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The Role of Calmodulin in PKC-Regulated DAT Surface Expression

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The Role of Calmodulin in PKC-Regulated DAT Surface Expression

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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January 17, 2014

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Abstract

The dopamine (DA) transporter (DAT) is a sodium symporter (NSS) transporter protein that removes dopamine from the synaptic cleft to the pre-synaptic nerve terminal. This DAT function regulates dopaminergic signaling and controls brain functions such as motor control, behavior, and learning. DAT is regulated by signal and protein cascade pathways, and thus is trafficked to and from the nerve terminal cell surface to modulate DA uptake. This project investigates the role of calmodulin (CaM) in the signaling pathway affecting DAT trafficking, and specifically focuses on whether CaM is required for PKC-mediated DAT surface expression. DA transport assays conducted in the presence of a CaM inhibitor, CGS 9343B, revealed a trend toward CaM's ability to increase DAT function, although no conclusive results were established due to time constraints. Preliminary results suggest that CaM is able to increase DAT function, but whether it affects DAT surface levels is the foundation for further investigations.

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Background

Dopamine

Dopamine (3,4-dihydroxyphenethylamine) (DA) is a catecholamine neurotransmitter, named after the similarly structured molecular precursor L-DOPA, distinguished by the removal of a carboxyl group from the nitrogen bound carbon. Dopamine is derived from the amino acid tyrosine and is a precursor to the monoamines norepinephrine and epinephrine (GHR.NLM.NIH.gov, 2014). Two scientists considered the pioneers of DA research, Arvid Carlsson and Paul Greengard, were awarded the Nobel Prize in Physiology and Medicine in 2000 for their basic research on DA. Arvid Carlsson discovered DA as a neurotransmitter in the brain approximately 60 years ago using the brains of reserpinized animals treated with L-DOPA along with the then-recent technology of spetrophotofluorimetry. Using this discovery, Carlsson was able to map regional distributions of DA, and show where the highest concentrations were located (nobelprize.org, 2014).

Dopamine Pathways

Dopamine is found in its highest concentrations in the basal ganglia and substantia nigra regions of the midbrain (Cheramy et al., 1981; Cheramy et al., 1988). Using fluorescence histochemical methods, three major trajectories (pathways) of DA neurons have been outlined from the dopaminergic neurons of the midbrain to the: dorsal striatum, ventral striatum including the nucleus accumbens, and prefrontal cortex (Ungerstedt, 1976; Moore and Bloom, 1978). Projecting from the substantia nigra to the striatum, the nigro–striatal pathway is one of the main dopaminergic projections involved in simple or complex, conditioned or unconditioned motor

behaviors (Barnes et al., 2005). An important feature of this pathway is that it has been elucidated to contain coordinated, multiple, parallel, and recurrent anatomical loops which link the midbrain, basal ganglia, thalamus, and cortex with respect to motor behavior (Haber, 2003; Alexander et al., 1990). The use of dopamine grafts by Döbrössy and Dunnett (2001) showed the organization of the dorsal striatal area and the cortex to be vital areas for the development and learning of complex motor behavior in rats. Upon the initial realization that the nucleus accumbens has a strong relation to the limbic system (Heimer et al., 1997), it was proposed that mesolimbic dopamine pathways could have an important role in motivation, specifically between certain actions and their produced outcomes (Mogenson et al., 1980). Projections to the ventral striatum are significant for goal-directed behavior-based learning and reinforcement. This is based on the animal's learned ability to predict outcomes based on their actions and the value of these outcomes, which has been termed the "cognitive value system" (Dickinson and Balleine, 1994). In addition, Balleine and Dickinson (1991) named another value system, the "pleasure system", as a result of the proposed hypothesis of the role of ventral striatal dopaminergic pathways in reward prediction. Dopamine input to the nucleus accumbens core has been shown in a recent study to possess information about the salience of perceived objects, defined as a certain quality that makes them memorable in order to facilitate learning (Kapur, 2004). At a functional level, increases in DA release into the synapse and phasic bursts of DA neurons firing have been proposed as signals at postsynaptic neurons that is relevant to promoting reward prediction or incentive salience (Waelti et al., 2001). Kapur (2004) postulated that abnormal function of this type of neuronal firing and synaptic release of DA could cause psychotic delusions in schizophrenic patients. The accumbens shell possesses the control of DA input to a variety of areas in the striatum as a result of differences in neuronal pattern organization and

efferent targets, which is thought to stimulate behaviors between the accumbens core and dorsal striatum (Zabrokszky et al., 1985; Usuda et al., 1998; Voorn et al., 1989). The mesolimbic DA neurons transmit DA from the ventral tegmental area (VTA) to the limbic system through the nucleus accumbens, and is thought to be essential for associative learning. The disruption of this pathway is speculated to potentially enhance some of the negative effects of schizophrenia and ADHD (Rosenkranz and Grace, 2002). Finally, the mesocorticol pathway contains mostly D1 type DA receptors which are modulators of cognitive behavior and spatial working memory (Desimone, 1995). D2 DA receptors are also present, in fewer numbers, but have been found to correlate with quick movements, or saccades, that are memory guided (Wang et al., 2004).

Dopamine Diseases

Since DA is involved in a variety of brain functions including reward, motor control, and learning, it is reasonable to speculate that any variance in normal DA function can lead to diseases in the nervous system. Three well documented diseases associated with DA dysfunction are Parkinson's, schizophrenia, and attention deficit hyperactivity disorder (ADHD). Parkinson's is a motor system disorder than generally develops in people over the age of 50. It is caused by a rapid loss of dopamine-producing brain cells in the substantia nigra, in addition to depleted levels of dopamine in the basal ganglia, which cause symptoms like tremors, rigidity, and increased difficulty in starting movements/tasks. The DA precursor L-DOPA has been given as a treatment to increase levels of DA, and is now combined with other drugs to prevent L-DOPA metabolism (NINDS.NIH.gov, 2014). Schizophrenia is a severely chronic and crippling brain disorder that generates delusional ideas and sounds (voices) in the minds of the affected. The causes of this disease are unknown, but are speculated to be a result of a multitude of factors

such as genes (passed from previous generations predisposed to the disorder) and differences in brain chemistry and structure (imbalances in intertwined chemical reactions involving DA and possibly other neurotransmitters) (NIH.gov Schizophrenia, 2014). ADHD is a disorder that causes difficulty in paying attention to tasks at hand, hyperactivity, and controlling one's behavior. The neurological basis of this disorder is currently a subject of research and debate, although some of the prospective reasons include genes, environmental factors, brain injuries, nutrition, and social environments (NIH.gov ADHD, 2014). Psychostimulants are commonly used to treat symptoms of this disorder such as Ritalin and Adderal (amphetamine), which act on the dopamine transporter (DAT) to have an end effect of increasing synaptic levels of dopamine (Iversen, 2006).

Dopamine Transporter (DAT)

The dopamine transporter (DAT) is a monoamine neurotransmitter transporter that belongs to the sodium symporters (NSS) family, also termed the solute carrier 6 (SLC6) family. The family includes the norepinephrine, serotonin, glycine, and GABA (gamma-aminobutyric acid) transporters. Around five decades ago, Julius Axelrod discovered monoamine reuptake in sympathetic nerve terminals, which he proposed was an essential process for the termination of released neurotransmitters (Hertting and Axelrod, 1961). Soon after, the mechanisms for dopamine uptake were elucidated, and were similar but distinct from noradrenaline (Iversen, 1971), and lead to the realized importance of monoamine reuptake and functional properties of these pre-synaptic sites. DAT, a 620 residue protein, exists as a polytopic membrane protein containing twelve transmembrane domains (TMDs) with both amino (N) and carboxy (C) termini residing in the cytoplasm of the cell (Bruss et al., 1995; Hersch et al., 1997; Chen et al.,

1998; Androutsellis-Theotokis and Rudnick, 2002). The N and C termini of mammalian NSS proteins are highly conserved among species, but are not well conserved between different types of NSS transporters. This differing conservation pattern is observed to play a role in regulation of specific, targeted transporters within a cell through protein-protein interactions or post-translational modifications (Eriksen et al., 2010). A high resolution crystal structure of LeuT, a bacterial homologue of mammalian NSS proteins, was used to confirm the DAT structure (Yamashita et al., 2005) and eventually led to further studies showing that NSS proteins are oligomeric assemblies in the plasma membrane (Sitte et al., 2004). The actual crystal structure of *Drosophila* DAT was revealed in 2013 (Penmatsa et al., 2013) and was an important breakthrough in the field of neuroscience. Although the actual role of oligomerization has not been fully clarified, it has been proposed by Torres et al. (2003) to play a part in DAT trafficking to the cell surface. For DAT specifically, the transporter protein dimerizes from symmetrical crosslinking of cysteine residues located on the sixth TMD's extracellular face (Hastrup et al., 2001).

DAT is located in the same regions of the brain where DA is secreted in high concentrations, such as the substantia nigra, the ventral tegmental area (VTA), and the basal ganglia (Hoffman et al., 1998). Immunoreactivity experiments have shown projections of dopaminergic neurons to the: striatum, nucleus accumbens, olfactory tubercle, nigrostriatal bundle, lateral habenula, and prefrontal cortex (Hersch et al., 1997; Freed et al., 1995; Ciliax et al., 1995). These dopaminergic neurons occupy many regions of the brain in subpopulations of the midbrain where their protein expression levels and projections distinguish them from one another (Greene, 2006; Lammel et al., 2008). Interestingly enough, DAT is also found in regions outside of the brain such as the stomach, pancreas, and kidney (Eisenhofer, 2001).

Localized on the pre-synaptic plasma membrane nerve terminals of dopaminergic neurons, DAT regulates the uptake of dopamine from the synapse into the pre-synaptic neuron. This allows DAT to regulate signaling from dopaminergic neurons and to mediate how much extracellular dopamine is maintained (reviewed in Eriksen et al., 2010). SCAM (substituted cysteine accessibility method) analysis suggested a role of the third extracellular loop of DAT to be a vital element in the translocation pathway of binding substrates, for DA reuptake or pharmacological drugs (Ferrer and Javitch, 1998; Chen et al., 2000). Hydrophobic and charged residues of TMDs have received much attention as these amino acids might play a role in interactions with the aromatic ring or amine group of DA. An aspartic acid residue in the first TMD is necessary for substrate recognition, thought to work through interactions of the carboxy group of aspartic acid and the positive charge of DA's amino group (Torres et al., 2003). These NSS transporters function using the sodium ion gradient generated by the plasma membrane protein Na^+/K^+ ATPase (reviewed by Torres et al., 2003), between the plasma membrane along with co-transport of chloride ions to translocate substrates transporters. DAT specifically requires two sodium ions outside the cell and one chloride ion to transport a DA substrate, while the rest of SLC6 transporters need only one of each ion (reviewed by Torres et al., 2003).

DAT Regulation

In addition to the aforementioned DAT oligomerization, the transporter is also subject to dynamic regulation mediated by post-translational modifications and protein-protein interactions. DAT substrates such as amphetamine and DA are capable of regulating surface levels and directing DAT internalization, while inhibitors like cocaine increase DAT surface levels (Saunders et al., 2000; Chi and Reith, 2003). One of the most studied post-translational

modifications with respect to all monoamine transporters is phosphorylation, which possesses the ability to alter the function of these transporters in a variety of methods (Torres et al., 2003). Activated protein kinase C (PKC) has some of the best documented effects on the downregulation of DAT activity, which operates by decreasing the surface expression levels of DAT (endocytosis) by the administration of the phorbol ester PMA (phorbol 12-myristate 13-acetate) (Zhu et al., 1997; Daniels and Amara, 1999; Melikian and Buckley, 1999; Chi and Reith, 2003). PKC, as proposed by Foster et al. (2002), primarily phosphorylates at a cluster of serine residues located at the N terminus of DAT. It is important to note that truncation of these sites on DAT have shown that phosphorylation by PKC is not directly coupled to PMA-induced internalization (Granas, et al., 2003). Ubiquitination, a common modification for regulating protein homeostasis (Raiborg and Stenmark, 2009), is another important post-translational modification that has been proposed to be involved in PKC-activated downregulation (Miranda et al 2007). DAT is constitutively ubiquitinated at three lysines at its N-terminus, and introduction of PMA increases the frequency of ubiquitination. Miranda et al (2007) showed these lysines were pivotal in ubiquitination of DAT when mutation of the trio to arginines stopped constitutive ubiquitination and PMA-stimulated downregulation, as a result of modification by the E3 ubiquitin ligase NEDD4-2 (Neuronal precursor cell expressed, developmentally down-regulated 4-2). In another study by Miranda et al. (2005), ubiquitination was found to be prevalent in endosomes by fluorescence resonance energy transfer (FRET) experiments, pointing to the idea that ubiquitination could be an endocytic signal. Holten et al. (2005) elucidated an internalization motif on the C terminus of DAT, a 10 residue sequence (FREKLAYAIA) that regulates constitutive and PKC-regulated DAT internalization. A basal level of DAT trafficking between intracellular endosomal vesicles and the surface has been

measured at an endocytic rate of 3-5% of DAT surface levels per minute in PC12 cells (Loder and Melikian, 2003), this rate will vary in different cell. PKC-mediated DAT sequestration was defined by Loder and Melikian (2003) as increasing basal DAT endocytic rates and slowing DAT cell-surface delivery. Through the interleukin2-alpha receptor (Tac) chimeras with DAT, certain parts of the FREKLAYAIA internalization motif were discovered to have different roles. The distal five carboxy residues (AYAIA) were revealed to contain information required for constitutive DAT internalization, especially the nonpolar residues within this region as mutations to alanines resulted in a significant reduction in endocytic rates (Holten et al., 2005). FREKLAYAIA was shown to be well conserved among the SLC6 family members, as mutagenesis experiments with the cognate region in the norepinephrine transporter (NET) yielded a similar drop in endocytic rates (Holten et al., 2005). In addition to a constitutive DAT internalization signal, the first five residues of FREKLAYAIA contain an independent and distinct signal for PKC-mediated DAT sequestration. Point mutation disrupting basal DAT internalization did not yield any effect on PKC-stimulated DAT internalization, confirming the idea of independent and distinct signals on the FREKLAYAIA motif (Holten et al., 2005).

DAT Protein Interactions

Besides DAT regulation by post-translational modifications, protein to protein interactions have been postulated to regulate monoamine transporters based on two observations: monoamine transporters undergo trafficking in cells with the help of interacting proteins, and monoamine transporters are driven to specific locations on the presynaptic sites of nerve terminals under the direction of intracellular proteins (Torres et al., 2003). One of the proteins that plays a role in DAT trafficking is a member of the Rit subfamily of Ras-like small GTPases,

Rin (Ras-like protein in neurons) or also termed Rit2 (Melikian et al., 2011). Ras-related GTPases are molecular switches that, based on external signals, swap GTP for bound GDP. These GTP-bound active proteins interaction with a multitude of target effector proteins turning on intracellular signaling cascades (Campbell et al., 1998; Takai et al., 2001). Rin is expressed in high concentrations, particularly in neuronal cells (Zhou et al., 2011), and is expressed in non-neuronal cell lines like rat muscle and liver cells, primary cultured rat astrocytes, and mouse embryonic kidney cells (Melikian et al., 2011). Specific co-localization was observed with DAT and Rin (using FRET microscopy) at specific focal points in the plasma membrane (Melikian et al., 2011). These co-localization points were shown to be significantly higher at lipid raft microdomains within the plasma membrane than non-lipid raft microdomains (Melikian et al., 2011). Rin is required for PKC-mediated DAT trafficking, as shown by Melikian et al. (2011) by using reversible biotinylation to measure DAT internalization rates with co-expression of DAT and Rin mutants. In addition, shRNA-mediated Rin knockdown showed that Rin is required for PKC-mediated DAT internalization. Since Rin is required for PKC-mediated DAT trafficking, it is required to down-regulate the functional uptake of DA through DAT via administration of PMA (Melikian et al., 2011). Rin dissociates from DAT based on the guanyl nucleotide exchange, as revealed from cellular imaging and co-immunoprecipitation experiments of Rin binding with the DAT C terminus (Melikian et al., 2011). Additional results from Melikian et al. (2011) suggest a negative regulatory mechanism controlling DAT endocytic rates in residues 587-590 (REK), and that PKC activation regulates DAT/Rin interactions. An endocytic brake has been proposed to exist here that slows DAT endocytic rates under normal conditions, yet is released during PKC activation (Boundanova et al., 2008) or mutations of residues (REK) 587-590 (Melikian et al., 2011). Since Rin also binds with the C terminus, it has

been suggested that Rin provides a synergistic interaction between DAT's C and N termini, regulating DAT's endocytic rate (Melikian et al., 2011). Recent evidence from Heo et al. (2006) outlines a polybasic charged domain conserved at the C terminus that facilitates Rin's association with the plasma membrane, as the C-terminus lacks a prenylation box (CaaX) which distinguishes this protein from others in its Ras-like GTPase family. This polybasic region of Rin also serves as a calmodulin (CaM) binding site (Lee et al., 1996) and has been shown to bind *in vitro* with CaM with the possibility of being regulated by Ca^{2+} levels *in vivo* (Lee et al., 1996; Hosino and Nakamura, 2003). Hoshino and Nakamura have also shown CaM association to be necessary for Rin function. Thus, a question remaining is whether calmodulin function upstream of Rin activation plays a role in PKC-stimulated DAT trafficking and regulation.

Calmodulin

Calmodulin (CaM) is a ubiquitously expressed intracellular protein that is an important sensor of Ca^{2+} levels resulting in the regulation of a diverse group of intracellular proteins (Hoshino and Nakamura, 2003; Holten et al., 2005). Interaction of CaM with these proteins is dependent on the intracellular levels of Ca^{2+} ions which bind CaM. Protein kinases, cyclic nucleotides, cytoskeleton proteins, and ion channels are subjected to CaM regulation through varying Ca^{2+} levels. This allows CaM to regulate cellular processes such as cell growth, proliferation, movement, and sensory transduction (Beckingham et al., 1998; Chin and Means, 2000). One of CaM's most important roles in neurons and their targeted muscles is synaptic development and transmission, neuronal survival, and axonal outgrowth and path direction (Burgoyne and Weiss, 2001). This multitude of important roles that CaM plays in neurons and other cells led to the identification of RIC (Ras-related protein which interacts with calmodulin, a

Drosophila homologue of Rin) as a potentially important compound in the development of neurons and sensory signaling (Wes et al., 1996). RIC, one of the few proteins detected to interact directly with CaM, was the stepping stone to discovering a functional similarity in Rin (Harrison et al., 2005). CaM was shown to inhibit the function of RIC in *Drosophila*, paralleling another small GTPase named Gem, which was reported as also being negatively regulated by CaM (Fischer et al., 1996). CaM association with Gem displayed inhibited GTP binding (Fischer et al., 1996) and also disturbed association of Gem with downstream effector proteins preventing Gem function (Beguin et al., 2001). These are functions of other small GTPase proteins that are related to or homologous with Rin.

Project Purpose

The purpose of this project was to investigate whether CaM is required for the PKC-stimulated decrease in DAT surface expression. Through a protein signaling pathway, PKC activation from the phorbol ester PMA inhibits CaM function effectively releasing Rin and allowing for Rin-DAT interactions stimulating the internalization of DAT. Since CaM is a negatively regulating protein involved in this signaling pathway, the role of CaM on DAT function was explored using the CaM inhibitor CGS 9343B in SK-N-MC cells stably expressing DAT. Although the data acquired was not statistically significant, high doses above 1 μM appeared to decrease DAT uptake, while low concentrations CGS 9343B showed an increased specific uptake of DA (0.1 μM-10 μM). This can either be a result of two processes: an increase of DAT function by causing it to function more efficiently thus increasing DA uptake, or by increasing the amount of DAT that is present on the cell surface. To distinguish between these two possibilities, further biotinylation experiments will elucidate whether DAT SK-N-MC cells under certain CGS 9343B concentrations cause a difference in DAT cell surface expression levels. In order to test whether the CaM inhibitor CGS 9343B could block PKC-induced DAT functional loss, DAT SK-N-MC cells were pre-treated with various doses of CGS before activating PKC using the phorbol ester PMA. This data was also not significant, but it will be interesting to see if the CaM inhibitor can block the PMA effect at low concentrations. Understanding these DAT trafficking molecular mechanisms can provide information into DA dysregulation and psychostimulant addiction, and may shed light on future therapeutic treatments for disorders pertaining to DAT.

Methods

Cell Culture Techniques. Stably transfected SK-N-MC cells expressing wild-type DAT were maintained at 37°C, 5% CO_2 in MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 10^2 U/mL penicillin/streptomycin, and under selective pressure from 0.2 mg/mL G418 (Invitrogen).

[^{3}H]-DA Uptake Assays. Stable DAT SK-N-MC cells were plated on Falcon Multiwell 24-well polystyrene, non-pyrogenic plates at densities of $2x10^5$ cells/well 24 hours prior to the experiment being conducted. Indicated drugs were prepared just before the start of the uptake assay, where the cells in 24-well plates were washed twice at room temperature in KRH buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.2 mM $CaCl_2$, 10 mM HEPES, pH 7.4) and then pre-incubated in KRH buffer along with specified drugs for 60 minutes (DMI = desipramine, to block uptake of DA by endogenously expressed NET) (GBR12909 = Vanoxerine, defines nonspecific uptake acting as a dopamine reuptake inhibitor "DRI") (1μM PMA added at 30 minutes pending assay). Dopamine uptake commenced with the addition of 1 μM [^{3}H]-DA (3,4-[ring-2,5,6,-^{3}H]DA, Perkin Elmer) in the presence of 10^{-5}M pargyline and ascorbic acid. Uptake proceeded for 10 minutes at 37°C, which was halted by washing three times with ice cold KRH buffer. 250 μL of scintillation fluid was added to each well to solubilize cells, and plates were shaken for 15 minutes. The radioactivity levels of each well were measured by liquid scintillation counting using a Wallac Microbeta scintillation plate counter. Microsoft Excel and GraphPad Prism Software were used to conduct the data analysis, including ANOVA and unpaired T tests.

Results

The purpose of this project was to investigate whether calmodulin (CaM) is required for PKC-mediated down-regulation of DAT surface expression. To test the possibility of CaM's effect on DAT function, SK-N-MC cells stably expressing DAT were used in DA uptake assays to generate a dose-response curve using the CaM inhibitor CGS 9343B. Initially, CGS 9343B was used at a range of 0.1 µM-10 µM, which was established using the IC_{50} of 3.3 µM reported by Norman et al. (1987). Pre-treatment with CGS 9343B for 60 minutes at 37°C resulted in a dose dependent loss in DA uptake with an IC_{50} of 10.39 µM (**Figure-1**). Visible is a downward trend of specific DA uptake starting after 1 µM. The dose-response curve shows documented effects of CGS 9343B at reported selective CaM inhibiting concentrations.

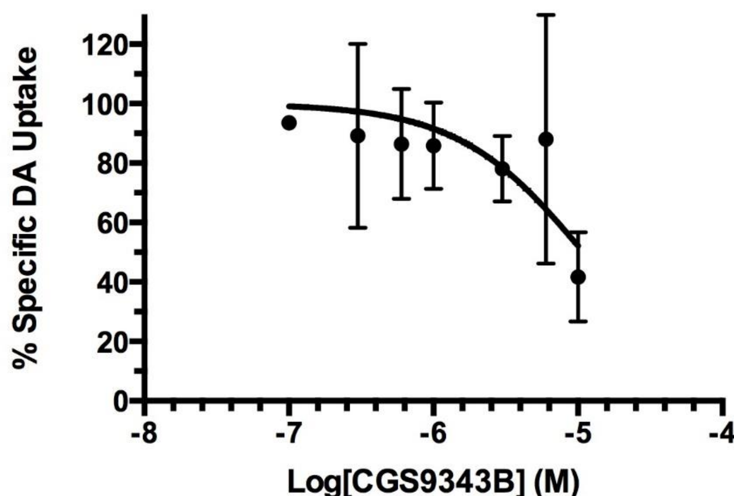

Figure 1: High Doses of CaM Inhibitor CGS 9343B Decreases DAT Function. DA uptake assay was performed in presence of CaM inhibitor CGS 9343B at various concentrations. Average data was fit using log (inhibitor) vs. normalized data best fit line. IC_{50} = 10.39 µM, n=2-3.

Additional DA uptake assays were conducted using concentrations in a lower range (data not shown). The first CGS 9343B dose response DA uptake assay [0.6 nM-30 nM] yielded an

increased specific DA uptake compared to that of the first tested range [0.1 μM-10 μM]. Seeing this unexpected increase in DA uptake led to further investigating the effects of CGS 9343B at a lower concentration range of 3 nM-100 nM (**Figure-2**). CGS 9343B at 0.6 nM provided little effect on specific DA uptake, giving a good baseline for increasing concentrations above that level to show potential deviations from 100% uptake (horizontal dotted line, Fig. 2). The trend continues upwards over the CGS 9343B range and begins to decrease after the peak at 60 nM. The most effective increase in specific DA uptake was at a concentration of 60 nM while the second most effective was at 300 nM. This finding provided two good concentrations to use in conjunction with PMA to further test whether CGS 9343B could block PMA-induced DAT down-regulation.

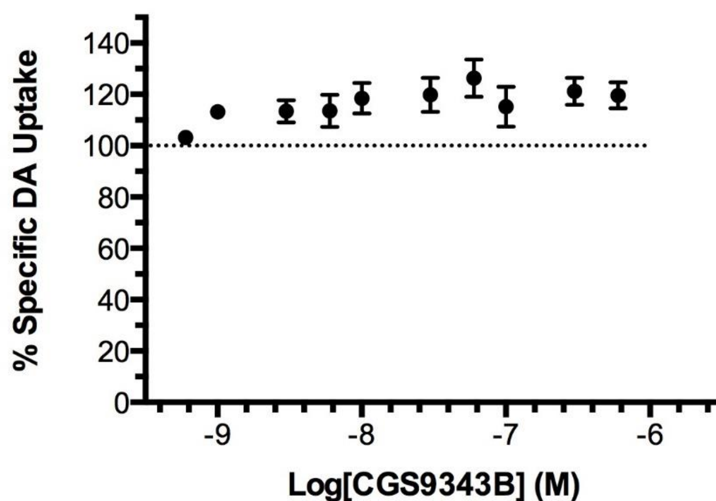

Figure 2: Low Doses of Drug CGS 9343B Increase DAT Function. n=1-7.

In order to further explore whether CGS 9343B could block the effects of PMA-induced DAT function down-regulation, a DA uptake assay was conducted involving the addition of CGS at 60 nM and 300 nM with or without 1 μM of PMA (**Figure-3**). Treatment with PMA gave about a 22% decrease in specific DA uptake compared to vehicle conditions. As opposed to the

results from Figure 2, this experiment shows pretreatment of 300 nM of CGS yields the highest increase in specific DA uptake, a 14% increase over the vehicle, with 60 nM of CGS only having a 6% increase. The magnitudes of the PMA effect on vehicle and 300 nM CGS pretreatment are quantified in **Figure 4**. Unfortunately the experiment has not been completed enough times in order to show statistical significance between all of the treatments. This leaves an opportunity to continue these DA uptake assays in the future to hopefully show a significant effect between the conditions of CGS ± PMA.

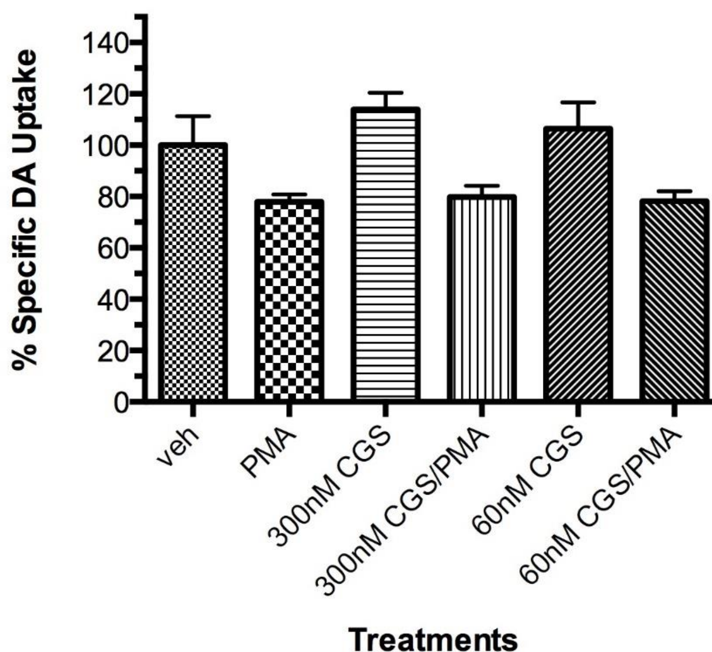

Figure 3: Summary of Various Treatments on DA Uptake. PMA appears to lower DA uptake as expected, while CGS 9343B+PMA enlarges the decrease in specific uptake in DAT. n=3-5.

Further expanding the results from Figure 3, the magnitude of the PMA effect was calculated between vehicle vs. PMA, and 300 nM CGS vs. 300 nM CGS/PMA (Figure 4). PMA decreases DAT function relative to vehicle conditions or to pre-incubated cells with 300 nM

CGS 9343B. The magnitude of the PMA effect when pretreated with CGS is about 10% higher than without CGS (vehicle vs. PMA).

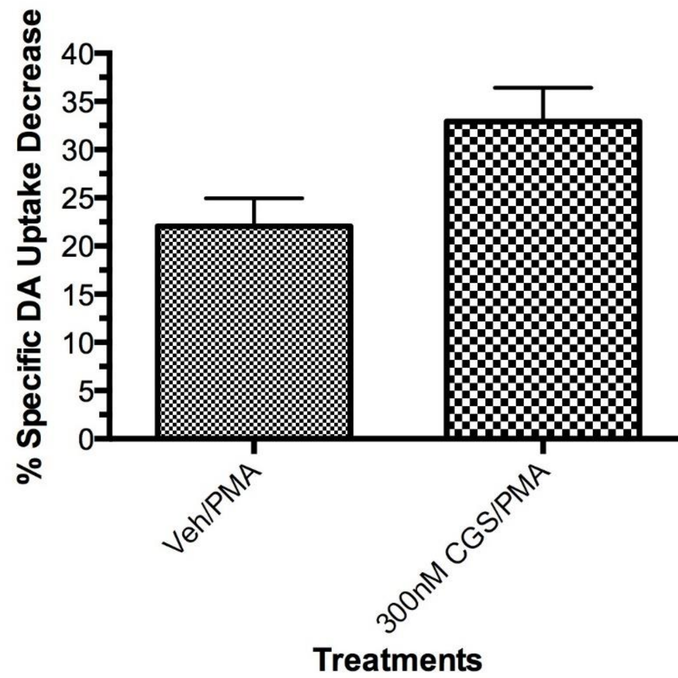

Figure 4: Pre-incubation with 300 nM CGS Increases the Magnitude of PMA-Induced Down-Regulation of DAT Function. n=4

Discussion

This project sought to determine whether calmodulin (CaM) is required in the signaling pathway of PKC-induced DAT downregulation. DAT function was assayed using a [^{3}H]-DA uptake assay. The main experiment conducted was a DA uptake assay under two different parameters: a dose-response using varying concentrations of CaM-inhibitor CGS 9343B on DAT function, and the effect of CGS 9343B at two different concentrations with or without PMA, to test whether CGS can block a PMA-induced DAT downregulation. Figure 1 shows the effects of CGS 9343B on DAT-stable SK-N-MC cells using a concentration range of 0.1 µM-10 µM. A best fit log of inhibitor line was generated using the analyze function of the GraphPad Prism program yielding an experimentally calculated IC_{50} of 10.89 µM. Comparing this with the reported IC_{50} of CGS 9343B is 3.3 µM (Norman et al., 1987) reaffirms the fact that this CGS 9343B dose response DA uptake assay was not performed enough times, otherwise the experimentally calculated IC_{50} value would have approached its reported value. It is important to note that another lab has demonstrated that CGS 9343B is not entirely specific for CaM in concentrations that were used for this uptake assay. Neuhaus & Reber (1992) showed that CGS 9343B inhibits current amplitudes at a concentration of 3.0 µM, and is reversible when washed at a concentration of 10 µM. CGS also blocks the transient increase of Ca^{2+} during depolarization at a concentration of 10 µM, and after a five minute incubation it completely blocked this. At 10 µM, CGS also causes a reversible inhibition of K^+ and Na^+ voltage-gated channels. The authors made a point to stress that the rapid reversibility of blocking currents argues against CGS's involvement in the Ca^{2+} and CaM complex (Neuhaus and Reber, 1992). These initial Figure-1 experiments were a good introduction to the lab, and they also served to show the inhibitory

effects of CGS 9343B on CaM activity, and its downregulating effect on the function of DAT's specific DA uptake.

The next experiment was a dose response of CGS 9343B, but using lower concentrations ranging from 0.6 nM-300 nM. The horizontal line shows where basal levels of DA uptake are (100%), and all the concentrations at this range are above this level. CGS, for some reason, in this range appears to cause an increased amount of specific DA uptake. 60 nM has the highest specific DA uptake at 128.0±9.2% (n=6) and 300 nM has the second highest at 121.2±5.3% (n=2). These two values are especially important because according to Figure 2 they have the biggest increase in DAT function which gives them credibility to use in other experiments assessing CGS 9343B regarding DAT function. These results potentially showing elevated levels of DAT function says two things: 1) that CGS in this concentration range may increase the function of DAT by causing more DA to be taken up into the cell, or that 2) CGS is causing the level of DAT surface expression to increase allowing more DATs to bring in more DA. Since CGS 9343B is generally not used at this low of concentrations, it is interesting that these effects are occurring. What CGS is actually doing is unknown, and is an area that will be explored next using surface biotinylations of SK-N-MC cells to quantify the surface levels of DAT. This experiment will allow us to determine how much DAT is on the surface of these cells under: basal conditions, PMA, CGS, and PMA/CGS pretreatments. Once the levels of DAT surface expression are calculated from these experiments, it will answer the question whether CGS in this concentration range causes an increase of DAT surface expression. Using these results in conjunction with the DA uptake assay, a CGS 9343B dose response will start to elucidate the effect that CGS is having on DAT in SK-N-MC cells.

The last DA uptake assay conducted for this project tested the effects of pre-incubated CGS (at 60 nM and 300 nM) in conjunction with PMA incubation for 30 minutes. A 30 minute PMA incubation yielded a 22.1±2.9% decrease in specific DA uptake, but has increased effects when combined with CGS pretreated cells. 300 nM of CGS had the highest average specific DA uptake (note it is not consistent with data from Figure 2 where 60 nM has the highest average specific DA uptake) of 114±6.5% while 60 nM had an uptake of 106.5±10.3%. CGS at 300 nM also had the largest average magnitude of the PMA effect (Fig. 4) at a difference of 32.9±3.5%. Due to the low number of experiments conducted (n-value ranging from 3 to 5), the only pair of treatments that were shown to be significant (data not shown) were 300 nM CGS vs 300 nM CGS/PMA. More experiments will need to be conducted to show statistical significance according to ANOVA using Šídák's multiple comparisons tests among the other desired pairs (vehicle vs. 300 nM CGS or 60 nM CGS; 60 nM CGS vs. 60 nM CGS/PMA). Additionally, Figure 4 quantifies the magnitude of the PMA effect between vehicle conditions and pretreatment with 300 nM CGS. According to an unpaired T test (results not shown), the two magnitudes are not significantly different (n = 4). Conducting more of the aforementioned CGS and PMA DA uptake assays will generate more data to conclude whether or not the difference in PKC-stimulated decrease in DAT function is significant. If it is, this will show that PMA's DAT down-regulating effects combined with CGS' functional increase in DA uptake are causing an increased magnitude of PMA-induced decrease in DA uptake. We speculate that this could mean CGS 9343B possesses the capability to increase PMA's effect on DAT function, allowing it have an increased magnitude of decreased specific DA uptake. Since CGS 9343B acts through the inhibition of the CaM-stimulated cAMP phosphodiesterase (PDE1) to inhibit CaM (Norman et al., 1987), it is possible that removing CaM's inhibitory effect on Rin with CGS 9343B allows

the protein pathway that contains protein kinase C (PKC) to also inhibit CaM, thus allowing an even larger PMA-induced downregulation of DAT surface expression. Since CaM interacts with Rin in order to negatively regulate DAT trafficking through inhibition of Rin, the increased PMA effect could potentially inhibit more CaM in conjunction with CGS 9343B leading to more DAT-Rin interactions, effectively internalizing more DAT. This would mean that the same concentration of PMA (1 μM) is causing more DAT to be internalized, leading to the increased magnitude of DAT decrease in specific DA uptake seen in Figure 4. Although these are just speculations, repeating these experiments, in addition to conducting surface biotinylations, will start to give an answer to the question of whether CaM is required for PKC-induced DAT surface loss. It will also shed light on possible mechanisms for how CGS 9343B at low concentrations is increasing DAT function. All of this information is relevant to revealing the protein cascade pathways that orchestrate DAT internalization from varying signal pathways.

The importance of these conclusions could be applied to discoveries of therapeutic treatments for disorders pertaining to DAT dysfunction and an increased understanding of psychostimulant addiction and overall knowledge of the DAT and its regulatory mechanisms.

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