999). In fact, agonist-mediated endocytosis of $D_1$ receptors has been demonstrated in a number of cellular preparations. Treatment of $D_1$ receptor expressing CHO cells with 50μM dopamine for 15 minutes leads to significant incorporation of the stryl dye FM1-43 into intracellular lipid bilayers of vesicular organelles, indicative of endocytosis (Ariano, Sortwell et al. 1997). Incubation of cultured striatal neurons with the $D_1$ receptor agonist SKF 82958 and alexa-488-labeled transferrin results in significant co-localization of $D_1$ receptors and transferrin 10 minutes after agonist treatment (Martin-Negrier, Giselle et al. 2006). This indicates that $D_1$ receptors can be found in early endosomes within 10 minutes of agonist treatment. Intrastriatal or intraperitoneal injection of SKF 82958 and intraperitoneal injection of amphetamine (known to release dopamine within the striatum) all led to intense redistribution of $D_1$ receptor immunoreactivity from the plasma

membrane to endocytic vesicles in cell bodies and dendrites of rat striatal neurons within 40 minutes of injection (Dumartin, Caille et al. 1998). This suggests that agonist-induced endocytosis of $D_1$ receptors occurs *in vivo*.

After endocytosis, GPCR membrane trafficking is thought to have important consequences for cellular signaling. Sorting of endocytosed GPCRs to a lysosomal degradation pathway can lead to prolonged desensitization of signaling whereas receptor sorting to recycling endosomes is thought to facilitate dephosphorylation of desensitized receptors, restore functional receptors back to the plasma membrane and promote recovery of signaling (Hanyaloglu and Zastrow 2008; Marchese, Paing et al. 2008). This recovery of GPCR-mediated signaling is commonly referred to as receptor resensitization. The classical receptor recycling/resensitization paradigm was first established for the $\beta_2$-adrenergic receptor (Pippig, Andexinger et al. 1995; Lefkowitz 1998). The $D_1$ receptor also undergoes sequence-directed recycling after endocytosis in HEK 293 cells (Vargas and von Zastrow 2004). Following agonist-induced endocytosis in cultured striatal neurons, $D_1$ receptors show a similar distribution to un-stimulated $D_1$ receptors within 20 minutes of agonist washout (Martin-Negrier, Giselle et al. 2006). This change in receptor distribution is sensitive to treatment with monensin, a compound known to disrupt organelle acidification and prevent recycling of other GPCRs.

Unlike the $\beta_2$-adrenergic receptor, previous studies have reached differing conclusions about how, if at all, $D_1$ receptor membrane trafficking might regulate dopaminergic signaling. In C6 glioma cells, $D_1$ receptors show increased phosphorylation and

desensitization with respect to $D_1$ receptor-mediated cAMP accumulation after dopamine treatment (Gardner, Liu et al. 2001). The phosphorylation state of the receptor returns to basal levels within 20 minutes of agonist washout. Interestingly, although the authors demonstrate $D_1$ receptor endocytosis after 30 minute incubation with 50μM dopamine, they also demonstrate that endocytosis is not necessary for receptor dephosphorylation. Furthermore cells treated with dopamine for 60 minutes did not recover $D_1$ receptor-mediated cAMP accumulation for several hours after agonist washout. These findings suggest that endocytosis of $D_1$ receptors does not contribute to the recovery of dopaminergic signaling over this time course. Another study reported that a $D_1$-mutant receptor truncated after residue 351 of the cytoplasmic tail is defective in its ability to undergo dopamine-induced endocytosis and subject to significantly less desensitization than its wild-type counterpart (Jackson, Rafal et al. 2002). Although this could suggest that endocytosis inhibits dopaminergic signaling, it is important to point out that this receptor is missing a number of candidate phosphorylation sites thought to be important for the initiation of receptor desensitization. Despite the numerous contributions that these studies have made to our understanding of the association between $D_1$ receptor membrane trafficking and dopaminergic signaling, a causal relationship between $D_1$ receptor trafficking and signaling has yet to be defined. It is also not known whether $D_1$ receptor trafficking can regulate signaling in physiological models of dopaminergic transmission such as cortical and striatal neurons. Furthermore, previous studies have failed to examine trafficking and signaling over a time course that is relevant to observed dopaminergic transmission within the brain.

The present body of work examines the relationship between $D_1$ dopamine receptor trafficking and dopaminergic signaling in HEK 293 cells, cortical neurons, striatal neurons and an intact striatal slice preparation. In chapter 2, we employ immunofluorescence techniques to observe endocytosis and recycling of the $D_1$ dopamine receptor over a time course that is consistent with classic biochemical cAMP signaling assays. We demonstrate that $D_1$ receptors undergo significant endocytosis in both HEK 293 cells and cultured cortical neurons after 30-minute agonist treatment. We also show that this duration of agonist exposure leads to decreased cellular sensitivity with respect to $D_1$-stimulated cAMP accumulation (desensitization). Agonist-washout promotes efficient recycling of $D_1$ receptors back to the plasma membrane of HEK 293 cells and cortical neurons, but does not lead to the recovery of $D_1$-stimulated cAMP accumulation (resensitization). Further, we demonstrate that 30-minute incubation with 10μM dopamine is capable of inhibiting agonist-induced cAMP accumulation via another $G_s$-coupled receptor, the $\beta_2$-adrenergic receptor. Together these findings indicate that the $D_1$ receptor membrane trafficking is not a major regulatory mechanism of dopaminergic signaling along this time scale.

The high concentrations of dopamine thought to activate $D_1$ receptors within the brain are thought to be quite transient in duration (Heien and Wightman 2006; Goto, Otani et al. 2007). As such, we examine $D_1$ receptor trafficking and signaling with far greater temporal resolution in chapter 3. We employ total internal reflection (TIRF) microscopy and a pH-sensitive variant of green fluorescent protein (GFP) to observe $D_1$ receptor trafficking in live HEK 293 cells and striatal neurons. We observe individual $D_1$

endocytic events that were initiated within 15 seconds of agonist addition and $D_1$ receptor exocytic events, consistent with previous descriptions of receptor recycling (Yudowski, Puthenveedu et al. 2007), within seconds of agonist washout. We also employ a FRET-based biosensor to observe $D_1$ receptor-mediated cAMP dynamics and establish that $D_1$ receptor trafficking and acute G protein-mediated signaling occur with overlapping kinetics. We ask whether a direct, causal relationship exists between $D_1$ receptor endocytosis and dopaminergic signaling by inhibiting endocytosis and measuring $D_1$ receptor-stimulated cAMP or action potential firing in neurons of the dorsal lateral striatum. We find that rapid endocytosis is essential for $D_1$ receptor signaling in HEK 293 cells, isolated medium spiny neurons and an intact striatal slice preparation. These results establish a novel role of endocytosis in promoting dopamine neurotransmission.

# Chapter 2: Investigation of $D_1$ Dopamine Receptor Trafficking and Signaling

# 2.1 Abstract

Dopaminergic signaling is essential for a number of important physiological functions. The $D_1$ receptor mediates the majority of all excitatory dopamine transmission within the brain. In this study we examine agonist-induced membrane trafficking of $D_1$ receptors in both HEK 293 cells and cultured cortical neurons. We also measure $D_1$ receptor-mediated cAMP accumulation in these systems on a similar time scale. We establish that $D_1$ receptors undergo robust endocytosis in the presence of agonist and efficiently recycle back to the plasma membrane after agonist washout. Cellular sensitivity to acute $D_1$ receptor-stimulated cAMP accumulation is decreased after pre-exposure to $D_1$ receptor agonists. Surprisingly, this inhibition persists up to 60 minutes after agonist washout, in spite of efficient $D_1$ receptor recycling. We also demonstrate that this decreased sensitivity after prolonged agonist treatment is not specific to the $D_1$ receptor and likely reflects a more general change in the signaling pathway. Our results suggest that $D_1$ receptor trafficking does not promote recovery of dopaminergic signaling in HEK 293 cells or cortical neurons along this time scale.

## 2.2 Introduction

Dopamine is a catecholamine neurotransmitter in the central nervous system (CNS) known to influence a number of important processes including movement, learning and memory, emotion and goal-directed behavior (Missale, Nash et al. 1998). Excessive dopaminergic transmission has been associated with a number of pathological states including schizophrenia and addiction. As such, precise regulation of dopaminergic signaling is crucial for normal physiology. All of the cellular actions of dopamine are mediated by a family of G-protein coupled receptors (GPCRs), the dopamine receptors. The $D_1$ receptor is the major mediator of excitatory dopaminergic signaling in the brain. Elucidating the cellular mechanisms that modulate $D_1$ receptor-mediated signaling is important for understanding the complex regulation of dopamine physiology.

Upon agonist binding, $D_1$ receptors undergo a conformational change that contributes to the activation of their associated heterotrimeric G-proteins, $G_s/G_{olf}$. Activated $G\alpha_{s/olf}$ go on to stimulate adenylyl cyclase in the plasma membrane and increase the cytoplasmic concentration of cAMP. This elevation of cellular cAMP is thought to underlie all of the $D_1$ receptor-mediated signaling responses in the CNS (Greengard 2001; Neve, Seamans et al. 2005). Shortly agonist activation, several GPCRs are subject to a series of regulatory events that reduce both the number and the activity of receptors at the plasma membrane. These events are initiated by agonist-dependent phosphorylation of GPCRs, followed by receptor endocytosis (Lefkowitz 1998; Marchese, Paing et al. 2008). Post-

endocytic receptor trafficking is thought to affect cellular sensitivity by controlling the number of functional GPCRs that are on the cell surface able to transduce a signal. GPCR trafficking to lysosomes leads to receptor degradation and a prolonged inhibition of cellular signaling, whereas GPCR trafficking via recycling endosomes is thought to restore cellular sensitivity by returning functional receptors back to the plasma membrane (Hanyaloglu and Zastrow 2008).

The $D_1$ dopamine receptor undergoes agonist-induced endocytosis in a number of cellular systems (Ariano, Sortwell et al. 1997; Dumartin, Caille et al. 1998; Vickery and von Zastrow 1999; Bloch, Bernard et al. 2003). In addition, $D_1$ receptor recycling has been demonstrated in HEK 293 cells and striatal neurons after agonist washout (Vargas and von Zastrow 2004; Martin-Negrier, Giselle et al. 2006). It is not known if the $D_1$ receptor exhibits similar membrane trafficking properties in cortical neurons. Furthermore, the functional consequences of $D_1$ receptor membrane trafficking with respect to cellular signaling are not clear. In this study, we examine $D_1$ receptor membrane trafficking and signaling in HEK 293 cells as well as physiologically relevant cortical neurons. Prolonged incubation with agonist leads to both $D_1$ receptor endocytosis and decreased cellular sensitivity to $D_1$ receptor-stimulated accumulation of cellular cAMP. We also find that although $D_1$ receptors recycle after agonist washout, this recycling does not lead to the recovery of $D_1$ receptor-mediated cAMP production. Thus, membrane $D_1$ receptor membrane trafficking does not promote dopaminergic signaling on this time scale.

## 2.3 Results

**The $D_1$ receptor undergoes dopamine-stimulated endocytosis and recycles following agonist washout in HEK 293 cells.**

To begin to investigate the relationship between membrane trafficking and dopaminergic signaling, we examined the trafficking of the $D_1$ receptor in HEK 293 cells. It has been previously established that $D_1$ receptors undergo robust endocytosis within 30 minutes of agonist treatment and efficiently recycle back to the plasma membrane within 60 minutes of agonist removal (Vickery and von Zastrow 1999; Vargas and von Zastrow 2004). We first verified these results by immunofluorescence microscopy. In the absence of agonist, labeled FD1 receptors showed a smooth plasma membrane distribution **(Figure 1A, top)**. Treatment with 10μM dopamine and subsequent stripping of surface labeled receptors in non-permeabilizing conditions revealed a profound redistribution of FD1 into endosomal structures **(Figure 1A, center)**. Agonist washout and incubation in fresh media for 60 minutes led to recovery of labeled FD1 receptors to the cell surface that were recognized by a Cy3-conjugated secondary antibody, consistent with recycling **(Figure 1A, bottom)**.

In order to quantify endocytosis and recycling of $D_1$ receptors, we used a previously described flow cytometric assay to measure both the decrease and recovery of labeled FD1 fluorescence in response to dopamine treatment and subsequent washout (Tsao and von Zastrow 2000). Total surface fluorescence of untreated, FD1-expressing HEK 293 cells was defined as 100%. Incubation with 10μM dopamine for 30 minutes led to a

significant decrease in surface fluorescence that recovered back to baseline levels after agonist washout **(Figure 1B)**. This recovery of fluorescence indicates that FD1 receptors are efficiently recycled back to the plasma membrane within 60 minutes after removal of dopamine.

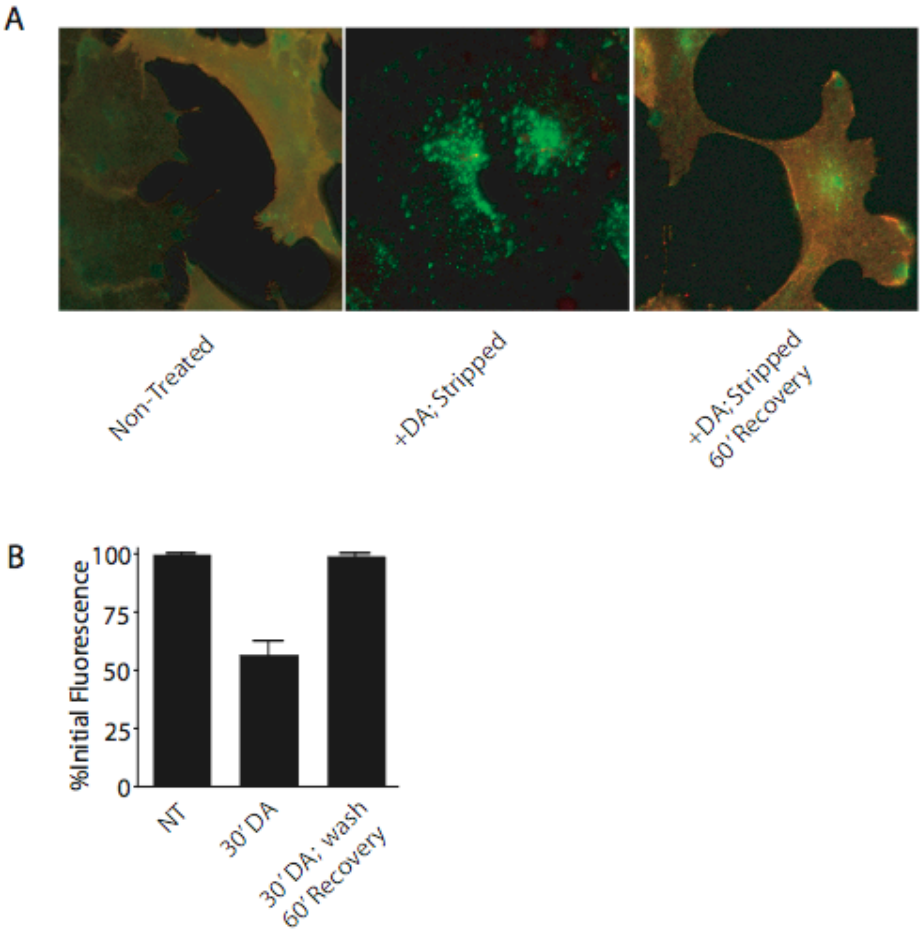



**Figure 1. Regulated endocytosis and recycling of $D_1$ dopamine receptors in HEK 293 cells.** **(A)** FD1 receptors present in the plasma membrane of stably transfected 293 cells were labeled with Alexa488-conjugated M1 anti-FLAG (green). Cells were either fixed without any drug treatment (Non-treated), treated with 10µM dopamine (DA) for 30 minutes then stripped of remaining surface antibody with a PBS/EDTA wash (+DA; Stripped), or treated with 10µM DA for 30 minutes, surface stripped, placed in fresh media containing 1µM SCH23390 and returned to the incubator for 60 minutes (+DA; Stripped; 60' Recovery) before fixation in non-permeabilizing conditions. Cells were then incubated with Alexa594-conjugated donkey anti-mouse secondary (red). **(B)** FD1-expressing cells were analyzed using fluorescence flow cytometry to measure dopamine-induced effects on surface receptor number. The amount surface fluorescence measured with no agonist treatment was defined as 100% (NT). Incubation with DA for 30 min. (30' DA) reduced surface fluorescence to 57.2 +/- 5.7%. Agonist washout and recovery (30'DA; wash; 60' Recovery) resulted in a recovery of FD1 surface fluorescence to 99.4 +/- 1.1%. Data represent mean surface fluorescence +/- SEM relative to non-treated for four separate experiments, 10,000 cells counted/condition in duplicate.

**Previous exposure to dopamine inhibits $D_1$-stimulated accumulation of cellular cAMP.**

We next wished to examine whether the membrane trafficking of $D_1$ receptors that we observed might correlate with changes in cellular sensitivity to $D_1$ receptor-mediated signaling. We did this by measuring dopamine-induced activation of adenylyl cyclase with a whole cell cAMP assay. Given that a significant fraction of surface $D_1$ receptors undergo endocytosis in response to dopamine treatment, we asked if previous exposure to dopamine might inhibit $D_1$ receptor-stimulated cAMP accumulation **(See experimental schematic, Figure 2A)**. We first measured cAMP accumulation in FD1-expressing HEK 293 cells after acute exposure to 10μM dopamine. The amount of cellular cAMP generated over 15 minute interval in the presence of 10μM dopamine, 1mM ascorbic acid and the phosphodiesterase (PDE) inhibitor IBMX was defined as 100% Max cAMP **(Figure 2B, t=0)**. FD1-expressing cells were treated with dopamine for 10, 30 or 60 minutes prior to washout and subsequent stimulation with dopamine. Previous exposure to dopamine led to a significant decrease in the amount of cellular cAMP generated by an acute challenge with dopamine **(Figure 2B)**.

It is possible that the presence of PDE inhibitors contributed to the decreased cellular sensitivity to $D_1$ receptor-stimulated cAMP accumulation observed after previous exposure to dopamine. To examine this possibility, we measured dopamine induced changes in cellular cAMP in individual living cells with a FRET-based cAMP biosensor, Epac1-cAMPs (Nikolaev, Bunemann et al. 2004). HEK 293 cells expressing FD1 and Epac1-cAMPs showed a robust decrease in normalized FRET emission ratio, indicating

elevated cytoplasmic cAMP concentration, within 60 seconds of addition of 10μM dopamine **(Figure 2C, naïve)**. Pre-treating cells with 10μM dopamine for 30 or 60 minutes prior to washout and imaging led to a decrease in $D_1$ receptor-stimulated cAMP accumulation and an increase in the time required to achieve a maximal signaling response **(Figure 2C, inset).** These data, in combination with the results of biochemical cAMP assays, suggest that a correlation may exist between $D_1$ receptor endocytosis and $D_1$ receptor mediated signaling. Acute, agonist-stimulated cAMP accumulation is inhibited after cells are incubated with dopamine for a period of time known to cause pronounced $D_1$ receptor endocytosis.

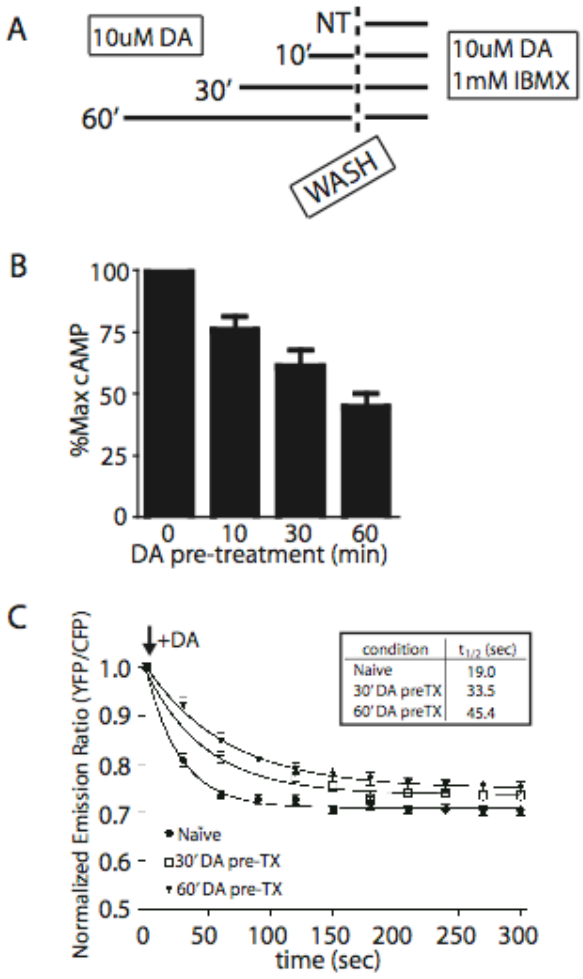



**Figure 2. Pre-exposure to dopamine inhibits agonist-stimulated cAMP accumulation in D1 receptor expressing HEK 293 cells.**
**(A)** Schematic representation of experimental procedure to assay effects of dopamine pretreatment on dopamine-stimulated cAMP accumulation in FD1 expressing 293 cells. **(B)** DA pretreatment decreased cAMP accumulation observed in response to a subsequent 15 min. DA challenge. The amount of cAMP generated by acute application of 10µM DA in the presence of IMBX in cells seeing no DA pretreatment was defined as 100% Max cAMP. 10 min. DA pretreatment reduced the DA-stimulated cAMP accumulation to 75.2 +/- 5.6%, 30 min. pretreatment reduced cAMP to 60.6 +/- 6.7% and 60 min. pretreatment reduced cAMP to 43.9 +/- 5.8%. Data represent mean +/- SEM, normalized to 100% Max cAMP from 3 experiments, each condition in triplicate. **(C)** Change in normalized Epac1-cAMPs FRET in response to stimulation with 10µM DA in naïve (closed circles), 30 min. DA pretreated (open squares), or 60 min. DA pretreated (inverted triangles) cells. Data represent mean +/- SEM normalized FRET emission ratio (See methods for calculations) at each time point for cells in each group. (n=21-32 cells per group). t(1/2) values were calculated by fitting data to one-phase exponential decay with Graph Pad software.

**Prolonged attenuation of cellular signaling after dopamine washout.**

Previous studies have demonstrated that recycling of GPCRs after agonist-induced endocytosis is necessary to restore functional receptors back to the plasma membrane that are capable of generating a signaling response (Pippig, Andexinger et al. 1995; Lefkowitz 1998). In addition, our trafficking data indicate that internalized $D_1$ receptors efficiently recycle back to the cell surface after agonist washout. This led us to question whether the decreased cellular cAMP accumulation we observed after pre-exposure to dopamine could recover after agonist washout **(See experimental schematic, Figure 3A)**. As previously demonstrated, acute dopamine stimulation of FD1-expressing HEK 293 cells generated robust cAMP accumulation and 30-minute dopamine pre-treatment significantly reduced the amount of cAMP generated by an acute dopamine challenge. Surprisingly, dopamine-stimulated cAMP accumulation did not recover either 30 minutes or 60 minutes after dopamine washout **(Figure 3B)**. These data suggest that in spite of efficient recycling of $D_1$ receptors within 60 minutes after agonist washout, cellular sensitivity to dopamine-stimulated cAMP accumulation remains inhibited on a similar time scale.

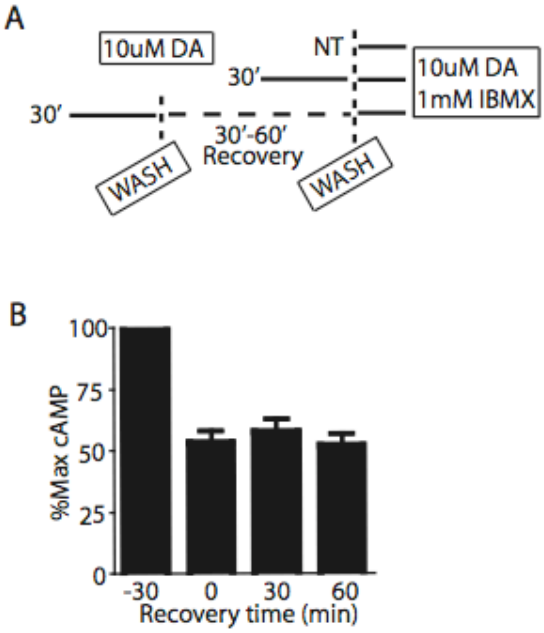



**Figure 3. Prolonged inhibition of dopamine-stimulated cAMP accumulation after agonist washout.**
**(A)** Schematic representation of experimental procedure to assay effects of agonist washout on recovery of dopamine-stimulated cAMP accumulation.
**(B)** Dopamine stimulated cAMP accumulation does not recover after agonist washout. Cells receiving no DA pretreatment were stimulated as described in figure 2 and the amount of cAMP generated was defined as 100% Max cAMP (-30 min recovery time). 30 min. DA pretreatment and 0 min recovery time after washout decreased cAMP to 52.8 +/- 4.9%. 30 min after DA washout, cAMP remained reduced to 57.4 +/- 5.0%, 60 min after washout, cAMP remained reduced to 51.8 +/- 4.7%. Data represent mean +/- SEM normalized to 100% Max cAMP from 3 experiments, each condition in duplicate.

**Prolonged attenuation of cellular signaling is not specific to the $D_1$ receptor.**

Given that $D_1$ receptor-mediated signaling remained attenuated following essentially complete recovery of surface receptor number, we next asked if the reduction in cAMP responsiveness after pre-exposure to dopamine is specific to the $D_1$ receptor, or if it might reflect a more general change in the signaling pathway. HEK 293 cells endogenously express adrenergic receptors, which are also $G_s$-coupled and activate adenylyl cyclase upon agonist stimulation. Similar to the experiment outlined in figure 3A, we treated $D_1$ expressing 293 cells with 10uM dopamine for 30 minutes, washed out agonist then returned cells to fresh media for 0, 30, or 60 minutes before applying a 10uM challenge dose of the adrenergic agonist isoproterenol **(See experimental schematic, Figure 4A)**. When compared to the amount of cAMP generated by acute stimulation with isoproterenol challenge (100% Max cAMP), dopamine pre-exposure significantly inhibited isoproterenol-stimulated cAMP accumulation **(Figure 4B)**. In addition, isoproterenol-stimulated cAMP accumulation did not recover after dopamine washout and 30 or 60-minute incubation in fresh media. We verified that dopamine was not directly stimulating endogenous adrenergic receptors by measuring both isoproterenol and dopamine-stimulated cAMP accumulation in untransfected 293 cells. While isoproterenol generated robust accumulation of cAMP, dopamine-stimulated cAMP levels were not significantly different than un-stimulated controls **(Figure 4C)**. These findings strongly suggest that regulation of cellular cAMP accumulation seen with prolonged dopamine exposure is not specific to the $D_1$ receptor and likely reflects a more general change in the signaling pathway. Furthermore, they support the hypothesis that

$D_1$ receptor trafficking is not a major regulatory mechanism of dopaminergic signaling after prolonged agonist treatment.

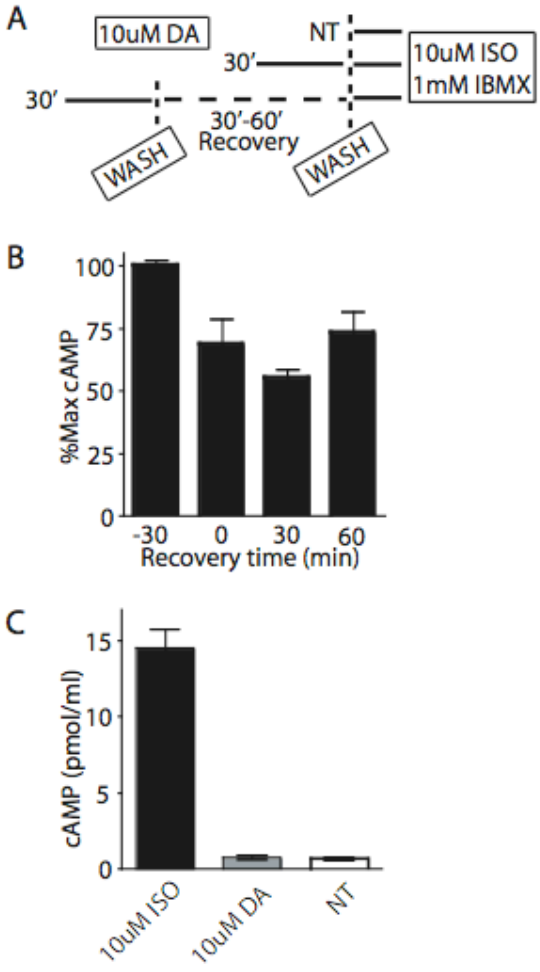



**Figure 4. Prolonged inhibition of cAMP accumulation after dopamine pretreatment is not specific to $D_1$ receptors.**
**(A)** Schematic representation of experimental procedure to assay effects of dopamine pretreatment on isoproterenol-stimulated cAMP accumulation. **(B)** Pretreatment with dopamine leads to a persistent decrease of isoproterenol (ISO) stimulated cAMP accumulation in FD1 expressing 293 cells. The amount of cAMP generated by 10µM ISO in 15 min. was defined as 100% Max cAMP. 30 min. DA pretreatment and 0 min recovery after washout resulted in a reduction of ISO-stimulated cAMP to 68.6 +/- 9.8%. 30 min. after washout, cAMP remained reduced to 55.2 +/- 3.2%, 60 min. after washout cAMP was at 73.1 +/- 8.5%. Data represent mean +/- SEM normalized to 100% Max cAMP from 3 experiments, each condition in triplicate. **(C)** cAMP production in untransfected HEK 293 cells. 15 min. incubation with 10µM ISO generated 14.5 +/- 1.2 pmol/ml cAMP, 10µM DA generated 0.75 +/- 0.12 pmol/ml cAMP and 0.67 +/- 0.10 pmol/ml cAMP could be detected in untreated cells (NT).

**Agonist induced endocytosis and recycling of $D_1$ receptors in cortical neurons.**

Although HEK 293 cells are a useful model system for studying the mechanisms of $D_1$ receptor trafficking and signaling, we wished to examine these processes in cells that natively express $D_1$ receptors in the brain. $D_1$ receptors are highly expressed within mammalian cortex (Levey, Hersch et al. 1993; Bergson, Mrzljak et al. 1995; Bordelon-Glausier, Khan et al. 2008). We expressed FD1 in dissociated cultures of rat cortical neurons and to observe $D_1$ receptor membrane trafficking in a more physiologically relevant cellular system. As cortical neurons are known to express a number of dopamine receptor subtypes, we used the $D_1/D_5$ receptor specific agonist SKF 81297 for all experiments in cortical neurons. FD1 receptors were present in a smooth, plasma membrane distribution on both the soma and dendrites of cortical neurons prior to agonist treatment **(Figure 5, top panel)**. Incubation with 1μM SKF 81297 led to a redistribution of labeled FD1 into internal structures **(Figure 5, middle panel)**. Antibody stripping of any remaining labeled FD1 from the cell surface in non-permeabilizing conditions confirmed that these structures were internal, consistent with endocytosis. The majority of internalized, FD1 receptors returned to the plasma membrane within 60 minutes after agonist washout **(Figure 5, bottom panel)**. These receptors were recognized by a secondary antibody, confirming that they were previously on the plasma membrane and underwent endocytosis prior to recycling. Our findings confirm that $D_1$ receptors undergo efficient endocytosis and recycling in cortical neurons.

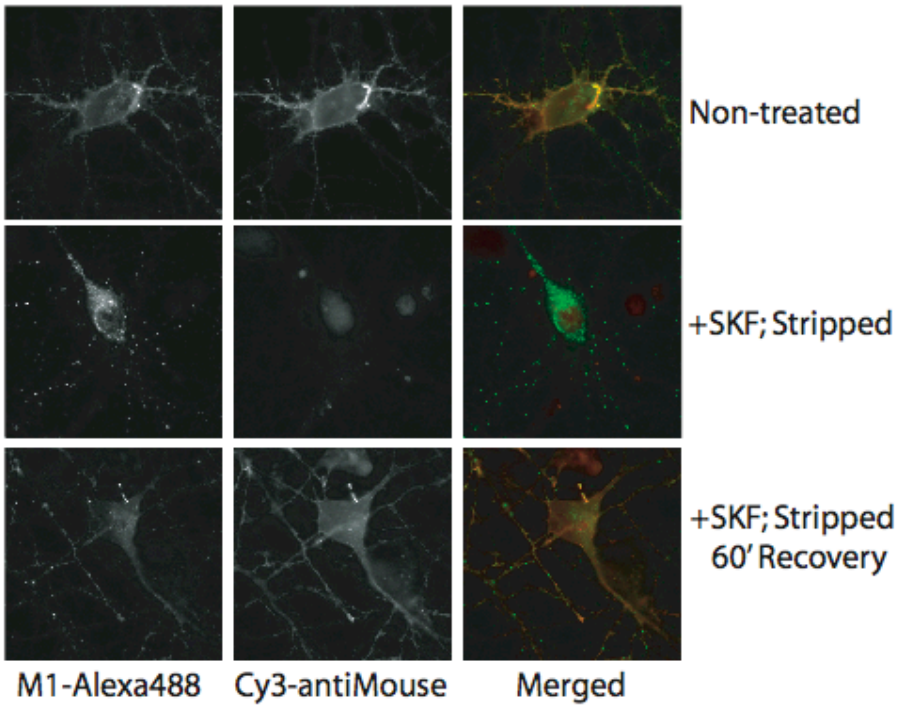



**Figure 5. Regulated endocytosis and recycling of $D_1$ receptors in cortical neurons.**
FD1 receptors present in the plasma membrane of dissociated cortical neurons were labeled with Alexa488-conjugated M1 anti-FLAG (green). Cells were either fixed without any drug treatment (Non-treated), treated with 1µM SKF 81297 (SKF) for 30 minutes then stripped of remaining surface antibody with a PBS/EDTA wash (+SKF; Stripped), or treated with 1µM SKF 81297 for 30 minutes, surface stripped, placed in fresh media containing 1µM SCH23390 and returned to the incubator for 60 minutes (+SKF; Stripped; 60' Recovery) before fixation in non-permeabilizing conditions. Cells were then incubated with Cy3-donkey anti-mouse secondary (red).

**$D_1$ receptor-mediated signaling is attenuated after prolonged agonist exposure in cortical neurons.**

We also wished to determine how previous exposure to agonist might affect $D_1$ receptor-mediated signaling in a more physiologically relevant system. We employed the previously described biochemical cAMP assay to examine $D_1$ receptor-mediated cAMP accumulation cortical neurons. Acute stimulation of FD1 expressing cortical neurons with 1μM SKF 81297 in the presence of IBMX led to marked accumulation of cAMP. Similar to our findings in HEK 293 cells, incubating neurons with 1uM SKF 81297 for 10, 30, or 60 minutes prior to washout and acute agonist challenge significantly reduced $D_1$ receptor-mediated generation of cAMP **(Figure 6A)**. In addition, allowing neurons to recover in fresh media for 30 or 60 minutes after 30-minute incubation with SKF 81297, did not lead to the recovery of cellular sensitivity to acute $D_1$ receptor-stimulated cAMP accumulation **(Figure 6B)**. Thus, in spite of efficient recycling of $D_1$ receptors back to the plasma membrane on this same time scale, $D_1$ receptor-mediated signaling in cortical neurons remains inhibited.

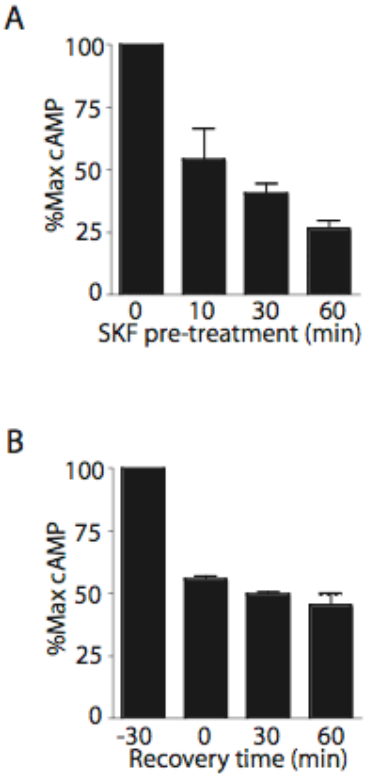



**Figure 6. Agonist-stimulated cAMP accumulation is inhibited by previous agonist exposure and fails to recover after washout in cortical neurons.**
**(A)** SKF 81297 pretreatment decreased cAMP accumulation observed in response to a subsequent 15 min. agonist challenge in FD1-expressing cortical neurons. The amount of cAMP generated by acute application of 1µM SKF 81297 in the presence of IMBX in cells seeing no agonist pretreatment was defined as 100% Max cAMP. 10 min. agonist pretreatment reduced the SKF-stimulated cAMP accumulation to 53.9 +/- 12.1%, 30 min. pretreatment reduced cAMP to 39.9 +/- 4.0% and 60 min. pretreatment reduced cAMP to 26.0 +/- 3.3%. Data represent mean +/- SEM, normalized to 100% Max cAMP from 3 experiments, each condition in triplicate. **(B)** The amount of cAMP generated by acute application SKF 81297 was determined as described above and defined as 100% Max cAMP (-30 min recovery time). 30 min. agonist pretreatment led to cAMP accumulation upon SKF stimulation of 55.4 +/- 1.3% of max. 30 min after agonist washout, SKF-stimulated cAMP levels were at 49.3 +/- 0.83%, and 60 min after washout SKF-stimulated cAMP levels were at 44.8 +/- 4.9%. Data represent mean +/- SEM normalized to 100% Max cAMP from 3 experiments, each condition in duplicate.

## 2.4 Discussion

In this study we demonstrate extensive $D_1$ receptor endocytosis following 30-minute agonist treatment in HEK 293 cells and cortical neurons. Prolonged agonist pre-treatment (10-60 minutes) also leads to an inhibition of acute dopamine or SKF 81297-stimulated cellular cAMP accumulation. This inhibition occurs regardless of whether PDE inhibitors are present during agonist pre-treatment. We show efficient recycling of internalized $D_1$ receptors back to the plasma membrane within 60 minutes of agonist washout. Interestingly, cellular sensitivity to $D_1$ receptor-mediated cAMP accumulation remains inhibited after washout, despite recovery of nearly the full complement of surface $D_1$ receptors. Further, we show that the persistent reduction in cellular sensitivity occurring after prolonged incubation with dopamine is not specific to the $D_1$ receptor. These findings support the hypothesis that membrane trafficking of $D_1$ receptors, on this time scale, does not promote recovery of dopaminergic signaling in HEK 293 cells or cultured cortical neurons.

Following agonist activation, many GPCRs become phosphorylated and can no longer efficiently signal via trimeric G proteins. Receptor phosphorylation by G protein coupled receptor kinases (GRKs) or other protein kinases can also recruit arrestins and promote clathrin-mediated endocytosis. After endocytosis, a number of GPCRs are delivered to a recycling pathway that facilitates receptor dephosphorylation and returns functional receptors to the cell surface. This has been shown for a number of GPCRs, perhaps the

best characterized being the beta-2 adrenoreceptor (Pippig, Andexinger et al. 1995; Lefkowitz 1998). Surprisingly, our results suggest that this is not the case for the $D_1$ receptor. Although this receptor is efficiently recycled back to the plasma membrane after agonist washout, $D_1$ receptor-mediated cellular signaling is not restored. Interestingly, this decreased cellular sensitivity cannot be solely attributed to the $D_1$ receptor, as signaling via another $G_s$-coupled receptor is also inhibited after prolonged dopamine treatment and agonist washout. This raises a number of questions for future study. First, what are the cellular mechanisms that lead to decreased cAMP production after prolonged incubation with dopamine and second, what role does $D_1$ receptor membrane trafficking play in the regulation of dopaminergic signaling? Although we do not further address the first question in this body of work, one can imagine that regulation could occur anywhere in the signaling cascade between $D_1$ receptor activation and cAMP production. It is also formally possible that the $D_1$ receptor could be recycled back to the plasma membrane without being dephosphorylated, resulting in cell surface accumulation of desensitized receptors that are unable to generate a signal in response to dopamine. Although, one study has reported dephosphorylation of activated $D_1$ receptors in the absence of endocytosis or recycling (Gardner, Liu et al. 2001). As for what role membrane trafficking plays in the regulation of dopaminergic signaling, that question will be addressed in the following chapter.

Improper regulation of dopaminergic signaling has been implicated in a number of pathologies including Parkinson's disease, addiction and schizophrenia. The persistent reduction of $D_1$ receptor-mediated signaling that we observed in spite of efficient receptor

recycling likely represents an important homeostatic mechanism that neurons employ to limit dopaminergic signaling within the brain. To our knowledge, this study is the first to demonstrate agonist-mediated endocytosis and recycling of $D_1$ receptors in cultured cortical neurons. We also believe it is the first to show that $D_1$ receptor-mediated signaling is inhibited after prolonged agonist treatment and washout, despite efficient receptor recycling over the same time course. Finally, this study presents the novel finding that this inhibition in signaling is not specific to the $D_1$ receptor in HEK 293 cells. These results provide insight into how neurons might cope with excessive dopaminergic transmission in the brain and also further our understanding of how a key signaling pathway in the CNS is regulated.

## 2.5 Experimental Procedures

### *cDNA and Constructs*

The N-terminally FLAG (DYKDDDD) epitope-tagged human D1 dopamine receptor (F-D1R) and Epac1-camps have been previously described (Vickery and von Zastrow 1999; Nikolaev, Bunemann et al. 2004). F-D1R and Epac1-camps were subcloned into pCAGGS (Niwa, Yamamura et al. 1991) for expression in cultured cortical neurons.

### *Cell Culture and Transfections*

HEK 293 cells (ATCC) were maintained in Gibco Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin (University of California, San Francisco, Cell Culture Facility). Cells were plated on 6 cm dishes and grown to 50-80% confluence prior to transfection using a cationic lipid reagent (Effectene; Qiagen). 1-2 ug of DNA was added to each dish per manufacturer instructions. 12-24 hours after transfection, cells were lifted with PBS with 0.04% EDTA (University of California, San Francisco, Cell Culture Facility) and re-plated onto poly-D-lysine (Sigma) coated, flame-polished glass coverslips (Corning) for recycling assays, 35mm glass-bottom dishes (Matek) for live FRET-imaging and 12 well dishes for cAMP immunoassays. All assays were done 48-72 hours post transfection.

Cortical neurons were taken from embryonic day 17-18 Sprague Dawley rats. Cortices were dissected in ice cold Hank's buffered saline solution (University of California, San Francisco, Cell Culture Facility) and tissue dissociated in 1x trypsin/EDTA (Invitrogen) for 25 minutes at 37˚C. Cells were washed in Gibco Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (University of California, San Francisco, Cell Culture Facility) and mechanically separated with a flame-polished Pasteur pipette. Cortical neurons were transfected via electroporation (Rat Neuron Nucleofector Kit; Lonza) per manufacturer's instructions and plated on poly-D-lysine coated 35mm glass bottom dishes (Matek) for imaging or 12 well plates for cAMP immunoassays. All experiments in cortical neurons were carried out 10-14 days post-transfection.

***Whole cell biochemical cAMP Assays***

To measure the effects of agonist pre-exposure on signaling, HEK 293 cells stably expressing FD1 and FD1-expressing cortical neurons were pretreated with 10uM dopamine (HEK 293) or 1μM SKF 81297 (Sigma) (cortical neurons) for 0, 10, 30 or 60 minutes and washed 3x with PBS (University of California, San Francisco, Cell Culture Facility) prior to challenge with the same agonist for 15 minutes in the presence of 1mM phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylisoxanthine (IBMX, Sigma) and 100uM ascorbic acid (Sigma). After dopamine challenge, cells were washed with ice-cold PBS and lysed with 0.1M HCl with 0.1%Triton-X100. Lysates were cleared of particulates via 10-minute centrifugation at 20,000xg at 4˚C. cAMP concentrations of cleared lysates were determined using the Correlate EIA Direct Cyclic AMP Enzyme

Immunoassay kit (Assay Designs), per manufacturer's instructions. The amount of cAMP generated by cells receiving only a challenge dose of dopamine was defined as 100% Maximum cAMP accumulation with all other experimental conditions normalized and compared to this value.

To assess the recovery of cAMP production after prolonged agonist treatment and washout, FD1 expressing cells were pretreated for 30 minutes with 10uM dopamine or 1μM SKF 81297, washed with PBS, placed in fresh media and returned to a 37˚C/5%$CO_2$ for 0, 30 or 60 minutes before 15 minute challenge with agonist in the presence of ascorbic acid and IBMX. Relative cAMP accumulation was determined as previously described.

To determine whether dopamine pre-exposure could inhibit isoproterenol-stimulated cAMP accumulation, FD1-expressing HEK 293 cells were treated with 10μM dopamine for 30 minutes prior to washout and 15-minute challenge with 10uM isoproterenol in the presence of IBMX and ascorbic acid. Cell were processed and cAMP levels determined as previously described.

Cyclic AMP production in non-transfected 293 cells was determined by incubating cells with 10uM isoproterenol, 10uM dopamine or no drug for 15 minutes in the presence of 1mM IBMX and 100uM ascorbic acid. Cells were then washed and lysed and absolute cAMP levels measured as previously described. All samples were normalized for protein concentration.

***Immunocytochemistry and Qualitative analysis of D1 receptor trafficking.***

FD1 expressing 293 cells or cortical neurons were incubated with 1ug/ml Alexa488 conjugated M1 anti-FLAG monoclonal antibody for 20 minutes to label surface D1 receptors. A first group of cells (Non-Treated) were washed 3x with cold TBS, fixed with 4% paraformaldehyde and 5% sucrose in PBS for 15 minutes and washed again with TBS prior to incubation with a 1:500 dilution of Cy3-conjugated donkey anti-Mouse IgG for 20 minutes under non-permeabilizing conditions (3% BSA in PBS). A second group of cells (+DA/+SKF; Stripped) was treated with 10uM dopamine or 1uM SKF 81297 (neurons) at 37˚C for 30 minutes then stripped of any remaining surface Alexa488 conjugated M1 by washing 3x with $Ca^{2+}$/$Mg^{2+}$-free PBS +0.04% EDTA (University of California, San Francisco, Cell Culture Facility) prior to fixation and incubation with secondary antibody. A third group of cells (+DA/+SKF; Stripped, 60' Recovery) was treated with dopamine or SKF and stripped as described above, but returned to fresh media containing 1uM SCH23390 to prevent further receptor activation and placed back in the incubator for 60 minutes in order to allow for recycling of Alexa488 labeled FD1 receptors prior to fixation and incubation with secondary antibody. All cells were washed with mTBS and covered with Fluoromount-G (Southern Biotech) to preserve signal for fluorescence microscopy.

Epifluorescence microscopy was performed using a Nikon TE2000E inverted microscope equipped with a 60x/numerical aperture (NA) 1.2 objective, xenon arc lamp with Lambda LS excitation and Lambda 10-3 emission filter wheels controlled with a SmartShutter

(Sutter Instruments) and NIS-Elements Advanced Research software (Nikon). Standard S470/30x (Alexa488) and S565/55 (Cy3) excitation and emission S510/30m (Alexa488) and S650/75m (Cy3) filters were used. Images were captured with a CoolSnap HQ2 CCD camera (Photometrics) and analyzed using ImageJ software (http://rsbweb.nih.gov/ij).

***Quantification of D1 endocytosis and recycling with Fluorescence Flow Cytometry.*** Surface fluorescence of FD1 expressing cells was used to measure receptor endocytosis and recycling. Non-treated cells were washed with ice-cold PBS and mechanically lifted prior to incubation with 1ug/ml Alexa647-congugated M1 anti-FLAG monoclonal antibody at 4˚C for 1-2 hours. For endocytosis, cells were treated with 10uM dopamine for 30 minutes prior to cold PBS wash and antibody incubation. For recycling, cells were treated with 10uM dopamine for 30 minutes, washed with PBS and returned to fresh media at 37˚C for 60 minutes prior to cold PBS wash and incubation with Alexa647-conjugated M1. Mean fluorescence intensity for 10,000 cells/condition was collected using a flow cytometer (Becton Dickson). Each condition was performed in duplicate with a minimum of 3 experiments.

***Live cell imaging and FRET Measurements of cAMP with Epac1-cAMPs*** FD1/Epac1-cAMPs expressing 293 cells were washed 2x with warm PBS and imaged in Dulbecco's modified Eagle's medium without phenol red (University of California, San Francisco, Cell Culture Facility), supplemented with 1% fetal bovine serum and 30mM Hepes (Sigma) at pH 7.4. Cells were maintained at 37˚C using a temperature controlled

stage (Bioscience Tools) and an objective heater (Bioptechs). A series of 3 sequential images were taken for each time point (every 30 seconds) in a given experiment. The "FRET" image was obtained with an ET430/24x nm (CFP) excitation filter and an ET535/30m nm (YFP) emission filter in place. The "CFP" image was obtained with the CFP excitation filter and an ET470/24m nm (CFP) emission filter in place. The "YFP" image was obtained with ET500/20x nm (YFP) excitation filter and the YFP emission filter in place. Exposure times for FRET, CFP and YFP images were 100msec, 100msec and 30msec respectively. Manufacturer's estimate of the time between sequential images is approximately 40msec. Correction values for bleed through of CFP into the YFP channel ($BT_{DONOR}$) and direct excitation of YFP from the CFP setting ($DE_{ACCEPTOR}$) were obtained by expressing CFP alone or YFP alone in cells and measuring the ratio of FRET to CFP emission ($BT_{DONOR}$) or FRET to YFP emission ($DE_{ACCEPTOR}$). These measurements were performed weekly throughout the course of FRET experiments. Intensity values ($I_X$) were calculated by drawing an ROI around each individual cell and measuring integrated fluorescence intensity and in a given channel at each time point. Background values ($BG_X$) were measured by drawing a similarly sized ROI in an area where no cells were present and collecting integrated fluorescence intensity values in a given channel at each time point. A corrected FRET ratio could be obtained for each cell at each time point using the following equation: $NFRET = [(I_{FRET}-BG_{FRET})-(I_{CFP}-BG_{CFP})BT_{DONOR}-(I_{YFP}-BG_{YFP})DE_{ACCEPTOR})] / I_{CFP}$ and normalizing the corrected FRET value obtained at the first time point to 1. All analysis was done using ImageJ.

FRET changes in response to agonist stimulation were obtained by adding 2µl of 1000x dopamine stocks in ascorbic acid or 2µl of 1000x SKF 81297 stocks to cells in 2ml of imaging media as indicated. Naïve cells were acutely stimulated with dopamine, whereas cells in the +30' preTX and +60' preTX groups were treated with agonist and placed in a 37ºC/5% $CO_2$ incubator for the indicated period of time, prior to 3x wash with warm, fresh media and imaging.

# Chapter 3: Endocytosis Promotes Rapid Dopaminergic Signaling

# 3.1 Abstract

$D_1$ dopamine receptors are the major transducers of excitatory dopamine signaling in the central nervous system (CNS). These receptors undergo agonist-mediated endocytosis via clathrin-coated pits, but the functional consequences of this regulation remain unclear. Here we apply real-time fluorescence imaging and a FRET-based cAMP biosensor to analyze rapid $D_1$ receptor endocytosis and signaling in both HEK 293 cells and striatal neurons. We observe remarkably fast $D_1$ receptor trafficking, and establish that acute G protein-mediated signaling occurs with overlapping kinetics. We also find that rapid endocytosis is essential for $D_1$ receptor signaling in isolated medium spiny neurons and an intact striatal slice preparation. These results establish a novel role of endocytosis in promoting dopamine neurotransmission.

# 3.2 Introduction

Dopamine is a major catecholamine neurotransmitter that controls many physiological processes (Missale, Nash et al. 1998; Sibley 1999) Improper regulation of dopaminergic signaling has been implicated in pathological states including Parkinson's disease, schizophrenia, attention-deficit/hyperactivity disorder and addiction. It is therefore not surprising that dopaminergic signaling in the CNS is highly regulated and subject to precise temporal control. All of the known cellular actions of dopamine are mediated by a subfamily of G protein coupled receptors (GPCRs), the dopamine receptors. The $D_1$ dopamine receptor represents the major excitatory dopamine receptor expressed in the CNS (Missale, Nash et al. 1998). The pharmacological properties of this receptor indicate that it is particularly important for transducing responses to transient bursts of high extracellular dopamine concentration that are characteristic of phasic release (Heien and Wightman, 2006; Richfield et al., 1989). Upon binding dopamine, $D_1$ receptors activate adenylyl cyclase (AC) through coupling to specific heterotrimeric G-proteins ($G_s$ or $G_{olf}$), thereby increasing cytoplasmic cyclic AMP (cAMP) concentration. In turn cAMP mediates the vast majority of $D_1$ receptor-dependent signaling effects in neurons (Greengard 2001; Neve, Seamans et al. 2005).

In order for neurons to respond to rapid changes in extracellular dopamine concentrations, $D_1$ receptors must be able to reliably transduce and sustain dopamine-dependent increases in cellular cAMP concentration over an appropriate period of time.

However, shortly after agonist activation, $D_1$ receptors are subject to a linked series of regulatory events that culminate in endocytic removal of receptors from the plasma membrane (Ng, Mouillac et al. 1994; Tiberi, Nash et al. 1996; Ariano, Sortwell et al. 1997; Dumartin, Caille et al. 1998; Mason, Kozell et al. 2002; Bloch, Bernard et al. 2003; Martin-Negrier, Giselle et al. 2006) This process has been shown to occur in the intact brain, yet its functional significance remains unknown (Dumartin, Caille et al. 1998; Muriel, Orieux et al. 2002).

The present study investigates the relationship between endocytosis and $D_1$ receptor signaling in both striatal medium spiny neurons and HEK 293 cells. Our results establish that regulated $D_1$ receptor endocytosis occurs rapidly within both systems and that the kinetics of endocytosis are comparable to those of $D_1$ receptor-mediated signaling induced by acute exposure to dopamine. Remarkably, we also demonstrate that rapid endocytosis is essential for dopaminergic signaling in dissociated striatal neurons and an intact slice preparation. These results identify a critical, and previously unanticipated, role of endocytosis in neuronal dopamine signaling.

## 3.3 Results

**Real-time analysis of $D_1$ receptor endocytosis and recycling with live cell imaging.** Regulated endocytosis of $D_1$ dopamine receptors is mediated primarily by clathrin-coated pits (Vickery and von Zastrow 1999). Previous studies have examined $D_1$ receptor endocytosis over a time scale of 10 to 30 minutes, but studies of other membrane cargo suggest that clathrin-mediated endocytosis can occur far more rapidly (Kirchhausen 2005; Perrais and Merrifield 2005). Furthermore, studies of phasic dopaminergic signaling in the brain suggest that dopamine release is quite transient in nature (Heien and Wightman 2006; Schultz 2007). This information led us to question whether $D_1$ receptor membrane trafficking might regulate cellular dopaminergic signaling on a more rapid time scale.

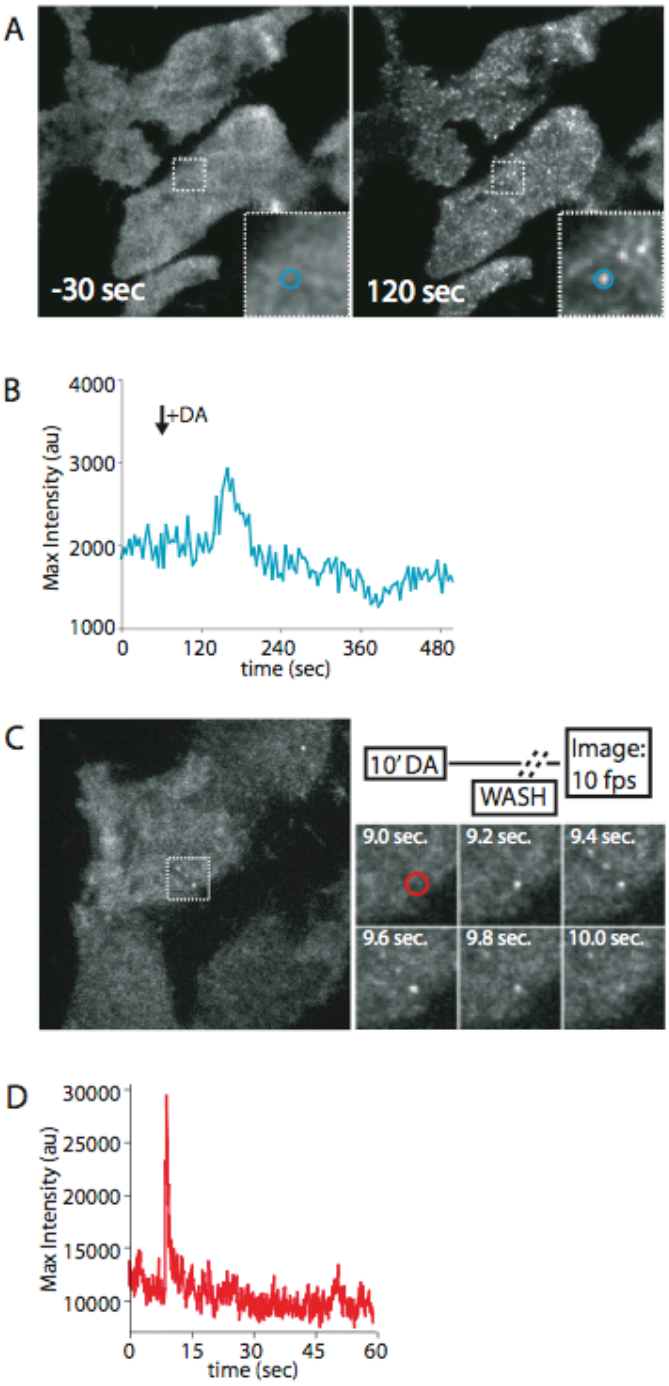
We first investigated this question using HEK293 cells. Regulated endocytosis of FLAG-epitope tagged $D_1$ dopamine receptors (FD1) in this model system was dose-dependent and detectable by fluorescence flow cytometry within 5 minutes after dopamine addition **(Figure S1)**. To observe $D_1$ receptor endocytosis with greater temporal resolution, we employed TIRF microscopy and the pH-sensitive GFP variant superecliptic pHluorin (SpH, or SEP in some studies) fused to the N-terminal extracellular region of $D_1$ receptor (SpH-D1R). SpH is highly fluorescent at pH 7.4, allowing us to detect SpH-D1R at the cell surface that is in contact with the extracellular media (Miesenbock, De Angelis et al. 1998; Sankaranarayanan, De Angelis et al. 2000).

This fluorescence is rapidly quenched in more acidic environments, such as those of the endocytic pathway. We made use of these properties to observe individual endocytic events in SpH-D1R expressing HEK 293 cells. In the absence of dopamine, SpH-D1R fluorescence was present with a uniform distribution on the plasma membrane **(Figure 1A, left)**. Strikingly, we were able to see an initial wave of SpH-D1R clustering and endocytosis that began as soon as 15 seconds after agonist addition **(Movie S1)**. Within minutes after dopamine addition, SpH-D1R appeared clustered in numerous puncta on the plasma membrane then rapidly endocytosed **(Figure 1A, right and Movie S1)**. The maximum fluorescence intensity of these puncta decreased to baseline or lower within 30 seconds to 1 minute of their appearance **(Figure 1B)**, consistent with previous descriptions of endocytosis of other signaling receptors (Puthenveedu and von Zastrow 2006; Yudowski, Puthenveedu et al. 2006).

We were also able to use SpH-D1R to observe receptor insertion into the plasma membrane. Quenched SpH fluorescence is rapidly reversed when the protein comes back in contact with the more neutral pH environment of the extracellular media. This allowed us to view individual exocytic events by imaging cells at 10 frames/second, both in the continuous presence of dopamine (data not shown) and after dopamine washout **(Figure 1C and Movie S2)**. Maximum intensity measurements revealed these events to be both of greater-fold intensity over background and of much shorter duration than the receptor clustering events that were observed immediately after dopamine addition **(Figure 1D)**. Both the lifetime and intensity of these distinct insertion events are in line with

descriptions of exocytosis for other signaling receptors (Yudowski, Puthenveedu et al. 2006; Yudowski, Puthenveedu et al. 2007).

Total surface measurements of SpH-D1R fluorescence in individual cells over time confirmed substantial $D_1$ receptor endocytosis within minutes of dopamine addition **(Figure 1E)**. Furthermore, dopamine washout facilitated complete recovery of SpH-D1R fluorescence within a matter of minutes, indicating that $D_1$ receptors were rapidly recycled back to the plasma membrane **(Figure 1F)**.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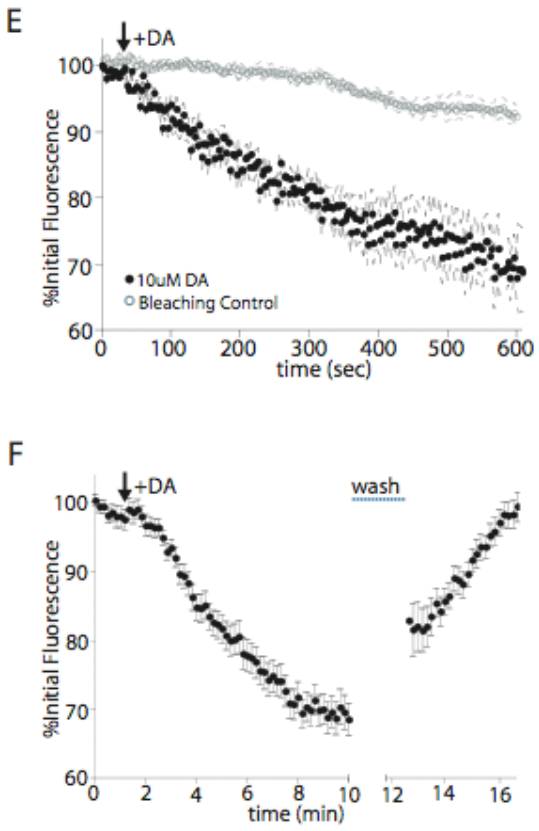



**Figure 1. Rapid dopamine-stimulated endocytosis and recycling of D1 dopamine receptors.** **(A)** Representative images of SpH-D1R surface fluorescence as visualized by TIRF microscopy prior to agonist addition (left, inset) and 120 sec. after DA addition (right, inset). **(B)** Maximum intensity trace of representative SpH-D1R cluster vs. time. Trace represents area outlined with blue circle in (A), maximum intensity measurements taken every 3 sec. **(C)** Experimental schematic and representative images of SpH-D1R insertion events visualized by TIRF microscopy after dopamine washout. Images were taken every 100 msec. **(D)** Maximum intensity trace of representative SpH-D1R insertion event vs. time. Trace represents area outlined with red circle in (C), maximum intensity measurements taken every 100 msec. **(E)** Average surface fluorescence of SpH-D1R expressing HEK 293 cells measured every 3 sec. in the absence of agonist treatment (Bleaching Control, n=5 cells) or in response to 10µM DA addition (10µM DA, n=20 cells). Initial fluorescence values of each cell normalized to 100%, data represent mean surface fluorescence +/- SEM. **(F)** Average surface fluorescence of SpH-D1R expressing cells measured every 10 sec. in response to 10µM DA addition and after agonist washout (n=10 cells). Initial fluorescence values of each cell normalized to 100%, data represent mean surface fluorescence +/- SEM.

**Real-time measurement of cAMP dynamics using a FRET-based biosensor.**

Given that endocytosis of $D_1$ receptors was observed within one minute of dopamine addition, we were interested in examining $D_1$ receptor-mediated signaling over a similar time scale. Conventional biochemical cAMP assays are not sufficient to achieve this temporal resolution. We employed the FRET-based cAMP biosensor, Epac1-cAMPs, in order to measure dopamine stimulated cAMP production in real time, in individual cells, without the requirement of phosphodiesterase inhibitors (Nikolaev, Bunemann et al. 2004). Cells expressing FD1 and Epac1-cAMPs showed a robust decrease in normalized FRET emission ratio, indicating elevated cytoplasmic cAMP concentration, within 60 seconds of dopamine addition **(Figure 2A)**. Dopamine application produced both a sustained decrease of YFP emission and a corresponding increase in CFP emission, verifying that the observed changes were indeed due to decreased FRET **(Figure 2B)**. Incubation of FD1/Epac1-cAMPs expressing cells with a range of dopamine concentrations resulted in a dose-dependent decrease in Epac1-cAMPs FRET that was evident as soon as 20 seconds after adding agonist **(Figure 2C)**. We verified that co-expression of Epac1-cAMPs did not alter $D_1$ receptor trafficking by dual-channel fluorescence microscopy **(Figure 2D)**.

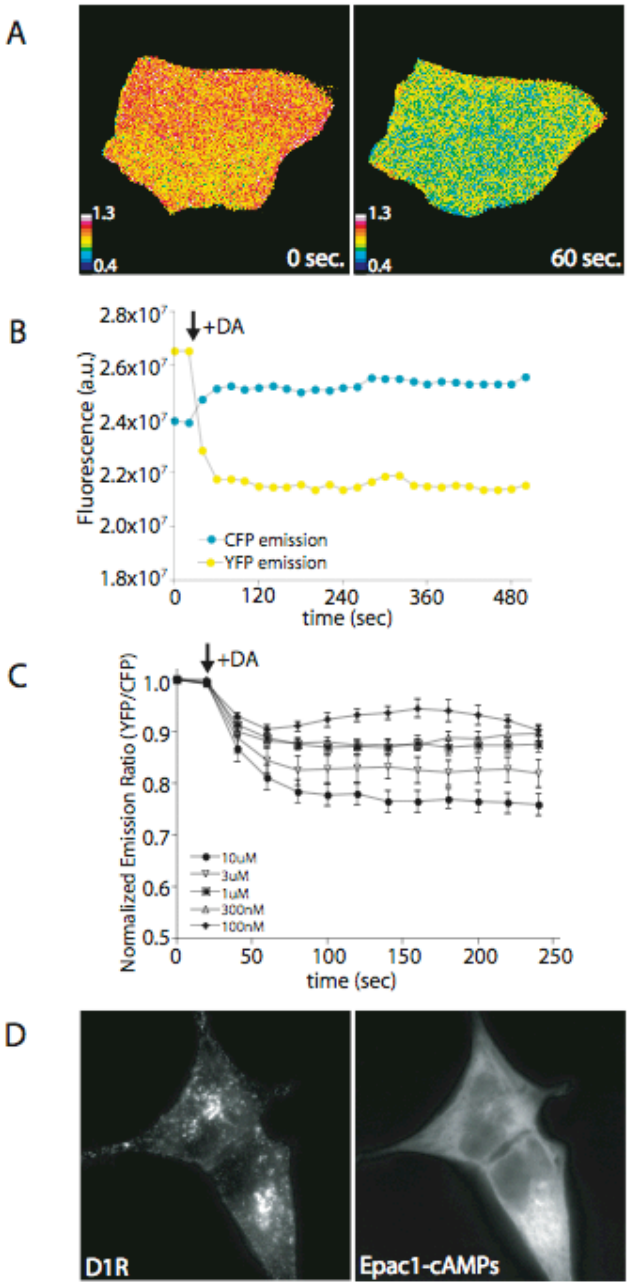



**Figure 2. Real-time examination of D1 receptor-stimulated changes in cellular cAMP using Epac1-cAMPs.**

**(A)** Pseudo-color representation of Epac1-cAMP FRET values in a representative D1/Epac1-cAMPs expressing 293 cell in the absence of DA (left) or 60 sec. after addition of 10µM DA (right). Look up tables represent bleed through/direct excitation corrected absolute (not normalized to 1) FRET values. See methods for calculations. **(B)** Changes in Epac1-cAMPs emission at 470nm (CFP) and 535nm (YFP) in a response to DA. Data represent direct excitation/bleed through corrected values of a single representative D1/Epac1-cAMPs expressing cell over time. **(C)** Change in normalized Epac1-cAMPs FRET in response to stimulation over a range of DA concentrations. Data represent mean +/- SEM normalized FRET emission ratio (See methods for calculations) at each time point for cells treated with a given dose of DA. (n= 8-16 cells per dose) **(D)** Subcellular distribution of Alexa594 surface labeled FD1 receptors in response to 10µM DA treatment (left) in the presence of Epac1-cAMPs (right).

**Endocytosis promotes acute $D_1$ receptor-mediated signaling.**

In light of the substantial temporal overlap between $D_1$ receptor trafficking and $D_1$ receptor-mediated signaling, we wished to determine if there is a causal relationship between the two. To do so, we employed two manipulations that acutely inhibit clathrin-dependent endocytosis. First, hypertonic sucrose is well known to inhibit clathrin-mediated endocytosis of a number of membrane proteins, including the $D_1$ receptor (Vickery and von Zastrow 1999; Gardner, Liu et al. 2001). This inhibitory effect is thought to occur via disruption of the normal clathrin lattice structure (Heuser and Anderson 1989). We confirmed that sucrose inhibited $D_1$ receptor endocytosis via immunofluorescence microscopy. Cells pretreated with hypertonic sucrose showed far less dopamine-stimulated redistribution of labeled FD1 into internal vesicles than cells not subject to treatment with this inhibitor **(Figure 3A)**. To assess the effect that inhibition of endocytosis has on $D_1$ receptor-mediated signaling, we pretreated FD1/Epac1-cAMPs expressing cells with hypertonic sucrose. Hypertonic sucrose significantly reduced the dopamine-stimulated change in Epac1-cAMPs FRET compared to cells receiving no pretreatment, indicative of impaired cAMP accumulation **(Figure 3B)**.

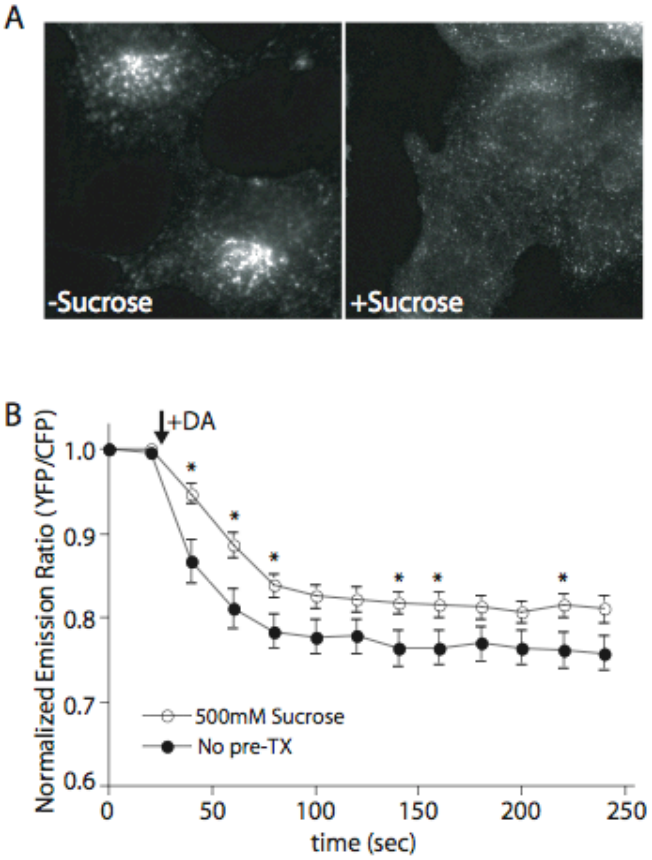
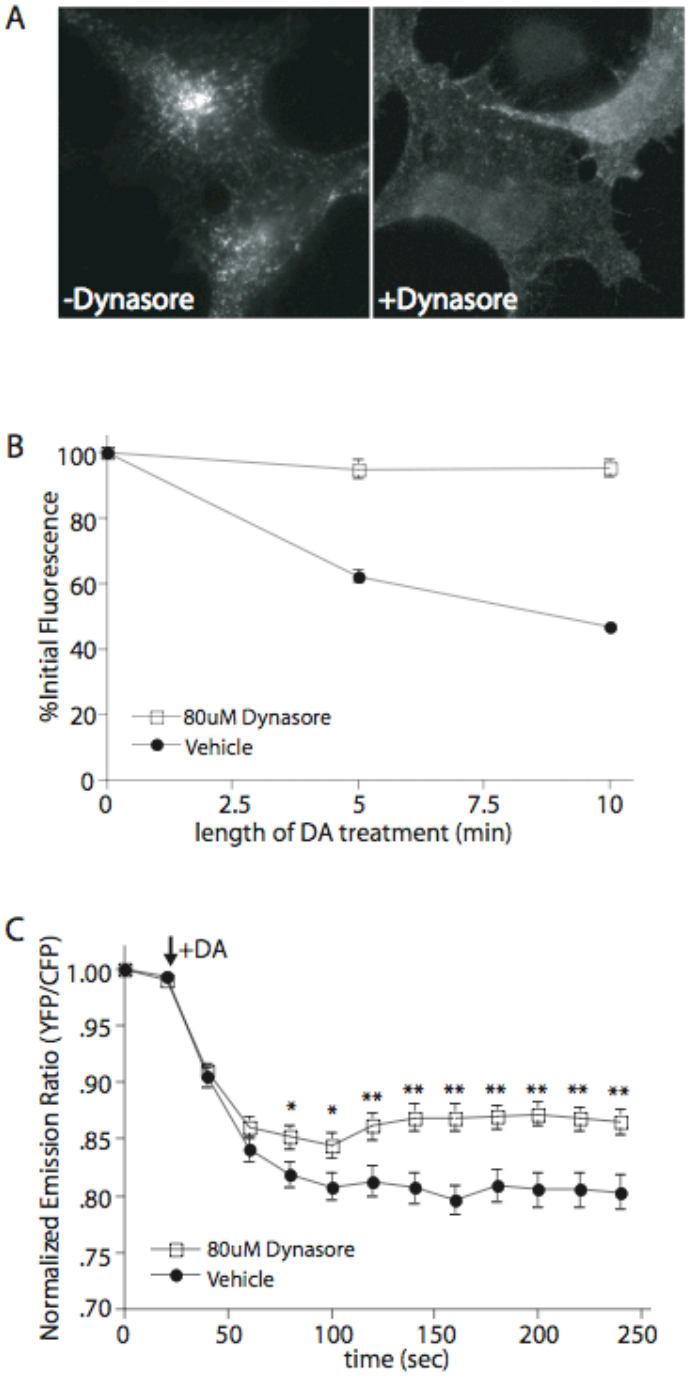



**Figure 3. Inhibition of endocytosis with hypertonic sucrose inhibits dopamine stimulated cAMP accumulation in HEK 293 cells.**
**(A)** Subcellular distribution of Alexa594 surface labeled FD1 receptors in response to 10µM DA treatment in the absence (left) or presence (right) of 500mM sucrose. **(B)** Change in normalized Epac1-cAMPs FRET in response to 10µM DA in the presence of 500mM sucrose (open circles) or absence of any pretreatment (filled black circles). Data represent mean +/- SEM normalized FRET emission ratio (See methods for calculations) at each time point for cells treated with sucrose (n=19) or cells receiving no pretreatment (n=16). (*$p<0.05$, **$p<0.01$)

Second, to achieve a more specific block of endocytosis, we employed the small molecule inhibitor dynasore. Dynasore inhibits clathrin-dependent endocytosis by interfering with the GTPase activity of dynamin, thereby preventing scission of endocytic vesicles (Kirchhausen, Macia et al. 2008). Dynasore effectively blocked dopamine-induced internalization of labeled FD1 receptors, as visualized by fluorescence microscopy **(Figure 4A)**. We verified this inhibition quantitatively using fluorescence flow cytometry. Vehicle pretreated, FD1 expressing 293 cells showed a significant decrease in surface fluorescence after incubation with dopamine for 5 or 10 minutes, indicative of endocytosis. Dynasore prevented this reduction in surface fluorescence, suggesting a near complete inhibition of $D_1$ receptor endocytosis **(Figure 4B)**. Dynasore also inhibited the dopamine-stimulated increase in cAMP accumulation, as evidenced by a decreased reduction in Epac1-cAMPs FRET **(Figure 4C)**. Importantly, dynasore did not affect the decrease in Epac1-cAMPs FRET in response to receptor-independent activation of adenylyl cyclase with forskolin **(Figure 4D)**. This indicates that blocking endocytosis specifically inhibits adenylyl cyclase-mediated signaling by activated $D_1$ receptors.

Interestingly, the initial dopamine-induced change in Epac1-cAMPs FRET was similar for both dynasore and vehicle pretreated cells, with the greatest discrepancy in cAMP levels becoming apparent around 120 seconds after dopamine addition. This corresponds to a time at which robust receptor clustering and endocytosis of SpH-D1R can be seen with TIRF microscopy **(Movie S1, Figure 1A)**. We quantified the change in signal produced by inhibition of endocytosis and both dynasore and sucrose showed a

significant difference 120 seconds after dopamine addition in comparison to controls **(Figure 4E)**. These data demonstrate that endocytosis promotes dopamine-stimulated generation of cellular cAMP on a remarkably short time scale.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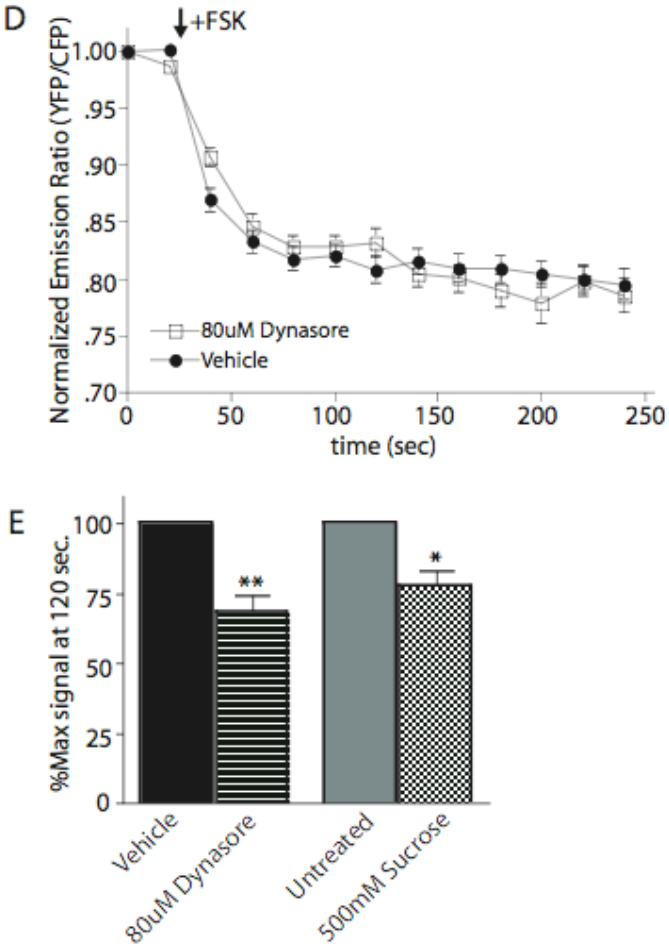


**Figure 4. Inhibition of endocytosis with dynasore inhibits dopamine stimulated cAMP accumulation in HEK 293 cells.**

**(A)** Subcellular distribution of Alexa594 surface labeled FD1 receptors in response to 10µM DA treatment in the absence (left) or presence (right) of 80µM dynasore. **(B)** FD1-expressing cells were analyzed using fluorescence flow cytometry to measure dopamine-induced effects on surface receptor number at 0, 5 and 10 minutes in cells pretreated with 0.2%DMSO (Vehicle; filled black circles) or 80µM dynasore (open squares). The amount surface fluorescence measured at time 0 was defined as 100%. Data represent mean surface fluorescence +/- SEM, n=3 experiments, each time point done in duplicate. **(C)** Change in normalized Epac1-cAMPs FRET in response to 10µM DA in the presence of 80µM dynasore (open squares) or 0.2% DMSO (Vehicle; filled black circles). Data represent mean +/- SEM normalized FRET emission ratio (See methods for calculations) at each time point for cells treated with dynasore (n=21) or vehicle (n=22). (*$p<0.05$, **$p<0.01$) **(D)** Change in normalized Epac1-cAMPs FRET in response to 1uM Forskolin in the presence of 80µM dynasore (open squares) or 0.2% DMSO (Vehicle; filled black circles). Data represent mean +/- SEM normalized FRET emission ratio (See methods for calculations) at each time point for cells treated with dynasore (n=23) or vehicle (n=18). **(E)** Comparison of Dynasore and Sucrose mediated inhibition of maximum normalized FRET changes seen 120 sec. after DA addition. The average DA-stimulated change in normalized FRET seen 120 sec. after DA addition in vehicle pretreated (solid black bar) or untreated (solid grey bar) cells was defined as 100% maximum signal. Pretreatment with 80µM dynasore reduced this signal to ~68% of vehicle treated (striped bar) and pretreatment with 500mM sucrose (checkered bar) reduced this signal to ~78% of untreated. (*$p<0.05$, **$p<0.01$)

**Rapid endocytic trafficking of $D_1$ receptors in cultured striatal neurons.**

We next asked whether this relationship between $D_1$ receptor endocytosis and receptor-mediated signaling exists in physiologically relevant neurons. The striatum is a major target of dopaminergic projection neurons within the brain. $D_1$ receptor expression is enriched in the GABAergic medium spiny neurons (MSNs) that make up 99% of this region (Kreitzer 2009). As a first step, we used an immunofluorescence assay to examine $D_1$ receptor endocytosis in dissociated striatal cultures. Anitbody-labeled FD1 showed a smooth plasma membrane distribution in both the soma and dendrites of these neurons **(Figure 5A, left)**. Given that a substantial fraction of striatal neurons express $D_2$ dopamine receptors, we used the $D_1$-specific agonist SKF 81297 instead of dopamine for experiments in cultured neurons. SKF 81297 caused a pronounced redistribution of labeled FD1 from the plasma membrane to endocytic vesicles of striatal neurons within 10 minutes **(Figure 5A, right)**. These labeled receptors were resistant to cell surface stripping confirming their endocytic localization.

To observe endocytic trafficking in neurons with greater temporal resolution, we expressed SpH-D1R in dissociated striatal neurons and used TIRF microscopy to image receptor dynamics at the cell surface. SpH-D1R fluorescence could be observed on the plasma membrane of both the cell body and dendrites of these neurons **(Figure 5B, left)**. SpH-D1R appeared robustly clustered within 120 seconds after agonist addition **(Figure 5B, center and Movie S3)**. These clusters are consistent with those previously described as receptor containing, clathrin-coated pits (Yudowski, Puthenveedu et al. 2006). Although most clusters disappeared within 3 minutes of their appearance, indicative of

endocytic scission **(Figure 5B, arrows)**, some clusters remained on the plasma membrane throughout the duration of the experiment **(Figure 5B, arrowhead)**. SpH-D1R clustering occurred remarkably rapidly, within seconds after the addition of SKF 81297. A kymograph of SpH-D1R fluorescence shows the lifetimes of two receptor clusters that appeared shortly after agonist addition and subsequently endocytosed **(Figure 5C)**. Measurements of integrated SpH-D1R fluorescence intensity averaged over multiple striatal neurons verified the rapid kinetics of receptor endocytosis induced by SKF 81297 **(Figure 5D)**.

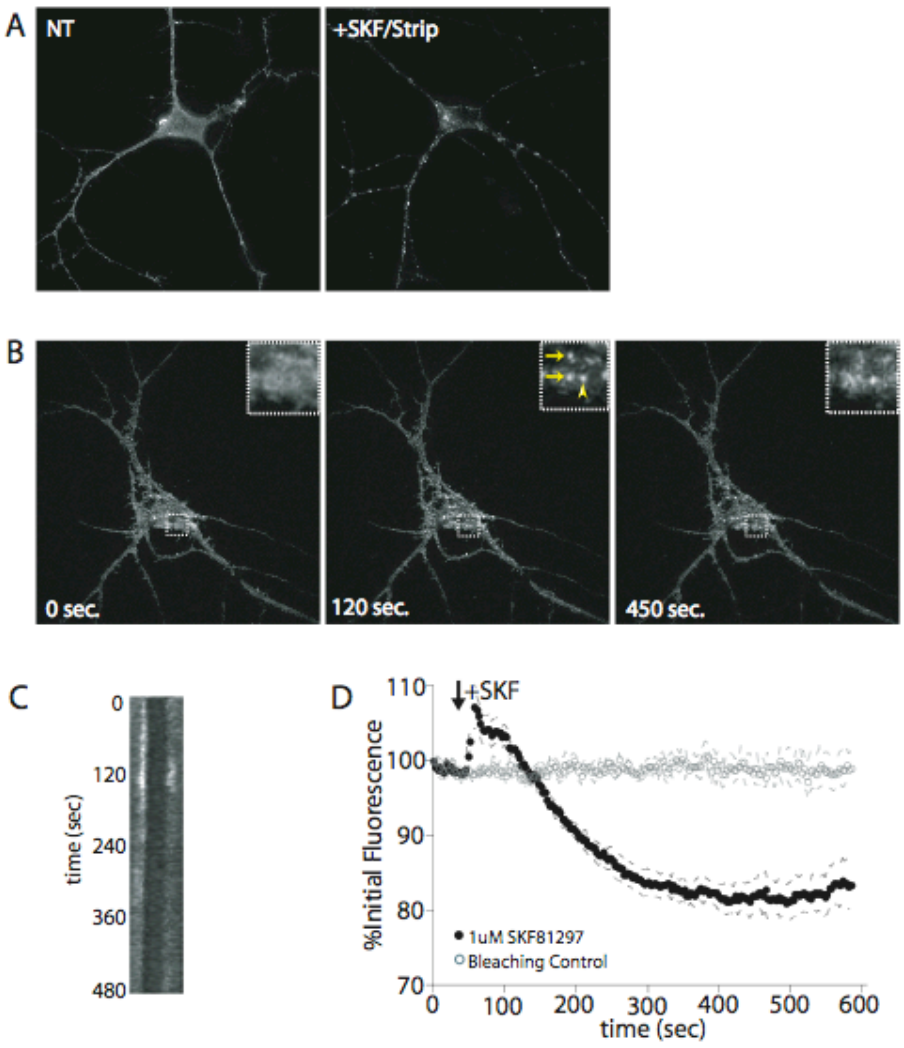


**Figure 5. Rapid endocytosis of D1 receptors in striatal neurons.**
**(A)** Surface D1 receptors in FD1-expressing striatal neurons were labeled with Alexa594-conjugated M1 anti-FLAG. Cells were either fixed without any drug treatment (NT) or treated with 1µM SKF 81297 for 10 minutes then stripped of remaining surface antibody with a PBS/EDTA wash (+SKF/Strip) prior to fixation in non-permeabilizing conditions. **(B)** Representative images of SpH-D1R surface fluorescence in striatal neurons as visualized by TIRF microscopy prior to agonist treatment (left, inset), 120 sec. after SKF 81297 addition (center, inset) and 450 sec. after SKF 81297 addition. Arrows indicate clusters of SpH-D1R surface fluorescence that appeared after agonist addition and returned to background levels within the time course of imaging, arrowhead indicates a cluster of SpH-D1R surface fluorescence that appeared after agonist addition and persisted throughout the duration of imaging. **(C)** Kymograph of SpH-D1R fluorescence for endocytic events indicated by arrows in **(B)**. Kymograph depicts surface fluorescence over 8 minutes after agonist addition. **(D)** Average surface fluorescence of SpH-D1R expressing striatal neurons measured every 3 sec. in the absence of agonist treatment (Bleaching Control, n=5 neurons) or in response to 1µM SKF 81297 addition (n=9 neurons). Initial fluorescence values of each cell normalized to 100%, data represent mean surface fluorescence +/- SEM. Endocytosis of SpH-D1Rs is indicated by decreased fluorescence intensity.

**Endocytosis supports acute $D_1$ receptor-mediated signaling in striatal neurons.**

To assess cAMP signaling in striatal neurons in real time, we applied the same FRET-based technology previously described for HEK 293 cells. Due to the typically lower expression of Epac1-cAMPs in neurons compared to HEK 293 cells, we used TIRF microscopy to achieve greater signal-to-noise ratio in this system (Steyer and Almers 2001). Incubation of striatal neurons with SKF 81297 caused a rapid decrease in the normalized (YFP/CFP) emission ratio of Epac1-cAMPs, consistent with a rapid increase in cAMP concentration in the peripheral cytoplasm **(Figure 6A)**. This effect persisted in the continuous presence of $D_1$ receptor agonist **(Figure 6B)**. These results indicate that, similar to what we observed in HEK 293 cells, agonist-stimulated $D_1$ receptor trafficking and signaling occur on a rapid and overlapping time scale in cultured striatal neurons.

To investigate if endocytosis plays any causal role in $D_1$ receptor mediated signaling in neurons, we again used dynasore to acutely inhibit clathrin-mediated, dynamin-dependent endocytosis. Dynasore significantly inhibited the rapid increase in cAMP elicited by SKF 81297 in striatal neurons **(Figure 6C)**. These data suggest that receptor endocytosis is indeed necessary to promote or sustain elevated levels of cellular cAMP generated in response to activation of $D_1$ receptors in dissociated striatal neurons on a rapid time scale.

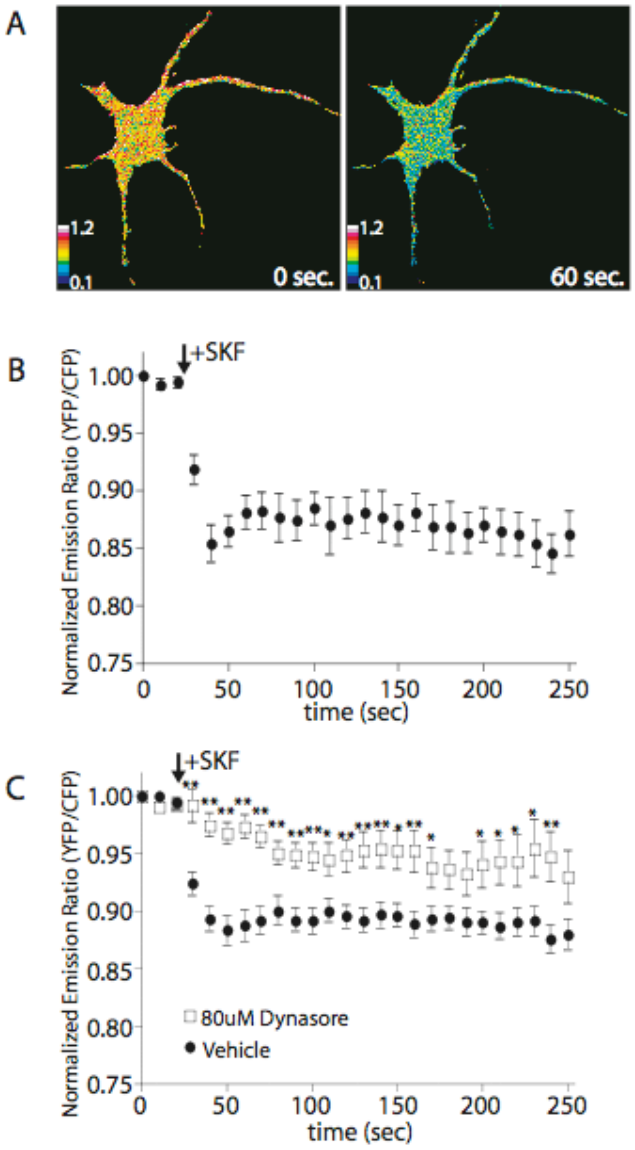


**Figure 6. Dynasore inhibits D1 receptor-mediated cAMP accumulation in striatal neurons.** **(A)** Pseudo-color representation of Epac1-cAMP FRET values in a representative D1/Epac1-cAMPs expressing striatal neuron in the absence of SKF 81297 (left) or 60 sec. after addition of 1µM SKF 81297 (right). Look up tables represent bleed through/direct excitation corrected absolute (not normalized to 1) FRET values. See methods for calculations. **(B)** Change in normalized Epac1-cAMPs FRET in response to stimulation with 1µM SKF 81297. Data represent mean +/- SEM normalized FRET emission ratio (See methods for calculations) at each time point for individual neurons at each time point (n=7 neurons). **(C)** Change in normalized Epac1-cAMPs FRET in response to 1µM SKF 81297 in the presence of 80µM dynasore (open squares) or 0.2% DMSO (Vehicle; filled black circles). Data represent mean +/- SEM normalized FRET emission ratio (See methods for calculations) at each time point for neurons treated with dynasore (n=13) or vehicle (n=18). (*$p<0.05$, **$p<0.01$)

**Endocytosis is required for $D_1$ receptor-mediated regulation of action potential firing in dorsolateral striatum.**

We next sought to investigate the functional role of endocytosis in a more integrated model of $D_1$ receptor-mediated neural signaling. It has previously been reported that agonist stimulation of $D_1$ receptors can increase firing rates of striatal MSNs via PKA-dependent enhancement of L-type calcium currents (Surmeier, Bargas et al. 1995; Hernandez-Lopez, Bargas et al. 1997; Abdallah, Bonasera et al. 2009). To examine whether endocytosis contributes to this signaling response, we performed whole-cell patch-clamp electrophysiology in intact brain slices containing the lateral dorsal striatum. Neurons were brought to a resting membrane potential of ~-90mV by passage of DC current via the patch amplifier and subsequently exposed to a series of 300msec current pulses to depolarize neurons and generate action potentials (APs). In neurons pre-exposed to vehicle (0.2%DMSO), SKF 81297 significantly enhanced AP generation **(Figure 7A top, Figure 7B)**. In contrast, pre-exposure to dynasore completely prevented the SKF 81297-stimulated increase in AP firing **(Figure 7A bottom, Figure 7B)**. These data further support the hypothesis that endocytosis is essential for rapid $D_1$ receptor-mediated signaling.

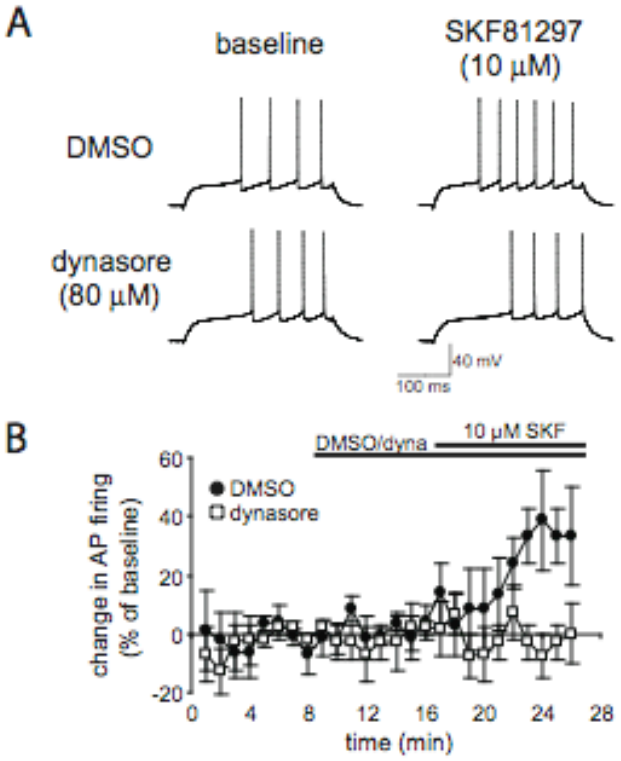


**Figure 7. Dynasore pre-exposure prevents $D_1$ receptor-mediated enhancement of firing in lateral dorsal striatal neurons in brain slice.**
**(A)** Example traces showing increased firing with the $D_1$ receptor agonist SKF81297 (10 µM) after pre-exposure to vehicle (0.2% DMSO), but no increase in firing after pre-exposure to 80 µM dynasore.
**(B)** Grouped data showing significant increase in firing with SKF81297 after pre-exposure to vehicle (34.7 +/- 12.8%, n=5) but not dynasore (-2.78 +/- 4.9%, n=5). $p<0.05$ The percent change in number of action potentials (APs) generated relative to baseline was determined at the current step at baseline with 4 APs, or 5 APs. If no current steps at baseline had 4 APs, the baseline number of APs was determined by averaging each min for the 4 minutes before addition of DMSO or dynasore.

## 3.4 Discussion

To our knowledge, the present results provide the first analysis of the relationship between $D_1$ receptor trafficking and signaling in neurons, on a time scale that is within the range of physiological dopaminergic neurotransmission. $D_1$ receptors undergo endocytic trafficking with remarkably rapid kinetics. In both medium spiny neurons and HEK 293 cells, we demonstrate $D_1$ receptor endocytosis beginning within 1 minute of agonist-induced activation. We also show receptor-mediated accumulation of cellular cAMP occurring on a similar time scale. Most importantly, we establish that endocytosis is essential for rapid dopaminergic signaling, both in cultured cells and in a brain slice preparation that enables us to observe an integrated electrophysiological response.

Previous studies of $D_1$ receptor-mediated signaling over longer time periods ($\geq$ 30 minutes) have suggested that endocytosis either inhibits (Jackson, Rafal et al. 2002; Zhang, Vinuela et al. 2007) or has no effect on dopaminergic signaling (Gardner, Liu et al. 2001). Endocytosis has been shown to regulate signaling by other classes of receptors, in addition to GPCRs. Interestingly, endocytosis is generally thought to attenuate cellular or synaptic responsiveness, as in the case of AMPA receptor endocytosis and LTD (Malenka 2003) or restore cellular responsiveness after a significant refractory period, as in the case of the beta-2 adrenoreceptor (Pippig, Andexinger et al. 1995). As such, we believe that presently identified role of endocytosis in supporting acute $D_1$ receptor-mediated signaling is without precedent. Furthermore,

this finding identifies a new relationship between signaling and endocytosis that pertains to the major pathway of excitatory dopaminergic signaling in the CNS.

How might endocytosis support rapid dopaminergic signaling? One possibility is that this occurs by an unusually rapid recycling/resensitization mechanism. Many GPCRs are phosphorylated following agonist-induced activation, resulting in decreased signaling via trimeric G proteins. Endocytosis is thought to deliver receptors to a recycling pathway that enables dephosphorylation and returns functional receptors to the plasma membrane. This classical receptor recycling/resensitization paradigm has been demonstrated for number of GPCRs, most notably the beta-2 adrenoreceptor, albeit on a significantly longer time scale (Pippig, Andexinger et al. 1995; Lefkowitz 1998). Interestingly, we were able to observe individual $D_1$ receptor-containing membrane insertion events both in the presence of dopamine (data not shown) and after dopamine washout **(Supplemental Movie S2)**. Thus it is plausible that the receptor recycling/resensitization mechanism could support or maintain dopaminergic signaling on this rapid time scale. Another possibility is that $D_1$ receptors continue to signal via trimeric G-proteins after being removed from the plasma membrane. While GPCR signaling via trimeric G proteins is traditionally thought to occur only from the plasma membrane, emerging evidence suggests that G protein signals can also originate from endosomes (Slessareva and Dohlman 2006). A recent study has reported TSH receptor-mediated activation of adenylyl cyclase from endosomes in mammalian cells (Calebiro, Nikolaev et al. 2009). Our results are also potentially consistent with this mechanism.

A variety of complex functions including learning and memory, locomotion and goal-directed behaviors such as food or drug seeking require precise regulation of dopaminergic signaling via $D_1$ receptors (Sibley 1999; Kelley 2004). Our findings propose that endocytosis of $D_1$ receptors could represent one of the mechanisms involved in accomplishing this regulation in neurons receiving dopaminergic input. Recent studies in awake, behaving animals have shown transient spikes in dopamine concentrations that last on the order of seconds (Heien, Khan et al. 2005; Roitman, Wheeler et al. 2008; Tsai, Zhang et al. 2009). Our data indicate that the $D_1$ receptor is capable generating a robust increase in cellular cAMP and undergoing endocytosis on a time scale that approaches this reported physiology. Importantly, our data also demonstrate that endocytosis is essential for sustained dopaminergic responsiveness in both cultured striatal neurons and brain slices.

In vivo measurements have shown that extracellular dopamine transients can vary in peak intensities from nano- to micromolar concentration. We have demonstrated that the $D_1$ receptor undergoes rapid endocytosis in the upper range these concentrations **(see Figure S1)**. Interestingly, the peak concentration measured in each of these studies varied substantially depending on the experimental paradigm that elicited dopamine transients. Rewarding taste stimuli evoked dopamine transients in the nucleus accumbens with peak concentrations near 50nM (Roitman, Wheeler et al. 2008), whereas electrical stimulation of dopaminergic VTA neurons could elicit dopamine transients in the nucleus accumbens with peak amplitudes greater than 0.5 μM (Heien, Khan et al. 2005). In vivo microdialysis measurements of dopamine in the striatum of non-human primates showed

dopamine concentrations greater than 1uM after self-administration of cocaine (Bradberry, Barrett-Larimore et al. 2000). Our data predict that low extracellular dopamine concentrations within the brain would stimulate little $D_1$ receptor endocytosis, while higher extracellular dopamine concentrations would elicit robust $D_1$ receptor endocytosis and promote ongoing dopaminergic signaling. Thus, endocytosis of $D_1$ receptors may reflect a novel mechanism by which signal strength, signal duration and perhaps even the salience of a given stimulus could be rapidly encoded at the cellular level.

In conclusion, the present study examines $D_1$ receptor-mediated signaling and endocytic trafficking with unprecedented temporal resolution and identifies a causal role of endocytosis in supporting rapid dopaminergic signaling. We believe that these findings are the first to establish such an intimate relationship between endocytosis and signaling for any GPCR and the first to demonstrate this relationship in neurons that naturally respond to dopamine. We propose that endocytosis-supported signaling by $D_1$ receptors likely represents a fundamental principle by which the nervous system shapes and maintains rapid dopaminergic responsiveness at the level of the individual neuron.

## 3.5 Experimental Procedures

### *cDNA and Constructs*

Both the N-terminally FLAG (DYKDDDD) epitope-tagged human D1 dopamine receptor (F-D1R) and Epac1-camps have been previously described (Vickery and von Zastrow 1999; Nikolaev, Bunemann et al. 2004). An N-terminal superecliptic pHluorin version of the human D1 dopamine receptor (SpH-D1R) was constructed by subcloning a signal-sequence flanked superecliptic pHluorin cassette (Yudowski, Puthenveedu et al. 2006) into the existing F-D1R construct. Briefly, EcoRI sites were inserted in frame 3' to the signal-sequence flanked superecliptic pHluorin in SpH-B2AR and 5' to the human D1 receptor in F-D1R. The signal-sequence flanked superecliptic pHluorin cassette was excised using EcoRI and NheI and subcloned into F-D1R (NheI/EcoRI digested) to create SpH-D1R. F-D1R, Epac1-camps and SpH-D1R were all subcloned into pCAGGS (Niwa, Yamamura et al. 1991) for expression in cultured striatal neurons.

### *Cell Culture and Transfections*

HEK 293 cells (ATCC) were maintained in Gibco Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin (University of California, San Francisco, Cell Culture Facility). Cells were plated on 6 cm dishes and grown to 50-80% confluence prior to transfection using a cationic lipid reagent (Effectene; Qiagen). 1-2 ug of DNA was added to each dish per manufacturer instructions. 12-24 hours after transfection, cells were lifted with PBS with 0.04% EDTA (University of California, San Francisco, Cell Culture Facility) and re-plated onto poly-

D-lysine (Sigma) coated 35mm glass-bottom dishes (Matek) for imaging. All assays were done 48-72 hours post transfection.

Striatal neurons were dissected from embryonic day 17-18 Sprague Dawley rats. The striatum, including the caudate-putamen and nucleus accumbens, was identified as described by Ventimiglia and Lindsay (Ventamiglia and Lindsay, 1998). Striata were dissected in ice cold Hank's buffered saline solution (University of California, San Francisco, Cell Culture Facility) and tissue dissociated in 1x trypsin/EDTA (Invitrogen) for 25 minutes at 37˚C. Cells were washed in Gibco Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (University of California, San Francisco, Cell Culture Facility) and mechanically separated with a flame-polished Pasteur pipette. Striatal neurons were transfected via electroporation (Rat Neuron Nucleofector Kit; Lonza) per manufacturer's instructions and plated on poly-D-lysine coated 35mm glass bottom dishes (Matek) for imaging. All experiments in striatal neurons were carried out 10-14 days post-transfection.

***Live Cell Imaging of SpH-D1R with TIRF Microscopy***

SpH-D1R expressing 293 cells were washed 2x with warm PBS and imaged in Dulbecco's modified Eagle's medium without phenol red (University of California, San Francisco, Cell Culture Facility), supplemented with 1% fetal bovine serum and 30mM Hepes (Sigma) at pH 7.4. Striatal neurons (7-10 D.I.V.) were washed 3x with warm $Ca^{2+}$/$Mg^{2+}$-free PBS and imaged in a physiological solution consisting of 130mM NaCl, 2mM KCl, 4mM $CaCl_2$, 10mM glucose and 30mM Hepes pH 7.35, osmolarity: 290-

300mosM. Cells were maintained at 37˚C using a temperature controlled stage (Bioscience Tools) and an objective heater (Bioptechs). TIRF microscopy was performed using a Nikon 2000E inverted microscope equipped with Perfect Focus, 100x/NA1.49 TIRF objective, Nikon 488 laser TIRF illuminator and standard 488/516 excitation cube, Lambda 10-3 emission filter wheel (520/50m filter) controlled via SmartShutter (Sutter Instruments) and interfaced to a PC running NIS-Elements Advanced Research software (Nikon). To visualize endocytic events cells were treated with 10µM dopamine + 100µM ascorbic acid (Sigma) or 1µM SKF 81297 and images taken every 3 seconds with 100-200msec exposure times. For fluorescence recovery after washout, images were taken every 10 seconds with 100-200msec exposure times. To visualize exocytic events, cells were treated with 10µM dopamine + 100µM ascorbic acid for 10 minutes, washed 4x with warm imaging media and imaged continuously at 10 frames per second for 1-2 minutes. Bleaching controls were obtained by performing identical experiments in the absence of any agonist treatments. Images were captured using a Cascade II EMCCD camera (Photometrics). Fluorescence changes were analyzed by drawing and ROI around cell bodies and measuring integrated intensity minus background intensity over time. Background fluorescence intensity was determined by drawing a similarly sized ROI in an area where no cells were present and measuring these values over time. Background intensity was calculated separately for each dish of cells measured. All analysis was performed using ImageJ software (http://rsbweb.nih.gov/ij).

***Real-time FRET Measurements of cAMP Changes with Epac1-cAMPs***

Epac1-cAMPs/FD1 expressing 293 cells were rinsed, placed in imaging media and visualized with the live-cell fluorescence microscopy set-up that was previously described. A series of 3 sequential images were taken for each time point (every 20 seconds) in a given experiment. The "FRET" image was obtained with an ET430/24x nm (CFP) excitation filter and an ET535/30m nm (YFP) emission filter in place. The "CFP" image was obtained with the CFP excitation filter and an ET470/24m nm (CFP) emission filter in place. The "YFP" image was obtained with ET500/20x nm (YFP) excitation filter and the YFP emission filter in place. Exposure times for FRET, CFP and YFP images were 100msec, 100msec and 30msec respectively. Manufacturer's estimate of the time between sequential images is approximately 40msec. Correction values for bleed through of CFP into the YFP channel ($BT_{DONOR}$) and direct excitation of YFP from the CFP setting ($DE_{ACCEPTOR}$) were obtained by expressing CFP alone or YFP alone in cells and measuring the ratio of FRET to CFP emission ($BT_{DONOR}$) or FRET to YFP emission ($DE_{ACCEPTOR}$). These measurements were performed weekly throughout the course of FRET experiments. Intensity values ($I_X$) were calculated by drawing an ROI around each individual cell and measuring integrated fluorescence intensity and in a given channel at each time point. Background values ($BG_X$) were measured by drawing a similarly sized ROI in an area where no cells were present and collecting integrated fluorescence intensity values in a given channel at each time point. A corrected FRET ratio could be obtained for each cell at each time point using the following equation: $NFRET = [(I_{FRET}-BG_{FRET})-(I_{CFP}-BG_{CFP})BT_{DONOR}-(I_{YFP}-BG_{YFP})DE_{ACCEPTOR})] / I_{CFP}$ and normalizing the corrected FRET value obtained at the first time point to 1. All analysis was done using ImageJ.

FRET measurements in striatal neurons were obtained using the live-cell TIRF microscopy set-up described above, with the following exceptions: FRET and CFP excitations were performed with a 440nm Nikon Laser TIRF illuminator. YFP excitation was performed with a 514nm Nikon Laser TIRF illuminator. TIRF and YFP emissions were collected through a 545/40m emission filter. CFP emission was collected through a 485/30m emission filter. The same exposure times were used for striatal neurons and 293 cells, but images were taken every 10 sec for neurons. Intensity values were obtained by drawing an ROI around the cell bodies of individual neurons and performing the same measurements and calculations described for 293 cells.

FRET changes in response to agonist stimulation were obtained by adding 2µl of 1000x dopamine stocks in ascorbic acid or 2µl of 1000x SKF 81297 stocks to cells in 2ml of imaging media as indicated. Bleaching control estimates were obtained by performing identical experiments in the absence of any agonist treatment. Hypertonic sucrose was used to inhibit endocytosis by pre-treating cells for 10 minutes with a final concentration of 500mM sucrose (Sigma) before imaging. Dynasore inhibition of endocytosis was performed by adding a 1:500 dilution of 40mM dynasore (Sigma) in DMSO (80µM dynasore/ 0.2% DMSO final) to cells 10 minutes before imaging. Vehicle control values were obtained by incubating cells in a final volume of 0.2% DMSO prior to imaging. Normalized FRET values were corrected for bleaching in the presence of dynasore by adding back the average change in normalized FRET at a given time point in dynasore pretreated, unstimulated controls.

***Immunocytochemistry and Qualitative analysis of D1 dopamine receptor endocytosis.***

FD1 expressing 293 cells or striatal neurons were first incubated with 1ug/ml Alexa594-conjugated M1 anti-FLAG monoclonal antibody for 20 minutes to label surface D1 receptors. For sucrose or dynasore experiments, drugs were added 10 minutes prior to any agonist treatment. Cells receiving no agonist (NT) were then washed 3x with cold TBS and fixed with 4% paraformaldehyde and 5% sucrose in PBS for 15 minutes. Agonist treated cells were surface labeled, treated with 10μM dopamine + 100μM ascorbic acid or 1μM SKF 81297 at 37˚C for 10 minutes to drive internalization, then stripped of any remaining surface Alexa594-conjugated M1 by washing 3x with $Ca^{2+}$/$Mg^{2+}$-free PBS +0.04% EDTA (University of California, San Francisco, Cell Culture Facility) prior to fixation. All cells were washed with mTBS and covered with Fluoromount-G (Southern Biotech) to preserve signal for fluorescence microscopy.

Epifluorescence microscopy was performed using a Nikon TE2000E inverted microscope equipped with a 60x/numerical aperture (NA) 1.2 objective, xenon arc lamp with Lambda LS excitation and Lambda 10-3 emission filter wheels controlled with a SmartShutter (Sutter Instruments) and NIS-Elements Advanced Research software (Nikon). Standard S470/30x (Alexa488) and S565/55 (Alexa594) excitation and emission S510/30m (Alexa488) and S650/75m (Alexa594) filters were used. Images were captured with a CoolSnap HQ2 CCD camera (Photometrics) and analyzed using ImageJ software.

***Quantification of D1 receptor endocytosis with Fluorescence Flow Cytometry.***

Surface fluorescence of FD1 expressing 293 cells was used to measure receptor endocytosis. Cells were treated with 80μM dynasore or 0.2%DMSO (vehicle) prior to agonist addition. Cells were then incubated with 10μM dopamine + 100μM ascorbic acid for 0 (100% surface fluorescence), 5 or 10 minutes prior to wash with ice-cold PBS, then mechanically lifted and incubated with 1ug/ml Alexa647-congugated M1 anti-FLAG monoclonal antibody at 4˚C for 1-2 hours. Mean fluorescence intensity for 10,000 cells/condition was collected using a flow cytometer (Becton Dickson). Each condition was performed in duplicate with a minimum of 3 experiments.

***Slice preparation and Electrophysiology***

All animal methods were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and the Ernest Gallo Clinic and Research Center's Institute for Animal Care and Use Committee. P20-P28 male Sprague-Dawley rats were briefly anesthetized with halothane then decapitated and the brain quickly removed. Brain slices (250-300 mM) containing the dorsal striatum were cut in a modified artificial cerebrospinal fluid (aCSF) containing (in mM): 225 sucrose; 119 NaCl, 2.5 KCl, 1.0 $NaH_2PO_4$, 4.9 $MgCl_2$, 0.1 $CaCl_2$, 26.2 $NaHCO_3$, 1.25 glucose; 1 ascorbic acid; and 3 kynurenic acid, chilled to ~4$^{o}$C. Slices then recovered at 32$^{o}$C in carbogen-bubbled aCSF (126 mM NaCl, 2.5 mM KCl, 1.2 mM $NaH_2PO_4$, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 18 mM $NaHCO_3$, 11 mM glucose, with pH 7.2-7.4 and milliosmolarity 301-305), with 1 mM ascorbic acid added just before the first slice. Brain slices recovered for 45 min to 6 hr before use in experiments. During experiments, slices were submerged and continuously perfused (~2 ml/min) with carbogen-bubbled aCSF

warmed to 31-32$^{o}$C, and supplemented with picrotoxin (50 μM, to block GABA-A receptors) and CNQX (10 μM, to block AMPA-type glutamate receptors). All other drugs were bath applied. Experiments were restricted to GABAergic medium spiny neurons, which represent more than 90% of the neurons within the NAcb core, and other cell types can easily be distinguished by a large soma (Bennett, Callaway et al. 2000) or by very high rates of firing and a larger AHP (Bracci, Centonze et al. 2002).

All electrophysiology experiments were performed using whole-cell recording and visualized infrared-DIC with 2.5 to 3.5 MΩ electrodes. The internal solution was potassium methanesulfonate-based, containing (in mM): 130 mM KOH, 105 mM methanesulfonic acid, 17 mM HCl, 20 mM HEPES, 0.3 mM EGTA, 2.8 mM NaCl, 2.5 mg/ml MgATP, 0.25 mg/ml GTP, pH 7.2-7.4, 275-285 mOsm. Current-clamp data were recorded using Clampex 9.2 or 10.1 and an Axon 700A or 700B patch amplifier (Axon Instruments, Foster City, CA), and were acquired at 20 KHz and filtered at 2 KHz. After breaking into a neuron, the resting membrane potential was set to ~ –90 mV by injecting DC current through the patch amplifier. To measure firing, current pulses were applied using a patch amplifier in current-clamp mode, and a series of 7-8 current pulses (300 ms duration, 20 pA apart) were applied every 30 seconds, where the minimum current amplitude was set for each cell so that the first pulse was just sub-threshold for spike firing. Depolarizing pulses were alternated with a 33.3 pA hyperpolarizing pulse to examine the input resistance. Voltage values were corrected for the liquid junction potential, estimated to be 10 mV using the Junction Null Calculator in Clampex 9.2, and also by direct measurement of the potential difference between internal and external

solutions present after zeroing the pipette current. Bridge balance was used to compensate 60-80% of the series resistance. The percent change in number of action potentials (APs) generated relative to baseline was determined at the current step at baseline with 4 APs, or 5 APs if no current steps at baseline had 4 APs. The baseline number of APs was determined by averaging each min for the 4 minutes before addition of DMSO or dynasore.

## 3.6 Acknowledgements

This work was supported in part by grants from the National Institutes of Health (DA-010711 and DA-010154 to M.Z., MH-24468 to S.J.K., AAA-015358 to F.W.H.) and funds provided by the State of California for medical research on alcohol and substance abuse through the University of California, San Francisco (AB). Data for this study were collected at the Nikon Imaging Center at the University of California, San Francisco. We thank Kurt Thorn, Guillermo Yudowski and Kit Wong for valuable advice and assistance.

# 3.7 Supplemental Data

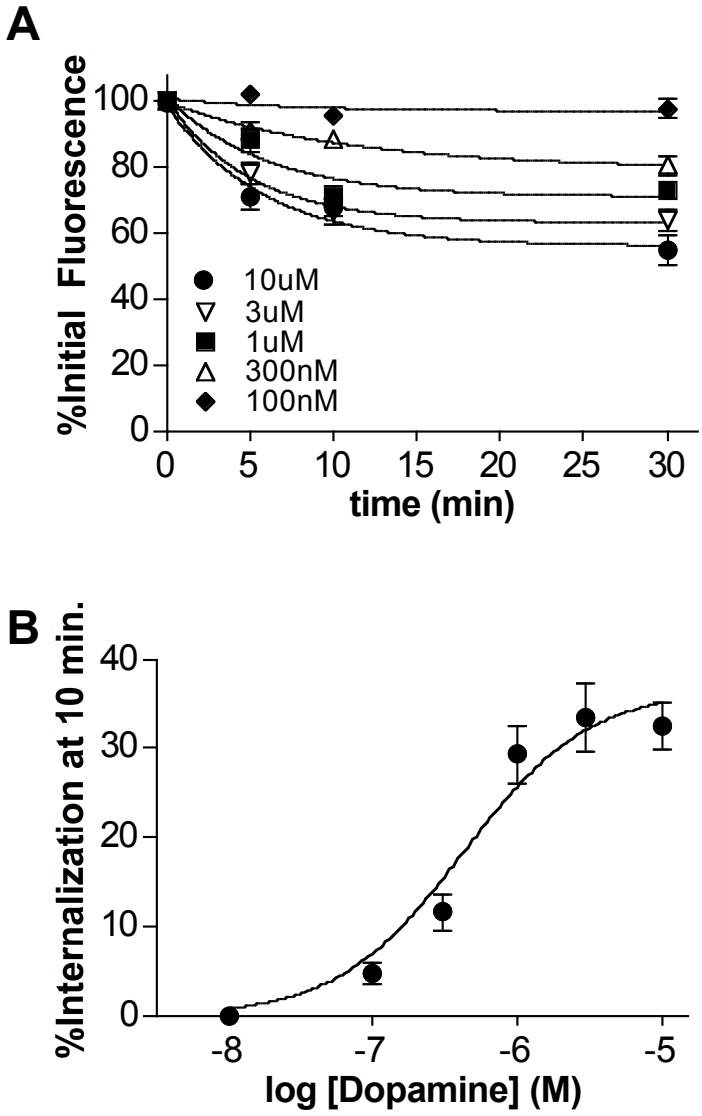


**Figure S1. Dose dependent dopamine-stimulated endocytosis of $D_1$ receptors.**
**(A)** FD1-expressing cells were analyzed using fluorescence flow cytometry to measure agonist-induced effects on surface receptor number at 0, 5, 10, and 30 minutes after DA addition, for a range of dopamine concentrations. The amount surface fluorescence measured at time 0 was defined as 100%. The $t_{1/2}$ for loss of surface fluorescence in response to 10μM DA was ~3.9 minutes. Data represent mean fluorescence values +/- SEM for 3 experiments per concentration, with each time point done in duplicate.
**(B)** Surface fluorescence data from (A) at 10 min. after DA treatment were fit to a sigmoidal dose-response curve. $EC_{50}$ for $D_1$ receptor internalization was estimated at $4.17 \times 10^{-7}$M.

# Chapter 4: Discussion

The present body of work represents a significant contribution to the understanding of regulation of dopaminergic signaling via membrane trafficking of $D_1$ receptors. The following discussion will address the implications of these findings and their relevance to both the fields of GPCR trafficking and dopamine transmission in the CNS.

## 4.1 $D_1$ receptor trafficking and signaling after prolonged agonist exposure

In chapter 2 of this study we examine $D_1$ receptor membrane trafficking and signaling in HEK 293 cells and cortical neurons after 10-60 minute agonist incubation. We demonstrate significant, agonist-mediated endocytosis of $D_1$ receptors in both cell types, within 30 minutes. Similar agonist pre-treatments (10-60 minutes, 10uM dopamine or 1μM SKF 81297) lead to inhibition of acute agonist-stimulated cellular cAMP accumulation, a condition commonly referred to as cellular desensitization. This desensitization occurs regardless of whether PDE inhibitors are present during agonist pre-treatment. Agonist washout promotes efficient recycling of internalized $D_1$ receptors back to the plasma membrane within 60 minutes. Interestingly, cellular sensitivity of $D_1$ receptor-mediated cAMP accumulation does not recover, even though nearly the full complement of cell surface $D_1$ receptors returns to the plasma membrane. These findings suggest that membrane trafficking of $D_1$ receptors, on this time scale, does not contribute to recovery of dopaminergic signaling in HEK 293 cells or cultured cortical neurons. To our knowledge, these data are the first to demonstrate this result in cortical neurons.

We observe significant attenuation of $D_1$ receptor-mediated cAMP accumulation after exposing HEK 293 cells and cortical neurons to an experimental paradigm known to induce pronounced receptor endocytosis. Although we do not directly examine arrestin recruitment or the clathrin dependence of endocytosis in this study, previous findings suggest that activated $D_1$ receptors can recruit arrestins (Oakley, Laporte et al. 2000; Kim, Gardner et al. 2004) and that $D_1$ receptor endocytosis in HEK 293 cells is clathrin-mediated (Vickery and von Zastrow 1999). Our results are potentially consistent with clathrin-mediated endocytosis playing a role in cellular desensitization. However, these findings do not prove that endocytosis is a requirement for desensitization. Previous studies have demonstrated that significant agonist-mediated receptor phosphorylation can occur in the absence of endocytosis, suggesting that desensitization may not depend upon receptor endocytosis (Gardner, Liu et al. 2001). Endocytosis could represent the final step in an arrestin-mediated process that begins by preventing the interaction of receptors and G-proteins and culminates in receptor sequestration away from the plasma membrane. Previous studies have reported that mutant $D_1$ receptors unable to undergo agonist-mediated endocytosis do not experience significant desensitization with respect to cAMP accumulation (Jackson, Rafal et al. 2002). These findings do not agree with our own observations of the relationship between $D_1$ receptor endocytosis and signaling on this time scale, but it is important to mention that this mutant receptor was also missing a number of residues implicated in receptor desensitization via agonist-mediated phosphorylation.

Our results suggest that unlike the $\beta_2$-adrenergic receptor, the classical recycling/resensitization paradigm does not apply to the $D_1$ dopamine receptor over a similar time course (Pippig, Andexinger et al. 1995; Lefkowitz 1998). As mentioned in chapter 1, shortly after agonist stimulation, many GPCRs become phosphorylated and lose the ability to efficiently signal through G-proteins (Ferguson 2001; Gainetdinov, Premont et al. 2004). Endocytosis and recycling are thought to enable receptor dephosphorylation and promote recovery of cellular signaling (resensitization) (Lefkowitz 1998; Hanyaloglu and Zastrow 2008). The apparent lack of association between $D_1$ receptor trafficking and recovery cellular sensitivity to dopaminergic signaling was also reported in a previous study (Gardner, Liu et al. 2001). The authors demonstrate that significant $D_1$ receptor phosphorylation occurs after stimulating C6 glioma cells with dopamine for 10 minutes and that endocytosis is not required for the dephosphorylation of $D_1$ receptors after agonist washout. More relevant to our studies, they show that desensitization of dopamine-mediated cAMP accumulation persists for several hours after agonist washout. This is particularly interesting as our membrane trafficking results predict that $D_1$ receptors would undergo efficient endocytosis and recycling within their experimental paradigm. A number of mechanistic possibilities might account for these findings. First, it is possible that recycled plasma membrane $D_1$ receptors exist in a conformation that prevents them from efficiently coupling to G-proteins and signaling in response to agonist. Although the study by Gardner et al, suggests that the population of $D_1$ receptors returns to basal levels of phosphorylation after agonist washout, their results provide no information about phosphorylation states of individual receptors at the plasma membrane. Thus, we cannot entirely rule out the

possibility that recycled $D_1$ receptors cannot efficiently activate the second-messenger cascade. Interestingly, in chapter 2 we also demonstrate that prolonged dopamine treatment is able to inhibit cellular cAMP accumulation mediated by acute stimulation of another $G_s$-coupled receptor. HEK 293 cells endogenously expressing $\beta_2$-adrenergic receptors produce significantly less isoproterenol-stimulated cAMP after incubation with dopamine than naïve controls. This finding indicates that the regulation of cAMP accumulation seen after prolonged dopamine exposure is not specific to the $D_1$ receptor. It is therefore quite likely that the lack of cellular resensitization seen in spite of efficient $D_1$ receptor recycling can be accounted for by down-regulation of some other component of the second messenger cascade. Adenylyl cyclase and $G\alpha_s$ could both be targets of down-regulation in response to prolonged cellular exposure to dopamine. Our finding that cellular desensitization occurred both in the absence and presence of PDE inhibitors seem to rule out up-regulation of PDEs as a potential explanation for this phenomenon.

Precise regulation of dopaminergic signaling is thought to be critical for maintaining physiological homeostasis. This is supported by the strong physiological and behavioral effects of drugs that either inhibit or increase dopaminergic signaling. Psychostimulants such as cocaine and amphetamine generate prolonged, elevated levels of extracellular dopamine within the CNS (Bradberry, Barrett-Larimore et al. 2000; Hyman, Malenka et al. 2006). Additionally, some studies have reported a correlation between certain symptoms of schizophrenia or Tourette's syndrome and excessive $D_1$ receptor activity in the cortex (Campbell, McGrath et al. 1999; Hirvonen, van Erp et al. 2006). The regulation of cellular signaling that we observed in response to high concentrations of $D_1$

receptor agonists for an extended duration of time most likely reflects an important homeostatic mechanism for limiting dopaminergic signaling within the brain. It is possible that some component of this homeostatic mechanism is defective in the aforementioned pathologies. Importantly, our findings indicate that $D_1$ receptor recycling over this time course is not sufficient to restore dopaminergic signaling in cortical neurons. Also, the observed down-regulation of cAMP accumulation is likely not specific to the $D_1$ receptor. This suggests that therapies aimed at reversing or reproducing this particular mechanism of decreased cellular sensitivity to dopamine should not focus on direct modulation of $D_1$ receptors. Focusing on targets downstream of the receptor may prove to be more therapeutically useful. Although the regulation of dopaminergic signaling is considerably more complex that the basic cell biology that we present in this study, these results may shed light on how neurons limit excessive dopaminergic transmission in the brain and also further our understanding important molecular mechanisms involved in the regulation of GPCR-mediated signals.

## 4.2 Endocytic regulation $D_1$ receptor signaling: timing matters.

In chapter 3 we examine $D_1$ receptor trafficking and signaling with much greater temporal resolution. To our knowledge, our findings represent first analysis of the relationship between $D_1$ receptor trafficking and dopaminergic signaling, on a time scale that is within the range of physiological dopamine neurotransmission. We demonstrate that $D_1$ receptor endocytosis begins within 1 minute of agonist stimulation in HEK 293

cells and striatal neurons. Robust, receptor-mediated accumulation of cellular cAMP occurs with overlapping kinetics. We are able to inhibit clathrin-mediated endocytosis of $D_1$ receptors and measure the dynamics of cAMP accumulation to establish a causal relationship between endocytosis and dopaminergic signaling. Our results indicate that rapid endocytosis is critical for $D_1$ receptor-mediated cAMP accumulation in HEK 293 cells and in cultured striatal neurons. Further, inhibition of endocytosis also prevents a $D_1$ receptor-mediated increase in AP firing of MSNs of the dorsolateral striatum, suggesting it is essential for integrated electrophysiological responses.

Previous studies, including our own, have examined $D_1$ receptor-mediated signaling over much longer time periods. These studies conclude that after prolonged agonist-treatment, endocytosis is correlated with the inhibition of dopaminergic signaling (see chapter 2) or has no effect on dopaminergic signaling (Gardner, Liu et al. 2001). As for the regulation of other signaling receptors, most GPCR endocytosis is thought to contribute to attenuated cellular responsiveness (Ferguson 2001). Likewise, AMPA receptor endocytosis has been shown to decrease synaptic activity and contribute to long term depression (LTD) at glutamatergic synapses (Malenka 2003). Previous reports of endocytosis contributing to the restoration of cellular responsiveness have been shown to involve a significant refractory period (Pippig, Andexinger et al. 1995). Certainly our finding that rapid endocytosis promotes $D_1$ receptor mediated signaling in neurons is unexpected.

As of yet, we are unsure how endocytosis might enable rapid dopaminergic signaling. One possibility is that this occurs by an unusually rapid recycling/resensitization mechanism. This mechanism is discussed in detail in previous sections of this work, but it is worth mentioning that it has never been demonstrated within the time frame that we measure $D_1$ receptor signaling and trafficking in chapter 3. Interestingly, we see individual $D_1$ receptor-containing membrane insertion events within minutes of the addition of dopamine or SKF 81297 (data not shown). We are able to observe these events in HEK 293 cells in the continuous presence of dopamine, suggesting that rapid endocytosis and recycling of $D_1$ receptors likely occur with overlapping kinetics. This supports the hypothesis that a rapid recycling/resensitization mechanism could, at least in theory, promote dopaminergic signaling within a sufficiently rapid period of time.

It is also possible that $D_1$ receptors continue to signal efficiently via trimeric G-proteins after agonist-induced endocytosis. Although GPCR-mediated signaling via heterotrimeric G proteins has traditionally been demonstrated at the plasma membrane, emerging evidence suggests that G protein signaling can also occur from endosomes (Slessareva and Dohlman 2006). In fact, thyroid stimulating hormone (TSH) receptor-mediated activation of adenylyl cyclase from endosomes was recently reported in mammalian cells (Calebiro, Nikolaev et al. 2009). Signaling from endosomes may also potentially explain the manner in which rapid endocytosis promotes dopaminergic signaling. For this hypothesis to be viable, a number of conditions would have to be met. First, $D_1$ receptors, $G_s$ proteins and adenylyl cyclase all need be present on the endosomal membrane in an active confirmation. As previously mentioned, $D_1$ receptors have been

reported to undergo rapid agonist-mediated phosphorylation and arrestin recruitment that decreases their ability to efficiently signal via G-proteins (Ng, Mouillac et al. 1994; Tiberi, Nash et al. 1996; Gardner, Liu et al. 2001; Kim, Gardner et al. 2004). Although this regulation is thought to occur prior to clathrin-mediated GPCR endocytosis, it is possible that active, signaling $D_1$ receptors could undergo rapid endocytosis and continue to signal from within endosomes. Conversely, phosphorylated/desensitized $D_1$ receptors could theoretically return to an active conformation while on endosomal membranes, though we are unaware of any precedence for this mechanism.

Precise regulation of dopaminergic signaling is required for a number of complex physiological processes including learning and memory, locomotion and goal-directed behaviors such as food or drug seeking (Sibley 1999; Kelley 2004). $D_1$ receptor endocytosis is likely contributes to this regulation in neurons that respond to dopamine. Recent studies in the brains of awake, behaving animals have demonstrated that transient spikes in dopamine can achieve concentrations in the micromolar range and last on the order of milliseconds to a minute (Heien, Khan et al. 2005; Schultz 2007; Roitman, Wheeler et al. 2008; Tsai, Zhang et al. 2009). Although the ability to detect transient local changes in dopamine has undergone marked improvement in the last few years, it is still not possible to measure dopamine concentrations directly at the synapse or at the level of $D_1$ receptors. Therefore, the actual concentration and duration of dopamine at a given $D_1$ receptor is going to depend, at a minimum, on the distance of that receptor from the site of release, the area available for dopamine diffusion, as well as the proximity and activity of nearby DAT proteins. Regardless of the exact biophysical predictions for

dopamine concentration and duration of action at individual receptors, our data predict that $D_1$ receptors are capable generating a robust increase in cellular cAMP and undergoing endocytosis at dopamine concentrations observed in the intact brain. In fact, *in vivo* endocytosis of striatal $D_1$ receptors has been previously reported (Dumartin, Caille et al. 1998).

It is well established that *in vivo* measurements of extracellular dopamine vary in peak intensities from nano- to micromolar concentrations, depending on the behavior or experimental paradigm that elicits dopamine release. Sucrose administration, considered a mildly appetitive stimulus, produces dopamine transients with peak concentrations near 50nM in the nucleus accumbens of awake behaving rats (Roitman, Wheeler et al. 2008). Whereas in primates, an *in vivo* microdialysis study reported striatal dopamine concentrations greater than 1uM after these animals self-administer cocaine (Bradberry, Barrett-Larimore et al. 2000). This suggests that the magnitude of peak dopamine concentrations elicited by given stimuli may be correlated with either its hedonic value or incentive salience (Berridge and Robinson 1998). Interestingly, our data predict these differences in peak dopamine concentrations would lead to varying amounts of $D_1$ receptor endocytosis (see Chapter 3, Figure S1). Lower extracellular dopamine concentrations stimulate little or no $D_1$ receptor endocytosis and would therefore be expected to attenuate dopaminergic signaling, while higher extracellular dopamine concentrations elicit robust $D_1$ receptor endocytosis and promote ongoing dopaminergic signaling. When we attempt to place our findings within the scope of neural information processing, we hypothesize dopamine-induced $D_1$ receptor endocytosis may represent a

mechanism by which the strength, duration and even salience of stimuli are rapidly encoded at the level of the individual neuron.

Although we acknowledge that there is a large gap between the cell biological basis of our research and precise regulation of dopaminergic signaling within the human brain, our finding that endocytosis is required for high fidelity dopaminergic signaling in neurons may have implications for both disease research and potential therapies. In diseases such as Parkinson's, where there is a deficiency in dopaminergic transmission, therapies that augment $D_1$ receptor endocytosis might lead to better disease management. Further, it may be worthwhile to examine the link between genes and proteins involved in receptor endocytosis the etiology of Parkinson's disease. As for pathologies and symptoms that are thought to result from excessive $D_1$ receptor-mediated dopaminergic transmission, such as the negative symptoms of schizophrenia and tics of Tourette's syndrome, finding a way to selectively disrupt $D_1$ receptor endocytosis could be a useful therapeutic target.

## 4.3 Future directions

Currently, our results cannot distinguish between the two proposed mechanisms by which $D_1$ receptor endocytosis promotes dopaminergic signaling: rapid recycling/resensitization or signaling from endosomes. We do not believe that these mechanisms are necessarily mutually exclusive, but examining their roles in dopaminergic signaling will provide a useful contribution to the field. In the future we hope to develop an assay that can

determine if rapid recycling is necessary for effective dopaminergic signaling. Our attempts to inhibit $D_1$ receptor recycling and measure cAMP accumulation on this time scale have so far been fruitless. Monensin, a compound known to interfere with vesicular acidification of early endosomes and inhibit the recycling of other GPCRs, did not prevent $D_1$ receptor recycling when assayed via flow cytometry (data not shown). Additionally, a $D_1$ mutant receptor previously shown to exhibit recycling deficits (Vargas and von Zastrow 2004) is also deficient in agonist-mediated endocytosis, making it difficult to understand individual contributions of recycling versus endocytosis in promoting dopaminergic signaling.

If endocytosis does promote $D_1$ receptor signaling via endosomes, it will be worthwhile to examine whether this has differential effects on the activation of distinct signaling pathways. It is generally thought that cAMP is spatially restricted in its ability to mediate downstream effectors due in part to localization in specific signaling domains and rapid breakdown by PDEs. If $D_1$ receptors are able to generate cAMP from endosomes, it could potentially mediate a completely different profile of effectors than cAMP liberated at the plasma membrane. In fact, this potential consequence has been proposed for endosomal signaling of the TSH receptor (Calebiro, Nikolaev et al. 2009). Thus far, our studies provide information on an integrated cellular cAMP response. Improving the spatial resolution of our assay, perhaps by targeting the Epac1-cAMPs FRET sensor to particular membrane domains, could lead to insight about regionally specific cAMP signals. Additionally, although the majority of the cellular effects of $D_1$ receptor activation are thought to arise via cAMP-dependent mechanisms (Neve, Seamans et al.

2005), it is possible that $D_1$ signaling from endosomes could regulate completely different signaling pathways. In fact, there is evidence that another $G_s$-coupled receptor, the $\beta_2$-adrenergic receptor, is capable of crosstalk to mitogen activated protein kinase (MAPK) cascades from endosomal membranes (Sorkin and von Zastrow 2009).

Recent findings in the GPCR signaling field that challenge classical concepts of receptor pharmacology, may prove particularly important for the design of future $D_1$ receptor based therapies. The concepts of functional selectivity, agonist-directed trafficking and biased agonism all suggest that specific agonists can initiate functionally distinct cellular signaling profiles despite activating the same receptor (Urban, Clarke et al. 2007). Our data suggest that the ability of an agonist to drive endocytosis of the $D_1$ receptor will be a particularly important predictor of its efficacy with respect to cAMP signaling. Given the diversity of downstream effectors that $D_1$ receptor signaling can modulate in neurons (ion channels, ionotropic receptors, enzymes and transcription factors), it seems plausible that even slight differences in receptor activation produced by distinct $D_1$ receptor agonists could lead to vastly different integrated responses.

Finally, as the technology for dissecting the circuitry of specific behaviors and the technology for imaging individual proteins the intact brain become more and more sophisticated, it may someday be possible to examine $D_1$ receptor trafficking and determine its relative contribution to behaviors in awake behaving animals (Svoboda and Yasuda 2006; Zhang, Aravanis et al. 2007). Progress in both the fields of both GPCR membrane trafficking and dopaminergic signaling will demand that we continue to apply

the latest technologies to examine these processes and better understand their relevance to human physiology. In conclusion, this body of work represents a significant advancement in the understanding of how $D_1$ receptor membrane trafficking contributes to the regulation of neuronal dopamine signaling.

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