

Characterization of a Mutant Dopamine Transporter in HEK-293 Cells

Michael Yetman

University of Texas at Austin
Dean's Scholars Honors Thesis
2009

Supervising Professor: R. Dayne Mayfield

Signature Date

Second Reader: Ruth Buskirk

Signature Date

Dean's Scholars Adviser: Arturo De Lozanne

Signature Date

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Abstract:

Recently, research has demonstrated potentiation of the dopamine transporter's function by ethanol. This, coupled with data showing that the modulation of transporter uptake is determined by changes in trafficking and not function, implied that ethanol increases the number of transporters on the cell surface. To identify which amino acid sites may be targets for ethanol, hybrid transporters were constructed that share different ratios of amino acid sequences with the dopamine transporter and the norepinephrine transporter, a similar protein that lacks ethanol-mediated potentiation. Dopamine transporter mutants were created at the four most promising amino acid residues using site directed mutagenesis. Two mutants expressed in *Xenopus* oocytes showed some sensitivity to ethanol and two did not. The quadruple transporter mutant, which contains all four amino acid mutations, demonstrated the most interesting phenotype of inability to take up dopamine.

The present study confirms this lack of function in human embryonic kidney (HEK) cells, a mammalian expression system, and addresses whether or not the IGLF dopamine transporter is on the surface, yet not functional, or is simply not trafficked to the cell surface. To accomplish this, HEK cells stably expressing the wild-type or quadruple mutant transporter were created. Radioactive uptake assays were used to determine the extent to which each cell type was able to take up dopamine. Cell surface biotinylation and Western blots were then used to identify surface transporters. Both preliminary results and the current study showed lack of uptake in HEK cells expressing the mutant, while the latter determined that the mutant protein was present on the cell surface, albeit to a lesser extent than wild-type protein. By demonstrating that the

transporter is indeed being trafficked to the surface, the implication that the mutant is simply not functional as a dopamine transporter becomes the more likely possibility.

Background:

The annual cost of alcohol abuse to society at large has been estimated at 185 billion dollars (U.S. Department of Health and Human Services, 2000). Alcohol related deaths rank number three, just under tobacco usage and poor diet, on the list of unnatural causes of death in the U.S. (Marshall, 2004). Although the molecular targets of ethanol in the central nervous system are widespread and include many of the prominent neurotransmitter systems (glycine, GABA, 5-HT), the most interesting, from an addiction research perspective, is the dopaminergic circuitry that makes up the reward pathway in the brain (Vengeliene, *et al*, 2008). Drugs of abuse directly and indirectly change the firing patterns, metabolism, and gene expression of neurons in these brain regions, most notably the neurons projecting from the ventral tegmental area in the midbrain to the nucleus accumbens (the mesolimbic circuit) (Bowirrat and Oscar-Berman, 2005). These actions disrupt the homeostasis of the system and can lead to the aberrant behavioral phenotypes seen in addicts.

Dopamine is secreted from vesicles by presynaptic dopaminergic neurons onto neurons expressing dopamine receptors on their cell surface. When dopamine is released into the space between the two neurons (known as the synaptic cleft), this neurotransmitter binds to the postsynaptic receptors. This can lead to a wide range of actions on the postsynaptic cell such as inhibition, excitation, or even changes in gene

expression (as mediated by more complex signaling cascades). These effects alter the neurons' ability to depolarize and fire, which in turn determines the physiological output of the cell.

Dopamine signaling is terminated by its removal from the synaptic cleft. Otherwise, it would constantly be bound to its postsynaptic receptors, leading to a sustained, non-variable signal. Although some of the neurochemical diffuses away into areas where it can no longer act on receptors, the primary means by which dopamine is removed from the synapse is via the dopamine transporter (DAT). This protein tightly controls the temporal and spatial activity of dopamine neurotransmission and is absolutely vital to the proper functioning of these circuits.

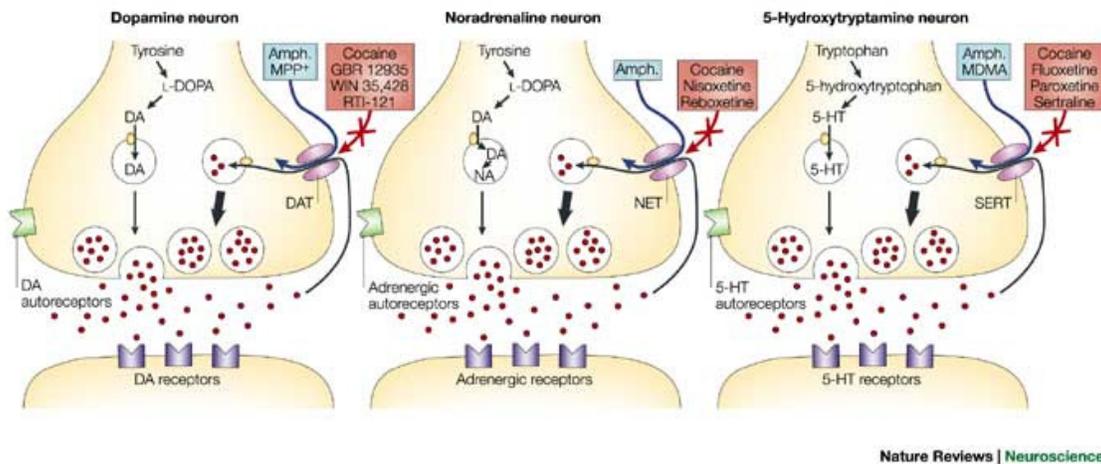


Figure 1. Pre- and perisynaptically located monoamine transporters are the primary means by which the spatial and temporal activity of dopamine transmission is controlled (Torres, et al, 2003).

The dopamine transporter belongs to a family of high-affinity, Na⁺-dependent integral membrane proteins that remove monoamines from the cleft (Torres, *et al*, 2003). Norepinephrine and 5-hydroxytryptamine (serotonin) transporters are examples of two other members of this family. The dopamine and norepinephrine transporters can transport each other's substrates (norepinephrine and dopamine, respectively) (Gu, *et al*, 1994). All three transporters are located pre- and perisynaptically, allowing for exquisite control over transmission (Fig. 1). They also all pass through the lipid bilayer twelve times (Fig. 2b).

Prior research on *Xenopus laevis* oocytes has shown that DAT function is potentiated by ethanol in a concentration-dependent manner, while the function of NET (norepinephrine transporter) is inhibited (Mayfield, *et al*, 2001). These opposing effects and the similarity of the two protein sequences led researchers to create hybrid proteins called chimeras in which regions of DAT and NET were combined. After testing the effects of ethanol on these chimeras, a 76-amino acid stretch encompassing the first intracellular loop was demonstrated to be necessary for the ethanol effect (Maiya, *et al*, 2002). Only four amino acid residues in this loop lack sequence homology between the two transporters, so site directed mutagenesis was used to change each of the four DAT residues (glycine 130, isoleucine 137, phenylalanine 123, and leucine 138, with the number corresponding to location in the primary protein structure) to their corresponding NET residues (threonine, phenylalanine, tyrosine, and phenylalanine respectively). In addition, a quadruple mutant containing all four mutations and dubbed IGLF was created and examined. In *Xenopus* oocytes transfected with each of the five cRNA types, it was

demonstrated that the G130T and I137F mutants lacked ethanol potentiation, while the function of F123Y and L138F showed slight potentiation when compared to wild-type transporter.

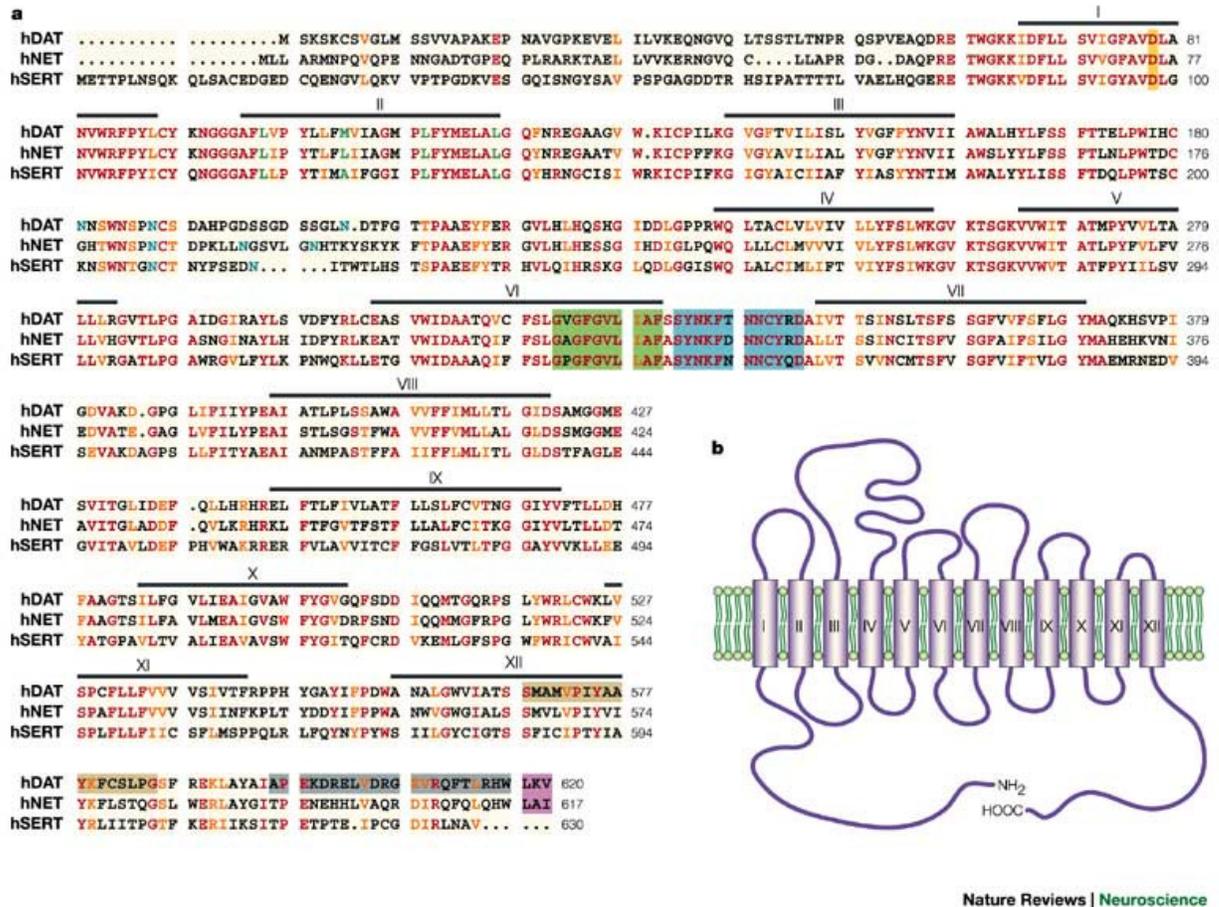


Figure 2. Sequence homology of DAT, NET, and SERT and schematic structure for members of this transporter family (Torres, et al, 2003).

The quadruple mutant showed no signs of transporter activity in radioactive dopamine uptake assays and also showed drastically reduced surface expression, as assayed by tritiated WIN 35,428 binding studies. The latter uses the binding ability of a radioactive isotope to determine surface density. However, these studies were performed

in *Xenopus* oocytes, and it was yet to be determined if the same held for mammalian expression systems. The goal of the current study was to confirm that IGLF DAT was nonfunctional and to determine whether or not it was present on the surface of human embryonic kidney (HEK 293) cells.

Materials and Methods:

Cell culture

HEK 293 cells were purchased from ATCC (Manassas, VA). They were maintained in a humidified atmosphere at 37 degrees Celsius and 5% CO² in Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 0.1% Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO). For uptake experiments, stably expressing WT and IGLF DAT cells were split into poly D-lysine-coated 12-well plates (Becton-Dickinson, Bedford, MA) at a density of 2.0×10^5 cells per well and used two days later.

Stable transfections

HEK 293 cells were stably transfected with IGLF human dopamine transporter cDNA. This construct was obtained from glycerol stocks (Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin) using a Maxi prep kit (QIAGEN) and contained enhanced green fluorescent protein (eGFP) which allowed expressing cells to be identified by their green fluorescent glow. HEK cells were transfected with 20 ug cDNA and 192 ul Polyfect transfection agent (QIAGEN), and selected using 0.6 mg/ml G418 antibiotic (Sigma-Aldrich) until cells were expressing protein in 80-90 % of cells

based on fluorescent readings. Cells were treated with 0.3 mg/ml G418 whenever a change of media was required in order to prevent the proliferation of non-expressing cells. GFP labeled wild type (WT) DAT expressing HEK 293 cells were obtained from active stocks in the Mayfield lab at the Waggoner Center.

[³H]DA uptake assays

WT and IGLF DAT expressing HEK 293 cells were plated in 12-well poly d-lysine coated plates and incubated for two days at 37 degrees Celsius and 5% CO². The cells were washed with serum and antibiotic free DMEM. After letting cells acclimate to 25 degree Celsius temperature for 20 min in an incubator, half of the WT and IGLF cells (3 wells each) were treated with 10 uM 3-hydroxyphenyl N-propyl piperidine (Sigma-Aldrich), a cocaine analog that blocks DAT action, to define nonspecific uptake. 100 nM [³H]DA was added to each for a 3-min time period.

The cells were washed three times with Krebs-Ringer-HEPES (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, pH 7.4) before being lysed in 500 ul of 1% sodium dodecyl sulfate (SDS) for 20 minutes at room temperature with gentle shaking. The lysates were used to quantify [³H]DA uptake for each condition using a liquid scintillation counter.

Cell surface biotinylation

HEK 293 cells stably expressing WT and IGLF DAT were grown to 85-90% confluency in two T-150 culture flasks. The cells were washed twice with ice cold

phosphate buffer saline , pH 7.4 (Gibco) supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS/Mg/Ca), and incubated with 10 ml of 1.0 mg/ml sulfosuccinimidyl-2-(biotinamido)ethyl-1,2-dithiopropionate (EZ-link Sulfo-NHS-SS-Biotin) (Pierce, Rockford, IL) for 30 min at 4 degrees Celsius. Excess biotin was quenched with two washes of ice cold PBS/Ca/Mg supplemented with 100-mM glycine, followed by two additional washes with ice cold PBS/Ca/Mg. Biotinylated cells were scraped into the final wash, collected by centrifugation at 500g for 5 min, broken by ultrasound sonification, and lysed in 0.5 ml radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, and protease inhibitor cocktail) for 30 min on ice with intermittent agitation. The lysate was cleared of insoluble material by centrifugation at 12,000g for 10 min at 4 degrees Celsius, and 1:10 aliquot of the lysate was reserved to be analyzed later as total protein sample (total). Sample (50 uL) was added to 200 uL of Streptavidin coated Dynabeads (Invitrogen, Carlsbad, CA) at 4 degrees Celsius to separate biotinylated (cell surface) from nonbiotinylated (intracellular) proteins via affinity chromatography.

Unbound proteins were removed by 1,000g centrifugation at 4 degrees Celsius for 2 min. After three washes with PBS, pH 7.4, the beads were incubated with 2X Laemmli buffer for 1 h at room temperature while rocking to elute biotinylated (cell surface) proteins bound to Dynabeads. The protein concentration of the lysates was determined using DC Protein Assay kit (Bio-Rad, Hercules, CA), and an equal amount of protein was separated by SDS-PAGE in a 7.5% Tris-HCl Ready gel (Bio-Rad). After the proteins were transferred to polyvinylidene difluoride (PVDF) membrane, the blots were blocked overnight at 4 degrees Celsius in Tris-buffered saline (TBS-T) (10 mM Tris-Cl, pH 8,

150 mM NaCl, 0.01% Tween-20) containing 5% nonfat dried milk (Bio-Rad). Dopamine transporter was detected with a rat monoclonal antibody specific for the N-terminus of DAT (MAB 369, Millipore-Chemicon, Billerica, MA) for 2 h at room temperature while rocking in TBS-T containing 1% nonfat dried milk and 1% bovine serum albumin. After three 10 min washes in TBS-T, blots were visualized by incubating blots in horseradish peroxidase conjugated rat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and exposing to enhanced chemiluminescence solution (Perkin Elmer, Boston, MA). Immunoreactivity was detected using a Kodak Image Station 2000 mm, and band intensities were quantified with NIH Image J software.

Data analysis

Specific [³H]DA uptake was calculated as the difference between total and nonspecific uptake. DAT surface densities were determined by quantifying the optical densities (ODs) from the Western analysis of cell surface and total populations of the transporter. The relative amount of surface DAT was calculated by normalizing surface population ODs to their respective total population ODs of the transporter to obtain a ratio of surface expression. The student's t-test was used to compare WT to IGLF surface-total ratios and specific uptake counts.

Results:

IGLF DAT cells show no specific uptake of [³H]DA

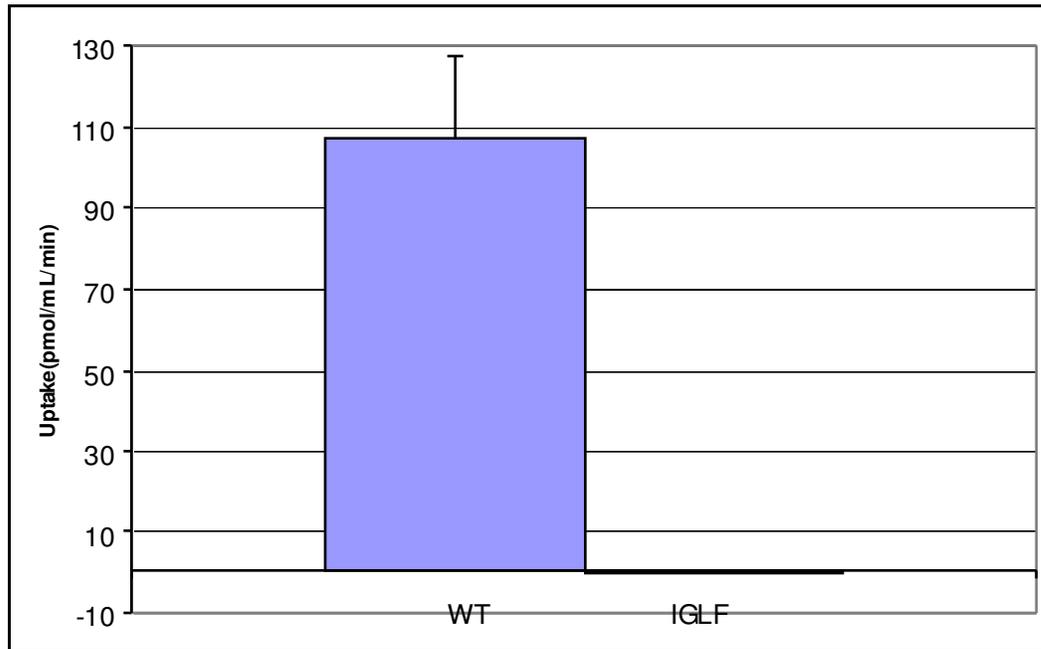


Figure 3. IGLF DAT cells show no uptake of [3H] dopamine.

As shown above in Figure 3, IGLF DAT expressing HEK 293 cells showed no specific uptake of [³H]DA, while WT DAT expressing HEK 293 cells demonstrated uptake capability (WT, 107.040 pmol/ml/min, S.E.M 20.697 pmol/ml/min; IGLF, -0.224 pmol/ml/min, S.E.M 0.311 pmol/ml/min; $p < 0.01$, $n = 3$).

IGLF DAT present of surface of HEK 293 cells

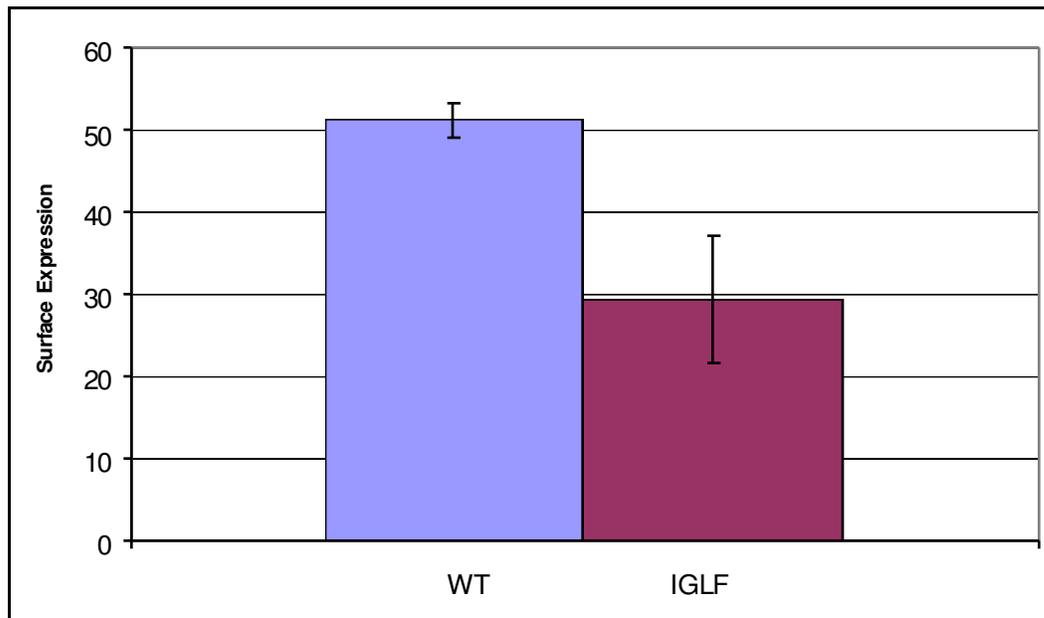


Figure 4. Surface Expression of WT DAT vs. IGLF DAT in HEK Cells as measured in percent of total protein on the surface.

The biotinylation assays confirmed the presence of IGLF DAT on the surface of HEK 293 cells. Although the mean of IGLF DAT relative surface expression was lower than that of WT DAT, they were not significantly different from one another (WT, 51.215%, S.E.M 2.217%; IGLF, 29.292%, S.E.M. 7.775%; $p>0.05$, $n=3$).

Discussion:

Prior to this study, the working hypothesis was that IGLF was unable to take up dopamine due to lack of expression on the cell surface. Lack of surface expression was demonstrated in *Xenopus* oocytes by performing radioactive WIN 35,428 binding assays (Maiya, *et al.*, 2002). These experiments quantified the surface expression levels of DAT by exposing WT and IGLF DAT expressing cells to [3H]WIN 35,428 which binds to the protein at the dopamine binding site. This means that it shows the amount of surface DAT that has an exposed binding site. However, our results in HEK 293 cells showed that the IGLF mutant was indeed expressed on the surface; and, in fact, the expression level was not significantly different from WT DAT. The lack of uptake ability was conserved between the two model systems.

It is possible that this quadruple mutant could cause a structural change that obstructs one of the areas that are associated with substrate translocation (TMD 4 through 8) (Buck and Amara, 1995). Earlier studies dismissed large scale misfolding as a possible explanation by using antibodies that can bind to different regions of DAT (anti-GFP instead of MAB 369) by showing that surface and total expression levels did not change between the two antibodies (Bjornstal, unpublished data). However, both of the antibodies that were used bind to large, relatively isolated regions of the transporter such as the intracellular C-terminus, so we cannot rule out the possibility of smaller scale protein misfolding as causing the lack of function. If the DA translocation domain was disrupted by this mutation, then the disparity between the [3H]WIN 35,428 binding assay data and these results could be reconciled. For if this TMD 4 through 8 region were disrupted, the WIN 35,428 could not bind at all, so the results would seem to imply that

there was no IGLF on the surface, whereas our biotinylation assay more directly measures the surface levels.

It would be interesting to determine if DAT mutant shows any concentration dependent trafficking by ethanol like WT DAT (Riherd *et al.*, 2008). If so, the fact that ethanol does not enhance G130T activity would further complicate the issue (as the G130T mutation is contained in the IGLF construct). This could be easily tested by incubating the WT and IGLF DAT expressing HEK 293 cells in graded concentrations of ethanol prior to biotinylation. Also, testing IGLF DAT's ability to take up norepinephrine, which is readily taken up by WT DAT might help to determine if IGLF really has a disrupted binding site. If significant NE transport takes place, then it is less likely that a folding error is the problem, as it is unlikely the mutation would make the TMD 4 through 8 region amenable to uptake of a different catecholamine.

Although IGLF DAT is trafficked to and stably expressed on the surface of HEK 293 cells in quantities not significantly different from those of WT DAT, in order to determine the mechanism behind this, further tests need to be completed. The hypothesis that misfolding of the TMD 4 through 8 region, which has been implicated in dopamine and norepinephrine translocation, could be further elucidated by testing IGLF DAT expressing cells' ability to take up [³H]NE, while testing whether surface expression is potentiated by the addition of ethanol could help us to understand the puzzling fact that a quadruple mutant (IGLF) containing a single mutation (G130T) with a functionally significant phenotype would lack said phenotype.

In conclusion, this study set out to determine the location of the nonfunctional quadruple mutant IGLF DAT and found that it was indeed present on the surface of HEK

293 cells. Coupled with its lack of ability to translocate dopamine from the extracellular space, this implies that the mutant is “broken” in some way. We have concluded that the most likely explanation is that the four amino acid changes making up this mutant misfold the protein in such a way as to obstruct the substrate binding site. This explains the disparity between previous studies with WIN 35,428 and the present study’s cell surface biotinylation results.

Acknowledgments:

I thank Drs. Dayne and Jodi Mayfield, Nicole Riherd, and Olaf Bjornstal for mentorship and contributions to this project. Funding for this project was provided in part by the University of Texas Undergraduate Research Fellowship.

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