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Regulation of the Dopamine Transporter: A Role for Ethanol and Protein Interactions

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Regulation of the Dopamine Transporter: A Role for Ethanol and Protein Interactions

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Regulation of the Dopamine Transporter: A Role for Ethanol and Protein Interactions

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The dopamine (DA) transporter (DAT) serves to clear released DA from the synaptic cleft and is an important part of the mesolimbic DA system, which mediates the rewarding and reinforcing effects of various drugs of abuse. Several studies suggest that the function of DAT is regulated by protein-protein interactions and signaling systems that alter cellular trafficking of DAT. Ethanol potentiates DAT function in Xenopus oocytes expressing DAT in a manner consistent with altered cellular trafficking. In contrast to ethanol's effects on DAT, the function of the related norepinephrine transporter (NET) is inhibited by ethanol. To delineate mechanisms of ethanol action on DAT, chimeras were generated between DAT and NET. The results of these as well as site directed mutagenesis experiments revealed ethanol sensitive sites in the first

intracellular loop of DAT. The absence of consensus phosphorylation sites in this loop led to the hypothesis that ethanol modulates the interaction between DAT and a putative regulatory protein important for ethanol-induced trafficking of DAT and that this interaction occurs at the first intracellular loop. To identify proteins and signaling pathways that might regulate DAT function, an interaction proteomics based approach was used to isolate and identify proteins associated with DAT. These studies revealed that DAT is part of a large multiprotein complex consisting of 21 proteins that can be classified as ion channels, trafficking proteins, extracellular matrix associated and cytoskeletal proteins. Finally, the effects of ethanol on DAT trafficking were ascertained by examining ethanol-induced changes in DAT function in several cell types. Studies on MDCK cells stably expressing GFP-DAT suggest that ethanol potentiates DAT function in this cell type. SH-SY5Y cells stably expressing DAT were also examined for ethanol effects on DAT function. Ethanol produced a 25% enhancement in DAT function in these cells, which was not statistically significant. The effects of ethanol on DAT trafficking in neuronal cells were observed by using a sindbis viral construct encoding GFP-DAT. The experiments outlined above have led to the identification of a novel role for DAT in ethanol-induced neuroadaptation and in the identification of several novel proteins that could modulate DAT function.

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List of Abbreviations

DAT	Dopamine transporter
DA	Dopamine
[3H]WIN 35,428	2β Carbomethoxy-3β-(4-fluorophenyl)[^{3}H] tropane,
NET	Norepinephrine transporter
SERT	Serotonin transporter
GLYT	Glycine transporter
PKC	Protein kinase C
PKA	Protein kinase A
NMDA	N-Methyl D-Apartate
$5HT_{2C}$	Serotonin 2C receptor
TMD	Transmembrane domain
G130T	Glycine at position 130 mutated to threonine
F123Y	Phenylalanine at position 123 mutated to tyrosine
I137F	Isoleucine at position 137 mutated to phenylalanine
L138F	Leucine at position 138 mutated to phenylalanine
VTA	Ventral tegmental area
NAc	Nucleus accumbens
aCSF	Artificial cerebrospinal fluid
PICK1	Protein interacting with C Kinase 1
Hic-5	Hydrogen peroxide-inducible protein 5

PCA	Principal component analysis
GFP	Green fluorescent protein
MDCK	Madin Darby canine kidney cells
HEK	Human embryonic kidney cells

Chapter 1

Introduction

Neurons rely upon chemical neurotransmitters for rapid (order of milliseconds) and precise communication with each other and with target organs. Neurotransmitters released into the synaptic cleft activate specific receptors on the postsynaptic neuron, which in turn causes opening of ion channels and activation of signal transduction cascades in the post-synaptic cell (Kandel et al., 1991), resulting in transmission of the signal. The lifetime of the neurotransmitter released into the synaptic cleft is predominantly regulated by specific proteins that mediate their reuptake into the presynaptic cell. Julius Axelrod was the first to introduce the concept of reuptake by demonstrating that synaptically released noradrenaline is taken up by specific transporters in sympathetic nerve terminals (Hertting and Axelrod, 1961). Subsequently, reuptake mechanisms have been discovered for serotonin, dopamine (DA), γ-amino butyric acid (GABA), glycine and a number of other neuromodulators. Neurotransmitter transporters have been shown to play a very important role in sculpting the temporal and spatial aspects of neurotransmission. They are also the sites of action of various psychostimulants and therapeutic agents used for treating attention deficit hyperactivity disorder and depression (Torres et al., 2003b).

The DA transporter (DAT) belongs to the family of Na^+ and Cl^- dependent transporters and functions to clear released DA from the synaptic cleft and effectively reduce the concentration of dopamine at both pre- and post-synaptic dopamine receptors

(Nelson, 1998) . Thus, DAT regulates the spatial and temporal aspects of dopaminergic synaptic transmission and is an integral part of the mesostriatal DA system. This system plays a central role in mediating the rewarding and reinforcing effects of various drugs of abuse such as ethanol (Brodie and Appel, 1998; Brodie et al., 1990; Weiss et al., 1993). DAT is also the site of action for various psychostimulants like cocaine, mazindol, methylphenidate and amphetamine (Nelson, 1998). These findings underscore the importance of DAT in mediating the effects of these drugs of abuse.

1.1 Cellular localization of DAT

DAT is exclusively expressed in midbrain dopaminergic neurons where it is expressed in the cell bodies, dendrites and axonal membranes. Midbrain dopaminergic neurons originate in the substantia nigra and ventral tegmental area (VTA) region and project mainly to the striatum, nucleus accumbens (NAc), amygdala, and the prefrontal cortex (Bonci et al., 2003; Wise, 2002). DAT is localized to the plasma membrane and smooth endoplasmic reticulum of dendrites and dendritic spines in the substantia nigra (Nirenberg et al., 1996). The subcellular distribution of DAT in the VTA is very similar to that in the substantia nigra, with most of the labeling found to be associated with plasma membranes of dendrites and dendritic spines (Nirenberg et al., 1997b). DAT is expressed highly in the striatum. It is estimated that there are 150-300 molecules μm^{-3} of DAT in the striatum (Cragg and Rice, 2004). Immunogold labeling of DAT in the dorsolateral striatum was localized to the axons and axon terminals (Nirenberg et al., 1996). In the NAc, differential expression of DAT is observed in the NAc shell region as

compared to the core. A majority of the labeled DAT was found to be associated with the cytoplasmic surfaces of axons, however small amounts of DAT immunoreactivity were also associated with small synaptic vesicles in this brain region (Nirenberg et al., 1997a). The presence of DAT in intracellular compartments suggests that targeting mechanisms exist to transport DAT to specific perisynaptic sites.

1.2 Structure and function of DAT

Monoamine transporters including DAT are integral membrane proteins. Hydrophobicity analysis predicts that DAT contains 12 transmembrane domains (TMD) (Giros and Caron, 1993). The N- and C-termini of the transporter are intracellular and a large extracellular loop between TMD3 and TMD4 contains several consensus glycosylation sites (Figure 1). DAT mediates uptake of DA as well as NE, although the uptake of NE is inefficient compared to DA (Chen et al., 2004). Other substrates include amphetamine and the neurotoxin 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP). An overview of the mechanism of DA uptake as well as the amino acid residues implicated in substrate binding and translocation is included in this section.

1.2.1 Structural basis of DA uptake

Transport of DA by DAT is dependent upon extracellular Na^+ and Cl^- ions. During a transport cycle, two Na^+, one Cl^- and one charged molecule of DA bind to the transporter and are translocated resulting in the net movement of two positive charges per

molecule of DA. The driving force for DA uptake by DAT is generated by the plasma-membrane protein Na^+, K^+-ATPase, which pumps Na^+ out and K^+ into the cell (Chen and Reith, 2003). The exact mechanism and regions of the transporter involved in this coupled transport process remain unclear. A crystal structure is not available currently for any member of the Na^+/Cl^-dependent neurotransmitter transporter family. A wide variety of techniques including substituted cysteine accessibility method and zinc site engineering have been employed to delineate amino acid sequences and mechanisms important for the transport process. Electrophysiological studies indicate that some aspects of DAT function can be compared to ion-channel like mechanisms. Several models have been proposed to explain the channel-like properties of DAT. The most accepted of these is the alternating access model. In this model a transporter is thought to resemble a channel but with gates at both the cytoplasmic and the extracellular end. The gates open sequentially to allow binding of substrates and ions and hence produce coupled transport (Lester et al., 1996). The transport rates for DA transport by DAT are relatively modest and estimated to be 2-5 molecules of DA per second per molecule of DAT (Cragg and Rice, 2004).

Studies of transporter-associated currents in heterologous systems suggest at least three different conductances: 1) a substrate coupled conductance 2) a constitutive leak conductance that is blocked by both substrates and inhibitors of the transporter and 3) uncoupled transport-associated conductance (Ingram et al., 2002). A physiological role for these DAT associated conductances was recently highlighted by two studies. The first study suggests that synaptic activation of somatodendritic DA release in the substantia

nigra is due to DAT-mediated reverse transport of DA. However, the mechanism by which DAT function is reversed to cause DA efflux remains unclear (Falkenburger et al., 2001). A second study describes an endogenous DAT-mediated anion conductance in rat midbrain DA neurons in culture. The anion conductance promotes excitability of DA neurons in culture and suggests an alternative mechanism for DAT-mediated release of DA (Ingram et al., 2002). Clearly, the demonstration of multiple conducting states of the transporter hints at the complexity of the DA uptake process.

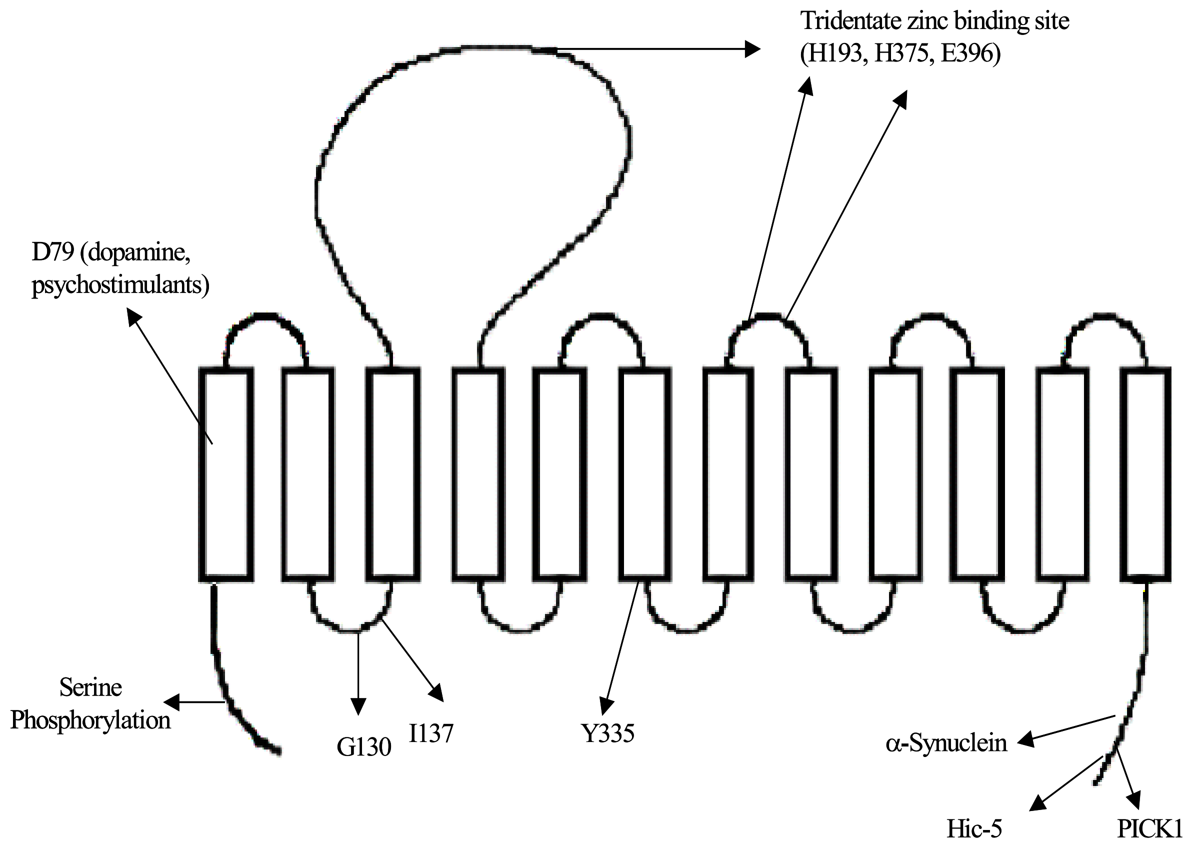

Figure 1: Schematic of the DAT with positions of amino acid residues important for substrate and Zn^{2+} binding, ethanol modulation of DAT function, PKC phosphorylation, and interaction with proteins that modulate DAT function.

DA uptake by DAT has been shown to be dependent upon the membrane potential (voltage dependent) in expression systems. In *Xenopus* oocytes expressing DAT, DA uptake increases with hyperpolarization and decreases with depolarization (Sonders et al., 1997). However, a correlation between membrane potential and DAT function has not been observed under more physiological conditions in cultured dopamine neurons (Prasad and Amara, 2001). Activation of the dopamine D2 autoreceptor has also been shown to inhibit DAT and affect DA clearance. D2 antagonists have been shown to inhibit DAT function in brain (Cass and Gerhardt, 1994) and in slice preparations (Meiergerd et al., 1993). D2 regulation of DAT function has been shown to be voltage-independent and likely involves second messenger systems (Mayfield and Zahniser, 2001).

1.2.2 Structural basis for substrate binding and translocation.

Chimeric constructs between DAT and NET were utilized to identify regions on the transporter important for substrate binding, translocation, and cocaine affinity (Buck and Amara, 1994; Buck and Amara, 1995; Giros and Caron, 1993). These studies suggest that a region of the transporter encompassing TMD6 through TMD8 is important for cocaine affinity while amino acid residues spanning TMD9 through the carboxyl-terminal tail are important for high affinity substrate binding. The regions of the transporter important for substrate translocation are somewhat controversial with one study implicating a region spanning TMD4 through TMD8 (Giros et al., 1994) and another study suggesting that the first five transmembrane domains are important for this function

(Buck and Amara, 1994). Studies utilizing chimeras between human and bovine DAT also implicate TMD3 in DA uptake. Amino acid residue D79 in the first TMD also seems to be important for substrate binding (Loland et al., 2002).

Accessibility of native cysteines to thiol-reactive componds (MTSET, MTSEA) and Zn^{2+}-site engineering have been used to examine the conformational changes associated with substrate binding and translocation. Experiments with thiol-specific reagents have revealed that the amino acid residue at position 342 is involved in conformational changes that accompany substrate translocation. Mutation of a tyrosine (Y335) residue in the third intracellular loop has also been shown to abolish DA uptake suggesting a role for this loop in substrate translocation (Chen et al., 2000). Experiments have also revealed that Zn^{2+} in μM concentrations is a potent inhibitor of DA transport. Three residues have been implicated in forming a tridentate Zn^{2+} binding site on DAT. These include histidine at position 193 in the second extracellular loop, histidine at position 375 in TM7 and glutamate at position 396 in TM8. Although these residues appear to be far apart their role in Zn^{2+} binding suggests that these residues are in close proximity in the tertiary structure of DAT. Recent studies also suggest that Zn^{2+} can also enhance an uncoupled chloride conductance associated with DAT in Xenopus oocytes expressing DAT (Bjerggaard et al., 2004; Meinild et al., 2004).

1.2.3 Oligomerization of DAT

Recent studies indicate that DAT at the cell membrane can exist as a multimer

(Hastrup et al., 2001). Several regions of the transporter have been implicated in oligomerization of DAT. A glycophorin-like motif in TMD6 has been implicated in the formation of DAT dimers. This was demonstrated by symmetrical crosslinking of cysteine residues in the extracellular face of TMD6 (Hastrup et al., 2001). It has been shown that mutations in the leucine repeat region of TMD2 can abolish plasma membrane delivery of DAT and these mutants also display a dominant negative phenotype when co-expressed with wild type DAT (Torres et al., 2003a). Fluorescence resonance energy transfer (FRET) studies of tagged human DAT molecules have shown that DAT can exist as an oligomer in the endoplasmic reticulum (ER) and that DAT is internalized as an oligomer upon treatment with drugs like amphetamine, cocaine, and phorbol esters (Sorkina et al., 2003). Recent cysteine cross-linking experiments have shown that DAT may exist at the cell surface as a tetramer composed of two symmetrical homo-dimers (Hastrup et al., 2003). These results suggest that discrete regions on the transporter are involved in transporter oligomerization and reiterate the importance of DAT oligomerization for steady state trafficking of DAT to the cell surface.

1.2.4 Glycosylation of DAT

DAT is a heavily glycosylated (glycosylation accounts for ~ 30% of the molecular weight of DAT) protein *in vivo*. The large extracellular loop between TMD3 and TMD4 has three asparagine residues at position 181, 188, and 205 which are glycosylated (Giros et al., 1994). Functional analysis of a mutant transporter in which all three of the

asparagine residues were mutated to alanine revealed that these residues are important for proper trafficking of the transporter to the cell surface. However, the fraction of the mutant transporters that were expressed on the cell surface were able to function normally suggesting that glycosylation is important for cell surface expression but not substrate affinity (Torres et al., 2003a).

1.2 Effects of psychostimulants on DAT function

Several psychostimulants and drugs of abuse mediate their effects by interfering with the DA uptake process. Cocaine is a potent inhibitor of DAT function (Loland et al., 2002). Cocaine competes with DA for DAT binding and hence competitively inhibits DA transport. Amino acid residues on DAT that mediate cocaine binding are not fully understood. An aspartate residue at position 79 is important for cocaine affinity of DAT (Loland et al., 2002). Amphetamine is a DAT substrate and is transported into the cell. Once inside the cell, amphetamine can, by mechanisms that are not clearly understood, cause efflux of DA from vesicular stores into the cytoplasm, and release of DA by DAT by reverse transport (Torres et al., 2003b). Hence, both cocaine and amphetamine can cause an increase in extracellular DA concentrations which is thought to mediate the rewarding and reinforcing properties of these drugs (Torres et al., 2003b). Cocaine has also been shown to be rewarding in DAT-knockout animals suggesting the presence of alternative sites of action for this drug (Gainetdinov et al., 2002).

1.4 Regulation of DAT

A number of studies in recent years suggest that cell surface DAT levels can be regulated acutely by a number of different second messenger pathways both in vitro and *in vivo*. This mechanism of regulation of transporter function could potentially play an important role physiologically because the uptake of DA by DAT is a slow process (Cragg and Rice, 2004) and an acute increase in the cell surface expression of DAT may facilitate the clearance of DA released during burst firing (Mortensen and Amara, 2003).

1.4.1 Regulation by PKC and other kinases

Activation of protein kinase C (PKC) by phorbol esters has been shown to induce a rapid downregulation of DAT in several cells and striatal synaptosomal preparations (Daniels and Amara, 1999; Melikian and Buckley, 1999; Zhu et al., 1997). This has been shown to be a dynamin- and clathrin-dependent process (Daniels and Amara, 1999). The fate of the internalized transporters is somewhat controversial with one study reporting that the internalized transporters are targeted to lysosomes for degradation (Daniels and Amara, 1999) and another study reporting that transporters are internalized into endosomes that are recycled to the cell surface (Melikian and Buckley, 1999). In PC12 cells transporters are constantly in a state of flux, constitutively endocytosing and then recycling back to the cell surface. Upon activation of PKC, endocytosis of DAT is enhanced and the recycling of endocytosed DAT to the cell surface is inhibited (Loder

and Melikian, 2003). The DAT sequence has several consensus sites for phosphorylation by PKC and protein kinase A (PKA, (Giros and Caron, 1993)). Mutagenesis studies have shown that these consensus sites are not essential for PKC-mediated internalization of DAT, suggesting a role for accessory proteins in PKC-regulation of DAT function (Chang et al., 2001). A recent study suggests that deletion of the N-terminus of the transporter results in a mutant transporter with normal DA uptake characteristics but impaired PKC regulation (Granas et al., 2003). Acute exposure to PKC inhibitors have also shown to inhibit amphetamine-mediated DA efflux in rat striatal slices in a calcium- and DAT-dependent manner (Kantor and Gnegy, 1998).

Stimulation of adenylyl cyclase by forskolin has been shown to stimulate DA transport in rat striatal tissue in a PKA-dependent manner (Batchelor and Schenk, 1998). Protein tyrosine kinases have also been implicated in regulating DAT function in a manner consistent with altered cellular trafficking. Inhibition of protein tyrosine kinases results in a decrease in DAT function. Phophatidylimosital-3-kinase and mitogen activated protein kinase (MAPK) pathways have also been implicated in regulating DAT function (Carvelli et al., 2002; Lin et al., 2003).

1.4.2 Regulation by substrates and drugs of abuse

Drugs of abuse like amphetamine can also regulate DAT function by causing internalization of cell surface transporters in a dynamin- and clathrin-dependent manner (Saunders et al., 2000). Experiments carried out in human embryonic kidney (HEK) cells

demonstrated that acute exposure to cocaine enhances DAT activity in a time-dependent manner by increasing the number of functional transporters at the cell surface (Daws et al., 2002). Cocaine also increases the number of DAT binding sites in neuro 2A (N2A) cells by altering the intracellular trafficking of DAT (Little et al., 2002). Dopamine has also been shown to regulate DAT expression levels. In HEK cells expressing DAT, exposure to DA has been shown to cause internalization of DAT (Saunders et al., 2000). Repeated exposure to DA has been shown to cause a decrease in DAT associated currents in Xenopus oocytes expressing DAT. DAT mediated DA clearance *in vivo* was also inhibited by repeated exposure to substrates (Gulley and Zahniser, 2003). The results outlined in this section suggest that alterations in membrane trafficking predominantly regulate DAT function at the cell surface. Hence, an understanding of signaling pathways and proteins that modulate DAT trafficking would aid in the development of therapeutic agents that could modulate DAT function.

1.5 Protein interactions that modulate DAT function

Thus, there is substantial evidence that DAT undergoes regulated trafficking both *in vitro* and *in vivo*, and this may be important for the functional and pharmacological sensitivity of the transporter. It is likely that transporter-interacting proteins play a key role in these modes of regulation. The DAT sequence contains numerous motifs for protein-protein interactions, including a PDZ binding domain at the C-terminus, an N-terminal leucine repeat and two di-leucine motifs (Torres et al., 2003b). Currently, three

proteins have been identified that alter DAT function by interacting directly with the transporter. These proteins were identified by the yeast two-hybrid approach using the C-terminal tail of DAT as bait. These are α-synuclein, the PDZ domain containing protein PICK1 (protein interacting with C kinase 1) and Hic-5 (hydrogen peroxide-inducible protein 5). PICK1 potentiates DAT function by enhancing cell surface distribution of the transporters in cultured neuronal cells and in HEK293 cells. PICK1 was originally identified as a protein that interacts with the catalytic domain of PKC-α (Torres et al., 2001). A recent study however suggests that the PDZ domain of DAT (and hence its interaction with PICK1) is not necessary for normal surface targeting of DAT in HEK293 cells and in N2A cells (Bjerggaard et al., 2004). It is possible that PICK1 could play a modulatory role in PKC-mediated regulation of DAT function. The interaction of DAT with Hic5 results in inhibition of DAT function by downregulating the number of cell surface transporters. Hic5 is a LIM-domain containing adaptor protein that has been shown to interact with several kinases like focal adhesion kinase and fyn kinase. Hence, Hic5 could play a role in linking DAT to diverse cell signaling pathways (Carneiro et al., 2002). α-Synuclein was the first protein that was shown to interact with the c-terminal tail of DAT (Lee et al., 2001). The functional consequence of DAT-α-synuclein interaction remains controversial with one group suggesting that DAT-interaction with α-synuclein enhances clustering of DAT and cellular uptake of DA (Lee et al., 2001); however, one study suggests that α-synuclein inhibits DAT function (Wersinger and Sidhu, 2003). Recent studies suggest that synaptic proteins function as part of multiprotein complexes. For example, the NMDA receptor is part of a protein complex

comprising of 77 proteins (Husi et al., 2000). It is likely that DAT is also part of a large multiprotein complex and a proteomics-based approach could aid in elucidating components of the DAT proteome.

1.6 Mass spectrometry-based proteomics

The term proteome refers to the set of all proteins expressed in a cell at a given time under a given set of conditions (Mann et al, 2003). Mass spectrometry is an analytical technique used to identify proteins by accurately determining the mass of fragments obtained by proteolytic digestion of the proteins and comparing the mass obtained to theoretical digests of known and predicted proteins in databases. Proteomics can be classified into two types: expression and interaction proteomics. Expression proteomics refers to large-scale studies of variations in protein expression. Interaction proteomics refers to the identification of protein–protein interactions within a cell (Aebersold and Mann, 2003). Protein-protein interactions can be enriched by antibody based or affinity purification techniques. The classical method for identifying protein-protein interactions is the yeast two-hybrid method, in which the bait proteins are expressed as a fusion to the DNA-binding domain of a yeast transcription factor and the cDNA library insert is expressed as a fusion protein to the activation domain of the transcription factor. Interaction between the bait and library proteins results in the activation of the transcription factor and a reporter gene. The yeast two-hybrid technique has been used successfully in biology to identify protein-protein interactions and is

amenable for high throughput applications. Mass spectrometry based approaches has advantages over the classical yeast two hybrid approaches in that the interactions are identified in authentic cellular contexts, and hence do not require extensive validations (Mann et al, 2003). Mass spectrometry is very sensitive and highly accurate and can identify proteins whose abundance is in the low femtomole levels.

Two most commonly used mass spectrometry approaches are MALDI (Matrix Assisted Laser/Desorption Ionization) and ESI (Electrospray Ionisation) mass spectrometry. The techniques differ in the methods used for ionization of peptides. MALDI analysis requires the digested sample to be mixed in a UV-absorbing matrix and the peptides are ionized with a UV laser beam. In case of ESI, the peptide solution is ionized by high voltages to form multiply protonated species (Rappsilber et al., 2003). Thus, ESI has higher mass accuracy than MALDI. The first step in identifying a protein by mass spectrometry is to digest the protein with a specific protease such as trypsin. The resulting peptides are then introduced into a mass spectrometer where they are ionized and their masses are measured. Sequence information is generated by tandem mass spectrometry where ions of interest are selected, fragmented, and the masses of the fragmented ions are used to generate an MS/MS or daughter spectrum. Complete sequence information of the entire protein is not determined by mass spectrometry but sequence information obtained from a few peptides is used to query a database and identify the protein (Perkins et al., 1999). Mass spectrometry based approaches have been used successfully to elucidate the NMDA receptor proteome (Husi et al., 2000) and the 5HT2c receptor proteome (Becamel et al., 2002). Novel interaction partners for the Ca^{2+}-

activated potassium channel were also identified using an interaction-proteomics based approach (Becamel et al., 2002).

1.7 Ethanol and dopaminergic systems

A number of studies suggest that ethanol can affect the firing of midbrain DA neurons (Brodie and Appel, 1998; Brodie et al., 1990; Weiss et al., 1993). The resulting increase in extracellular DA is thought to mediate the rewarding and reinforcing effects of ethanol. However, recent studies also suggest that increase in extracellular DA concentrations may not correlate with reward (Cannon and Bseikri, 2004). Self-administration of ethanol in alcohol-preferring rats and genetically heterogeneous wistar rats has been shown to increase levels of extracellular DA (Gonzales and Weiss, 1998; Katner et al., 1996). Since, DAT is the primary mechanism for DA uptake, an ethanol-induced change in DAT function could also profoundly affect dopaminergic synaptic transmission. Ethanol affects the function of several members of the Na^+ and Cl^- dependent family of transporters. Experiments in HEK-293 cells stably transfected with glycine transporters (GLYT1 and 2) have shown that relatively high concentrations of ethanol (100-200 mM) inhibit uptake of [^{3}H]glycine by GLYT2 and potentiate [^{3}H]glycine uptake by GLYT1 (Nunez et al., 2000). Also, acute exposure to ethanol has been shown to enhance serotonin transporter (SERT) activity in rat cortical, hippocampal and brainstem synaptosomes (Alexi and Azmitia, 1991; Eshleman et al., 1994).

High-speed chronoamperometry studies examining the effect of ethanol on clearance of locally applied DA in the dorsal striatum of rats suggest that ethanol enhances the rate of clearance of DA (Sabeti et al., 2003). Electrochemical studies also suggest that ethanol alters DA clearance *in vivo* (Lin et al., 1993). These results suggest that ethanol could alter DAT-mediated DA uptake *in vivo*. Polymorphisms in DAT gene have been linked to alcohol and drug addiction (Hitri et al., 1994; Tiihonen et al., 1995). DAT density is altered in human alcoholics experiencing relapse to alcohol abuse (Tiihonen et al., 1995). DAT density is increased in ethanol preferring monkeys (Mash et al., 1996). Chronic ethanol exposure also produces changes in DAT density in rat striatal membranes (Hamdi and Prasad, 1993). In COS-7 cells pre-loaded with DA, ethanol has been shown to enhance amphetamine-mediated efflux of [^{3}H] DA (Eshleman et al., 1994). Acute ethanol (10-100 mM) enhances DAT-mediated [^{3}H]DA uptake and transporter-associated currents in a time- and concentration-dependent manner (Mayfield et al., 2001). This potentiation of transporter function was accompanied by an increase in the number of functional cell surface transporters suggesting that ethanol affects transporter function by altering DAT trafficking.

1.8 Research Aims

Electrochemical experiments suggest that local application of ethanol inhibits NET function in the rat cerebellar cortex (Lin et al., 1993). Ethanol also inhibits NET function in HeLa cells that express NET. Experiments using DAT/NET chimeras and site

directed mutagenesis studies suggest that discrete amino acid residues in the first intracellular loop of DAT are important for ethanol regulation of DAT function. The absence of consensus sites for PKC or PKA phoshorylation in the first intracellular loop suggest that ethanol regulation of DAT function may not be due to increased phosphorylation of the protein. Hence, it is hypothesized that ethanol modulates the interaction between DAT and a putative regulatory protein important for ethanol-induced trafficking of DAT and that this interaction occurs at the first intracellular loop. However, not much is known about proteins that interact with DAT. The objective of this study is to identify regions on DAT that confer ethanol sensitivity and to delineate the mechanism of ethanol action on DAT. The specific aims of this project are as follows.

Aim 1. *To characterize DAT function in the ethanol insensitive mutant G130T DAT.*
Preliminary results have shown that the mutations G130T, I137F and L138F in the first intracellular loop of DAT render the transporter insensitive to ethanol effects. The objective of this aim is to elucidate the kinetics of dopamine uptake in the mutant G130T. Also, the first intracellular loop of DAT will be replaced with that of norepinephrine transporter (NET) and the effect of ethanol on this mutant will be analyzed.

Aim 2. *To determine whether ethanol alters cell surface trafficking of DAT.*
Experiments to address this question will be carried out in mammalian cell lines. GFP-tagged wild type and G130T DAT will be transfected into mammalian cell lines and ethanol-induced trafficking of DAT will be visualized both in real time (using live cells)

and in fixed cells by confocal microscopy. Cell surface biotinylation and immunofluoresence techniques will also be used to study ethanol-induced changes in transporter trafficking in these cells.

Aim 3. *To identify novel proteins interacting with the first intracellular loop of DAT.* Preliminary results have shown that the first intracellular loop of DAT is important for ethanol's enhancement of DAT function. An interaction proteomics based approach will be used to investigate proteins that interact differentially with the first intracellular loop of DAT.

1.9 Chapter Overview

The second chapter describes the methodologies employed to address the questions raised in the specific aims. They include isolation and injection of Xenopus oocytes, [^{3}H]DA uptake assays in oocytes and in different cell lines, mass spectrometry based proteomics, immunoisolation of DAT-interacting proteins from the striatum etc.

The third chapter addresses questions raised in Aim 1. Previous studies have shown that ethanol enhanced [^{3}H]dopamine uptake in *Xenopus* oocytes expressing the dopamine transporter (DAT). This increase in DAT activity was mirrored by an increase in the number of transporters expressed at the cell surface. In the present study, ethanol potentiated the function of DAT expressed in HeLa cells but inhibited the function of the related norepinephrine transporter (NET). Chimeras generated between DAT and NET

were examined for ethanol sensitivity and demonstrated that a 76 amino acid region spanning transmembrane domains (TMD) 2 and 3 was essential for ethanol potentiation of DAT function. The first intracellular loop between TMD2 and 3 of DAT, which differs from that of NET by four amino acids, was explored for possible sites of ethanol action. Site directed mutagenesis was used to replace each of these residues in DAT with the corresponding residue in NET and the resulting cRNA's were expressed in *Xenopus* oocytes. We found that mutations G130T or I137F abolished ethanol potentiation of DAT function, whereas the mutations F123Y and L138F had no significant effect. These results identify novel sites in the first intracellular loop important for ethanol modulation of DAT activity.

The fourth chapter describes the isolation of proteins associated with DAT and *in silico* analysis of these DAT-associated proteins. Monoamine transporters play a key role in neuronal signaling by mediating reuptake of neurotransmitters from the synapse. The function of the dopamine transporter (DAT), an important member of this family of transporters, is regulated by multiple signaling mechanisms which result in altered cell surface trafficking of DAT. Protein-protein interactions are likely critical for this mode of transporter regulation. In this study we identified proteins associated with DAT by immunoprecipitation followed by mass spectrometry. We identified 19 proteins with diverse cellular functions that can be classified into trafficking proteins, cytoskeletal proteins, ion channels and extracellular matrix-associated proteins. DAT was found to associate with the potassium channel Kv2.1 and synapsin Ib, a protein involved in regulating neurotransmitter release. In addition, we have used our proteomics data to

query mouse genetic and genomic databases to provide a novel *in silico* assessment of the biological relevance of these interactions.

Experiments done using the *Xenopus* expression system suggest that ethanol alters DAT function by a mechanism consistent with altered cell surface trafficking of DAT. To visualize these trafficking events, ethanol effects on DAT function was examined in several different cell lines transiently or stably expressing GFP-tagged DAT. Sindbis viral particles expressing GFP-tagged DAT were generated and used to infect neuronal cells in culture as well as explants from the VTA. The results of these experiments will be summarized in chapter 5.

The sixth chapter is an overall discussion of the results obtained and conclusions that can be drawn from the results.

Chapter 2

Materials and methods

This chapter describes the methods used in addressing specific aims 1, 2, and 3.

2.1 Transporter chimeras:

Chimeras between the human NET (Pacholczyk et al., 1991) and rat DAT (Nelson, 1998) were constructed using a restriction site independent method as previously described (Buck and Amara, 1994; Buck and Amara, 1995). Sequence analysis (partial) identified the precise location of each chimera junction and confirmed that the junction was in frame. Previous data indicate that most junctions within conserved regions of DAT and NET are not disruptive of transporter function (Blakely et al., 1991; Fuerst et al., 1987).

2.2 Substrate uptake in transfected mammalian cells:

Wildtype and chimeric transporter cDNAs were expressed in HeLa cells using the vaccinia/T7 transient expression system as previously described (Blakely et al., 1991; Fuerst et al., 1987). This method employed a recombinant vaccinia virus strain that encodes a bacteriophage T7 RNA polymerase and allows rapid high-level expression of proteins encoded by plasmids bearing T7 promoters (Blakely et al., 1991; Fuerst et al.,

1987). Briefly, HeLa cells were plated ($2x10^5$ cells/well) into 24-well tissue culture plates and infected the following day. The recombinant vaccinia virus strain VTF-7 was used to infect cells at 10 plaque-forming units/cell in 100 μl of growth medium. T7 promoter-driven plasmids with cDNA inserts encoding wildtype NET, DAT or chimeric transporters were added 30 min later as liposome suspensions (1 μg DNA and 3 μg Lipofectin; GIBCO-BRL) in a total volume of 350 μl /well. Sixteen hours after transfection, the virus/liposome suspension was removed by aspiration, and the cells were washed once with 37^0C KRTH medium containing (in mM): 120 NaCl, 4.7 KCl, 2.2 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 5 Tris, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4. Cells were preincubated for 1 min at 37^0C in 500 μl KRTH in the absence or presence of ethanol (20, 40, 60, or 100 mM). Uptake was initiated by the addition of [^{3}H]DA or [^{3}H]NE (10, 100, or 1000 nM) in KRTH containing L-ascorbate (100 μM final). Uptake was terminated after 20 min at 37^0C by washing twice with 1 ml of ice-cold KRTH medium, cells were solubilized with 0.5N NaOH, and the accumulated radioactivity was determined by scintillation spectrometry. Nonspecific transport was determined by assays of cells transfected with the plasmid vector (pBluescript SKII-) on the same plate and subtracted from total uptake.

2.3 cRNA preparation and oocyte injection:

cDNAs encoding the human DAT was provided by S.G. Amara and M.S. Sonders (Vollum Inst, Oregon Health & Science University, Portland, OR). Capped cRNAs were transcribed from linearized plasmids using standard *in vitro* transcription reactions (Stratagene). After manual isolation, *Xenopus laevis* oocytes were injected with water-diluted cRNA (~10 ng/oocyte), and maintained in Frog Ringers Buffer (FRB), containing (in mM): 96 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, pH 7.5 supplemented with 2.5 mM Na pyruvate, 0.5 mM theophylline, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin. Uninjected or water-injected oocytes were used to define nonspecific uptake and binding in all experiments.

2.4 Site directed mutagenesis:

Site directed mutagnesis of DAT was performed on cDNA subcloned in pBK-CMV vector (Stratagene) using the Quick Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutagenesis was verified by sequence analysis.

2.5 [^{3}H]DA uptake in Xenopus oocytes:

For [^{3}H]DA uptake assays, DAT expressing oocytes were incubated in 0.5 ml FRB containing 100 nM [^{3}H]DA for 10 min at 21^{0}C. Oocytes were exposed to 100 mM

ethanol (12) for different time periods in tightly sealed 12 well plates. Oocytes were washed three times in FRB and [^{3}H]DA uptake into individual oocytes was quantitated by liquid scintillation spectroscopy.

2.6 [^{3}H]WIN 35,428 binding in Xenopus oocytes:

Whole cell radioligand binding was performed in 0.5 ml FRB containing 4 nM [^{3}H]WIN 35,428 for 15 minutes at 4^{0}C. Radioactivity was quantitated using liquid scintillation spectroscopy. Uninjected oocytes were used to specify non-specific binding. Oocyte homogenates were prepared by sonicating 6 DAT (wildtype or mutant) expressing oocytes in 0.5 ml ice-cold FRB. Binding to oocyte homogenates was performed in 0.5 ml FRB containing 4 nM [^{3}H]WIN 35,428. Non-specific binding was determined using 3-PPP (R(+)-3-(3-Hydroxyphenyl)-N-propylpiperidine hydrochloride). Binding was terminated by rapid filtration and washing using a vacuum manifold.

2.7 Materials for proteomics:

DBA/2J mice were obtained from Jackson Laboratories (Bar Harbor, Maine). DAT (monoclonal) and neurocan antibodies were from Chemicon (Temecula, CA). Synapsin, dynamin, NR1 and Brca2 antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Kv2.1 antibody was from Sigma (St. Louis, MO). Protein G Plus/ Protein A agarose suspension (referred to as agarose beads) was from Oncogene research

products (San Diego, CA). HA antibody was from Roche Diagnostics Corporation (Indianapolis, IN)

2.8 Co-immunopreciptation of the DAT complex:

Brains obtained from DBA/2J mice (100 days old) were solubilized in ice-cold buffer (1% (w/v) sodium deoxycholate, 0.1% (v/v) Triton X-100, and Tris–HCl (500 mM, pH 9.0) containing protease inhibitors (10 μg/ml of the protease inhibitors leupeptin, chymostatin and pepstatin A, Sigma, St. Louis, MO). The homogenate was incubated on ice for 1h and insoluble proteins removed by centrifugation at 10,000 x g for 10 mins. The soluble protein fraction was dialyzed (Spectrapor M_WCO 1200–1400 Da, Spectrum Laboratories, Rancho Dominguez, CA) against 500 ml immunoprecipitation (IP) buffer (50 mM Tris–HCl, pH 7.4, 0.1% Triton X-100). The dialysate was centrifuged at 15,000xg for 10 mins and the supernatant used for all IP's. The lysates (supernatant, 2mg protein) were pre-cleared for 2 hrs with protein A/G agarose beads (50 μl) and used for IP. IP's were carried out overnight using 10-25μg of DAT antibody. The beads were washed with 60 volumes of IP buffer, and proteins eluted by addition of 50 μl Laemmli sample buffer and boiling for 5 mins. IP's under denaturing conditions were carried out by solubilizing the protein in 2% SDS, 2% Triton-X 100 and 50 mM Tris-HCl, pH 7.5. Solubilized proteins were diluted with five volumes of IP buffer prior to incubating with the antibody. For Western Blots samples were separated

on 10% SDS PAGE and transferred onto nitrocellulose membranes. Peroxidase conjugated secondary antibodies were used for chemiluminiscence detection.

2.9 Crude synaptosome preparation:

Mice striata were dissected into 10 volumes of ice-cold homogenization buffer comtaining (in mM) 0.32 Sucrose, 10 HEPES pH 7.4, 2 EDTA. The tissue was homogenized using 10-15 strokes of a glass-teflon homogenizer and spun at 1000 x g for 15 minutes. The supernatant was spun again at 17, 000 x g for 30 minutes and the crude membrane pellet was resuspended in HEPES lysis buffer containing 50 mM HEPES, 2 mM EDTA and protease inhibitors (10 μg/ml of the protease inhibitors leupeptin, chymostatin and pepstatin A, Sigma, St. Louis, MO).

2.10 Preparation of protein samples:

DAT-associated proteins were separated by 10% SDS-PAGE and the bands were visualized by coomassie staining. Individual bands ranging from 30 kDa-300 kDa were excised and digested with trypsin and used for mass spectrometry.

2.11 Mass Spectrometry:

Experiments were performed using a microcapillary LC system (Agilent 1100 series) on-line with an Esquire ion trap mass spectrometer (Bruker Instruments). Peptides were separated using a 30 x 0.15 mm C18 column and a short linear gradient; 2-65% buffer B (0.1 % formic acid, 0.01% trifluoroacetic acid in 10% HPLC grade water, 12% isopropyl alcohol and 68% acetonitrile) in 5 mins at a flow rate of 2 μl/min. All peptides were eluted off the column within 35 minutes. For optimal results, up to 9 precursor ions were automatically selected for fragmentation. A list of the masses containing precursor ion and fragmentation data was generated and used for database searches.

2.12 Strategy for Protein Identification:

Protein identifications were performed using the search engine MASCOT (45). A non-redundant database was searched without applying any constraint on molecular weight. Proteins were identified based on at least 2 peptide matches and were classified into high and medium probability hits using the following criteria: high probability hits were proteins identified by MASCOT as having a high probability based Molecular Weight Search (MOWSE) score (Perkins et al., 1999). Medium probability hits were proteins with a low probability based MOWSE scores, but were reliably immunoprecipitated in multiple experiments and for which good fragmentation patterns were detected (Husi et al., 2000).

2.13 *In silico* analysis of DAT transcriptome:

To evaluate the relevance of the identified proteins, we investigated their relationships at the transcriptional level using data from a public web-based database (the WebQTL Project, www.webqtl.org). This database contains mRNA abundance values for over 12,000 genetic transcripts measured by Affymetrix® microarrays in 32 BXD recombinant inbred (RI) strains, their progenitor strains – C57BL/6J (B) and DBA/2J (D), and the BXD F1 hybrids. In addition to transcriptome information, WebQTL contains published data on hundreds of phenotypical traits studied in some of the BXD RI.

Forebrain mRNA abundance values for DAT proteome genes were extracted from UTHSC Brain mRNA U74Av2 (Dec03) MAS5 database (see http://webqtl.org/dbdoc/U74Av2Mas5_December03.html for sample preparation details). To investigate gene expression patterns principal component analysis (PCA) was carried out using STATISTICA package (Version 6.1, StatSoft, Inc). WebQTL databases were also used to obtain Pearson's product moment correlations between several published BXD phenotypes functionally related to DAT and expression values of the DAT proteome genes.

2.14 Generation of Sindbis viral constructs encoding GFP-DAT:

The hDAT-EGFP cDNA containing EGFP fused to the carboxyl terminus of DAT was excised from the plasmid hDAT- pEGFP (3) at the Kpn1-Xba1 sites and inserted into the Pml1 site of the plasmid pSINREP5 (Invitrogen, CA). In vitro transcription of the

resulting construct and that of the helper virus construct DH(26S)5'SIN was carried out using SP6 polymerase (mMessage mMachine Kit, Ambion, Austin, TX).

2.15 [^{3}H]DA uptake in BHK cells and MDCK cells expressing GFP-DAT:

BHK cells were grown to confluence on 12-well dishes and infected with pseudovirions. The cells were assayed 12-18 hours post infection. Uptake was initiated by the addition of 100 nM [^{3}H]DA (NEN life science products) and allowed to proceed for 10 min at room temperature. Nonspecific uptake was determined in uninfected cells and in cells treated with 3-PPP (R(+)-3-(3-Hydroxyphenyl)-N-propylpiperidine hydrochloride).

2.16 Preparation and infection of explant cultures with virions:

Mesolimbic explants were obtained from Sprague-Dawley pups. Slices containing the VTA/NAc were placed in 30 mm Millipore CM membrane inserts housed in six well plates with 1.2 ml per well of HEPES-buffered 75% MEM, 25% horse serum, 3 mM L-Glutamine, 100 units/ml penicillin/streptomycin and 5.5 mg/ml dextrose and incubated in 5% CO_2 at 37^{0}C. Explants were infected following the basic methods as described (4). Concentrated virus was injected manually using a micromanipulator at 3-5 sites/slice via borosilicate glass pipette electrode broken to about 20 μm diameter. Approximately 50 nl was injected into each site.

2.17 Preparation and infection of midbrain DA neurons in culture:

Primary neuronal cultures from the midbrain were made from Sprague-Dawley pups (1-3 days old) under IACUC guidelines. Dissociated neurons were plated onto chambered coverglasses (Fisher Scientific). The culture medium (Neurobasal media, 1x B-27, 100 Units/ml Pen Strep, Amphotericin B) was serum-free and contained Neurobasal-A supplemented with B-27 to maintain pure populations of neuronal cells. Medium was changed every 3 days. Cells were imaged 24 hours after infection with sindbis viral particles (40 µl of unpurified GFP-DAT). Media was replaced with aCSF (in Mm: 120, NaCl; 3.3 KCl; NaH_2PO_4, 1.23; $NaHCO_3$, 25; $CaCl_2$, 2.0; $MgSO_4$, 0.9; dextrose, 10; continuously gassed with 95% O_2/ 5% CO_2, Ph 7.2).

2.18 Fluorescence Imaging:

BHK cells and cultured neuronal cells were grown in chambered coverglass or 3.5 mm petridishes and imaged using an Olympus IX-70 microscope. GFP-fluorescence in explants was induced by two-photon excitation using a mode-locked Spectra-Physics Tsunami titanium:sapphire laser pumped by a millenia 5.5W argon source. Scans were controlled using an Olympus Fluoview scan head mounted on an Olympus BX-50W1 microscope.

2.19 Generation of stably transfected cell lines:

NG108, SKNSH, and SH-SY5Y cells were obtained from ATCC (Va). The cells were grown to 80% confluence in media (recommended by ATCC) containing 10% FBS. The cells were transfected using 1.5ug of GFP-tagged wildtype and G130T DAT cDNA. Five hours after transfection the cationic lipid-containing media was replaced with medium containing 10% FBS. 48 hours post-transfection the media was replaced with media containing .25 (SH-SY5Y)-0.5mg/ml (NG108 AND SKNSH) G418 (Invitrogen, CA). Selection in the presence of G418 was carried out for 2-3 weeks after which individual colonies were picked using a cloning cylinder and assayed for expression of DAT.

Chapter 3

Ethanol modulation of the human dopamine transporter function*

3. 1 Introduction

The family of Na^+ and Cl^- dependent transporters, which include the dopamine (DA) [1] and norepinephrine (NE) transporters (DAT and NET, respectively), function to clear released neurotransmitters from the synaptic cleft (Nelson, 1998). DAT regulates the spatial and temporal aspects of dopaminergic synaptic transmission and is an integral part of the mesostriatal DA system. This system plays a central role in mediating the rewarding and reinforcing effects of various drugs of abuse including ethanol (Brodie and Appel, 1998; Brodie et al., 1990; Weiss et al., 1993). DAT is also the site of action for various psychostimulants like cocaine and amphetamine (Nelson, 1998). The function of monoamine transporters at the cell membrane is regulated by multiple second messenger systems, and this regulation involves redistribution of the transporters at the cell surface rather than changes in rate of flux of substrate. For example, activation of protein kinase C (PKC) and drugs of abuse like amphetamine inhibit DAT function by causing internalization of cell surface transporters in a dynamin- and clathrin-dependent manner (Daniels and Amara, 1999; Melikian and Buckley, 1999; Saunders et al., 2000).

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* Portions of this manuscript was published in Journal of Biological Chemistry, volume 277, pages 30724-9, 2001, and is reprinted with permission from the American Society for Biochemistry. Kari Buck generated the DAT/NET chimeras and tested them for ethanol sensitivity. The reminder of the experiments were performed by Rajani Maiya.

Experiments carried out in human embryonic kidney (HEK-293) cells demonstrated that acute exposure to cocaine enhances DAT activity in a time-dependent manner by increasing the number of functional transporters at the cell surface (Daws et al., 2002). Cocaine also increases the number of DAT binding sites in neuro2A (N2A, derived from mouse neuroblastoma) cells by altering the intracellular trafficking of DAT (Little et al., 2002).

Ethanol has been shown to affect the function of several members of the Na^+ and Cl^- dependent family of transporters. Experiments in HEK-293 cells stably transfected with glycine transporters (GLYT1 and 2) have shown that relatively high concentrations of ethanol (100-200 mM) inhibit uptake of [^{3}H]glycine by GLYT2 and potentiate [^{3}H]glycine uptake by GLYT1 (Nunez et al., 2000). Also, acute exposure to ethanol has been shown to enhance serotonin transporter activity in rat cortical, hippocampal and brainstem synaptosomes (Alexi and Azmitia, 1991). Acute ethanol (10-100 mM) enhances DAT-mediated [^{3}H]DA uptake and transporter-associated currents in a time- and concentration-dependent manner (Mayfield et al., 2001). This potentiation of transporter function was accompanied by an increase in the number of functional cell surface transporters suggesting that ethanol affects transporter function by altering the steady state trafficking of DAT to the cell surface. In contrast, electrochemical experiments suggest that NET function is inhibited by ethanol (Lin et al., 1993).

DAT shares a high degree of sequence homology with NET but results outlined above suggest that ethanol may have different effects on DAT and NET function. In the present study, we use the contrasting effects of ethanol on DAT and NET function to

identify critical amino acids in DAT that are important for ethanol action. DAT/NET chimeras were expressed in HeLa cells to identify discrete structural domains on DAT and NET that are important for ethanol regulation of transporter function. Site directed mutagenesis experiments were then carried out and mutant transporters were functionally analyzed to pinpoint individual amino acid residues that may be crucial for ethanol enhancement of DAT function.

3.2 Effect of ethanol on wildtype DAT and NET expressed in HeLa cells:

Wildtype DAT and NET were expressed in HeLa cells and the effect of ethanol on [^{3}H]DA and [^{3}H]NE accumulation into these cells was measured. In DAT expressing cells, ethanol (20-100mM) significantly increased [^{3}H]DA uptake in a concentration-dependent manner, with 60 mM ethanol producing an ~50% increase in DA uptake (Figure 2a). In contrast, in NET expressing cells, ethanol (40-100mM) inhibited [^{3}H]NE uptake by as much as 22% (Figure 2b) but had no effect on NET mediated [^{3}H]DA uptake (Table 1).

3.3 Structural domains essential for ethanol potentiation of DAT function:

DAT and NET chimeras were generated and eight chimeras (i.e. four sets of approximately reciprocal chimeras) having junctions in or near transmembrane domains (TMD) 1, 3, 9, and 10 were used in this study (Figure 2). The chimeras were referred to

as NET/DAT or DAT/NET according to their relative orientations and are numbered to indicate a TMD near their junction. Maximally effective concentrations of ethanol (40-100 mM) were chosen for each chimera. DA uptake was similar to that observed for wildtype transporters (Vmax > 90% of wildtype DAT) in most of the chimeras used in this study. However, chimeras NET/DAT3, DAT/NET1, and DAT/NET3 showed DA uptake that was 60-80% of that observed for wildtype DAT (16, 17).

Functional analyses of the chimeric transporters indicate that chimeras that possess DAT sequence elements within the region including TMD 1-3 all demonstrate enhancement of [^{3}H]DA uptake by ethanol as did DAT (Table 1). In contrast, in chimeras with sequence elements within TMD 1-3 from NET, [^{3}H]DA uptake was not enhanced by ethanol, and thus resembled NET in this regard. These results indicate that ethanol enhancement of DAT function requires the TMD 1-3 region of DAT. Chimeras NET/DAT1 and DAT/NET1 with junctions in TMD1 (F78 and W63, respectively) were also tested for ethanol effects. Ethanol responsiveness of these two chimeras was similar to DAT and NET respectively (Table 1). Furthermore, we also tested chimeras DAT/NET9, DAT/NET10, NET/DAT10, and NET/DAT11 with junctions downstream of TMD3 (TMD9, TMD10, and TMD11 respectively), for ethanol effects. These chimeras demonstrated ethanol sensitivities comparable to that of NET/DAT3 and DAT/NET3, and further delineated the TMD 1-3 region to 76 amino acid residues spanning positions 78-154.

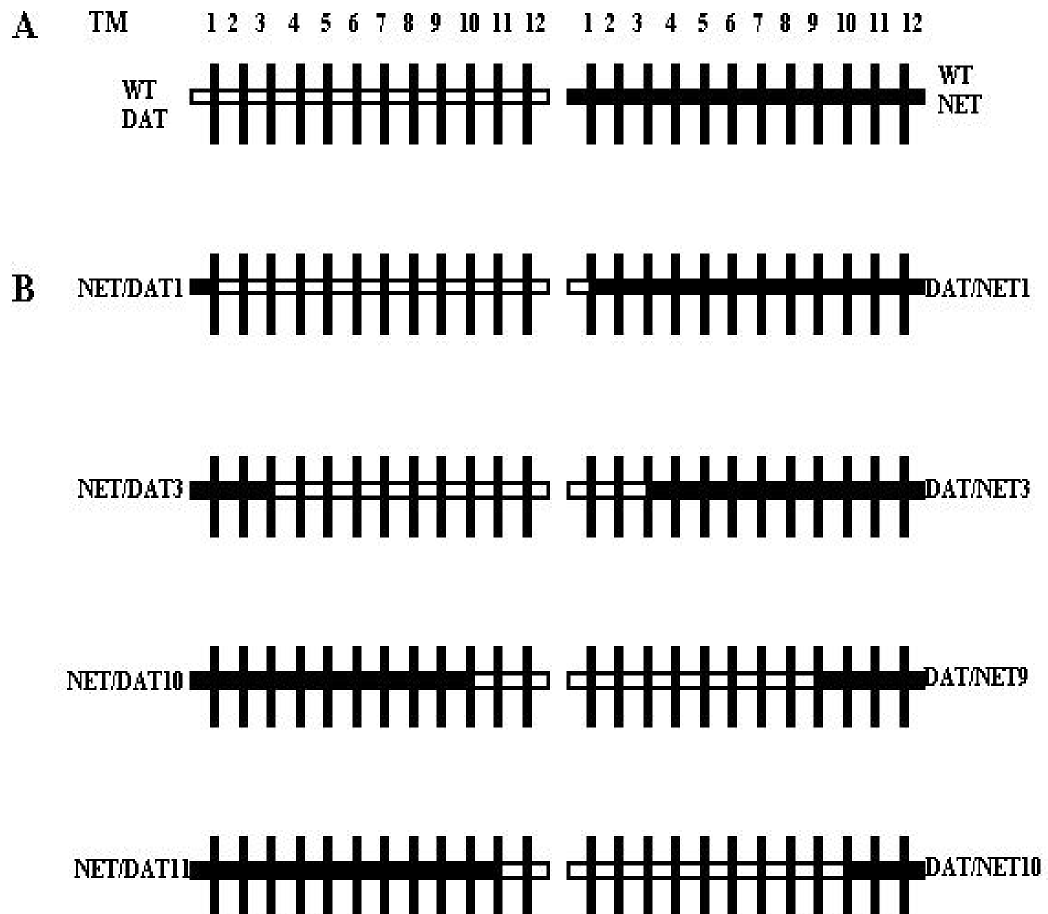

Figure 2: Schematic representation of wildtype (A) and chimeric (B) DAT and NET. Approximately reciprocal chimeras made from combinations of NET (black) and DAT (white) are shown. Each chimera is designated as DAT/NET or NET/DAT to identify orientation and numbered to indicate the TMD nearest its junction. These chimeras depict DA uptake characteristics similar to wildtype DAT.

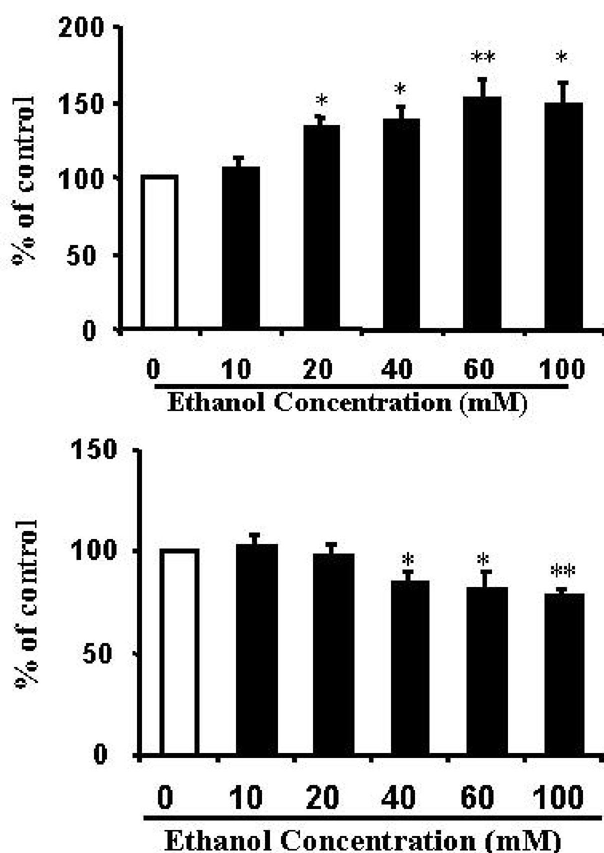

Figure 3: Ethanol effects on [^{3}H]DA uptake and [^{3}H]NE uptake in HeLa cells expressing wildtype DAT and NET, respectively. Cells were preincubated for 1 minute at 37^{0}C with different concentrations of ethanol. Uptake was initiated by the addition of 10nM [^{3}H]DA or [^{3}H]NE for 20 minutes. (A) Ethanol potentiated [^{3}H]DA uptake in DAT expressing HeLa cells. Maximum potentiation (50%) was observed with 60mM ethanol. (B) [^{3}H]NE uptake was dose-dependently inhibited by ethanol. Maximum inhibition (22%) was observed in the presence of 100mM ethanol. Mean values $\pm$SEM are shown for six independent experiments. *$p<.05$, **$p<.01$ one way ANOVA followed by Neumans-Keuls test.

Table 1: Effect of ethanol on [^{3}H]DA uptake in HeLa cells expressing wildtype DAT, NET and chimeric transporters

Maximal effective concentrations of ethanol (40-100 mM) were chosen for each chimera. Uptake was initiated by the addition of 10nM [^{3}H]DA for 20 minutes. *$p<.05$ as compared to their respective control.

	Enhancement of DA uptake by ethanol: % of control	Km DA (μM)	Km NE (μM)
WT DAT	178±19*	3.0	5.0
WT NET	103±5	0.2	0.4
ND1	147±15*	3.0	3.0
DN1	99±5	0.2	0.4
ND3	101±7	0.8	1.7
DN3	143±16*	1.0	2.0
ND10	112±20	0.6	0.9
DN9	172±25*	2.0	2.0
ND11	116±21	0.3	0.4
DN10	158±18*	2.0	2.0

3.4 Site directed mutagenesis of DAT:

Mutagenesis studies were carried out in human DAT to pinpoint sites of ethanol action within the TMD 1-3 region. Since intracellular loops are more accessible for proteins that modulate trafficking (20), we explored the intracellular loop between TMD 2 and TMD 3 for possible sites of ethanol action. The first intracellular loop differs between DAT and NET by only four amino acids at positions 123, 130, 137, and 138. Each of these amino acids in DAT was replaced with the corresponding amino acid from NET. The resulting cDNAs were transcribed *in vitro* and expressed in *Xenopus* oocytes.

Oocytes expressing wildtype and G130T mutant DAT were exposed to ethanol for 1 or 4 hours and [^{3}H]DA uptake was measured. The time periods were chosen to examine the effects of ethanol on [^{3}H]DA uptake as a function of ethanol exposure time. Basal [^{3}H]DA uptake in this mutant was 1.2 fmol/sec/oocyte which was comparable to that of wildtype DA uptake (1.0 fmol/sec/oocyte). Wildtype DAT expressing oocytes showed a 50% potentiation of [^{3}H]DA uptake after 4 hours of ethanol exposure. In contrast, the potentiating effects of ethanol were abolished in the G130T DAT mutant after pre-incubation with ethanol for 4 hours (Figure 4).

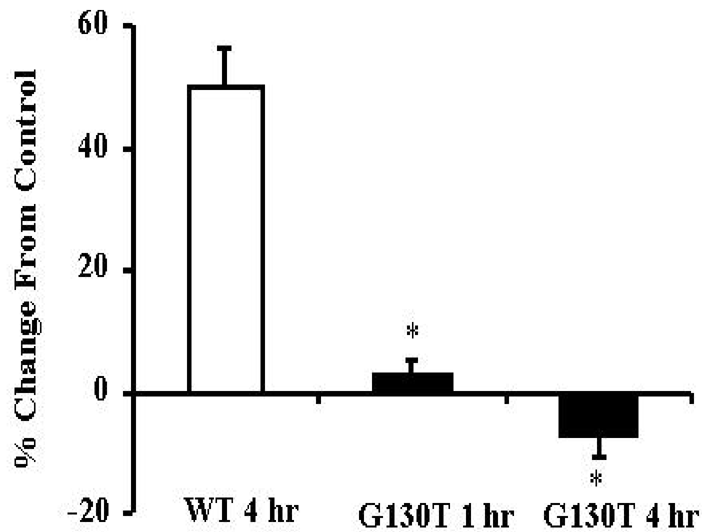

Figure 4: Ethanol had no effect on [^{3}H]DA uptake in G130T DAT. [^{3}H]DA (100nM) uptake was measured in wildtype (WT) and G130T DAT expressing oocytes after a 4 hour exposure to either ND96 (white bar) or 100 mM ethanol (black bar). Ethanol potentiated [^{3}H]DA uptake (~ 50%) after 4 hours of exposure, in wildtype DAT expressing oocytes. In contrast, ethanol potentiation of DAT activity was not observed in the G130T DAT mutant. Mean values $\pm$ SEM are shown for n=6-10 oocytes per condition from 3-4 batches of oocytes. *$p<0.05$ as compared to ethanol potentiation of wildtype DAT, one way ANOVA followed by Neuman-Keuls test.

Amino acids phenylalanine at position 123, isoleucine at position 137, and leucine at position 138 were also mutated to the corresponding amino acids in NET (F123Y, I137F, and L138F, respectively). [^{3}H]DA uptake in these mutants was comparable to that of wildtype DAT (data not shown). Maximum potentiation of transporter function was observed after 1 hour of ethanol exposure in oocytes expressing wildtype transporter (Figure 5). After exposure to 100 mM ethanol for 1 hour, potentiation of [^{3}H]DA uptake was not observed in the mutant I137F (Figure 4). The mutant F123Y showed ethanol sensitivity comparable to that of wildtype DAT (Figure 4). Ethanol sensitivity of the mutant L138F was reduced but not significantly as compared to that of wildtype DAT (Figure 5).

Previously, we showed that ethanol potentiation of DAT activity was mirrored by an increase in the density of cell surface transporters (12). Upon exposure to ethanol for 1 and 4 hours [^{3}H]WIN 35,428 binding was significantly increased by 40% and 53% respectively (12). We investigated cell surface transporter numbers in the mutant G130T DAT in the presence and absence of ethanol by measuring [^{3}H]WIN 35,428 binding. G130T DAT cell surface density was approximately 20 fmol/oocyte. Ethanol had no significant effect on the number of cell surface transporters in the mutant G130T (Figure 6).

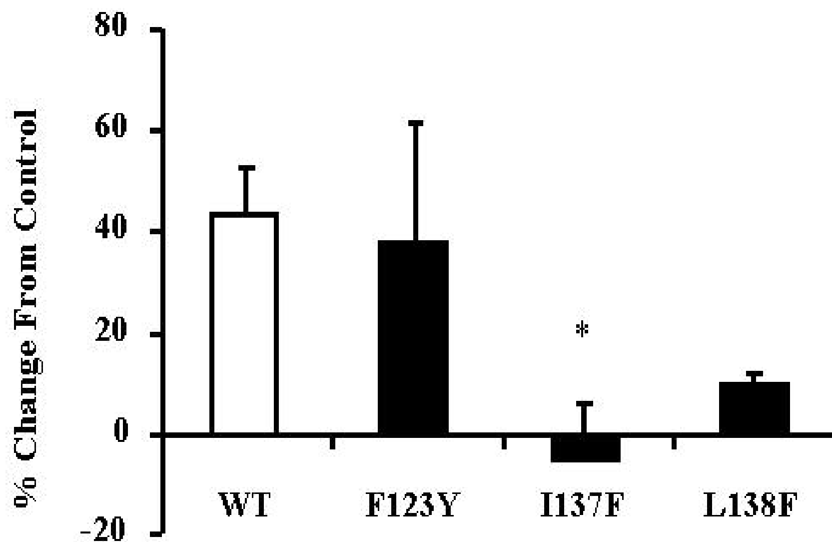

Figure 5: Effects of ethanol on F123Y, I137F, and L138F mutants. [^{3}H]DA uptake was measured in oocytes expressing wildtype versus mutant DAT. Oocytes expressing WT and mutant transporters were exposed to 100 mM ethanol for 1 hour and [^{3}H]DA (100 nM) uptake was measured. Enhancement of [^{3}H]DA uptake was observed in wildtype (white bar) and F123Y (black bar) expressing oocytes, but not in the mutants I137F and L138F. Mean values $\pm$ SEM are shown for n=8-10 oocytes per condition from 4-5 batches of oocytes. *$p<0.05$ as compared to wildtype potentiation, students t-test.

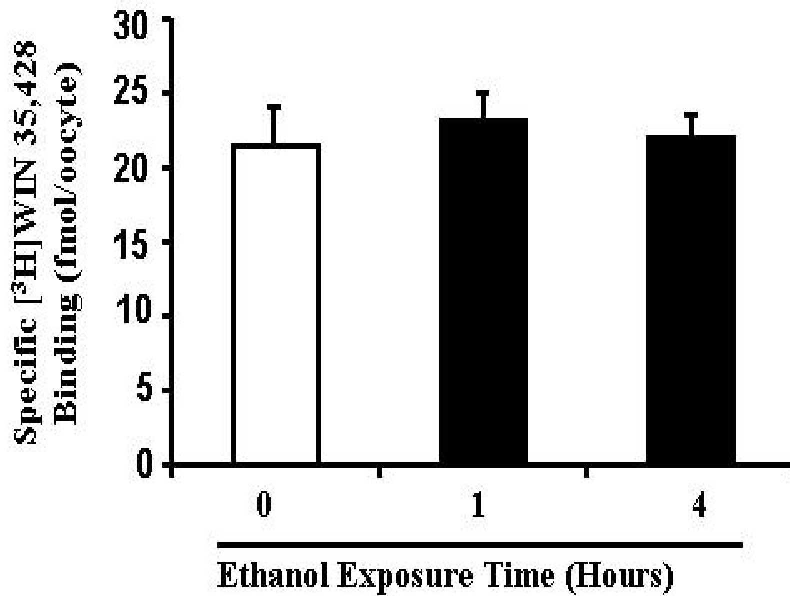

Figure 6: Ethanol does not change [^{3}H]WIN 35,428 binding in G130T expressing oocytes. [^{3}H]WIN 35,428 (4nM) binding was measured in oocytes expressing G130T DAT in the absence (0 hr) and presence of ethanol (1-4hr). [^{3}H]WIN 35,428 binding in oocytes exposed to ethanol (black bars) did not differ significantly from untreated controls (white bars). Mean values $\pm$ SEM are shown for n=6-10 oocytes per condition from 3-4 batches of oocytes.

To test whether ethanol inhibits DAT function when the first intracellular loop is replaced with that of NET, we substituted the first intracellular loop of DAT with that of NET (by sequentially mutating positions F123, G130, I137 and L138 to the corresponding amino acids in NET). This loop replacement mutant is termed IGLF. [^{3}H]DA uptake and [^{3}H] WIN 35,428 binding was measured in this mutant and compared to wildtype. [^{3}H]DA uptake was not observed in the mutant IGLF (Figure 7A). [^{3}H]WIN 35,428 binding in the mutant IGLF was significantly reduced as compared to that of wildtype DAT (Figure 7B). We next carried out [^{3}H]WIN 35,428 binding studies on oocyte homogenates (Methods). We found that the mutant IGLF is synthesized but not inserted to the cell surface (Figure 7C).

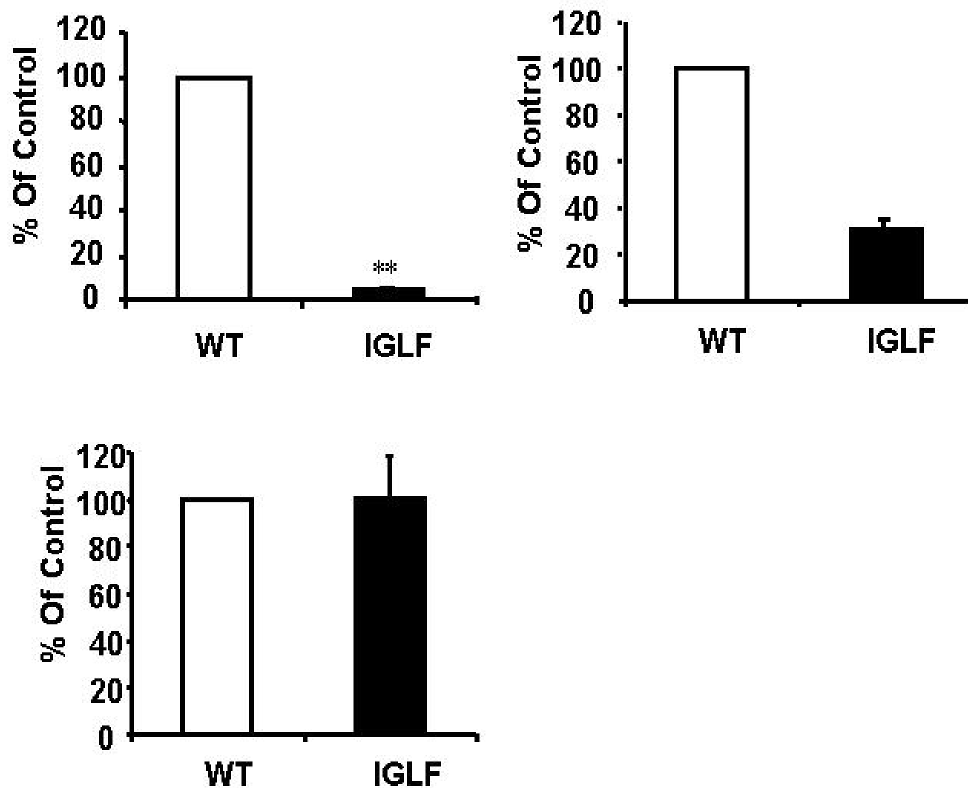

Figure 7: The mutant IGLF is expressed but not trafficked to the cell surface. [^{3}H]DA uptake and [^{3}H]WIN 35,428 binding studies were carried out in the quadruple DAT mutant IGLF. (A) [^{3}H]DA uptake was not observed in oocytes expressing the transporter IGLF . (B) Cell surface transporters were measured in oocytes expressing both wildtype and mutant transporters using 4nM [^{3}H]WIN 35,428. (C) Binding was not detected in the mutant IGLF. Homogenates of oocytes expressing wildtype and IGLF transporters assayed for [^{3}H]WIN 35,428 binding . Similar levels of binding were detected in both wildtype and mutant expressing oocytes. Mean values $\pm$ SEM are shown for n=6 oocytes per condition from 3-4 batches of oocytes. * *p<0.05,* ***p<0.005* as compared to wildtype, students t-test.

3.5 Discussion

Previous studies carried out in the *Xenopus* oocyte expression system have shown that acute ethanol (10-100mM) enhances [^{3}H]DA uptake and transporter-associated currents in a time- and concentration-dependent manner. Ethanol-induced increases in cell surface DAT were not associated with increased protein synthesis (Mayfield et al., 2001), but were associated with increased cell surface binding. These results suggest that ethanol enhancement of transporter function may involve redistribution of DAT at the cell surface. The goal of this study was to determine regions on DAT that are critical for ethanol action. NET, which also belongs to the Na^+ and Cl^- dependent family of neurotransmitter transporters, shares a high degree of homology (64%) with DAT (Mayfield et al., 2001); but, *in vivo* electrochemical studies have shown that ethanol inhibits rather than enhances NET function (Lin et al., 1993).

We used chimeras between DAT and NET and site directed mutagenesis studies to define critical ethanol sensitive sites in the first intracellular loop of the transporter. The kinetic parameters of DA uptake (Km and Vmax) in most of these chimeras were nearly identical to wildtype DAT. These chimeras showed that ethanol enhancement of DAT-mediated [^{3}H]DA uptake required the presence of TMD 1-3. Replacing this region of DAT with the corresponding region from NET resulted in abolition of ethanol effects. Chimeras NET/DAT1 and DAT/NET1 with junctions in TMD1 had ethanol sensitivities comparable to DAT and NET, respectively. In contrast, chimeras NET/DAT3 and DAT/NET3 with junctions at TMD3 have ethanol sensitivities comparable to NET and

DAT. Also, chimeras with junctions downstream of TMD3 (NET/DAT10 and 11, DAT/NET9 and 10) resemble NET/DAT3 and DAT/NET3 in their ethanol sensitivities. Taken together, these results indicate that a 76 amino acid region spanning TMD 1-3, including the first intracellular loop, is critical for mediating ethanol enhancement of DAT function.

Since intracellular loops are accessible at all times to modifying enzymes and accessory proteins that modulate trafficking (Quick et al., 1997), we explored the first intracellular loop between TMD2 and TMD3 for possible sites of ethanol action. This loop in DAT differs from that of NET by four amino acids. Site directed mutagenesis was carried out to substitute glycine at position 130 to threonine, the corresponding amino acid in NET. [^{3}H]DA uptake in this mutant was comparable to that of the wildtype DAT, however, ethanol potentiation of [^{3}H]DA uptake was abolished completely in this mutant. Using [^{3}H]WIN 35,428 binding, we examined cell surface expression of this mutant before and after ethanol exposure. [^{3}H]WIN 35,428 binding to the wildtype DAT is Na^+-dependent and low intracellular concentrations of sodium ions in the oocyte prevent [^{3}H]WIN 35,428 from binding to intracellular DAT (Barish, 1983; Reith and Coffey, 1993). Ethanol had no effect on the number of cell surface G130T mutant transporters indicating that this amino acid is critical for ethanol-mediated increases in [^{3}H]DA uptake. This suggests that ethanol affects DAT function by altering cell surface distribution of the transporters.

We also mutated amino acids F123, I137 and L138 to the corresponding amino acids in NET. We found that I137F abolished ethanol potentiation of [^{3}H]DA uptake,

whereas the other mutations demonstrated ethanol sensitivities comparable to the wildtype DAT. Robust changes in ethanol sensitivities were observed in the mutant G130T and I137F, which are nonconservative amino acid changes and no change in ethanol sensitivity was observed in the F123Y mutant, which is a conservative amino acid change. However, the mutant L138F did not demonstrate significant attenuation of ethanol sensitivity despite being a nonconservative mutation. We generated the mutant transporter IGLF to investigate whether replacing the first intracellular loop of DAT with that of NET would result in ethanol inhibition of [^{3}H]DA uptake. However, this mutant transporter does not demonstrate [^{3}H]DA uptake and expresses a significantly lower number of functional transporters at the cell surface. Oocyte fractionation studies indicate that the transporters are synthesized but not trafficked to the cell surface. This result, though unexpected, supports the hypothesis that the first intracellular loop is important for steady state insertion of DAT to the cell surface.

There is now an emerging literature on mutations that affect ethanol actions on ion channels and it is of interest to compare our analysis of catecholamine transporters with studies of other proteins. Discrete amino acid residues located either in TMD or in the cytoplasmic regions are required for ethanol action on ion channels (Harris, 1999). For example, mutation of serine 267 at TMD2 of the glycine receptor (GlyR) $\alpha 1$ subunit to isoleucine results in an alcohol insensitive receptor (Mihic et al., 1997). A transmembrane phenylalanine residue is important for ethanol inhibition of *N*-methyl-*D*-aspartate receptors (Ronald et al., 2001). Alcohol modulation of G-protein coupled inwardly rectifying potassium channel (GIRK) function requires a 48 amino acid region

in the intracellular C-terminal region of the protein (Lewohl et al., 1999). Similarly, ethanol action on voltage sensitive potassium channels (Kv1) requires the presence of a discrete amino acid residue in the putative cytoplasmic region of the protein (Covarrubias et al., 1995). Ethanol affects the function of metabotrophic glutamate receptors (mGluR1) indirectly by enhancing PKC-mediated receptor phosphorylation (Minami et al., 1998). Mutation of a consensus PKC phosphrylation site, serine 890, abolishes ethanol regulation of receptor function. None of these sites appear to be important for protein trafficking; in contrast, our results identify novel amino acid residues in the first intracellular loop of DAT that are necessary for ethanol modulation of DAT function by a mechanism that is consistent with altered trafficking of the protein to the cell surface.

A common theme in the functional regulation of the Na^+/Cl^--dependent family of transporters is the redistribution of cell surface transporters. Several agonists and antagonists of gamma-aminobutyric acid and serotonin transporters have been shown to alter subcellular distribution of these transporters (Whitworth et al., 2002; Whitworth and Quick, 2001). PKC activators like PMA downregulate DAT function by causing internalization of cell surface DAT (Daniels and Amara, 1999; Melikian and Buckley, 1999; Zhu et al., 1997). Protein tyrosine kinase A (PKA) inhibitors have been shown to inhibit DAT function by internalization of cell surface DAT (Doolen and Zahniser, 2001). Drugs of abuse have also been shown to alter cell surface DAT densities in some but not all studies. For example, amphetamine decreases cell surface DAT levels by endocytosing the transporter in a dynamin- and clathrin-dependent manner in HEK-293 cells (Saunders et al., 2000). More recently two independent studies have shown that

cocaine causes an increase in the cell surface DAT levels (Daws et al., 2002; Little et al., 2002).These studies, carried out in N2A cells and HEK-293 cells demonstrate that cocaine-induced enhancement in cell surface DAT levels is due to increased rates of insertion of DAT at the cell surface. Also, dopamine D_2 receptor activation affects DAT function by increasing the number of functional cell surface transporters (Mayfield and Zahniser, 2001). Our results, which suggest that ethanol-mediated functional regulation of DAT involves redistribution of cell surface transporters and that this redistribution involves discrete regions on the transporter, fits very well with this common theme in transporter regulation.

The absence of consensus sites for PKC or PKA phoshphorylation in the first intracellular loop suggests that ethanol regulation of DAT function may not be due to increased phosphorylation of the protein. Furthermore, it has been demonstrated that direct phosphorylation of the transporter is not required for functional regulation by PKC (Chang et al., 2001). It is likely that accessory proteins aid in the functional regulation of DAT, but these remain largely unknown. Recent studies using yeast two-hybrid techniques have identified candidate proteins that interact with the C-terminal tail of DAT and regulate transporter function. For example, α-Synuclein interacts with DAT in neurons and the resulting DAT-α-synuclein complex has been shown to be essential for clustering of DAT at the cell surface and thereby accelerating cellular DA uptake and DA-induced apoptosis (Lee et al., 2001). The C-terminal tail of DAT also contains a conserved PDZ domain that interacts with the protein PICK1 and this interaction is crucial for proper targeting and functioning of DAT (Torres et al., 2001). Based on our

mutants, we hypothesize that ethanol modulates the interaction between DAT and a putative regulatory protein important for ethanol-induced trafficking of DAT and that this interaction occurs at the first intracellular loop. The ethanol insensitive mutants described in the present work will aid in testing this hypothesis.

Chapter 4

Defining the Dopamine Transporter Proteome by Convergent Biochemical and *In Silico* Analyses*

4.1 Introduction

Monoamine transporters modulate neurotransmission in the brain by mediating reuptake of released neurotransmitter from the extracellular space. This family of proteins includes the serotonin (SERT), norepinephrine (NET), and dopamine transporter (DAT)[1]. DAT, which is responsible for removing released dopamine from extracellular space, is a particularly important site of action for the clinical effectiveness of drugs used to treat attention defecit hyperactivity disorder (Volkow et al., 2002). In addition, inhibition of DAT function is also highly correlated with the abuse potential of drugs like cocaine and amphetamine (Gainetdinov et al., 2002).

DAT is an integral part of the brain reward pathway and is abundantly expressed exclusively in dopaminergic neurons. DAT is localized to distal dendrites and perisynaptic terminals of dopaminergic cells in the substantia nigra and striatum respectively (Nirenberg et al., 1997a). DAT function at the cell surface is regulated by second messenger systems, which can alter the subcellular distribution of the transporter. Drugs of abuse such as amphetamine have been shown to down regulate transporter

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* Dr. Klaus Linse performed the mass spectrometry analysis. Dr. Igor Ponomarev carried out the *in silico* analysis.

function by a dynamin- and clathrin-dependent mechanism (Zahniser and Doolen, 2001). In contrast to inhibition of DAT function by amphetamine, cocaine and ethanol enhance DAT function by a mechanism consistent with altered cellular trafficking of DAT (Maiya et al., 2002; Mayfield et al., 2001; Zahniser and Doolen, 2001). Regulated DAT trafficking would require the transporter to interact with specific proteins involved in cell surface targeting. However, little is known about proteins that interact with and regulate DAT function. The DAT sequence possesses several consensus sites for protein-protein interactions, including a PDZ binding domain at the C-terminus (Torres et al., 2001). Currently, three proteins have been identified that alter DAT function by interacting directly with the transporter. These proteins were identified by the yeast two-hybrid approach using the C-terminal tail of DAT as bait. These are α-synuclein (Lee et al., 2001), the PDZ domain containing protein PICK1 (protein interacting with C kinase 1, 7), and Hic-5 (hydrogen peroxide-inducible protein 5, (Carneiro et al., 2002)).

An emerging theme in neuronal cell signaling is the assembly of receptors and channels into large macromolecular complexes via protein-protein interactions. For example, 77 proteins involved in mediating a variety of cellular functions have been found to associate with the NMDA receptor (Husi et al., 2000). The goal of this study was to identify proteins that exist in a complex with DAT using an interaction-proteomics based approach.

4.2 Isolation and validation of the DAT proteome

The DAT proteome was isolated from mouse striatum by IP (Figure 8A, 8B). A band (approximately 70-80 kDa) corresponding to DAT was readily detected by mass spectrometry. Numerous bands were detected in the DAT complex when compared to proteins that were immunoprecipitated using a monoclonal anti-HA (haemagglutinin) antibody (Figure 8B). The IP procedure was validated by examining the association between DAT and α-synuclein, which was readily detected in the DAT complex isolated from the striatum but not the cerebellum (Figure 8C). The DAT antibody did not immunoprecipitate the NMDA receptor subunit, NR1 (Figure 8D) suggesting that the components of the DAT proteome were specific and discrete.

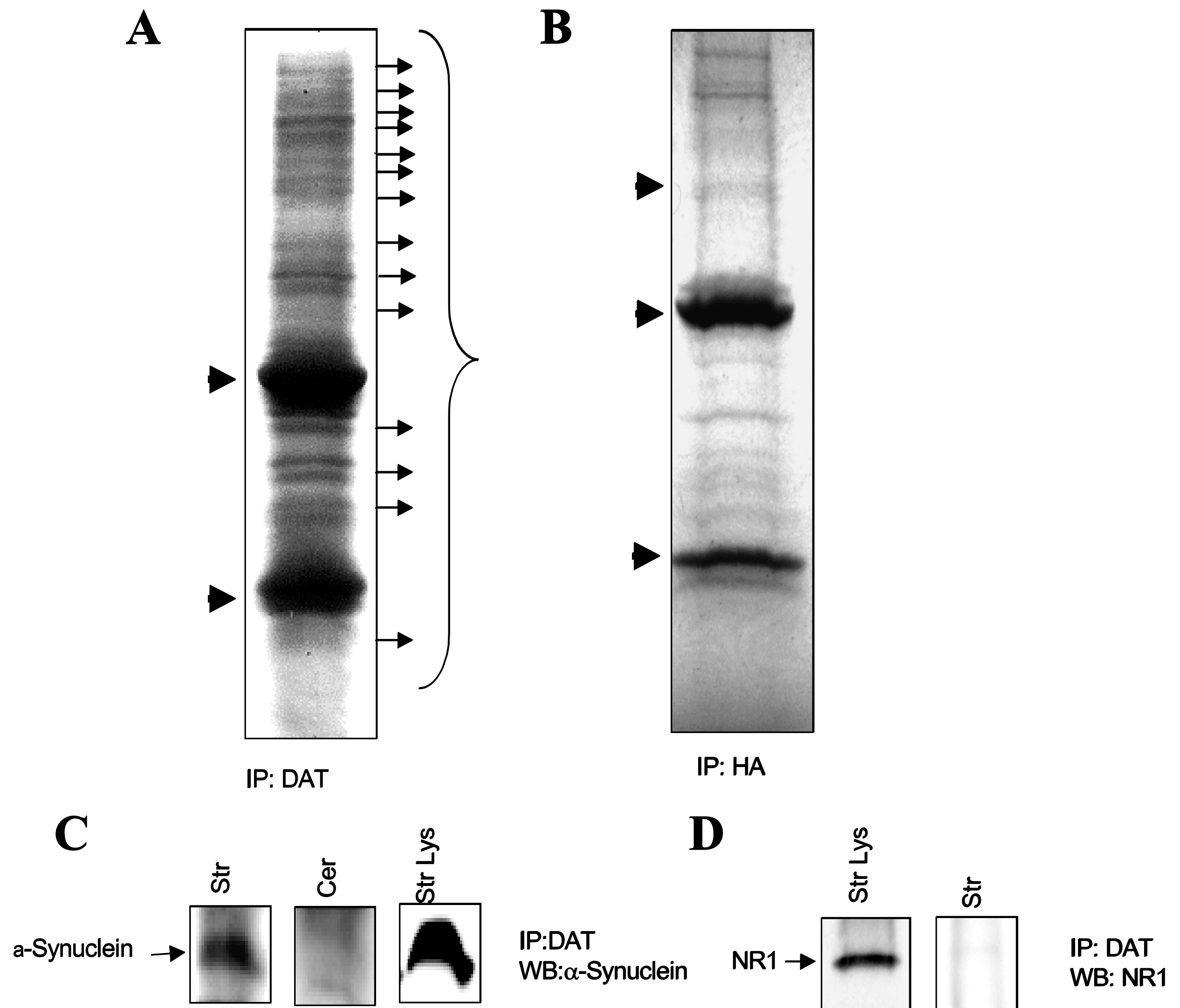

Figure 8: Co-immunoprecipitation of DAT-associated proteins. A) DAT-associated proteins were immunoprecipitated from the striata of male DBA/2J mice and the proteins were separated using SDS-PAGE (10%) and visualized with coomassie staining. B) Nonspecific immunoprecipitation of proteins using same amount of protein as in (A) using monoclonal HA antibody. C) DAT-associated proteins were immunoprecipitated from the striatum and the blot was probed for α-synuclein (n=2). D) IP using DAT antibody and Western Blot using anti-NR1 antibody. Str-Striatum IP, Cer-Cerebellum IP (negative control), Str Lys-Striatal Lysate (positive control). Small arrows indicate approximate positions of bands excised and large arrows indicate IgG heavy and light chains.

4.3 Mass Spectrometry:

Approximately 20-25 bands ranging from 35 to 300 kDa were excised from the coomassie-stained gel and the proteins identified by LC-ESI-MS. 20 Candidate proteins were identified and classified as high and medium probability hits (Methods, Table 2). The presence of five of these proteins in the DAT complex was confirmed by co-IP experiments.

Table 2: Classification of high- and medium-probability proteins.

Proteins were grouped according to known cellular function. Proteins were classified into high and medium probability (italics) hits as described. Fragmentation patterns and sequence information were analyzed for medium probability hits. The maximum numbers of peptides detected for each protein are reported.

Proteins/Accession numbers	Number of peptides detected
Signaling Proteins	
Ras GRF2 (P70392)	6
Rho GEF (T30867)	3
Traffficking Proteins	
Synapsin I (AAD08933)	8
Dynamin I (Q61358)	5
Synaptojanin 2(AAC40153)	*4*
Adapter protein 1 beta (Q922E2)	*3*
Cell Adhesion molecules	
Neurocan (S52781)	11
Brevican precursor (S57653)	4
Ion Channels	
KV 4.3M (AAD16974)	*3*
KV 2.1 (I56529)	*4*
Cystic Fibrosis Transmembrane conductance Regulator (A39901)	*14*
Cytoskeletal/Motor Proteins	
Tubulin (I77426)	4
Actin (CAA27396)	17
Kinesin related protein KIF3b (A57107)	*6*
Aczonin (T42215)	*6*
Metabolic Enzymes	
Similar to Mitochondrial aconitase (Q99KI0)	6
Fructose bis phosphate aldolase (ADMSA)	14
Triose Phosphate Isomerase (ISMST)	2
Miscellaneous	
Par-3 (Q99NH2)	2
Brca 2 (Q9BTL1)	*10*

4.4 Confirmation of members of the DAT proteome:

Eight peptides corresponding to synapsin 1b were detected by mass spectrometry (Table 1). To confirm the presence of synapsin Ib in the DAT complex, co-immunoprecipitation experiments were carried out using DAT and synapsin I (both a and b) antibodies under non-denaturing and denaturing conditions. Under non-denaturing conditions, DAT antibody could immunoprecipitate synapsin from the striatum (Figure 9B). Only a faint band could be detected in the cerebellum, a region devoid of DAT. Synapsin antibody could also IP DAT from the striatum (Figure 9A). DAT was not immunoprecipitated by an antibody directed against the NMDA receptor subunit, NR1 (Figure 9H). Association between DAT and synapsin was disrupted when IP's were performed under denaturing conditions, thereby ruling out non-specific association of synapsin with the protein A/G agarose or antibody used for IP (data not shown). Slightly lower amounts of DAT were immunoprecipitated under denaturing conditions when compared to immunoprecipitations under non-denaturing conditions (data not shown). The presence of dynamin in the DAT complex was also similarly confirmed using DAT and dynamin antibodies (Figure 9C, 9D). These interactions were also disrupted under denaturing conditions (data not shown). Synapsin and dynamin were also immunoprecipitated by a polyclonal antibody directed against DAT (data not shown).

Four peptides corresponding to Kv2.1, a potassium channel, were detected in two out of four experiments; hence Kv2.1 was classified as a medium probability hit. Kv2.1 antibody was used to IP Kv2.1-associated proteins from the striatum and the blot was

probed with DAT antibody (Figure 9E). A band corresponding to DAT was observed in the striatum but not in the cerebellum. Association between DAT and Kv2.1 was not observed under denaturing conditions (data not shown). The presence of Brca2 and neurocan was also confirmed by Western Blots using Brca2 and neurocan antibodies. DAT was immunoprecipitated under non-denaturing conditions using the Brca2 and neurocan antibodies (Figure 9F, 9G). This interaction was disrupted under denaturing conditions (data not shown). We were not able to unambiguously detect the presence or absence of Kv2.1, Neurocan or Brca2 by immunoprecipitating DAT using the monoclonal DAT antibody followed by Western Blots using antibodies directed against these candidate interactors.

The subcellular localization of Kv2.1 has not been examined in the striatum. Crude synaptosomal membranes were prepared and examined for the presence of Kv2.1 and DAT, to verify that Kv2.1 was localized to the same cellular compartment as DAT. As illustrated in Figure 3, Kv2.1 (top panel) is enriched in synaptosomes as DAT (middle panel). β-actin was used as a loading control (bottom panel).

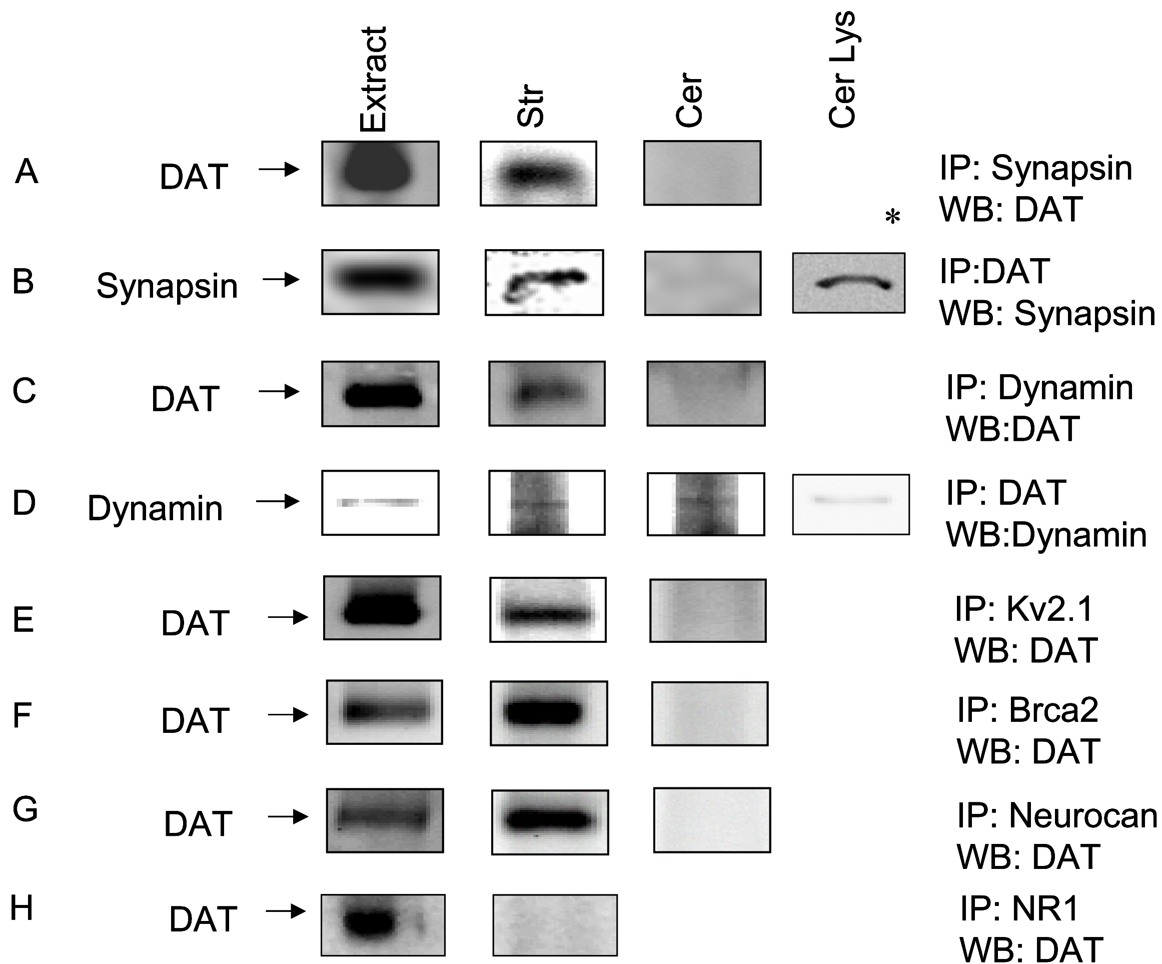

Figure 9: IP under non-denaturing conditions followed by Western Blots was performed to confirm the presence of candidate proteins in the DAT complex. A) IP with synapsin and Western Blot using DAT antibody B) IP with DAT antibody and Western Blot using synapsin antibody C) IP with dynamin antibody and Western Blot with DAT antibody D) IP with DAT antibody and Western Blot using dynamin antibody E) IP with Kv2.1 antibody and Western Blot with DAT antibody F) IP with Brca2 antibody and Western Blot with anti DAT antibody G) IP using anti neurocan antibody and Western Blot using anti DAT antibody. H) IP with anti-NR1 antibody and Western Blot using anti-DAT antibody. Str-Striatum IP, Cer-Cerebellum IP; negative control, Str Lysate-Striatal Lysate; positive control**.** Equal amounts of cerebellar and striatal lysates were used for each immunoprecipitation. All of the images depicted for a particular protein were obtained from the same experiment except when indicated by an "*" symbol.

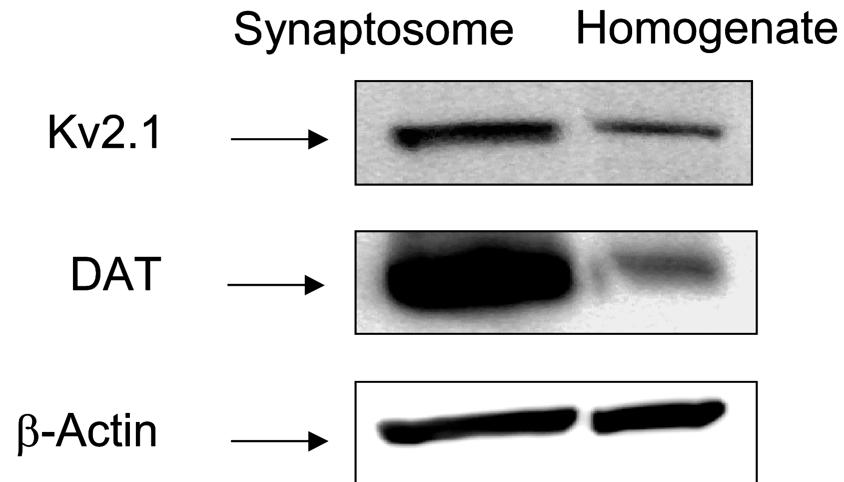

Figure 10: Enrichment of Kv2.1 in the synaptosomal fraction. Equal amounts of (60 μg) striatal homogenate and synaptosomal preparation were loaded on a gel and probed for the presence of Kv2.1 and DAT. Both Kv2.1 and DAT were enriched in the synaptosomal fraction (top and middle panel respectively). β-Actin was used as a loading control (bottom panel).

4.5 *In silico* analysis of DAT proteome:

Studies suggest that genes encoding interacting proteins have similar mechanisms of transcriptional regulation (Ge et al., 2003). WebQTL (Chesler et al., 2004; Chesler et al., 2003; Wang et al., 2003) databases were used to answer two questions: 1) Does the expression of 21 DAT-associated proteins (listed in Table 2 excluding actin, including DAT and α-synuclein) co-vary? Co-variation would imply a common pattern of gene expression, which would in turn, suggest a functional interaction of these proteins. 2) Do transcript abundance values of these proteins correlate with phenotypes functionally related to the dopamine transporter? To investigate the first question we carried out principal component analysis (PCA). PCA is a dimensionality reduction procedure often used for searching common pattern among investigated variables (Landgrebe et al., 2002). PCA is based on a correlation matrix of variables of interest. Thus, it reduces the dimensionality of the data matrix while capturing the underlying variation and relationships among the variables. Principal component 1 (PC1) is a new PCA variable that accounts for the largest proportion of data variance. The proportion of variance explained by PC1 generally reflects how well variables correlate with each other. Principal component 1 (PC1) of the set of 21 DAT proteome genes accounted for about 34% of data variation (Figure 11). By comparison, the mean value of PC1 of 100 different sets of 21 randomly selected genes from the forebrain expression dataset (total of 12,422 expression traits) was 24% $\pm$ 4% (SD). Thus DAT proteome PC1 deviates more than 2.5 SD from the estimated population mean, indicating a non-random

intercorrelation pattern among the 21 DAT proteome genes. PCA of 21 DAT proteome genes also distinguished a group of 9 genes. These genes had high loading scores (a measure of the contribution of the variables to the principal component) for PC1 and clustered in a 2-dimensional (PC1 vs PC2) space (encircled in black, Figure 11). Most correlations among members of this subset were highly significant (data not shown).

The second question of functional relevance was addressed by calculating genetic correlations between expression values of 21 DAT proteome genes and 7 phenotypes listed in BXD Published Phenotypes database (www.webqtl.org). The 7 phenotypes are listed in Table 3, supplementary information. These phenotypes could be classified as functionally related to DAT and were chosen as representatives from a number of related traits from the same studies. Both genetic transcript and phenotype data including record numbers for phenotypes and Affymetrix probeset ID numbers for the 21 genes are listed in (Table 3). Two of the phenotypes studied were DAT density in male Nucleus Accumbens and Caudate Putamen of male mice. Interestingly, the only significant correlation between any transcript and DAT density detected was a negative correlation between DAT mRNA and DAT density in Caudate Putamen ($r=-0.59$, $p<0.01$), suggesting a negative feedback regulation of DAT abundance. In addition, mRNA levels of Rasgrf2, Brca2, Pard3 and Ap1b1 were also correlated with some cocaine-induced behavioral traits (Table 4).

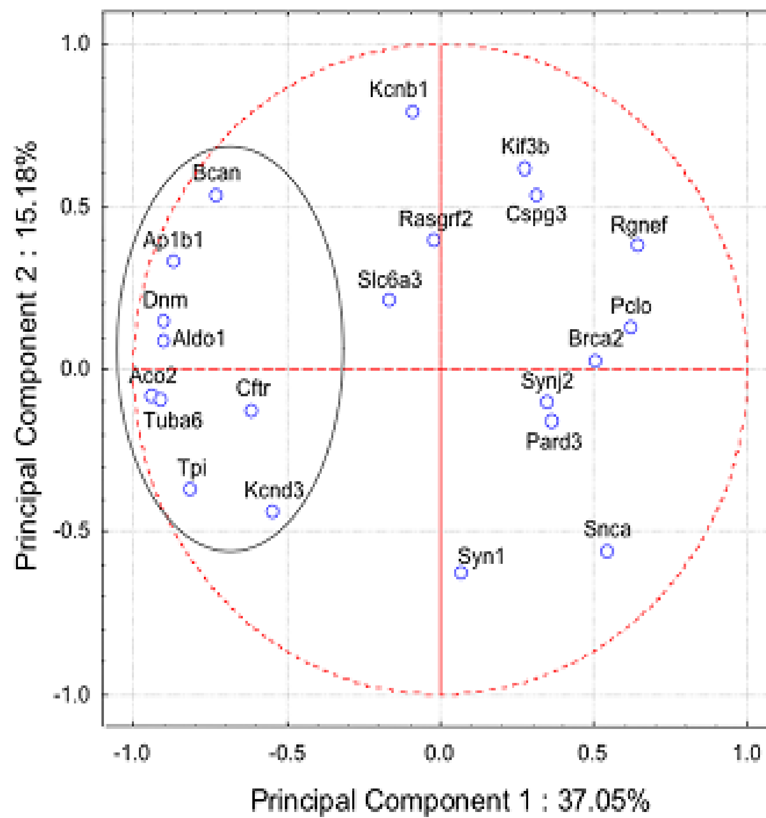

Figure 11: Results of *in silico* analysis using WebQTL database. A) Results of PCA on the 21 DAT-related genes are plotted on a 2-dimensional diagram, with PC1 and PC2 loadings for each gene being shown. 9 genes with similar loading patterns are encircled in black. Bcan-Brevican, Kcnb1-KV2.1, Aco2-Similar to mitochondrial aconitase, Dnm-Dynamin, Kif3b-Kinesin related protein Kif3b, Cspg3-Neurocan, Tuba6-Tubulin, Snca-α-Synuclein, Rasgrf2-Ras GRF2, Cftr-Cystic fibrosis transmembrane conductance regulator, Aldo1-Fructose bis phosphate Aldolase, Syn1-Synapsin1, Rgnef-Rho GEF, Kcnd3-Kv4.3M, Pclo-Piccolo, Ap1b1-Adaptor protein 1 beta, Synj2-Synaptojanin2, Slc6a3-Dopamine Transporter, Pard3-Par3, Tpi-Triose Phosphate Isomerase

Table 3: Genes and phenotypes extracted from the WebQTL project and used for PCA and/or correlational analysis. See Methods for details

Gene/Phenotype description	Phenotype/ Gene Symbol	ID # in WebQTL
Dopamine transporter expression in Nucleus Accumbens - males	**DAT_NA**	**10591541-25-BXD**
Dopamine transporter Caudate Putamen - males	**DAT_CP**	**10591541-28-BXD**
Cocaine stereotypy - number of movements 45 mg/kg cocaine ip (Difference from saline) - males	**S_45**	**10591541-65-BXD**
Cocaine Open Field Activity - total distance 45 mg/kg cocaine (Difference from saline) - males	**O_45**	**10591541-68-BXD**
Locomotor activity post cocaine (32 mg/kg) [activity counts/hr]	**L_32**	**7932176-01-BXD**
Sensitization of locomotor response to cocaine (32 mg/kg) [% initial response]	**SE_32**	**7932176-02-BXD**
Stereotypical behavior frequency post daily cocaine injection (32 mg/kg), day 8	**S_32**	**7932176-03-BXD**
Potassium channel, delayed rectifier, subtype 1, gene 1	**Kcnb1**	**101308_at**
RAS protein-specific guanine nucleotide-releasing factor 2	**Rasgrf2**	**102572_at**
Breast cancer 2	**Brca2**	**102911_at**
Dynamin	**Dnm**	**103031_g_at**
Aldolase 3, fructose-bisphosphatase	**Aldo1**	**160546_at**
Par-3 (partitioning defective 3) homolog (C. elegans)	**Pard3**	**160607_at**
Kinesin family member 3b	**Kif3b**	**161003_at**
Brevican	**Bcan**	**92700_at**
Rho-guanine nucleotide exchange factor	**Rgnef**	**92710_at**
Synuclein, alpha	**Snca**	**93273_at**
Synapsin 1	**Syn1**	**93730_at**
Cystic fibrosis transmembrane conductance regulator	**Cftr**	**94757_at**
Adaptor-related protein complex AP-1, beta 1 subunit (beta-prime adaptin)	**Ap1b1**	**95704_at**
Aconitase 2, mitochondrial	**Aco2**	**96870_at**
Piccolo (presynaptic cytomatrix protein)	**Pclo**	**97753_at**
Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	**Slc6a3**	**97787_at**
Synaptojanin 2	**Synj2**	**97962_at**
Tubulin, alpha 2	**Tuba6**	**98759_f_at**
Chondroitin sulfate proteoglycan 3	**Cspg3**	**98799_g_at**
Potassium voltage gated channel, Shal-related family, member 3	**Kcnd3**	**99371_at**
Triosephosphate isomerase 1	**Tpi**	**99566_at**

Table 4: Data from the WebQTL project. Pearson's correlation coefficients among the 28 variables of interest.

Phenotypes/Gene symbols are shown in the left column (See Table 2 for variable details). Shown in bold are the correlations between DAT-related phenotypes and DAT-related transcripts.

	DAT_NA	DAT_CP	S_45	O_45	L_32	SE_32	S_32	Kcnb1	Rasgrf2	Brca2	Dnm	Aldo1	Pard3	Kif3b	Bcan	Rgnef	Snca	Syn1	Cftr	Ap1b1	Aco2	Pclo	Slc6a3	Synj2	Tuba6	Cspg3	Kcnd3
DAT_CP	0.47																										
S_45	0.27	0.27																									
O_45	0.23	0.14	0.52																								
L_32	-0.15	-0.30	0.24	-0.10																							
SE_32	-0.19	-0.29	0.01	0.11	-0.67																						
S_32	0.14	-0.06	0.04	-0.03	0.49	-0.77																					
Kcnb1	0.27	0.44	0.14	-0.11	0.15	-0.42	0.20																				
Rasgrf2	0.13	-0.08	0.06	-0.18	0.43	**-0.64**	0.34	0.49																			
Brca2	0.02	-0.21	-0.32	-0.40	-0.44	**0.57**	**-0.52**	-0.12	0.15																		
Dnm	-0.02	-0.01	-0.15	0.03	0.20	-0.23	0.04	-0.20	0.05	-0.33																	
Aldo1	-0.18	-0.06	-0.33	-0.07	-0.05	-0.14	0.00	-0.19	0.00	-0.20	0.86																
Pard3	0.18	-0.10	0.22	**0.51**	-0.06	0.45	-0.14	-0.32	-0.52	-0.12	-0.11	-0.26															
Kif3b	0.23	0.35	0.05	-0.12	-0.10	-0.26	0.09	0.58	0.28	0.08	-0.38	-0.27	-0.41														
Bcan	-0.32	0.10	0.07	0.30	0.20	-0.48	0.20	0.14	0.18	-0.35	0.63	0.47	-0.18	0.00													
Rgnef	0.10	0.32	0.09	0.17	-0.34	0.10	0.12	0.26	-0.07	0.05	-0.63	-0.61	0.24	0.51	-0.17												
Snca	0.38	0.09	-0.02	-0.12	-0.11	0.39	-0.11	-0.03	-0.01	0.40	-0.49	-0.53	0.18	-0.08	-0.58	0.31											
Syn1	-0.14	0.25	0.14	0.08	-0.25	0.24	0.10	-0.13	-0.12	-0.27	0.18	0.30	0.08	-0.23	-0.05	-0.05	-0.04										
Cftr	0.32	-0.05	-0.13	0.17	-0.02	0.11	-0.13	-0.14	0.07	-0.09	0.62	0.53	0.06	-0.26	0.18	-0.35	0.18	0.12									
Ap1b1	-0.06	-0.07	-0.08	0.07	0.25	**-0.57**	0.26	0.14	0.18	-0.52	0.73	0.72	-0.37	0.03	0.70	-0.50	-0.79	0.03	0.29								
Aco2	-0.09	-0.17	-0.16	0.06	0.16	-0.18	0.01	-0.28	0.10	-0.27	0.96	0.88	-0.18	-0.35	0.59	-0.67	-0.49	0.18	0.66	0.74							
Pclo	0.35	0.01	-0.23	-0.11	-0.08	0.27	-0.08	0.05	0.05	0.39	-0.52	-0.43	0.23	0.00	-0.70	0.29	0.72	-0.19	0.09	-0.64	-0.49						
Slc6a3	-0.41	**-0.59**	-0.05	-0.05	0.49	-0.46	0.26	0.13	0.37	-0.15	0.12	0.08	-0.42	0.08	0.26	-0.33	-0.30	-0.23	-0.12	0.39	0.20	-0.26					
Synj2	0.12	0.06	0.19	0.17	0.04	-0.10	0.12	-0.10	0.06	-0.21	-0.13	-0.36	0.13	-0.13	-0.04	0.25	0.37	-0.04	0.12	-0.24	-0.15	0.08	-0.15				
Tuba6	-0.04	-0.18	-0.29	-0.10	0.20	-0.12	-0.05	-0.32	0.10	-0.18	0.86	0.81	-0.18	-0.41	0.30	-0.74	-0.28	0.18	0.66	0.58	0.87	-0.25	0.19	-0.22			
Cspg3	0.08	0.13	0.14	-0.27	-0.12	0.13	-0.27	0.10	0.31	0.33	-0.20	-0.21	-0.38	0.44	-0.12	0.27	-0.06	-0.22	-0.31	-0.16	-0.17	-0.02	0.10	-0.13	-0.23		
Kcnd3	-0.09	-0.07	-0.17	-0.38	0.32	-0.10	0.00	-0.10	-0.10	-0.21	0.65	0.53	-0.25	-0.14	0.29	-0.63	-0.15	0.08	0.47	0.46	0.63	-0.37	0.16	-0.17	0.67	-0.25	
Tpi	-0.01	0.00	0.07	0.01	0.25	-0.14	0.04	-0.16	0.03	-0.43	0.84	0.64	0.00	-0.49	0.44	-0.59	-0.28	0.36	0.58	0.54	0.79	-0.53	0.05	0.18	0.72	-0.28	0.63

4.6 Discussion

There is growing evidence that protein-protein interactions play a vital role in regulating transporter function. For example, the function of the GABA and SERT are regulated by syntaxin and protein phosphatase 2A respectively, two proteins that have been shown to exist in a complex with the transporters (Bauman et al., 2000; Deken et al., 2000). However, global studies of proteins that exist in a complex with and regulate monoamine transporter function are lacking, and our work is the first to use an interaction proteomics based approach to elucidate a transporter proteome.

Antibody and affinity purification based approaches are widely used to elucidate protein-protein interactions (Becamel et al., 2002; Ho et al., 2002; Husi et al., 2000). In this study we used an antibody directed towards the N-terminus of DAT to isolate proteins associated with DAT. This approach was validated by confirming the presence of α-synuclein in the DAT complex (Lee et al., 2001). Our approach identified 20 candidate proteins which were involved in various cellular functions including cell surface trafficking, cell adhesion, scaffolding proteins, ion channels and metabolic enzymes. It is, of course, possible that some of the proteins that are found to be associated with DAT are artifactual interactions caused by detergent-based extraction used to isolate these protein complexes.

Two proteins, DAT and synapsin 1b, were identified as high probability hits with peptides derived from a band of approximately 70-80 kDa. Synapsin 1b belongs to a family of phosphoproteins that are involved in regulating neurotransmitter release (Greengard et al., 1993). It has been shown previously that syntaxin 1A, a protein also

involved in regulating neurotransmitter release, interacts directly with the GABA transporter and regulates its function (Deken et al., 2000) providing an elegant mechanism to couple neurotransmitter release to reuptake. Thus, it is tempting to speculate that synapsin Ib plays a similar role in coupling transmitter release to DAT function. Synapsin also interacts with actin (which is a component of the DAT complex) and it is possible that interaction of DAT with synapsin 1b could be indirectly mediated through actin.

Four peptides corresponding to Kv2.1 alpha subunit, a potassium channel, were detected by mass spectrometry in the DAT complex. Kv2.1 alpha subunit is widely expressed in the brain and its expression is restricted to the soma and proximal dendrites in cortical and hippocampal neurons (Murakoshi and Trimmer, 1999). There is also evidence for expression of Kv2.1 in axons and neuronal terminals in rat cerebellar basket cells (Tan and Llano, 1999), but the subcellular localization of Kv2.1 in the striatum has not been examined. Our study suggests that Kv2.1 is localized to striatal synaptosomes and is enriched in the synaptosomal fraction. This implies a common subcellular localization for DAT and Kv2.1 in the striatum, although this needs to be confirmed by immunohistochemical studies. In globus pallidus (GP) neurons, the ratio of Kv2.1 to Kv3.1/3.2 channels determines the discharge frequency of these neurons. It is postulated that an alteration in the expression levels of these channel types could lead to burst-firing observed in GP neurons after treatments or due to diseases that reduce dopamine levels (Baranauskas et al., 1999). Recent studies suggest that DAT-associated currents can modulate neuronal excitability (Ingram et al., 2002) and association of DAT with Kv2.1

could provide a novel mechanism by which DAT could control neuronal excitability and modulate neurotransmitter release. Although, functional association of neurotransmitter receptors with ion channels has been established (Law et al., 2000), this is the first report of association between a neurotransmitter transporter and an ion channel.

We also identified dynamin I, a protein involved in synaptic vesicle recycling. Dynamin I is a GTPase involved in pinching off vesicles from the presynaptic membrane (Danino and Hinshaw, 2001). There is evidence that endocytosis of DAT is a dynamin-dependent process (Zahniser and Doolen, 2001). Hence, the association of dynamin I with DAT could also have a functional role in regulating DAT trafficking *in vivo.*

Brca2 was detected as a medium probability hit in our screen for DAT interacting proteins. Brca2 is a nuclear protein involved in the maintenance of chromosome structure and mutations in the gene encoding Brca2 render susceptibility to breast and ovarian cancers (Venkitaraman, 2002), but our results point to an additional role for this protein in brain, perhaps by regulating DAT function. We were unable to detect Brca2 in the synaptosomal fraction. A number of factors may account for this finding. Due to its large size (~400 kDa), the Western transfer conditions may need to be altered substantial for Brca2 to be detected. Interaction between DAT and large proteins such as Brca2 may not be stable in this preparation. It is also possible that DAT/Brca2 interactions may be localized to neuronal compartments that are absent in synaptosomal preparations.

Neurocan was detected as a high probability hit in the DAT complex. Neurocan is a chondroitin sulphate proteolycan whose expression is developmentally regulated. In adults, neurocan is proteolytically cleaved to a 130 kDa core protein with an average

molecular mass of ~200 kDa (Rauch et al., 2001). Neurocan is an extracellular matrix associated protein that is a ligand for tenascin-R and NCAM (Dityatev and Schachner, 2003). Neurocan has been implicated in the organization of striatal compartments *in vivo* (Charvet et al., 1998).

The cytoskeletal proteins, actin and tubulin were detected as high probability hits in our analysis. We also detected kinesin related protein 3b (KIF3b), a motor protein involved in vesicular transport and highly enriched in the brain (Yamazaki et al., 1995). KIF3b is part of the kinesin II complex, which plays an important role in insulin-stimulated glucose transporter 4 (GLUT4) trafficking (Imamura et al., 2003). Hence, it is possible that KIF3b is involved in the transport of vesicles containing DAT to the cell surface. The metabolic enzymes, aldolase, triose phosphate isomerase and similar to mitochondrial aconitase were also immunoprecipitated with the DAT complex. These enzymes are highly expressed in the brain and there is evidence that these metabolic enzymes interact with tubulin (Walsh et al., 1989). It is possible that these proteins exist in a complex with DAT via their interactions with tubulin.

The scaffolding proteins PAR-3 and aczonin were detected in our analysis. Scaffolding proteins are important because of their ability to nucleate multiprotein complexes. Aczonin is a presynaptic cytomatrix protein that is involved in synapse assembly. Aczonin possesses numerous protein interaction motifs including a C-terminal PDZ domain and is thought to play a role in localizing exo and endocytic machinery (Garner et al., 2000). PAR-3 is an 180kDa adaptor protein containing 3 PDZ domains. It

is highly expressed in the embryonic and adult striatum and known to play a role in the development of neuronal polarity (Lin et al., 2000).

The C-terminal tail of DAT has been shown to interact with PICK-1 (via its PDZ domain), α-synuclein, and Hic-5. While α-synuclein was readily detected in our screen by Western Blotting neither PICK-1 nor Hic 5 were detected. A possible reason for this could be the co-migration of PICK1 and Hic5 with the IgG heavy chain. It is also possible that PICK1 and Hic 5 do not associate with DAT under the conditions used for isolating the DAT complex in this study.

We also compared the components of the DAT proteome with the recently published PSD-95 (Peng et al., 2004) and the NMDA receptor proteome (Husi et al., 2000). Of the 347 proteins identified as components of the PSD-95 proteome, there are only 8 proteins that are also detected in the DAT proteome. There are only 3 proteins that overlap between the DAT and the NMDA receptor proteome. This further illustrates that members of the DAT proteome are discrete and do not show significant overlaps with that of two other widely receptor protein complexes, the PSD-95 and NMDA receptor proteomes.

The relationships between mRNA and protein levels are complex. Depending on the specific gene class, genetic system and treatment conditions the mRNA-protein correlations can range from 0 to 0.9 (Greenbaum et al., 2003). Interestingly, several studies suggest a robust correlation between gene expression profiles and protein interaction datasets (see (Ge et al., 2003)for review). For example, some studies carried out using yeast and *C.elegans* proteome and transcriptome data suggest that genes

encoding interacting proteins more likely belong to common gene expression clusters (Ge et al., 2003). Hence it can be hypothesized that the gene expression patterns of the 21 DAT-associated genes are part of a common transcriptome cluster. To test this hypothesis we used the WebQTL Project (web-based databases) that has been recently introduced to scientific community and proved to be a powerful tool for hypothesis driven investigations (Chesler et al., 2004; Chesler et al., 2003; Wang et al., 2003). Specifically, the WebQTL databases are useful for studies of regulation of gene expression and genetic mechanisms of complex traits. *In silico* investigation using these databases provided evidence for potential functional relationships among the 21 DAT-associated proteins detected by mass-spectrometry in the present study. PCA suggested a co-regulation of expression of these genes, while correlational analysis of gene expression and DAT-related phenotypes suggested involvement of several genes in DAT function. However, these *in silico* analyses do not substitute for a formal validation of the results. There are several limitations of the WebQTL data sets. For example, the brain region used for mRNA extraction in the WebQTL project was much larger than the one used in the present study. Therefore, expression profiles detected in BXD RI strains could represent a general global pattern that could not be specific for striatum of DBA mice. Another limitation concerns the relationships between gene expression and behavior. A correlation between mRNA abundance and behavior only suggests but does not indicate mechanistic involvement of this transcript in the behavior of interest because of multilayer level of cellular regulation. Nonetheless, the current *in silico* approach proved

to be an important hypothesis-generating tool that assisted greatly in potential target lists for future investigation.

Studies indicate that DAT function is sensitive to modulation by ethanol (Maiya et al., 2002; Mayfield et al., 2001). Hence we examined if any of the DAT-associated proteins map to QTL's for alcohol-related behaviors. Kv2.1 and Kif3b also map to a QTL for alcohol-induced loss of righting reflex (Markel et al., 1997). Dynamin I maps to a QTL for alcohol preference drinking (Melo et al., 1996).

In summary, the mass spectrometric approach identified a network of functionally diverse proteins that exist in a complex with DAT and may play a role in regulating DAT activity. Our work suggests that combining proteomics approaches with emerging genomic and genetic databases can facilitate such studies. Future studies will investigate the functional consequences of these interactions and examine how this network is perturbed by psychiatric conditions, drug treatments and drug addiction.

Chapter 5

Characeterization of ethanol effects in mammalian cells expressing GFP-DAT*

5.1 Introduction

The goal of specific aim 2 was to examine ethanol effects on cell surface trafficking of DAT. GFP-tagged wild type and G130T DAT were generated and transfected into mammalian cell lines to visualize ethanol-mediated trafficking events. Ethanol induced changes in DAT function and localization were monitored by [^{3}H]DA uptake experiments as well as by monitoring changes in localization of GFP-tagged transporters in real time. Characterizing ethanol effects on DAT function in a mammalian cell line will also enable us to address part of Aim 3, which is to identify proteins that associate differentially with wildtype and G130T DAT. The results from these experiments will be summarized in this chapter.

5.2 Effects of Ethanol on Madin Darby Canine Kidney (MDCK) Cells Stably Expressing DAT.

MDCK cells stably expressing the human DAT were obtained from Dr. Susan Amara's laboratory (Vollum Institute, Oregon). The cells were grown to 60-80% confluence. These cells were exposed to ethanol (100 mM) for 1 hour in KRH buffer

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* Experiments showing ethanol modulation of GFP-DAT function in midbrain slices were performed by Angela Ozburna. David Galindo performed the experiments showing ethanol modulation of DAT function in NG108 cells. All other experiments were performed by Rajani Maiya.

(Methods) at room temperature. DAT function was assayed by exposing these cells to [^{3}H]DA for 10 minutes in the presence of ethanol. Ethanol potentiated DAT function by 80% (Figure 12). These results indicate that ethanol potentiation of DAT function is not restricted to the *Xenopus* expression system.

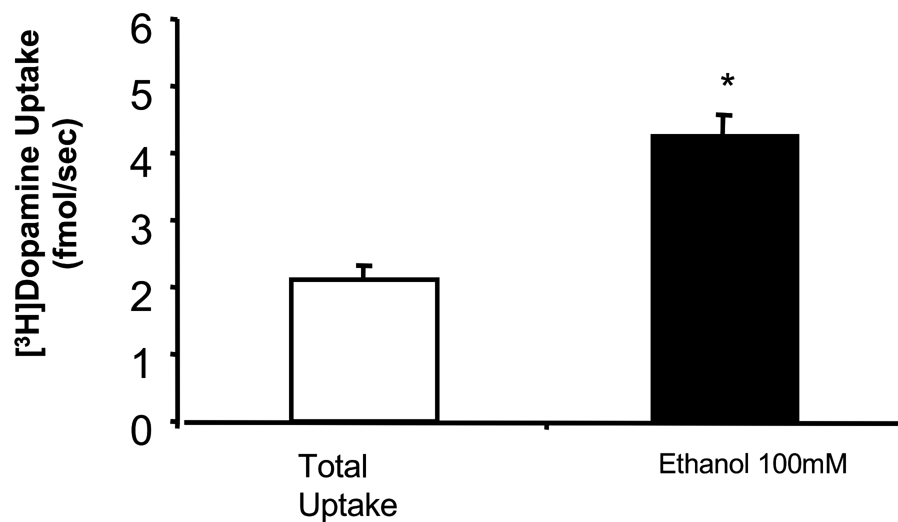

Figure 12: Effect of ethanol on MDCK cells stably expressing GFP-tagged DAT. MDCK cells stably expressing GFP-labeled DAT were exposed to 100 mM ethanol for 1 hour and [^{3}H]DA uptake was measured. Ethanol enhanced [^{3}H]DA uptake by 80% in these cells (n=3). * $p<.05$, two tailed t-test.

5.3 Effect of ethanol on SH-SY5Y cells stably expressing the human DAT

Recent site directed mutagenesis experiments in the Xenopus oocyte expression system suggest that discrete amino acids in the first intracellular loop are important for ethanol modulation of DAT function (Maiya 2002). In addition, proteomics data suggests that DAT belongs to a complex of interacting proteins. These proteins may play a role in regulating cell surface trafficking of DAT. Identification proteins associated with wildtype and ethanol insensitive mutant transporters will provide valuable insight into ethanol induced alterations in DAT function. An interaction-proteomics based approach, using antibodies directed to wildtype and G130T DAT could be used to identify differences in the proteins that associate with these transporters. Thus ethanol's effects on DAT function in human neuroblastoma SH-SY5Y cells were investigated. SH-SY5Y cells resemble dopaminergic neurons and hence DAT regulation in these cells might closely mimic that in midbrain DA neurons (Jiang et al., 2004; Shamoto-Nagai et al., 2004). Human neuroblastoma cells were used because they are more amenable to proteomics-based approaches (because of a fully sequenced genome). Since stable cell lines have the advantage of expressing consistent levels of protein over time, SH-SY5Y cells stably expressing GFP-tagged wildtype and G130T DAT were generated.

SH-SY5Y cells stably expressing DAT were generated as described (Methods). Seven clones were isolated and screened for DAT expression and four stably expressing DAT clones were selected based on the level of expression of DAT. The clones selected could be classified as high, medium, and low expressors (based on their [^{3}H]DA uptake

characterestics). The ability of ethanol to alter DAT function in these clones was tested by exposing these cells to ethanol for 1 hour in KRH buffer at room temperature. The cells were then exposed to [^{3}H]DA in the presence of ethanol for 10 minutes and the amount of radioactivity taken up was measured by liquid scintillation spectroscopy. The clones were all insensitive to ethanol. One of the clones (clone# 13) displayed a trend towards potentiation (25%) but this was not statistically significant (Figure 13). It is possible that longer exposure times to ethanol and/or incubation at 37^{0}C are required for ethanol to potentiate DAT function in these cell lines.

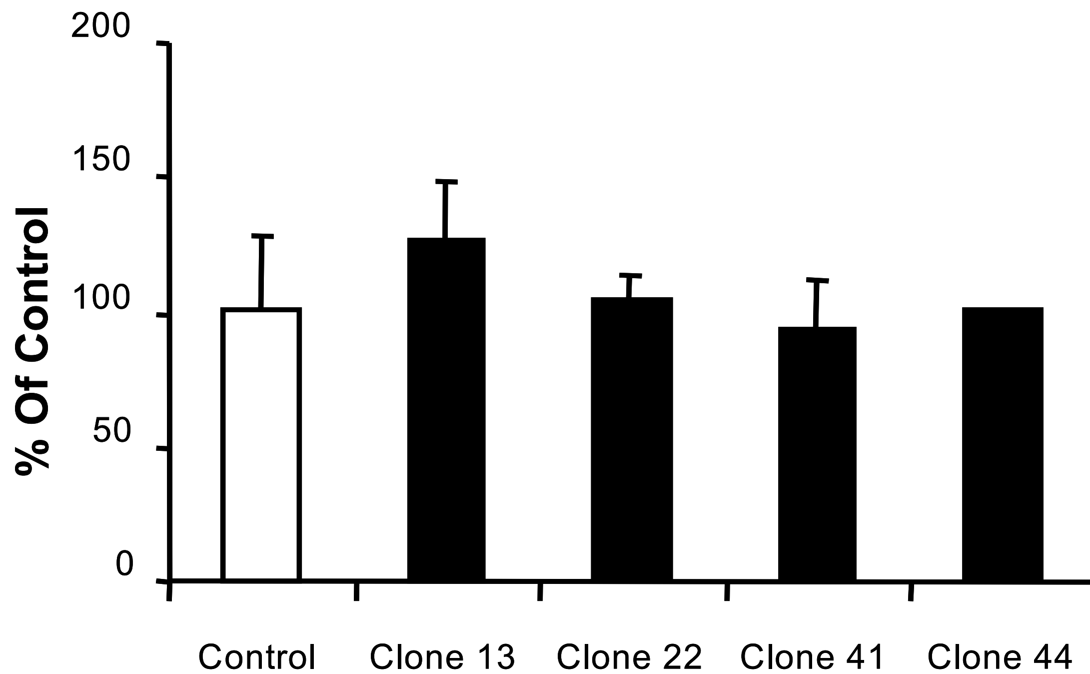

Figure 13: Ethanol effects on SH-SY5Yclones stably expressing GFP-DAT.
Four clones stably expressing GFP-DAT were exposed to ethanol for 1 hour and $[^3H]$DA uptake values were measured. Values are expressed as % of control with the control set to 100%. Clone 13 shows slight potentiation (25%) in DAT function upon ethanol exposure but this was not statistically significant. Mean values $\pm$ SEM are shown. N=7 for clone 13 and N=4 for clones 22, 41, and 44.

5.4 Generation of sindbis viral particles encoding GFP-tagged DAT

Protein trafficking regulates the function of a number of synaptic proteins (Buckley et al., 2000). Numerous studies suggest that the function of neurotransmitter transporters at the cell membrane is also regulated by trafficking events (Zahniser and Doolen, 2001). This mode of regulation plays a vital role in controlling the dynamics of neurotransmission. The aim of the experiments discussed in this section was to examine in real time the trafficking of DAT as a mechanism underlying the synaptic neuroadaptation to ethanol. The experimental design involved observation of GFP-DAT trafficking in primary cultures from mouse mesolimbic neurons using confocal and real time two-photon fluorescence imaging.

To study trafficking events that regulate transporter function in mouse mesolimbic neurons, sindbis virus-mediated gene delivery system was generated and used to infect neuronal cells with enhanced green fluorescent protein (GFP)-tagged DAT viral particles. Sindbis virus is a plus strand RNA virus belonging to the *Alphavirus* genus of the *Togaviridae* family. The sindbis virus has been shown to preferentially infect neuronal cells. The sindbis viral gene delivery system has several advantages as compared to other viral vectors. They have a higher efficiency and specificity of infection of neuronal cells and also demonstrate a rapid onset of gene expression (Ehrengruber, 2002).

BHK cells were electroporated with mRNA encoding GFP-tagged DAT as described (Methods). Fluorescence was monitored 12-24 hours post transfection. 70% of the cells were transfected with GFP-tagged DAT (Figure 14A). To determine the

functionality of the GFP-tagged DAT, [^{3}H]DA uptake experiments were carried out 24 hours post transfection. Significantly higher [^{3}H]DA uptake was observed in cells expressing the GFP-tagged DAT suggesting that the transporters were functional (Figure 14B).

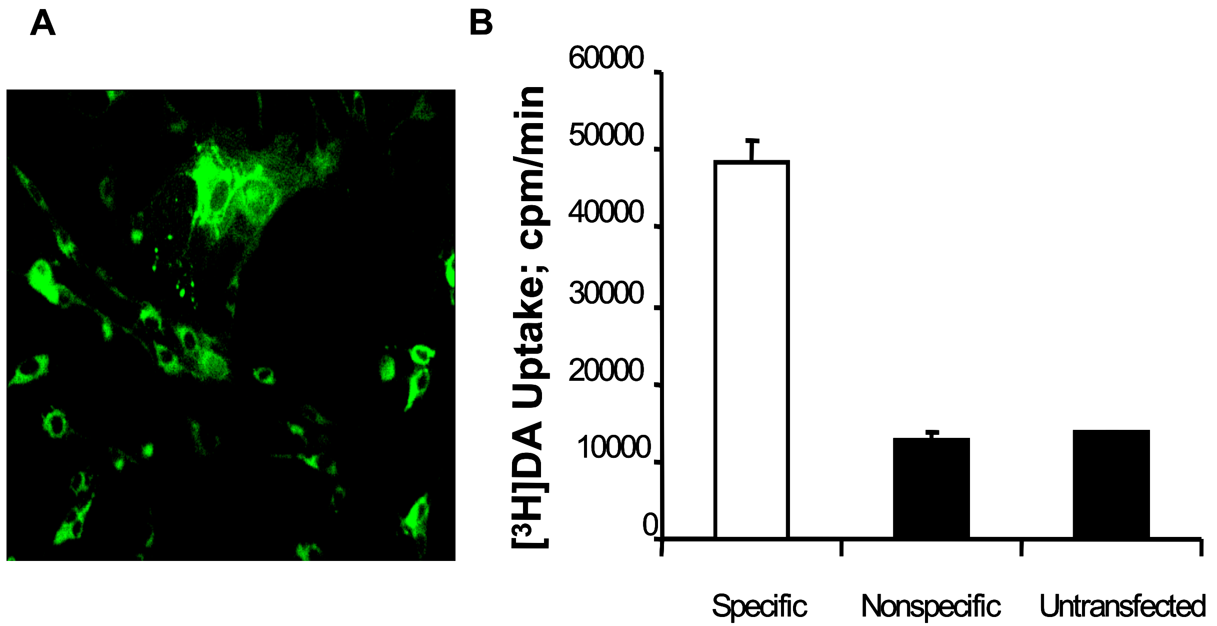

Figure 14: Expression of functional GFP-tagged transporters in BHK cells. A) BHK cells were electroporated with RNA encoding EGFP-DAT and helper virus RNA. Cells were imaged 12-24 hours post transfection. Greater than 70% of the cells expressed GFP-DAT. B) Virions were harvested and used to infect a fresh monolayer of BHK cells and [^{3}H]DA uptake was measured as described. Specific [^{3}H]DA uptake was measured in cells infected with virions. In contrast, [^{3}H]DA uptake was not observed in uninfected cells.

The viral particles generated were also tested for their ability to infect neuronal cells in culture. Cortical neurons were cultured and infected with GFP-tagged viral particles as described. The neurons were imaged 24 hours post-infection. The neurons were uniformly labeled with GFP-tagged transporters. Fluorescence could be visualized in dendrites as well as in the terminals (data not shown). The sindbis viral particles expressing GFP-tagged DAT were successfully used to infect cultured cortical neurons.

Next, the viral particles were used to infect intact neurons in slices. Explants were generated from VTA and infected with sindbis viral particles as described (Methods). The explants were visualized using a 2-photon microscope 24 hours post-infection (Figure 15). GFP-labeling could be observed in both the explants in the cell body and terminal regions. Hence, the sindbis viral particles generated could be used to infect a variety of cell and neuronal preparations.

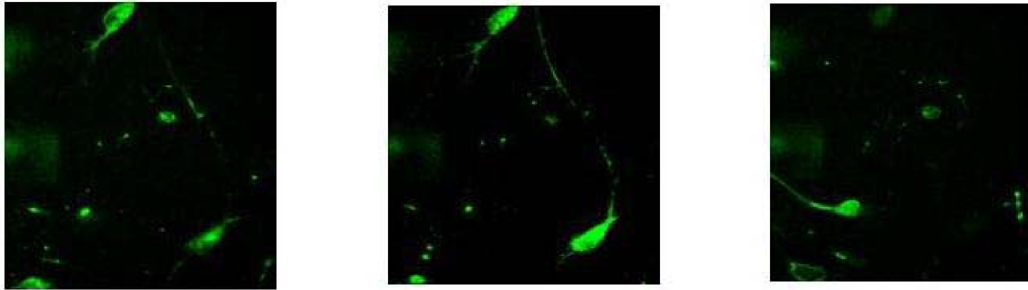

Figure 15: Expression of GFP-DAT in mesolimbic slice explants. Mesolimbic explants were prepared and infected with recombinant sindbis virus particles as described. The slices were imaged 24 hours post-injection using a two-photon microscope. Z-scans of infected neurons in mesolimbic slice explants are depicted. In both types of explants fluorescence was observed in the cell body, axons, and in distal dendrites.

5.4.1 Effects of ethanol on GFP-DAT expressed in mouse midbrain DA neurons

Primary cultures of midbrain neurons were prepared and plated to a density of 100,000 cells/ml on glass coverslips in 24-well culture dishes (methods). Cultures were maintained for 1 week before infection with recombinant sindbis viral particles encoding GFP-DAT. Purified virus was added directly to the media 24 hours prior to ethanol treatment. Under these conditions approximately 80% of the neuronal population expressed the fusion protein. Simultaneous z-section scanning for GFP fluorescence was performed in the absence and presence of ethanol (100 mM). The media was replaced with artificial cerebrospinal fluid (aCSF) and control images were taken after 120 and 150 minutes of equilibration. The cells were then exposed to ethanol (100 mM) and imaged after 10 and 30-minute exposure. Each cell served as its own control. Computer generated control and experimental images of two representative neurons were compared to determine GFP-DAT redistribution (Figure 16). Fluorescence was observed in the cell body, axons, and in distal dendrites of infected neurons**.** The image series B-D (top and bottom panel) shows ethanol-induced redistribution of GFP-DAT as a function of time. These changes are represented by the graded color changes indicated by the arrows. A change in the distribution of fluorescence intensity could be observed around the cell body and dendrites of both cells (top and bottom panel) after 10-minute ethanol exposure. The z-series of each image has been arithmetically compressed so that composite fluorescence can be visualized in a single plane. Thus, 10-15 images contribute to the apparent heavy fluorescence observed.

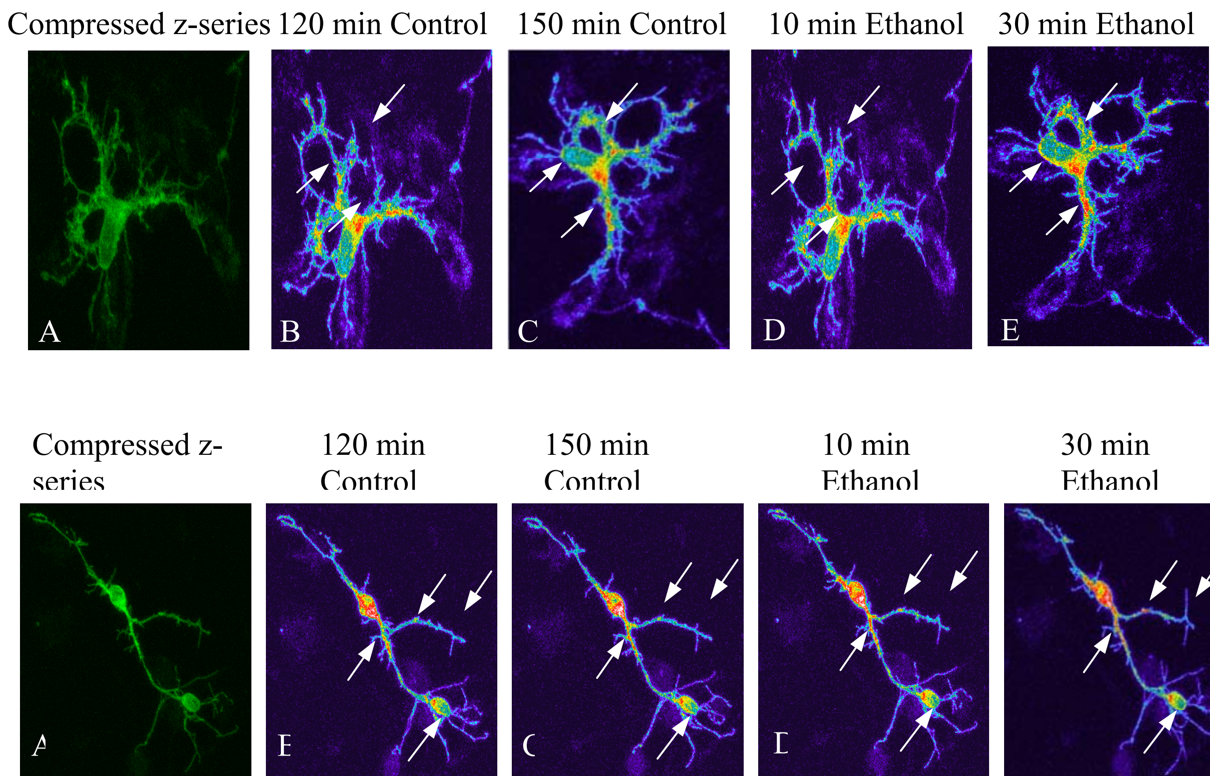

Figure 16: Effects of ethanol on GFP-DAT expressed in midbrain DA neurons. Midbrain neurons were cultured and infected with Sindbis viral particles encoding GFP-DAT. Simultaneous z-section scanning for GFP fluorescence were performed in the absence (control) and presence of ethanol (100 mM). Control images were taken 120 and 150 min after equilibration with aCSF. Cells were then exposed to ethanol (100 mM) and imaged after 10 and 30 min exposure. Metamorph analysis package was used to compare patterns of GFP-DAT distribution. Raw and pseudocolored z-scans of two infected neurons (top and bottom panel) imaged in the presence and absence of ethanol are shown in Figure 6. Ethanol-induced redistribution of DAT (arrows) can be observed in panels B-D.

5.6 Discussion

Ethanol's effects on DAT function in mammalian cells expressing DAT were examined. Results obtained from MDCK cells stably expressing DAT and HeLa cells transiently expressing DAT show that ethanol potentiates DAT function in mammalian cells (by 80% and 30% respectively). These results suggest that ethanol's effects on DAT function is not restricted to the *Xenopus* oocyte expression system, where ethanol caused an approximately 30% enhancement in DAT function. It is still unclear whether the mechanism by which ethanol potentiates DAT function in MDCK cells is similar to that in *Xenopus* oocytes. Attempts to monitor changes in the cell surface distribution of DAT by confocal microscopy in MDCK cells have been inconclusive. Cell surface biotinylation techniques may be needed to observe the subtle changes in transporter density caused by ethanol exposure. It is also possible that the mechanism of ethanol action in MDCK cells might differ from that in oocytes and occur through a trafficking independent mechanism. The regulation of DAT in MDCK cells, which are epithelial in origin, may differ from that in neurons. Hence, ethanol effects on Dat function were tested in neuroblastoma cell lines that are derived from neurons.

Ethanol's effects on DAT function in SH-SY5Y cells stably expressing DAT were examined. SH-SY5Y cells are neuroblastoma cells and resemble DA neurons in culture. Four clonal SH-SY5Y cell lines with different expression levels (classified as medium, high and low) of DAT were chosen for ethanol studies. Ethanol had no effect on DAT function in the four clones tested. Clone # 13 showed a trend towards potentiation (25%) but this was not significantly different from the control. Numerous reasons could

account for the observed discrepancy in ethanol effects between MDCK cells and SH-SY5Y cells. It is possible that longer ethanol exposure times are required to potentiate DAT function in SH-SY5Y cells. It is also possible that the temperature of incubation with ethanol plays a crucial role in ethanol potentiation of DAT function. Preliminary results using SK-N-SH and NG108 cells transiently transfected with DAT suggest that 2-hour exposure to ethanol at 37^0C causes robust potentiation of DAT function (Mayfield and Galindo, personal communication). It is possible that a similar paradigm of ethanol exposure is necessary to potentiate DAT function in the SH-SY5Y cells stably expressing DAT and experiments are currently underway to standardize these conditions.

To study changes in DAT trafficking in cultured neurons and neuronal explants, a sindbis viral construct expressing GFP-tagged DAT was generated. The viral particles generated were used to successfully infect neurons in culture as well as VTA explants. These viral particles were also used to deliver GFP-tagged DAT into the VTA of mice. Midbrain DA neurons were infected with sindbis viral particles encoding GFP-DAT and imaged in the presence and absence of ethanol (100 mM) in real time. Preliminary analysis of fluorescence distribution patterns in the presence and absence of ethanol suggest that even a 10-minute exposure to ethanol can cause changes in pattern of distribution and intensity of fluorescence in these neurons. These changes need to be quantitated to evaluate if they are significantly different from the control images.

The results outlined above validate the approach of using viral delivery systems to monitor GFP-DAT trafficking in a variety of neuronal preparations. Currently studies are underway to quantitate the changes in fluorescence observed in these cells upon ethanol

exposure. Cell surface biotinylation experiments could also aid in dissecting the mechanism by which ethanol alters cell surface trafficking in these cells. Techniques developed for viral mediated gene delivery and confocal imaging through the course of these studies will be used in future studies on ethanol-induced neuroadaptation in the laboratory.

Chapter 6

Discussion

The midbrain DA system comprises of neurons originating in the substantia nigra and VTA and projecting on to the striatum and NAc area respectively. This circuitry has been widely implicated in mediating the rewarding and reinforcing effects of various drugs of abuse (Wise, 2002). DAT is an important member of this pathway and controls the spatio-temporal dynamics of DA neurotransmission by mediating reuptake of DA into the presynaptic neuron. DAT is also the site of action of various psychostimulants like cocaine and amphetamine. These drugs function by inhibiting DA uptake by DAT and the resulting increases in extracellular DA are thought to mediate in part the rewarding and reinforcing effects of these drugs of abuse (Gainetdinov et al., 2002).

6.1 Ethanol's effects on dopaminergic synaptic transmission

Effects of ethanol, unlike most drugs of abuse involve multiple neurotransmitter and receptor systems in multiple brain regions (Harris, 1999). The effects of ethanol on various properties of midbrain DA neurons have been examined and the results are controversial. Ethanol has been shown to enhance the firing rates of VTA DA neurons and cause increases in extracellular DA concentrations in the NAc (Di Chiara and Imperato, 1988). Ethanol has also been shown to enhance DA release in the NAc of rats self-administering ethanol. Pharmacological intervention with DA transmission in the

VTA or NAc has been shown to reduce ethanol-reinforcement (Weiss and Porrino, 2002). These and other studies support a role for DA in the reinforcing actions of ethanol. However, DA lesion studies in the NAc suggest that ethanol responding or ethanol-reinforcement is independent of DA systems (Ikemoto et al., 1997).

Evidence for ethanol's effects on DAT function *in vivo* have been controversial. Numerous studies have examined the effects of ethanol on dialysate DA concentration in the brain. One study examining ethanol's effects on DA clearance in the striatum in rats suggest that local exposure to ethanol enhances DA clearance (Wang et al., 1997). Chronoamperometric studies on DA clearance in the dorsal striatum of rats injected with ethanol also suggest that DAT function is upregulated (Sabeti et al., 2003). These studies are consistent with our findings in Xenopus oocytes and MDCK cells that acute exposure to ethanol can enhance DAT function. However, a recent voltametry study suggests that ethanol decreases extracellular DA in the striatum and this effect occurs through decreased release and not increased reuptake (Budygin et al., 2001). This effect is however sensitive to GBR12909, a DAT inhibitor, suggesting that DAT maybe involved in mediating decreased DA levels in the striatum. Numerous microdialysis studies also suggest that ethanol enhances synaptic DA concentrations presumably by a mechanism involving enhanced release of DA (Yim and Gonzales, 2000). These results appear to conflict with our findings suggesting that ethanol enhances DA uptake by DAT. However, these results were obtained using microdialysis techniques, which do not directly measure DA uptake. One study (Yim and Gonzales, 2000) has simultaneously quantitated dialysate DA levels and extracellular ethanol concentrations. The results from

this suggest indicate that dialysate DA levels return to baseline even when ethanol levels continue to remain high. This temporal mismatch between dialysate DA and ethanol levels could result from ethanol enhancement of DAT function (Mayfield et al., 2001).

Ethanol-mediated increases in DAT function may represent a compensatory mechanism that occurs to counter-balance the increase in extracellular DA concentrations mediated by ethanol. This mechanism could contribute to acute functional tolerance that is observed for DA-mediated responses (Holman and Snape, 1985; Mullin and Ferko, 1983). It is also possible that enhanced DAT function could contribute to the decrease in mesolimbic dopaminergic activity (Shen and Chiodo, 1993) and decrease in extracellular DA concentrations (Diana et al., 1993) observed during ethanol withdrawal. Hence, changes in DAT density may also contribute to ethanol withdrawal symptoms and may motivate ethanol seeking in dependent individuals.

Relatively few studies have focused on the role of presynaptic neurotransmitter transporters in ethanol-induced neuroadaptation. The significance of this work was the elucidation of a novel mechanism by which ethanol could inactivate the dopaminergic pathways. In addition, effects of ethanol on protein interactions and trafficking defined by these studies could also apply to additional receptor systems. The use of gene delivery and proteomics techniques is very limited in alcohol research and this work provides applications of these techniques to this field. This is also the first study to carry out a comprehensive analysis of the DAT proteome. Defining this important complex will give us valuable insight into the signal transduction pathways that regulate the function of DAT and other neurotransmitter transporters.

6.2 Ethanol' s effects on DAT expressed in Xenopus oocytes

Ethanol enhances [^{3}H] and cold DA uptake by DAT in Xenopus oocytes expressing DAT. This enhancement was accompanied by an increase in the number of cell surface transporters. Treatment of oocytes with cycloheximide, a protein synthesis inhibitor did not alter ethanol-induced enhancement of [^{3}H]DA uptake suggesting that enhancement of DA uptake by ethanol is not protein synthesis dependent (Mayfield et al., 2001). Ethanol has been shown to alter trafficking of neuronal proteins; for example, ethanol exposure causes translocation of the catalytic subunit of PKA (subunit C alpha) from the golgi to the nucleus (Dohrman et al., 1996). Ethanol exposure also induces trafficking of receptor for activated C kinase 1(RACK1) from the cytoplasm to the nucleus (Ron et al., 2000). The data discussed above suggests that ethanol may also affect DAT function in a similar manner by altering DAT trafficking.

Experiments also suggest that ethanol has contrasting effects on DAT and NET function. Studies on human DAT and NET function expressed in HeLa cells as well as *in vivo* electrochemistry studies suggest that ethanol enhances DAT function but inhibits NET function. Ethanol sensitivity of chimeras generated between DAT and NET were examined in order to delineate regions on DAT important for ethanol enhancement. These chimeras suggest that a region of the transporter spanning regions TMD1-3 was important for ethanol potentiation. Site directed mutagenesis experiments further narrowed this region down to two amino acids G130T and I137 in the first intracellular loop. Replacement of these individual amino acid residues to the corresponding residue

in NET resulted in a mutant transporter that displayed normal DAT function but was insensitive to ethanol. Replacement of the first intracellular loop of DAT with that of NET resulted in a mutant transporter that did not traffic to the cell surface, reiterating the role of these residues in steady state trafficking of DAT to the cell surface.

The mechanism by which these residues mediate ethanol potentiation of DAT function is not clear. There are no consensus phosphorylation sites for PKC or PKA in this region of the transporter. Further, it has been shown that direct phosphorylation of the transporter is not required for PKC–mediated regulation of the transporter. These results suggest that ethanol-mediated regulation of DAT occurs through a mechanism independent of protein kinases and led to the hypothesis that ethanol modulates the interaction between DAT and a putative regulatory protein important for ethanol-induced trafficking of DAT and that this interaction occurs at first intracellular loop. Based on the results outlined above, it is possible to outline a model for ethanol modulation of DAT function (Figure 1). Ethanol can alter the trafficking of DAT to the cell surface by possibly modulating the interaction of DAT with an unknown protein at the first intracellular loop. It is still not clear whether ethanol alters DAT function by enhancing trafficking of DAT to the cell surface or by inhibiting DAT endocytosis. Cell surface biotinylation experiments could aid in addressing this important question.

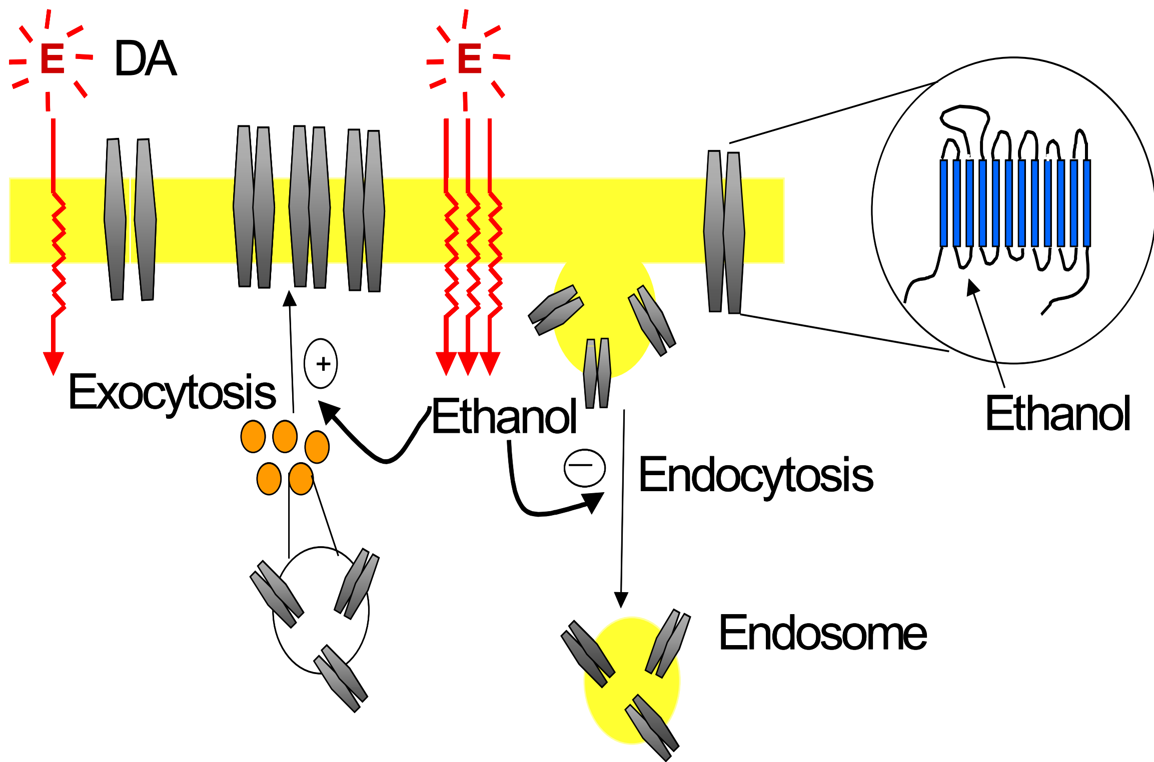

Figure 17: A schematic illustrating mechanisms of ethanol action on DAT. Ethanol could be affecting DAT function. Ethanol potentiates DAT function by enhancing trafficking of DAT to the cell surface or by preventing internalization of cell surface DAT.

6.3 Ethanol effects on DAT function in mammalian cells expressing DAT

Experiments in the Xenopus oocyte expression system suggest that ethanol affects DAT function by altering cell surface trafficking of DAT. To visualize such trafficking events, GFP-tagged wildtype and G130T DAT transporters were generated and used to transfect a variety of mammalian cell lines.

Ethanol effects were monitored in MDCK cells stably expressing GFP-tagged human DAT. Exposure to 100mM ethanol potentiated DAT function in MDCK cells by 80%. The mechanism by which ethanol potentiated DAT function in these cells is not known.

SH-SY5Y cells stably expressing GFP tagged wildtype and G130T DAT were generated. Four different stable cell lines expressing different amounts of wildtype GFP-DAT were chosen and ethanol effects on DAT function were examined in these cells. DAT function was unaffected by ethanol in all four of the cell lines tested. One of the cell lines clone # 13 showed a 25% enhancement in DAT function on ethanol exposure. This potentiation was not significantly different from that of the control. The ethanol exposure paradigm used for SH-SY5Y cells was similar to that used for MDCK cells and Xenopus oocytes which was 100 mM ethanol for 1 hour at room temperature. It is possible that these conditions need to be altered substantially in order to observe ethanol enhancement of DAT function in SH-SY5Y cells. Preliminary observations in NG108 cells, and SK-N-SH cells transiently transfected with GFP-DAT suggest that 2-hour exposure to 100 mM ethanol at 37^0C causes robust potentiation (34% and 59% respectively) of DAT function.

Exposure of SH-SY5Y cells stably expressing DAT to similar conditions may result in potentiation of DAT function.

The enhancement in DAT function reported in this study is modest (30%) and dependent on cell types. The best experiment to determine whether DAT plays a role in ethanol-induced neuroadaptation would be to manipulate DAT expression levels *in vivo* and monitor changes in ethanol consumption. The hypothesis to be tested would be that changes in DAT expression levels would alter ethanol consumption. Two groups have examined ethanol consumption in recombinant mice lacking DAT and have reached opposing conclusions. One study suggests that ethanol consumption is decreased in female DAT knockout mice (Savelieva et al., 2002) while another study suggests that ethanol preference and consumption is enhanced in female DAT knockout animals and decreased in males (Hall et al., 2003). The DAT knockout mice also display developmental abnormalities and hence may not be well suited for drinking studies. An alternative to the DAT knockout mice is the DAT knockdown mice where only 10% of DAT is expressed compared to wildtype (Zhuang et al., 2001). These hyper-dopaminergic mice do not display any of the developmental abnormalities observed in the DAT knockouts.

The two strategies discussed above involve reducing cell surface DAT expression levels. An attractive approach to monitor consequences of enhanced DAT expression on ethanol consumption would be to express DAT in specific brain regions using viral expression systems. As a first step towards achieving this goal, a sindbis virus system that is capable of delivering viral particles encoding GFP-DAT into a variety of neuronal

preparations including cultured neuronal cells, slice preparations and *in vivo* was generated. Preliminary results of ethanol exposure in neuronal cells expressing GFP-DAT suggest that a 10-minute exposure to ethanol can induce redistribution and enhance fluorescence intensity of GFP-DAT in these cells. These results are however preliminary and efforts are underway to quantitate these differences and evaluate the functional significance of the observed changes.

6.4 The dopamine transporter proteome

Experiments performed in the Xenopus oocyte expression system led us to the hypothesis that ethanol modulates the interaction between DAT and a putative regulatory protein important for ethanol-induced trafficking of DAT and that this interaction occurs at the first intracellular loop. However not much is known about proteins that interact with and modulate DAT function. So far, yeast two hybrid screens using the C-terminal tail of DAT as bait has led to the isolation of three proteins that can directly interact with DAT and modulate its function: PICK1, α-synuclein and Hic5. All three of these proteins interact with the C-terminal tail of DAT (Torres et al., 2003b). An emerging theme in neuronal signaling is the organization of receptors into multiprotein complexes. Recently, the NMDA receptor has been shown to exist as part of a protein complex comprising of more than a 75 components (Husi et al., 2000). $5HT_{2c}$ receptor also exists as part of a multiprotein complex (Becamel et al., 2002). DAT has numerous consensus sites for protein-protein interactions including a C-terminal PDZ domain and leucine repeat motifs

(Torres et al., 2003b). Hence, DAT could also be part of a multiprotein complex. An interaction proteomics strategy was used to to purify DAT-associated proteins from the striatum. An antibody directed towards the N-terminus of DAT was used for this purpose. The resulting complex was resolved on a one-dimensional gel. Mass spectrometric analysis resulted in the identification of 21 proteins that associate with DAT. These proteins could be classified into different functional classes: ion channels, synaptic vesicle trafficking proteins, extracellular matrix proteins etc.

The network of proteins thought to exist in a complex with DAT is schematically illustrated in Figure 5. This network was assembled by combining our data with database searches. Proteins that are shaded in grey are “missing links”, that is, not detected in our screen but have been shown in other studies to interact with proteins that are found in the DAT complex in our analysis (Greengard et al., 1993), (Dityatev and Schachner, 2003; Lutchman et al., 2002; Robinson and Bonifacino, 2001; van Horck et al., 2001; Yamazaki et al., 1995; Yamazaki et al., 1996).

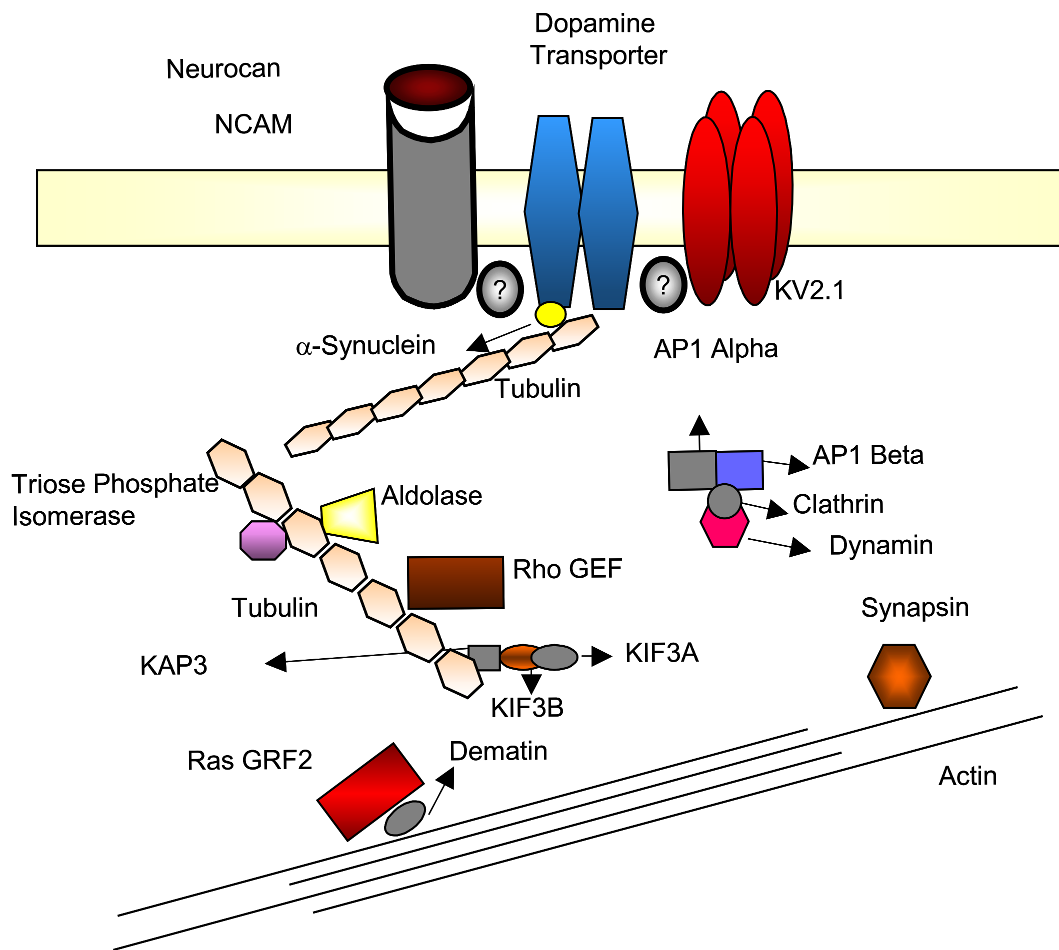

Figure 18: Schematic representation of the dopamine transporter proteome: Proteins found in the dopamine transporter complex are shown. Proteins shown in gray were not found in our studies but are known to exist in a complex with one or more proteins that were identified in our screen.

The identification of DAT associated proteins can give us valuable insight into signal transduction pathways that regulate DAT function. For example, one of the proteins identified as part of the DAT complex is the small G protein RasGRF2. RasGRF2 is a Ca^{2+} -responsive guanine nucleotide exchange factor. RasGRF2 contains a calmodulin-binding site that is thought to regulate its Ca^{2+} sensitivity. The function of various monoamine transporters is regulated by intracellular Ca2+ levels. There is also evidence that DAT function is rapidly upregulated by intracellular Ca^{2+} (Uchikawa et al., 1995). The mechanism of this upregulation is currently unknown. A hypothesis that could be formulated and easily tested based on our proteomics data is that Ca^{2+}-induced upregulation of DAT function is dependent upon RasGRF2.

Correlation between gene expression profiles and protein interaction datasets has not been very well studied in complex multicellular organisms. Studies in yeast suggests that a correlation exists between gene expression profiles and protein interaction datasets (see (Ge et al., 2003) for review). Recently this finding in yeast was extended to the C.*elegans* proteome and transcriptome data. It was found that genes encoding interacting proteins more likely belong to common gene expression clusters (Ge et al., 2003). Hence it can be hypothesized that the gene expression patterns of the 21 DAT-associated genes are part of a common transcriptome cluster. To test this hypothesis we used the WebQTL database. *In silico* investigation using WebQTL databases provided evidence for potential functional relationships among the 21 DAT-associated proteins detected by mass-spectrometry in the present study. Principal component analysis suggested the expression of the 21 DAT-associated genes is co-regulated. WebQTL can also be used to investigate

correlations between gene expression profiles and behavior. A correlational analysis of gene expression profiles of the 21 DAT-associated genes and DAT –related phenotypes (like DAT density in different brain regions, cocaine –induced locomotor activity and cocaine induced stereotopy) suggested involvement of several genes in DAT function. These are correlational studies and hence need to be interpreted cautiously. The benefit of WebQTL analysis is that it can potentially help in narrowing down the list of candidate proteins for future investigations.

A comparison of the components of the DAT proteome with that of the other brain proteomes published revealed very few overlaps. There are only 8 proteins that overlap between the DAT proteome and that of the PSD-95 proteome (comprised of a network 375 proteins). DAT and the NMDA receptor proteome have only 3 proteins in common. Further, while the NMDA receptor proteome is composed of both pre- and post-synaptic components, no proteins that were exclusively post-synaptic were detected in the DAT proteome. This is significant because it implies that members of the DAT proteome are discrete and specific.

The identification of the components of the DAT proteome has raised several important questions. The molecular size of the DAT complex isolated needs to be determined by techniques like gel filtration. This information is important since it could reveal if DAT is part of a single large network of proteins or composed of several smaller networks. It is also important to determine if members of the DAT proteome co-localize to the same cellular compartment. Our studies suggest that Kv2.1and DAT are localized to the presynaptic compartment. The subcellular localization of the remaining

components of the DAT proteome needs to be examined. One of the caveats of detergent based extractions to isolate protein complexes is that they could lead to potential artifactual interactions. One way to overcome this problem is to isolate the DAT protoeme from synaptosomal membranes which are prepared free of detergents. The number of DAT–associated proteins reported in this study is a conservative estimate and is likely to increase with more sophisticated protein separation and sensitive mass spectrometry techniques**.** It would be of interest to compare the DAT proteome with that of other neurotransmitter transporters to evaluate if common signaling pathways regulate the function of these transporters. DAT function is known to differ between brain regions. It would also be of interest to examine if differences in the components of the DAT proteome in different brain regions could account for these observations. Several strains of mice and rats differ in their ability to consume alcohol: for ex. the alcohol preferring (P) and non preferring (NP) rats. It would also be interesting to examine if DAT proteome differs between different strains and if the differences in DAT proteome can account for behavioral differences observed. Another question that remains to be answered is whether the DAT proteome differs between wildtype and G130T transporters and if the underlying differences can explain their contrasting ethanol sensitivities.

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