Published in final edited form as:

J Neurosci Methods. 2012 August 15; 209(2): 357–366. doi:10.1016/j.jneumeth.2012.06.002.

A fluorescent-based assay for live cell, spatially resolved assessment of vesicular monoamine transporter 2-mediated neurotransmitter transport

Alison I. Bernstein^{1,2}, Kristen A. Stout^{1,2}, and Gary W. Miller^{1,2,3,4}

¹Center for Neurodegenerative Disease, Emory University, Atlanta, GA 30322, United States

²Department of Environmental Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322, United States

³Department of Neurology, Emory University, 1518 Clifton Road, Atlanta, GA 30322, United States

⁴Department of Pharmacology, Emory University, 1518 Clifton Road, Atlanta, GA 30322, United States

Abstract

The vesicular monoamine transporter 2 (VMAT2; Slc18a2) packages monoamines into synaptic vesicles. Monoamine homeostasis is highly regulated and dysfunction may play a role in Parkinson's disease, Huntington's disease, drug addiction, and neuropsychiatric disorders. The primary function of VMAT2 is to sequester monoamine neurotransmitters into vesicles for subsequent release; it also sequesters toxicants away from cytosolic sites of action. Identification of compounds that modify the action of VMAT2 may be useful as therapeutic agents for preventing or reversing monoamine-related toxicity. Current methods for measuring VMAT2 function are unable to assess uptake in intact cells. Here, we adapted the Neurotransmitter Uptake Assay (Molecular Devices) to develop a measure of VMAT2 function in live whole cells. This assay contains a fluorescent compound, which is transported into cells by the plasma membrane monoamine transporters and has been marketed as a rapid, high-throughput, plate reader based assay for function of these plasma membrane transporters. We demonstrate a modified version of this assay that can be used to visualize and measure transport into vesicles by VMAT2. HEK293 cell lines stably expressing the dopamine transporter and a mCherry-VMAT2 fusion protein were generated. Confocal microscopy confirmed that the fluorescent compound is transported into mCherry-positive compartments. Furthermore, the VMAT2-specific inhibitor tetrabenazine (TBZ) blocks uptake into the mCherry-positive compartment. Confocal images can be analyzed to generate a measure of VMAT2 activity. In summary, we demonstrate a method for spatially resolved analysis of VMAT2-mediated uptake in live intact cells.

© 2012 Elsevier B.V. All rights reserved.

Gary W. Miller (corresponding author): gary.miller@emory.edu, 404-712-8582.

Alison I. Bernstein: abernstein@emory.edu

Kristen A. Stout: kastout@emory.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The vesicular monoamine transport 2 (VMAT2; SLC18A2) is predominantly localized to the central nervous system in monoaminergic brain regions, where it packages free monoamines (dopamine, serotonin, norepinephrine, epinephrine, and histamine) in the cytosol into small synaptic and dense core vesicles (Nirenberg et al., 1998). Proper packaging of these monoamines, in particular dopamine (DA), is critical to the function and survival of these neurons. Cytosolic DA is neurotoxic but this toxicity must be balanced with the need for DA to facilitate essential behavioral functions like motor movements, learning, and the acquisition of natural rewards. Thus, greater than 90% of intracellular DA is sequestered into vesicles, preventing its cytosolic accumulation and subsequent transformation to neurotoxic species (Eisenhofer et al., 2004). The critical role of vesicular storage of DA and the effects of both pharmacological and genetic disruption has been extensively reviewed (Caudle et al., 2008; Guillot and Miller, 2009; Sulzer et al., 2005). Our laboratory has found that VMAT2-deficient mice undergo progressive degeneration of monoaminergic brain regions (the substantia nigra, locus coeruleus, and dorsal raphe) and exhibit motor and non-motor symptoms similar to those seen in Parkinson's disease (Caudle et al., 2007; Taylor et al., 2011; Taylor et al., 2009). Disruption of vesicular storage is also implicated in drug abuse (Eiden and Weihe, 2011; Sulzer, 2011). Additionally, the VMAT2-specific inhibitor tetrabenazine (TBZ) is used to treat Huntington's disease and other hyperkinetic disorders (Ondo et al., 2002; Paleacu et al., 2004). Environmental toxicants, including a variety of pesticides, organochlorine compounds, and brominated flame retardants also disrupt vesicular packaging of dopamine by VMAT2 (Bemis and Seegal, 2004; Caudle et al., 2006; Chaudhry et al., 2008; Fonnum and Mariussen, 2009; Hatcher et al., 2008; Hatcher et al., 2007; Richardson and Miller, 2004; Richardson et al., 2005). Taken together, these studies demonstrate the proper regulation of vesicular storage of monoamines is critical to the health and function of these neurons. Furthermore, the weight of evidence indicates that VMAT2 is a target of environmental contaminants and other man-made chemicals and that its study is an issue of importance to public health.

Currently, radioactive uptake assays of ^3H -DA in synaptic vesicles isolated from rat or mouse brain are often used to directly assess VMAT2 function. Vesicles can be prepared from animals treated with various drugs or toxicants to determine *in vivo* effects (Caudle et al., 2007; Chu et al., 2010; Guillot et al., 2008; Hatcher et al., 2008; Volz et al., 2009). Alternatively, isolated vesicles can be treated directly to determine the pharmacokinetics, such as IC_{50} or K_i , of a compound. While this is a powerful technique to determine the actions of compounds at VMAT2, these experiments require a large amount of tissue material that necessitates the use of many animals. The amount of material obtained also limits the number of doses and time points that can be assessed in each experiment. Two alternate techniques in cell lines bypass these limitations. In the first method, cells are treated with detergent treatment to permeabilize the plasma membrane while leaving the vesicle membrane and machinery intact (Erickson et al.). The second method involves isolation of a post-nuclear fraction from cell lines stably expressing VMAT2; tetrabenazine (TBZ, a specific VMAT2 inhibitor)-sensitive uptake can be detected in this fraction (Bellocchio et al., 2000; Parra et al., 2008). However, none of these vesicular uptake assays provides an understanding of the action of these compounds in a whole cell. They do not allow for assessment of access to the vesicle, combined action at plasma membrane and vesicular transporters or indirect mechanisms of regulation. For example, a compound that affects VMAT2 function in isolated vesicles may not be able to cross the plasma membrane; such a compound would have no effect on VMAT2 function in a whole cell. In addition, these methods are not amenable to a high throughput format primarily due to their use of radioactivity. Furthermore, adhering an isolated vesicle fraction to a plate is technically challenging and the partial permeabilization method requires multiple washing steps.

Therefore, we sought to develop a method that could measure vesicular uptake in whole cells and be suitable for adaptation to a high-throughput format.

We modified the Neurotransmitter Uptake Assay (Molecular Devices), which is marketed to measure uptake through the plasma membrane monoamine transporters. This assay consists of a proprietary fluorescent compound that is transported into cells by the plasma membrane monoamine transporters and a non-permeable masking dye to quench fluorescence in the media. Here, we demonstrate that this dye is transported by VMAT2 and can be adapted to measure uptake in real time in intact cells.

2. Methods

2.1 Cell culture

HEK293 cells (ATCC) and HEK293 lines stably expressing hDAT, hVMAT2, mCherry-hVMAT2, hDAT and hVMAT2, or hDAT and mCherry-VMAT2 constructs were cultured at 37°C and 5% CO₂ in DMEM with 10% FBS. All constructs were made in pcDNA3.1 (Life Technologies). hDAT and mCherry-hVMAT2 expressing constructs contained a neomycin or zeocin resistance gene, respectively. Plasmids were transfected into HEK293 cells with Lipofectamine 2000. Stable cell lines were generated by repetitive rounds of limiting dilutions in selective media. Double stable cell lines were created by transfecting HEK-hDAT stable cells with the hVMAT2 or mCherry-hVMAT2 construct and selecting for both plasmids with both neomycin and zeocin.

2.2 Neurotransmitter Uptake Assay (Molecular Devices)

Cells were plated in 16 well chambered coverslips (Life Technologies) one day before assay. Cells were plated to be 70% confluent on the day of assay. Reagent was reconstituted in Hank's Balanced Salt Solutions (HBSS), 20 mM HEPES according to manufacturer's instructions. Cells were preincubated with HBSS, 20 mM HEPES, 0.1% BSA, 50 mM glucose with or without the indicated dose of inhibitor for 15 minutes. Inhibitors (GBR12909, nomifensine and tetrabenazine) were purchased from Sigma. Uptake assay reagent was added after incubation with vehicle or inhibitor and the reaction was imaged on a Nikon A1R confocal (dye: 405 nm excitation, 525/50 emission filter; mCherry: 561 nm excitation, 595/50 emission filter). Multipoint image capture was used to collect images at multiple time points and multiple sites.

2.3 MitoTracker staining

Cells were incubated with 50 nM MitoTracker Deep Red (Life Technologies) for 10 minutes. Media was replaced with HBSS, 0.1% BSA, 50 mM glucose with or without the indicated dose of inhibitor and the Neurotransmitter Uptake Assay was performed as above.

2.4 Image analysis

Images were analyzed in ImageJ (Rasband). Regions of interest were defined by thresholding the mCherry channel and creating a region of interest around mCherry-positive extensions of the cells (Figure 6). This region of interest was applied to the green channel and standard deviation of pixel intensity within those regions was measured. A punctate-diffuse index was calculated by dividing each standard deviation value by the average of the values acquired for the vehicle treated images (Goldstein et al., 2000). Data were analyzed in Graph Pad Prism 5.0.

2.5 Whole cell ^3H -DA Uptake

Cells were plated in 48-well plates one day before uptake was performed. Cells were washed with 0.5 ml uptake buffer (4 mM Tris, 6.25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM CaCl_2 , 1.2 mM MgSO_4 , 5.6 mM D-glucose, 1.7 mM ascorbic acid, and 1 μM pargyline, pH 7.4). Cells were incubated with 225 μl uptake buffer with or without the indicated dose of inhibitor for 15 minutes. After incubation, 25 μl uptake buffer containing ^3H -DA and DA for a final concentration of 20 nM ^3H -DA and 1 μM DA was added. Cells were incubated at 37°C for 20 minutes or time indicated. Nonspecific uptake was determined in the presence of 10 μM nomifensine. Uptake was terminated by aspirating uptake buffer and washing each well twice with 0.5 ml ice-cold uptake buffer. Cells were lysed in 0.1 N NaOH and transferred to vials containing 3 ml scintillation cocktail. Radioactivity was measured by and counted using a Beckman LS6500. Data were analyzed in Graph Pad Prism 5.0.

2.6 Vesicular ^3H -DA Uptake

Cells were plated in 10 cm dishes and grown to 100% confluency. Cells were washed with warm PBS without Ca^{2+} or Mg^{2+} and resuspended in uptake buffer (25 mM HEPES, 100 mM potassium tartrate, 100 μM EDTA, 50 μM EGTA, pH 7.4). Cells were homogenized with a glass/Teflon homogenizer 30 times on ice. Homogenate was centrifuged at 8000 $\times$ g for 8 minutes at 4°C. Protein content of the resulting supernatant was determined by BCA assay. Uptake assays utilized 100 μg of protein in complete uptake buffer (uptake buffer with 1.7 mM ascorbate, 2 mM Mg^{2+} -ATP salt, pH 7.4) and 20 μM TBZ to define specific uptake. Samples were incubated for 10 minutes at 30°C followed by addition of 1 μM dopamine with a 2% tracer of [^3H]-dopamine. Samples were incubated for 5 minutes at 30°C with gentle shaking. The assay was terminated by addition of 5 ml ice-cold assay buffer before filtration through 0.5% PEI-soaked Whatman GF/F filters (Brandel Inc., Gaithersburg, MD). Filters were then placed in vials containing 3 ml scintillation fluid and counted using a Beckman LS6500. Data were analyzed in Graph Pad Prism 5.0.

3. Results

3.1 The fluorescent substrate is transported by DAT and VMAT2

The fluorescent reporter dye is marketed as a substrate of the plasma membrane monoamine transporters (the dopamine, norepinephrine and serotonin transporters; DAT, NET and SERT). Since this dye is an analog of MPP $^+$, which is transported by DAT and VMAT2, we hypothesized that this dye is also a VMAT2 substrate. To test this, we generated HEK293 cell lines stably expressing human DAT, human VMAT2 or both (HEK-DAT, HEK-VMAT2, or HEK-DAT/VMAT2, respectively). These cells were incubated with the assay reagent (dye concentration, 4 μM) for 1 hour and images were collected by confocal microscopy (Figure 1). Cells that do not express DAT (HEK cells and HEK-VMAT2 cells; Figure 1A,B) do not take up the dye and show no fluorescence. However, cells that express DAT (HEK-DAT and HEK-DAT/VMAT2; Figure 1C,D) are able to take up the compound and fluoresce. In addition, the presence of VMAT2 in the cells changes the localization of the dye. In HEK-DAT and HEK-DAT/VMAT2 cells, the dye localizes to the cell body in a similar pattern as is seen in HEK-DAT cells, but there is also additional punctate staining in extensions from the cell body. This punctate localization is seen only in the presence of active VMAT2 (Figure 1D, Figure 4). The extensions contain a concentration of mCherry-VMAT2-positive puncta. These puncta represent a vesicle-like compartment that contains functional VMAT2. Their localization to extensions from the cell body likely reflects the partial neuronal profile of HEK293 cells (Shaw et al.). Despite their renal origin, these cells express many genes characteristic of neuronal cells. In addition, during the creation of this stable cell line, we selected cells for further propagation that developed such extensions.

Additionally, since the dye is a substrate for DAT and VMAT2, we assessed whether the dye impairs dopamine uptake of dopamine by these transporters. In HEK-DAT/VMAT2 cells and in vesicles isolated from HEK-VMAT2 cells, preincubation with the dye (4 μ M) did not impair uptake of 3 H-DA (data not shown). Therefore, competition of the dye with dopamine is unlikely to be a concern at concentrations of dye used in the assay.

We also tested the toxicity of the reagent by WST-1 assay since the dye is an analog of MPP⁺. There was no toxicity observed 24 hours after the cells were treated with the concentration of dye used in the assay (4 μ M) according to the protocol (15 minute incubation with HBSS buffer, 2 hour incubation with assay reagent, wash and replace with media) (data not shown).

3.2 Characterization of HEK-DAT/mCherry-VMAT2 stable cell line

To create a method for measuring this VMAT2-dependent difference in localization, we created a stable cell line expressing hDAT and a fusion of mCherry and hVMAT2 (HEK-DAT/mCherry-VMAT2). We verified that these cells contained functional DAT and VMAT2 by radioactive uptake assays. These assays showed that inhibition of DAT by GBR12909 almost completely blocked uptake of 3 H-DA into the cells, as expected (Figure 2A). They also demonstrate that inhibition of VMAT2 by tetrabenazine (TBZ) lowers the capacity of the cell to take up DA (Figure 2A). This is not due to an inhibition of DAT by TBZ, as TBZ does not inhibit uptake in HEK-DAT cells (Figure 2D). The radioactive uptake assays verified that the cells contain functional DAT and VMAT2 and that they respond to the appropriate inhibitors. Vesicles isolated from these cells have TBZ-dependent uptake (IC_{50} = 54 nM; 95% CI: 40 – 75 nM) (Figure 2B). In intact cells, inhibition of DAT by nomifensine blocks uptake in HEK-DAT (IC_{50} = 2.4 μ M; 95% CI: 1.7 – 3.3 μ M) and HEK-DAT/mCherry-VMAT2 cells (IC_{50} = 5.5 μ M; 95% CI: 3.8 – 8 μ M) (Figure 2C). TBZ inhibits uptake in HEK-DAT/mCherry-VMAT2 cells (IC_{50} = 219 nM; 95% CI: 144 – 331 nM) but not in HEK-DAT cells (Figure 2D). The IC_{50} for TBZ in whole cells is higher than the IC_{50} in purified vesicles (Figure 2B, 2D). This is likely due to low permeability of TBZ, such that the concentration inside the cell at the vesicle is much lower than the concentration of TBZ applied in the media. The concentration of TBZ inside the cell, at the vesicle, is most likely closer to the values calculated for purified vesicles. This difference between purified vesicle fractions and whole cells underscores the need for an assay to assess vesicular function in an intact cellular environment.

3.3 Subcellular localization of dye

To characterize the localization of the dye, we initially examined uptake of the fluorescent substrate in HEK-DAT cells. In these cells, uptake of the dye is blocked by nomifensine, as expected (Figure 3A). In addition, the dye primarily localized to the mitochondria, shown by colocalization with MitoTracker (Figure 3B). Mitochondrial localization is expected based on the structural similarity of the dye to MPP⁺. In contrast, in HEK-DAT/mCherry-VMAT2 cells, the dye only partially localizes to the mitochondria as indicated by colocalization of the dye with MitoTracker (Figure 4A). In these cells, the dye also localizes to mCherry-positive puncta in extensions from the cell body as indicated by colocalization of the dye with mCherry (Figure 4A). This punctal localization is TBZ-dependent and can be visualized as a loss of colocalization between mCherry and the reporter dye (Figure 4B). When VMAT2 function is blocked by TBZ, the localization of the dye is primarily mitochondrial, similar to HEK-DAT cells. Uptake in these cells (HEK-DAT/mCherry-VMAT2) is also blocked by nomifensine (Figure 4B). Together, this demonstrates that the reporter dye is transported by VMAT2 into a VMAT2-containing compartment and that in the absence of VMAT2 activity, either in cells that do not express VMAT2 or by pharmacological inhibition with TBZ, localization to this compartment is lost. Similar

mitochondrial localization is seen in HEK-DAT cells and TBZ-treated HEK-DAT/mCherry-VMAT2 cells (Figure 3, Figure 4B).

3.4 Quantification of TBZ-dependent uptake

As mentioned above, the localization of the reporter dye to mCherry positive puncta is TBZ-dependent. Therefore, we carried out experiments to determine if this dependence could be quantified and used to generate dose-response curves and calculate IC_{50} values. Confocal images clearly show a loss of the fluorescence from the dye in mCherry-positive puncta with increasing concentrations of TBZ, indicated by a loss of green fluorescence in the mCherry-positive regions of the cells (Figure 5A). This change was quantified by thresholding the red channel, defining regions of interest based on that threshold, applying those regions to the green channel and measuring the standard deviation of pixel intensities in the green channel within those regions (Figure 6). By defining a region of interest based on mCherry staining, the areas of mitochondrial staining are excluded from the analysis. Thus, this strong mitochondrial localization does not complicate the data analysis. Standard deviation of pixel intensities within these mCherry-defined regions of interest provides a measure of the punctate fluorescence. When VMAT2 activity is high, fluorescence within these regions is highly punctate and a high standard deviation of pixel values is calculated. As VMAT2 activity is inhibited by increasing concentrations of TBZ, fluorescence in these regions becomes less punctate and a low standard deviation of pixel values is calculated. From these values, a punctate-diffuse index was calculated by dividing the standard deviation in each image by the mean of the standard deviations in all control (vehicle treated) images (Goldstein et al., 2000). The IC_{50} calculated by this method ($IC_{50} = 142$ nM; 95% CI: 33 – 608 nM) is similar to the IC_{50} calculated from the radioactive uptake assays ($IC_{50} = 219$ nM; 95% CI: 144 – 331 nM).

4. Discussion

4.1 A fluorescent, spatially resolved assay for vesicular packaging in live cells

Radioactive neurotransmitter uptake by isolated synaptic vesicles has produced many insights into the pharmacology of VMAT2. However, the amount of brain tissue required limits the number of time points, doses and compounds that can be measured. The fluorescent-based assay described here overcomes this limitation because cell lines are a practically unlimited resource. Assay conditions are not limited by the amount of material required as cell numbers adequate for multiple replicates, concentrations and time points can easily be generated. In addition, in a whole cell assay, the presence of glucose in the media and functioning mitochondria ensure a steady supply of ATP, as degradation of ATP in the assay buffer can be a concern for reconstituted vesicular assays. Additionally, microscopy offers other advantages. The same cells can be imaged over time to generate time course data, whereas in a vesicular assay, a different sample is needed for each time point. Use of a motorized stage also allows imaging of multiple sites in multiple wells for dose response experiments. Images can be acquired at multiple time points with multipoint acquisition so that time course and dose response experiments can be carried out simultaneously.

Importantly, this assay provides a way to observe modulation of vesicular packaging in an intact cell. The actions of a compound at VMAT2 in an isolated vesicle are not necessarily the same as its action at VMAT2 in an intact cell due to permeability of the compound at the plasma membrane, action of the compound at plasma membrane transporters, or through indirect interactions. This is poignantly illustrated by the different IC_{50} values of TBZ calculated for isolated vesicles (54 nM) and whole cells (219 nM) (Figure 2B,D). In whole cells, the IC_{50} must be calculated based on the concentration of TBZ in the media, as the concentration at the interface of the vesicular compartment is unknown. Thus, a greater

concentration of inhibitor is needed in whole cells, because only a fraction of the inhibitor enters the cell and gains access to VMAT2. Experiments with radiolabeled TBZ confirm that only a small percentage of TBZ applied in the media enters the cell (data not shown). Other compounds may inhibit VMAT2 in isolated vesicles but be impermeable to the plasma membrane. Such a compound would be identified as an inhibitor in a vesicular uptake assay but not in the whole cell assay. Thus, the whole cell assay more accurately represents the action of such a compound *in vivo*.

An additional advantage is the ability of this assay to identify compounds that affect vesicular packaging by VMAT2 indirectly. For example, VMAT2 is known to be regulated by G-protein signaling (Brunk et al., 2006; Brunk et al., 2008; Hölting et al., 2003; Jagow et al., 2000). These pathways are unlikely to be intact in a vesicle preparation and compounds that modify VMAT2 function through such an indirect mechanism would not be identified in assays performed in those preparations. In contrast, these pathways may be intact in certain cell lines so that a whole cell assay would be capable of identifying compounds that modify them. Overall, this fluorescent whole cell assay is more flexible and can test a larger range of conditions and compounds than radioactive uptake assays. While this assay does not allow calculation of exact pharmacokinetic data of compounds at VMAT2 in isolation, as a radioactive uptake assay in a vesicular preparation does, it provides a more complete picture of the interplay of plasma membrane, vesicular transporters and other cellular pathways than the isolated vesicular uptake assays. This assay can provide information that is not possible or is cumbersome to gather from traditional radioactive uptake assays. Overall, the low assay cost, low animal usage, and the flexibility of this assay make it an invaluable tool in the investigation of monoaminergic systems.

4.2 Inhibiting VMAT2 activity in whole cells decreases uptake

In the course of developing this assay and characterizing the HEK-DAT/mcherry-VMAT2 cell lines, we found that inhibition of VMAT2 with TBZ in whole cells reduces the total uptake of DA into the cell (Figure 2D). This is consistent with uptake experiments performed in synaptosomes (Meyer et al., 2011). However, the authors concluded that the ability of TBZ to inhibit uptake in synaptosomes is due to action at DAT. However, we showed that TBZ does not inhibit uptake in the HEK-DAT cells (Figure 2D), indicating that TBZ does not act at DAT, either by direct inhibition of DAT or displacement of DA. Instead, results suggest that inhibition of VMAT2 by TBZ prevents the sequestration of cytosolic DA into vesicles, leading to the maintenance of a high concentration of cytosolic DA, leading to an overall decreased capacity to store DA. Our data demonstrate that TBZ is not active at DAT and suggest that it is action by TBZ at VMAT2 that reduces the total DA capacity of the cell or synaptosome.

4.3 Future applications

4.3.1 Adaptation to a high content format—We have demonstrated here that this assay can be used to measure vesicular function in intact cells in a low to medium throughput format. This assay is amenable to a high content format, as the separation of values between vehicle and high dose of TBZ translates to a Z-factor of 0.62. Generally, an assay with a z-factor between 0.5 and 1 is considered to be a good candidate for a high throughput assay. For adaptation to a high content format, conditions for a 96-well plate need to be established. Fortunately, the well size of the chambered coverslips used for confocal imaging is the same as a single well of a 96-well plate. Therefore, the assay conditions should translate easily to a high content format. Additionally, many high content software packages require a nuclear stain or a whole cell dye to delineate cells boundaries prior to image analysis. The dye utilized in this assay bleeds through into the blue channel generally used by nuclear stains. However, many whole cell stains that emit in the far red region of the

spectrum are available; these do not bleed through into the wavelengths used to visualize the assay dye.

In the current or high content format, this assay can be used to screen for drugs or environmental contaminants that modulate VMAT2. Screening of compounds on even a small scale has previously been hindered by the large amounts of material required in experiments from vesicles isolated from animal brain tissue, by the labor intensive protocols required to isolate vesicles from animal brain tissue and cell lines, and by the use of radioactivity. Development of this fluorescent, non-radioactive assay that can be performed in cell lines bypasses these limitations and will allow testing of compounds on a scale that was not previously possible.

4.3.2 Adaptation to fluorescent false neurotransmitters—Fluorescent dopamine analogs, known as fluorescent false neurotransmitters (FFNs) have been developed to visualize dopamine uptake and release (Gubernator et al., 2009; Lee et al., 2010). We did not use the initial compound (FFN511) to develop this assay since it was developed for use in slice preparations and is not taken up by cells in culture (Gubernator et al., 2009). A subsequent series of dyes are potentially appropriate for cellular analysis of VMAT2 function (Lee et al., 2010). However, these dyes display pH sensitive fluorescence. VMAT2 is powered by an ATPase-established proton gradient that results in acidification of the vesicular lumen. Thus, pH sensitive dyes are potentially valuable fluorescent tools for studying vesicular function. Yet, it is important to note that pH change can be independent of VMAT2 function and, therefore, the assessment of these compounds is likely more complicated than the dye used in our assay. Though FFNs are valuable as a substrate for real-time spatial analysis of VMAT2 function, these characteristics would limit the usability of the assay and the amenity of FFN for high content assay development. However, future series of fluorescent false neurotransmitters that are taken up in cell culture may be suitable for use in this assay.

4.3.3 Adaptation to other cell lines and primary culture—In addition, adaptation of this assay to neuronal cell lines and primary neuronal cultures may be possible. Our assay was performed in HEK cell lines to isolate the functions of DAT and VMAT2. However, in some cases, it may be preferable to use a cell line or primary cultures with a more complete complement of vesicular proteins. In these systems, there would not be a fluorescently tagged vesicular transporter. Thus, the analysis method would need to be adapted to differentiate subcellular structures. Another possibility would utilize newly developed Qdot based vesicular probes (Park et al., 2012). These probes could potentially be used in place of a tagged transporter and would bypass the need for transfection of a fluorescently tagged vesicular transporter, expanding the cell types that can be utilized for this assay. Use of this dye in primary cultures or cell lines that synthesize and store monoamines (unlike HEK cells) may be problematic if the dye, like MPP⁺, induces vesicular release of monoamines. If this is a complication, fluorescent false neurotransmitters may be more suitable in these cells.

4.3.4 Adaptation to other vesicular transporters—This method can also easily be adapted to assess vesicular storage of other neurotransmitters. An analogous cell line that expresses the appropriate plasma membrane transporter and a fluorescently tagged vesicular transporter can be generated. Fluorescent analogs of the relevant neurotransmitter or other fluorescent compounds transported by its transporter can be used to monitor uptake into the cell and into the vesicular compartment. Image acquisition and analysis would be performed identically to the method described here.

4.4 Conclusion

We report the development of a fluorescent, spatially resolved assay for VMAT2 function that is amenable to a high content imaging format. This assay has greater flexibility and lower cost than currently utilized methods. In addition, this method can also be adapted to other neurotransmitter systems and other cell types. While this method cannot replace current *in vivo* and *ex vivo* techniques, it is better suited for larger scale screenings.

References

- Bellocchio EE, Reimer RJ, Freneau RT, Edwards RH. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science*. 2000; 289:957–60. [PubMed: 10938000]
- Bemis JC, Seegal RF. PCB-induced inhibition of the vesicular monoamine transporter predicts reductions in synaptosomal dopamine content. *Toxicological Sciences*. 2004; 80:288–95. [PubMed: 15115888]
- Brunk I, Blex C, Rachakonda S, Hölting M, Winter S, Pahner I, Walther DJ, Ahnert-Hilger G. The first luminal domain of vesicular monoamine transporters mediates G-protein-dependent regulation of transmitter uptake. *The Journal of Biological Chemistry*. 2006; 281:33373–85. [PubMed: 16926160]
- Brunk I, Blex C, Sanchis-Segura C, Sternberg J, Perreau-Lenz S, Bilbao A, Hörtnagl H, Baron J, Juraneck J, Laube G, Birnbaumer L, Spanagel R, Ahnert-Hilger G. Deletion of Go2alpha abolishes cocaine-induced behavioral sensitization by disturbing the striatal dopamine system. *The FASEB Journal*. 2008; 22:3736–46.
- Caudle WM, Colebrooke RE, Miller GW, Emson PC. Altered vesicular dopamine storage in Parkinson's disease: a premature demise. *Trends in Neurosciences*. 2008; 31:303–8. [PubMed: 18471904]
- Caudle WM, Richardson JR, Delea KC, Guillot TS, Wang M, Pennell KD, Miller GW. Polychlorinated biphenyl-induced reduction of dopamine transporter expression as a precursor to Parkinson's disease-associated dopamine toxicity. *Toxicological Sciences*. 2006; 92:490–9. [PubMed: 16702228]
- Caudle WM, Richardson JR, Wang MZ, Taylor TN, Guillot TS, McCormack AL, Colebrooke RE, Di Monte DA, Emson PC, Miller GW. Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007; 27:8138–48. [PubMed: 17652604]
- Chaudhry FA, Edwards RH, Fonnum F. Vesicular neurotransmitter transporters as targets for endogenous and exogenous toxic substances. *Annual Review of Pharmacology and Toxicology*. 2008; 48:277–301.
- Chu P-W, Hadlock GC, Vieira-Brock P, Stout K, Hanson GR, Fleckenstein AE. Methamphetamine alters vesicular monoamine transporter-2 function and potassium-stimulated dopamine release. *Journal of Neurochemistry*. 2010; 115:325–32. [PubMed: 20649837]
- Eiden LE, Weihe E. VMAT2: a dynamic regulator of brain monoaminergic neuronal function interacting with drugs of abuse. *Annals of the New York Academy of Sciences*. 2011; 1216:86–98. [PubMed: 21272013]
- Eisenhofer G, Kopin IJ, Goldstein DS. Leaky catecholamine stores: undue waste or a stress response coping mechanism? *Annals of the New York Academy of Sciences*. 2004; 1018:224–30. [PubMed: 15240373]
- Erickson JD, Schafer MK, Bonner TI, Eiden LE, Weihe E. Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93:5166–71. [PubMed: 8643547]
- Fonnum F, Mariussen E. Mechanisms involved in the neurotoxic effects of environmental toxicants such as polychlorinated biphenyls and brominated flame retardants. *Journal of Neurochemistry*. 2009; 111:1327–47. [PubMed: 19818104]
- Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nature Cell Biology*. 2000; 2:156–62.

- Gubernator NG, Zhang H, Staal RGW, Mosharov EV, Pereira DB, Yue M, Balsanek V, Vadola PA, Mukherjee B, Edwards RH, Sulzer D, Sames D. Fluorescent false neurotransmitters visualize dopamine release from individual presynaptic terminals. *Science*. 2009; 324:1441–4. [PubMed: 19423778]
- Guillot TS, Miller GW. Protective actions of the vesicular monoamine transporter 2 (VMAT2) in monoaminergic neurons. *Molecular Neurobiology*. 2009; 39:149–70. [PubMed: 19259829]
- Guillot TS, Shepherd KR, Richardson JR, Wang MZ, Li Y, Emson PC, Miller GW. Reduced vesicular storage of dopamine exacerbates methamphetamine-induced neurodegeneration and astrogliosis. *Journal of Neurochemistry*. 2008; 106:2205–17. [PubMed: 18643795]
- Hatcher JM, Delea KC, Richardson JR, Pennell KD, Miller GW. Disruption of dopamine transport by DDT and its metabolites. *Neurotoxicology*. 2008; 29:682–90. [PubMed: 18533268]
- Hatcher JM, Di Monte DA, Richardson JR, Guillot TS, McCormack AL, Jones DP, Pennell KD, Miller GW. Dieldrin exposure induces oxidative damage in the mouse nigrostriatal dopamine system. *Experimental Neurology*. 2007; 204:619–30. [PubMed: 17291500]
- Höltje M, Winter S, Walther D, Pahner I, Hörtnagl H, Ottersen OP, Bader M, Ahnert-Hilger G. The vesicular monoamine content regulates VMAT2 activity through Galphaq in mouse platelets. Evidence for autoregulation of vesicular transmitter uptake. *The Journal of Biological Chemistry*. 2003; 278:15850–8. [PubMed: 12604601]
- Jagow BV, Pahner I, Lautenschlager M, Ho H, Jahn R, Ahnert-hilger G, Höltje M, von Jagow B, Hörtnagl H, Nürnberg B. The neuronal monoamine transporter VMAT2 is regulated by the trimeric GTPase Go(2). *Journal of Neuroscience*. 2000; 20:2131–41. [PubMed: 10704487]
- Lee M, Gubernator NG, Sulzer D, Sames D. Development of pH responsive fluorescent false neurotransmitters. *Journal of the American Chemical Society*. 2010; 132:8828–30. [PubMed: 20540519]
- Meyer AC, Horton DB, Neugebauer NM, Wooters TE, Nickell JR, Dwoskin LP, Bardo MT. Tetrabenazine inhibition of monoamine uptake and methamphetamine behavioral effects: locomotor activity, drug discrimination and self-administration. *Neuropharmacology*. 2011; 61:849–56. [PubMed: 21669212]
- Nirenberg MJ, Chan J, Liu Y, Edwards RH, Pickel VM. Ultrastructural localization of the vesicular monoamine transporter 2 in mesolimbic and nigrostriatal dopaminergic neurons. *Advances in Pharmacology*. 1998; 42:240–3. [PubMed: 9327888]
- Ondo WG, Tintner R, Thomas M, Jankovic J. Tetrabenazine treatment for Huntington's disease-associated chorea. *Clinical Neuropharmacology*. 2002; 25:300–2. [PubMed: 12469001]
- Paleacu D, Giladi N, Moore O, Stern A, Honigman S, Badarny S. Tetrabenazine treatment in movement disorders. *Clinical Neuropharmacology*. 2004; 27:230–3. [PubMed: 15602104]
- Park H, Li Y, Tsien RW. Influence of synaptic vesicle position on release probability and exocytotic fusion mode. *Science*. 2012; 335:1362–6. [PubMed: 22345401]
- Parra LA, Baust T, Mestikawy SE, Quiroz M, Hoffman B, Haflett JM, Yao JK, Torres GE, Curie M. The orphan transporter Rxt1/NTT4 (SLC6A17) functions as a synaptic vesicle amino acid transporter selective for proline, glycine, leucine, and alanine. *Molecular Pharmacology*. 2008; 74:1521–32. [PubMed: 18768736]
- Rasband, W. ImageJ. U. S. National Institutes of Health; Bethesda, Maryland, USA:
- Richardson JR, Miller GW. Acute exposure to aroclor 1016 or 1260 differentially affects dopamine transporter and vesicular monoamine transporter 2 levels. *Toxicology Letters*. 2004; 148:29–40. [PubMed: 15019086]
- Richardson JR, Quan Y, Sherer TB, Greenamyre JT, Miller GW. Paraquat neurotoxicity is distinct from that of MPTP and rotenone. *Toxicological Sciences*. 2005; 88:193–201.
- Shaw G, Morse S, Ararat M. Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *The FASEB Journal*. 2002; 16:869–71.
- Sulzer D. How addictive drugs disrupt presynaptic dopamine neurotransmission. *Neuron*. 2011; 69:628–49. [PubMed: 21338876]
- Sulzer D, Sonders MS, Poulsen NW, Galli A. Mechanisms of neurotransmitter release by amphetamines: a review. *Progress in Neurobiology*. 2005; 75:406–33. [PubMed: 15955613]

- Taylor TN, Caudle WM, Miller GW. VMAT2-deficient mice display nigral and extranigral pathology and motor and nonmotor symptoms of Parkinson's disease. *Parkinson's disease*. 2011; 2011
- Taylor TN, Caudle WM, Shepherd KR, Noorian A, Jackson CR, Iuvone PM, Weinshenker D, Greene JG, Miller GW. Nonmotor symptoms of Parkinson's disease revealed in an animal model with reduced monoamine storage capacity. *Journal of Neuroscience*. 2009; 29:8103–13. [PubMed: 19553450]
- Volz TJ, Farnsworth SJ, Hanson GR, Fleckenstein AE. Method development and validation of an in vitro model of the effects of methylphenidate on membrane-associated synaptic vesicles. *Journal of Neuroscience Methods*. 2009; 177:177–82. [PubMed: 18992277]

Highlights

- We report a method for spatially resolved analysis of VMAT2 mediated uptake in live cells.
- Uptake of the fluorescent substrate was measured in HEK293 cell lines stable expressing DAT and mCherry-VMAT2.
- Uptake in to the mCherry positive compartment is sensitive to tetrabenazine.
- VMAT2 activity can be quantified by analyzing confocal images.
- We have developed an assay amenable to high content imaging that is low cost, uses no animals, and is flexible compared to radioactive assays.

Figure 1. The fluorescent substrate is transported by DAT and VMAT2

Untransfected HEK cells (A), HEK cells stably expressing hVMAT2 (HEK-VMAT2) (B), HEK cells stably expressing hDAT (HEK-DAT) (C), and HEK cells stably expressing both hDAT and hVMAT2 (HEK-DAT/VMAT2) (D) were incubated with HBSS/BSA/glucose for 10 minutes before addition of the uptake assay reagent. Images were acquired one hour after addition of reagent. Arrows indicate extensions with high concentration of punctate staining with dye.

Figure 2. HEK-DAT/mCherry-VMAT2 cells contain functional DAT and VMAT2

(A) Whole cell uptake in HEK cells stably expressing hDAT and the mCherry-hVMAT2 fusion (HEK-DAT/mCherry-VMAT2 cells). Cells were incubated with inhibitor (vehicle, 20 μ M TBZ or 10 μ M GBR12909) for 10 minutes prior to the addition of dopamine. Uptake was terminated after 20 minutes. (****, $p < 0.0001$) (B) Vesicular uptake in vesicles isolated from HEK-DAT/mCherry-VMAT2 cells. Vesicle fractions were incubated with indicated concentration of TBZ for 10 minutes prior to addition of dopamine. Uptake was terminated after 5 minutes. (C) Nomifensine dose response of whole cell uptake in HEK-DAT and HEK-DAT/mCherry-VMAT2 cells. Cells were incubated with indicated concentration of nomifensine for 10 minutes prior to addition of dopamine. Uptake was terminated after 20 minutes. (D) TBZ dose response of whole cell uptake in HEK-DAT and HEK-DAT/mCherry VMAT2 cells. Cells were incubated with indicated concentration of TBZ for 10 minutes prior to addition of dopamine. Uptake was terminated after 20 minutes. All graphs show the average of 3 independent experiments.

Figure 3. Uptake of the fluorescent substrate in HEK-DAT cells

(A) Uptake in HEK-DAT cells with vehicle or 10 μ M nomifensine. Cells were incubated in HBSS/BSA/glucose with or without nomifensine for 10 minutes prior to addition of the uptake assay reagent. Images were collected one hour after addition of reagent. (B) Uptake in HEK-DAT cells with MitoTracker. Cells were incubated with 50 nM MitoTracker Deep Red for 10 minutes. Media was the replaced with HBSS/BSA/glucose for 10 minutes. Uptake assay reagent was added and cells were imaged one hour after addition of reagent.

Figure 4. Uptake of the fluorescent substrate in HEK-DAT/mCherry-VMAT2 cells
 (A) Uptake in HEK-DAT/mCherry-VMAT2 cells with MitoTracker. Cells were incubated with 50 nM MitoTracker Deep Red for 10 minutes. Media was the replaced with HBSS/BSA/glucose for 10 minutes. Uptake assay reagent was added and cells were imaged 1 hour after addition of reagent. (B) Uptake in HEK-DAT/mCherry-VMAT2 cells. Cells were incubated with HBSS/BSA/glucose with or without inhibitor (TBZ or nomifensine, as indicated) for 10 minutes. Uptake assay reagent was added and cells were imaged one hour after addition of reagent.

Figure 5. TBZ sensitive uptake in HEK-DAT/mCherry-VMAT2 cells

(A) TBZ dose response of uptake in HEK-DAT/mCherry-VMAT2 cells. Cells were incubated with indicated concentration of TBZ for 10 minutes prior to addition of uptake assay reagent. Images were acquired one hour after addition of reagent. (B) Images were analyzed in ImageJ and the punctate-diffuse index was calculated. Curves were analyzed in GraphPad Prism. Data are combined from six independent experiments.

Figure 6. Analysis method for image quantification

(A) Flow chart with example images describing image analysis technique. First, the red channel (B) is thresholded to identify the mCherry-positive extensions, while excluding more centrally localized regions that may include mitochondria (C). Next, a selection is made based on this threshold and an ROI is defined from the selection (D,E). The green channel is then made the active image and the ROI created in the red channel is applied to the green channel (F,G). The standard deviation of pixel intensity within the ROI is measured. This value for each image is used to calculate the punctate-diffuse index (H). To calculate this index, the values obtained for all control images are averaged; the value of each image is then divided by this mean. This produces an index from 0 to 1, where 0 is more diffuse and 1 is more punctate relative to the control images.