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Development of a screening assay for vesicular dopamine transport.

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Advisor: Dr. Gary Miller

An Abstract of a Thesis

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Abstract

Development of a screening assay for vesicular dopamine transport.

Author: Bethany Wilson

The vesicular monoamine transporter 2 (VMAT2; Slc18a2) packages monoamines into vesicles for subsequent release and neurotransmission and is critical for sequestering both exogenous and endogenous toxicants away from their sites of action inside the neuron. We have previously found that genetic reduction of VMAT2 in a mouse model reproduces many of the features of Parkinson's disease and shows increased neurotoxic vulnerability. Other studies demonstrate that environmental chemicals can inhibit VMAT2 and that VMAT2 is dysfunctional in Parkinson's disease. Conversely, we have found that genetic increase of VMAT2 expression in mice results in elevated levels of dopamine in the brain, increased vesicular packaging of dopamine, increased dopamine release, and resistance to toxic insult. Thus, VMAT2 appears to be a valid target of interest for pharmacological treatment of Parkinson's disease and other monoamine-related disorders, as well as a measure of the impact of environmental exposures. We have developed and optimized an assay suitable for the pharmacological and toxicological screening of compounds, which positively or negatively affect vesicular dopamine transport. This assay is designed to evaluate modulators of VMAT2 function, but will also identify compounds that affect the expression or trafficking of VMAT2 and the function of other vesicular proteins; specific effects can be elucidated through secondary screens. The assay is based on the new fluorescent false neurotransmitter dye FFN206, which is selectively transported by VMAT2. The assay is tetrabenazine-sensitive and amenable to the 96-well format. Furthermore, we have optimized the assay for multiple conditions including cell line, plating density, plating technique, plate material and coating, well size, reaction volume, experimental media, negative control, reagent handling and storage conditions, test compound concentration and incubation time, FFN concentration and incubation time, FFN incubation temperature, wash temperature and volume, reading volume, plate reader type and temperature, and plate reading dimensions. In so doing, we have achieved a z-factor of 0.93, which is suitable for high throughput screens, and scalable to robotic adaptation and miniaturization in a 384 or 1536 well format.

Development of a screening assay for vesicular dopamine transport.

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Table of Contents

Introduction…………………………………………………Page 1

Materials and Methods………………………………………Page 11

Results…………………………………………………………Page 14

Discussion……………………………………………………..Page 19

Figure Legends………………………………………………..Page 24

References……………………………………………………..Page 30

Figures…………………………………………………………Page 38

Introduction

The vesicular monoamine transporter (VMAT) packages monoamines into vesicles for subsequent release and neurotransmission and is critical for sequestering both exogenous and endogenous toxicants away from their sites of action inside cells. Many neurological movement disorders (including Parkinson's Disease, dystonia, and Huntington's disease) and psychiatric disorders (including addiction, depression, schizophrenia, post traumatic stress disorder, and attention deficit hyperactivity disorder), have been associated with function disruption of monoaminergic signaling (Taylor 2000, Russell 2002, Picconi 2003, Schwartz 2003, Howell 2008, Song 2012). Therefore, identifying both environmental toxicants that inhibit VMAT and potential pharmacological compounds to enhance VMAT function holds tremendous value. This thesis describes the significance of VMAT as a target and the optimization of a high throughput assay to assess VMAT function.

Evidence for the role of VMAT in Parkinson's disease. Parkinson's disease has historically been defined by motor characteristics (tremor, bradykinesia, rigidity, and postural instability) mediated by the progressive loss of dopaminergic transmission (Ehringer 1960). Therapies aimed at restoring dopamine function have been only partially satisfactory. In fact, Parkinson's disease results in a complex constellation of autonomic, cognitive, and neuropsychiatric symptoms mediated by multiple monoaminergic transmitters (Halliday 1990, Zarow 2003). VMAT is the common transporter for all monoamines, and alterations in vesicle integrity are fundamental to the dysfunction observed in Parkinson's disease. Thus, therapeutic interventions aimed at restoring or enhancing VMAT function are more likely to address the full spectrum of Parkinson's disease symptoms.

The role of VMAT in Parkinson's disease was first hinted with early trials of reserpine (a VMAT inhibitor) as an antipsychotic. Although the mechanism of action is unknown (reserpine was isolated from the Indian snakeroot *Rauwolfia serpentine*), the medication caused severe side effects closely resembling Parkinson's disease (Richman 1955). If the function of VMAT is disrupted, neurotransmitters are not packaged into vesicles, and therefore remain in the cytosol. Cytoplasmic monoamines produce toxic oxidative products (through metabolism by enzymatic deamination or autoxidation) which contribute to neurodegeneration (Burke 2004, Eisenhofer 2004, Rees 2009, Wey 2012, Goldstein 2011, Zahid 2011, Alter 2013). Similarly, the results of genetic impairment of VMAT has also been observed in humans. A mutation causing a single nucleotide substitution in the coding region results in severe symptoms, including infant onset parkinsonism and autonomic, cognitive, and mood disorders (Rilstone 2013). Conversely, gain of function mutations in the promoter region have been observed to be protective against Parkinson's disease (Glatt 2006, Brighina 2013).

Evidence from animal studies. Studies in animals show that dopamine remaining in the cytoplasm is toxic to the cell, and VMAT is responsible appropriately sequestering dopamine inside the vesicle (Graham 1978, Hastings 1996). Neurons in mice which can take up dopamine but lack VMAT2 to package it have severe oxidative stress induced neurodegeneration which results in profound motor deficits (Chen 2008). A spectrum of VMAT expression levels in various mouse models shows a dose response effect for VMAT ranging from deleterious to protective. VMAT knockout is lethal soon after birth, and mice with very low VMAT expression (less than 5% of wildtype levels) model parkinsonism with oxidative stress induced neurodegeneration and motor symptoms as the mice age (Colebrooke 2006, Taylor 2009, Ulusoy 2012). VMAT heterozygotes (50% expression level of wildtypes) do not show overt Parkinson's symptoms but are highly susceptible to toxicant damage (Takahashi 1997, Wang 1997, Gainetdinov 1998). Mice

with increased VMAT expression (200% of wildtype level) are protected from neurotoxic insult and show increased resilience associated with measures of anxiety and depression (Lohr 2014). Furthermore, enhancing VMAT function does not seem to increase the risk for addictive behaviors. Therefore, enhancing VMAT function is a promising target for treating Parkinson's disease as well as other disorders of monoaminergic signaling.

Evidence from environmental studies. Further, the profound effect of VMAT inhibition is supported by epidemiological evidence (Tanner 1990, Semchuk 1991, Semchuk 1992, Priyadarshi 2000, Priyadarshi 2001, Ascherio 2006, Steenland 2006, Ritz 2006, Elbaz 2009, Gatto 2009) . In addition to packaging dopamine, VMAT also sequesters other toxicants into vesicles which protects the neuron from their toxic effects. Environmental toxicants (including organochlorine insecticides, polychlorinated biphenals, and brominated flame retardants) are associated with Parkinson's disease through epidemiological exposure studies and have been reported in post mortem brain tissue and serum of Parkinson's disease patients (Fleming 1994, Corrigan 1998, Miller 1999, Corrigan 2000, Weisskopf 2010, Caudle 2012, Hatcher-Martin 2012). Investigation of the mechanisms of these compounds to determine if their deleterious effects are exclusively through VMAT inhibition or other pathways leading to similar oxidative stress will provide valuable information. This knowledge can lead to protective treatments to prevent damage from exposure to dangerous compounds. Further the assay proposed here can evaluate compounds which should no longer be used (and their putative replacements) for predicted safety with long term human exposure, as the consequences of exposure to these chemical classes has only been observed long after irreparable harm has occurred to generations of humans.

VMAT structure and function. VMAT can be found in two isoforms. The VMAT2 isoform (*Slc18a2,* solute carrier family 18 member 2) is found in the nervous system (as

well as platelets and the gastric epithelium), and it has a much higher affinity than VMAT1 for monoamines, which contributes to the ability to concentrate monoamines in the vesicle at levels 10-100,000x more than the cytoplasmic level (Peter 1994, Peter 1995, Erikson 1996, Lesch 1993). Vesicular monoamine transporter 1 (VMAT1) is an isoform found primarily in the periphery, particularly in the neuroendocrine secretory granules of chromaffin cells of the adrenal medulla (Liu 1992). VMAT2 has twelve transmembane domains, and the 3D shape coils around, so the first transmembrane domain interacts with the tenth and eleventh domains (Merickel 1997). The sequence shows remarkable similarity to bacterial proteins responsible for exporting toxins (and contributing to drug resistance) (Chaudhry 2008).

VMAT has two major inhibitory binding sites, one for tetrabenazine in the tenth transmembrane domain, and one for reserpine adjacent to the substrate binding site, and most other newly discovered inhibitors bind to one of these two sites (Chaudhry 2008, Wimalasena 2010). Interestingly, tetrabenazine also inhibits reserpine binding through an allosteric mechanism (Chaudhry 2008, Wimalasena 2010). There are no known sites characterized for positive allosteric modulators. The N and C terminus are both on the cytoplasmic side, as potential sites of regulation from cell signaling pathways, and the long loop between transmembrane domain one and two is the most promising site for intra-vesicular regulation (Parsons 2000).

VMAT regulation. This thesis focuses on the optimization of an assay of VMAT function. Briefly, cells stably expressing VMAT2 are incubated with a compound of interest, then a fluorescent false neurotransmitter (FFN206) is added which is loaded into vesicles by VMAT. Excess fluorophore is washed off the cells and fluorescence is read in a platereader. FFN206 only fluoresces in acidic compartments, so any fluorescent activity should be exclusive to FFN206 that has been taken up by VMAT and sequestered into an acidic compartment or vesicle. Therefore, more fluorescence

equals more uptake of FFN206, as a proxy for uptake of neurotransmitters. Ideally, a compound that causes a drop in fluorescence is a VMAT inhibitor, and a compound that causes an increase in fluorescence is a novel positive allosteric modulator of VMAT. In reality, alterations in fluorescence level could occur by direct regulation of VMAT or indirect effect on the regulation of vesicular filling.

The vesicular ATPase pump sets up a proton gradient so that pH inside the vesicle is approximately 5.6 (Liu 1997). VMATs act as proton exchangers (two protons exchanged for each molecule of neurotransmitter), so their function is dependent on the existence of the correct proton gradient (Johnson 1988, Knoth 1981, Parsons 2000). Consequently, anything that affects the proton pump would also show as a hit on this assay. Certainly, VMAT itself is a better target, since indiscriminately affecting ATPases would have broad reaching consequences, and this pump is highly conserved across other non-monoamine neurotransmitter types as well.

Synaptic vesicles also express chloride channels, and although chloride channels are not as critical in the normal regulation monoamine filling (compared to glutamate regulation), changes in chloride channel permeability would also alter the assay outcome (Johnson 1988, Hell 1990, Maycox 1990, Schuldiner 1995, Reimer 1998). Opening channels would increase vesicular chloride, increase the proton gradient, and decrease the electrical gradient. Decreases in chloride intake would increase the electrical gradient and decrease the proton gradient due to reduced proton accumulation, since positive charges in vesicular lumen would not be neutralized by chloride (Maycox 1990, Reimer 2001). Outright inhibiting or removing chloride channels on vesicles would reduce acidification, and proton gradient, and increase vesicular neurotransmitter content. These pH considerations are particularly salient for interpreting the results of this assay, since FFN206 is a pH sensitive dye. Furthermore, anything that totally disrupts the vesicle membrane might look like an inhibitor, since

there would be no acidic compartment in which it could fluoresce (Liefferinge 2013). Lipophilic weak bases (such as chloroquine) would reduce VMAT-dependent uptake by collapsing the required pH gradient across the vesicle membrane (Hu 2013). Similarly, ATPase inhibitors would also lead to false hits. Using a secondary screen which measures alkalinization of the vesicles, such as acridine orange or a LysoTracker probe would efficiently eliminate these false positives.

To consider the possible targets of indirect regulation of vesicle filling, it is necessary to consider the regulators of VMAT itself. Building evidence suggests that VMAT activity is itself regulated by vesicle content. The exact mechanism isn't completely parsed out yet, but there's very convincing evidence that G proteins are involved (Brunk 2006). Although G proteins are commonly thought of as located on the plasma membrane, they can also be located on endomembranes, including the synaptic vesicle (Nurnberg 1996, Ahert-Hilger 2003). The G αo class of G proteins are the most abundant in the brain (about 1% of the total brain protein), but scarce information is available about their distribution or their up or down stream targets (Jiang 1998, Dhingra 2002). G proteins are localized to the synaptic vesicle, however, as functional heterotrimers, activated on the luminal side of organelles (Ahert-Hilger 1998). Most interestingly, different vesicular G sub units exist for specific transmitter types, and different G vesicular subunits occur for different tissue types, further supporting their role as specific regulators of vesicular filling (Pahner 2002). G αo_2 inhibits both VMAT 1 and 2, and studies show non hydrolysable GTP analogue or activated G αo_2 inhibit vesicular filling (Alhert-Hilger 1998, Holtje 2000). Also, this downregulation is blocked by preincubation with pertussis toxin (which is known to cleave G α proteins) (Alhert-Hilger 1998). Remarkably, VMAT regulation by G proteins is dependent on the presence of monoamines. Therefore, when no monoamines are present in the vesicle, or the monoamine content is drastically reduced, VMAT is not inhibited by G protein activation

(Alhert-Hilger 2003). If vesicles are preloaded, the G protein inhibition is recovered. Therefore, although G protein coupled receptors are historically prime targets, this assay will be unlikely to identify these indirect modulators of vesicular filling, since the target compounds are incubated while the vesicles are empty.

Important for the viability of the positive effects of a newly identified positive allosteric modulator of VMAT, it is known that vesicular fusion is completely independent of the contents of the vesicle (they are equally likely to fuse even if they are empty), so vesicles are not cycled out to fuse once they have reached a set point in total monoamine content or concentration (Croft 2005). Rather than a predetermined set point, it seems that this consistent concentration is simply the result of the steady state of the normal levels of VMAT expression and neurotransmitter expression (Bruns 2000, Pothos 2000, Pothos 2002). So, by directly enhancing VMAT function, or by adding more VMAT to the vesicle, it should increase the amount of transmitter per vesicle, without running into other regulatory blocks. Even given the evidence of G protein inhibition, any ceiling effect would be above the normal steady state. Alternatively, increasing the number of VMATs per vesicle might change the rate of filling, but not necessarily the total final concentration, which may still have a meaningful physiological effect *in vivo* of sequestering toxins more efficiently or aiding in synaptic transmission during rapid firing rates.

VMAT trafficking is not well characterized, but *in vivo* estimates range from 1 – 14 VMATs per vesicle (Takamori 2006). In neurons, VMAT is expressed on both synaptic vesicles and large dense core vesicles (LDCV) and therefore, must contain amino acid sequence tags that target it to both locations (Nirenberg 1998). Known alternative splicing of these trafficking domains can change the vesicle type to which the VMAT is sent, or whether VMAT is sent to a vesicle at all (Yao 2007). A di-lucine signal very close to a transmembrane domain signals to send VMAT to an endosomal type

compartment (Tan 1998). VMAT is also regulated by post-translational modifications. VMAT has acidic clusters that are phosphorylated by CK2, which binds PACS-I, which is necessary for proper trafficking to the vesicle and determines to which vesicle type it is sent. Reducing PKA signaling also sends VMAT to synaptic vesicles preferentially over LDCV (Yao 2004).

Glycosylation is a particularly interesting modification. VMAT exists in three forms, a glycosylated, a partially glycosylated, and a native form. In old age, there is decrease in the glycosylated form, and an increase in the partially glycosylated form (Cruz-Muros 2008). This loss in glycosylation results in the trafficking away from the membrane (Cruz-Muros 2008). This could explain the increased risk of Parkinson's disease with aging, or it could be a good compensatory effect that preferentially places the remaining effective transporters on the vesicles for use, and is part of the method by which signaling is preserved for so long in degenerative disease. In addition to all the classical effects of stimulants on VMAT, stimulants also appear to have a significant effect of redistribution of VMAT away from the membrane. The drug induced redistribution may also occur through this glycosylation effect, and shows that it can be modified in a short time scale, and drugs that act through a similar mechanism might easily be picked up through screening in this assay (Brown 2001).

Further, regulation of VMAT, like all proteins, can occur at the levels of transcription and translation, but the short time scale of this assay (under two hours total) makes these indirect mechanisms unlikely to show large scale effects in that time frame (Watson 1999, Watson 2000). Furthermore, these more general regulations of fundamental cell functions yield poor drug targets due to their broad reaching effect. Though, if these forms of regulation were of special academic interest, the stability of the FFN206 fluorophore means that the assay could be modified to read the plates long after washing off excess FFN206 to more readily address those questions.

Additionally, it must be acknowledged that many proteins are located on the vesicle in addition to VMAT. This assay may also be suited to pick up effects of the role of specific other proteins of interest by co-expression with VMAT2. To some extent, all those hits are valuable, because if the overall premise is that enhanced vesicular function (not just specifically VMAT function) is protective, having a screen capable of casting a broader net is a benefit. For each of the other proteins, however the other effects of modulations will have to be carefully assessed, since a body of evidence as exists for VMAT supporting the benefits of enhancing function without detrimental side effects is rare. Therefore, VMAT2 is selected as the primary target for high throughput screening.

Previous Methods. Historically, radioactive uptake assays using tagged dopamine in synaptic vesicles isolated from rodent brains have been used to assess VMAT function (Caudle 2007, Guillot 2008, Hatcher 2008, Volz 2009, Chu 2010,). Vesicles can be prepared from brains from animals treated with environmental toxicants or pharmacological compounds of interest *in vivo*, or vesicles can be treated directly *in vitro*, but both methods are resource intensive due to the high volume of animal tissue required. Two previous cell-based assays utilized vesicular fractions from cell lines expressing VMAT, but could not address the actions of compounds in the whole cell, including the ability to effectively cross the plasma membrane to act on the vesicle (Erickson 1996, Bellocchio 2000, Parra 2008). A previous whole cell dye based assay improved on this situation, but required co-staining vesicles for subcellular localization of the dye (due to extraneous mitochondrial staining), therefore could not be read as total fluorescence in a plate reader, therefore limiting the number of compounds, doses, and time points which could feasibly be examined (Bernstein 2012). A novel fluorescent false neurotransmitter (FFN206) based assay was proposed by Hu et al. (2013) and the

protocol described here was optimized for high throughput screening at Emory's Chemical Biological Discovery Center.

High throughput design. High throughput assays require a high degree of accuracy and sensitivity, and therefore demand a wide dynamic range and minimal variability within the datasets. The feasibility of using an assay is described by the z factor (Zhang 1999). The z factor is calculated as shown, where: positive control mean ($\hat{\mu}_p$), positive control standard deviation ($\hat{\sigma}_p$), negative control mean ($\hat{\mu}_n$), and negative control standard deviation ($\hat{\sigma}_n$):

$$z = 1 - \frac{3(\hat{\sigma}_p - \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}.$$

This calculation ensures that assays with favorable z values (as close to 1 as possible) will have a large band of separation between the distributions of the data for the positive and negative control (described as means plus or minus three standard deviations). The methods described in this thesis iteratively increased the z factor from 0.278 to consistently greater than 0.9, and therefore the optimized assay is suitable for use in high throughput screening.

Materials and Method

Protocol from Hu et al. (2013) was replicated to determine the viability of using the novel fluorescent false neurotransmitter (FFN206) as an assay of VMAT2 function. After successful proof-of-principle experiments were conducted, iterative experiments were conducted to refine the protocol for selection of the cell line, plating technique, reaction protocol, and analysis, until a z factor > 0.9 was consistently achieved, indicating the protocol was then suitable for high throughput screening. The final protocol, described here, was used to test a selection of compounds of interest to confirm feasibility.

Cell Culture and Plating Human Embryonic Kidney cells (HEK-293, ATCC CRL-1573) stably transfected with VMAT2 were cultured in 10 cm tissue culture dishes (Corning 430167) in HEK media [Dulbecco's Modified Eagle Medium (DMEM, Corning 10-013-CV) + 10% Fetal Bovine Serum (FBS) + 1% Penicillin-Streptomycin (Pen Strep, Corning 30-002-CI) + 0.1% Zeocin (Invivogen ant-zn-1) for selection] and incubated at 37°C with 5% CO_2 to 80% confluency. Media was aspirated and cells were incubated with 2 ml 0.25% trypsin/ 2.21mM EDTA in HBSS (Corning 20-053-CI) for 2 minutes at 37°C. Cells were gently washed off of the plate with the addition of 8 ml of HEK media. Cells were diluted 1:3 in HEK media in a 50 ml trough, mixed thoroughly by gentle trituration through a 10 mL stripette ten times.

Cells were plated with a multichannel pipette, 100 µl of diluted cells per well, into a half volume, solid black wall, poly-D-lysine (PDL) coated (Sigma P0899) 96 well plate (Grenier Bio One 675090). Between plating each column, cell solution was gently triturated three times to prevent cell settling. Cells were plated into the inner 60 wells, with sterile PBS added to the outer ring of wells to prevent evaporation. Cells were incubated at 37°C with 5% CO_2 for 36-48 hours until 90% confluent.

Assay Sterile phosphate-buffered saline (PBS) and experimental media [eDMEM, DMEM without phenol red (Corning 17-205-CV) + 1% 200mM L-glutamine (Life

Technologies 1187509)] were pre-heated to 37°C in a water bath. All experimental drugs were solubilized and diluted in experimental media with 2% DMSO for maintaining solubility. Culture media was aspirated and 90 µl of experimental media was added to each well. Next, 5 µl of drug treatment was added to each well, using 5 µl of 2% DMSO in experimental media as vehicle control, and 5 µl of 200 µM tetrabenazine in experimental media as negative control. Plates were incubated with drug treatment for 30 minutes at 37°C with 5% CO_2.

FFN206 (Abcam ab144554) was stored in 10 mM stock at -20°C. FFN206 was diluted in experimental media to 20 µM. After incubation with treatment compound, 5 µl of 20 µM FFN206 was added to experimental wells (final concentration of FFN206 was 1 µM in each well), with 5 µl of experimental media used as a vehicle control. Total reaction well volume was 100 µL. Plates were incubated with FFN206 for 75 minutes in the dark at 37°C with 5% CO_2.

After incubation with FFN206, experimental media was aspirated and wells were washed by the addition of 180 µl warm PBS. PBS was aspirated and 90 µl warm PBS was added for plate reading.

Plates were read from the top, without lids, on a Perkin Elmer Victor 3 Plate Reader, with plate stage pre-heated to 37°C. Plate reading settings were as follows: CW Lamp Energy 3431, Stabilized Energy, CW Lamp Filter: F355 (FFN206 peak excitation = 369 nm), Emission Filter: F460 (FFN peak emission = 464 nm), normal emission aperture, counting time 0.1 seconds.

Experimental Set-up. Trials were also conducted to determine the most efficient methods of personally completing this protocol. Plates were pre-coated with PDL before use. Plates were coated with 75µL of PDL per well, and incubated at 37 °C for 1 hour to set PDL coating. PDL was aspirated off, and wells were washed three times with sterile water. Plates were allowed to dry in the hood, with lids slightly ajar, then wrapped with

ethanol sterilized plastic wrap and stored at 4 °C for future use. Plates were coated in batches of 8 plates (as shipped in sterile packets of 8 plates), with the entire process repeated if more plates were needed, rather than coating more plates at once. To suit this method, 10X PDL was stored in 5mL aliquots in 50mL conical tubes at -20 °C and then diluted with sterile water in the same tube at the time of use.

Assay was conducted in batches of three plates, to ensure the time difference between adding reagents to the first well of the first plate and the last well of the last plate was less than ten minutes. Each plate represented a different experiment, so that the three replicates of one experiment were conducted on three separate days to ensure cells and reagents were independent samples. If more than three plates were conducted on the same day, batches of three were repeated morning, afternoon, and evening. Alternatively, batches were staggered by starting the second batch 50 minutes after the beginning of the first batch, and the third batch 90 minutes after the beginning of the first batch. The plate reader stage was pre-heated at least seven minutes before reading, to allow the plate to reach 37 °C from room temperature.

Analysis. The data were analyzed by t-test, ANOVA, and z factor analysis as appropriate. No significant difference between conditions was determined when $p > 0.05$. Optimization experiments shown in the results to follow were conducted with single factor deviation from the protocol described above. Optimization decisions were based on z factor analysis, rather than p value alone, since alterations in variability were also necessary to consider. All experiments (both optimization experiments and compound testing) were conducted in triplicate wells, with 3 replicate plates. Triplicate measures were collapsed, and replicates were used to calculate sample averages and sample standard deviations for the following equation:

$$z = 1 - \frac{3(\hat{\sigma}_p - \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}.$$

RESULTS

Pilot Data. Initial proof-of-principle data for FFN206 feasibility (shown in Figure 1) found that FFN206-specific fluorescence can be detected, and is tetrabenazine sensitive. FFN206 fluorescence is significantly different than FFN206 fluorescence suppressed by tetrabenazine ($p < 0.01$). No significant difference was observed between DMSO vehicle control for FFN206 and tetrabenazine-suppressed FFN206 fluorescence. The initial trials found $z' = 0.278$, which was unsuitable for use in high throughput screening. Further pilot studies showed that FFN206 fluorescence is dose-dependent (Figure 2), and tetrabenazine suppression of FFN206 fluorescence is also dose-dependent (Figure 3).

FFN206 and tetrabenazine dose selection. To select the tetrabenazine dose for use as negative control, a narrower selection of the initial dose response curve was tested (1, 5, 10, 20, and 50 μM), but no significant difference was found (Figure 4). Initially, 10 μM tetrabenazine was selected as the starting dose for the negative control. Subsequently, a broad range of FFN206 concentrations (0.1 - 10μM) were tested against the selected concentration of the negative control (Figure 5). For doses as high as 1 μM, FFN206 is exquisitely sensitive to 10 μM tetrabenazine suppression of VMAT activity (no significant difference found between tetrabenazine-suppressed fluorescence and water blanks). For further experiments, a midlevel dose of FFN206 was chosen (below 10 μM) to ensure the assay has the dynamic range necessary to detect increases in VMAT function. Next, a narrow range of optimized FFN206 concentrations (1, 2, and 5 μM) was tested against a narrow range of optimized tetrabenazine concentrations (10, 50, 100 μM) to determine FFN206 and tetrabenazine dose interactions (Figure 6). Lower variability was observed in lower doses of both FFN206 and tetrabenazine, which improves z factor analysis. Finally, a narrower range of optimized FFN206 concentrations (1, 1.5, 2, and 2.5 μM) was tested against 10 μM tetrabenazine as the

negative control (Figure 7). Although higher doses of FFN206 result in higher observed florescence, the variability scales with the increase in signal strength, so does not yield an overall improvement in z factor. Therefore, 1 μM FFN206 was selected based on z factor analysis and cost management. The protocol to determine best practices for tetrabenazine and FFN206 reagent handling and storage was also tested to set the storage stock concentrations of 200 μM tetrabenazine and 10mM FFN206. FFN206 stock aliquots were diluted to a working concentration of 20 μM, and neither storage nor working stock was re-frozen for later use, due to degradation of fluorophore (data not shown). Parallel experiments were conducted with reserpine as a possible alternative negative control (instead of tetrabenazine), but due to difficulty with consistent solubilization at higher concentrations, the results were much more variable, and reserpine was rejected as a suitable control for high throughput screens (data not shown).

Variables related to cell selection and plating. This dose protocol was conducted on multiple cell lines (HEK, HEK + DAT, HEK +VMAT2, and HEK + DAT +VMAT2). FFN206 fluorescence was found to be VMAT specific but not DAT dependent for cell entry (Figure 9). Fluorescence observed in HEK + VMAT2 cells was more than four times greater than observed in the HEK + DAT + VMAT2, so HEK cells with only the addition of the VMAT2 construct were selected for further use in the assay.

To determine if fluorescence scaled with cell density, cells were plated at multiple dilutions (1:2 1:3, 1:4, and 1:5) 24 hours before the assay. As expected, fluorescence was found to scale with cell density (Figure 9). As another proxy to determine if FFN206 fluorescence scales with VMAT function, varying amounts of VMAT2 construct were transfected, beginning with the optimized transfection protocol, and decreasing by a factor of 2 (100%, 50%, 25%, and 12.5%). As expected, FFN206 fluorescence scales in a dose-dependent manner with transfected VMAT copy number (Figure 10).

Next, trials were conducted to optimize the plating protocol. Clear-walled plates were compared to black-walled plates (all plates had clear bottoms), and data were normalized to water blanks to account for differences in auto-fluorescence of different plastics. Black-walled plates were found to have a significantly higher fluorescence count and a significantly higher z factor (Figure 11). The protocol was conducted using black plates purchased pre-coated with PDL and compared to coating black plates in house with PDL as described in methods. Data shown is DMSO subtracted, to account for the difference in auto-fluorescence of the plate plastics. Hand coating was not significantly inferior to commercially available coated plates, $p = 0.97$ (Figure 12).

The initial pilot experiments had shown a trend for higher reads on the left of the plate, and the inner wells of the plate. Therefore, cell plating technique was adjusted as described in methods to ensure even pipetting into each well, with trituration to prevent cell settling; new multichannel pipettes were purchases to ensure consistent calibration and even performance for each tip; and plate reader reading dimensions were adjusted to ensure no overlap with the well reads until no significant difference was found between wells in different plate locations, $p=0.63$ (final trial shown in Figure 13). Next, the protocol was conducted in PDL-coated black plates in original volume and half volume to determine if smaller wells/ fewer reagents could be used without affecting the z factor. No significant difference was found between reaction volumes ($p = 0.63$) (Figure 14). Therefore, the protocol was updated to half-volume PDL-coated black plates with a 100 µL total reaction volume.

The protocol was tested with various media to determine which was most conducive for assay. Media tested included DMEM, DMEM + 10% FBS + 1% Pen Step (HEK media), HEK media + Zeocin (selection media for the VMAT2 construct), Opti-MEM (commercial reduced serum media marketed for transfections), DMEM without phenol red + 10% FBS, and DMEM without phenol red and without FBS. Media without

phenol red, and media without serum were found to be best for the assay (Figure 15). Antibiotics (Pen Strep and Zeocin were used during cell passage and initial plating, but were not used during the brief assay. All factors considered, experimental DMEM (eDMEM), as described in the methods section, was found to be the best choice for the assay.

To determine if the improved fluorescence in media without FBS was due to interference from FBS during the assay or cell function changes from short-term serum starvation, the protocol was conducted in full serum media, and with prior serum starvation of 0, 6, or 24 hours, then assayed in serum-free experimental media. Results show that although removing FBS from the media during testing improves assay results, additional serum starvation diminishes results (Figure 16).

Variables related to experimental reaction. To determine the ideal incubation time for FFN206, a time course was conducted. Fluorescence peaked at an incubation time of 75 minutes, which was selected for the optimized protocol (Figure 17). Additionally, no significant difference was found between room temperature and 37 °C for FFN206 incubation, $p = 0.542$ (Figure 18). To determine the stability of fluorescence, a time course of wash time (time between washing to remove fluorophore and reading the plate in the plate reader) was conducted. No significant degradation was observed by 2 hours (Figure 19).

To investigate the robustness and reproducibility of the protocol, plates were read on three different plate reader brands, in three different laboratories. Once normalized by range, no significant difference was found between results from different plate readers, showing the consistency of this protocol (Figure 20).

Optimized Protocol. After optimizing the protocol, the optimized z' was consistently > 0.9. As shown in Figure 21, z' = 0.947.

The optimized assay was used to test a variety of pharmacological VMAT inhibitors to demonstrate the utility of the assay for evaluating the effect of pharmacological compounds on VMAT activity. Dose dependent VMAT inhibition was found for reserpine, tetrabenazine, methamphetamine, and methylphenidate (Figure 22). Reserpine and tetrabenazine show essentially complete inhibition at 1μM doses, while methamphetamine and methylphenidate show inhibition at high doses (10, 100 μM). The optimized assay was also used to test a variety of environmental toxicants of interest due to the association with Parkinson's disease risk. Rotennone, Paraquat, Arochlor 1254, and Aroclor 1260 each show dose dependent inhibition of VMAT activity.

Discussion

In the design of protocols to be used for high throughput screens, a resolute focus on z factor is necessary to ensure that the methods will be suitable for high throughput use, which has different demands than smaller-scale use in the laboratory. High throughput assays require a high degree of accuracy and sensitivity, and therefore demand a wide dynamic range and minimal variability within the datasets. In calculating the z factor, only four variables are used in the equation (Zhang 1999): positive control mean ($\hat{\mu}_p$), positive control standard deviation ($\hat{\sigma}_p$), negative control mean ($\hat{\mu}_n$), and negative control standard deviation ($\hat{\sigma}_n$), as shown by

$$z = 1 - \frac{3(\hat{\sigma}_p - \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}.$$

This calculation ensures that assays with favorable z values (as close to 1 as possible) will have a large band of separation between the distributions of the data for the positive and negative control (described as means plus or minus three standard deviations). Therefore, to optimize the z factor, it is fundamentally necessary to enhance the separation between the negative and positive control means (increasing the positive control mean while decreasing the negative control mean) and decrease the variability of both datasets. Most optimization decisions were based on these goals, as shown in Table 1. For example, black walls prevent overflow fluorescence between wells and increase the difference in fluorescence count between positive and negative controls.

The combined effect of these strategic alterations improved the z factor from ~0.2 (an assay suitable for small-scale lab use, but impossible for high volume screens) to > 0.9 (an excellent assay, nearly ideal for high throughput use).

Furthermore, other factors affect the realities of conducting assays. Some experiments showed that cheaper options could be used without negatively affecting the z factor, including decreasing the reaction volume (decreasing the required cost for cells,

media, fluorophores, control reagents, and test reagents), and producing some materials in house (hand coating plates with PDL and creating experimental media from component ingredients). Other tests showed that although a certain protocol may be optimal, the assay demonstrates a robust tolerance for deviations on some points. This knowledge, although not used within the optimal protocol, is valuable from two perspectives. First, for the demands of robotic handling in high throughput screens, variations, such as timing or temperature, may be introduced by the logistical constraints of equipment setup. This assay is not overly delicate, and such variations are unlikely to cause the loss of a valuable target hit. Second, this assay will continue to be used to directly answer specific questions of significant interest to the lab, and alterations unsuitable for high throughput use may be perfectly suitable within the lab, such as adjusting the incubation time to complement other experimental demands, to times ranging from 60 to 90 minutes, instead of demanding rigid adherence to the optimal 75 minute protocol. By knowing the optimal values, rather than arbitrary but empirically functional values, deviations clustered around those values are more likely to be tolerated. Knowing which aspects are somewhat flexible (plate coating, temperature, incubation and wash timings and volumes, plate reader settings, etc.) and which alterations would be detrimental (negative control, plating technique, cell confluence, media ingredients, etc.) can save valuable time and prevent wasted effort and resources.

Some protocol decisions were made by weighing the relative costs and benefits. For example, cells at the highest density showed greater variability due to visible patches of cell loss due to multiple media changes and wash steps. Although it causes a slight decrease in possible maximum fluorescence value, cells at ~90% confluence were found to be best suited for assaying due to decreased variability. Below ~80-85% confluence, the loss of potential maximum was too severe a sacrifice. Repeated trials

using cells from different stocks or passage number revealed that due to changes in the rate of proliferation, neither a set cell number at plating nor a set time between plating and assay produced optimal results. Rather, confluence at the time of the assay was critical, so when cells were not at ~85-90% confluence at the time of scheduled experiment, the experiment was rescheduled, rather than assaying sparse plates. This results in reliably high-quality data, at the sacrifice of scheduling convenience.

Another carefully considered decision was the cell line used for screening. Since multiple known pharmacological compounds take advantage of DAT's transporter function to enter the cell, using a DAT expressing cell for the screen was seriously considered. Unfortunately, the positive control mean for the DAT and VMAT expressing HEK cells was only 25% of the mean value for HEK cells expressing VMAT only. This resulted in a z factor below 0, therefore the HEK + DAT + VMAT cell line was entirely unsuitable for high throughput use. Lower fluorescence in this cell line is likely due to intrinsic factors of cell line and lower transfection efficiency for the double construct. Although the exclusion of DAT-dependent entry is not ideal, the improved z factor based on the selection of the VMAT cell line will allow for the screening of orders of magnitudes more potential compounds. Additionally, potential therapeutic compounds that can enter without the dependence on DAT presence or function may be particularly well suited for treating neurons damaged by neurodegeneration.

The test of exemplar pharmacological and environmental compounds shows the range and utility of this assay. Various doses and compounds show a range of inhibition, spanning from no inhibition with graduations all the way to complete inhibition. This allows for the creation of dose response curves for a single compound, and the comparison of the relative inhibitory effects of multiple compounds, as the well as the construction of IC_{50} tables (half maximum inhibitory concentration) for previously untested compounds. These results match previously reported values for

methamphetamine determined by inhibiting uptake of radioactively-tagged dopamine into rat brain vesicles, in a far more efficient and cost-effective assay (Partilla 2006).

The association between pesticides and Parkinson's disease is well established, but the specific mechanism for each pesticide has not yet been confirmed. These results for VMAT inhibition by rotenone provide more direct evidence for the specific mechanism of alteration of dopamine metabolism in pesticide exposure (Goldstein 2015). Similarly, these data provide the first evidence of the immediate effect of paraquat on VMAT function, improving upon indirect measures of alterations of VMAT expression (Singhal 2011, Kumar 2010). The effect of Aroclor 1254 on dopamine synaptosomal concentrations has been examined, showing a similar moderate inhibition which prevents the calculation of IC_{50} values for direct comparison, but Aroclor 1260 was previously not examined (Bemis 2004). The efficiency of this assay will allow for the direct and speedy comparison of a large library of compounds of interest.

In a large scale screen for compounds which alter VMAT function, secondary screens will be necessary to eliminate false positives. Lipophilic weak bases (such as chloroquine) would reduce VMAT-dependent uptake by collapsing the required pH gradient across the vesicle membrane (Hu 2013). Similarly, ATPase inhibitors would also lead to false hits. Using a secondary screen which measures alkalinization of the vesicles, such as acridine orange or a LysoTracker probe would efficiently eliminate these false positives. After secondary screening, promising hits could be further evaluated through progressively more labor-intensive methods, including radioactive dopamine uptake, voltammetry, microdialysis, long-term animal exposure studies, and human exposome data. This assay provides a highly amenable format for both directed and high throughput evaluation of VMAT inhibitors and novel VMAT enhancers.

Table 1. Rationale for key protocol decisions

FFN dose of 1 µM	Decreased variability of positive control Preserve dynamic range of assay
Tetrabenazine as negative control	Decreased fluorescence of negative control Decreased variability of negative control
Tetrabenazine dose of 10 µM	Decreased variability of negative control
HEK + VMAT2 cell line	Increased fluorescence of positive control
90% confluence at testing	Increased fluorescence of positive control Decreased variability of positive control
Black plate plastic	Increased fluorescence of positive control Decreased fluorescence of negative control
Hand coating with PDL	Increased fluorescence of positive control Decreased variability of positive control Cost savings
Pre-coating PDL in batches of 8	Experimental efficiency
PDL storage aliquots	Experimental efficiency
Reaction volume of 100 µL	Cost savings
Eppendorf Research Plus multichannel pipettes	Decreased variability of positive control
50 mL trough for cell dilution	Decreased variability of positive control
10x trituration with 10 mL stripette	Decreased variability of positive control
3x trituration with multichannel	Decreased variability of positive control
Experimental media	Increased fluorescence of positive control Cost savings
Serum starvation only during experiment	Increased fluorescence of positive control
200 µM tetrabenazine stock	Experimental efficiency
10 mM FFN206 stock	Experimental efficiency
20 µL FFN206 working stock	Experimental efficiency
FFN206 single use aliquots	Decreased variability of positive control
FFN206 incubation for 75 minutes	Increased fluorescence of positive control
FFN206 incubation at 37°C and 5% CO_2	Physiological relevance
Wash volume	Experimental efficiency
Wash time	Experimental efficiency
Plate reader brand	Experimental efficiency
Plate reading emission and excitation filters	Equipment limitation

Figure Legends

FIGURE 1: FFN206 Pilot Data Initial proof of principle data for FFN 206 feasibility, bars indicate Standard Error of the Mean (SEM). FFFN 206 specific fluorescence can be detected, and is tetrabenazine (TBZ) sensitive. FFN 206 fluorescence is significantly different than FFN 206 fluorescence suppressed by TBZ. ($p < 0.01$). No significant difference between vehicle control and TBZ suppressed fluorescence. Z ' = 0.278 (not suitable for high through put screening).

FIGURE 2: FFN 206 Dose Response. FFN 206 fluorescence is dose-dependent. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 3: TBZ Dose Response. TBZ suppression of FFN 206 fluorescence is dose-dependent. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 4: Selection of TBZ dose. No significant different was found across a narrower range of TBZ doses. 10 uM was selected as starting dose for TBZ suppression. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 5: FFN 206 Dose Selection. Broad range FFN 206 concentration was tested against selected concentration of 10 uM TBZ. For doses as high as 1 uM, FFN 206 is exquisitely TBZ sensitive. A midlevel dose of FFN 206 is chosen (below 10 uM) to ensure the assay has the dynamic range necessary to detect increases in VMAT functions. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 6: FFN 206 and TBZ dose interaction. A narrow range of optimized FFN 206 concentrations was tested against a narrow range of optimized TBZ concentrations.

Lower variability observed in lower doses of both FFN 206 and TBZ improves z factor analysis. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 7: FFN 206 and TBZ dose interaction. A narrower range of optimized FFN 206 concentrations was tested against 10 uM TBZ. 1 uM FFN 206 was selected based on z factor analysis and cost management. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 8: Cell Line Comparison. Protocol was conducted on multiple cell lines. FFN 206 fluorescence was found to be VMAT specific and not DAT dependent for cell entry. Although putative drugs targeting VMAT may be DAT dependent for cell entry, further experiments were conducted in VMAT only cell line due to DAT/VMAT z factor below 0, therefore unsuitable for high through-put use. Lower fluorescence in this cell line is likely due to intrinsic factors of cell line and lower transfection efficiency for the double construct. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 9: Cell Density. To determine if fluorescence scaled with cell density, cells were plated at multiple dilutions 24 hours before the assay. Fluorescence was found to scale with cell density. Cells at the highest density showed greater variability due to visible patches where cells lifted off the plates during multiple media changes and wash steps. Cells at ~90% were found to be best suited for assaying. Time between plating and assay was found to be far more flexible than final density at the time of assay, so when cells were not at 85-90% at the time of scheduled experiment, the experiment was rescheduled, rather than assaying sparse plates. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 10: VMAT Dose Response. As another proxy to determine if FFN206 fluorescence scales with VMAT function, varying amounts of VMAT2 construct were transfected, beginning with the optimized transfection protocol, and decreasing by a factor of 2. As expected, FFN206 fluorescence scales with VMAT copy number. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 11: Plate type. Clear walled plates were compared to black walled plates (both with clear bottoms). Data shown were normalized to water blanks. Black walls prevent overflow fluorescence between wells and increase the difference in fluorescence count between positive and negative controls. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 12: PDL coating. Protocol was conducted using plates purchased pre-coated with PDL and compared to coating plates in house with PDL as described in methods. Data shown is DMSO subtracted, to account for the difference in autofluorescence of the plate plastics. Hand coating was found to be both superior and more cost effective. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 13: Well Position Within Plate. Initial pilot experiments had shown a trend for higher reads on the left of the plate, and the inner wells of the plate. Cell plating technique was adjusted to ensure even pipetting into each well, with trituration to prevent cell settling; new multichannel pipettes were purchases to ensure even performance for each tip; and plate reader reading dimensions were adjusted to ensure no overlap with well reads until no significant difference was found between wells in different plate locations. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 14: Reaction Volume Reduction. Protocol was conducted in original volume and half volume to determine if smaller wells/ fewer reagents could be used without affecting the z factor. No significant difference was found between reaction volumes ($p = 0.63$). Protocol was then used with 100 µL total reaction volume. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 15: Media. Protocol was tested with various media to determine which was most conducive for assay. Media varied by color due to the presence of phenol red (indicated by darker bar color), by the presence of FBS (indicated by hatching), and the presence of zeocin as selection antibiotic (indicated by cross hatching). eDMEM, as described in the methods section, was found to be the best choice for the assay. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 16: Serum Starvation. To determine if the improved fluorescence in media without FBS was due to interference from FBS during the assay or cell function changes from short-term serum starvation, the protocol was conducted in full serum media, and with prior serum starvation of 0, 6, or 24 hours then assayed in serum-free experimental media. Results show that although removing FBS from the media improves assay results, additional serum starvation diminishes results, likely due to decreased cell proliferation or decreased packaging of FFN into vesicle compartments. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 17: FFN Time. To determine the ideal incubation time for FFN206, a time course was conducted. Fluorescence peaked at an incubation time of 75 minutes, which was selected for the optimized protocol. The relative stability of the data shows that

while 75 minutes is ideal, for less sensitive tasks, 60-90 minutes may be chosen as a suitable incubation time. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 18: FFN Incubation Temperature. Incubation at 37 ^{o}C was compared to room temperature. Although 37 ^{o}C is preferred for its smaller variability and physiological relevance, no significant difference was found between incubation temperatures, indicating that the assay will be robust to possible variations in temperature during plate handing in high throughput use. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 19: Wash Time. To determine the stability of fluorescence, a time course of wash time (time between washing to remove fluorophore and reading the plate in the plate reader) was conducted. No significant degradation was observed out to 2 hours. Although plates were read immediately after washing when trials were conducted by hand, the stability of the fluorophore makes this assay well-suited for high throughput screens where delays may be introduced by the logistics of handling large numbers of plates. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 20: Plate Reader Comparison. To investigate the robustness and reproducibility of the protocol, plates were read on three different plate reader brands, in three different laboratories. Once normalized by range, no significant difference was found between results from different plate readers, showing the consistency of this protocol. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 21: Optimized Protocol. After optimizing the protocol, the optimized z' was consistently > 0.9. Shown here, z = 0.947. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 22: Optimized TBZ dose response curve. The optimized assay was used to create dose response curves (TBZ shown here as an example) to demonstrate the utility of the assay for evaluating the effect of pharmacological compounds on VMAT activity. Fluorescence is shown here as percentage of uninhibited VMAT activity. Error bars indicate Standard Error of the Mean (SEM).

FIGURE 23: Pharmacological VMAT Inhibitors. The optimized assay was used to test a variety of pharmacological VMAT inhibitors to demonstrate the utility of the assay for evaluating the effect of pharmacological compounds on VMAT activity. Relevant dosages of four known VMAT inhibitors are shown here as percentage of uninhibited VMAT activity (marked with dashed line). Error bars indicate Standard Error of the Mean (SEM).

FIGURE 24: Environmental Toxicants. The optimized assay was used to test a variety of environmental toxicants of interest due to association with Parkinson's disease risk. This demonstrates the utility of the assay for evaluating the effect of environmental toxicants on VMAT activity. Relevant dosages of four environmental toxicants are shown here as percentage of uninhibited VMAT activity (marked with dashed line). Error bars indicate Standard Error of the Mean (SEM).

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