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ETHANOL POTENTIATES DOPAMINE UPTAKE AND INCREASES CELL SURFACE DISTRIBUTION OF DOPAMINE TRANSPORTERS EXPRESSED IN SK-N-SH AND HEK-293 CELLS

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Abstract

Ethanol increases dopaminergic release in the reward and reinforcement areas of the brain. The primary protein responsible for terminating dopamine (DA) neurotransmission is the plasma membrane-bound dopamine transporter (DAT). *In vitro* electrophysiological and biochemical studies in *Xenopus laevis* oocytes have previously shown ethanol potentiates DAT function and increases transporter binding sites. The potentiating effect of ethanol on the transporter is eliminated in *Xenopus* oocytes by the DAT mutation glycine 130 to threonine. However, ethanol's action on DAT functional regulation has yet to be examined in mammalian cell expression systems. To further understand the molecular mechanisms of ethanol's action on DAT, we determined the direct mechanistic action of short-term (≤ 2 hours) ethanol exposure on transporter function and cell surface distribution in non-neuronal human embryonic kidney cells (HEK-293) and neuronal SK-N-SH neuroblastoma cells expressing the transporter. Wild-type or G130T mutant DAT were overexpressed in HEK-293 and SK-N-SH cells. Ethanol potentiated DAT mediated [3 H]DA uptake in a dose (25, 50, 100 mM), but not time dependent manner in cells expressing wild-type DAT. Ethanol-induced potentiation of uptake was significantly reduced in cells expressing the G130T mutant. Analysis of DA uptake kinetic parameters indicates 100 mM ethanol exposure increased [3 H]DA uptake velocity ($V_{\max}$), while affinity for DA (K_m) remained unchanged. The effect of ethanol on wild-type DAT surface expression was measured by biotinylation cell surface labeling. DAT surface expression increased 40–50% after 1 hour 100 mM ethanol exposure. These studies show ethanol potentiates DAT functional regulation in both neuronal and non-neuronal cells, suggesting a direct mechanistic action of ethanol on transporter trafficking in mammalian systems. Our findings demonstrate ethanol's action on DAT function and regulation is consistent across multiple model systems.

Keywords

dopamine transporter; ethanol; HEK-293; SK-N-SH; dopamine uptake; cell surface

Introduction

Activation of the dopaminergic system in the mesolimbic areas of the brain controls a variety of physiological functions, including locomotion, emotion, and reward and reinforcement learning. The duration and strength of dopamine (DA) neurotransmission is dependent on the concentration of the neurotransmitter in the synaptic cleft. Re-uptake by the plasma membrane

dopamine transporter (DAT), located on the peri-synaptic terminal of dopaminergic neurons, predominately terminates dopaminergic neurotransmission and maintains homeostatic transmitter levels (Gainetdinov and Caron, 2003; Giros et al., 1991; Melikian and Buckley, 1999). DAT is a member of the Na^+/Cl^- family of neurotransmitter transporters that includes glycine, norepinephrine (NET), serotonin (SERT), and γ -aminobutyric acid (GAT1) transporters (Nelson, 1998; Rudnick, 1998). The transporters have similar topological arrangements and serve to terminate neurotransmission by removing transmitter from the extracellular space (Rudnick, 1998).

Neurotransmitter transporters undergo rapid and dynamic trafficking to and from the cell surface in order to regulate neurotransmission. (Chi and Reith, 2003; Li et al., 2004; Melikian and Buckley, 1999; Zhang et al., 1997). The functional regulation of DAT, via alterations in the cell surface expression of the transporter, can occur through signaling cascades and exposure to DAT substrates. Phorbol ester activation of protein kinase C, tyrosine kinase inhibition, and N-glycosylation prevention decreases the transporter activity by down-regulating DAT surface expression (Hoover et al., 2007; Li et al., 2004; Melikian and Buckley, 1999; Miranda et al., 2005; Vaughan et al., 1997; Zhang et al., 1997). When expressed in *Xenopus Laevis* oocytes, stimulation of the D_2 receptor induces a voltage-independent upregulation of DAT activity and [^3H]WIN 35428 surface binding, dependent on G(i/o) protein activation (Mayfield and Zahniser, 2001). Similar studies in midbrain primary culture and human embryonic kidney cells (HEK-293) reveal D_2 receptor stimulation increases DAT activity and surface expression via direct protein-protein interaction and ERK 1/2 pathways (Bolan et al., 2007; Lee et al., 2007). Stimulation of D_3 receptors expressed in HEK-293 cells also upregulates DAT activity and surface expression through MAPK and PI3K pathways (Zapata et al., 2007). Exposure to DAT substrates, inhibitors and other pharmacological reagents, such as psychostimulants can also alter DAT surface expression levels altering dopaminergic signaling in the brain.

Ethanol and psychostimulants such as cocaine and amphetamine induces cellular and molecular maladaptive changes in dopaminergic reward pathways, including alterations in DA release and/or DAT mediated DA uptake (Di Chiara and Imperato, 1988; Hyman et al., 2006; Jayanthi and Ramamoorthy, 2005; Koob and Weiss, 1992; Zahniser and Doolen, 2001; Zahniser and Sorkin, 2004). Acute or chronic (or both) exposure to cocaine, d-amphetamine, and ethanol has been reported to alter DAT cell surface densities (Chen and Reith, 2007; Chi and Reith, 2003; Daws et al., 2002; Gulley and Zahniser, 2003; Jayanthi and Ramamoorthy, 2005; Johnson et al., 2005; Kahlig et al., 2006; Mayfield et al., 2001; Zahniser and Sorkin, 2004). Cocaine rapidly increases DAT surface expression in mammalian cells and synaptosomes from rat striatum (Chen and Reith, 2007; Chi and Reith, 2003; Daws et al., 2002; Little et al., 2002), and competitively inhibits DAT-mediated DA uptake (Ritz et al., 1988). Other studies have shown chronic cocaine administration does not change the number of DAT binding sites, but causes a dose-dependent reduction in DAT mRNA expression (Letchworth et al., 1997). Amphetamines also increase dopaminergic signal strength by reducing DAT-mediated DA uptake. D-amphetamine acts as a substrate for DAT and the vesicle transporter, VMAT2 causing competitive inhibition of DA binding, and reverse DA transport (Johnson et al., 2005; Kahlig et al., 2006; Saunders et al., 2000; Sulzer et al., 1995; Sulzer et al., 1993).

It is generally accepted that ethanol elevates synaptic DA concentrations by activating mesolimbic dopaminergic neurons (Brodie and Appel, 1998; Imperato and Di Chiara, 1986; Yoshimoto et al., 1992). However, ethanol's action on DAT is currently under debate. *In vitro* studies suggest ethanol has a potentiating effect on the transporter. After a 24 hour exposure, ethanol and cocaine increase DAT-mediated DA uptake both individually and combined in neuronal, but not non-neuronal, cell lines expressing the transporter (Ho and Segre, 2001). These studies suggest ethanol and cocaine alters DAT function through a

common neural pathway. However, electrophysiological and biochemical studies in transporter-expressing *Xenopus laevis* oocytes have also shown ethanol exposure potentiates DAT function and increases [³H]WIN 35428 surface binding (Maiya et al., 2002; Mayfield et al., 2001). Two amino acids in the first intracellular loop of DAT, glycine 130 and isoleucine 137, mediate ethanol sensitivity of the transporter (Maiya et al., 2002).

In vivo studies examining the effects of chronic ethanol exposure on DAT suggest ethanol increases the number of DAT binding sites in alcohol-preferring vervet monkeys (Mash et al., 1996), the striatum of violent alcoholics (Tiihonen et al., 1995), and in specific brain regions of Wistar-Kyoto rats. (Jiao et al., 2006). Chronic *in vivo* ethanol exposure has also been reported to potentiate DAT-mediated dopamine uptake (Budygin et al., 2007; Carroll et al., 2006). However, the acute action of ethanol *in vivo* is less clear. Electrochemical studies in mice and rats suggest ethanol has no effect on the transporter (Budygin et al., 2001; Budygin et al., 2005; Jones et al., 2006; Mathews et al., 2006). However, other electrochemical studies have shown acute ethanol either increases (Sabeti et al., 2003; Wang et al., 1997) or decreases dopamine uptake (Robinson et al., 2005).

The present studies aimed to investigate the hypothesis that short-term (≤ 2 hours) ethanol exposure alters DAT trafficking and increases cell surface distribution of DAT, potentiating transporter-mediated DA uptake in heterologous expression systems. We chose a neuronal mammalian cell line, catecholaminergic SK-N-SH neuroblastoma cells (Liu et al., 2001; Richards and Sadee, 1986), and non-neuronal, HEK-293 cells, to express the transporter. Biochemical methods were used to characterize the effects of ethanol on transporter function and trafficking. We found ethanol potentiates DAT-mediated DA uptake in neuronal and non-neuronal cells expressing the transporter. This potentiation appears to be associated with an increase in expression of the transporter on the cell surface. Our results suggest ethanol has a direct mechanistic action in modulating DAT functional regulation in mammalian cell systems. These findings demonstrate ethanol's potentiating effect on the transporter is consistent across multiple expression systems, and furthers our understanding of the molecular mechanisms of ethanol's action in mammalian systems.

Materials and methods

Site-Directed Mutagenesis

The ethanol insensitive mutant (G130T DAT) (Maiya et al., 2002) was constructed from wild-type human DAT cDNA subcloned in a pBK-CMV vector (Stratagene, La Jolla, CA) using the QuikChange site-directed mutagenesis kit (Stratagene) to replace the glycine at position 130 with a threonine. Both wild-type and G130T DAT cDNA constructs were subcloned into the mammalian expression vector pEGFP-C1, and transfected into HEK-293 or SK-N-SH mammalian cells. The constructs contained an enhanced green fluorescent protein (eGFP) marker to confirm DAT expression by visualization with fluorescent microscopy.

Cell cultures and transient transfections

HEK-293 and SK-N-SH cells were purchased from ATCC (Manassas, VA). Cells were maintained in a humidified atmosphere at 37°C and 5% CO₂ in HEK-293 growth media (Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 0.1% Penicillin / Streptomycin (Sigma-Aldrich, St. Louis, MO)) or SK-N-SH growth media (Modified Eagle Medium (MEM) (Gibco, Carlsbad, CA) supplemented with 10% FBS and 0.1% Penicillin / Streptomycin). For uptake and kinetics assays, cells were split 24 hours prior to transfection into poly-D-lysine-coated 12-well plates (Becton-Dickinson, Bedford, MA) at a density of 0.5×10^5 per well. Cells were transiently transfected with either eGFP wild-type human DAT

or eGFP G130T DAT cDNA for 2 hours using Lipofectamine/PLUS reagents (Invitrogen, Carlsbad, CA). HEK-293 and SK-N-SH cells were transfected with 0.4 μ g and 0.2 μ g cDNA per well, respectively. Subsequent uptake or kinetics assays were performed 24 hours post-transfection. Unless noted otherwise, cells lines transiently expressing wild-type or mutated DAT cDNA were used for all experiments.

Stable Expression of Wild-type DAT

To generate monoclonal cell lines stably expressing wild-type DAT (DAT HEK cells), 70% confluent HEK-293 cells were transfected with 10 μ g of eGFP wild-type human DAT cDNA for 2 hours using Lipofectamine/PLUS reagents (Invitrogen). The transfected cells were split at 48 hours post-transfection into 100 mm cell culture dishes at a 1:10 dilution. G418 (1 mg/ml) (Sigma-Aldrich) antibiotic was added to the culture media 24 hours post-split for selection. After seven days of selection, G418-resistant colonies were plucked with cloning disks (Bel-Art, Pequannock, NJ), and the individual colonies were transferred into 24 well plates containing growth media. The colonies grew to 80% confluence, and those fluorescing eGFP were transferred to a larger culture flask for expansion. HEK-293 cells stably expressing eGFP wild-type DAT were maintained in growth media supplemented with 0.3 mg/ml G418 antibiotic.

Uptake Assays

HEK-293 or SK-N-SH cells were seeded in twelve well plates and transiently transfected with wild-type or G130T DAT as described above. Cells were then preincubated in serum-free media with or without ethanol (25, 50, 100 mM) for 60 or 120 min (3 wells per a condition). The ethanol pre-treatment media was quickly removed, and replaced with either the cell's respected growth media or KRH buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, pH 7.4) containing 100 nM [³H]DA and corresponding ethanol treatment (25, 50, 100 mM) for a 5 min incubation at 37°C to initiate [³H]DA uptake. For the 5 min time point, cells were exposed to ethanol only during the uptake assay. Control cells, expressing wild-type or G130T DAT, were incubated in growth media containing 100 nM [³H]DA without ethanol. Uptake was terminated by quickly removing media, and washing the cells three times in 1 ml of ice-cold KRH buffer. Cells were then lysed with 500 μ l of 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature with gentle shaking. The whole-cell lysate was used to measure [³H] DA uptake and protein concentration. Accumulation of [³H]DA in the wild-type DAT or G130T DAT expressing cells was quantified in a liquid scintillation counter. Protein concentration (DC protein assay, Bio-Rad, Hercules, CA) was used to normalize the uptake assays, and nonspecific [³H]DA uptake was determined by measuring uptake in the presence of the DAT inhibitor, cocaine (100 μ M) (Sigma-Aldrich).

Kinetics Assay

To determine the kinetics parameters (K_m and V_{max}) of DAT-mediated [³H]DA uptake, HEK-293 cells were seeded in twelve-well plates (3 wells per condition), and transfected with wild-type DAT, as described above. Cells were treated with ethanol under maximal uptake conditions, determined by the dose and time dependent experiments (100 mM ethanol in serum-free media for 60 min). Kinetics of DAT-mediated dopamine uptake was evaluated by measuring uptake of [³H]DA (100 nM) in the presence of increasing non-radioactive DA concentrations (0.025–1.5 μ M) and in the presence or absence of ethanol (100 mM).

Cell Surface Biotinylation

Stably expressing DAT HEK cells were grown to 85–90% confluence in two T-150 culture flasks. The cells were washed twice with room temperature phosphate buffer saline (PBS), pH 7.4 (Gibco), and incubated at 37°C for 60 min with 10 ml of KRH buffer in the presence or

absence of 100 mM ethanol. Cells were quickly washed twice with ice-cold PBS supplemented with 0.1 mM CaCl_2 and 1 mM MgCl_2 (PBS/Ca/Mg), and incubated with 10 ml of 1.0 mg/ml sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (EZ-link Sulfo-NHS-SS-Biotin) (Pierce, Rockford, IL) for 1 h at 4°C. Excess biotin was quenched with two washes of ice-cold PBS/Ca/Mg supplemented with 100 mM glycine, followed by two additional washes with ice-cold PBS/Ca/Mg. Biotinylated cells were scraped into the final wash, collected by centrifugation at 1000×g for 5 min, broken by ultrasonic sonication, and lysed in 0.5 ml of radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, and protease inhibitor cocktail) for 30 min on ice with intermittent agitation. The lysate was cleared of insoluble material by centrifugation at 10,000×g for 10 min at 4°C, and 1:10 aliquot of the lysate was reserved to be analyzed later as total protein sample (total). The remainder of the cleared lysate was rocked overnight with 500 μl of UltraLink Immobilized NeutrAvidin Protein Beads (Pierce) at 4°C to separate biotinylated (cell surface) from non-biotinylated (intracellular) proteins via affinity chromatography. Unbound proteins were removed by 1000×g centrifugation at 4°C for 2 min. After three washes with PBS, pH 7.4, the beads were incubated with 2× Laemmli buffer for 1 hr at room temperature while rocking to elute biotinylated (cell surface) proteins bound to NeutrAvidin beads. The protein concentration of the lysates was determined using DC Protein Assay kit (Bio-Rad, Hercules, CA), and an equal amount of protein was separated by SDS-PAGE in a 7.5% Tris-HCl Ready gel (Bio-Rad). After the proteins were transferred to polyvinylidene difluoride (PVDF) membrane, the blots were blocked overnight at 4°C in Tris-buffered saline (TBS-T) (10 mM Tris-Cl, pH 8; 150 mM NaCl; 0.01% Tween-20) containing 5% non-fat dried milk (Bio-Rad). DAT was detected with a rat monoclonal antibody specific for the N-terminus of DAT (MAB 369; Millipore-Chemicon, Billerica, MA) for 2 hr at room temperature while rocking in TBS-T containing 1% non-fat dried milk and 1% BSA. After three 10 min washes in TBS-T, blots were visualized using a horseradish peroxidase conjugated rat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with enhanced chemiluminescence solution (Perkin Elmer, Boston, MA). Immunoreactivity was detected using Kodak Image Station 2000 mm, and band intensities were quantified with NIH Image J software. Blots were stripped and reprobed with antibodies against an intracellular marker, calnexin, an endoplasmic reticulum-resident protein. The absence of calnexin in the biotinylated protein sample indicates the integrity of the cell membrane was maintained during the biotinylation process, and only cell surface proteins were labeled.

Data Analysis

Specific uptake was calculated as the difference between total and non-specific uptake. Maximal ethanol potentiation was determined by pooling maximum [^3H]DA uptake data from each ethanol dose / time dependence experiments and calculating the mean for each cell type. Kinetic parameters (K_m and V_{max}) were determined by non-linear regression line analysis using Graphpad Prism, version 4.0 software. Ethanol-induced changes in DAT cell surface density was determined by quantifying the optical densities (OD) from the Western analysis of cell surface and total populations of the transporter. The relative amount of surface DAT was calculated by normalizing surface population OD to their respective total population OD of the transporter to obtain a ratio of surface expression. The ratio of surface expression in the ethanol treated sample was then normalized to the untreated ratio. The student's *T* test was used to compare ethanol treated samples to untreated controls, and one-way ANOVA with post-hoc analysis for dose and time comparisons.

Results

Ethanol exposure enhances dopamine uptake in SK-N-SH and HEK-293 cells transiently expressing DAT

We examined ethanol actions on DAT function in SK-N-SH and HEK-293 cells expressing the transporter by [^3H]DA uptake assays. SK-N-SH and/or HEK-293 cells expressing wild-type or ethanol insensitive mutant (G130T) were pre-treated with ethanol (25, 50, 100 mM) for 0, 60 or 120 min, followed by an incubation in 100 nM [^3H]DA and corresponding ethanol treatment for 5 min at 37°C. Dopamine uptake was measured as the accumulation of [^3H]DA into DAT expressing cells. The 5 min time point includes cells exposed to ethanol only during the course of the uptake assay. Baseline dopamine uptake (untreated control cells) by SK-N-SH and HEK-293 cells expressing G130T DAT was not significantly different from the baseline uptake in cells expressing wild-type DAT (data not shown). Ethanol enhanced dopamine uptake in wild-type expressing SK-N-SH cells in a dose, but not time, dependent manner (Figure 1A). The dose-response curve differed slightly from assay to assay, but a clear dose response pattern was observed for each individual assay (representative assay, 13, 27, and 30% potentiation for 25, 50, and 100 mM ethanol, respectively) (Figure 1A, *inset*). In SK-N-SH cells expressing the G130T DAT mutant, dose response patterns also differed between assays (Figure 1B); however, there was no dose or time response relationship observed in individual assays (representative assay, 27, 14, and 18% potentiation for 25, 50, 100 mM ethanol, respectively) (Figure 1b, *inset*).

Maximal ethanol effects (see methods) on transfected HEK-293 cells were similar to those seen in transfected SK-N-SH cells (Figure 2). For SK-N-SH and HEK-293 cells expressing wild-type DAT, ethanol maximally potentiated dopamine uptake at 51% and 55%, respectively. In both cell lines expressing G130T DAT, ethanol significantly potentiated DAT-mediated [^3H]DA 29% in SK-N-SH and 28% in HEK-293 (Figure 2). However, ethanol-induced potentiation of uptake was significantly reduced in G130T DAT expressing SK-N-SH and HEK-293 cells compared to cells expressing the wild-type transporter. As seen previously, the untransfected SK-N-SH cells exhibited a low-level of transporter activity that could be blocked by 100 μM cocaine (Liu et al., 2001), and these cells were potentiated 13% by ethanol compared to untreated, untransfected SK-N-SH cells, while untransfected HEK-293 cells exhibited no specific uptake (data not shown).

Ethanol enhances dopamine uptake velocity in HEK-293 cells expressing wild-type DAT

Kinetics parameters (V_{max} and K_{m}) of DAT-mediated [^3H]DA uptake were determined to further evaluate the mechanistic action of ethanol on the transporter. Cells were exposed to ethanol under maximal uptake conditions (100 mM ethanol for 60 min) determined by the dose / time dependent uptake assays described above. Ethanol enhanced maximal dopamine uptake velocity (V_{max}) 30–35% while the affinity for dopamine (K_{M}) remained unchanged (Figure 3).

Ethanol increases cell surface density of DAT

To investigate if the ethanol-induced increase in dopamine uptake (Figures 1 and 2) in DAT expressing mammalian cells was due to an increase in transporter expression on the cell surface, DAT surface density was examined by cell-surface biotinylation assays. Stably transfected HEK-293 cells from the same monoclonal populations were used in all biotinylation experiments to increase immunoblot signal intensity and reduce variance between experiments. HEK-293 cells adhere strongly to the surface of the flasks, reducing the loss of cells during the multiple washes of these biochemical experiments. Expression of total and cell surface population of wild-type DAT was determined in DAT HEK cells incubated in the presence or absence of ethanol (100 mM) for 1 hr at 37°C (Figure 4A). Total wild-type DAT was detected

at ~55–125 kDa when assessed by anti-DAT (MAB 369; Chemicon). The presence of the eGFP tag on the N-terminal tail of the transporter increased the size ~25 kDa from the expected size of DAT (~55–90 kDa). The lower bands (~55–90 kDa) reflect lysosomal-degraded and unglycosylated DAT. The upper bands (~90–125 kDa) are glycosylated forms of the transporter, expected at the cell surface or in recycling pools of DAT. Ethanol (100 mM) had no effect on the total population of the transporter in cells; however, ethanol treatment significantly increased the surface density of DAT (Figure 4B) by 40–50% (Figure 4B, *inset*). Ethanol had no effect on the expression of the intracellular endogenous control protein, calnexin (Figure 4A). Less than 2% of calnexin was biotinylated (data not shown), indicating the integrity of the cell membrane was maintained throughout the surface labeling process.

Discussion

The goal of this study was to determine the direct action of ethanol on DAT functional regulation in mammalian cell expression systems. We found a short-term ethanol exposure potentiates dopamine uptake in both mammalian neuronal (SK-N-SH) and non-neuronal (HEK-293) wild-type DAT expressing cells. This potentiation was significantly reduced in cells expressing the ethanol insensitive mutant, G130T DAT. Ethanol-induced enhancement of DAT activity was associated with an increase in transporter expression on the cell surface. Our results in mammalian systems are in agreement with similar biochemical studies examining the effects of ethanol on DAT functional regulation in other *in vitro* systems (Maiya et al., 2002; Mayfield et al., 2001). Ethanol alters the functional regulation of DAT across multiple expression systems, suggesting a direct mechanistic action of ethanol on the transporter trafficking system.

The reward and reinforcing effects of ethanol are predominately mediated through the mesolimbic dopaminergic system (Carboni et al., 2000; Di Chiara and Imperato, 1988; Engleman et al., 2000; Gonzales et al., 2004; Katner and Weiss, 2001; Tang et al., 2003). Maladaptive changes in various properties of the dopaminergic system, including regulation of neurotransmission, contribute to ethanol addiction (Gonzales et al., 2004). Mammalian cell lines serve as important model systems to study the molecular mechanisms of drug and alcohol action. In this study, catecholaminergic SK-N-SH neuroblastoma and non-neuronal HEK-293 cells served as our mammalian cell model systems. Both cell lines were transfected with human wild-type or ethanol-insensitive G130T DAT to detect the effects of short-term ethanol exposure on DAT function and cell surface expression. These studies allowed us to identify potential molecular mechanisms of ethanol action on the transporter.

We found ethanol exposure potentiates dopamine uptake in neuroblastoma SK-N-SH and non-neuronal HEK-293 cells expressing wild-type DAT. Ethanol increased DAT activity in a clear dose-dependent manner within each individual assay. The dose response relationship was more variable across assays. This variation was likely due to differences in transfection efficiencies, growth rates, or passage number. Previous studies examining the effects of a 24 hour exposure to ethanol and cocaine (individually or combined) in neuronal and non-neuronal cell lines resulted in an increase in DAT-mediated DA uptake only in neuronal cell lines (Ho and Segre, 2001). Their results suggest ethanol and cocaine act on DAT through common neural-specific pathways to alter transporter function. We found ethanol alters DAT function in both non-neuronal and neuronal cell lines. This contradiction could be due the differences in ethanol exposure time and/or cell type. Our results suggest ethanol may have a direct mechanistic action on the transporter trafficking system. The SK-N-SH cell line used in these studies endogenously express NET and DAT. These transporters are responsible for a low level of uptake, which was not blocked during these assays. However, this endogenous uptake is negligible compared to the uptake by the wild-type or G130T DAT overexpressed in these cells, thus any contribution of endogenous transporters to our reported effects is minimal. Our results

demonstrating the enhancement of DAT activity by ethanol are consistent with previous electrochemical, chronoamperometry, and biochemical experiments in rats (Sabeti et al., 2003; Wang et al., 1997), and *Xenopus* oocytes (Maiya et al., 2002; Mayfield et al., 2001).

Chimera and site-directed mutagenesis studies in the *Xenopus* oocyte expression system identified the site of ethanol action at the first intracellular loop between transmembrane domains two and three of DAT. Specifically, a mutation of glycine 130 for threonine, a nonconservative amino acid change, eliminated the potentiating effects of ethanol on transporter action and cell surface binding in a *Xenopus* oocyte expression system (Maiya et al., 2002). As seen in oocytes, basal [³H]DA uptake (0 mM ethanol) in SK-N-SH and HEK-293 cells expressing G130T DAT is similar to wild-type DAT. However, in the mammalian cell models the mutation reduced, but did not eliminate ethanol sensitivity. While the G130T mutant demonstrated a significant potentiation of function after ethanol exposure, potentiation was also significantly reduced by half compared to wild-type DAT expressing cells. This ethanol-induced increase in G130T DAT activity was not dose-dependent. The lack of a dose-response effect is not due to variance in transfection efficiency, growth rates, or passage number, because none of the individual assays demonstrated a dose-response pattern. It is more likely due to the reduced sensitivity of the DAT mutant to ethanol. Alternatively, ethanol could be acting as an allosteric modulator of DAT, as seen with the GABA(A) receptor. This would suggest the G130T mutation disrupts a critical allosteric modulation site for ethanol. However, the potentiation of DAT function is associated with an ethanol-induced increase in surface expression of the transporter, as seen in the biochemical studies described above (Figure 4) and in oocytes (Maiya et al., 2002; Mayfield et al., 2001). We hypothesize the G130T mutation is disrupting a protein-protein interaction site critical for ethanol-regulated trafficking of DAT to the cell surface. The elimination of ethanol sensitivity in G130T DAT expressing oocytes was attributed to a lack of change in cell surface distribution of the transporters (Maiya et al., 2002). The differences in ethanol potentiation between oocytes and mammalian cells are most likely due to differences in trafficking mechanisms and/or cellular machinery in the model systems. Studies are in progress to examine the differences in ethanol-induced trafficking of G130T versus wild-type DAT in mammalian cell expression systems.

Trafficking and alterations in surface distribution of DAT is the predominant mechanism to rapidly regulate transporter function (Jayanthi and Ramamoorthy, 2005; Maiya et al.; Torres, 2006). Psychostimulants such as cocaine and amphetamine have been shown to modulate DAT activity by redistributing the transporter to the cell surface. Our kinetics data demonstrated DAT activity was potentiated by ethanol (increased V_{max}) with no change in transporter binding affinity (K_m). The potentiation of uptake velocity parallels an increase number of transporters on the cell surface in DAT HEK cells. While stably expressing DAT HEK-293 cells have been reported to have higher levels of [³H]DA uptake compared to transiently expressing cells (Chen and Reith, 2007), our results are in agreement with other *in vitro* studies using DAT expressing *Xenopus* oocytes where potentiation of DAT activity by ethanol also correlated with an increase in [³H]WIN 35428 surface binding (Mayfield et al., 2001). *In vivo*, ethanol has been reported to differentially modulate the expression of a variety of proteins in mesolimbic areas of the brain (Bell et al., 2006), including DAT (Jiao et al., 2006; Mash et al., 1996; Tiitonen et al., 1995). Abstinent alcohol-preferring vervet monkeys have an increase number of cell surface DAT, and chronic alcohol consumption down-regulates surface DAT expression (Mash et al., 1996). However, chronic ethanol has also recently been shown to increase DAT binding in specific brain regions of Wistar-Kyoto rats, including areas of the mesolimbic system involved in addiction (Jiao et al., 2006), while differential expression of surface DAT has been seen in violent versus non-violent alcoholics (Tiitonen et al., 1995). These changes could reflect ethanol-induced neuroadaptations in the brain, but further research is needed to compare expression differences between various brain regions, drinking models, and model systems.

Ethanol-mediated increases in DAT activity and cell surface expression after a short-term exposure, could represent a mechanism that underlies an acute functional tolerance to ethanol (Mayfield et al., 2001). Another amine transporter of the Na⁺/Cl⁻ family, GAT1, has been shown to have an acute tolerance to ethanol (Byas-Smith et al., 2004). Although GAT1 does not directly interact with ethanol, transporter activity increased in response to acute ethanol exposure. Interestingly, the DAT inhibitor, GBR 12909 and ethanol cross-sensitize in DBA/2J mice (Broadbent et al., 2005). These experiments suggest DAT inhibition unmasks increased basal levels of dopamine due to ethanol sensitization, which is normally compensated for by DAT uptake activity.

It is generally accepted ethanol exposure increases dopamine release in the mesolimbic areas of the brain (Gonzales et al., 2004; Yim and Gonzales, 2000). However, ethanol action on DAT regulation is currently under debate. *In vivo*, chronic ethanol exposure has been shown to increase DAT-mediated uptake (Budygin et al., 2007; Carroll et al., 2006), while acute ethanol has been reported to increase (Sabeti et al., 2003; Wang et al., 1997), decrease (Robinson et al., 2005) or have no effect on DAT activity (Budygin et al., 2001; Budygin et al., 2005; Jones et al., 2006; Mathews et al., 2006). Electrochemical studies using the 'no net flux' microdialysis method suggested acute ethanol-induced increases in dopamine levels were not due to an inhibition in transporter function, but an increase in release (Yim and Gonzales, 2000). *In vitro* electrophysiological and biochemical experiments in a *Xenopus* oocyte model system, which measured DAT activity in a more direct manner, suggested ethanol exposure increases DAT-mediated uptake through a redistribution of transporter expressed on the cell surface (Maiya et al., 2002; Mayfield et al., 2001). Many of the electrochemical studies described above indirectly examine transporter activity and regulation as a function of alterations in extracellular dopamine levels. The contradiction between findings could be contributed to inherent differences between model systems and/or techniques.

In conclusion, we determined ethanol induced a potentiation of DAT activity in both DAT-expressing neuroblastoma SK-N-SH and non-neuronal HEK-293 mammalian cells. This potentiation appears to be associated with an increase in the number of transporters expressed on the cell surface. These findings are in agreement with similar biochemical experiments in other *in vitro* systems, suggesting ethanol may have a direct action on the transporter's trafficking system thus modulating DAT functional regulation. In this study, we established and characterized working mammalian cell lines for future mechanistic studies involving the transporter, and compared ethanol's effect on DAT function and regulation. Experiments are in progress to define the components of ethanol-induced alterations in transporter trafficking, and to identify the proteins involved in the DAT trafficking interactome. These data further our understanding of the molecular mechanism of intracellular ethanol action, and provides insight to potential sites of maladaptive changes involved in alcoholism.

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Figure 1.

Dose and time dependent effects of ethanol pre-treatment on [3 H]DA (100 nM) uptake in DAT expressing SK-N-SH cells. Cells were incubated in the presence (25, 50, or 100 mM) or absence of ethanol (control) for 5, 60 or 120min. Data represents the average of four uptake assays (three wells per condition) for each ethanol dose and time parameter in SK-N-SH cells expressing wild-type or G130T DAT. (a) Wild-type DAT-mediated [3 H]DA uptake. Ethanol (25, 50 and 100 mM) significantly enhanced uptake compared to untreated controls for all pretreatment times, but not in a time dependent manner. (*inset*) Dose response relationship was variable between assays, but evident in individual assays. (representative 60 min ethanol pretreatment assay shown). (b) G130T DAT-mediated [3 H]DA uptake. Ethanol (25, 50 and 100

mM) significantly enhanced uptake compared to untreated controls for all pre-treatment times, but not in a time dependent manner. (*inset*) Dose response relationship was variable between assays, and in individual assays (representative 60min ethanol pre-incubation assay shown). Data are expressed as means $\pm$ SEM of four experiments. * $p < 0.01$ compared with untreated control (one-way ANOVA for dose and time comparison tests).

Figure 2.

Maximum ethanol potentiation (see methods) from pooled [^3H]DA uptake data in SK-N-SH and HEK-293 cells expressing wild-type or G130T mutant DAT. Control cells, expressing wild-type or G130T DAT, were incubated in growth media with 100 nM [^3H]DA without ethanol. Ethanol significantly potentiated transporter-mediated [^3H]DA uptake compare to untreated controls in SK-N-SH cells expressing wild-type DAT or G130T DAT 51% AND 29%, respectively. Potentiation was significantly reduced in SK-N-SH cells expressing the G130T mutant compared to wild-type DAT expressing cells. Ethanol potentiated transporter-mediated [^3H]DA uptake in HEK-293 cells expressing wild-type DAT or G130 DAT 55% and 28%, respectively. Potentiation was significantly reduced in HEK-293 cells expressing the G130T mutant compared to wild-type DAT expressing cells. Data are expressed as mean $\pm$ SEM of maximal uptake values from three experiments. * $p < 0.05$ compared with untreated control. ** $p < 0.05$ compared with wild-type DAT (Student's t test).

Figure 3.

Ethanol pre-treatment (100 mM; 60 min) increased dopamine uptake velocity. Uptake of [³H] DA (10 nM-10 μM) was measured after a 60 min exposure to either KRH (control; filled circles) or ethanol (100 mM; open circles) in DAT expressing HEK-293 cells. V_{max} (control 78.2 pmol/min/well; ethanol 107.9 pmol/min/well) and K_m (control 1.06 μM; ethanol 1.12 μM) parameter estimates were obtained from the nonlinear regression curve fits. The graph is representative of four experiments. Each data point represents an average of three wells $\pm$ SEM.

Figure 4.

Ethanol increases surface density of DAT. Ethanol-induced changes in the cell surface density of wild-type DAT, containing an N-terminal eGFP tag, in DAT HEK cells was determined using cell surface biotinylation assays. Cells were incubated in KRH buffer in the absence or presence of 100 mM ethanol for 1h at 37°C. (a) Representative immunoblots of DAT and calnexin in biotinylated DAT HEK cell lysate from four separate experiments. Shown are the total extracts (total DAT 55–125 kDa) and biotinylated extracts (cell surface DAT 90–125 kDa) from DAT HEK cells with indicated treatments. Blots were stripped and reprobed with the antibody for endogenous intracellular control protein, calnexin. (b) Mean optical density (O.D.) of total DAT in ethanol-treated (empty bars) DAT HEK cells did not significantly

change compared to total DAT in untreated cells (filled bars), while the cell surface DAT O.D. was significantly increased in ethanol treated cells. $**p < 0.01$ compared to untreated surface DAT O.D. (Student's *t* test). (*inset*) Quantification of the percent change in cell surface DAT populations between ethanol treated and untreated cells. Ethanol significantly increases surface DAT populations 40–50% (expressed as a percentage relative to the total amount of DAT in the respective lysate (untreated or ethanol-treated) and normalized to untreated surface DAT). Data are expressed as normalized values $\pm$ SEM of four experiments. $*p < 0.05$ compared with untreated surface DAT population (Student's *t* test).