Effects of Methamphetamine and Lobeline on Vesicular Monoamine and Dopamine Transporter-Mediated Dopamine Release in a Cotransfected Model System

Clare J. Wilhelm, Robert A. Johnson, Paul G. Lysko, Amy J. Eshleman, and Aaron Janowsky

Research Service, Veterans Affairs Medical Center, and Departments of Physiology and Pharmacology (C.J.W., A.J.E., A.J.), Psychiatry (A.J.), and Behavioral Neuroscience (A.J.), Oregon Health and Science University, Portland, Oregon; and GlaxoSmithKline (P.G.L.), King of Prussia, Pennsylvania

Received February 19, 2004; accepted April 21, 2004

ABSTRACT

Dopamine (DA) retention and drug-induced release kinetics were characterized in human embryonic kidney (HEK)-293 cells stably coexpressing the human DA transporter (hDAT) and human vesicular monoamine transporter (hVMAT2). Cofunction of hDAT and hVMAT2 caused greater retention of [3H]DA at 20 min (37°C), or 45 min (22°C) compared with cells that were treated with dihydrotetrabenazine (DHTB) to block the hVMAT2. In hDAT- and hVMAT2-coexpressing cells treated with DHTB during [3H]DA loading, methamphetamine (METH)-induced efflux was only 20% of preloaded [3H]DA, compared with 50 to 60% efflux in the absence of DHTB. Interestingly, the presence of DHTB (during release only) increased the potency and efficacy of METH at inducing [3H]DA release (without DHTB: EC50 = 33.8 μM, maximal release 51%; release with DHTB: EC50 = 3.2 μM, maximal release 61%), suggesting that the effects of METH and DHTB on vesicular storage are additive. High concentrations of lobeline induced a statistically significant release of [3H]DA from HEK-hDAT-hVMAT2 cells, but only in the absence of DHTB, suggesting an hVMAT2-mediated effect. Likewise, lobeline did not induce a significant release of [3H]DA from HEK-hDAT cells. The substrates DA and p-tyramine induced robust release of preloaded [3H]DA from cotransfected cells. Cocaine was somewhat effective at blocking substrate-induced [3H]DA efflux. These results suggest that coexpression of the hDAT and hVMAT2 can be used as a model system to distinguish functional pools of DA and to quantify differences in drug effects on DA disposition. In addition, cotransfected cells can be used to determine mechanisms of simultaneous drug interactions at multiple sites.

All drugs of abuse, including METH, either directly or indirectly increase DA neurotransmission (Grace, 2000; Di Chiara, 2002). The DAT and VMAT2 terminate neurotransmission after stimulated release by reuptake and repackaging of DA into vesicles. The interaction of these proteins in a simple, stable system has not been described in detail. However, Pifl et al. (1995), using a superfusion apparatus, reported increases in DA uptake and amphetamine-induced release of [3H]DA in cells coexpressing the hDAT and rat VMAT2, compared with cells expressing only the hDAT. There was also an apparent decrease in the potency and efficacy of amphetamine-induced release from DAT cells versus hDAT/rVMAT2 cells, although no direct comparisons were made.

METH is an efficacious and relatively potent releaser of DA and preloaded 1-methyl-4-phenylpyridinium via the hDAT (Eshleman et al., 1994; Sulzer et al., 1995; Johnson et al., 1998; Dwoskin and Crooks, 2002). The amphetamines exert their actions through a number of mechanisms, including inhibition of reuptake, redistribution of DA between vesicle stores and cytoplasm, reversal of transport, and possibly by collapsing proton gradients driving vesicular uptake (Cubells et al., 1994; Sabol and Seiden, 1998; Jones et al., 1999; Schmitz et al., 2001). METH also exerts regulatory effects on both the DAT and VMAT2, affecting long-term DA homeosta-

This work was supported by the Department of Veterans Affairs Merit Review and Research Career Scientist Programs (to A.J.), a National Institutes of Health/Veterans Administration interagency agreement (to A.J. and A.E.), and by the Portland Alcohol Research Center (P50AA10760) (to A.E.). Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.104.067314.

ABBREVIATIONS: METH, methamphetamine; DA, dopamine; DAT, dopamine transporter; VMAT, vesicular monoamine transporter; hVMAT, human vesicular monoamine transporter; rVMAT, rat vesicular monoamine transporter; HEK, human embryonic kidney; DHTB, dihydrotetrabenazine; MSR, macrophage scavenger receptor; RTI-55, 3β-(4-iodophenyl)tropane-2β-carboxylic acid methyl ester; MAO, monoamine oxidase; COMT, catechol-O-methyl transferase; ANOVA, analysis of variance.
lobeine on DA release. For example, lobeline interacts with the VMAT2 and affects DA homeostasis.

There are no reports describing effects of METH and other substrates, or lobeline on DA efflux in cells stably expressing both hDAT and hVMAT2. To further explore the possible shift in affinity during drug-induced release (Pill et al., 1995) and to examine in more detail DA homeostasis and the potency and efficacy, HEK-293 cells stably expressing hDAT and hVMAT2 were developed.

We now report that retention of [3H]DA within stably transfected hDAT cells is increased by the presence of the hVMAT2 and that blockade of hVMAT2 (during drug-induced DA release only) increases the apparent potency and efficacy of METH-induced release. Importantly, blockade of hVMAT2 during [3H]DA loading results in a decrease in the magnitude of METH-induced release. The differences in [3H]DA retention between HEK-hDAT and HEK-hDAT-hVMAT2 cells suggest that the expressed hVMAT2 is functional and that non-neuronal cotransfected cells possess the biochemical intermediates necessary to provide the proton gradient required for hVMAT2 function (Erickson et al., 1992; Merickel et al., 1995). In neurons, vesicles make use of an ATP-dependent proton pump to create a pH gradient that is used by the hVMAT2 to concentrate neurotransmitter. In the model system used here, the hVMAT2 is likely associated with early endosomes (Eshleman et al., 2002) that have an acidic internal pH that aids in the dissociation of receptor-ligand complexes (Sheff et al., 1999). Likewise Liu and Edwards (1997), using transfected Chinese hamster ovary cells, reported the comigration of a vesicular monoamine transporter (VMAT1) with transferrin receptor, a commonly used early endosomal marker, in Western blots after differential centrifugation. The acidic pH of early endosomes could drive the uptake of neurotransmitter by the hVMAT2. Our results indicate that lobeline induces [3H]DA release and seems to exert its effects exclusively through interaction with the hVMAT2. DHTB, a drug that binds with high affinity to the hVMAT2 (Sievert et al., 1998; Thiriot and Ruoho, 2001), completely blocks the effect of lobeline on [3H]DA release and blocks 60–70% of the effect of METH. Thus, similarly to METH effects on efflux, the majority of the lobeline effect is on [3H]DA that is sequestered by the hVMAT2. Unlike METH, the endogenous substrate DA and the trace amine p-tyramine do not exhibit a shift in potency or efficacy in response to hVMAT2 blockade during release. These drugs also have the largest maximal effect (about 75% release), compared with that of METH (50–60%). This simple model system can be used to quantify the drug-induced disposition of DA into two sequestered compartments within the cell and to determine the specific mechanism of action of abused drugs and potential pharmacotherapeutics.

Materials and Methods

Materials. [3H]DA (3,4-[7-3H]diaryloxyphenylethylamine, 5.8–9.7 Ci/mmol) was purchased from Amersham Biosciences Inc. (Piscataway, NJ). Eco-Lume scintillation fluid was purchased from ICN Biochemicals, Inc. (Aurora, OH). DHTB and [3H]DHTB were purchased from American Radiolabeled Chemicals (St. Louis, MO). All water used in these experiments was purified by a Milli-Q system (Millipore, Bedford, MA). Methamphetamine, lobeline, pargyline, tropolone, and most other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture. HEK-293 cells were transfected with the hDAT and characterized as described previously (Eshleman et al., 1999). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 0.05 U of penicillin/streptomycin. Stock plates were grown on 150-mm-diameter tissue culture dishes in 10% CO2 at 37°C. The SR-AI macrophage scavenger receptor (MSR) cDNA was originally derived from human placenta as described previously (Lysoo et al., 1999). The MSR cDNA was subcloned into pcDNA3.1 (with zeocin resistance) and approximately 1 μg of DNA was transfected into HEK-hDAT cells using LipofectAMINE. Expression of the MSR increased cell adherence, a necessity for the rigorous washing procedures and prolonged release experiments (Robbins and Horlick, 1998; Saunders et al., 2000). The hVMAT2 cDNA was generously supplied by Dr. Robert Edwards (University of California, San Francisco, CA) and subcloned into pcDNA3.1 (with G418 resistance). Approximately 1 μg of DNA was transfected into HEK-hDAT cells using LipofectAMINE.

Binding Assay. The binding of [125I]3β-(4-isopropyl)tropane-2β-carboxylic acid methyl ester ([125I]RTI-55), or [3H]DHTB to the hDAT or hVMAT2, respectively, in HEK-hDAT and HEK-hDAT-hVMAT2 cells was performed as described previously, with minor modifications (Eshleman et al., 1999). In short, cells were grown to confluence and washed with 5 ml of phosphate-buffered saline buffer. Cells were scraped from plates, resuspended, and homogenized to 5 ml of 0.32 M sucrose with a Polytron homogenizer at setting 7 for 5 to 10 s. For RTI-55 binding assays, the homogenate was centrifuged at 400g for 5 min. at 4°C. The supernatant was decanted and centrifuged at 22,000g for 15 min. at 4°C. The resulting pellet was then resuspended in 3 ml of buffer and homogenized with a Polytron homogenizer at setting 7 for 5 to 10 s. The assays contained approximately 75 to 150 μg of membrane protein for DHTB binding (less than 15% of the radioactivity added was bound), or 25 to 50 μg of membrane protein for RTI-55 binding (less than 25% of radioactivity added was bound), drug, and [125I]RTI-55 (60 pM final concentration), or [3H]DHTB (6 nM final concentration) in a final volume of 250 μl. Krebs-HEPES buffer (25 mM HEPES, 122 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2,1 mM pargyline, and 10 μM tropolone see [3H]DA uptake assay below), 2 mM of glucose/ml, and 0.2 mM of calcium/ml, pH 7.4 were used for all assays, except as indicated. Specific binding was defined as the difference in binding observed in the absence and presence of 10 μM mazindol ([125I]RTI-55 binding), or 2 μM DHTB ([3H]DHTB binding). The membranes were incubated for 90 min (equilibrium) at room temperature in the dark. Assays were terminated by filtration through Wallac Filtermat A filters using a 96-well Tomtec cell harvester. Scintillation fluid (50 μl) was added to each filtered spot, and the radioactivity remaining on the filters was determined using a Wallac 1205 Bqplate scintillation counter. Saturation binding experiments were conducted with duplicate determinations at each ligand concentration, which included radiolabeled and nonradiolabeled ligand for final concentrations ranging from 6 to 200 nM for DHTB, or 60 pM to 20 nM for RTI-55. Protein concentrations for this and all other experiments requiring protein normalization were performed using a modified bicinchoninic acid protein assay.

Time Course of [3H]DA Uptake. Cells were plated on poly-lysine-coated 24-well plates and grown to confluence. Media were decanted and cells were prepared for [3H]DA uptake. Cells were incubated in the presence or absence of DHTB (1 μM) or mazindol (10 μM for nonspecific) for at least 10 min before addition of [3H]DA. The uptake assay (final volume 0.5 ml) was initiated by the addition of 50 nM [3H]DA and 2 μM DA in Krebs-HEPES buffer. Pargyline [monoamine oxidase (MAO) inhibitor] and tropolone [catechol-o-
methyl transferase (COMT) inhibitor] were included in the buffer so that results could be compared with studies done using tissue from animals, which required the addition of MAO and COMT inhibitors.

In previous studies, Vindis et al. (2000) found no evidence for MAO activity in HEK-293 cells; however, other studies suggest the presence of COMT activity in HEK-293 cells (Echleman et al., 1997). Pifl et al. (1995) using African green monkey kidney (COS-7) cells, in the absence of MAO or COMT inhibitors, reported that 70 to 80% of the tritium released from hDAT and 80 to 90% of the tritium released from hDAT/rVMAT2 cells was \([^{3}H]DA\), suggesting that DA metabolites comprise only a small part of the recovered tritium. Therefore, recovered tritium will be referred to as \([^{3}H]DA\) here.

Assays were carried out at the temperatures indicated for time points ranging from 5 to 90 min. Experiments were terminated by aspiration and treatment with 250 \(\mu l\) of 0.1 \(M\) HCl. Radioactivity remaining in each well was determined using liquid scintillation spectrometry. Experiments were conducted with duplicate determinations.

\([^{3}H]DA\) Saturation Curve. Experiments were carried out as described for time course of \([^{3}H]DA\) uptake experiments with the following exceptions. Experiments were conducted with concentrations of unlabeled DA ranging from 0 to 25 \(\mu M\). Cells were preincubated with drug(s) for 10 min. Assays were initiated by the addition of \([^{3}H]DA\) (approximately 40 nM) and carried out at 22°C for 5 min.

Time Course of \([^{3}H]DA\) Retention. Experiments were carried out as described for time course of \([^{3}H]DA\) uptake experiments with the following exceptions. The uptake assay (final volume 0.5 ml) was initiated by the addition of 20 nM \([^{3}H]DA\) in Krebs-HEPES buffer at 37°C. Mazindol was not present during \([^{3}H]DA\) uptake in retention or release assays. \([^{3}H]DA\) uptake continued for 60 min, the time required to reach steady state, and was terminated by decanting the buffer.

After uptake, cells were washed once with 400 \(\mu l\) of buffer. The release assay was initiated by the addition of 400 \(\mu l\) of buffer with or without 1 \(\mu M\) DHTB. The buffer was aspirated at time points ranging from 0 to 90 min, 250 \(\mu l\) of 0.1 \(M\) HCl was added to the wells, and radioactivity remaining in each well was determined by liquid scintillation spectrometry. All experiments were conducted with duplicate determinations, unless otherwise noted.

Drug-Induced \([^{3}H]DA\) Release. Cells were prepared as described for \([^{3}H]DA\) release time course experiments, with the following exceptions. The release assay was initiated by the addition of 400 \(\mu l\) of vehicle (Krebs-HEPES buffer with 0.75% dimethyl sulfoxide for lobeline) with or without 1 \(\mu M\) DHTB and with or without drug (METH, lobeline, dopamine, and \(p\)-tyramine, concentration ranges as indicated). This concentration of dimethyl sulfoxide had no effect on \([^{3}H]DA\) uptake or release (data not shown). For assays using cocaine to block drug effects on \([^{3}H]DA\) release, cells were incubated for 10 min with cocaine before initiation of release. The release assay was terminated after 30 min, the buffer was decanted, and 250 \(\mu l\) of 0.1 \(M\) HCl was added to each well.

Data Analysis. Prism software (GraphPad Software Inc., San Diego, CA) was used to analyze all kinetic, retention, and drug-induced release data, and analysis of variance (ANOVA) with Bonferroni post tests. Data shown are mean \(\pm\) S.E.M., except as indicated. \(t\) tests (two-tailed, unpaired) were performed using Microsoft Excel (Microsoft, Redmond, WA).

Results

HEK-hDAT cells were transfected with cDNA for the hVMAT2 and characterized for expression of hDAT and hVMAT2 using \([^{125}I]RTI-55\) or \([^{3}H]DHTB\), respectively (Fig. 1). Analysis of \([^{125}I]RTI-55\) binding to the hDAT indicated a \(B_{\text{max}}\) of 5.03 \pm 0.44 pmol/mg protein for the parent HEK-hDAT cell line, whereas HEK-hDAT-hVMAT2 cells had a \(B_{\text{max}}\) of 5.99 \pm 1.52 pmol/mg protein. No statistical difference between the parent and the cotransfected cell line, as measured by two-tailed Student’s \(t\) test, was observed for the affinity or \(B_{\text{max}}\) of \([^{125}I]RTI-55\) binding (HEK-hDAT \(K_{d} = 2.96 \pm 0.70 \text{nM}\); HEK-hDAT-hVMAT2 \(K_{d} = 3.13 \pm 1.07 \text{nM}\)). Cotransfected cells had a \(B_{\text{max}}\) of 2.80 \pm 0.51 pmol/mg protein and a \(K_{d}\) of 8.91 \pm 0.98 nM for \([^{3}H]DHTB\) binding to the hVMAT2. HEK-hDAT cells also had specific \([^{3}H]DHTB\) binding, with a \(K_{d}\) of 98.2 \pm 22 nM and a \(B_{\text{max}}\) of 0.93 \pm 0.32 pmol/mg protein. The binding to hDAT cells has a significantly lower (\(p < 0.05\) affinity and \(B_{\text{max}}\) (\(p < 0.05\)) compared with the cotransfected cells.

HEK-hDAT cells were further characterized to determine the presence or absence of functional hVMAT2 expression. \([^{3}H]DA\) uptake time courses (2.02 \(\mu M\) DA total concentration; 20 nM \([^{3}H]DA\)) at both 37 and 22°C were carried out using HEK-hDAT and HEK-hDAT-hVMAT2 cells (Fig. 2). This concentration of DA was chosen because it is close to the \(K_{m}\) for \([^{3}H]DA\) uptake in attached cells (Saunders et al., 2000). At 37°C, but not at 22°C, HEK-hDAT-hVMAT2 cells exhibited increased \([^{3}H]DA\) uptake compared with DHTB-treated cells. \([^{3}H]DA\) uptake in HEK-hDAT cells was not affected by 1 \(\mu M\) DHTB. This concentration of DHTB was chosen to block the hVMAT2 because it is approximately 100-fold higher than the \(K_{d}\) for DHTB at the hVMAT2 (see binding data above) and because hDAT function at this concentration was not affected.

No difference in the \(K_{m}\) values were found between HEK-hDAT cells or HEK-hDAT-hVMAT2-transfected cells under
any of the conditions used to measure [3H]DA uptake (Fig. 3; Table 1). HEK-293 cells expressing only the hVMAT2 did not exhibit any specific uptake in saturation experiments (data not shown). Furthermore, 1 μM DHTB did not affect the \( V_{\text{max}} \) for [3H]DA uptake by HEK-hDAT cells. These data suggest that the low-affinity [3H]DHTB binding to HEK-hDAT cells is not a measure of hVMAT2 expression. Untreated HEK-hDAT-hVMAT2 cells had significantly increased [3H]DA uptake at a DA concentration of 10 μM, compared with DHTB-treated cells (Fig. 3).

Retention of [3H]DA by HEK-hDAT-hVMAT2 cells and HEK-hDAT cells over time (Fig. 4) was also assessed. HEK-hDAT-hVMAT2 cells had a significant decrease in [3H]DA retention over time in the presence of DHTB (Fig. 4). However, DHTB had no effect on [3H]DA retention in HEK-hDAT cells (Fig. 4, inset).

The effects of hVMAT2 expression on [3H]DA disposition were further investigated by inducing [3H]DA release with METH under four conditions involving DHTB-induced blockade of the hVMAT2. As seen in Fig. 2, when DHTB is present, there is a significant decrease in [3H]DA uptake after a 60-min incubation at 37°C. For release experiments, the 30-min time point was chosen because it was the time at which there was a significant and reliable difference in [3H]DA retention between untreated and DHTB-treated HEK-hDAT-hVMAT2 cells (Fig. 4). There was no significant difference in METH-induced release between hDAT-hVMAT2-transfected cells treated with DHTB during both uptake and release, compared with cells treated with DHTB during uptake only.

In both groups, 30 min of exposure to METH induced the release of only about 20% of the [3H]DA (Fig. 5; Table 2). In contrast, METH induced the release of 61% of the [3H]DA in the cells that were treated with DHTB during the release phase of the experiment. This is a significant (\( p < 0.05 \)) increase in [3H]DA release compared with METH-induced [3H]DA release from cells in the absence of DHTB. Significant differences in release between cells exposed to buffer during uptake and release, and cells treated with DHTB only during release, were observed at concentrations of METH above 2.5 μM (Fig. 5; Table 2). The potency of METH shifted from 33.8 to 3.2 μM with the addition of DHTB during release. Thus, DHTB treatment only during release increases both the potency and the efficacy of METH-induced [3H]DA release (Fig. 5; Table 2).

To determine whether another drug that had previously been reported to interact with the VMAT2 had similar effects on efflux, the effects of lobeline were also examined (Fig. 5; Table 2). At the highest concentration of lobeline tested (750 μM) and in the absence of DHTB, there was a 27% release of the [3H]DA from the cells. The difference between untreated cells and cells treated with DHTB only during the release phase of the experiment reached statistical significance (\( p < 0.05 \)) at concentrations of lobeline above 250 μM. Under any conditions where DHTB was present, lobeline did not induce [3H]DA release (Fig. 5; Table 2). An endogenous substrate and a trace amine were also tested for their effects on [3H]DA retention in this system. DA and p-tyramine had similar maximal effects on [3H]DA release, but differed in their potencies by roughly an order of magnitude, with p-tyramine being more potent than DA. Maximal effects for these drugs, in the absence of DHTB during uptake, were about 75% (Fig. 5; Table 2). Unlike METH, DHTB treatment during release did not affect the potency or efficacy of DA or p-tyramine. Blockade of the hVMAT2 during [3H]DA uptake resulted in a significantly decreased maximal effect of both DA and p-tyramine on [3H]DA release, down to about 20–25%, compared with cells that were not pretreated with DHTB (Fig. 5; Table 2).

To further explore the effects of METH, lobeline, DA, and p-tyramine on [3H]DA disposition, HEK-hDAT cells were also examined for drug-induced [3H]DA release. When 60 min of [3H]DA uptake (steady state) were followed by 30 min of drug-induced release, higher concentrations of lobeline blocked [3H]DA efflux from HEK-hDAT cells (Fig. 6). Similar to METH, DA, and p-tyramine caused only slight increases in [3H]DA release (Fig. 6). All of these effects in HEK-hDAT cells were minimal (approximately 5%).

To determine the physiological nature of the putative VMAT2-expressing vesicles, drug-induced [3H]DA release was evaluated in the presence or absence of Ca\(^{2+}\) (Fig. 7).
The absence of Ca$^{2+}$ in the buffer had virtually no effect on drug-induced release. A relatively small increase in lobeline-induced $[^3]$H]DA release was observed in the absence of Ca$^{2+}$.

Cocaine, a reuptake blocker, was examined for its ability to block drug-induced $[^3]$H]DA release from HEK-hDAT-hVMAT2 cells. The concentration of cocaine chosen for these studies was approximately 30 times its IC$_{50}$ for blocking uptake (Eshleman et al., 1995). Two concentrations of each releasing drug were chosen, one near the EC$_{50}$ and one near the maximal effect on release (Fig. 8). Treatment with 10 $\mu$M cocaine resulted in a small but statistically significant loss of the maximal effect on release (Fig. 8). Treatment with 10 $\mu$M cocaine resulted in a small but statistically significant loss of the maximal effect on release (Fig. 8). Treatment with 10 $\mu$M cocaine resulted in a small but statistically significant loss of the maximal effect on release (Fig. 8).

A dose-response curve for cocaine’s effects on METH-induced release was also constructed. Cocaine (750 $\mu$M) by itself induced release of approximately 15% of the available $[^3]$H]DA. A high concentration of METH (100 $\mu$M) was used to elicit a maximal effect on release. A significant inhibition of METH-induced release was observed at concentrations of 25 $\mu$M cocaine and above (Fig. 9).

**Discussion**

HEK-293 or HEK-hDAT cells do not express a functional hVMAT2, as demonstrated by a lack of high-affinity $[^3]$H]DHTB binding sites, and the failure of DHTB to affect $[^3]$H]DA uptake in HEK-hDAT cells. There is a significant DHTB-induced decrease in $[^3]$H]DA uptake in HEK-hDAT-hVMAT2 cells in both the $[^3]$H]DA uptake time course and $[^3]$H]DA saturation experiments. This is not a result of functional cell surface expression of the hVMAT2; no specific uptake is seen in cells expressing only the hVMAT2 (data not shown). Therefore, the results suggest this difference is due to sequestration of intracellular $[^3]$H]DA by the hVMAT2, which decreases the “effective” cytoplasmic DA concentration, and results in an increase in the ability of the hDAT to accumulate more DA.

Previously, we characterized the ability of other cell expression systems to retain $[^3]$H]DA. Monkey kidney COS-7 cells and rat glioma C6 cells that expressed the hDAT were unable to retain $[^3]$H]DA. In C6-hDAT cells, the efflux was so rapid that the cells could not be used in $[^3]$H]DA release assays (Eshleman et al., 1994; Johnson et al., 1998). Coexpression of the hDAT and hVMAT2 in HEK-293 cells reveals both time- and temperature-dependent effects on $[^3]$H]DA retention (Fig. 4). Compared with untreated cells, DHTB-treated cells have significantly less $[^3]$H]DA after 20 min at 37°C ($p < 0.05$) and 45 min at 22°C ($p < 0.01$). Although the effect is small at 22°C, it is augmented by an increase in temperature. The temperature dependence of DHTB-mediated inhibition of $[^3]$H]DA retention may be due to a relative decrease in DAT and VMAT2 activity at temperatures that are significantly below physiological conditions (Vizi, 1998). Therefore, transporter-mediated efflux will be reduced by a decrease in temperature. Blockade of the hVMAT2 with DHTB inhibits hVMAT2-mediated sequestration, allowing more rapid release of $[^3]$H]DA. Furthermore, $[^3]$H]DA retention by HEK-hDAT cells resembles that of DHTB-treated HEK-hDAT-hVMAT2 cells. The efflux profiles of HEK-hDAT-hVMAT2 cells suggests that activity of the hVMAT2 results in sequestration of $[^3]$H]DA and the formation of a second pool of $[^3]$H]DA.


### Table 1


<table>
<thead>
<tr>
<th>Treatment</th>
<th>HEK-hDAT-hVMAT2</th>
<th>HEK-hDAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_{m}$</td>
<td>V$_{max}$</td>
<td>K$_{m}$</td>
</tr>
<tr>
<td>$\mu$M</td>
<td>pmol/mg/min</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>Buffer</td>
<td>2.41 ± 0.54</td>
<td>430 ± 70</td>
</tr>
<tr>
<td>DHTB</td>
<td>2.09 ± 0.52</td>
<td>340 ± 50</td>
</tr>
</tbody>
</table>
Fig. 5. Drug-induced release in HEK-hDAT-hVMAT2 cells. [3H]DA uptake was conducted for 60 min at 37°C in HEK-hDAT-hVMAT2 cells. There were four treatment groups in this experiment. Solid squares (□) denote untreated cells exposed to buffer (Krebs-HEPES) during uptake and treated with drug at the indicated concentrations during release. Solid diamonds (○) denote cells exposed to buffer only during uptake and treated with various drug concentrations and 1 μM DHTB during release. Open triangles (△) denote cells treated with 1 μM DHTB during uptake and with drug during release. Open squares (■) denote cells treated with 1 μM DHTB during uptake and with 1 μM DHTB and drug during release. Un-treated cells kept at 22°C are shown in solid triangles (●), and cells kept at 37°C are shown as solid squares (□). Lines were fit to a one-phase exponential decay. Inset, [3H]DA efflux at 37°C in the presence or absence of DHTB from HEK-hDAT cells. Solid circles (●) represent HEK-hDAT cells, and open circles (○) represent HEK-hDAT cells treated with DHTB (1 μM). Each curve represents the average of at least three independent experiments conducted with duplicate determinations. Statistical analysis was carried out by two-way ANOVA followed by Bonferroni post tests comparing release in the presence and absence of 1 μM DHTB at 37 or 22°C. *p < 0.05; **p < 0.01; ***p < 0.001.
There was also an apparent increase in the potency of METH-induced efflux when DHTB was present during the release portion of the experiment. During [3H]DA uptake when the hVMAT2 was blocked, [3H]DA could diffuse throughout the cell and become unavailable for release. In contrast, when the hVMAT2 was functioning, [3H]DA could be concentrated and stored. The pool of [3H]DA associated with hVMAT2 function may be more readily accessible to METH and other releasing drugs.

In the present study, the EC_{50} value of METH-induced [3H]DA release was high (34 μM), compared with about 20 nM reported by Rothman et al. (2000) and Partilla et al. (2000) who used synaptosomal preparations. Unlike the present study, the synaptosomal preparations did not include a COMT inhibitor. Therefore, DA metabolism may play a role in the results obtained using synaptosomes. Furthermore, reserpine, which alters DA disposition, was included in the synaptosomal studies. Other differences include the assay temperature (25 versus 37 °C here) and the use of attached cells here, versus the homogenized, centrifuged, and resuspended preparations in synaptosomal studies. Last, there are receptors, trace amines, and neuropeptides that may interact with METH or the preloaded [3H]DA in the synaptosomal preparation. The model system described here allows measurement of direct effects of drugs on the transporters of interest.
Lobeline released 27% of the available [3H]DA. The presence of DHTB completely blocked lobeline’s effects. Therefore, the mechanism of lobeline-induced [3H]DA release probably involves a direct interaction of the drug with the hVMAT2 and not with the hDAT. This hypothesis is supported by studies from Teng et al. (1998) who reported that lobeline displaces [3H]DHTB from rat striatal synaptic vesicles, and Miller et al. (2001) who reported that lobeline enhances tritium overflow from rat striatal slices preloaded with [3H]DA. Lobeline is a potent pharmacotherapeutic and has been used to treat nicotine addiction. Some nicotinic acetylcholine receptors are located on norepinephrine neurons (Rao et al., 2003). Thus, lobeline might aid individuals addicted to nicotine by reducing the amount of norepinephrine available for release from vesicles. Therefore, when nicotine stimulates nicotinic acetylcholine receptors, in the presence of lobeline, less norepinephrine will be released. Our results support this hypothesis and demonstrate a significant effect of lobeline on vesicular neurotransmitter storage.

The endogenous substrate DA and the trace amine p-tyramine had significant effects on [3H]DA release. Interestingly, these drugs exhibit higher maximal effects on release than METH. Unlike METH, however, their effects on release are not additive with the effects of DHTB; DHTB treatment during release did not increase the potency or efficacy of either DA or p-tyramine on [3H]DA release. METH might act as a weak base within vesicular structures to dissipate the proton gradient, thereby decreasing vesicular content (Cu-bells et al., 1994). The finding that neither DA, nor p-tyramine, which like METH, are substrates for both the hDAT and hVMAT2, do not have additive effects when coadministered with DHTB during release, suggests that METH may be acting through an additional mechanism.

Drug-induced release of [3H]DA was also carried out using HEK-hDAT cells. Lobeline did not enhance [3H]DA release in these cells. At higher concentrations it weakly blocked [3H]DA efflux. This finding confirmed the results from the HEK-hDAT-hVMAT2 studies, which suggested that lobeline interacts primarily with the hVMAT2. Likewise, METH, DA, and p-tyramine induced only a small amount of [3H]DA efflux (<10%) from HEK-hDAT cells, emphasizing the importance of the hVMAT2 in mediating the effects of METH. These data suggest that although efflux of [3H]DA by HEK-hDAT cells is robust, under these conditions, only a relatively small proportion of the [3H]DA taken up is available for drug-induced release via the hDAT.

Our results are comparable with those of Pifl et al. (1995) who found that transient coexpression of hDAT and rVMAT2 caused an apparent shift in METH-induced release, compared with cells expressing only the hDAT. Those authors also reported a similar rate of efflux between cells coexpressing both hDAT and rVMAT2, and cells expressing only hDAT. However, we found differences in [3H]DA retention between stably expressing HEK-hDAT and HEK-hDAT-hVMAT2 cells, as well as differences between control and DHTB-treated (hVMAT2-blocked) HEK-hDAT-hVMAT2 cells. This may be due to differences in methods or to differences in relative transporter expression between the studies. The use of a transient expression system (Pifl et al., 1995)
transfected cells, and the magnitude of the drug-induced release of DA from H9262 cells. [3H]DA uptake was conducted for 60 min at 37°C. Cells were incubated with cocaine (as indicated) for 10 min before treatment with METH. The solid column represents the effect of 100 μM METH. Solid squares (▴) represent cells exposed to cocaine only. Solid triangles (▾) represent the combined effect of 100 μM METH and cocaine. +, p < 0.05; **, p < 0.01; ***, p < 0.001.

may prevent consistent levels of hDAT and hVMAT2 protein expression across experiments.

To further determine the physiological significance of hVMAT2 expression in this cell line, drug-induced release, as well as nonstimulated efflux, was compared in the presence and absence of Ca2+ ions. In this model system, no component of drug-induced, or tonic [3H]DA efflux was Ca2+-dependent, in contrast to neurons, where vesicles fuse with the plasma membrane in a Ca2+-dependent process.

Drug-induced neurotransmitter release may be a distinct physiological process independent of the uptake mechanism. Despite cocaine’s ability to effectively block DA uptake, it may not be effective as an inhibitor of release (Eshleman et al., 1994). Cocaine was most effective at blocking release induced by the trace amine p-tyramine. There was also a slight inhibition of DA-mediated [3H]DA release by cocaine. Cocaine (10 μM) had no effect on METH-induced [3H]DA release. However, higher concentrations of cocaine blocked the METH effect. The mechanism through which cocaine antagonizes METH-induced release is not clear. It is possible that cocaine blocks METH-induced [3H]DA efflux through the DAT. Another possibility, however, is that cocaine simply blocks the ability of METH to interact with the hDAT, thereby limiting METH’s ability to interact with the hVMAT2.

Our data indicate that stable coexpression of hDAT and hVMAT2 resulted in formation of a sequestered [3H]DA pool that was sensitive to METH, lobeline, DA, and p-tyramine. In this simple model system, the overwhelming majority of METH-, DA- and p-tyramine-induced [3H]DA release seems to come from the sequestered pool of [3H]DA. In release experiments where DHTB is applied during the uptake portion of the experiment, [3H]DA is not retained well by cotreated cells, and the magnitude of the drug-induced release is either completely blocked (lobeline), or reduced (METH, DA, and p-tyramine), suggesting that sequestration of [3H]DA is the result of functional expression of hVMAT2 and is important for the releasing effects of these drugs.

METH is a substrate for both the hDAT and hVMAT2. Our data suggest that the ability of the hVMAT2, in this model system, to sequester [3H]DA at high concentrations makes it an essential part of the mechanism of METH effects on DA disposition. This compares favorably with studies by Jones et al. (1998), who found that both the hVMAT2 and hDAT were important for amphetamine-induced release from striatal mouse slices but that the rate-limiting step was the interaction of amphetamine with the hVMAT2. Thus, blocking METH interactions with the hVMAT2 may be a more relevant target for developing pharmacotherapies that effectively treat symptoms of METH intoxication, abuse, and withdrawal.

Acknowledgments

We thank Dr. Robert Edwards for generously supplying the cDNA for the hVMAT2.

References


prefrontal cortex by neuronal nicotinic acetylcholine receptor agonists. *Brain Res* 996:203–208.


Address correspondence to: Dr. Aaron Janowsky, Research Service (RD-22), Veterans Affairs Medical Center, 3710 S.W. U.S. Veterans Hospital Rd., Portland, OR 97239. E-mail: janowsky@ohsu.edu