Regulation of Dopamine Transporter Trafficking by Substrates, Rabs and SNAREs

by

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Dedication

I would like to dedicate this thesis to my parents Ronald and Robyn Furman who have been my strongest source of support throughout my life and always believed in me more than I believed in myself. I could not have done it without their prayers and support. I'd also like to dedicate this to my Aunt Marsha Wilson who didn't get to see me finish graduate school but gave me so much love and support throughout my life.

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List of Abbreviations

Amphetamine – AMPH

Botulinum Neurotoxin C – BoNT/C

Dopamine – DA

Dopamine Transporter – DAT

Syntaxin 1A – SYN1A

Protein kinase C – PKC

Abstract

Regulation of Dopamine Transporter Trafficking by Substrates, Rabs and SNAREs

The plasmalemmal dopamine transporter (DAT) regulates dopaminergic neurotransmission by facilitating reuptake of extracellular dopamine (DA). The psychostimulant amphetamine (AMPH) is a substrate of DAT which stimulates DA efflux through DAT. DAT undergoes both constitutive and substrate-regulated trafficking and this trafficking further regulates DA action. To date, most trafficking studies focus on DAT endocytosis following prolonged (10 – 60 min) substrate treatment. However, little is known about the mechanisms regulating DAT exocytosis or trafficking to the plasma membrane.

The purpose of this thesis was to elucidate the mechanisms by which constitutive and rapid stimulated DAT trafficking occur. Specifically, the role of Rab and SNARE (N-ethylmaleimide sensitive factor attachment protein receptor) proteins in constitutive DAT trafficking was examined. Additionally, the mechanisms of rapid DAT trafficking to the surface in response to substrates were examined. Our results provide a new model whereby (1) Rab 11 and syntaxin 1A (SYN1A) are required for proper tethering and fusion of DAT to the plasma membrane; (2) Rapid substrate treatment induces DAT exocytosis in a PKC-β-dependent, D2R-independent manner and; (3) AMPH and SYN1A regulate one another's action on DAT trafficking and function.

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Taken together DAT trafficking and function are rapidly and specifically regulated by intracellular stimuli including protein kinase activation and GTPase protein regulation as well as extracellular stimuli including the physiological substrate DA and the psychostimulant amphetamine. These data confirm the importance of DAT in maintaining proper dopaminergic neurotransmission and demonstrate that homeostatic mechanisms are in place to rapidly modulate DAT function in response to exogenous stimuli.

Chapter I

Introduction

The dopamine system

Dopaminergic neurotransmission plays an important role in normal physiological functions such as the regulation of mood, reward and behavior (Amara & Kuhar 1993, Iversen 1971). It is also considered to be a crucial pathway in the development of neurological and psychiatric diseases including Parkinson's disease, and schizophrenia (Giros & Caron 1993). There are two predominant dopaminergic pathways, A9 and A10, which originate in the ventral midbrain and project to the striatum (Figure 1.1).
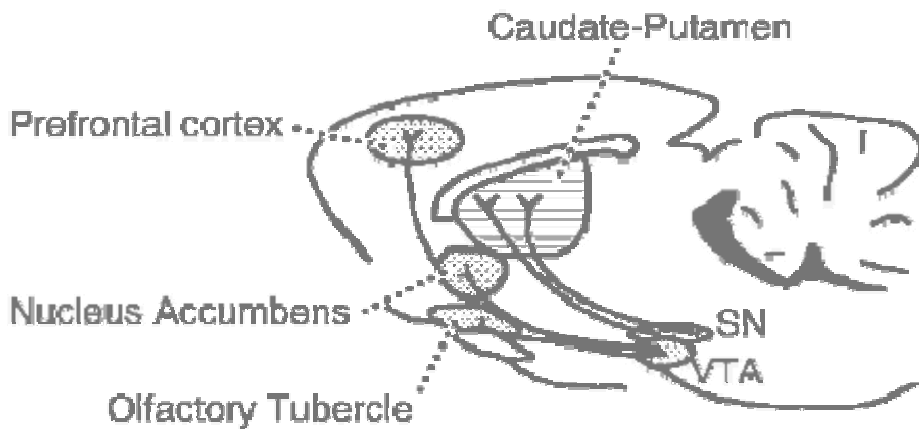
Figure 1.1 A9 and A10 Dopaminergic Pathways. Image source:
www.cellscience.com/cca.files/image003.gif

Specifically, A9 dopamine (DA) cell bodies originate in the substantia nigra and project to the caudate nucleus and putamen, which is also referred to as the dorsal striatum

(nigrostriatal pathway), whereas A10 DA cell bodies originate in the ventral tegmental area and project to the nucleus accumbens, also known as the ventral striatum (mesolimbic pathway).   Another pathway which originates from A10 DA cell bodies is the mesocortical pathway which projects to hippocampus, cerebral cortex, lateral septum and the anterior olfactory nucleus. The mesolimbic pathway is thought to be important for setting the 'emotional tone' therefore the nucleus accumbens is a key area of study in psychiatric disorders including schizophrenia and depression. The mesocortical pathway is important in cognition since the frontal cortex is a major site of these neuron projections. It is thought that the combination of these two pathways, i.e, a mesocorticolimbic pathway contributes to psychiatric and cognitive diseases. Overall, the A9 and A10 DA pathways constitute a principal component in what is known as the "reward" pathway. It is accepted that dopaminergic neurotransmission through these pathways functions is a signal that a reward, be it natural (food, sex) or unnatural (synthetic drugs) (Koob 2009). This "reward" element of the DA pathway makes it extremely important in all types of addiction. In addition, the reinforcing effects and habit development leading to drug addiction involves the neurocircuitry of these A9 and A10 dopamine pathways (Koob 2009).

Dopamine is synthesized by conversion of tyrosine to L-DOPA by the rate-limiting enzyme tyrosine hydroxylase. L-DOPA is then converted to DA by the enzyme aromatic amino acid decarboxylase (AADC). In noradrenergic terminals, DA can be converted to norepinephrine (NE) by the enzyme dopamine-β-hydroxylase. Although this thesis focuses solely on the brain, it is important to note that DA is also a crucial neurotransmitter in kidney function.

Stimulation of a DA neuron causes a calcium-triggered exocytosis of DA vesicles and following vesicle fusion, DA is released into the synaptic cleft. DA is metabolized by monoamine oxidase which is located on the outer mitochondrial membrane. It can also be metabolized extra-neuronally by catechol-O-methyl transferase enzyme. Extracellular DA acts on pre- and postsynaptic DA receptors, whose subtypes are labeled D1 – D5. Dopamine receptors are seven transmembrane G-protein coupled receptors. The D1-like family of receptors include D1 and D5 receptors which couple to G_s proteins that stimulate signal transduction through activation of adenylyl cyclase and subsequent cyclic AMP production. In contrast D2-like receptors including D2, D3 and D4, couple to $G_{i/o}$ proteins and are inhibitory to signal transduction. Both D1 and D2 receptors are located in the whole striatum (Girault & Greengard 2004). D3 receptors are located in limbic areas of the brain and are considered potential therapeutic targets for treatment of drug addiction (Heidbreder *et al.* 2005). D2 receptors exist in two forms, short and long. The short form D2 receptors (D2S) are thought to predominantly be auto-receptors. D2 auto-receptors are located on the presynaptic membrane and are involved in the regulation of exocytotic DA release. For example, when DA is released into the synaptic cleft following exocytosis, it acts on D2 auto-receptors which inhibit both the synthesis of DA and exocytosis of DA-containing vesicles.

Dopamine Transporter (DAT) – structure, function and location

The amount of extracellular DA in the synapse dictates the strength and endurance of dopaminergic neurotransmission, thus it is critical that DA levels be tightly regulated (Amara & Kuhar 1993, Giros *et al.* 1996). The primary mechanism by which

extracellular DA is removed from the synapse is through uptake into the nerve terminal

by the dopamine transporter (DAT). DAT mRNA is located in areas containing DA cell

bodies and nerve terminals including the substantia nigra pars compacta and the basal

ganglia (Ciliax *et al.* 1995). DAT protein is expressed in the dopaminergic neurons in the

substantia nigra and ventral tegmental area as well as in the axons and nerve terminals to

which these neurons project (Ciliax et al. 1995, Freed *et al.* 1995). Differential

expression of DAT in the striatum is observed; 40-60% less DAT is found in the nucleus

accumbens than in the dorsal striatum (Marshall *et al.* 1990). DAT proteins are not

directly located in "active zones" but instead are perisynaptic (Nirenberg *et al.* 1997).

DAT is a Na^+/Cl^- dependent transporter belonging to the solute carrier SLC6 class

of transporters which includes the norepinephrine (NET), serotonin (SERT) and GABA

(GAT) transporters (Amara & Kuhar 1993). Giros & Caron (1993) proposed the

structure of DAT as a 12 transmembrane domain protein with the N- and C-terminus on
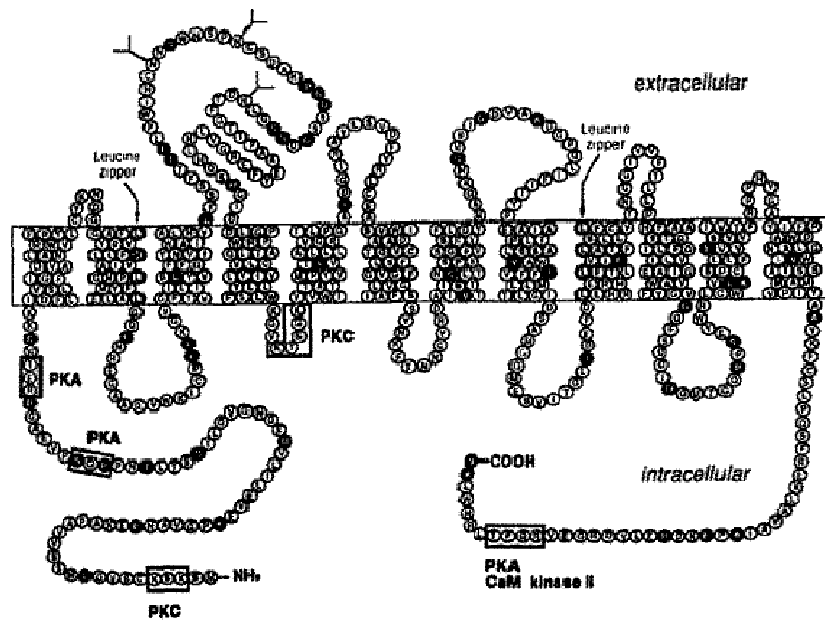
Figure 1.2 Primary amino acid sequence of the human
Dopamine Transporter. Image source: Giros & Caron 1993

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the intracellular side based on the amino acid sequence and hydropathy plot (Figure 1.2).

Both termini contain consensus sites for various protein kinases. Consensus sequences

for protein kinase C (PKC) and protein kinase A (PKA) are located at the N-terminus and

consensus sequences for PKA and calcium/calmodulin-dependent kinase II (CAMKII)

sites are located at the C-terminus (Giros & Caron 1993). Phosphorylation of the N-

terminus of DAT is observed (Foster *et al.* 2002), and mutating serines on the N-terminus

of DAT has functional consequences (Khoshbouei et al., 2004). However, to date, no

studies have identified the specific sites of phosphorylation on DAT thus it is not known

whether these consensus sites are phosphorylated or whether there is an indirect

phosphorylation. DAT has a large second extracellular loop that is heavily glycosylated.

Although no crystal structure for monoamine transporters currently exists, a bacterial

homologue of Na^+/Cl^- dependent transporters was recently crystallized (Yamashita *et al.*

2005). This crystal structure confirmed the notion that monoamine transporters have a

conformation that allows for substrate binding from the extracellular space (outward

facing), a transitional state (substrate occluded) and lastly a conformation that allows for

substrate binding from the intracellular space (inward facing).

In 1996, Giros and colleagues used DAT knock-out mice to demonstrate the role

of DAT in DA clearance (Giros *et al.* 1996). Mice lacking the dopamine transporter have

increased locomotor activity as compared to wild-type mice and high levels of DA in the

synapse. These knock-out mice also have reduced DA tissue content, DA release and DA

receptors relative to WT controls, underscoring the importance of DAT in maintenance of

DA levels.

Experiments done in cellular models with cloned transporters demonstrated that cell systems were a reasonable model for the physiological DA system (Giros *et al.* 1992). As a result, the DAT has been studied in a variety of heterologous cell lines including, but not limited to, pheocromocytoma PC12, human embryonic kidney (HEK), madin-darby canine kidney (MDCK), and neuroblastoma (N2A) cells. The intracellular location of DAT varies in heterologous cell systems. For example, in PC12 cells, DAT is located in endosomes which are less than 50 nm (Loder and Melikian, 2003). In this cell line, DAT was absent from large dense-core vesicles and recycled with transferrin (Loder & Melikian 2003). However, in MDCK cells, DAT is located in lysosomes as well as endosomes (Daniels & Amara 1999, Melikian 2004). Interestingly, these two cell lines show differential regulation of DAT trafficking which may be due to the difference in intracellular localization.

DAT in neurological and psychiatric disorders

<u>Psychosis</u>

Psychosis is broadly defined as an impaired contact with reality. One prominent type of psychosis is schizophrenia which affects 1.1% of the U.S. population over age 18 (National Institutes of Mental Health). "Positive symptoms" in psychosis include auditory hallucinations, delusions, hyperactivity and combativeness and are generally treatable. "Negative symptoms" in psychosis include flat affect and severe withdrawal and are much more difficult to treat. A wealth of pharmacological evidence suggests that the phenotype of excessive behavior (positive symptoms) in psychosis occurs as a result of greater dopaminergic activity in the basal ganglia and has led to the so-called "DA

hypothesis of schizophrenia" (Lieberman *et al.* 1997). One study which provided important evidence for the DA hypothesis of schizophrenia was by Laruelle et al., (1994) who found that AMPH-stimulated DA release through DAT as measured indirectly by single photon emission computed tomography (SPECT) imaging was greater in drug-free schizophrenic patients than in age matched controls. These data suggest that there are alterations in DAT activity that may contribute to schizophrenia.

Parkinson's Disease

Parkinson's Disease is a neurological disorder with hallmark symptoms including cogwheel rigidity of motion, tremor, shuffling gait and bradykinesia. The molecular hallmark symptoms of the disease include pronounced loss of dopaminergic tone (Girault & Greengard 2004). Dopamine neuron loss may be more or less severe depending on the brain region. The relative loss of DA neurons correlates with the amount of DAT expressed in these specific brain regions; the higher the amount of DAT expression, the more vulnerable the DA neurons (Bannon 2005). Furthermore, imaging studies using DAT markers show that there is a decrease in DAT labeling in Parkinson's Disease patients as compared to control that correlates with the severity of Parkinson's Disease (Kugaya *et al.* 2000). DAT may contribute to the neurotoxicity associated with Parkinson's Disease since it can take up certain neurotoxins such as MPP^+ into the cell which leads to cell death (Storch *et al.* 2004). Taken together, these data suggest that DAT may be an important target for treatment of Parkinson's Disease. Current symptomatic treatments include L-DOPA (precursor to dopamine), as well as DA agonists pramipexole and ropinirole. To date, there is no cure for Parkinson's Disease; therefore much research has focused on understanding the disease.

Attention Deficit Hyperactivity disorder (ADHD)

ADHD is a disorder characterized by inattentiveness and hyperactivity/impulsivity (Mazei-Robinson & Blakely 2006). The DAT blocker methylphenidate (Ritalin®) and DAT substrate and releaser D-amphetamine (Adderall®) are commonly used in the treatment of ADHD. It is somewhat paradoxical that a drug that acts as a psychostimulant in normal subjects would be used to treat a disorder characterized by hyperactivity. However, these psychostimulants have been shown to ameliorate hyperactive symptoms in humans with ADHD as well as animal models of ADHD (Mazei-Robinson & Blakely 2006). The differential action of these drugs in ADHD subjects v. normal subjects points to a difference in the functional characteristics of DAT and/or other monoamine transporters between these groups. Several studies have demonstrated a modest but significant increase in DAT labeling in adults with ADHD as compared to controls (Mazei-Robinson & Blakely 2006). Furthermore, studies have demonstrated a link between genetic polymorphisms of DAT and ADHD (Hahn & Blakely 2007). Specifically the 3'VNTR (variable number of tandem repeats) DAT mutation is a prevalent mutation whose characteristics may shed light on the mechanism by which dopaminergic tone is set to 'normal' following psychostimulant treatment. In addition, specific DAT protein variants have been identified in ADHD cohorts and these are currently being examined.

Drug addiction

Although both cocaine and AMPH target various monoamine transporters including SERT, NET and DAT, the dopaminergic system in particular is thought to be a major component of AMPH and cocaine psychostimulant effects. Numerous studies

have demonstrated the importance of the dopaminergic pathway in the reinforcing effects of psychostimulants (for review, see (Howell & Kimmel 2008, Koob 2009)). One prominent finding was in the use of DAT knock-out mice. Intra-peritoneal administration of high doses of cocaine (40 mg/kg) or AMPH (10 mg/kg) to DAT knock-out mice failed to produce any locomotor activity (Giros et al. 1996). Further evidence discussed below support a major role of DAT in AMPH and cocaine psychostimulant effects.

Amphetamines

According to the National Survey on Drug use and Health, there were an estimated 731,000 users of methamphetamine in 2007. Structural homologs of AMPH include methamphetamine (METH) and methlyene-dioxy-methamphetamine (MDMA, ecstasy) which are both highly abused illicit substances. Psychostimulant effects of amphetamines include euphoria, hyperactivity, insomnia and loss of appetite. Imaging studies using [^{11}C] WIN 35428 as a DAT marker showed that METH users have decreased striatal DAT binding as compared to control groups (McCann *et al.* 2008). Similarly, another study using SPECT imaging showed that long term (4 – 12 years) METH users showed decreased DAT binding as compared to controls (Chou *et al.* 2007). Interestingly, following a two week abstinence, DAT binding in METH users was not statistically different from controls (Chou *et al.* 2007).

Cocaine

A 2006 survey from the National survey on Drug use and health showed 43.8 million people reported use of crack or powder cocaine at some time in their life. Although it has been reported that DAT knock-out mice still exhibit conditioned place preference to cocaine (Rocha *et al.* 1998, Sora *et al.* 1998), these findings are strongly contested. A knock-in mouse of a cocaine-insensitive DAT was generated and tested for psychostimulant characteristics of cocaine (Chen *et al.* 2006). These knock-in mice did not display any conditioned place preference to cocaine or cocaine-induced locomotion over a wide range of doses (Chen et al. 2006, Tilley *et al.* 2009) strongly indicating that DAT is the site mediating the primary reinforcement effects of cocaine. Clinical evidence from cocaine abusers that were acutely abstinent showed that DAT binding was elevated by 20% as compared to controls (Malison *et al.* 1998).

DAT substrates and inhibitors

Dopamine is the major substrate for DAT as described above. Norepinephrine is also a substrate for DAT, just as DA is a substrate for NET. The psychostimulant AMPH is a well-known substrate of DAT which competes for DA uptake. The name amphetamine is a condensation of α-methyl-phenethyl-amine. AMPH is structurally similar to DA except that it lacks the hydroxyl groups on the phenyl ring that DA has and is therefore not a catechol. This has important metabolic consequences since enzymes like catechol-O-methyl transferase (COMT) can't breakdown AMPH. AMPH also acts as a competitive inhibitor of DA breakdown by monoamine oxidase since it binds the enzyme but is not itself metabolized by it.

Inhibitors of DAT include cocaine and methylphenidate. Inhibitors of DAT prevent reuptake of DA by blocking the site of DA transport. The exact site of inhibitor binding is not yet known and is an area of intense investigation. Blockade of DA transport causes an accumulation of extracellular DA, leading to increased dopaminergic neurotransmission and a psychostimulant effect.

AMPH-stimulated DA efflux

As previously discussed, AMPH elicits reversal of DAT resulting in DA efflux. There are several competing models for the mechanism of AMPH-stimulated DA efflux. The facilitated exchange diffusion model suggests that when AMPH is taken up by the transporter it increases the number of inward facing transporter binding sites, stimulating DA to bind to the inward-facing transporter and thus increasing reversal of DA transport. Under this model proposed by Stein in 1967 and specifically for catecholamine transporters by Bogdanski and Brodie in 1969, only one molecule can be released for every molecule that is taken up. The weak base model suggests that AMPH depletes DA vesicle pools by alkalinizing the vesicles, thereby increasing the cytosolic level of DA. The increased DA concentration then alters the electrochemical gradient such that it is more favorable for DAT to release DA into the synaptic cleft (Sulzer *et al.* 1992, Sulzer & Rayport 1990). Based on this model, AMPH-stimulated DA efflux is independent of any interaction between AMPH and DAT (Sulzer *et al.* 1995). Data from Galli and colleagues suggest that when AMPH is taken up by DAT, there is an increase in intracellular sodium availability which alters the charge gradient thereby reversing the transporter (Khoshbouei *et al.* 2003).

AMPH-stimulated DA efflux is regulated by protein kinase C (PKC) activity. PKC inhibition blocks AMPH-stimulated DA efflux in heterologous cells and rat striatum (Johnson *et al.* 2005b, Kantor *et al.* 2001). Activation of PKC with the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) mimics the effect of AMPH on reverse transport of DA by eliciting an efflux of DA that is cocaine-sensitive and independent of extracellular calcium (Kantor & Gnegy 1998).

DAT trafficking regulation

Since surface DAT expression shapes dopaminergic neurotransmission, DAT trafficking can efficiently regulate neurotransmission. Indeed, DAT undergoes both constitutive and regulated trafficking and this trafficking is often directly related to its function (Kahlig & Galli 2003, Melikian 2004). A common technique used to assess DAT trafficking includes biotinylation in which cell surface proteins are labeled with the cell impermeant molecule biotin followed by lysis and comparison of biotinylated (surface) DAT to lysate (total) DAT. Subcellular fractionation followed by western blot analysis is also a method used to monitor localization of proteins based on their co-fractionation with marker proteins. A more pharmacological method of detecting surface expression is binding of [^{3}H]WIN35428, a cocaine analog that specifically inhibits DAT. Finally, a method used to visualize and quantify DAT distribution is confocal microscopy in which one can visualize the amount of DAT surface protein v. DAT intracellular protein. Confocal microscopy can be performed with live cells provided a fluorescent tag such as green fluorescent protein or yellow fluorescent protein is fused to the protein of interest. Each of these methods has pitfalls and strengths. For example, although confocal microscopy allows for visualization of protein distribution, it is difficult to

accurately quantify protein levels relative to the amount of fluorescence that is detected. Often, researchers use [³H]DA uptake as a measure of DAT surface expression.

Constitutive DAT trafficking

Approximately 30 – 40 % of total DAT is located on the plasma membrane of DAT-N2A and DAT-PC12 cells (Loder & Melikian 2003, Little *et al.* 2002). DAT constitutively recycles at a rate of 3-5 % per min in DAT-PC12 cells (Loder & Melikian 2003). DAT likely exists in the plasma membrane in some oligomeric state which is necessary for proper DAT trafficking and function (Sorkina et al., 2003; Torres et al., 2003). Both basal and PKC-induced DAT internalization was found to be clathrin-dependent in a study which utilized knock-down of clathrin or dynamin II proteins (Sorkina *et al.* 2005). A specific ten amino acid sequence (FREKLAIYA) in the DAT C-terminus was found to rescue (promote) internalization of an endocytosis-defective reporter protein (Holton *et al.* 2005). This sequence was conserved across the SLC6 family representing an endocytic signal specific to neurotransmitter transporters yet distinct from classical endocytic signals. In contrast, there was no rescue of internalization when the N-terminus was fused to the same endocytic-defective reporter proteins (Holton et al. 2005).

An extensive body of work demonstrates regulation of DAT trafficking in response to substrates, inhibitors and phosphorylation (For extensive reviews: (Kahlig & Galli 2003, Zahniser & Doolen 2001).

DA-stimulated DAT trafficking

Decreased DAT surface expression is observed following prolonged treatment of DA in heterologous cell lines (Chi & Reith 2003, Little et al. 2002, Saunders *et al.* 2000). One hour of DA treatment (10 µM) reduced [^{3}H]DA uptake to 73% of control and surface DAT redistribution to the cytosol as measured by confocal microscopy in hDAT-HEK cells (Saunders et al. 2000). In contrast to the above studies, no DA-induced DAT internalization was detected in MDCK cells (Daniels & Amara 1999). DA-induced internalization has also been demonstrated in *Xenopus laevis* oocytes (Gulley *et al.* 2002). In this study, DA was administered for 40 – 60 min and DAT associated currents were reduced along with the decrease in surface expression.

All of the above studies utilized prolonged DA treatment ranging from ~ 40 – 60 min in a variety of systems. One study looked at rapid treatment of DA (30 µM, 30 sec) in rat striatal synaptosomes and no change in DAT surface expression was observed (Johnson *et al.* 2005a).

AMPH-stimulated DAT trafficking

Prolonged AMPH treatment

Fleckenstein et al (1997b) first demonstrated that METH acutely alters DAT function (Fleckenstein *et al.* 1997a). In this study, METH significantly decreased DAT function (measured by [^{3}H]DA uptake) 1 hr after a 5, 10 or 15 mg/kg injection. Similar studies were performed using high doses of AMPH (10 mg/kg) and MDMA (15 mg/kg) and these were also found to reduce DAT function (Fleckenstein *et al.* 1999). These studies did not determine whether changes in DAT surface expression were concomitant with decreased DAT function. Sandoval et al., 2001 demonstrated that METH (10 µM)

decreased [^{3}H]DA uptake in striatal synaptosomes following 5 – 30 minutes of treatment with no effect on [^{3}H]WIN35428 binding.

In heterologous cells, studies were done to determine whether changes in [^{3}H]DA uptake correlated with DAT surface expression. FLAG-hDAT HEK cells were treated with AMPH (2 µM, 1 hr) and a significant decrease in [^{3}H]DA uptake and DAT surface expression was observed (Saunders et al. 2000).

Rapid AMPH treatment

We demonstrated rapid AMPH-stimulated DAT trafficking to the surface at times corresponding to AMPH -stimulated DA efflux. A 1-min treatment of rat striatal synaptosomes with 3 µM AMPH induced a significant increase (~150% of control) in DAT cell surface expression (Johnson *et al.* 2005a) which was abolished in the presence of cocaine. The AMPH-stimulated increase was short-lived lasting for the first 2.5 minutes and returning to baseline by 10 minutes of treatment. After 30 min of treatment DAT surface expression was reduced to approximately 50% of control which corresponds to data from other researchers (Kahlig *et al.* 2004). When synaptosomes were pretreated with AMPH followed by washout and measured for [^{3}H]DA uptake, no change in uptake was seen as compared to vehicle (Johnson et al. 2005a). However, this rapid AMPH -stimulated increase in DAT cell surface expression functionally enhanced AMPH-stimulated DA efflux. These results suggest that the DAT that is brought to the surface following short AMPH stimulation is primarily functioning to release DA as opposed to taking up DA. In order to determine whether DAT surface expression increased due to increased trafficking towards the surface as opposed to decreased trafficking away from the surface, we performed reversible biotinylation studies. In these

experiments, surface DAT molecules were first labeled with biotin and then allowed to internalize. Following internalization, labeled DAT that remained on the surface was stripped. Treatment with AMPH was then performed for one minute. If AMPH brings DAT molecules from the cytosol to the surface, there should be an increase in the number of labeled DAT molecules on the surface and a corresponding decrease in labeled DAT molecules in the cytosol as compared to vehicle treated cells. Indeed, the results indicate less cytosolic labeled DAT following AMPH treatment versus vehicle treatment indicating that AMPH facilitates exocytosis of DAT as opposed to inhibiting endocytosis of DAT (Johnson et al. 2005a).

Cocaine-induced DAT trafficking

Several studies have been performed to determine whether chronic cocaine use results in alterations of dopaminergic neurotransmission, particularly in DAT activity (for extensive review see (Kahlig & Galli 2003). Studies using post-mortem brain tissue from chronic cocaine users show increased [^{3}H]WIN 35428 binding in the caudate putamen and nucleus accumbens compared to age-matched controls (Little *et al.* 1993). Similar results were found in a report by Mash et al. (2002) which showed both an increase in [^{3}H]WIN 34428 binding and in Vmax of [^{3}H]DA uptake (Mash *et al.* 2002). Studies in heterologous cell lines demonstrate acute regulation of DAT upon cocaine exposure. DAT-HEK cells treated with 10 μM cocaine for 10 min increased [^{3}H]DA uptake by 30% compared to control (Daws *et al.* 2002). Another report found an increase in surface DAT as measured by [^{3}H]WIN35428 binding upon 1 μM cocaine treatment after 12 and 24 hours, however, no rapid induction was found (Little et al. 2002).

Protein kinase C-induced DAT trafficking

There are several consensus sequence sites for PKC and PKA phosphorylation on the N- and C-termini of DAT. Therefore, much work has gone into trying to uncover the role of PKC activity in DAT function. It is now well established that PKC activity plays a role in DAT function, particularly DAT-mediated AMPH-stimulated DA efflux and DAT trafficking, however the precise mechanism is still unknown. PKC activation by phorbol esters such as phorbol myristate acetate (PMA) downregulates DAT activity and/or DAT surface expression in heterologous cells: MDCK, PC12, Sf9, Cos7 and PAE as well as primary *Xenopus laevis* oocytes (Daniels & Amara 1999, Loder & Melikian 2003, Melikian & Buckley 1999, Pristupa *et al.* 1998, Sorkina et al. 2005, Zhu *et al.* 1997) for review see (Melikian 2004, Zahniser & Doolen 2001). These effects of PKC are generally observed from 10 – 60 min of treatment. For example, in DAT-PC12 cells, PMA activation of PKC for 30 min resulted in DAT internalization into transferrin receptor positive endosomes (Melikian & Buckley 1999). The extent to which PKC downregulates DAT seems to depend on the amount of DAT expressed in the cell (Loder & Melikian 2003).

Insulin-stimulated DAT trafficking

Based on behavioral studies from diabetic mice, anatomical similarities between DAT and insulin receptors, and biochemical data from other monoamine transporters, insulin regulation of DAT has been hypothesized (Carvelli *et al.* 2002). Carvelli et al., (2002) demonstrated that insulin rapidly (2-5 min) increases [^{3}H]DA uptake and DAT surface expression in a phosphatidyl inositol 3 kinase (PI3K)-dependent manner. Blockade of PI3K with LY294002 in the absence of stimuli (i.e., insulin) caused a

reduction in basal DA uptake and DAT surface expression (Carvelli et al. 2002). This group also demonstrated that AMPH-induced DAT internalization could be reversed by insulin treatment in an Akt-dependent manner (Garcia *et al.* 2005). Inhibition of Akt, similar to inhibition of PI3K, resulted in decreased DAT surface expression and [^{3}H]DA uptake. Taken together, these data demonstrate a mechanism by which insulin activates PI3K which phosphorylates Akt and allows for normal recycling of DAT to the plasma membrane. In addition, the ability for the insulin signaling cascade to affect AMPH-induced DAT internalization, suggests a complimentary or converging pathway.

D2-mediated DAT trafficking

In addition to regulating the amount of exocytotic DA that is released, D2 receptors modulate the surface expression and function of DAT (Mayfield & Zahniser 2001, Meiergerd *et al.* 1993). Dopamine treatment of *Xenopus laevis oocytes* significantly upregulated [^{3}H]DA uptake and DAT surface expression as measured by [^{3}H]WIN35428 binding. These data correspond with recent studies by Bolan et al., (2007) which demonstrate that D2 regulates DAT surface expression in an agonist-dependent manner. In these studies, the D2 agonist quinpirole rapidly increased DAT surface expression. In contrast, Lee et al., (2007) identified a protein-protein interaction between D2 and DAT which occurred independently of agonist treatment. This interaction appeared to be functional since co-expression of D2 and DAT resulted in increased surface expression of DAT indicating that the direct interaction allowed for co-trafficking of the two proteins to the plasma membrane. Taken together, there may be multiple mechanisms including physical interaction and/or signaling pathway activation by which D2 receptors regulate DAT function.

Modulators and Regulators of transporter trafficking

The mechanisms of transporter trafficking are still poorly understood. Insights from receptor trafficking may provide information about yet unidentified transporter trafficking properties. There is now evidence that transporter trafficking has similar mechanistic properties to that of receptor trafficking. DAT undergoes constitutive internalization into early and recycling endosomes as indicated by its co-localization with endosomal markers (Melikian & Buckley 1999, Sorkina *et al.* 2003, Sorkina et al. 2005). Little is known about regulation of DAT trafficking and specifically how DAT undergoes trafficking to the plasma membrane. Two prominent protein families discussed in this section may be key regulators of DAT trafficking to the plasma membrane. Rab and SNARE proteins may work in conjunction to facilitate the translocation, tethering and fusion events necessary to increase DAT surface expression.

Rab proteins

Function of Rabs

Rab proteins are a large family within the Ras superfamily. Rabs are GTPase proteins that cycle between a GTP-bound state in which they are able to bind effector proteins and a GDP-bound state in which they are inactive. Rabs facilitate protein trafficking from one compartment to another. As shown in Figure 1.3, there are numerous Rab proteins involved in various stages of protein trafficking.

Figure 1.3 Transport pathways and localization of selected Rabs Image source: Stenmark and Olkonnen, 2001
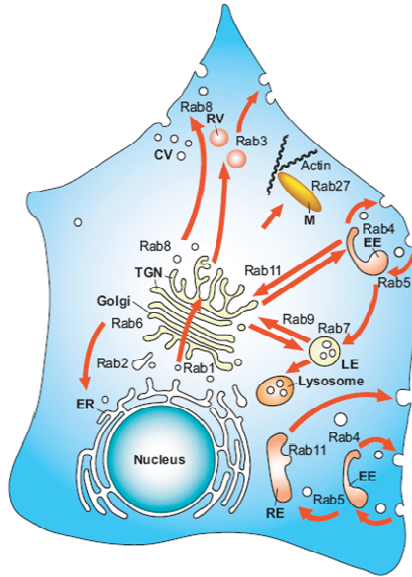
Rab proteins regulate the tethering and/or docking of vesicles to target

membranes including specific endocytic compartments and the plasma membrane (Zerial

& McBride 2001). Fusion of secretory vesicles is regulated by Rab 3a and 27 (Fukuda et

al., 2008). Rab 8 localizes to the trans golgi network (TGN) and regulates the trafficking

of proteins from the TGN to the plasma membrane (Ang *et al.* 2003, Peranen *et al.* 1996).

Rab 11 regulates neurotransmitter release in neurons as well as neurosecretion in PC12

cells (Fukuda et al., 2008). Rab 11 predominantly localizes to the pericentriolar recycling

endosomes and is thought to regulate trafficking of proteins from the recycling

endosomes to the plasma membrane (Sonnichsen *et al.* 2000, Stenmark & Olkkonen

2001, Zerial & McBride 2001). One of the best studied transporters that undergoes rapid

plasma membrane fusion similar to DAT is the insulin-sensitive glucose transporter 4

(GLUT4). GLUT4 has striking similarities to DAT in its trafficking regulation and

intracellular localization. For example, both DAT and GLUT4 rapidly translocate to the

plasma membrane in response to substrate (AMPH or DA for DAT and insulin for

GLUT4). Both GLUT4 and DAT are localized to the endocytic recycling compartment and both co-localize with Rab 11.

Rab and DAT

The DAT recycles into the endocytic recycling compartment in DAT-PC12 cells (Melikian & Buckley 1999). Loder and Melikian (2003) demonstrated that DAT proteins recycle into Rab 5 containing endosomes in PC12 cells. Treatment with the PKC activator PMA or the DAT substrate AMPH for 1-2 hours induced DAT internalization into Rab 11- and Rab 5-containing compartments (Sorkina et al. 2003). Notably, the authors in that study found a small amount of DAT co-localized in Rab 11-containing endosomes in the absence of treatment indicating constitutive trafficking to these endosomes. Furthermore Rab proteins have been implicated in trafficking of other transporters such as the GLUT4 (Zaid *et al.* 2008). The role of Rab protein function in DAT trafficking and activity has not been investigated.

SNARE proteins

In order for protein recycling between intracellular and membranous compartments to occur, fusion events must take place. Of particular relevance to this thesis is how membrane bound proteins such as DAT are trafficked from intracellular endocytic compartments to the plasma membrane. The fusion process typically involves tethering, docking (discussed in the previous section) and then fusion. Soluble NSF-sensitive attachment protein receptor (SNARE) proteins are essential in classical fusion events. A SNARE complex consists of two plasma membrane bound proteins syntaxin and SNAP25 and the vesicle bound protein VAMP (vesicular associated membrane protein; synaptobrevin). Based on the crystal structure of the SNARE complex, a four-

helix bundle composed of two SNAP25 alpha-helices, one syntaxin alpha-helix and one

VAMP alpha-helix (Sutton *et al.* 1998) form the core protein complex (Figure 1.4).
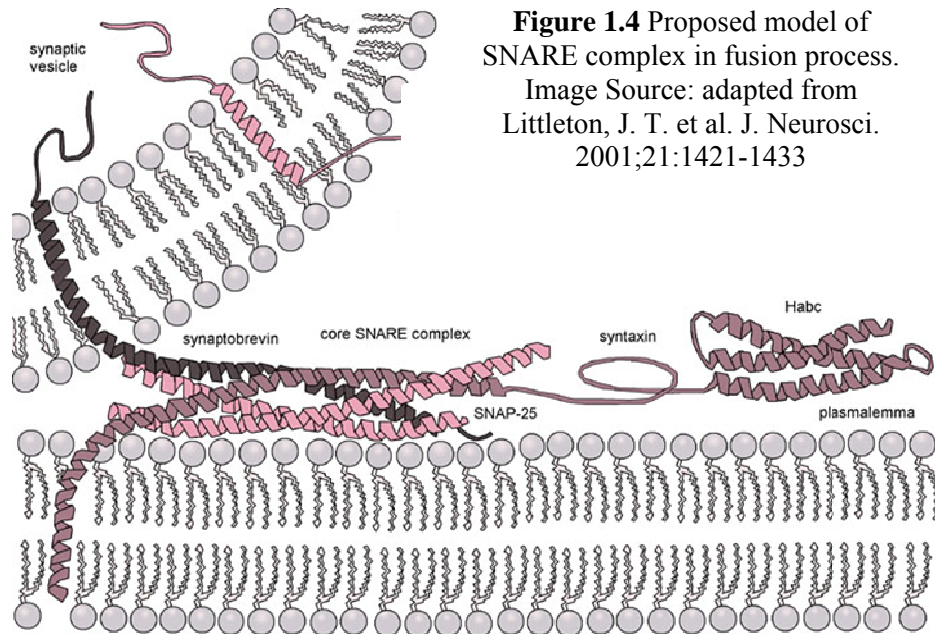
Figure 1.4 Proposed model of SNARE complex in fusion process. Image Source: adapted from Littleton, J. T. et al. J. Neurosci. 2001;21:1421-1433

Syntaxin has a small transmembrane domain and a large cytosolic domain. The cytosolic

domain of syntaxin contains an H3 domain (residues 189-258) which is the portion of

syntaxin that interacts with other SNARE proteins. Syntaxin adopts two conformations,

an open conformation in which the H3 domain is exposed and available to bind other

SNARE proteins, and a closed conformation in which the N-terminus occludes the H3

domain and prevents SNARE protein interaction (Dulubova *et al.* 1999).

Important regulators of exocytosis include Sec/Munc proteins. Munc18 binds to syntaxin

and is thought to hold it in a 'closed' conformation preventing it from binding to other

SNARE binding partners (Burgoyne *et al.* 2009, Dulubova et al. 1999). However, it has

been demonstrated that munc18 is still able to bind to syntaxin in an open conformation

in vivo albeit weakly (Liu *et al.* 2004).

Thesis goals and hypotheses

As described above, there are several mechanisms by which DAT trafficking may be regulated. It is still not completely understood how various mechanisms may converge into complimentary or opposing pathways. Many of the trafficking studies described above with the exception of the Gnegy laboratory's work (Johnson et al. 2005a) demonstrate prolonged (min – hr) treatments of substrates to induce DAT internalization. While DAT internalization is an important component of dopaminergic neurotransmission, an equally important component is DAT trafficking that occurs simultaneous to physiological responses. Therefore, Aim I of this thesis is to characterize the kinetics and mechanism of rapid AMPH-stimulated DAT trafficking using real-time measurements and determine whether this effect is substrate specific. The hypothesis for Aim I is that rapid DAT trafficking to the surface is not specific to AMPH, but may be extended to the natural substrate DA as well. Furthermore, based on evidence from Johnson et al., 2005 which shows a role of PKC-β in AMPH-stimulated DA efflux, I hypothesize that PKC-β contributes to the translocation of DAT to the surface upon substrate treatment.

In order to understand the mechanism of action of psychostimulants acting on DAT, much work has gone into elucidating the mechanism of stimulated DAT trafficking. However, it is important to also delineate the mechanism by which DAT recycles in a basal or constitutive state. Indeed, based on data from Loder and Melikian, 2003, we now know that DAT constitutively recycles with a $t_{1/2}$ of 13 min in heterologous DAT PC12 cells. The endocytic pathway through which DAT recycles is still poorly understood. Furthermore, it is likely that there are trafficking proteins that facilitate the recycling of DAT as well as the tethering of DAT to the plasma membrane.

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In Aim II, Rab proteins are investigated as potential regulators of constitutive DAT recycling. Based on the role of Rab 11 in endocytic recycling towards the plasma membrane (Zerial & McBride 2001), and the current literature on DAT recycling, I hypothesized that Rab 11, and possibly Rab 8 may play a role in DAT trafficking to the surface.

Various proteins may be involved in the trafficking of DAT to and from the plasma membrane. Particularly for DAT fusion to the plasma membrane, SNARE proteins are likely candidates as they are involved in fusion of transporters within the same class as DAT including NET and GAT1 (Quick 2006). The SNARE protein syntaxin is known to directly interact with the N-terminus of DAT and regulate AMPH-stimulated DA efflux through DAT (Binda *et al.* 2008, Lee *et al.* 2004). However, it is not known how syntaxin regulates constitutive DAT trafficking and function. Aim III will determine the role of syntaxin in basal DAT trafficking and function. In addition, the specific conformation of syntaxin when bound to DAT will be determined in order to elucidate the characteristics of the syntaxin/DAT interaction. I hypothesize that DAT trafficking towards the plasma membrane requires binding to syntaxin in its open conformation which would allow for syntaxin binding to other SNARE proteins and facilitate DAT fusion.

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Chapter II

Dopamine and Amphetamine Rapidly Increase Dopamine Transporter Trafficking to the Surface: Live Cell Imaging using Total Internal Reflection Fluorescent Microscopy

Abstract

Rapid treatment (1 min) of rat striatal synaptosomes with low dose amphetamine (AMPH) increases surface expression of the dopamine transporter (DAT). Using mouse neuroblastoma N2A cells stably transfected with GFP-DAT we demonstrate the real-time substrate-induced rapid trafficking of DAT to the plasma membrane using total internal reflection fluorescence microscopy (TIRFM). Both the physiological substrate, dopamine (DA), and AMPH began to increase surface DAT within 10 sec of drug addition and steadily increased surface DAT until removal 2 min later. The substrate-induced rise in surface DAT was dose-dependent, was blocked by cocaine and abated after drug removal. While individual vesicle fusion was not visually detectable, exocytosis of DAT was blocked using both tetanus neurotoxin and botulinum neurotoxin C to cleave SNARE proteins. Notably, the DA-induced increase in surface DAT was cocaine-sensitive but D_2-receptor independent. TIRFM data were confirmed in human DAT-N2A cells using biotinylation and similar effects were detected in rat striatal synaptosomes. A specific inhibitor of protein kinase C-β blocked the substrate-mediated increase in surface DAT in both DAT-N2A cells and rat striatal synaptosomes. These

data demonstrate that the physiological substrate, DA, and AMPH rapidly increase the trafficking of DAT to the surface by a mechanism dependent upon SNARE proteins and protein kinase C-β but independent of dopamine D_2 receptor activation. Importantly, this study suggests that the reuptake system is poised to rapidly increase its function upon DA secretion in order to tightly regulate dopaminergic neurotransmission.

Introduction

Dopaminergic neurotransmission is crucial for normal physiological functions as well as neurological and psychiatric diseases including drug addiction (Giros & Caron 1993) and is regulated by the level of synaptic DA. Extracellular DA is removed from the synapse by uptake into the nerve terminal through the Na^+/Cl^- dependent dopamine transporter (DAT). The psychostimulant AMPH is a DAT substrate which, when taken up into the nerve terminal, causes reversal of DAT resulting in DA efflux while cocaine blocks reuptake of dopamine.

Approximately 30 – 40 % of total DAT is located on the plasma membrane of heterologous cells (Loder & Melikian 2003, Little *et al.* 2002). Since surface DAT expression shapes dopaminergic neurotransmission, DAT trafficking can efficiently regulate neurotransmission. DAT undergoes both constitutive and substrate-mediated trafficking which alter its function (Kahlig & Galli 2003, Melikian 2004). Substrate-induced DAT trafficking has, until recently, focused on demonstrations of DAT internalization following prolonged, higher-dose substrate treatment. Both DA, the physiological substrate, and AMPH induce DAT internalization in heterologous cells, *Xenopus laevis* oocytes and striatal synaptosomes after 40 – 60 min of exposure (Chi & Reith 2003, Gulley *et al.* 2002, Saunders *et al.* 2000). Similarly, a high-dose AMPH

injection reduced DAT function in rat striatum (Fleckenstein *et al.* 1999). Of interest, however, is how substrates alter DAT trafficking at times commensurate with their initial action at the transporter. To that end, our lab demonstrated rapid AMPH-induced DAT trafficking to the surface at times corresponding to AMPH-stimulated DA efflux. A 1-min treatment of rat striatal synaptosomes with 3 μM AMPH induced a significant increase in DAT cell surface expression (Johnson *et al.* 2005a). By 30 min of treatment DAT surface expression was reduced. Importantly, this rapid AMPH-induced increase in DAT cell surface expression functionally enhanced AMPH-induced DA efflux.

Although biotinylation and confocal microscopy are commonly used to monitor trafficking, they are limiting in temporal and spatial resolution, respectively. Investigation of plasma membrane processes is aided by live cell imaging using total internal reflection fluorescence microscopy (TIRFM), which provides real time resolution coupled with the ability to sensitively detect and analyze cytosol to plasmalemmal membrane movement of vesicles and granules. Total Internal Reflection Fluorescence Microscopy (TIRFM, depicted in Figure 2.1) is a unique tool that allows one to focus primarily on events occurring close to the membrane.

In the present study we investigated, utilizing the high temporal and spatial resolution available in TIRFM, the dynamics of DAT trafficking to the plasma membrane. We found that within seconds of addition of AMPH or, notably, the physiological substrate DA, there were changes in surface expression of DAT with maximal increases within one minute. The rapid increase in DAT was blocked by transfection of the light chains of botulinum neurotoxin C (BoNT C) and tetanus neurotoxin (TeNT) which cleave t- and v-SNARE proteins, respectively, and prevent

vesicle fusion. The substrate-induced increase in DAT surface expression was modulated by a protein kinase C-mediated pathway and was independent of D_2 receptor function. These data suggest a substrate-mediated trafficking mechanism which directly and rapidly regulates DAT surface expression to increase DA clearance.

Materials and Methods

Materials.

D-amphetamine sulfate, dopamine, GBR12935, quinpirole and sulpiride were purchased from Sigma. LY379196 was a generous gift from Eli Lilly and Company.

Generation of GFP-DAT cDNA.

Rat dopamine transporter with a fluorescent tag (GFP-DAT) was created by fusing the coding region of enhanced green fluorescent protein (EGFP) from pEGFP-C2 vector (Clontech) to the N terminus of the rat DAT cDNA between EcoRI and KpnI sites in the multi-cloning site of the vector.

Cell culture and stable cell lines.

GFP-DAT cDNA was transiently transfected into the N2A cell line using the Lipofectamine Plus reagent kit (Invitrogen, Carlsbad, CA). A stable cell line was generated through selection with Geneticin (Invitrogen, Carlsbad, CA) over several weeks. Stable human DAT N2A cells were a generous gift of Dr. Karley Little (University of Michigan, VA hospital). N2A cell lines were grown in Opti-MEM I supplemented with 10 % Bovine Growth Serum, 1 % penicillin/streptomycin and 400 μg/ml geneticin for stable maintenance. D_2R short form DNA was kindly provided by Dr. Roger Sunahara (University of Michigan, Ann Arbor) and was transiently transfected into GFP-DAT N2A cells using the Lipofectamine Plus reagent kit. In control cells,

empty vector DNA was transiently transfected. Forty-eight hours after transfection, TIRFM experiments were performed. In experiments using neurotoxins, N2A cells were transiently co-transfected with GFP-DAT and the light chain form of TeNT or the light chain form of BoNT C. Control cells were co-transfected with GFP-DAT and empty vector.

TIRFM Image acquisition & Perfusion Experiments.

GFP-DAT N2A cells were plated onto No. 1.5 glass coverslips coated with 0.5 mg/ml poly-D-lysine (Sigma). Coverslips were placed into a closed chamber (Harvard apparatus) and KRH buffer composed of (in mM): 25 HEPES, 125 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 1.3 $CaCl_2$, 1.2 $MgSO_4$, and 5.6 glucose was added to cover the glass. Micro-perfusion of individual cells was performed at ~27°C using a six-chamber perfusion apparatus equipped with a quartz pipette (inner diameter: 0.2 μm) that was positioned near the cell being imaged. Cells were continuously perfused throughout image collection with KRH buffer or various drugs dissolved in KRH using a computer-controlled perfusion apparatus (model DAD-6VM; ALA Scientific Instruments, Westbury, NY). Total Internal Reflection Fluorescence was achieved using methods described previously with minor changes (Allersma *et al.* 2006). Prismless (through-the-objective) TIRFM was obtained using an Argon ion laser (488 line; Melles Griot [Carlsbad, CA] model 35-LAP-431-208 directed through a custom side port to a side-facing dichroic mirror Q495LPw/AR (Chroma Technology) and a HQ500 LP emission filter (Chroma Technology, Brattleboro, VT) on an Olympus IX70 (inverted) microscope (Olympus, Melville, NY). The beam was focused on the periphery of the back focal plane of an oil immersion objective (60X 1.49 numerical aperture, Olympus). The laser

beam was incident on the coverslip at an angle that resulted in an evanescent field with a decay constant of ~ 100 nm. Digital images were captured using a high sensitivity CCD camera (Andor iXon+, EM-CCD, BV, 512x 512 pixels). Images were acquired at 0.5 Hz with exposure times of 50 – 100 ms. In general, cells were perfused with three solution changes: 120 sec of KRH or test drug, 120 sec of DAT agonist with or without test drug, and 60 sec of KRH (150 frames total). For details, see individual experiments. For all TIRFM experiments, the first 100 sec of baseline perfusion are omitted in the graphs.

TIRFM Image Quantification.

Using the Image J program (NIH software), each cell was traced and analyzed for the mean fluorescence intensity over 150 frames. Background values were obtained by measuring a blank region from each frame and taking the average background of the frames. Data are plotted as mean arbitrary fluorescent units (AFU) = mean cell intensity minus average background intensity. Cells are normalized to 100 AFU at t = -30 sec before DAT agonist addition. Statistical significance was determined by 2-way analysis of variance using Prism 5 or by comparison of individual time points with buffer controls using 2-tailed Student's t test.

Biotinylation of human DAT-N2A cells.

Cells were washed with KRH and treated with the substrates AMPH or DA for 1 min at 37°C. In some cases a pretreatment of 200 nM LY379196 was given prior to substrate treatment (see figure legends for detail). The reaction was quenched using excess 4°C PBS/Ca/Mg. All remaining steps were performed at 4°C to prevent further trafficking. Cells were washed with PBS/Ca/Mg and surface expression of DAT was determined by reacting the surface proteins with 1.5 mg/ml sulfo-NHS-SS-biotin (Pierce,

Rockford, IL) at 4°C with constant rotation. Excess biotin was quenched by incubating

cells with 100 mM glycine, followed by two washes of 100 mM glycine in PBS/Ca/Mg

buffer. Cells were lysed in solubilization buffer (in mM: 25 Tris, 150 NaCl, 1

ethylenediaminetetraacetic acid (EDTA), 5 N-ethylmalemeide, phenylmethylsulfonyl

fluoride and 1% triton-X 100) containing a fresh protease inhibitor cocktail tablet

(Roche) and centrifuged at high speed to obtain soluble protein. Cells were analyzed for

protein content and ~100-200 μg protein was reacted with streptavidin beads (Pierce,

Rockford, IL) to isolate surface protein. Streptavidin beads were washed 3X with

solubilization buffer and protein was eluted in sample buffer composed of 250 mM Tris

pH 6.8, 25 mM EDTA, 10 % sodium dodecyl sulfate, 25 % sucrose, 0.5 % bromophenol

blue supplemented with 100 mM dithiothreitol. A portion of protein (~10-20 μg) was

saved for total DAT measurement (lysate). Surface and total DAT samples were resolved

using SDS-PAGE (10-12% Tris-glycine gel). Western blot analysis was performed using

a monoclonal anti-DAT antibody (MAB369, Chemicon). Protein was detected using

West Pico (Pierce, biotin) or Amersham (lysate) electrochemiluminescence detection.

Western blot bands were quantified using Scion Image software. Data are expressed as

the optical density (OD) units of the biotinylated fraction/OD of the lysate.

Quinpirole-induced DA uptake

Human DAT-N2A cells were treated with either KRH or 10 μM quinpirole for

15 min at 37°C. [^{3}H]DA (30 nM, 34.4 μCi/μl, Perkin Elmer) + unlabeled DA were added

to cells to initiate DA uptake in the presence or absence of the DAT blocker 10 μM

GBR12935 to determine non-specific binding. After 5 min of incubation, cells were

filtered onto GFC Whatman filters and washed three times with excess cold PBS. Filters

were dried and counted on a Beckman scintillation counter.

Biotinylation of rat striatal synaptosomes

Synaptosomes were prepared as previously described (Johnson et al. 2005a). Biotinylation of rat striatal synaptosomes was performed similarly in human DAT N2A cells but were conducted in KRB (in mM): 145 NaCl, 2.7 KCl, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 0.255 ascorbic acid, 24.9 $NaHCO_3$. Synaptosomes were pretreated with KRB, 3 μM AMPH, or 10 μM DA at 37°C (AMPH) or room temperature (DA) for 1 min. To test the role of PKC-β, synaptosomes were incubated with 100 nM LY379196 added with the AMPH. Reactions were stopped by adding 4°C PBS/Ca/Mg buffer and washed once with PBS/Ca/Mg to remove residual drugs. Synaptosomes were lysed in RIPAE buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Triton X-100, 1 % sodium deoxycholate and protease inhibitors). Surface DAT expression was determined as described above for human DAT-N2A cells. Immunoblot analysis was performed using MAB16, monoclonal anti-DAT antibody generously supplied by Dr. Roxanne Vaughan, Department of Biochemistry, University of North Dakota.

AMPH-stimulated DA efflux following AMPH pretreatmen.

Synaptosomes were pretreated with KRB, 3 μM AMPH, 100 nM LY379196 or 100 nM LY379196 + 3 μM AMPH for 1 min at 37°C and then washed three times to remove all residual AMPH. AMPH-stimulated DA efflux was performed in resuspended synaptosomes as previously described (Johnson et al. 2005a). Briefly, the resuspended synaptosomes were separated into 2 portions and incubated with either KRB or 3 μM AMPH for 5 min at 37°C. The reactions were stopped by filtering the samples through a

0.2 mm syringe into an internal standard solution (ISS) containing 0.05 M HClO4, 4.55 mM dihydroxybenzylamine, 1 M metabisulfate and 0.1 M EDTA. Dopamine was measured in the eluate by HPLC-EC analysis. Total DA was measured by lysing a portion of synaptosomes in ISS. Dopamine efflux was calculated as the percentage of total DA in the synaptosomes (DA in eluate/total DA in synaptosomes) following the pretreatment.

Statistical analysis was performed using Graph Pad Prism 5 (Graph Pad, San Diego, CA).

Results

AMPH increases surface DAT levels in DAT-N2A cells

Earlier biochemical studies demonstrated an increase in DAT surface expression within one minute of AMPH treatment in rat striatal synaptosomes using biotinylation (Johnson et al. 2005a). In this study, real-time DAT trafficking was visually monitored in live N2A neuroblastoma cells stably expressing GFP-DAT. We employed TIRFM to analyze the spatial and temporal resolution of rapid DAT trafficking. TIRFM revealed that AMPH had similar effects in GFP-DAT N2A cells as in synaptosomes. Individual cells were perfused with KRH for 120 sec (baseline) followed by 120 sec in the absence or presence of AMPH and lastly with KRH for 60 sec (washout). Figure 2.2 (upper panel) shows a representative cell under KRH perfusion 20 sec before AMPH addition (-20 sec), and 0, 60 and 120 sec after AMPH perfusion. Hot spots reflect high amounts of GFP-DAT and/or regions of the plasma membrane especially close to the glass interface. Fluorescence was stable for at least 90 sec before perfusion with AMPH. Upon perfusion with 5 μM AMPH, GFP-DAT intensity increased, shown at 60 sec and 120 sec post drug

application in Figure 2.2. Amphetamine addition increased GFP-DAT intensity in a dose-dependent manner. The curves were statistically different when analyzed by 2-way analysis of variance (see legend to Fig. 2.2). In post-hoc Bonferroni testing, perfusion with 1 μM AMPH significantly increased GFP-DAT intensity by 60 sec of treatment whereas a significant increase was attained after 42 sec of perfusion with 5 μM AMPH. GFP-DAT intensity remained significantly elevated with 5 μM AMPH for another 60 sec following washout by KRH whereas 1 μM AMPH treatment was not significantly different between KRH and 1 μM AMPH just 18 sec after washout.

To confirm that the increase in fluorescence in TIRFM reflected increased DAT insertion into the plasma membrane, we measured surface expression by biotinylation. Biotinylation of DAT-N2A cells, as previously shown in rat synaptosomes, demonstrated an AMPH-induced increase in DAT trafficking to the plasma membrane. Incubation with 3 μM AMPH for 60 sec at 37°C resulted in surface DAT that was 150 % of control (KRH treatment, $p < 0.01$ by paired 2-tailed t-test, n = 4). These data temporally correlate with the TIRFM studies which demonstrate an increase in GFP-DAT intensity within 40 – 60 sec of AMPH perfusion.

In order to determine whether substrate transport was required for substrate-induced DAT trafficking, biotinylation experiments using a buffer absent of sodium were performed. When NMDG-Cl was substituted for NaCl during the one minute treatment of AMPH or DA, no increase in DAT surface expression was detected (data not shown).

The physiological substrate DA increases surface DAT levels in DAT-N2A cells

The ability of the substrate, AMPH, to rapidly increase DAT trafficking to the plasma membrane raised the possibility that the normally occurring substrate, DA, could

have the same effect. Indeed, DA (10 μM) increased surface DAT with similar kinetics and efficacy as AMPH. Figure 2.3 (upper panel) shows a representative cell under KRH perfusion 20 sec before DA addition (-20 sec), and 0, 60 and 120 sec after DA perfusion. Perfusion with 10 μM DA increased GFP-DAT intensity, shown at 60 sec and 120 sec post DA (Figure. 2.3, upper panel). In post-hoc analysis of 2-way ANOVA, (see legend to Fig. 2.3), DA significantly increased GFP-DAT intensity within 80 sec of treatment although a noticeable increase can be seen ~ 40 sec after drug perfusion. Following washout of DA after 120 sec of treatment, the intensity decreases to control (KRH) levels within 34 sec of KRH addition. Comparison of the washout effects of DA with those of AMPH suggests that AMPH has the longer-lasting effect on DAT surface expression.

Since DA-induced DAT trafficking had not been previously demonstrated, we confirmed that DAT was physically being inserted into the plasma membrane using biotinylation. Biotinylation studies showed that 10 μM DA treatment resulted in surface DAT that was 250% of control ($p < 0.05$ by paired 2-tailed t-test, n = 5).

Amphetamine and dopamine increase GFP-DAT intensity through a DAT-dependent mechanism

In order to establish whether an interaction with DAT was required for the substrate-induced increase in GFP-DAT intensity, cells were pretreated with 30 μM cocaine for 2 min before addition of AMPH or DA. For simplicity, data are presented at three time points: immediately before the addition of substrate (0 time), and 60 sec and 120 sec after addition of substrate. At the 0 time, cells have been incubated for 2 min with either KRH or 30 μM cocaine. As shown in Figure 2.4A and 2.4B, cocaine itself had no effect on surface DAT intensity. However, cocaine effectively blocked the

increases in surface DAT fluorescence elicited by 5 μM AMPH (Figure 2.4A) or 10 μM DA (Figure 2.4B) at 60 and 120 sec.

SNARE proteins regulate GFP-DAT trafficking to the plasma membrane

While monoamine transporter recycling to and from the membrane has been demonstrated, few studies have focused on the exocytosis of monoamine transporters. To determine whether DAT was undergoing exocytosis upon substrate treatment, we utilized the specific neurotoxins, BoNT C and TeNT, which are known to proteolyze the soluble N-ethylmaleimide sensitive factor attachment proteins (SNARE) syntaxin 1A and vesicle-associated membrane protein (VAMP)/synaptobrevin, respectively. Cells were transiently co-transfected with GFP-DAT and the light chain of either BoNT C or TeNT. All cells were perfused with KRH for 2 min followed by 10 μM DA for 2 min. As shown in Fig. 2.5, in cells transfected with BoNT C (gray bars) or TeNT (black bars), DA-induced DAT trafficking to the plasma membrane was significantly abrogated as compared to cells transfected with vector alone. At no time did DA significantly increase the DAT intensity over the 0 time point in neurotoxin-treated cells. These data indicate that the substrate-induced increase in GFP-DAT intensity is an exocytotic event which is regulated by SNARE proteins.

Dopamine increases surface DAT through a D_2R independent mechanism

The experiments involving DA suggest that physiological levels of synaptic DA could interact with the DAT to rapidly regulate synaptic levels of DA. Rapid increases in surface DAT in response to DA D_2 receptor (D_2R) activation, however, have been demonstrated in human embryonic kidney cells co-expressing D_2R-short and human DAT (Bolan *et al.* 2007). It is unlikely that the rapid increase in surface DAT in our

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experiments is mediated by D_2Rs because the DA-stimulated increase was blocked by 30 μM cocaine. Nonetheless, we felt it important to examine whether any endogenous D_2Rs in the GFP-DAT-N2A cells could be mediating the effect of DA. We could not unambiguously prove the presence or absence of D_2Rs in the N2A cells using immunoblotting. A western blot of N2A cell lysate with a D_2 receptor antibody revealed a very faint band following overexposure (data not shown) suggesting there could be a small number of D_2Rs in these cells. If there are D_2Rs present, they appear not to be functionally coupled to DA transport. There was no increase in [³H]DA uptake in response to the D_2R agonist quinpirole (Figure 2.6A). In addition, we pretreated GFP-DAT-N2A cells with 10 μM sulpiride, a D_2R antagonist, for 2 min followed by 2 min of 10 μM DA and examined fluorescent intensity using TIRFM. Sulpiride alone did not affect GFP-DAT intensity and did not block the DA-induced increase when given simultaneously with DA (Figure 2.6B). Since previous experiments in DAT-N2A cells overexpressing D_2Rs demonstrated a D_2R-dependent increase in DAT activity (Bolan et al., 2007), we performed TIRFM experiments with cells overexpressing short form D_2Rs, D_2R-S. We confirmed surface presentation of D_2Rs by performing binding assays with the D_2R antagonist [³H]-spiperone (data not shown). TIRFM analysis demonstrated that there was no difference in the DA-induced increase in DAT surface expression between GFP-DAT cells transiently transfected with D_2-receptors *vs* control cells transiently transfected with empty vector (Table 2.1). These data, combined with the lack of quinpirole-induced increase in [³H]DA uptake and the blockade of the DA effect by cocaine, demonstrate that the DA-stimulated rapid DAT trafficking is D_2-receptor independent in the N2A cells within our experimental time frame.

Because the concept of a DA-induced, DAT-mediated increase in surface DAT is novel, we examined whether this could be demonstrated in a more physiologically relevant system, rat striatal synaptosomes. In a previous study, Johnson et al. (2005a) found no increase in surface DAT following treatment of rat synaptosomes with 30 μM DA at 37°C. At present, we chose to perform the experiments at room temperature, mimicking the conditions in which the TIRFM experiments were performed. Rat striatal synaptosomes were treated with KRB or 10 μM DA for 1 min and surface DAT was measured by biotinylation. As shown in Figure 2.7, there was a significant 30% increase in surface DAT as compared to vehicle following DA treatment that was blocked by simultaneous treatment with 1 μM of the DAT blocker, GBR12935. In some experiments GBR12935 appeared to reduce surface DAT, but no significant difference between GBR12935 and KRB-treated cells was found. Alternative statistical analysis using a one-way ANOVA which compared DA, GBR or DA + GBR treatments (excluding KRB alone) revealed a significant difference between DA v. GBR ($p < 0.05$) and DA v. DA + GBR ($p < 0.01$).

PKC-β inhibition blocks the substrate-induced increase in DAT surface expression

A previous report from our lab demonstrated that pretreatment with the specific PKC-β inhibitor, LY379196, attenuated AMPH-stimulated DA efflux in rat striatal slices (Johnson *et al.* 2005b). We wished to examine whether PKC-β was affecting substrate-induced DA efflux by altering rapid DAT trafficking to the surface. To determine whether a PKC-β dependent effect was also occurring in the N2A cell system, TIRFM and biotinylation experiments were performed. We found that these cells contain PKC-β1 (data not shown). Cells were pretreated for 5 min with KRH or 200 nM LY379196

and then treated with 5 μM AMPH or 10 μM DA in the presence of KRH or LY379196. As shown in Figure 2.8A, LY379196 slightly but significantly decreased the AMPH-induced increase in surface DAT after two minutes of treatment. Even though the zero time point in the LY + AMPH group appears lower than that of the AMPH alone group, none of the three zero time groups are significantly different from one another. LY379196 had similar effects on DA-induced increases of surface DAT although the blockade of the DA effect was more pronounced than with AMPH (Figure 2.8B). These data are further validated with biotinylation experiments which show complete blockade of AMPH or DA-induced increases in surface DAT expression following pretreatment of LY379196 in DAT-N2A cells (Figure 2.8C). Alternative statistical analysis using a one-sample t-test revealed that AMPH-stimulated increases in DAT surface expression following KRH pretreatment were nearly significant ($p = 0.051$ as compared to hypothetical value of 1). Similarly DA-stimulated increases in DAT surface expression following KRH pretreatment were nearly significant ($p = 0.052$ as compared to hypothetical value of 1). However, LY379196 pretreatment blocked AMPH and DA-stimulated increases (AMPH: $p = 0.61$; DA: $p = 0.74$ as compared to hypothetical value of 1).

The blocking effect of LY379196 appeared more complete in the biotinylation experiments than in the TIRFM experiments. This differential effect could indicate a delayed effect of the inhibitor when it is slowly perfused as opposed to being given as a bolus as in biotinylation.

We next examined whether PKC-β similarly affects substrate-induced DAT trafficking in rat synaptosomes. Synaptosomes were treated with vehicle, 3 μM AMPH,

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100 nM LY379196 or AMPH + LY379196 for 1 min and surface DAT expression was then measured using biotinylation (Figure 2.9A). In the absence of LY379196, AMPH increased DAT surface expression 1.5 fold over vehicle but in the presence of drug, this effect was attenuated. LY379196 alone did not alter basal DAT surface expression indicating that there is a blockade of AMPH-induced DAT trafficking as opposed to a change in basal DAT trafficking. To determine whether the inhibition of AMPH-induced DAT trafficking by PKC-β was sufficient to block AMPH-stimulated DA efflux, synaptosomes were pretreated with KRB, 3 μM AMPH, 100 nM LY379196 or AMPH + LY379196 for 1 min as described in the biotinylation experiment. Synaptosomes were subsequently washed at 4°C to prevent trafficking and AMPH-induced DA efflux was measured (see Materials and Methods). As we demonstrated previously (Johnson et al. 2005a), a one minute pretreatment with AMPH alone (followed by washout) significantly enhanced AMPH-stimulated DA efflux (Figure 2.9B). However, this enhanced efflux was blocked when AMPH and LY379196 were given simultaneously during the one minute pretreatment. This demonstrates that the enhanced AMPH-stimulated DA efflux is likely due to increased DAT surface expression that is PKC-β dependent. A one-minute pretreatment of LY379196 alone followed by washout, did not block subsequent AMPH-stimulated DA efflux (Figure 2.9B).

Discussion

Using total internal reflection microscopy we have documented real-time trafficking of DAT to the cell surface in response to the substrates, AMPH and DA. This is the first report to describe rapid trafficking of DAT in response to the physiological substrate DA independent of DA D_2R stimulation. To our knowledge, this is the first

report to demonstrate that rapid substrate-induced exocytosis of DAT is dependent upon the SNARE proteins, syntaxin 1A and VAMP/synaptobrevin. Moreover, the results obtained in the DAT-N2A cells are verified in rat synaptosomes using biotinylation. We have also demonstrated the involvement of PKC-β in the rapid trafficking of DAT in both systems.

Substrate-induced DAT trafficking to the plasmalemmal membrane occurred at times commensurate with activation of DAT by substrate. Previously, using biotinylation of rat striatal synaptosomes, we demonstrated that incubation with AMPH increased DAT surface expression within 30 - 60 sec (Johnson et al. 2005b). Functional significance of this rapid transit was established by an enhancement in AMPH-stimulated DA efflux, as shown again in this study. We also find that pretreatment of synaptosomes with AMPH, followed by washing out of the drug, elicits an increase in [^{3}H]DA uptake (Chen and Gnegy, *in press*). Taken together, these results suggest a mechanism by which a DAT substrate rapidly recruits the transporter to the surface, increasing both substrate uptake and, in response to AMPH, efflux. Upon continued exposure to substrate, the transporter undergoes internalization which reduces uptake of substrate.

We sought to determine the mechanism of DAT trafficking towards the surface by visualizing the movement of GFP-DAT containing vesicles near the plasmalemmal membrane. TIRFM provides real time resolution coupled with the ability to sensitively detect and analyze cytosol to plasmalemmal membrane movement of vesicles and granules. TIRFM selectively illuminates the aqueous phase immediately adjacent to a glass interface with an exponentially decaying excitation (Axelrod 1981, Axelrod 2003). TIRFM has been used to discern tethering of GLUT4 containing vesicles to the rat

adipocyte membrane before fusion (Lizunov *et al.* 2005) as well as the multiple tethering states and significant motion of chromaffin granules immediately preceding exocytosis (Holz & Axelrod 2008).

We were unable to detect fusion of individual puncta on the plasma membrane in these cells, which may be due to the diffuse pattern of DAT on the membrane, a low incorporation of DAT molecules into individual vesicles, or the small size of the individual vesicles. The GABA transporter, GAT1, for example, is found in clear synaptic vesicles with a diameter of 50 nm (Deken *et al.* 2003). Therefore, in order to determine whether the substrate-stimulated increase in DAT surface intensity was due to a fusion event, we used neurotoxins known to proteolyze SNARE proteins and inhibit exocytosis. A role for vesicle fusion in the substrate-induced increase in GFP-DAT intensity is strongly suggested by the fact that neurotoxins affecting either a t-SNARE (syntaxin 1A) or a v-SNARE (VAMP/synaptobrevin) abolished the substrate-induced increase in fluorescence. VAMP2 was detected as a component of small GAT-1-containing vesicles in rat cortices (Deken et al. 2003), and the present study suggests it is also a component of DAT-containing vesicles. In addition to playing an essential role in the docking and fusion of neurotransmitter-containing vesicles to the presynaptic membrane of neurons, syntaxin 1A regulates function and localization of transporters (Quick 2006). Furthermore, we demonstrated that AMPH increases the binding of syntaxin 1A to DAT and that syntaxin 1A promotes AMPH-stimulated DA efflux in hDAT-expressing cells (Binda *et al.* 2008).

Despite the sensitivity of TIRFM, we found distinct differences between TIRFM and biotinylation in quantitation of the degree of change in surface DAT following

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substrate treatment. Biotinylation showed AMPH and DA treatment to result in DAT surface expression that was 150 % and 250 % of control, respectively, whereas measurement by TIRFM showed ~112% of control for both substrates. One major factor that likely contributes to this difference is that biotin has access only to transporter that is physically within the membrane and not to compartments that are continuous with the membrane (invaginations). TIRFM, on the other hand, may detect DAT-containing compartments that reside immediately underneath the membrane and could contribute to background fluorescence. The evanescent field of our TIRFM setup is ~100 nm and the intensity of fluorescence falls off exponentially with distance from the surface. If the DAT-containing vesicles are similar in size to the ~50 nm GAT1-containing vesicles, background fluorescence could represent some DAT that is not integrally located in the plasmalemmal membrane. Fluorescence of GFP-DAT >200 nm from the plasma membrane would be attenuated at least 90% so the source of the rapidly-trafficked DAT is likely within 100 nm of the membrane. The increase in intensity following substrate, however, represents GFP-DAT that is moving closer to the membrane and would involve fusion events as suggested by the experiments with the neurotoxins. The 'background' fluorescence detected in TIRFM could potentially represent the source of the DAT that moves to the surface following substrate stimulation. More experiments are underway to determine where this rapidly translocated DAT is coming from within the cell. Carneiro & Blakely (2006) found that serotonin stimulation elicited a trafficking of the serotonin transporter, SERT, from cytoskeletal fractions to membrane fractions in platelets within 30 min (Carneiro & Blakely 2006). A similar, but more rapid, change in association could occur with DAT vesicles residing directly below the plasma membrane.

Other factors reported to increase surface DAT relatively rapidly are cocaine treatment and DA D_2R activation. Cocaine increased surface DAT in DAT overexpressed cells (Daws *et al.* 2002, Little et al. 2002). An increase in DA uptake was seen after 5 min of 10 µM cocaine treatment (Daws et al. 2002). Cocaine alone had no effect on DAT trafficking within our experimental time frame but blocked substrate-mediated DAT trafficking in both synaptosomes and heterologous cells demonstrating that, in our system, substrate binding to DAT was required to elicit trafficking. Activation of D_2Rs also regulate surface DAT and DA uptake (Meiergerd *et al.* 1993, Mayfield & Zahniser 2001). Bolan et al. (2007) demonstrated a D_2R-dependent increase in DAT surface expression in human DAT-N2A cells overexpressing the D_2R following 1 min of activation by the D_2R agonist quinpirole. Our human DAT-N2A cells were not overexpressing D_2Rs but may contain low but active levels of endogenous D_2 receptors. Our experiments clearly demonstrate, however, that the DA-induced increase in surface DAT in human DAT-N2A cells was not due to D_2R activation since D_2R overexpression in N2A cells did not change the DA-induced increase in DAT surface expression. Moreover, our experiments in rat striatal synaptosomes demonstrate that interaction of the substrate, DA, with DAT is required for the very rapid transport of DAT to the plasmalemmal membrane in a more physiological system. However, more experiments in rat synaptosomes using specific D_2R antagonists are needed to determine whether D_2Rs are directly or indirectly involved.

PKC appears to play an integral role in DAT trafficking. Analogous to substrate treatment, a more prolonged exposure of synaptosomes or DAT-overexpressed heterologous cells to PKC-activating phorbol esters induces a down-regulation of DAT

(Melikian 2004, Sorkina *et al.* 2005). However, evidence from our laboratory suggested that PKC could also enhance some DAT activities. PKC inhibitors blocked AMPH-stimulated DA efflux from rat striatum while phorbol esters themselves increased efflux (Kantor & Gnegy 1998, Cowell *et al.* 2000). Johnson et al. (2005b) identified PKC-β as a PKC isozyme contributing to AMPH-stimulated DA efflux (Johnson et al. 2005b). We have utilized the selective PKC-β inhibitor, LY379196, to demonstrate that PKC-β plays a role in the rapid substrate-induced trafficking in both DAT-N2A cells using TIRFM as well as biotinylation and in synaptosomes using biotinylation. Further, using synaptosomes, we demonstrated functional consequences of the PKC-β effect in that the enhancement in AMPH-stimulated DA efflux following AMPH pretreatment was blocked by LY379196.

Taken together, our results suggest that there are dual, biphasic regulations of DAT both by substrate and PKC activation. Within seconds of interacting with DAT, substrates induce exocytosis of DAT-containing vesicles into the plasmalemmal membrane. Substrate transport is likely required since experiments where sodium was removed failed to show increased DAT surface expression upon substrate exposure. The movement of DAT to the surface is dependent upon PKC-β activation. The source of DAT may be "readily releasable pools" in a subplasmalemmal membrane compartment that are primed for translocation into the plasmalemmal membrane during substrate stimulation. Work by Schmidt et al. (1997) revealed a synaptic-like microvesicle compartment that is continuous with the plasmalemmal membrane in neuroendocrine cells (Schmidt *et al.* 1997).

The rapid translocation of DAT-containing vesicles to the surface has functional

consequences (Johnson et al. 2005a). Longer treatment with substrate, or PKC activators,

leads to internalization of the transporter, although AMPH-stimulated internalization may

not require PKC (Boudanova *et al.* 2008). It is possible that different isozymes of PKC

could be responsible for the rapid transport and internalization of DAT, although it is

likely that a 'classical' Ca^{2+}-dependent PKC is also important for PKC-dependent

internalization (Doolen & Zahniser 2002). These studies are important for understanding

the responsiveness of the DA transporter to both the physiological substrate and to drugs

of abuse.

Figures

Figure 2.1 Total Internal Reflection Fluorescent Microscopy. TIRFM relies on a glass (coverslip) water (cell buffer) interface and cells are plated directly onto the coverslip. In epifluorescence, the excitation laser beam is positioned so that it hits the glass-water interface perpendicularly. This results in total refraction and infinite propagation of light through the cell sample which will allow for all fluorescent molecules within that path to be excited. Therefore, GFP-DAT near the center of the cell will fluoresce with the same intensity as GFP-DAT near the surface of the cell. However, TIR occurs when the critical angle defined by Snell's law is reached. At this stage, the light no longer propagates into the cell sample instead there is an evanescent field within the aqueous phase that decays exponentially as the distance from the glass-water interface increases. With our particular TIRF setup, GFP-DAT molecules within ~ 100 nm of the cell surface can be detected. Additionally, due to the exponential decay, as GFP-DAT molecules from further within the cell move closer to the glass-water interface (plasma membrane), they will increase in fluorescence intensity. Conversely, as GFP-DAT molecules move away from the plasma membrane, they will decrease in fluorescence intensity. This allows for a z-plane dimension to monitor and quantify DAT trafficking to and from the plasma membrane.
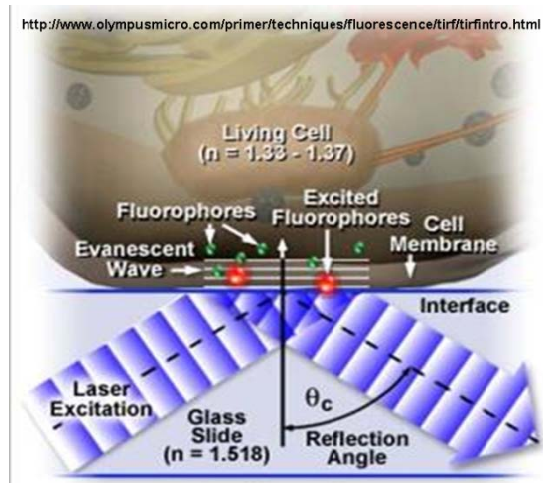
Figure 2.2. Rapid AMPH-induced DAT trafficking to the plasma membrane - TIRFM analysis. Top, pseudocolored images of a representative GFP-DAT N2A cell perfused with KRH for 120 sec followed by 5 µM amphetamine (AMPH) for 120 sec. Images show cell under KRH perfusion 20 sec before addition of AMPH (-20 sec) and at 0, 60 and 120 sec after addition of 5 µM AMPH. Bottom, Image J quantitation of AMPH-induced increases in GFP-DAT pixel intensity. Cells were treated with KRH for 120 sec (20 sec prior to AMPH addition shown), followed by KRH (n = 5), 1 µM AMPH (n = 11) or 5 µM AMPH (n = 13) for 120 sec. Data are expressed as mean Arbitrary Fluorescent Units (AFU) normalized to 100 AFU. Dashed lines represent addition of KRH or AMPH at 0 sec and perfusion is switched back to KRH at 120 sec. Error bars represent SEM. $p < 0.001$ for dose and $p < 0.0001$ for time and interaction of dose and time: $F_{(208, 2808)} = 6.61$ by 2-way ANOVA. In post-hoc Bonferonni testing, statistical significance ($p < 0.05$) is achieved from 60 sec to 138 sec with 1 µM AMPH, and from 42 sec to 180 sec with 5 µM AMPH as compared to KRH values.
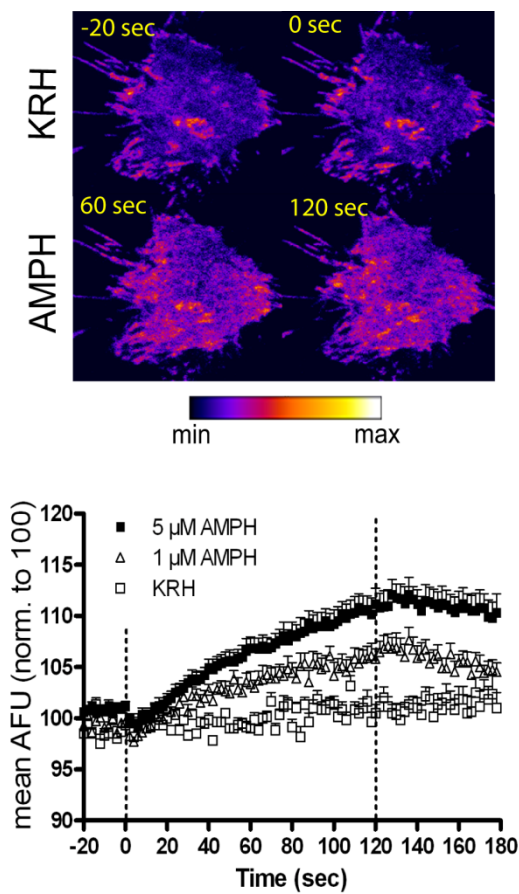
Figure 2.3 Rapid DA-induced DAT trafficking to the plasma membrane - TIRFM analysis. Top, pseudocolored images of a representative GFP-DAT N2A cell perfused with KRH for 120 sec followed by 10 µM dopamine (DA) for 120 sec. Images show cell under KRH perfusion 20 sec before addition of DA (-20 sec) and at 0, 60 and 120 sec after addition of 10 µM DA. Bottom, Image J quantitation of DA-induced increases in GFP-DAT pixel intensity. Cells were treated with KRH for 120 sec (20 sec prior to DA addition shown), followed by KRH (n = 5) or 10 µM DA (n = 14) for 120 sec. Data are expressed as mean Arbitrary Fluorescent Units (AFU) normalized to 100 AFU. Dashed lines represent addition of KRH or DA at 0 sec and perfusion is switched back to KRH at 120 sec. Error bars represent SEM. $p < 0.01$ for drug and $p < 0.0001$ for time and interaction of drug and time: $F_{(104, 1976)} = 6.73$ by 2-way ANOVA. In post-hoc Bonferroni testing, statistical significance ($p < 0.05$) is achieved from 80 sec to 154 sec with 10 µM DA as compared to KRH values.
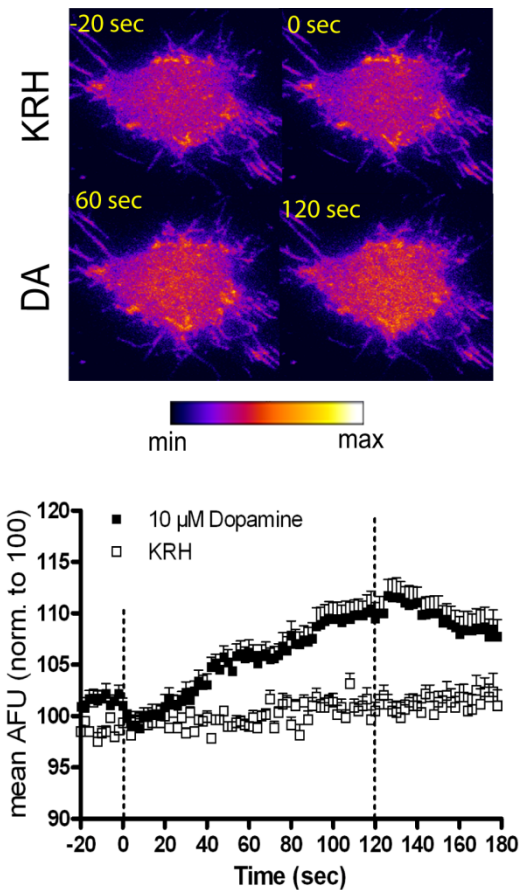
Figure 2.4 Cocaine blocks AMPH and DA-induced increases in GFP-DAT intensity in TIRFM. A, Cells were perfused for 2 min with KRH (white and black bars) or 30 μM cocaine (checkered bars) before addition of 5 μM amphetamine (AMPH, white bars, n = 5), 30 μM cocaine (black bars, n = 5) or 30 μM cocaine + 5 μM AMPH (checkered bars, n = 5) at t = 0. Data are expressed as mean arbitrary fluorescence units (AFU) and are normalized to 100 AFU (dashed line). Error bars represent SEM. Comparing across 3 drug groups, p < 0.0001 for drug and p < 0.05 for time by 2-way ANOVA. *p < 0.05 by post-hoc Bonferroni for 5 μM AMPH v. cocaine + AMPH at t = 60 sec and *p < 0.001 for 5 μM AMPH v. cocaine + AMPH at t = 120 sec. Comparing time points within each group, #p < 0.001 by 2-way ANOVA - post hoc Bonferroni for 5 μM AMPH at t = 0 sec v. 120 sec. B, Cells were pretreated for 2 min with KRH (white and black bars) or 30 μM cocaine (checkered bars) before addition of 10 μM dopamine (DA, white bars, n = 3), 30 μM cocaine (black bars, n = 5) or 30 μM cocaine + DA (checkered bars, n = 5) at t = 0. Comparing across 3 drug groups, p < 0.01 for drug and p < 0.05 for time by 2-way ANOVA. *p < 0.05 by post-hoc Bonferroni for 10 μM DA v. cocaine + DA at t = 120 sec. Comparing time points within each group, #p < 0.05 by 2-way ANOVA - post hoc Bonferroni for 10 μM DA at t = 0 v. 120 sec.
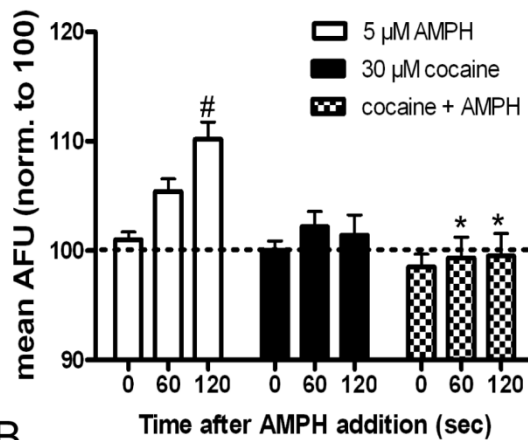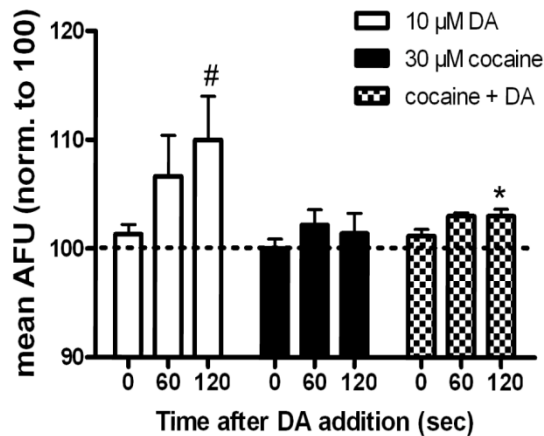
Figure 2.5 SNARE proteins regulate DA-induced DAT trafficking to the plasma membrane. N2A cells were co-transfected with GFP-DAT and empty vector (white bars, n = 13), the light chain of BoNT C (gray bars, n = 15) or the light chain of TeNT (black bars, n = 10). All cells were perfused for 2 min with KRH before addition of 10 µM DA for 2 min at t = 0. Data are expressed as mean arbitrary fluorescence units (AFU) and are normalized to 100 AFU (dashed line). Error bars represent SEM. $p < 0.05$ for interaction, and $p < 0.0001$ for time and treatment by 2-way ANOVA. Post-hoc Bonferroni analysis: *$p < 0.01$ for vector v. BoNT C at t = 120s, $p < 0.05$ for vector v. TeNT at t = 60s and $p < 0.001$ for vector v. TeNT at t = 120 s. Comparing time points within each group, #$p < 0.05$ for vector at t = 0 v. 60 sec and $p < 0.001$ at t = 0 v. 120 s. There was no significant difference between any time points in BoNT C or TeNT treated cells.
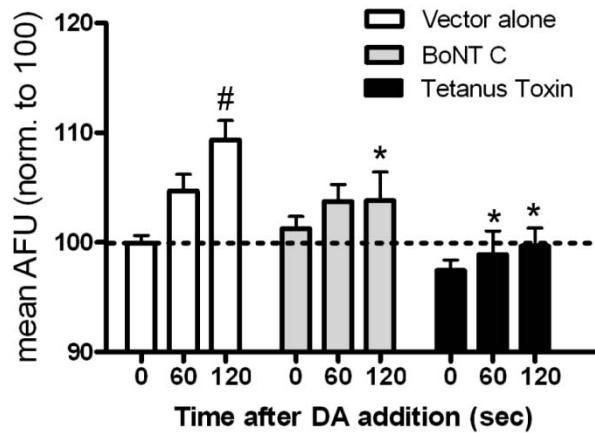
Figure 2.6 DA-induced DAT trafficking to the plasma membrane is D2R independent. A, Quinpirole-induced [3H]DA uptake. [3H]DA uptake (5 min) was measured following a 15 min treatment with vehicle (KRH) or 10 µM quinpirole. Data are expressed as pmol DA per mg protein. Error bars represent SEM. B, TIRFM - Image J quantitation of GFP-DAT N2A cells. Cells were pretreated for 2 min with KRH (white and black bars) or 10 µM sulpiride (checkered bars) before addition of 10 µM dopamine (DA, white bars, n = 15), 10 µM sulpiride (black bars, n = 5), or 10 µM sulpiride + 10 µM DA (checkered bars, n = 10) at t = 0. Error bars represent SEM. Comparing across 3 drug groups, $p < 0.0001$ for drug and time by 2-way ANOVA. *$p < 0.001$ by post-hoc Bonferroni for sulpiride + DA v. 10 µM sulpiride at 120 sec. No significant difference between 10 µM DA and sulpiride + DA treatment at any time point. Comparing time points within each group, #$p <0.01$ by 2-way ANOVA and post-hoc Bonferroni for 10 µM DA at t= 0 v. 60 s and $p < 0.001$ for 10 µM DA at t = 0 v. 120
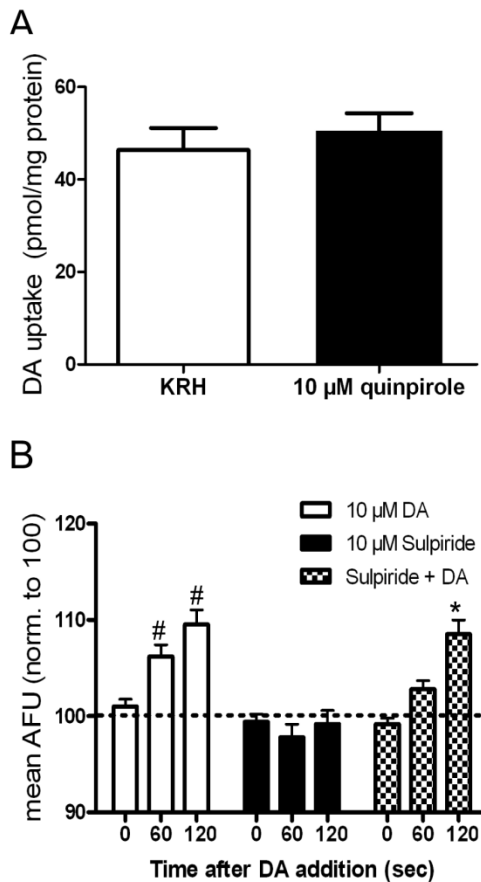
Table 2.1 Effect of D2R-overexpression in GFP-DAT N2A cells - TIRFM analysis. GFP-DAT N2A cells were transiently transfected with empty vector or D2R short DNA. Cells were perfused with 120 sec of KRH followed by 120 sec of 10 µM DA and finally 60 sec of KRH. The table depicts the mean arbitrary fluorescent units (AFU) at the time of drug addition (0 min), and 1 and 2 min after drug addition. No statistical difference was found between the two curves by 2-way ANOVA and post-hoc Bonferroni analysis.

mean AFU (norm. to 100)		
Time (min)	Vector	D_2R
0	97 ± 1.1	99 ± 0.5
1	105 ± 1.4	104 ± 1.1
2	109 ± 2.3	108 ± 0.9

Figure 2.7 DA-induced DAT trafficking in rat striatal synaptosomes. Top, Representative western blot of biotinylated (surface) and lysate (total) of rat synaptosomes treated for 1 min with KRB, 10 µM dopamine (DA), 1 µM GBR12935 (GBR) or GBR12935 + DA (G+D). Bottom, Quantitation of biotinylation. Western blots were scanned and quantitated using Scion Image software. Data are expressed as fold KRB (OD biotin/ OD lysate). Error bars represent SEM. $p < 0.01$ by 1-way ANOVA, n = 4. *$p<0.05$ by post-hoc Bonferroni, DA v. GBR and DA v. GBR + DA.
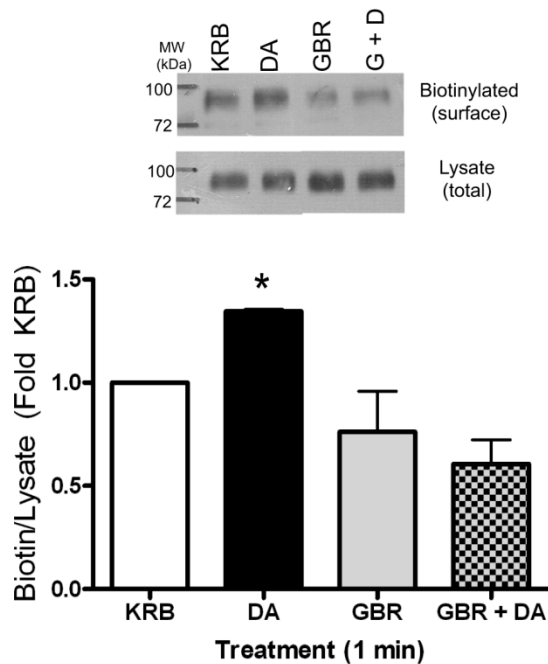
Figure 2.8 LY379196 inhibits substrate-induced DAT trafficking in DAT N2A cells. A, Image J quantitation of TIRFM. Cells were perfused for 2 min with KRH (white and black bars) or 200 nM LY379196 (checkered bars) before addition of 5 µM amphetamine (AMPH, white bars, n = 6), 200 nM LY379196 (black bars, n = 7) or 200 nM LY379196 + 5 µM AMPH (checkered bars, n = 12) at t = 0. Comparing across 3 drug groups, p < 0.0001 for drug and p < 0.05 for time by 2-way ANOVA. *p < 0.05 by post-hoc Bonferroni for 5 µM AMPH v. LY + AMPH at t = 120 sec. Comparing time points within each group, #p < 0.01 by 2-way ANOVA - post hoc Bonferroni for 5 µM AMPH at t = 0 sec v. 60 sec and p<0.001 at t = 0 sec v. 120 sec. Also, #p <0.001 by 2-way ANOVA - post hoc Bonferroni for LY + AMPH at t = 0 sec v. 60 sec and p<0.001 at t = 0 sec v. 120 sec. None of the zero time points were significantly different. B, Image J quantitation of TIRFM. Cells were perfused for 2 min with KRH (white and black bars) or 200 nM LY379196 (checkered bars) before addition of 10 µM dopamine (DA, white bars, n = 5), 200 nM LY379196 (black bars, n = 7) or 200 nM LY379196 + 10 µM DA (checkered bars, n = 6) at t = 0.. Comparing across 3 drug groups, p < 0.0001 for drug and p < 0.001 for time by 2-way ANOVA. *p < 0.01 by post-hoc Bonferroni for 10 µM DA v. LY + DA at t = 120 sec. Comparing time points within each group, #p < 0.05 by 2-way ANOVA - post hoc Bonferroni for 10 µM DA at t = 0 sec v. 60 sec and p<0.001 at t = 0 sec v. 120 sec. Also, #p <0.05 by 2-way ANOVA - post hoc Bonferroni for LY + DA at t = 0 sec v. 60 sec and p<0.01 at t = 0 sec v. 120 sec. None of the zero time points were significantly different. Data are expressed as mean arbitrary fluorescence units (AFU) and are normalized to 100 AFU (dashed line). Error bars represent SEM
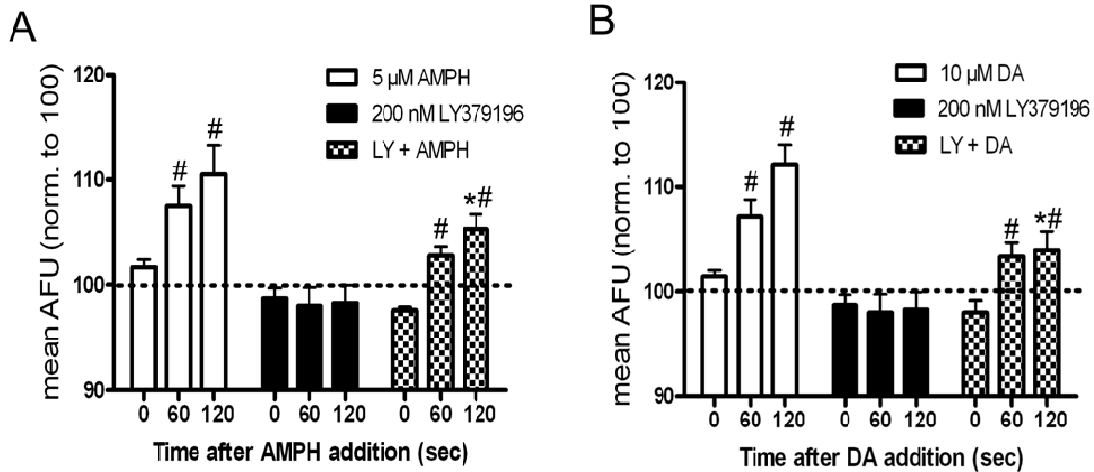
2.8C, Biotinylation. Cells were pretreated with Vehicle or 200 nM LY379196 for 5 min followed by 1 min of KRH (n = 5), 3 μM amphetamine (AMPH, n = 5) or 10 μM dopamine (DA, n = 6). Data are expressed as biotin/lysate and normalized to the respective pretreatment control (Veh or LY). p<0.05 by one-way ANOVA for drug treatment. *p<0.05 by post hoc Bonferroni for Veh v. AMPH and Veh. v. DA in vehicle pretreated cells. There was no significant difference between Veh (mean = 1.00 ± 0.27 OD) and LY37 (mean = 1.15 ± 0.32) raw values as measured by 2-tailed paired t-test (p = 0.455).
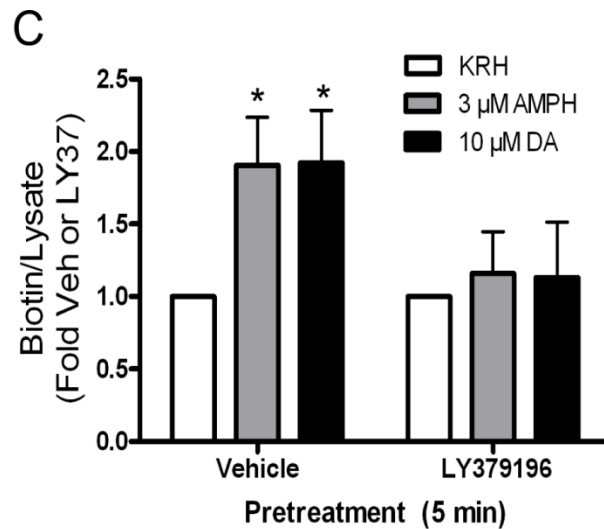
Figure 2.9 Role of PKC-ß in AMPH-induced DAT trafficking in rat synaptosomes. A, Biotinylation of DAT in rat synaptosomes treated for one minute with vehicle (Veh, KRB), 3 μM amphetamine (AMPH), 100 nM LY379196 (LY) or AMPH + LY379196. Error bars represent SEM. *p < 0.05 by 1-way ANOVA, n = 3. Post-hoc Bonferroni analysis shows a significant difference between AMPH and Veh (p < 0.05) B, Synaptosomes were pretreated using same protocol as in A. Synaptosomes were washed 3 times at 4°C then further separated and treated with vehicle or AMPH. Data are expressed as AMPH-induced dopamine (DA) efflux (fold KRB): DA efflux with AMPH / DA efflux with KRB. Error bars represent SEM.*p < 0.01 by 1-way ANOVA, n = 4. Post-hoc Bonferroni analysis shows a significant difference of AMPH v. Veh (p < 0.01), AMPH v. LY (p < 0.05) as well as AMPH v. LY + AMPH (p < 0.05).
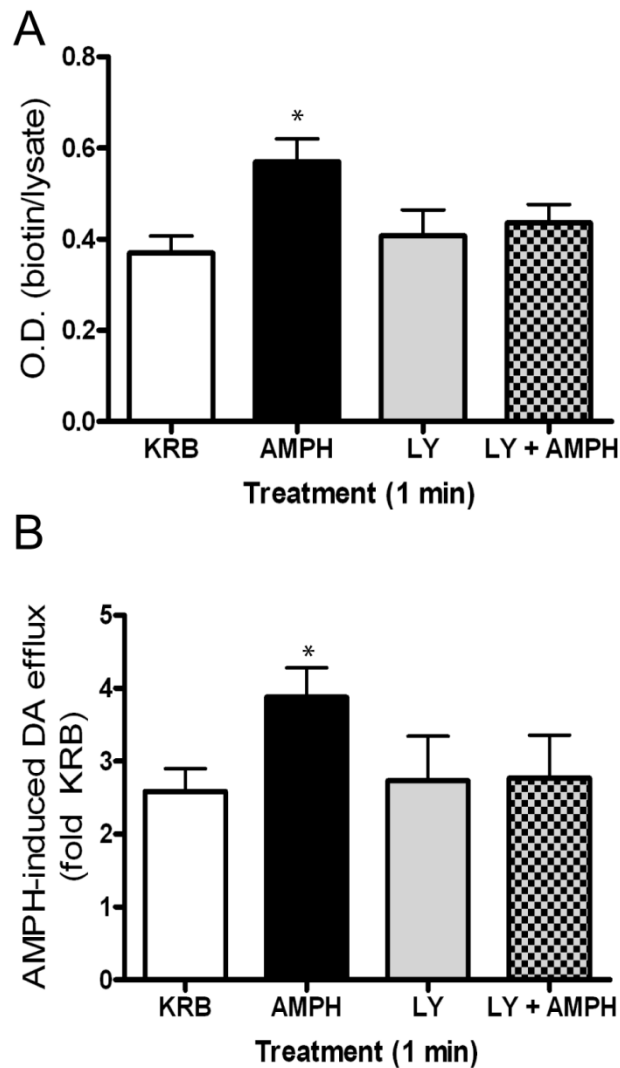
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Chapter III

Rab 11 Regulates Constitutive Dopamine Transporter Trafficking and Function in N2A Neuroblastoma Cells

Abstract

The dopamine transporter (DAT) is a crucial regulator of dopaminergic neurotransmission which undergoes constitutive and substrate-mediated trafficking to and from the membrane. Although, considerable research has been done to elucidate the regulation of substrate-stimulated DAT trafficking, less is known about which trafficking proteins are involved in constitutive DAT trafficking. Rab proteins are GTPases known to regulate the trafficking of proteins to and from specific endocytic compartments. Rabs 8 and 11, in particular, are involved in trafficking proteins from intracellular compartments to the plasma membrane. In this study, we sought to determine whether Rabs 8 and 11 would modulate DAT activity and trafficking in N2A neuroblastoma cells. We used Rab mutations known to confer constitutively active or dominant negative activity of these proteins to investigate the role of Rab activity in constitutive DAT trafficking and function. We found that constitutively active Rab 11 upregulates DAT function and surface expression while neither the constitutively active nor the dominant negative mutant of Rab 8 had any effect on DA uptake. Furthermore, immunofluorescence experiments revealed that dominant negative Rab 11 overexpression results in decreased surface DAT indicating a necessary function of Rab 11 in DAT

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trafficking to the plasma membrane. These data show for the first time a functional role of Rab proteins in the constitutive recycling of DAT to the plasma membrane.

Introduction

The neurotransmitter dopamine (DA) can efficiently be removed from the synaptic cleft by reuptake into the nerve terminal by the dopamine transporter (DAT). DAT is an important regulator of dopaminergic neurotransmission as it is the key mechanism by which dopamine signaling is terminated (Amara & Kuhar 1993). The DAT is a presynaptic plasma membrane protein with a predicted topology of twelve transmembrane domains with intracellular N and C tails (Giros & Caron 1993). Substantive evidence demonstrates dynamic regulation of DAT trafficking to and from the membrane which occurs constitutively and in the presence of DAT substrates (For review, see (Melikian 2004, Robertson *et al.* 2009)). Constitutive DAT internalization was found to be clathrin-mediated in various heterologous cell lines including madin-darby canine kidney (MDCK), human embryonic kidney (HEK) and porcine aortic endothelial (PAE) cells (Daniels & Amara 1999, Saunders *et al.* 2000, Sorkina *et al.* 2005). DAT undergoes constitutive internalization into early and recycling endosomes as indicated by its co-localization with endosomal markers (Melikian & Buckley 1999, Sorkina *et al.* 2003, Sorkina et al. 2005). Rab proteins regulate trafficking of proteins to specific endosomal compartments and are thought to mediate tethering or delivery to the plasma membrane (Zerial & McBride 2001). Rab 8 localizes to the trans golgi network (TGN) and regulates the trafficking of proteins from the TGN to the plasma membrane (Ang *et al.* 2003). Rab 11 predominantly localizes to the pericentriolar recycling

endosomes and is thought to regulate trafficking of proteins from the recycling endosomes to the plasma membrane (Sonnichsen *et al.* 2000, Stenmark & Olkkonen 2001, Zerial & McBride 2001). The DAT recycles into the endocytic recycling compartment (Melikian & Buckley 1999). Loder and Melikian (Loder & Melikian 2003) demonstrated that DAT proteins recycle into Rab 5 containing endosomes in PC12 cells. Treatment with the PKC activator phorbol myristate acetate or the DAT substrate amphetamine (AMPH) for 1-2 hours induced DAT internalization into Rab 11- and Rab 5-containing compartments (Sorkina et al. 2003). Notably, the authors in that study found a small amount of DAT co-localized in Rab 11-containing endosomes in the absence of treatment indicating constitutive trafficking to these endosomes. Furthermore Rab proteins have been implicated in trafficking of other transporters such as the insulin-sensitive glucose transporter GLUT4 (Zaid *et al.* 2008). The role of Rab protein function in DAT trafficking and activity has not been investigated. In the present study, we utilized constitutively active (CA, GTP-bound) or dominant negative (DN, GDP-bound) mutants of Rab to investigate their role in DAT function. Here we show in N2A neuroblastoma cells that a constitutively active form of Rab 11 upregulates DAT activity and trafficking to the surface while the dominant negative decreases DAT surface expression. These data indicate a sufficient and necessary role of Rab 11 in constitutive DAT trafficking.

Methods

GFP-Rab DAT-N2A cells

Rab GTPase DNA constructs with single point mutations known to confer either a GDP bound/dominant negative state or a GTP-bound/constitutively active state were

generated. N2A neuroblastoma cells stably expressing human DAT were transiently transfected with the constitutively active mutants of Rab 8 (Q67L) and 11 (Q70L) or the dominant negative mutant of Rab 8 (T22N) and Rab 11 (S25N) using Lipofectamine-PLUS reagent kit. These mutants have previously been described (Gerges *et al.* 2004). All Rab proteins contain an N-terminal Green Fluorescent Protein (GFP) tag for labeling. GFP-Rab fusion proteins correctly localize to membranes (Ang et al. 2003, Sonnichsen et al. 2000). As a transfection control, cells were transiently transfected with the GFP-vector alone.

[^{3}H]dopamine uptake assay

Twenty-four hours after transfection, cells were plated in quadruplicate (4 wells/transfection) onto coated 24-well plates and incubated for another 24 hours. Cells were washed twice with Krebs Ringers Hepes (KRH) buffer composed of (in mM): 25 HEPES, 125 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 1.3 $CaCl_2$, 1.2 $MgSO_4$, and 5.6 glucose. KRH was removed and uptake was initiated with the addition of [^{3}H]dopamine (10 nM, Perkin Elmer) + unlabeled dopamine in KRH at 25ºC in the presence or absence of the DAT blocker 10 μM GBR12935 to determine non-specific binding. Five minutes after treatment, cells were rapidly washed twice with KRH and 1% sodium dodecyl sulfate was added to solubilize cells. Lysed cells were collected and scintillation fluid was added to the vials before radioactive counting on a Beckman Liquid Scintillation counter. One well per transfection was assayed for protein content (Bio-Rad DC protein assay kit) and data were plotted as pmol DA uptake/mg protein. Specific uptake was determined in the presence and absence of the DAT inhibitor GBR12935 (10 μM).

[^{3}H]WIN35428 binding assay

Cells were plated as described for the [^{3}H]dopamine uptake assay. Cells were washed twice with 4°C KRH to stop constitutive DAT trafficking. [^{3}H]WIN35428 binding was initiated by the addition of 4 nM [^{3}H]WIN35428 + 70 nM unlabeled 2 beta-carbomethoxy-3- beta-(4-fluorophenyl)-N-methyltropane in 4°C KRH buffer in the presence or absence of 10 µM GBR12935 to determine non-specific binding. Thirty minutes after [^{3}H]WIN35428 treatment, cells were rapidly washed two times with cold KRH. Cells were counted for radioactivity as in the [^{3}H]dopamine uptake assay. One well per transfection was assayed for protein content and data were plotted as pmol [^{3}H]WIN35428 bound/mg protein.

Immuno-fluorescence experiments

N2A cells were transiently co-transfected with DAT containing a hemagglutinin (HA)-epitope in the extracellular loop (generously supplied by Dr. Jonathan Javitch, Columbia University) and GFP vector, GFP-Rab 11CA, or GFP-Rab 11DN. Twenty-four hours after transfection cells were replated onto coated glass coverslips and incubated at 37°C for 24 hours. Immuno-fluorescence assays were carried out at ~10-15ºC. Cells were washed twice with phosphate buffered saline supplemented with calcium and magnesium (PBS/Ca/Mg) and blocked with 2% normal goat serum. Surface labeling of DAT was achieved by incubating cells with 1:250 anti-HA (Covance) followed by 1:250 of secondary goat α-mouse AF594 (Invitrogen). In some cases, cells were permeabilized to determine total DAT content using 0.1% triton X-100 in PBS/Ca/Mg followed by anti-DAT (MAB369: Millipore Biomedical Research) or anti-HA and corresponding secondary antibody conjugated to AF594 (Goat anti-mouse) or AF647 (Goat anti-rat, Invitrogen). Cells were mounted onto glass slides using ProLong

Gold anti-fade reagent (Invitrogen). Cells were imaged on an Olympus FluoView 500 confocal microscope. Sequential scans were taken to prevent overlap of laser signal. Z-slices of cells were compressed and quantified using Image J software (NIH). Background was subtracted from each image. A total of 28 cells (28 for surface, 23 for total) were analyzed from three separate cultures.

Results

Rab 11CA increases [^{3}H]DA uptake

Both Rab 11 and Rab 8 have been shown to facilitate trafficking of proteins from intracellular compartments to the plasma membrane. If these proteins affect constitutive DAT trafficking and thus surface DAT, we would expect a change in DAT function reflected in the amount of [^{3}H]dopamine uptake. In order to determine whether Rab 11 or Rab 8 regulates DAT function, N2A-human DAT cells were overexpressed with the CA or DN forms of GFP tagged Rab 11 and 8. As shown in Figure 3.1A, overexpression of GFP-Rab 11CA increased [^{3}H]dopamine uptake to 127% of GFP vector control (p<0.01 by one-way ANOVA). Although GFP-Rab 11DN slightly decreased DA uptake to 84 ± 7% of control, there was no statistical significance when compared to GFP vector alone. Alternative statistical analysis using an unpaired t-test comparing Rab 11CA and Rab 11DN transfected cells revealed a significant difference between these two groups (p < 0.01). Transfection of either the constitutively active or dominant negative form of GFP-Rab 8 had no effect on [^{3}H]dopamine uptake as shown in Figure 3.1B.

Rab 11CA increases [^{3}H]WIN35428 binding

To determine whether this increase in function with GFP-Rab11CA was due to more DAT on the surface, [^{3}H]WIN35428 binding experiments were performed. WIN

35428 has been shown to measure DAT surface expression in numerous experiments and is presumed to specifically label plasma membrane DAT (Chen *et al.* 2004, Li & Reith 1999). Figure 3.2 demonstrates an increase in [³H]WIN35428 binding in cells transiently expressing GFP-Rab 11CA to 137% of control (GFP vector alone), $p<0.05$ by one-way ANOVA. No significant difference was found between GFP vector alone- and GFP-Rab 11DN-transfected cells. Alternative statistical analysis using an unpaired t-test comparing Rab 11CA and Rab 11DN transfected cells revealed a nearly significant difference between these two groups ($p = 0.054$). Changes in [³H]dopamine uptake and [³H]WIN35428 binding will only be reflected when there is a large effect because cells which do not contain overexpressed Rab 11 (i.e. cells which did not take up DNA) will also be measured in these assays. This may result in an under-representation of the effect that occurs in transfected cells.

Rab 11CA increases DAT surface expression

In order to measure effects of Rab 11 on DAT surface expression in individual cells, we performed immunofluorescence experiments. These experiments enabled us to exclude cells which did not contain a sufficient (visible) amount of GFP, GFP-Rab 11CA or GFP-Rab 11DN. In order to visualize and quantify surface DAT, a human DAT construct containing an HA tag in the extracellular loop was used. The HA-DAT construct used in these experiments has previously been described and has comparable function to wild-type DAT (Sorkina *et al.* 2006). Non-permeabilized cells were reacted with anti-HA to label surface DAT followed by a secondary antibody conjugated to a fluorescent (AF594, red) tag. To ensure that changes in surface DAT did not reflect total amounts of DAT within the cell, some cells were permeabilized and reacted with either

an anti-DAT antibody that binds to the N-terminal tail of DAT or anti-HA. Cells that

were permeabilized and reacted with anti-HA for total DAT were not previously exposed

to antibody. The cells were then reacted with a secondary antibody conjugated to a

fluorescent tag. Figure 3A shows the surface labeling (anti-HA) of representative cells

transfected with GFP-vector (left panel) or GFP-Rab 11CA (right panel). Cells

transfected with GFP-Rab 11CA show increased surface DAT fluorescence intensity as

compared to those transfected with GFP vector alone. GFP-Rab 11CA transfected cells

had a 2-fold increase in DAT surface expression as compared to GFP vector alone (GFP

$= 31 \pm 3.1\%$, 11CA $= 67 \pm 8.7$ %, Figure 3.3B).

Rab 11DN reduces DAT surface expression

GFP-Rab 11DN transfected cells demonstrated significantly decreased surface

staining as compared to GFP-vector alone as shown in figure 4. Figure 4A shows

representative cells co-transfected with HA-DAT and GFP vector (left) or GFP-Rab

11DN (right). Quantification of surface DAT images demonstrates a significant decrease

in surface labeling in GFP-Rab11DN transfected cells compared to GFP vector

transfected cells (GFP $= 38 \pm 3.3\%$, 11DN $= 23 \pm 4.5\%$, Figure 3.4B). Analysis of cells

permeabilized and stained for total DAT revealed lower but statistically insignificant total

DAT levels (optical density measurement) for 11CA and 11DN as compared to GFP; $p =$

0.14, by unpaired t-test for 11CA (303 ± 24, n $= 4$) v. GFP (419 ± 59, n $= 5$) and $p = 0.15$

for 11DN (363 ± 77, n $= 8$) v. GFP (612 ± 160, n $= 6$).

Discussion

This paper describes the novel finding that Rab 11 activity regulates DAT

function and trafficking. By utilizing mutants of Rab GTPase proteins, we demonstrated

that DAT trafficking and function was increased in cells expressing a constitutively active form of Rab 11. For Rab 11CA transfected cells, [3H]WIN35428 binding and [3H]dopamine uptake both increased to a similar degree (127 ± 9.7% of GFP vector for [3H]dopamine uptake and 136.6 ± 15% of GFP vector for [3H]WIN35428 binding). When looking at individual cells to ensure that all cells analyzed contained GFP-Rab 11 DNA, we found even greater effects with GFP-Rab 11CA as compared to GFP vector alone (Figure 3). The difference in magnitude of DAT surface expression as measured by [3H]WIN35428 binding versus immuno-fluorescence experiments is likely because in [3H]WIN35428 binding experiments, the measurement represents a net effect of untransfected cells and Rab 11-transfected cells where the untransfected cells likely dampen the effect of Rab 11CA-transfected cells. Although we did not see an effect of GFP-Rab 11DN in the [3H]dopamine uptake or [3H]WIN35428 binding assays, when measuring individual cell effects, we found decreased DAT surface expression in GFP-Rab 11DN-transfected cells as compared to GFP vector-transfected cells (Figure 3.4). While Rab 8 had no effect on [3H]dopamine uptake, it should be noted that we can't rule out a role of Rab 8 in DAT trafficking as immunofluorescence experiments with Rab 8 were not performed.

These data demonstrate a specific role for Rab 11 to regulate trafficking of DAT to the plasma membrane. Based on co-fractionation studies in DAT-PC12 cells, the predominant location of intracellular DAT at steady state is in the pericentriolar recycling endosome (Melikian & Buckley 1999), which is also the main location of Rab 11 (Zerial & McBride 2001). The most parsimonious explanation of our data, therefore, is that upregulation of Rab11 increased trafficking to the membrane of DAT which originated

from these recycling endosomes. Our data support the finding that intracellular DAT is localized to Rab 11-containing vesicles constitutively and in response to DAT substrates (Sorkina et al. 2003).

Historically, proteins known to be sorted by Rabs have been receptors such as transferrin receptor, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor and epidermal growth factor receptor (Ceresa 2006, Mohrmann & van der Sluijs 1999, Gerges et al. 2004). However, increasing evidence has showed that channels and transporters are also trafficked through Rab dependent pathways (Ishikura *et al.* 2008, McEwen *et al.* 2007). Since endosomal compartments can be somewhat fluid, it can be difficult to distinguish in which compartments a protein resides. Both Rabs 11 and 8 have been localized to the TGN as well as the pericentriolar recycling endosome (Ang et al. 2003, Sonnichsen et al. 2000, Zerial & McBride 2001) however Rab 8 is thought to be the predominant mediator of protein trafficking from the TGN to the plasma membrane (Zerial & McBride 2001). Differentiating the role of Rab 11 versus Rab 8 in affecting DAT function helps determine the specific compartment to which DAT is localized.

Numerous studies have focused on DAT internalization, usually following PKC activation or substrate treatment, while fewer studies demonstrate trafficking proteins necessary for constitutive DAT recycling. The mechanism of neurotransmitter transporter fusion to the plasma membrane has been a strong research area of interest. It is now evident that soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are important regulators of monoamine transporter trafficking (Quick 2006). The SNARE protein syntaxin 1A has been shown to interact with the N-terminus of DAT and regulate its activity (Binda *et al.* 2008, Lee *et al.* 2004). Furthermore, in the

norepinephrine transporter (NET), syntaxin has been demonstrated to regulate clearance capacity of norepinephrine in a calcium and PKC-dependent manner (Sung *et al.* 2003, Sung & Blakely 2007). We found that syntaxin 1A is important for the substrate-stimulated translocation of DAT to the plasma membrane (Furman *et al.* 2009). It is generally believed that SNARE proteins are necessary for the fusion of vesicles to the plasma membrane but that other proteins such as Rabs may be necessary to mediate the delivery and tethering of vesicles to allow for fusion. A potential role for Rab 11 could be to assist in fusion of DAT-containing vesicles to the plasma membrane by delivery or tethering. Since Rab 11CA mimics the rapid trafficking of DAT to the surface upon amphetamine (AMPH) treatment (Furman et al. 2009), it is tempting to speculate that AMPH may act through a Rab-11 dependent pathway to traffic DAT from the recycling endosome to the plasma membrane. A similar mechanism has been demonstrated with another transporter GLUT4 which requires Rab 11 for transport from the endocytic recycling compartment to the GLUT4 storage vesicles which are primed for GLUT4 translocation to the plasma membrane (Ishikura et al. 2008).

However, it remains to be determined whether Rab 11 functions to increase the rate of DAT exocytosis or slow down the rate of endocytosis. Also, further studies are needed to determine whether Rab 11 directly interacts with DAT or whether there is an indirect interaction via a Rab 11 binding protein.

Overall, these data demonstrate that Rab 11 is an integral part of constitutive DAT trafficking.

Figures

Figure 3.1 Rab 11CA increases [³H]DA uptake. A, DAT-N2A cells were transiently transfected with GFP-vector (GFP, n = 17), GFP-Rab 11CA (11CA, n = 9) or GFP-Rab 11DN (11DN, n = 14) and assayed for [³H]DA uptake (specific activity = 10 nM). $p < 0.0001$ by one-way ANOVA. *Post hoc* Bonferroni analysis shows a significant difference between GFP and11CA (**$p < 0.01$) and between 11CA and 11DN (***$p < 0.0001$, stars not shown) B, Cells were transiently transfected with GFP-vector (GFP, n = 10), GFP-Rab 8CA (8CA, n = 5) or GFP-Rab 8DN (8DN, n = 6). No significant difference by one-way ANOVA between any groups was seen ($p = 0.49$). Data are plotted as pmol DA uptake per mg protein as a percentage of GFP vector. Error bars represent SEM.

(A)

(B)
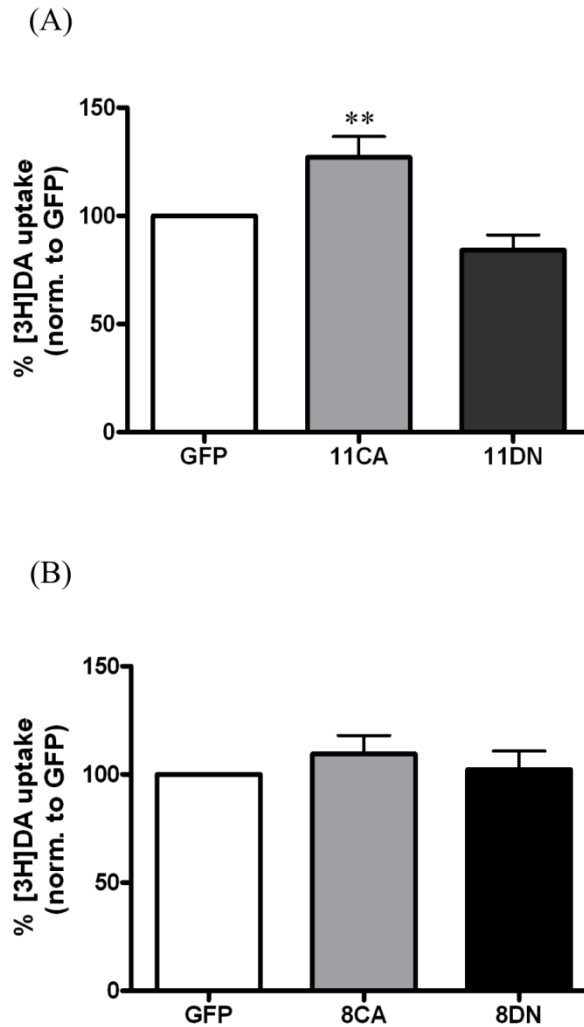
Figure 3.2 Rab 11CA increases [³H]WIN35428 binding. A, DAT-N2A cells were transiently transfected with GFP-vector (GFP, n = 5), GFP-Rab 11CA (11CA, n = 5) or GFP-Rab 11DN (11DN, n = 5) and assayed for [³H]WIN35428 binding (specific activity = 4 nM). $p < 0.05$ by one-way ANOVA. Post hoc Bonferroni analysis shows a significant difference between GFP and 11CA (*$p < 0.05$). Data are plotted as pmol [³H]WIN35428 bound per mg protein as a percentage of GFP vector. Error bars represent SEM.
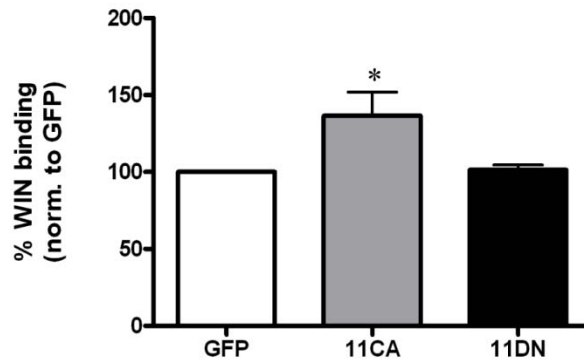
Figure 3.3 Rab 11CA increases DAT surface expression. A, Representative images of N2A cells co-transfected with HA-DAT and GFP-vector (left) or GFP-Rab 11CA (right). Surface labeling of DAT is shown (average of 10 z-slices in the middle of the cell). B, Quantitation of surface labeling of GFP vector (n = 7) or GFP-Rab 11CA (n = 9) using Image J software. Data are plotted as percent DAT surface expression: optical density (OD) of surface (HA) staining divided by the average total DAT staining. Error bars represent SEM. **p<0.01 by unpaired two-tailed t-test.
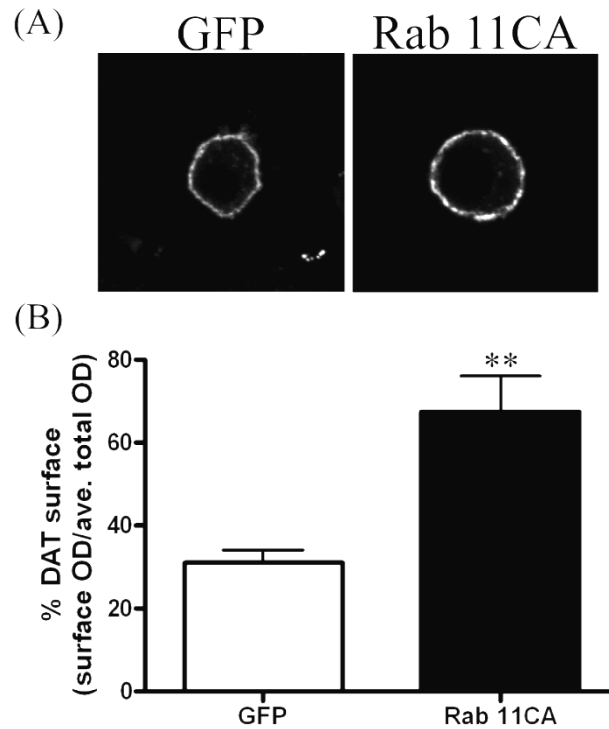
Figure 3.4 Rab 11DN reduces DAT surface expression. A, Representative images of N2A cells co-transfected with HA-DAT and GFP-vector (left) or GFP-Rab 11DN (right). Surface labeling of DAT is shown (average of 10 z-slices in the middle of the cell). B, Quantitation of surface labeling of GFP vector (n = 5) or Rab 11DN (n = 7) using Image J software. Data are plotted as percent DAT surface expression: optical density (OD) of surface (HA) staining divided by the average total DAT staining. Error bars represent SEM. *p<0.05 by unpaired two-tailed t-test.

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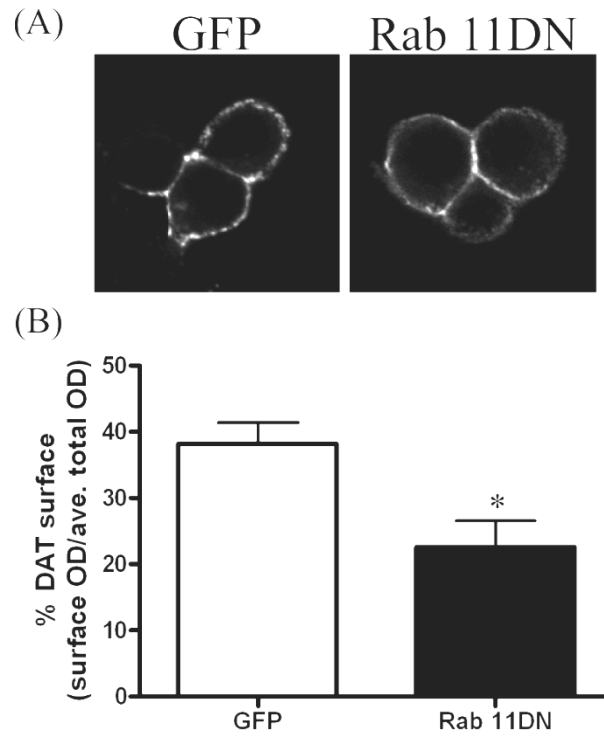
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Chapter IV

Syntaxin 1A regulates basal and stimulated dopamine transporter surface expression and function in rat synaptosomes and DAT-N2A cells

Abstract

The dopamine transporter (DAT) is a critical regulator of dopaminergic neurotransmission. DAT facilitates the reuptake of dopamine (DA) from the extracellular space thereby maintaining proper storage of DA into vesicles and terminating DA signaling. Regulation of DAT function is achieved through trafficking and direct or indirect protein-protein interactions. The psychostimulant amphetamine (AMPH) is a substrate for DAT which stimulates DA efflux. On a time scale commensurate with its effects on DA efflux (60 sec), AMPH increases trafficking of DAT towards the surface in both DAT-N2A cells and rat striatal synaptosomes. The AMPH-stimulated rapid trafficking of DAT is functionally expressed as an increase in [^{3}H]DA uptake and an increase in AMPH-stimulated DA efflux. Syntaxin 1A (SYN1A) is a soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) protein that directly binds to the N-terminus of DAT and regulates its activity. AMPH increases the DAT/SYN1A interaction in mouse synaptosomes and DAT-HEK cells overexpressing SYN1A. The goal of the current study was to determine the role of the DAT/SYN1A interaction on basal and AMPH-stimulated DAT activity and rapid trafficking.

Botulinum neurotoxin C (BoNT/C) was used to assess the role of SYN1A in basal

[³H]DA uptake, DAT surface expression, and AMPH-stimulated DA efflux in

synaptosomes. Basal and AMPH-stimulated DAT/SYN1A interactions were measured

by immuno-precipitation in rat synaptosomes and DAT-N2A cells which endogenously

express SYN1A. In addition, an 'open' form mutant of SYN1A was used to determine to

which form of SYN1A DAT binds.

Our results indicate that cleavage of SYN1A by BoNT/C reduced basal DAT

function, AMPH-stimulated DA efflux and DAT surface expression in synaptosomes to

approximately 50% of control. AMPH increased the DAT/SYN1A interaction in rat

synaptosomes and DAT-N2A cells to approximately 2-fold over control after only 30

seconds of treatment which correlated with the 2-fold increase over control in rapid DAT

trafficking stimulated by AMPH in these systems. In contrast, following 5 min of AMPH

treatment, surface DAT was at control levels and we saw no change in DAT/SYN1A

interaction. In addition, we found that DAT is able to bind to an open form of SYN1A to

the same degree as wild-type SYN1A. Therefore, our preliminary data suggest that

AMPH increases DAT/SYN1A interactions through a trafficking dependent mechanism.

Further studies are needed to determine whether AMPH can affect DAT/SYN interaction

in a non-trafficking environment.

Introduction

The dopamine signaling pathway is involved in the regulation of movement and

cognition as well as motivation and pleasure (Giros & Caron 1993, Iversen 1971). The

A9 and A10 dopamine pathways are thought to be the predominant 'reward' pathways

that are activated during natural rewards such as food and sex as well as 'unnatural'

rewards such as psychostimulant abuse (Koob 2009). The dopamine transporter (DAT) is crucial in terminating dopamine (DA) signaling by removing extracellular DA from the synapse following exocytosis. DAT is a twelve transmembrane spanning protein belonging to the SLC6 class which includes the norepinephrine (NET) and serotonin transporters (SERT) (Amara & Kuhar 1993). In addition to the natural substrate DA, the psychostimulant amphetamine (AMPH) is also a substrate and is taken up into the nerve terminal by DAT. Once taken up, AMPH can stimulate a reversal of the DAT and promote DA efflux into the extracellular space.

DAT as well as other neurotransmitter transporters traffics dynamically in a constitutive manner and in response to stimuli such as substrates (Kahlig & Galli 2003, Melikian 2004). Recently, researchers have focused on elucidating signaling mechanisms involved in transporter trafficking to and from the membrane. The cytosolic N-terminus of DAT is important for translocation to the plasma membrane or retention at the plasma membrane while the C-terminus of DAT is important for constitutive and PKC-mediated endocytosis of DAT (Holton *et al.* 2005, Sorkina *et al.* 2005, Boudanova *et al.* 2008, Sorkina *et al.* 2009). The N-terminus is also the site of many protein-protein interactions that can regulate DAT surface expression and activity (Torres 2006).

The SNARE (soluble N-ethylmaleimide associated protein receptor) protein syntaxin 1A (SYN1A) is known to bind directly to the N-terminus of DAT (Binda *et al.* 2008, Lee *et al.* 2004) and modulate AMPH-stimulated DA efflux through DAT. Additionally, rapid substrate induced exocytosis of DAT is blocked by botulinum neurotoxin C (BoNT/C) which cleaves SYN1A (Furman *et al.* 2009). Several studies investigating the role of SYN1A in NET demonstrate that SYN1A directly binds to the

N-terminus of NET and regulates constitutive NE uptake (Sung *et al.* 2003). Syn1A

modulation of NET function and trafficking is regulated by protein kinase C (PKC)

activity as well as calcium levels (Sung et al. 2003, Sung & Blakely 2007). Syntaxin 1A

interaction with other monoamine transporters including SERT, NET and GAT has also

been shown (Quick 2006). Our lab demonstrated that cleavage of either the t-SNARE

SYN1A by BoNT/C or the v-SNARE protein VAMP-2 by tetanus neurotoxin abolished

rapid substrate-stimulated DAT trafficking to the plasma membrane (Furman et al. 2009).

In this study, we sought to investigate the mechanism of SYN1A on DAT

function and surface expression in basal and AMPH-stimulated conditions. We found

that SYN1A is necessary for maintenance of proper DAT function and trafficking under

basal conditions and that the AMPH-stimulated DAT/SYN1A interaction may be

trafficking dependent.

Methods

[^{3}H]DA uptake following BoNT/C pretreatment

Rat synaptosomes were prepared as described previously (Johnson *et al.* 2005b)

and were incubated for 90 min at 37°C in the presence or absence of 200 nM BoNT/C.

DAT-mediated DA uptake was measured by treating samples with varying concentrations

(0.3 – 3 μM) of [^{3}H]DA (specific activity 23.5 Ci/mmol; Perkin Elmer) for 3 min at

37°C. Nonspecific DA uptake was measured in the presence of 30 μM cocaine.

Following incubation, synaptosomes were filtered onto glass fiber C Whatman filters and

washed three times with excess cold PBS. Filters were dried and counted on a Beckman

scintillation counter.

Biotinylation of rat synaptosomes following BoNT/C pretreatment

Rat synaptosomes were prepared and treated with BoNT/C as described above. Synaptosomes were washed 3 times in 4°C PBS/Ca/Mg and biotinylation experiments were performed as previously described with minor changes (Furman et al. 2009). Briefly, synaptosomes were treated with 1.5 mg/ml EZ link sulfo-NHS-SS-Biotin (Pierce, Rockford IL) for 1 hr at 4°C. The biotinylation reaction was stopped by treatment of 100 mM glycine, followed by 2 additional washes with 100 mM glycine in PBS/Ca/Mg. Synaptosomes were lysed in radioimmuno-precipitation assay buffer composed of: 25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS and centrifuged at high speed to rid of insoluble matter. Lysate was reacted with saturating amounts of streptavidin beads (Pierce, Rockford IL). Streptavidin beads were washed 3 times and protein was eluted in a sample buffer containing fresh dithiothreitol. Biotinylated (surface) and lysate (total) fractions were resolved by western blot analysis using a DAT antibody (MAB16, generously supplied by Dr. Roxanne Vaughan, University of North Dakota). Protein was detected using pico enhanced chemiluminescence reagents (Pierce) and the optical density of bands was quantitated using Scion Image Software.

AMPH stimulated DA release following BoNT/C pretreatment

Female Holtzman rats (~200 - 250g) were sacrificed and rat striatal slices were prepared by hand. Tissue was incubated for 90 min at 37°C in the absence or presence of 200 nM BoNT/C. Samples were loaded onto a perfusion system (Brandel SF-12; Brandel Inc., Gaithersburg, MD) and washed with oxygenated Krebs Ringer Buffer (KRB)

composed of (in mM): 145 NaCl, 24.9 NaHCO$_3$, 2.7 KCl 1.2 KH$_2$PO$_4$, 1.2 CaCl$_2$, 1.0 MgCl$_2$, 10 glucose, 0.05 ascorbic acid, and 0.05 pargyline. Perfusate was collected into vials containing a final concentration of 0.1 N HClO$_4$, 50 μM ethylenediamine tetraacetate, and 10 nM 2-aminophenol as an internal standard. Each fraction was collected for 2 min (0.4 ml/min) and a challenge of 3 μM AMPH at fraction number 5 was applied to measure AMPH-stimulated DA efflux and sample collections were continued for 24 additional minutes. DA was measured by high performance liquid chromatography with electrochemical detection (ESA biosciences).

Cell culture

Stable human DAT N2A cells were a gift from Dr. Karley Little (University). Cells were maintained in OPTI-mem (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Immuno-precipitation of DAT in synaptosomes and N2A cells

Rat striatal synaptosomes (as described above) or DAT-N2A cells were treated with 10 μM AMPH for 30 sec and/or 5 min at 37°C in KRB (synaptosomes) or Krebs-Ringer HEPES (KRH, cells) composed of (in mM): 25 HEPES, 125 NaCl, 4.8 KCl, 1.2KH$_2$PO$_4$, 1.3 CaCl$_2$, 1.2 MgSO$_4$, and 5.6 glucose. The reaction was stopped by addition of ice-cold buffer and samples were washed to deplete AMPH. Samples were then lysed in RIPAE buffer (synaptosomes) or solubilization buffer (DAT-N2A cells) composed of (in mM): 25 Tris, 150 NaCl, 1 ethylene-diamine-tetraacetic acid (EDTA), 5 N-ethylmalemide, phenylmethylsulfonyl fluoride and 1% triton-X 100 containing protease inhibitors (Roche). Equal amounts of lysate were loaded onto protein A sepharose (PAS) beads (Sigma) and rotated for 3 hours to preclear lysate samples.

Precleared lysate samples were then incubated overnight with a DAT antibody (synaptosomes - purified DAT ab; N2A samples – AB5802 (Chemicon)). Samples were then added to PAS beads and rotated for an additional 3 hours to immuno-precipitate DAT. PAS beads containing DAT were washed 3 times in buffer (RIPAE for synaptosomes; solubilization buffer for N2A cells) and eluted in sample buffer composed of (in mM) 250 Tris pH 6.8, 25 EDTA, 10 % sodium dodecyl sulfate, 25 % sucrose, 0.5 % bromophenol blue and supplemented with 100 dithiothreitol. Western blot analysis was performed using a monoclonal anti-syntaxin antibody (Sigma). A monoclonal anti-DAT antibody (MAB369, Chemicon) was used to verify pull-down of DAT. Protein was detected using enhanced chemiluminescence detection (Pierce). Western blot bands were quantified using Scion Image software.

Immuno-precipitation of DAT in DAT-N2A cells transiently transfected with cyan fluorescent protein (CFP)-syntaxin

DAT N2A cells were transiently transfected with wild-type CFP-SYN1A or 'open' CFP-SYN1A using the calcium-phosphate method. Forty-eight hours after transfection cells were washed twice in PBS and removed from the plate. Cells were lysed in solubilization buffer containing fresh protease inhibitor. Immuno-precipitation experiments were performed as described above.

Confocal microscopy

Cells transfected with wild-type CFP-SYN1A or open CFP-SYN1A were plated onto glass bottom dishes (mat-tek) and imaged on an Olympus FluoView 500 confocal microscope using a 405 nm laser.

Statistics

Graph Pad Prism 5 was used for statistical analysis. Values for the kinetic constants, Km and Vmax, for the hDAT and mutant DAT constructs were determined by nonlinear regression analysis of the mean values for each mutant using GraphPad Prism version 5. Statistical significance was determined using GraphPad Prism version 5 using an F-test by comparing fits in which selected values were constrained to be equal or were allowed to differ. The null hypothesis was that the best fit parameter for the value did not differ. A conclusion of statistical significance represents a rejection of the null hypothesis and indicates a difference between designated values.

Results

BoNT/C reduces [^{3}H]DA uptake and DAT surface expression in rat synaptosomes

To determine whether SYN1A regulates basal DAT function and surface expression, we treated synaptosomes with BoNT/C to cleave SYN1A. As shown in Figure 1, treatment with BoNT/C significantly reduced [^{3}H]DA uptake in rat striatal synaptosomes as compared to KRB treated synaptosomes (Fig 1A; unpublished data – Myung Kim). The maximal DA uptake velocity (V_{max}) was significantly lower (p < 0.001 by unpaired t-test) in BoNT/C treated synaptosomes (7.89 ± 0.31 pmol DA/mg protein/min) v. control-KRB (15.5 ± 0.55 pmol DA/mg protein/min). In addition, K_m values of BoNT/C treated cells were also significantly different from control (p < 0.01 by unpaired t-test for BoNT/C treated - 0.137 ± 0.03 µM v. control – 0.408 ± 0.08 µM). To determine whether the change in [^{3}H]DA uptake was due to surface expression changes, biotinylation experiments were performed. Biotinylation experiments revealed a reduction in DAT surface expression in synaptosomes pretreated with BoNT/C to 47% of control (Fig 4.1B; unpublished data – Myung Kim). Alternative statistical analysis using

a one-sample t-test revealed a significant decrease in BoNT/C treated cells ($p < 0.01$ as compared to a hypothetical value of 100).

BoNT/C reduces AMPH-stimulated DA efflux in rat striatal slices

Overexpression of SYN1A in DAT N2A cells resulted in increased AMPH-stimulated DA efflux (Binda et al. 2008). To determine the effect of endogenous SYN1A on AMPH-stimulated DA efflux, we used BoNT/C treated synaptosomes. AMPH-stimulated DA efflux was reduced in BoNT/C treated synaptosomes as compared to KRB-treated synaptosomes (Fig 4.2; unpublished data – Myung Kim).

AMPH stimulates DAT/SYN interactions

It has previously been demonstrated that AMPH stimulates the DAT/SYN1A interaction in mouse synaptosomes and DAT-HEK cells transiently over-expressing SYN1A after 5 minutes of treatment (Binda et al. 2008). To determine whether AMPH influences DAT and SYN1A interaction with times commensurate with rapid DAT trafficking, rat synaptosomes were treated in the absence or presence of 10 μM AMPH for 30 seconds. As shown in Figure 3A, AMPH significantly increased the DAT/SYN1A interaction in rat synaptosomes after 30 seconds of treatment (Figure 4.3A; unpublished data – Myung Kim). Alternative statistical analysis using a one-sample t-test revealed a significant increase in AMPH-pretreated synaptosomes ($p < 0.01$ as compared to a hypothetical value of 1). Neuroblastoma (N2A) cells endogenously express SYN1A as well as other SNARE proteins such as SNAP25 (data not shown). We previously found that substrate-induced DAT trafficking was SYN1A-dependent (Furman et al. 2009, Johnson *et al.* 2005a) in GFP-DAT N2A cells, therefore we wished to test whether DAT and SYN1A interact endogenously in this cell system. We found a baseline interaction of

DAT and SYN1A. As shown in figure 4.3B, AMPH stimulates DAT/SYN1A

interactions at 30 seconds of treatment; however, no change is seen after 5 minutes of

treatment (Figure 4.3B). Alternative statistical analysis using a one-sample t-test

revealed a significant increase in 30 second AMPH-pretreated cells ($p < 0.05$ as

compared to a hypothetical value of 1).

DAT interacts with an 'open' conformation of syntaxin

　　　While it is known that SYN1A binds to the N-terminus of DAT, the conformation

of SYN1A that binds to DAT is not known. In order for SYN1A to interact with other

SNARE proteins, it must be in an 'open' conformation in which the SNARE motif of

SYN1A is accessible (Dulubova *et al.* 1999). A double mutant of SYN1A (L165A,

E166A) located in the linker region creates an 'open' form of SYN1A which does not

bind to munc18 in *vitro*, and weakly binds munc18 *in vivo* (Dulubova et al. 1999, Liu *et*

al. 2004). We utilized this mutant to determine whether DAT and an open form of

SYN1A are able to interact. The open and wild-type forms of syntaxin utilized in this

experiment were tagged with a fluorescent tag (cyan fluorescent protein, CFP) in order to

detect the protein and determine its approximate location in the cell. As shown in figure

4.4A, both the wild-type CFP syntaxin (left) and the open mutant CFP syntaxin (right)

are expressed well in the DAT-N2A cells following transient transfection. While there is

a significant portion of syntaxin in the cytosol, there is also a considerable portion in the

plasma membrane as depicted by the thin blue line encircling the cell. The fact that we

can detect SYN1A at the plasma membrane suggests that proper trafficking is occurring.

Munc18 aids in the proper trafficking and localization of syntaxin (Medine *et al.* 2007,

Rowe *et al.* 2001) therefore the open mutant of syntaxin which does not bind munc18

well has some impairment of trafficking. This was noted in our immuno-precipitation studies where we found ~50 % less 'open' mutant CFP-SYN1A in the plasma membrane as compared to wild-type CFP-SYN1A (mean membrane total OD of 'open' syntaxin: 10.76 arbitrary units v. wild-type syntaxin: 20.57 arbitrary units; $p < 0.001$ by paired t-test). We are able to differentiate the exogenously added SYN1A from the endogenous SYN1A due to the CFP tag on the exogenous syntaxin which adds ~26 kDa to the molecular weight. Our immuno-precipitation studies of DAT and SYN1A show that both the wild-type and open form of CFP-SYN1A co-immuno-precipitate with DAT (Figure 4B). There was no significant difference ($p = 0.4$) between the mean OD of syn/mem syn/rel DAT value in the wild-type CFP-SYN 1A (0.576 ± 0.07 arbitrary units) v. 'open' mutant CFP-SYN1A (0.792 ± 0.23 arbitrary units).

Discussion

The aim of this study was to determine the role of SYN1A in basal DAT trafficking and function and determine whether the AMPH-stimulated increase in DAT/SYN interaction was trafficking dependent. Here we show that SYN1A is necessary for proper constitutive DAT trafficking and function since cleavage of SYN1A in rat synaptosomes significantly reduced [^{3}H]DA uptake, AMPH-stimulated DA efflux and DAT surface expression. We find that AMPH stimulates DAT/SYN interactions at times commensurate with its effect to induce rapid trafficking of DAT to the plasma membrane. Our data correlate with Sung et al., (2003, Sung & Blakely 2007) who found decreased norepinephrine transport through NET in rat cortical synaptosomes as well as other systems in the presence of BoNT/C. We found that AMPH-stimulated DA efflux was also reduced in the presence of BoNT/C suggesting that SYN1A regulates AMPH-

mediated effects. A decrease in AMPH-stimulated DA efflux could simply be due to the decrease in DAT surface expression which would limit the availability of DAT for AMPH to bind. However, since SYN1A targets the N-terminus of DAT which is known to regulate AMPH-stimulated DA efflux, it is also possible that SYN1A modulates AMPH-stimulated DA efflux through binding to the N-terminus. We found that AMPH increases the basal DAT/SYN1A interaction in rat synaptosomes and DAT-N2A cells to approximately ~2-fold after 30 seconds of treatment. The increase in DAT/SYN1A interaction following AMPH treatment correlated in time with the AMPH stimulated DAT trafficking to the plasma membrane observed in rat synaptosomes and DAT N2A cells (Furman et al. 2009, Johnson et al. 2005a). Therefore, it is possible that AMPH stimulates the increase in the DAT/SYN1A interaction by inducing DAT trafficking to the plasma membrane where DAT and SYN1A interact. In DAT-N2A cells, 5 minute treatment of 10 μM AMPH had no effect on DAT/SYN1A interactions. In contrast, Binda et al., (2008) found that 10 μM AMPH for 5 minutes could increase DAT/SYN1A interactions in mouse synaptosomes and DAT-HEK cells overexpressing SYN1A. These discrepancies may be due to variations in systems since N2A cells endogenously express SYN1A whereas HEK cells do not. In addition, N2A cells and HEK cells may have differences in DAT trafficking properties which may also contribute to differences in SYN1A regulation. In this study, AMPH-stimulated DAT/SYN1A interactions were not measured at 5 minutes in rat synaptosomes therefore we can't make any conclusions for this time point.

It is also of interest to know the biochemical characteristics of the DAT/SYN1A interaction. At this stage, it is known that SYN1A binds to the N-terminus of DAT but

the form of SYN1A is not known. Furthermore, it is not known whether other SNARE

proteins such as SNAP25 or regulators of SYN1A such as munc18 may also co-immuno-

precipitate with DAT. Use of an open mutant form of SYN1A has aided in the

understanding of conformational changes of SYN1A upon binding to SNARE proteins

and other regulatory proteins (Dulubova et al. 1999). We used the 'open' SYN1A mutant

in order to determine whether DAT can bind to an open form of SYN1A. Immuno-

precipitation studies demonstrate that the 'open' mutant of SYN1A co-immuno-

precipitates with DAT to the same degree as wild-type SYN1A. These results are

somewhat perplexing if we assume that wild-type SYN1A adopts an open conformation

50% of the time and a closed conformation 50% of the time. However, if wild-type

SYN1A adopts an open conformation the majority of the time, for example 80% or more,

then wild-type and open mutant SYN1A may bind to DAT to a similar degree. It is

possible that overexpression of wild-type SYN1A may result in more open forms of

SYN1A since proteins that keep it in a closed conformation such as munc18 are not

overexpressed. More experiments such as *in vitro* binding assays are needed to

determine the degree of open SYN1A binding to DAT.

Overall, we find that SYN1A is a necessary component of basal and stimulated

DAT trafficking and function and future studies should address the role of DAT

trafficking in this regulation

Figures

Figure 4.1 BoNT/C effect on basal DAT function and DAT surface expression. **A,** [³H]DA uptake: Rat striatal synaptosomes were pretreated with KRB (control) or 200 nM BoNT/C for 90 min at 37°C. Synaptosomes were then incubated with various concentrations of [³H]DA for 3 min to initiate uptake. Data are expressed as pmol DA per milligram protein per minute. Error bars show standard error. In analysis of the kinetic data by Graph Pad 5, the null hypothesis, that K_m and V_{max} did not differ between the groups, was rejected ($p < 0.0001$, F= 33.25 (2,31). **B,** Biotinylation of synaptosomes. Synaptosomes were pretreated as in (A), then biotinylated (see methods). Western blots of biotinylated *v.* total samples were scanned and quantified using Scion Image software. Data are plotted as optical density (OD) of biotin (surface) fraction divided by the OD of lysate (total) fraction normalized to KRB (control). Error bars represent standard error. **p<0.01 by paired two-tailed t-test (n=4).
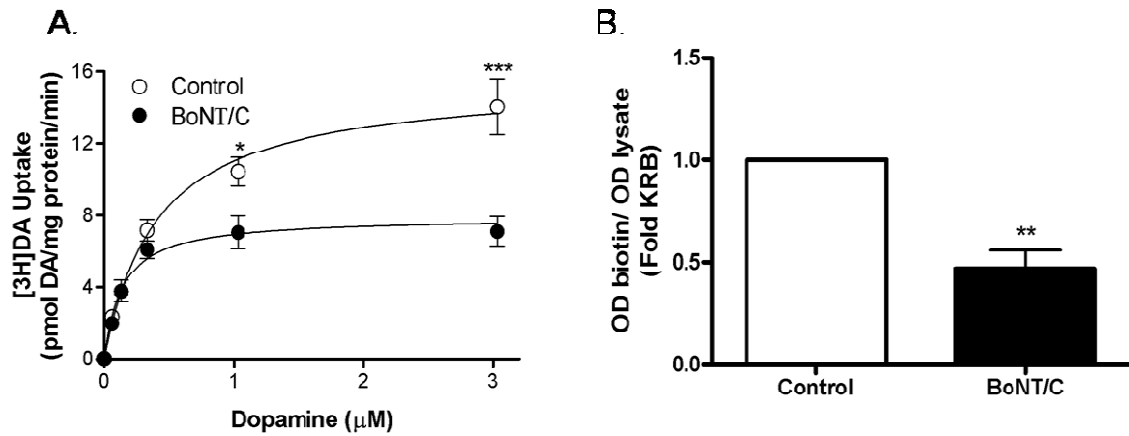
Figure 4.2 AMPH-stimulated DA efflux in BoNT/C-treated synaptosomes. Striatal slices were incubated with KRB (control) or 200 nM BoNT/C for 90 min. Slices were loaded onto the Brandel and perfused with KRB. Fractions were collected every 2 min (0.4 ml/min). At fraction 5 (arrow), 3 µM AMPH was introduced. Comparison of the two treatments by 2-way ANOVA showed an overall treatment effect ($p < 0.001$, $n = 3$). *$p<0.05$ by posthoc Bonferroni analysis at fraction 7 between control and BoNT/C treatments.
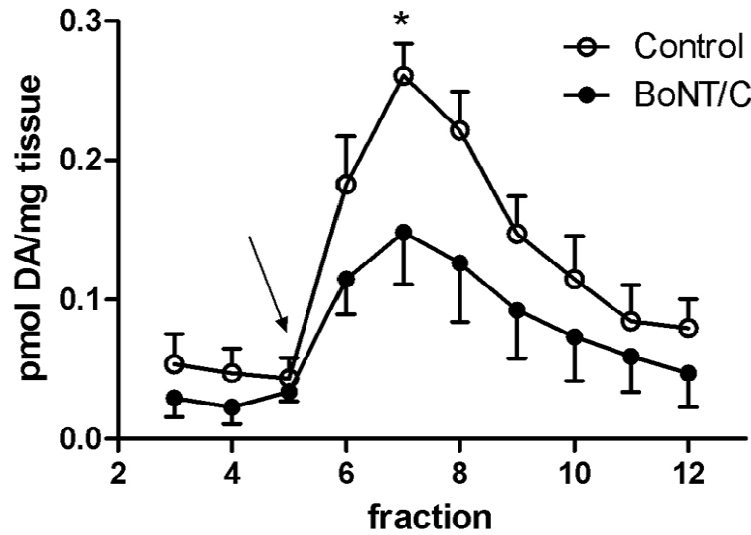
Figure 4.3 AMPH-stimulates DAT/SYN interactions at 30 sec. **A,** rat synaptosomes were treated with KRB or 10 μM AMPH for 30 sec at 37°C and immuno-precipitated for DAT. Western blots of SYN1A and DAT were performed. Data are plotted as average OD of SYN1A that co-immuno-precipitates with DAT divided by OD of immune-precipitated DAT normalized to KRB treatment. Error bars represent SEM (n = 4). AMPH significantly increased the OD SYN1A/OD DAT ratio as compared to KRB (**p<0.01 by paired t-test). **B,** DAT-N2A cells were treated with KRH (white bars) or 10 μM AMPH (black bars) for 30 sec or 5 min at 37°C and immuno-precipitated for DAT. Western blots of SYN1A and DAT were performed. Data are plotted as average OD of SYN1A divided by OD of DAT normalized to KRH (V30, V5). Error bars represent SEM (n = 4). AMPH significantly increased the OD SYN1A/OD DAT ratio as compared to KRB at 30 seconds of treatment (p<0.001 by one-way ANOVA, posthoc Bonferroni analysis: **p<0.01 for A30/V30 v. V30/V30).
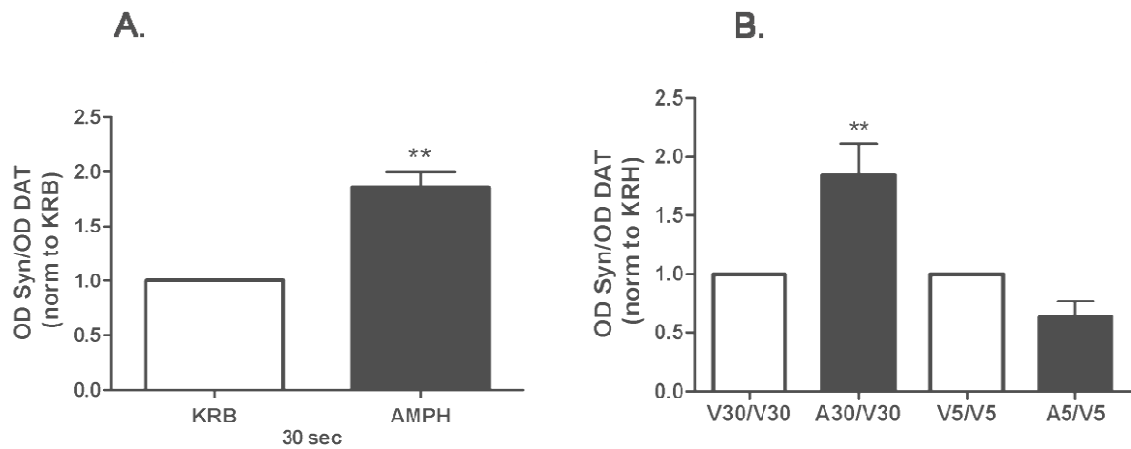

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Figure 4.4 DAT binds to open-SYN1A mutant. **A,** DAT-N2A cells were transiently transfected with CFP-WT SYN1A (left) or CFP-open SYN1A (right) and imaged on an Olympus fluoview 500 microscope 48 hours later. **B,** Membrane preparations of DAT-N2A cells transiently transfected with CFP- WT SYN1A (open circles) or CFP-open SYN1A (closed circles) were immuno-precipitated with DAT. Western blots for SYN1A and DAT were performed. Data are plotted as the average optical density (OD) of SYN1A that co-immuno-precipitates with DAT divided by membrane total of CFP-SYN1A divided by the relative amount of DAT immuno-precipitated.
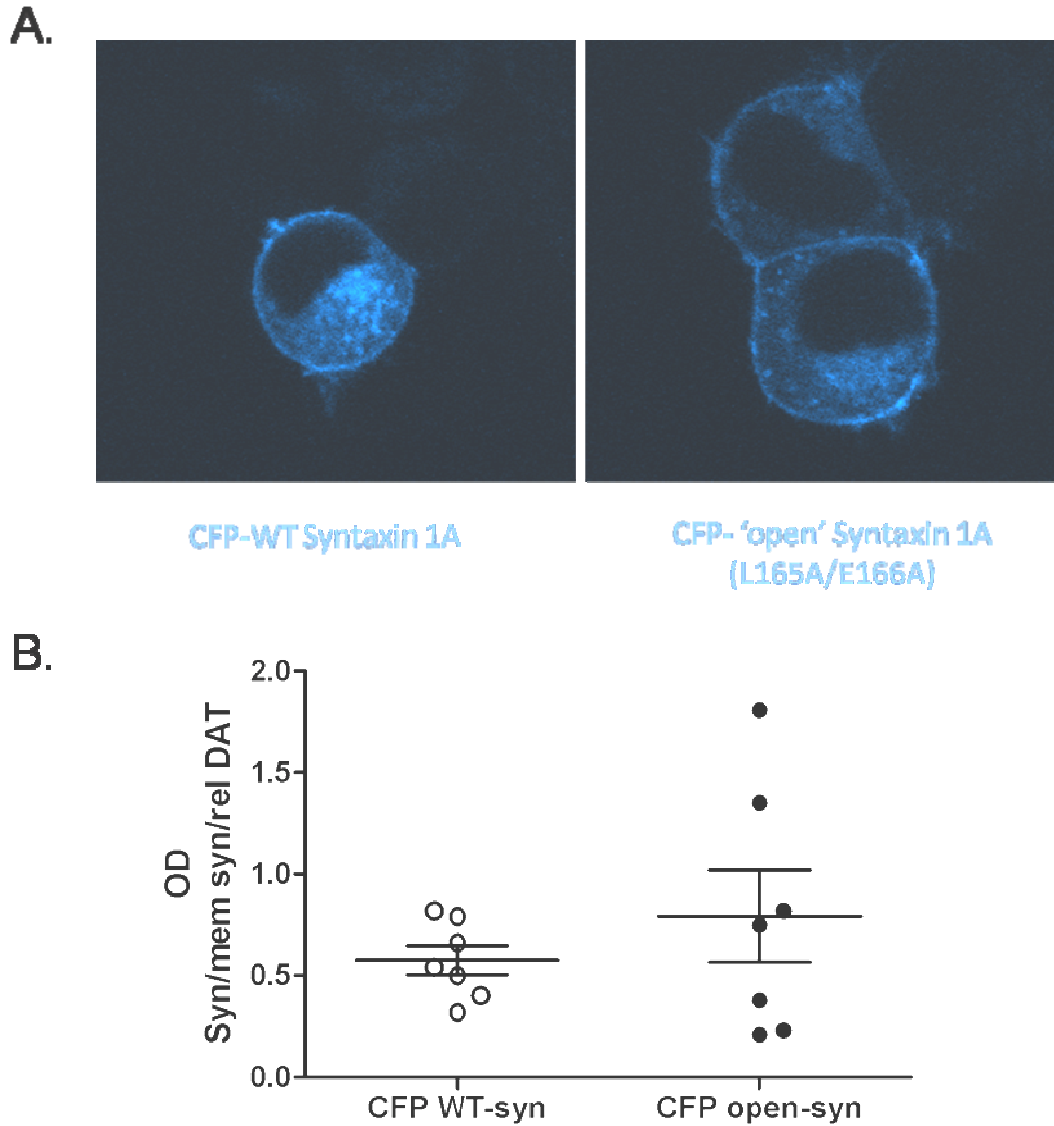
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Chapter V

Thesis Conclusions

Dopaminergic neurotransmission regulates normal physiological functions as well as disease states including psychosis, Parkinson's Disease, ADHD and drug addiction. In order to develop and improve treatments for these disease states, it is necessary to understand the basic neuropharmacological properties of dopaminergic neurotransmission in general and potential drug targets such as DAT in particular. The purpose of this thesis was to further characterize the regulation of DAT activity by constitutive and stimulated DAT trafficking. Previous research in the field of DAT trafficking has provided some insight into the regulation of DAT trafficking.

Based on previous research discussed in Chapter I, the following DAT trafficking model could be proposed whereby (1) DAT constitutively recycles from the plasma membrane into early and recycling endosomes; (2) In response to various stimuli including prolonged substrate treatment, and prolonged PKC activation, DAT endocytosis is predominant and a reduction in DAT surface expression occurs; (3) recent studies demonstrate that rapid treatment with various stimuli increases DAT trafficking towards the membrane leading to increased surface expression.

Based on research presented in this thesis (Chapters II, III and IV), an extended DAT trafficking model is proposed whereby (1) constitutive DAT recycling from the plasma membrane into early and recycling endosomes is regulated by Rab 11 and

syntaxin 1A (Chapters III and IV); (2) Rapid DAT trafficking to the plasma membrane occurs in response to both AMPH as well as the physiological substrate DA in a PKC-β dependent, D2 receptor independent manner (Chapter II); (3) As in the previous model, DAT endocytosis occurs in response to prolonged substrate treatment.

Constitutive DAT trafficking

Studies in DAT-PC12 and CFP-DAT PAE cells demonstrated that DAT constitutively recycles into early and recycling endosomes in a clathrin-dependent manner at an approximate rate of 3-5 %/min (Loder & Melikian 2003, Melikian & Buckley 1999, Sorkina *et al.* 2003, Sorkina *et al.* 2005). Recent studies in live ventral midbrain dopaminergic neurons demonstrate constitutive recycling in a physiological system (Eriksen *et al.* 2009).

Rab 11 was used as a marker of the recycling endosome in studies with PAE cells and DAT co-localized with Rab 11 under basal conditions (Sorkina et al. 2003). In DAT-N2A cells, we found that a constitutively active mutant of Rab 11 increases basal surface expression of DAT with a concomitant increase in [^{3}H]DA uptake (Chapter III). Furthermore, a dominant negative mutant of Rab 11 decreased DAT surface expression. These data suggest a role for Rab 11 in facilitating DAT trafficking to the plasma membrane. Rab 11 also regulates trafficking of the well-known recycling protein transferrin (Ren *et al.* 1998, Ullrich *et al.* 1996) which co-fractionates and co-localizes with DAT (Melikian & Buckley 1999, Sorkina et al. 2005). Based on substantial evidence from the Melikian and Sorkin groups, the carboxy terminus of DAT contains endocytic signals and is self-sufficient to undergo endocytosis whereas the amino terminus is not required for endocytosis and may contain membrane retention signals

(Holton *et al.* 2005, Sorkina et al. 2005, Sorkina *et al.* 2009, Boudanova *et al.* 2008b). Based on these data, it is possible that Rab 11 may act through the amino terminal to increase membrane retention or through the carboxy terminal to inhibit endocytosis. Alternatively, Rab 11 may act as a tether of DAT-containing vesicles to the plasma membrane and increase the likelihood of DAT fusion. Future studies with Rab 11 and DAT should address whether there is a direct or indirect interaction between these two proteins and whether Rab 11 increases DAT surface expression by increasing exocytosis or decreasing endocytosis.

SYN1A is a SNARE protein that regulates transport activity of several transporters including NET, GAT and DAT (Quick 2006). The mechanism of SYN1A regulation of neurotransmitter transporters is complex since SYN1A affects intrinsic activity and fusion of transporters such as NET (Sung *et al.* 2003, Sung & Blakely 2007). We found that under basal conditions, SYN1A cleavage decreases DAT surface expression, [^{3}H]DA uptake and AMPH-stimulated DA efflux in rat synaptosomes. Based on these data, SYN1A is required for basal DAT trafficking to the plasma membrane or retention at the plasma membrane. DAT and SYN1A primarily interact at the plasma membrane (Binda *et al.* 2008), so retaining DAT on the plasma membrane is a plausible mechanism for SYN1A regulation of DAT. However, since SYN1A is a critical SNARE protein it is equally as likely that DAT can't fuse to the plasma membrane in the absence of SYN1A. Data from chapter II (discussed below) suggest that SYN1A is required for DAT fusion to the plasma membrane. Future studies should address whether SYN1A modulates DAT activity through fusion regulation, a direct interaction, or both.

Thus far, it is clear that DAT endocytoses into early and recycling endosomes; however it is not known whether there are intermediate compartments to which DAT recycles prior to plasma membrane fusion. A 'readily releasable pool' (RRP) has been hypothesized for trafficking proteins in neurosecretory cells (Schmidt *et al.* 1997). The 'readily releasable pool' would likely be close to the plasma membrane. Based on images of DAT in TIRFM (discussed in Chapter II), it appears that there are many DAT molecules that are not at the plasma membrane but lie directly underneath the membrane. The RRP must also contain important proteins necessary for DAT fusion to the plasma membrane such as the SNARE protein VAMP2 (discussed in the next section) as well as Rab 11 assuming its function is to tether DAT-containing vesicles to the plasma membrane.

My proposed model for constitutive DAT trafficking (Figure 5.1) based on current literature as well as data presented in chapters III and IV is the following: DAT undergoes constitutive recycling to the plasma membrane via the endocytic recycling compartment/recycling endosome (ERC) and a yet unidentified readily releasable pool (RRP). Rab 11 mediates tethering of the RRP to the plasma membrane thus increasing the likelihood of DAT fusion. Once DAT is in the RRP, SYN1A facilitates DAT fusion by interacting with other essential SNARE proteins including VAMP-2 and SNAP25 (not depicted).
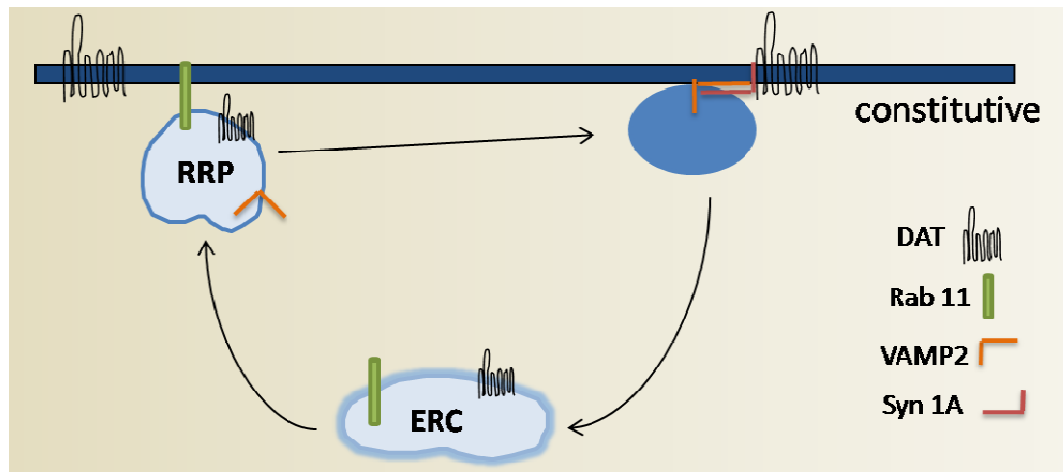
Figure 5.1 Proposed model for constitutive DAT trafficking. RRP: readily releasable pool; ERC: endocytic recycling compartment. Image source: CAF

Regulated DAT trafficking

A number of studies have demonstrated that DAT can rapidly traffic to the plasma membrane in response to various stimuli including AMPH, insulin, and the D2 receptor agonist, quinpirole (Bolan *et al.* 2007, Carvelli *et al.* 2002, Johnson *et al.* 2005). All of these stimuli increased DAT surface expression within 30 seconds to 5 minutes of treatment. Out of the three stimuli listed above, AMPH is the only one to show a biphasic action on DAT trafficking whereby short treatment (30 sec – 2 min) increases DAT surface expression and prolonged treatment (10 – 60 min) decreases DAT surface expression. This biphasic effect suggests that AMPH modulates DAT trafficking through two different pathways or the same pathway with differential time-dependent signaling. Chapter II provides evidence for AMPH-stimulated DAT trafficking through a PKC-β signaling pathway. We also found that the effect of AMPH to rapidly traffic DAT to the plasma membrane is not unique to AMPH but may also occur with rapid treatment of the physiological substrate of DA. Perhaps this finding is not too surprising since DA, like

AMPH, stimulates DAT endocytosis following prolonged treatment (Chi & Reith 2003, Gulley *et al.* 2002, Little *et al.* 2002, Saunders *et al.* 2000).

The SNARE protein SYN1A regulates basal DAT trafficking (described above) as well as stimulated DAT trafficking. We found that cleavage of either SYN1A or VAMP2 blocked substrate-stimulated DAT trafficking to the plasma membrane (Chapter II). Additionally, in Chapter IV, we found that AMPH stimulates the DAT/SYN1A interaction (previously described: (Binda et al. 2008, Lee *et al.* 2004) in DAT-N2A cells and rat synaptosomes at times commensurate with AMPH-stimulated DAT trafficking to the plasma membrane. Taken together these data suggest a synergistic effect whereby AMPH-stimulated trafficking of DAT requires SYN1A and SYN1A interaction with DAT is stimulated by AMPH. It remains to be determined whether SYN1A regulates DAT trafficking simply by regulating DAT fusion or whether the DAT/SYN1A interaction is necessary for AMPH-stimulated DAT trafficking to the plasma membrane. It is possible that AMPH could stimulate DAT/SYN1A interaction simply by increasing the number of DAT molecules that are at the plasma membrane thereby increasing the likelihood of DAT and SYN1A interaction. Alternatively, SYN1A could interact with DAT immediately prior to DAT fusion which would make the DAT/SYN1A interaction essential for AMPH-stimulated DAT trafficking. Elegant future studies should address these questions and determine whether AMPH-stimulated DAT trafficking can occur when SYN and DAT are unable to directly interact.

Figure 5.2 depicts a proposed model for rapid-substrate stimulated DAT trafficking based on data from chapters II, III and IV. This model assumes that proteins that regulate constitutive DAT recycling (ie., Rab 11) also regulate stimulated DAT

trafficking. Clearly, more studies are needed to determine whether these pathways

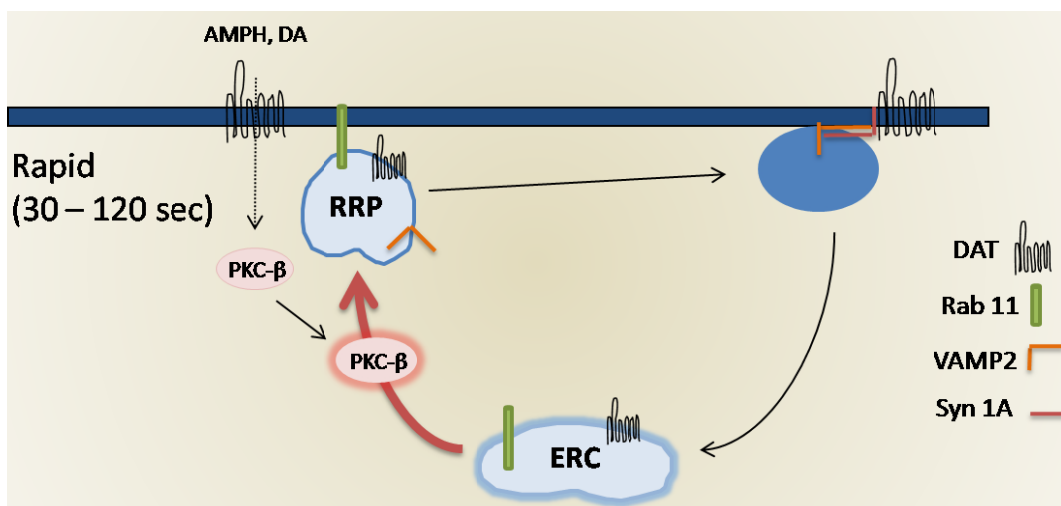actually converge and whether Rab 11 influences stimulated DAT trafficking.

Figure 5.2 Proposed model for rapid stimulated DAT trafficking. RRP: readily releasable pool; ERC: endocytic recycling compartment. Image source: CAF

Converging signaling pathways

A key issue in elucidating the mechanism of rapid stimulated DAT trafficking is

determining what signaling pathways converge given various stimuli. For example,

insulin stimulates DAT trafficking to the plasma membrane through a PI3K-dependent

pathway (Carvelli et al. 2002). This pathway converges with prolonged AMPH-

stimulated DAT trafficking since insulin can reverse the AMPH-stimulated endocytosis

of DAT and AMPH treatment causes phosphorylation of Akt, a kinase downstream of

PI3K (Garcia *et al.* 2005). AMPH and DA rapidly increase DAT surface expression

through a PKC-β dependent manner suggesting that these substrates must activate PKC-β

to cause DAT translocation. In fact, AMPH has been shown to increase PKC particulate

activity (Giambalvo 2003). The role of PKC β in DAT trafficking and function has been

further characterized in PKC- β knock-out mice. Mice lacking PKC- β exhibited

decreased basal DAT surface expression and [^{3}H]DA uptake as compared to wild-type

(WT) litter mates (Chen *et al.* 2009). Interestingly, AMPH-stimulated DAT trafficking showed opposite trends in PKC-β knock-out mice compared to WT. Rapid treatment of AMPH decreased DAT surface expression and prolonged AMPH treatment increased DAT surface expression in KO mice. These data support findings from Furman et al., 2009 and overall suggest a role of PKC-β in AMPH regulation of transporter trafficking. Based on these data, it may be hypothesized that AMPH and PKC regulate DAT trafficking through a common mechanism or pathway. This hypothesis is challenged by recent data that suggests that AMPH and PKC-mediated internalization operate through different pathways (Boudanova *et al.* 2008a). However, these data specifically look at prolonged AMPH and PKC-induced internalization and it is extremely possible that signaling pathways that regulate DAT trafficking to the plasma membrane are completely independent of pathways that regulate DAT endocytosis. Thus, PKC and AMPH may operate within the same pathway during DAT exocytosis but different pathways during DAT endocytosis. The D2 receptor agonist quinpirole increases DAT surface expression within one minute of treatment in DAT-N2A cells that overexpress D2 receptor (Bolan et al. 2007). Additionally, Lee et al., (2007) found a direct interaction between the D2 receptor and DAT. We found that rapid DA-stimulated DAT trafficking is not dependent on activation of D2 receptors, at least in DAT-N2A cells (Chapter II). However, it is highly likely that DA or AMPH treatment has some effect on D2 in systems that contain both DAT and D2R since DA can directly activate D2Rs and AMPH-stimulated DA efflux will lead to D2 receptor activation. Thus, it is entirely possible that DA stimulates a D2R-independent and D2R-dependent pathway.

Significance and Future Directions

In summary, this thesis demonstrates a role of Rabs and SNAREs in constitutive DAT trafficking to the plasma membrane as well as a PKC-dependent role of substrates in rapid DAT trafficking to the plasma membrane. Since much of the current and previous research has focused on the long-term substrate-induced DAT endocytosis, this research provides exciting new information about the process of DAT exocytosis.

Exogenous administration of the natural substrate DA rapidly increases DAT surface expression within one to two minutes of application. This finding suggests a homeostatic mechanism to regulate extracellular DA levels. An increase in DAT surface expression following exogenous application of dopamine would allow for increased reuptake of extracellular DA back into the nerve terminal which would help to restore the physiological levels of dopaminergic neurotransmission. The substrate AMPH may trigger the same homeostatic response since AMPH is structurally homologous to DA. Alternatively, in a physiological system, AMPH-stimulated DA efflux would lead to increased extracellular DA and DA may directly trigger the increase in DAT surface expression. The sensor for this DAT up-regulation is likely the transport of substrates through DAT since transport is required for the increase in surface expression. The mechanism of PKC-β regulation of substrate-stimulated DAT trafficking is an area of current investigation in the Gnegy lab. It is possible that PKC-beta affects DAT trafficking through indirect phosphorylation of a PKC substrate such as RACK1 (receptor of activated protein kinase C) which binds to the N-terminus of DAT.

The finding that Rab 11 function regulates DAT surface expression and function under basal conditions suggests that DAT trafficking to the plasma membrane is tightly regulated. Based on these data, we may start to consider DAT and other monoamine

transporters to undergo classic recycling similar to receptors such as the transferrin receptor. Further studies should address whether Rab proteins are involved in substrate stimulated DAT trafficking to the plasma membrane. Increased Rab 11 function leads to increased DAT surface expression and subsequently more re-uptake of DA indicating a negative regulation of Rab 11 on dopaminergic neurotransmission.

Our studies with SNARE proteins syntaxin 1A and VAMP-2 demonstrate a necessary role for these proteins in DAT trafficking and function. These findings extend the role of SNARE proteins from regulators of classic secretory vesicle fusion to regulators of transporter fusion to the plasma membrane. An important question for future research studies is identifying the source of DAT that trafficks to the plasma membrane constitutively and in response to substrates. It is likely that the source of DAT is small synaptic-like vesicles which contain Rab 11 and VAMP-2 to regulate tethering and fusion respectively.

Taken together DAT trafficking and function are rapidly and specifically regulated by intracellular stimuli including protein kinase activation and GTPase protein regulation as well as extracellular stimuli including the physiological substrate DA and the psychostimulant amphetamine. These data confirm the importance of DAT in maintaining proper dopaminergic neurotransmission and demonstrate that homeostatic mechanisms are in place to rapidly modulate DAT function in response to exogenous stimuli.

Overall conclusions

Over the last 10 years, there has been a boom of research on DAT trafficking. Much knowledge has been gained regarding constitutive and regulated DAT trafficking

in a variety of heterologous cell and primary culture systems. With the advent of advanced imaging techniques along with standard research tools, we are able to visualize and monitor living cells in action. The overall goal of research on constitutive DAT trafficking is to understand precisely how physiological dopaminergic neurotransmission works so that in circumstances in which there is injury, deficits or abnormal regulation, a treatment may be developed. Similarly, with regulated DAT trafficking, given that many current licit and illicit drugs target DAT as well as other monoamine transporters, it is absolutely essential that we understand the mechanism of these drugs so that new treatments and further development of current treatments can occur.

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