Trafficking of the Dopamine Transporter

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Abstract

Dysregulation of dopamine is indicated in multiple neurological disorders, including ADHD, addiction, and Parkinson's. The dopamine transporter (DAT) is one of the most important components of regulating extracellular dopamine levels. Typically, DAT is trafficked at a steady state. Previous research has shown that insertion of DAT into the cell membrane is stimulated by D2 activation. However, mutants such as DAT R615C are less stable on the surface and may facilitate dysregulated reuptake of dopamine into the presynaptic neuron. This may suggest that arginine at location DAT 615 is necessary for insertion of DAT into the cell membrane. To determine whether arginine is required for steady DAT trafficking, the mutant mDAT R615C was engineered and cultivated in human embryonic kidney cells. Immunoblotting was used to determine expression of cells with mDAT R615C, compared to wild-type mDAT. Trafficking was analyzed through biotinylation and immunoblotting. It was found that DAT R615C expresses 44% as much as wild-type DAT. Further, a significant 44% decrease in surface DAT was observed in DAT R615C compared to the wild-type. This suggests that DAT R615C is an unstable mutant of the dopamine transporter and may affect dopamine dependent behaviors. Findings support that arginine at DAT 615 is required for steady trafficking.

Trafficking of the Dopamine Transporter

Neurological disorders and diseases have a profound impact on global health, affecting over a billion people worldwide with nearly 7 million people dying each year due to their effects (Pan American Health Organization, 2007). Many of these conditions have been linked to dysregulation of the dopamine system, including Alzheimer's, Parkinson's, Autism Spectrum Disorder, Attention Deficit Disorder, schizophrenia, and addiction (Franco, et al., 2021). One of the most important components that regulates the dopamine system is the dopamine transporter (DAT). The dopamine transporter is a protein responsible for the reuptake of dopamine into the presynaptic neuron. Understanding the trafficking and regulation of the dopamine transporter is of great importance. Thus, this research aims to investigate the trafficking of the dopamine transporter. Specifically, the current work examines whether arginine at position DAT is required for insertion of DAT into the cell membrane.

Dopamine is a vital neurotransmitter that regulates mood, movement, and executive function (Wise, 2004). Dopamine is often referred to as the "reward" neurotransmitter, because of the critical role it plays for the rewarding feelings after a pleasurable event. Necessary human functions such as sleeping, eating, and sex both require and release dopamine (Iverson, 2007). Dopamine also plays a crucial role in the regulation of movement, as dopamine-producing neurons are responsible for the coordination and control of voluntary movement (Barter et al., 2015). Additionally, it modulates the activity of other neurotransmitters such as serotonin and norepinephrine, thereby influencing mood, cognition, and attention. Unfortunately, dopamine imbalances have strong medical implications (Iverson, 2007).

There are numerous neurological disorders linked to imbalanced dopamine levels, such as attention deficit hyperactivity disorder (ADHD), addiction, and Parkinson's disease (Wu et al.,

2012). The exact mechanism of action for dopamine's dysregulation and its role in these disorders is not well studied. For example, ADHD is defined as inattention and hyperactivity that significantly impacts a person's life. Symptoms often include low motivation, executive dysfunction, and anxiety (Guha, 2014). ADHD is thought to be the result of dopamine dysregulation in the striatum, which is a region primarily responsible for motivation and reward-based learning (Viggiano et al., 2004). However, the exact mechanisms of action are unknown.

Similarly, those with drug addictions have misfunctioning reward systems (Di Chiaro & Bassareo, 2007). Addictive drugs such as opioids drastically increase the dopamine levels in the brain, thus causing intense pleasurable feelings. Over time, the increased level of dopamine leads to decreased levels of dopamine receptors in the brain, through a mechanism that is not yet understood (Volkow, et al., 2004). This makes it necessary to take more drugs to produce the same high (Di Chiaro & Bassareo, 2007).

Comparably, Parkinson's is believed to be caused by a loss of dopamine producing neurons in the substantia nigra region of the brain (Damier et al., 1999). This region is largely responsible for coordination and movement; thus, loss of dopamine leads to tremors, rigidity, and involuntary movement, which are all symptoms of Parkinson's disease (Damier et al., 1999). Similar to those with ADHD and drug addiction, people with Parkinson's often cannot receive adequate help due to the lack of understanding surrounding the disorder.

Therefore, understanding dysregulated dopamine signaling is vital for developing proper and effective treatments of dopaminergic disorders. Dopamine signaling is a complex multi-step system. First, the dopamine must be synthesized from the amino acid tyrosine by the presynaptic neuron. An action potential triggers the release of dopamine from the presynaptic neuron into the synaptic cleft, which is the gap between a pre- and postsynaptic neuron. The postsynaptic neuron

takes up the dopamine using dopamine receptors D1-D5. The presynaptic neuron then takes up the dopamine in the synaptic cleft using the dopamine transporter (Girault & Greengard, 2004).

In addition, the dopamine transporter (DAT) is a critical component of dopamine signaling. It is a protein that facilitates reuptake of dopamine into the presynaptic neuron, thus altering both acute and chronic dopamine levels (Chen & Reith, 2000). Typically, there are dopamine transporters both within and on the surface of a neuron. DAT is trafficked at a steady state, meaning that the concentration of DAT on the surface of a cell membrane should be comparable at any given time. Essentially, it implies a homeostasis of DAT in and on the neuron (Melikian & Buckley, 1999). It has also been found that the insertion of DAT to the cell membrane is facilitated by stimulation of the D2 receptor (Lee et al., 2007). This suggests that D2 stimulation plays a key role in maintaining steady state trafficking.

Once DAT has bound to the dopamine, it is able to internalize into the presynaptic neuron. Finally, it releases the dopamine which will either be metabolized or rereleased into the synaptic cleft (Chen & Reith, 2000). Unsurprisingly, changes in DAT's trafficking alter dopamine levels in the brain (Bu & Farrer, 2021). Though DAT's general mechanism of action is understood, there needs to be more research done on how DAT can become dysregulated. Research shows that trafficking of DAT to and from the plasma membrane is a dynamic process that modulates dopamine neurotransmission. Previous studies have demonstrated that DAT trafficking is regulated by multiple pathways, including protein kinase C (PKC) (Foster & Vaughan, 2011), GTPase Rab11 (Daniels, 2004) and the dopamine receptor D2 (Gonzalez-Maeso, 2008).

Previous research has shown that alterations in the trafficking of the dopamine transporter can lead to dysregulation of the dopamine, which in turn contributes to various dopaminergic

disorders. For instance, in Parkinson's disease, reduced trafficking of DAT to the plasma membrane leads to a decrease in dopamine reuptake, contributing to the motor symptoms of the disease (Giros & Caron, 1993). Moreover, in ADHD, increased DAT trafficking to the plasma membrane leads to hyperactivity and impulsivity, two core symptoms of the disorder (Gainetdinov & Caron, 2003).

Therefore, based on past research that has found that insertion of DAT into the cell membrane is stimulated by D2 activation (Lee et al., 2007), as well as research that identified DAT R615C in people with dopaminergic disorders (Sakrikar et al., 2012), the current research examines whether arginine at DAT 615 is required for D2 stimulated insertion of DAT into the cell membrane. It was predicted that arginine would be required for D2 stimulated insertion of DAT and expected to see lower concentration of DAT onto the cell membrane. This was examined by quantifying DAT protein in Human Embryonic Kidney (HEK293T) cells transfected with wild-type mDAT and mDAT R615C, by biotinylating and immunoblotting.

Method

This research was conducted in human embryonic kidney (HEK293T) cells. These cells were used because HEK293T cells naturally have the D2 receptor. Thus, the cells are an ideal organism for experiments that question mechanisms surrounding the D2 receptor. Two samples were conducted, with N=2 for both samples. The protein of interest was mouse dopamine transporter (mDAT) R615C. Ideally, future experiments will use mDAT R615C to conduct behavioral studies in mice. Also, the mouse genome is about 70% identical to the human genome (National Institute of Health, 2015).

The independent variable was the coding variants of mDAT. The two possible conditions were: wild-type and mutant, thus giving an N of 2. The wild-type is the non-mutated gene that is found in nature. The mutant, mDAT R615C, is a mutated version of the wild-type. The dependent variable was the expression of surface and total DAT. This dependent variable was chosen because the amount of dopamine transporters on the surface of the synapse correlates with its speed of trafficking. All protocols are taken from the Melikian laboratory at UMass Chan Medical School unless directly stated otherwise.

Mutagenesis of Dopamine Transporter

To test if arginine at DAT 615 was necessary for D2 stimulated insertion, the arginine had to be mutated to a different amino acid. A single-point mutation was completed through mutant strand synthesis to change CGC (arginine) to TGC (cysteine). A reaction was set up with a preexisting mDAT vector, referred to as mDAT pcDNA 3.1+, and a sense and antisense primer with the desired mutation. The vector and primers underwent thermal cycling for 1 hour at room temperature, then a shift to 4 degrees Celsius overnight. Purified mDAT R615C was added to XL1-Gold Supercompetent cells and incubated on ice for thirty minutes. This was heat shocked at 46 degrees Celsius and recovered through Super Optimal broth with Catabolite repression (S.O.C.). These cells were plated onto agar plates and incubated at 37 degrees Celsius overnight. The plasmid DNA was purified from four separate colonies. The bacteria were incubated while shaking in Terrific Broth (TB) overnight. The extraction of DNA was completed according to the Wizard Plus SV Minipreps DNA Purification System (Promega Corporation, 2022).

The DNA was then sent to sequence to ensure that it contained the desired mutation. Once the sequence was confirmed, the DNA was transformed into DH5-alpha cells to get a higher concentration of DNA.

Subcloning Mutation into Wild-Type

The desired mutation, mDAT R615C, had to be subcloned into the wild-type DNA. This subcloning ensured that the desired mutation was the only mutation in the DNA sequence. Restriction enzymes were chosen to extract the desired segment; it was important that one enzyme be upstream and one be downstream from the mutation, and that each enzyme cut only once. Otherwise, unwanted fragments may have been cut from the sequence.

ALE1 and NOT1 were chosen due to their locations- ALE1 being an upstream restriction enzyme in mDAT and NOT1 being a downstream restriction enzyme in pcDNA 3.1+. A diagnostic digest was performed with the restriction enzymes and the wild type, as well as the restriction enzymes and the mutant. rCutsmart was used as a buffer for both digests. The enzymes were incubated with both the wild type and mutant for one hour; 0.7% gel electrophoresis was performed to ensure that the enzymes did cut the expected fragment by viewing the size of the fragments removed from the sequence. Due to insufficient separation of desired fragments, the NOT1 enzyme was replaced with XBA1. This yielded the desired fragments.

The desired fragments were located on the gel by using a UV light and marking with a sharp blade. These were then extracted and placed into two separate 3mL centrifuge tubes. The fragments were weighed to determine the amount of buffer needed

for each fragment; the ideal ratio was 1:3 v/w buffer to fragment. The fragments were extracted using the Qiagen gel extraction protocol (QIAGEN, 2019). Next, the DNA was ligated using the extracted segments and ligase. This was added to a PCR machine for 16 hours. Finally, the DNA was used in bacterial transformation of DH5-alpha cells. The plates grew an abundance of viable colonies for preparation of DNA. The DNA was extracted using the Qiagen maxi-prep protocol (Figure 2).

Cell Culture

The DNA had to be transfected, or artificially introduced, in living cells to test its activity *in vivo*. A cell line of human embryonic kidney cells (HEK293T) was cultured in Dulbeco's modified eagle medium (DMEM) complete with 10% fetal bovine serum (FBS). The culturing media contained 5mM glutamine to support the growth of cells. The media contained 10% penicillin streptomycin (Penn/Strep) to protect the cells from bacterial contamination. Cells were passaged when they reached 80% confluency, to prevent overgrowth and death. For passaging, the cells were washed with 10 mL of sterile phosphate buffered saline (PBS) and trypsinized with 2 mL trypsin for each passage. The cells were grown in a sterile 75 cm^2 flask with a vented cap, with 15 mL of culture media for each passage. The cells were incubated at 37 degrees Celsius at 5% CO_2.

Cells were counted for transfections using a hemocytometer, and 2 micrograms of DNA plasmid were used for transfection. Transfections were completed using Opti-MEM and Lipofectamine. Two hundred and fifty microliters of a Opti-MEM/Lipofectamine mixture were added to 250 microliters of Opti-MEM/DNA plasmid mixture. This mixture was added to 2 mL of cell culture media without Penn/Strep. 0.5 x 10^6 cells/well were plated in a 6 well cell culture

plate. The vector pcDNA 3.1+, wild type hDAT, wild type mDAT, and mDAT R615C were transfected for each experiment. For biotinylations, 0.75 mL of poly-d-lysine was added to each well and left to incubate for 30 minutes to ensure adequate adherence to wells. The poly-d-lysine was next washed off with 3 washes x 2mL of PBS. Transfection media was replaced with fresh media 24-hours post transfection. Cells were assayed 72 hours post transfection.

Protein Assays

Bicinchonic assays (BCA) were conducted to determine protein concentration. BSA standards were made in RIPA with the following concentrations: 5mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, and 0.3125 mg/mL. Ten microliters of each sample and standard were pipetted into 3 wells each. Two hundred microliters of BCA reagent were added to each well. The protein assay was measured after a 30-minute incubation at 37 degrees Celsius.

Immunoblotting

Samples were eluted with 2x sample buffer/DTT and rotated for 30 minutes. A 10% western blot was created according to the recipes of Sambrook & Maniatis (Sambrook & Maniatis, 1989). The samples were pipetted into the gel with SDS-page running buffer. Precision plus ladder was used. The gel ran at 100V through the stacking gel and 200V through the running gel. The gel was next transferred to nitrocellulose at 100V for one hour. Blocking solution was created with 20mL blot wash, 1 gram dry carnation milk (1% w/v) and 200 microliters 10% thimerosal. Ten mL blocking solution was added to the blot and left to shake for 45 minutes at room temperature. Primary antibody was then added to the blot. The antibody used

was rat anti-DAT. 5 microliters of this was added to 10 mL of blocking solution (1:2000 ratio). This shook overnight at 4 degrees Celsius.

The blot was vigorously washed with blot wash to remove all primary antibody. Secondary antibody was made using 2 microliters of goat anti rat in 10 mL blocking solution (1:5000). This was developed and imaged using ChemiDoc imaging system chemiluminescent blot after incubating for 45 minutes.

Determination of general expression

Cells were lysed and a protein assay was conducted. Fifty micrograms of protein were pulled down. This was eluted with 2x sample buffer/DTT and rotated for 30 minutes. Western blot was used to identify the proteins.

Determination of trafficking

Biotinylation was used to determine the amount of surface DAT as a percentage of total DAT in mDAT, and mDAT R615C. The following biotynilation procedures were performed at 4 degrees Celsius. Forty microliters of 200 microgram/microliter Sulfo-NHS-SS-biotin were added to 2 mL RIPA buffer. Next, 750 microliters of this solution were added to each well before cells were lysed, ensuring that the biotin only attached to DAT that lied on the surface. This was left to shake for 15 minutes on ice. Fresh solution was made and left to shake for 15 minutes. Cells were washed 3 x 2 mL with PBS^{2+} for 15 minutes to remove unreacted biotin. This was next washed 2 x 1 mL in Quench solution to react with unwanted molecules. The cells were lysed to release the internal DAT.

A BCA protein assay was conducted, and protein pulled down from 30 micrograms. Forty microliters of Streptavin beads were added to each sample. These beads attach to biotin, and thus only the surface DAT will be attached to the beads. The samples were rotated overnight. Cytivia columns were used to elute internal mDAT R615C from solution. The columns were prepared by blocking with 500 microliters blot wash and left to incubate overnight.

Blot wash was discarded from Cytivia columns and reservoirs washed 6 times with PBS. The columns were centrifuged with 500 microliters PBS and solution discarded. The beaded samples were centrifuged for 1 minute at 14,860 rpm. The supernatant was removed and added to the Cytivia columns. This was centrifuged for 10 minutes to separate the DAT protein from other unwanted molecules in the solution. Thirty microliters of 2x sample buffer/DTT was added to the beads whereas 25 microliters were added to the supernatant. Both samples were analyzed on a western blot.

Results

The aim of this study was to investigate whether arginine at location 615 is required for D2 stimulated insertion of the dopamine transporter (DAT) into the cell membrane. The hypothesis proposed that D2 stimulated insertion of DAT into the cell membrane is reliant on arginine at DAT 615. Further, the hypothesis states that mutants without arginine at this position are likely dysregulated, and in turn affect extracellular dopamine levels.

Expression of mDAT R615C

Western blots were imaged using the Bio-Rad ChemiDoc Imaging system (Figure 1). QuantityOne software was used to quantify the density of the bands (Table 1).

Figure 1

DAT Protein Expression of hDAT, mDAT, and mDAT R615C Imaged on a Western Blot

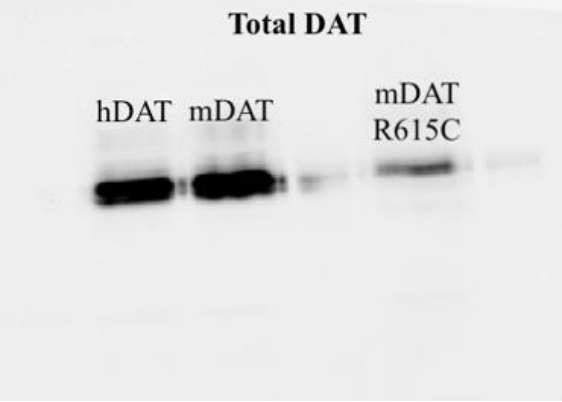

Table 1

Density of Protein Bands of hDAT, mDAT, and mDAT R615C

Name	Density (INT/mm^2)	Expression (%hDAT)
hDAT	32406.52	100
mDAT	31233.75	97
mDAT R615C	14349.55	44

Results showed that mDAT R615C expresses 43% of wild-type expression. Results also showed that the density of hDAT and mDAT was 100% and 99%, respectively. This implies that hDAT and mDAT are expressing fully, as hypothesized, with 100% of protein identified on the block. However, only 43% of DAT was expressed from mDAT R615C. This data suggests that

mDAT R615C does not express as well as the wild-type. In fact, its expression is drastically decreased.

Trafficking of mDAT R615C

Trafficking of mDAT R615C was identified through biotinylation and immunoblotting. Western blots were imaged using the ChemiDoc imaging system (Figure 2). Density of bands was measured using QuantityOne software (Table 2).

Figure 2

Surface vs Internal Proteins in hDAT, mDAT, and mDAT R615C Imaged on a Western Blot

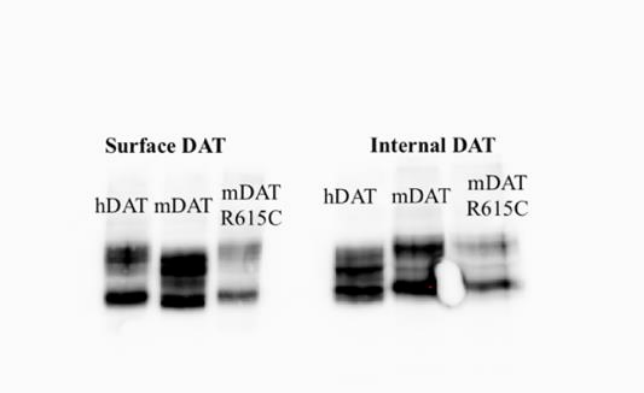

Table 2

Density of Protein Bands of surface vs total expression of hDAT, mDAT, and mDAT R615C

Name	Density of Surface Proteins (INT/mm^2)	Total Density (INT/mm^2)	Surface DAT (%total)
hDAT	33383.08	61696.22	45.89
mDAT	31322.27	66460.70	52.87
mDAT R615C	14548.49	44839.9	30.1

The wild type hDAT and mDAT showed 46% and 53% surface DAT expression, respectively. Contrastingly, mDAT R615C only had a surface expression of 30%. A two-tailed t-test was performed using PRISM software, where the independent variable was the DNA expressed (either wild-type hDAT, wild-type mDAT, or mDAT R615C) and the dependent variable was the density of the protein bands. The 2 cell conditions transfected with mDAT R615C (M = 33.10, SD = 2.95) compared to the 2 cell conditions transfected with mDAT (M = 53, SD = 0.24) demonstrated significantly decreased surface expression of DAT, $t(2) = 6.75$, p = .02. The effect size was calculated using Cohen's d, which yielded a value of 9.59 with a 95% confidence interval (-32.77 to -7.264). This supports the hypothesis that arginine is required for D2 stimulated insertion of DAT into the cell membrane, as the mutant mDAT R615C, which does not have arginine at DAT 615, shows significantly decreased surface expression.

Discussion

Dopaminergic disorders affect millions of people worldwide. Mutants of the dopamine transporter may be a cause of dopamine dysfunction (Herborg & Andreassen, 2018). Overall, in the current research, we found that a mutant of the mouse dopamine transporter, mdAT R615C significantly decreases expression of DAT. Further, surface expression is also decreased by half in the mutant compared to the wild-type. This implies that DAT mutants likely internalize DAT at an increased rate and are not trafficking efficiently to the cell surface. Past work has shown that dysregulation of the dopamine transporter may be linked to dopaminergic disorders (Sulzer, 2016), and this research suggests that arginine at DAT 615 may be required for stable trafficking. Thus, a mutation that gets rid of arginine at DAT 615 may cause dysregulation of dopamine and

thus these findings could have significant implications for dopaminergic signaling and for the development or exacerbation of dopaminergic disorders.

One of the main limitations of this study is that it was conducted using cells, which may not accurately reflect the in vivo effects of mDAT R615C. While cell-based experiments are a useful tool for investigating the molecular mechanisms underlying dopaminergic disorders, they cannot fully capture the complexity of the brain and its interactions with the rest of the body. Another limitation of this study is that the relatively small sample size of 6 may not generalize to all populations. Finally, while this study provides valuable insights into the molecular mechanisms underlying dopaminergic disorders, it is limited to a specific mutation and does not address the broader heterogeneity of these disorders. This is important to note because DAT trafficking can be affected by a multitude of factors outside of the R615C mutation, including PKC activation (Foster & Vaughan, 2011) and GTPase Rab11(Daniels, 2004). These mechanisms should be studied as well to provide a comprehensive understanding of DAT.

Future work should be conducted to analyze how this mutant affects dopaminergic behaviors in animal models, such as mice. Testing the effects of this mutation on dopaminergic behaviors, such as reward processing and motor coordination, could provide valuable insights into the role of mDAT R615C in the pathogenesis of dopaminergic disorders as it would contextualize findings from this paper and provide potential targets for pharmaceutical treatments. This could be achieved through a combination of behavioral assays, electrophysiological recordings, and imaging techniques to fully characterize the functional effects of this mutation in vivo. In addition, it would be interesting to investigate whether the impaired trafficking of mDAT R615C can be corrected with potential therapeutic drugs, which

could have important implications for the development of novel treatments for dopaminergic disorders. There exists pharmaceutical treatments target DAT, such as amphetamine and methylphenidate (Heal & Smith, 2013). However, these treatments work by inhibiting the reuptake of dopamine by the dopamine transporter (Heal & Smith, 2013). With the knowledge that arginine is required for D2 stimulated insertion of DAT into the cell membrane, treatments could try to target the D2 receptor itself to stabilize trafficking. Treating cells with the mutant with potential therapeutic drugs could be a useful initial step in this direction.

Another promising avenue for future research is to investigate the effects of other mutations in the dopamine transporter on dopaminergic signaling and behavior. The dopamine transporter is a complex protein with multiple domains that play important roles in its function, and mutations in different domains could have distinct effects on trafficking and signaling. For example, one study has found that SLC6A3 coding mutations are linked with dopamine transporter deficiency (Ng et al., 2014). Yet another study has found that DAT mutant DAT I312F may be linked with Parkinson's and ADHD (Hansen & Skjorringe, 2014). Investigating a broader range of mutations in the dopamine transporter could help to build a more comprehensive understanding of the molecular mechanisms underlying dopaminergic disorders and could lead to the identification of novel therapeutic targets. Furthermore, exploring the interactions between the dopamine transporter and other proteins involved in dopaminergic signaling, such as receptors and enzymes, could provide valuable insights into the complex interactions that underlie normal and pathological dopaminergic function.

In conclusion, this study provides evidence that arginine at DAT 615 is required for D2 stimulated insertion of DAT into the cell membrane, as mDAT R615C has a significantly

decreased expression and impaired trafficking compared to the wild-type. This indicates that there are less dopamine transporter proteins on cells with the DAT R615C mutation. Further, the decreased surface expression indicates that DAT R615C mutants internalize the dopamine transporter quicker than the wild-type. This indicates that DAT R615C mutants are clearing extracellular dopamine quicker than they are meant to, and thus are limiting the amount of dopamine taken up by the post-synaptic neuron (Bolan, 2007). These findings contribute to our understanding of the molecular mechanisms underlying dopaminergic disorders. They may also have important implications for the development of novel therapeutic interventions. Further research is needed to fully elucidate the role of mDAT R615C in the development of dopaminergic disorders and to explore potential strategies for correcting the trafficking defects associated with this mutation.

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