MECHANISMS OF VESICULAR MONOAMINE TRANSPORTER-2 DEGRADATION

by

Erin Kathleen Stachowski

B.A., University of Rochester, 2006

Submitted to the Graduate Faculty of

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Erin Kathleen Stachowski

It was defended on

November 7, 2016

and approved by

Edda Thiels, PhD, Associate Professor, Dept. Neurobiology

Alexander Sorkin, PhD, Professor, Dept. Cell Biology

Jeffrey Brodsky, PhD, Professor, Dept. Biological Sciences

Teresa Hastings, PhD, Associate Professor, Dept. Neurology

Habibeh Khoshbouei, PharmD, PhD, Associate Professor, Univ. Florida, Dept. Neuroscience

Dissertation Advisor: Gonzalo Torres, PhD, Associate Professor, Univ. Florida, Dept. Pharmacology and Therapeutics

MOLECULAR MECHANISMS OF VESICULAR MONOAMINE TRANSPORTER-2 DEGRADATION

Erin Kathleen Stachowski, PhD

University of Pittsburgh, 2016

The vesicular monoamine transporter-2 (VMAT2) packages monoamines into synaptic vesicles in the central nervous system. Not only vital for monoaminergic neurotransmission, VMAT2 protects neurons from cytosolic dopamine-related toxicity by sequestering dopamine into vesicles. This dissertation research is focused on determining the basic mechanisms of VMAT2 degradation—an unexplored aspect of VMAT2 regulation. The processes of protein synthesis and degradation balance to maintain proteostasis. As VMAT2 availability and function directly impact monoamine neurotransmission, its degradation is an important aspect of VMAT2 maintenance to study. While it has been proposed that VMAT2 is degraded by the lysosome, an acidic membrane-bound organelle, there is no direct evidence demonstrating this. In a PC12 cell model system stably expressing VMAT2-GFP, pharmacological tools were used to determine the impact of inhibiting pieces of cellular degradation machinery (the lysosome or the 26S proteasome) on VMAT2. Both mature and immature forms of VMAT2 accumulated following inhibition of the proteasome, but not the lysosome. In addition, at least a portion of the accumulated VMAT2 following proteasomal inhibition appears to be K48-linked polyubiquitinated—a post-translational modification associated with proteasomal degradation. Immature VMAT2 also accumulated following inhibition of endoplasmic reticulum-associated degradation (ERAD), a proteasome-dependent quality control mechanism for misfolded or damaged proteins undergoing processing in the ER. Demonstrated with immunocytochemistry

and confocal imaging, the proportion of VMAT2 overlapping with an ER marker increased following proteasomal inhibition. These results indicate that immature VMAT2 is likely subject to ERAD as it undergoes processing in the ER. Unexpectedly, mature VMAT2 accumulated following proteasomal, but not lysosomal inhibition. While this result could be due to secondary effects of proteasomal inhibition or changes in VMAT2 synthesis, it is also possible that mature VMAT2 can be degraded by the proteasome. This would be a unique finding, as glycosylated, transmembrane proteins, such as VMAT2, must overcome a large energy barrier for retrotranslocation from a membrane. As these experiments were done under basal conditions, the results don't exclude a role for lysosomal degradation under other circumstances. For example, there may be multiple pathways of VMAT2 degradation: proteasomal-dependent under basal conditions and lysosomal-dependent under conditions of synaptic activity.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

I would like to extend my deepest gratitude toward all members of my dissertation committee and my thesis advisor, Dr. Gonzalo Torres. In addition to being experts in their respective fields, everyone was helpful and genuine. I would especially like to thank Dr. Hastings and Dr. Thiels. Without them, the last year and a half of my dissertation work would have been far more difficult than it was.

I would also like to thank friends and family for their unwavering support. The road was made much smoother by them. I would like to especially thank Jocelyn Mauna and Tracy Baust; both gave me lab homes when I needed them.

1.0 INTRODUCTION

The goal of this project was to identify the basic mechanisms of vesicular monoamine transporter-2 degradation (VMAT2), a protein that has a vital role in monoaminergic signaling. Despite this importance, little is known regarding the regulation of VMAT2. Its degradation was chosen to investigate because the regulation of VMAT2 expression levels is crucial to normal dopamine transmission and disease states involving alterations in the dopamine system. In addition, to date, nothing has been published concerning this topic—an important part of proteostasis. Research regarding VMAT2 degradation not only provides information about VMAT2 regulation, but also may provide insight into the degradation of synaptic vesicular proteins in general. This dissertation project was broken into two specific aims: (1) to investigate what cellular machinery is involved in VMAT2 degradation and (2) to investigate the role of parkin, an E3 ligase, in VMAT2 degradation.

1.1 MONOAMINERGIC SIGNALING

Monoamine neurotransmitters are involved in a multitude of functions within the central nervous system. Classified because of their structure, the group includes neurotransmitters such as histamine, serotonin, epinephrine, norepinephrine and dopamine. These compounds are considered neuromodulators, sometimes modulating the direct effects or release of other

neurotransmitters (Pennartz et al., 1992, Harvey and Lacey, 1997, Guzman et al., 2003, Tecuapetla et al., 2009, Tritsch and Sabatini, 2012). As opposed to the classical neurotransmitters such as glutamate and GABA, neuromodulators act on metabotropic receptors, sometimes located extrasynaptically (Sesack et al., 1994, Yung et al., 1995, Beaulieu and Gainetdinov, 2011). The release of neuromodulators can be phasic (display firing bursts, driven by action potentials), like release of classic neurotransmitters (Grace and Bunney, 1984a). In addition, release, especially of dopamine, can also be tonic, meaning small amounts can be released independent of action potential in a slow, irregular pattern (Grace and Bunney, 1984b, Floresco et al., 2003).

While relatively small groups of neurons in the central nervous system synthesize different monoamines, they have wide-reaching processes that impact a number of processes throughout the brain. For example, the raphe nuclei are a cluster of nuclei in the midbrain that synthesize serotonin (Törk, 1990). Modestly sized, these nuclei have projections throughout the brain, impacting cognition, circadian rhythms, memory, and emotion—to name just a few (Puig and Gulledge, 2011, Versteeg et al., 2015, Zhang and Stackman, 2015, Bocchio et al., 2016). As a whole, monoamines influence almost every aspect of brain function in some way. Although there are several monoamines in the central nervous system, each with their own circuitry, this thesis work will be focusing on dopamine.

1.1.1 The dopamine system and molecular components

One of the most studied monoamines is dopamine (DA). The dopaminergic system is involved in a variety of systems such as movement, reward circuitry, and cognition. Dysfunction within the dopaminergic system is implicated in several diseases and disorders, including schizophrenia,

attention-deficit/hyperactivity disorder, Parkinson's disease, and addiction. Despite the involvement of DA in several circuits and systems, there are only a few locations in the brain that contain dopaminergic cell bodies. The most understood of these areas are located in the midbrain—the substantia nigra (SN) and ventral tegmental area (VTA). Neurons from these areas project to many areas of the brain, but here I will focus on a few main pathways and their primary roles. Dopaminergic neurons in the SN pars compacta project to the dorsal striatum to form the nigrostriatal pathway, which is primarily involved in control of movement (Haber et al., 2000). This group of neurons is notorious for their degeneration in Parkinson's disease, resulting in the motor symptoms typical of the disease. Dopaminergic neurons in the VTA, also located in the midbrain, project to a number of brain areas, including the amygdala and hippocampus. A group of neurons project from the VTA to the cortex (primarily the prefrontal cortex), forming the mesocortical pathway, which seems to play a role in cognition and motivation (Ungerstedt, 1971, Haber, 2014). Another group of neurons project from the VTA to the nucleus accumbens (Groenewegen et al., 1999). This pathway is heavily involved in the reward circuitry, and its role in the biological basis of addiction is often studied (Salamone, 1994, Sutton and Beninger, 1999, Baldo and Kelley, 2007). The roles these circuits play in brain functions are simplified here; more likely each circuit is involved in several functions and there is not a clear division of function between them (Verheij and Cools, 2008, Wise, 2009).

There are several molecular components/proteins that are essential for dopaminergic homeostasis. In addition to DA receptors, other components are involved in the synthesis, packaging, release, re-uptake, and degradation of DA. The synthesis of DA is regulated mainly by tyrosine hydroxylase (TH)—the rate-limiting step in the conversion of tyrosine to L-DOPA (Nagatsu et al., 1964a, b). Aromatic L-amino acid decarboxylase (AADC) then converts L-

DOPA into DA (Lovenberg et al., 1962). If the necessary enzymes are located in the neuron, DA can then be used to synthesize epinephrine and norepinephrine—previously mentioned monoaminergic neurotransmitters (Levin et al., 1960, Connett and Kirshner, 1970). DA is then packaged into vesicles for exocytosis by the vesicular monoamine transporter (VMAT). Following release, DA is taken back up into the presynaptic neurons via the dopamine transporter (DAT) located at the plasma membrane. DA can then be degraded (or re-packaged into vesicles) by catechol-O-methyl transferase (COMT) or monoamine oxidase (MAO) (Lachman et al., 1996, Edmondson et al., 2004). Dopamine receptors are metabotropic and can be divided into two families—D1-like (D1 and D5) or D2-like (D2, D3, D4) receptors. While all DA receptors are G-protein coupled, the ultimate result of being 'excitatory' or 'inhibitory' to the post-synaptic neuron depends on which receptor is activated (Stoof and Kebabian, 1981, Clark and White, 1987, Seeman and Van Tol, 1994, Beaulieu and Gainetdinov, 2011). Additionally, some D2 receptors are located on the presynaptic neuron and function as autoreceptors, regulating DA release from that neuron (Usiello et al., 2000).

1.2 THE VESICULAR MONOAMINE TRANSPORTER-2

The vesicular monoamine transporter (VMAT) is responsible for transporting dopamine into vesicles, readying them for exocytosis. As the name suggests, VMATs transport not only DA, but all monoamines. Although the existence of a monoamine transporter in synaptic vesicles had been known for decades, the VMAT cDNA wasn't cloned until the early 1990's (Erickson et al., 1992, Liu et al., 1992, Surratt et al., 1993, Takahashi and Uhl, 1997). There emerged two isoforms of the transporter, previously identified as CGAT (chromaffin granule amine

transporter) and SVAT (synaptic vesicular amine transporter). CGAT would become known as VMAT1, the isoform encoded by the *SLC18A1* gene, while SVAT would become known as VMAT2, encoded by the *SLC18A2* gene.

VMAT belongs to the MFS (Major Facilitator Superfamily) family of transporters and the SLC (solute carrier family of transporters) subfamily. The transporters included in this subfamily display little sequence homology with each other and are grouped based on function. The vesicular acetylcholine transporter (VAChT) shares the greatest sequence homology with VMAT, although the vesicular glutamate transporters (VGLUTs) also share some similarities—namely, basic structure and function. It was once thought that the roles of the vesicular transporters were very structured—VMATs transport monoamines, VGLUTs transport glutamate, and so on. However, there is some evidence that a small subset of dopaminergic neurons also co-release GABA in a VMAT-dependent manner, although this requires further confirmation (Tritsch et al., 2012). Glutamate is also released from a small subpopulation of dopaminergic neurons, but this is dependent on the VGLUT and not VMAT (Sulzer et al., 1998, Tecuapetla et al., 2010).

No crystallized structure of VMAT exists, although based on hydrophobicity plots, it is predicted to have 12 transmembrane domains. Both VMAT1 and VMAT2 have cytoplasmic N and C-termini and a large loop facing the interior of the vesicle. This large loop contains 3-4 residues capable of N-glycosylation. Figure 1 is the sequence and predicted transmembrane domains of human VMAT2, indicating putative N-glycosylation sites (adapted from various sources, namely Robert Edwards' group) (Erickson et al., 1992, Peter et al., 1996, Takahashi and Uhl, 1997, Hoffman et al., 1998). In addition to any role in folding or structural stability, these glycosylation sites are thought to be involved in sorting or trafficking (discussed in section

1.2.1), although knockdown of all VMAT1 glycosylation sites reduced vesicular uptake by 50% without altering gross cellular localization (Yelin et al., 1998, Yao and Hersh, 2007).

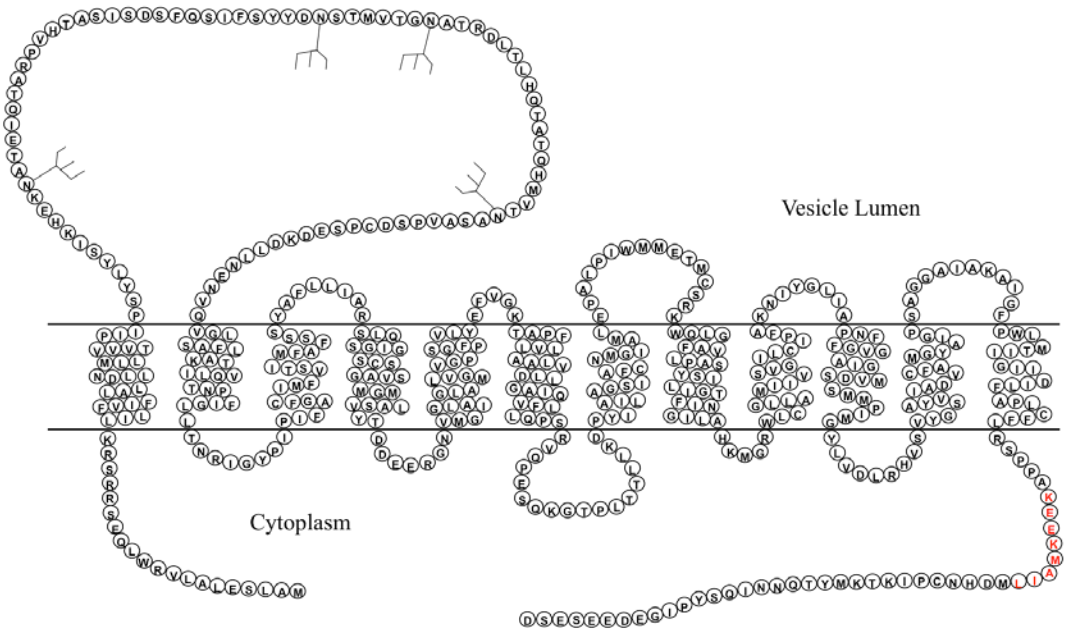


Figure 1. Human VMAT2 sequence and putative transmembrane domains. The amino acid human VMAT2 sequence from the N-terminus starting at the left. Putative N-glycosylation sites are represented by branches in the large, intravesicular loop. A sequence contained in the C-terminus that's involved in VMAT2 endocytosis, KEEKMAIL, is indicated in red.

1.2.1 Expression and localization

While the underlying function and protein structure of VMAT1 and VMAT2 are similar, there exist differences between these two transporter molecules. One of these differences is expression—VMAT1 is only located in the periphery, while VMAT2 exists in both the periphery and central nervous system. According to immunohistochemical studies performed with human tissue by Erickson et al. 1996, VMAT1 is primarily localized to chromaffin cells in the adrenal medulla and enterochromaffin cells of the small and large intestines. In the periphery, VMAT2

is also expressed in chromaffin cells in the adrenal medulla—the only location where VMAT1 and VMAT2 coexist. VMAT2 has been identified in enterochromaffin-like cells in the stomach and endocrine pancreatic cells (Erickson et al., 1996). Similar patterns of system-wide localization are seen in rats, although VMAT1 is more predominately expressed in chromaffin cells of the adrenal medulla; a smaller subset of these cells also contain VMAT2 (Peter et al., 1995). VMAT2 is expressed in both rat and human peripheral sympathetic ganglia. In contrast, VMAT1 is expressed in some human, but not rat, peripheral sympathetic ganglia.

VMAT2, but not VMAT1 is expressed in the central nervous system. VMAT2 can essentially be found in all brain regions that contain monoaminergic cell bodies or terminals. Nirenberg and colleagues used electron microscopic immunocytochemistry to view cellular localization of VMAT2 in the rat SN and VTA. They found VMAT2 was largely localized to tubulovesicular structures in the cell body, but also to the Golgi apparatus and endoplasmic reticulum. These tubulovesicular organelles are a proposed site of somatodendritic dopaminergic release (Hattori et al., 1979, Nirenberg et al., 1996). VMAT2 was rarely observed at the plasma membrane, in either cell bodies or axons (Nirenberg et al., 1996). Although frequently located on synaptic vesicles (SVs), VMAT2 is also found on dense core vesicles (DCVs). DCVs are distinct from SVs and typically contain peptides or hormones, instead of the classic neurotransmitters in SVs (like glutamate or GABA). Furthermore, DCVs and SVs have different biogenesis pathways and exocytosis mechanisms (Kelly, 1993).

Studies of VMAT2 trafficking to DCVs or SVs have largely been performed in neuroendocrine cell lines or PC12 cells, as they contain a larger proportion of DCVs (also called large dense core vesicles, LDCVs, or large dense core granules). In these cells, VMAT is preferentially localized to LDCVs instead of the smaller vesicles analogous to the SVs in the

brain (Liu et al., 1994). Both glycosylation and residues in the C-terminal have been implicated in trafficking to LDCVs (Varoqui and Erickson, 1998, Krantz et al., 2000, Li et al., 2005, Yao and Hersh, 2007). Specifically, phosphorylation of acidic residues located at the C terminus interferes with VMAT2 localization to LDCVs (Waites et al., 2001). It is unclear if these processes are retained in central nervous system neurons, where a much larger proportion of SVs exist.

1.2.2 Pharmacological properties

The primary function of the VMATs is to sequester monoamines into vesicles for exocytosis. VMAT does this by taking advantage of a proton gradient maintained by V-type ATPases. VMAT2 relies on this proton gradient and membrane potential to fully function. It is generally accepted that VMATs transport two protons out of the vesicle for every one monoamine molecule into the vesicle (Knoth et al., 1981). Recently, a "hinge" model has been proposed as a mechanism of transport. Yaffe and colleagues suggest that two 6 transmembrane helices contain several critical residues that act as "hinge points" to open and close the two helices, allowing for translocation of substrates (Yaffe et al., 2013).

While this basic mechanism is assumed to be true for both VMAT1 and VMAT2, the two forms differ somewhat in substrate affinity. The successful isolation and cloning of VMAT cDNAs has allowed for direct testing of VMAT1 and VMAT2 pharmacological properties. VMAT1 and VMAT2 have similar affinities for serotonin, dopamine, epinephrine, and norepinephrine, although VMAT2 has 2-4 times greater affinity for all of these substrates than VMAT1 (Peter et al., 1994, Erickson et al., 1996). VMAT2 displays a much higher affinity for histamine than VMAT1 (K_i in the mid to high micromolar range), suggesting histamine is

unlikely to be a typical substrate of VMAT1 (Peter et al., 1994, Merickel and Edwards, 1995, Erickson et al., 1996). Interestingly, there exists only approximately 60% homology between the human VMAT1 and human VMAT2 amino acid sequences, perhaps accounting for these differences in substrate affinity (Erickson et al., 1996, Wang et al., 1997). As shown in Figure 2, the transmembrane domains are the most conserved, while there exists sequence divergence in the N-terminus, the large intravesicular loop, and the C-terminus. In contrast, the VMAT2 amino acid sequence appears to be fairly well-conserved across mammalian species. As seen in Figure 3, there exists approximately 90% homology between rat, mouse, and human VMAT2, suggesting VMAT2 maintains similar pharmacological features across mammalian species.

Several inhibitors of the VMATs have been identified. Perhaps the most well studied inhibitor of VMAT1 and VMAT2 is reserpine. Selective and potent, reserpine is an irreversible, competitive inhibitor with a K_D in the low nanomolar range (Scherman and Henry, 1984, Erickson et al., 1996). Interestingly, reserpine and histamine poorly displace one another, suggesting the substrate binding site for histamine differs (at least partially) from other monoamine substrates (Merickel and Edwards, 1995). Another compound that inhibits VMAT is lobeline, which is a nonspecific, noncompetitive inhibitor with an IC_{50} in the low micromolar range (Teng et al., 1997, Teng et al., 1998). Finally, tetrabenazine (TBZ) is a reversible inhibitor of VMAT2, but not VMAT1. While VMAT2 has an IC_{50} for TBZ in the mid nanomolar range, concentrations up to 10 μM don't fully block VMAT1 activity (Pettibone et al., 1984, Peter et al., 1994, Erickson et al., 1996). Radiolabeled TBZ binding studies have been used to assess VMAT2 levels *in vitro*, as well as *in vivo* with PET (positron emission tomography), as TBZ is a relatively short-lived, noncompetitive inhibitor (Scherman and Henry, 1984, DaSilva and Kilbourn, 1992, DaSilva et al., 1994, Vander Borght et al., 1995).

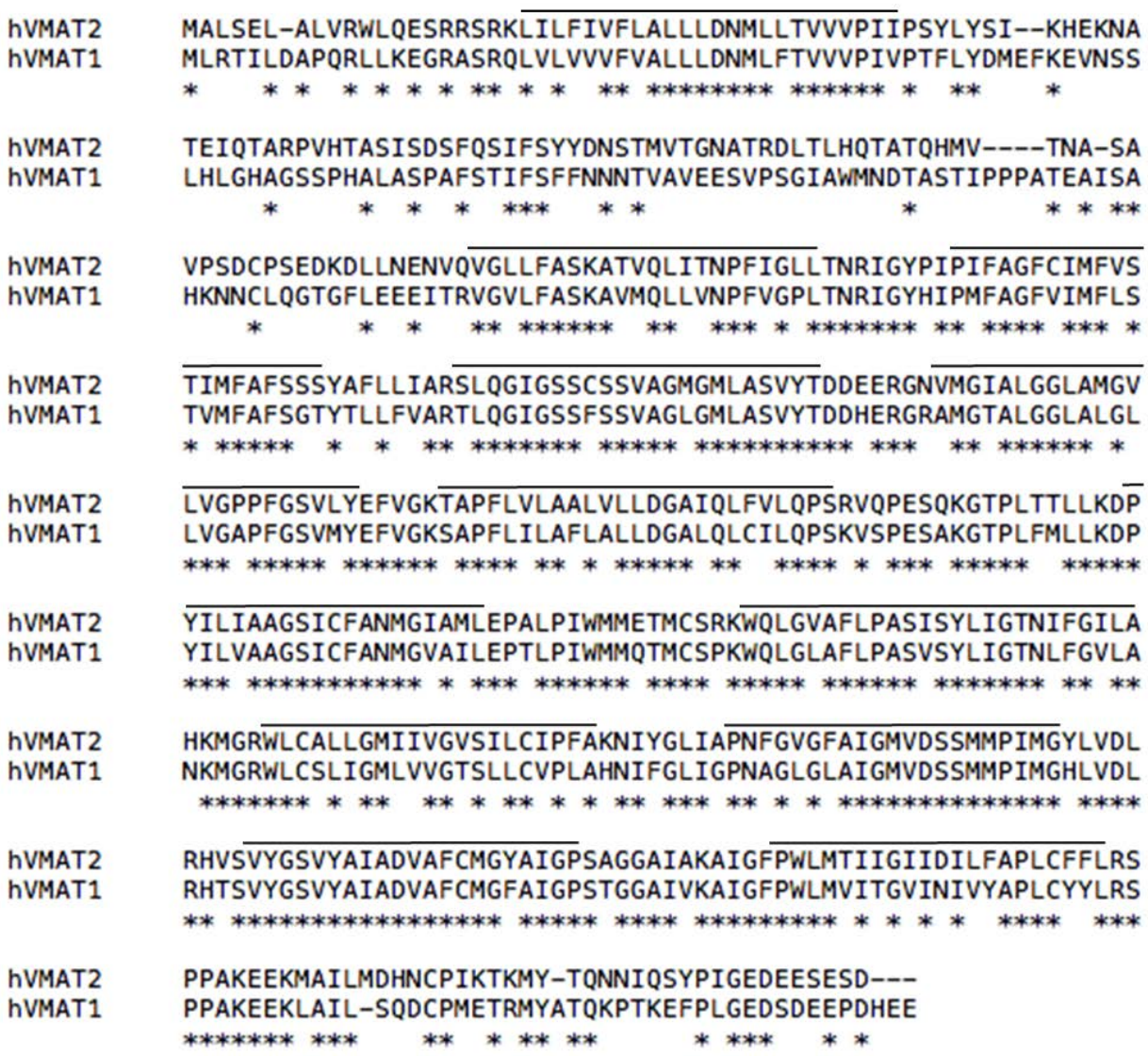


Figure 2. Sequence alignment of human VMAT2 and VMAT1. Predicted transmembrane domains are indicated by a line above hVMAT2. Asterisks located below hVMAT1 indicate residue homology. Note the greatest sequence divergence in the N- and C-termini, as well as the large intravesicular loop following the first transmembrane domain.

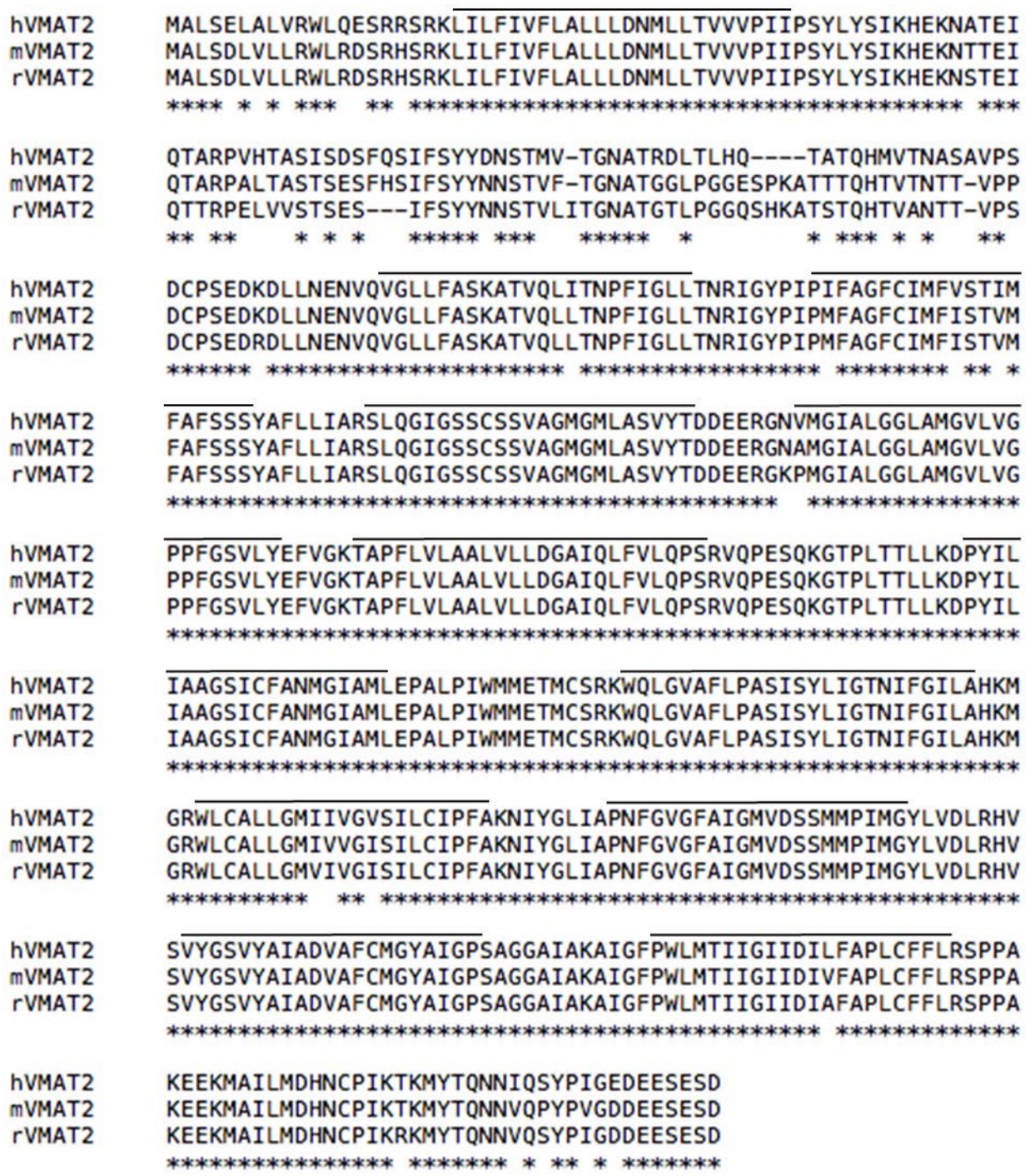


Figure 3. Sequence alignment of human, mouse, and rat VMAT2. Predicted transmembrane domains are indicated by a line above hVMAT2. Asterisks located below rVMAT2 indicate residue homology.

1.2.3 Role in determining quantal size

There's significant evidence indicating VMAT levels have the ability to confer quantal size, or the amount of monoamine packaged into a vesicle and released during a quantal event. While the number of VMATs within a vesicle remains somewhat unknown, there's indication that this number is a factor in determining how much monoamine is within a vesicle. A number of studies have been done in David Sulzer's lab utilizing PC12 cells, a rat pheochromocytoma cell line that contain vesicles and are capable of monoaminergic release. They demonstrate with amperometry that manipulation of VMAT2 levels alters the quantal size without altering the number of vesicles undergoing exocytosis (Pothos et al., 2000). A separate group obtained similar results, finding that L-DOPA (the dopamine precursor) and reserpine enhanced and diminished, respectively, vesicular volume and quantal size (Colliver et al., 2000).

Further evidence (albeit more indirect) that VMAT2 regulates quantal size is obtained from VMAT2 knockdown and over-expression animal models. Homozygous VMAT2 knockout mice do not have long-term viability and perish within a few days of birth (Takahashi et al., 1997, Wang et al., 1997). Heterozygous knockout mice have 50% of wild-type VMAT2 levels with a corresponding decrease in striatal DA and evoked DA release in primary neuronal cultures, indicating released DA is proportional to VMAT2 levels (Fon et al., 1997, Takahashi et al., 1997). A genetic line of mice expressing only about 5% VMAT2 have a similar 90-95% reduction in brain monoamine levels, including striatal DA (Colebrooke et al., 2006, Taylor et al., 2009). Recently, Gary Miller's group has begun to characterize a mouse model of VMAT2 over-expression. These mice express about 3 times more VMAT2 than wild-type mice and have increased striatal DA content. VMAT2 over-expression mice also have greater evoked DA release in striatal slices and interestingly, increased vesicle size (as measured using electron

microscopy) (Lohr et al., 2014). Together, these data strongly support the notion that VMAT2 levels can impact quantal size *in vivo*. Despite this data, it remains unclear what compensatory mechanisms take place when VMAT2 is genetically manipulated. There is some indication that post-synaptic dopamine receptors in heterozygous knockout mice are more sensitive than in wild-type mice, as heterozygous knockout mice have enhanced responses to apomorphine, a DA receptor agonist (Wang et al., 1997).

1.2.4 Role in neuroprotection

The primary role of VMAT is to transport monoamines into vesicles. Because some monoamines (notably, dopamine) can be harmful to the cell when remaining in the cytosol, VMAT also plays a role in neuroprotection. Free DA has the ability to auto-oxidize, forming DA quinones and reactive oxygen species (Graham, 1978). The metabolism of DA by monoamine oxidase (MAO) also creates a potentially harmful substance, H_2O_2, which can then form hydroxyl radicals. These products of DA metabolism can contribute to the overall level of oxidative stress within a neuron, making it more vulnerable to insults and subsequent neurotoxicity (Hastings et al., 1996, Linert et al., 1996, LaVoie and Hastings, 1999, Mosharov et al., 2009, Segura-Aguilar et al., 2014). Because of this, it is especially important to sequester DA into vesicles, where it won't be metabolized by MAO or auto-oxidized. In fact, there's evidence that TH and VMAT2 directly interact and are coupled with AADC (Cartier et al., 2010). In this way, levels of cytoplasmic DA are limited following synthesis. High levels of cytoplasmic DA are thought to play a role in neuronal cell death seen in Parkinson's disease, in addition to neurotoxicity induced by some psychostimulants (Cadet and Brannock, 1998, Sulzer and Zecca, 2000, Goldstein et al., 2014).

There are several lines of evidence indicating that VMAT2 over-expression or knockdown/inhibition affects vulnerability to toxic insult. For example, an *in vitro* study has demonstrated that over-expression of VMAT2 in PC12 cells protected against L-DOPA-induced toxicity, while reserpine treatment potentiated L-DOPA toxicity (Weingarten and Zhou, 2001). Presumably these effects are due to more or less VMAT2 being available to sequester the increased DA levels caused by L-DOPA treatment. Similarly, primary dopaminergic neurons derived from VMAT2 heterozygous knockout mice are also more vulnerable to L-DOPA toxicity (Kariya et al., 2005b). However, another group didn't observe the same, perhaps due to use of a different L-DOPA treatment paradigm (Reveron et al., 2002). VMAT2 inhibition has also been shown to increase vulnerability to BH4-induced toxicity (Choi et al., 2005). BH4 is a cofactor for tyrosine hydroxylase that can contribute to oxidative stress by undergoing auto-oxidation and generating toxic by-products that then increase formation of DA quinones. (Choi et al., 2000, Choi et al., 2003). It's proposed that an overall reduction in VMAT2 DA storage capability then potentiates an increase of BH4-mediated DA quinone production.

There are additional lines of evidence from *in vivo* studies that VMAT2 levels can affect oxidative stress-related toxicity. VMAT2 heterozygous knockout mice are more susceptible to dopaminergic cell death in paradigms of MPP^+/MPTP neurotoxicity—a pharmacological model of Parkinson's disease (Takahashi et al., 1997, Gainetdinov et al., 1998). Likewise, mice expressing lower VMAT2 levels also exhibit greater toxicity induced by high doses of methamphetamine (Fumagalli et al., 1999, Larsen et al., 2002, Guillot et al., 2008). Mice over-expressing VMAT2 are protected against terminal and cell loss due to neurotoxic doses of MPP^+ and methamphetamine, supporting this hypothesis (Lohr et al., 2014, Lohr et al., 2015).

In work done in *Drosophila* by David Krantz's group, loss of function VMAT mutants are more susceptible to rotenone and paraquat-induced neurotoxicity (pesticide models of Parkinson's disease). VMAT over-expression was partially protective against dopaminergic cell loss induced by rotenone, but not paraquat. The different results obtained between the two compounds are not understood, although it may be due to differences in mechanisms of action (Lawal et al., 2010). In a different model of Parkinson's disease, expression of mutant parkin in *Drosophila* can cause motor dysfunction and dopaminergic cell death. The effects are exacerbated by VMAT knockdown and ameliorated by VMAT over-expression (Sang et al., 2007). Again, it is unclear why VMAT impacts mutant parkin-mediated dopaminergic cell loss, although it is presumed cytosolic DA plays a role.

The ability of VMAT2 to modulate vulnerability to toxic compounds, such as MPP+, rotenone, or methamphetamine, could be due to a number of mechanisms. It is possible that altered VMAT2 levels impact neuronal vulnerability because the aforementioned compounds (a) are substrates of VMAT2, and more/less VMAT2 sequesters them, preventing/exacerbating their toxic actions (b) are inhibitors of VMAT2 function, either directly or by interfering with vesicular pH gradient or (c) alter cytoplasmic DA without impacting VMAT2 directly, but VMAT2 levels then modulate free DA levels, which could then in turn affect oxidative stress. In fact, several of the aforementioned toxic compounds influence VMAT2 activity directly. MPP^+ is believed to re-distribute dopamine from vesicles to the cytoplasm, increasing free DA; it is hypothesized that MPP^+ may do so by acting as a VMAT substrate (Daniels and Reinhard, 1988, Lotharius and O'Malley, 2000). Methamphetamine, a psychostimulant, is thought to do the same. Several research groups, notably David Sulzer's, have proposed and provided evidence for the "weak base hypothesis"—methamphetamine and amphetamine act as weak bases that

disrupt the vesicular pH gradient and re-distribute DA from the vesicle to the cytoplasm, contributing to levels of cytosolic DA and ultimately, oxidative stress and terminal loss (Maron et al., 1983, Sulzer and Rayport, 1990, Cubells et al., 1994, Pifl et al., 1995, Sulzer et al., 1995, Mundorf et al., 1999, Freyberg et al., 2016). Finally, it has been suggested that one of rotenone's actions is as a VMAT2 inhibitor, also resulting in accumulation of cytosolic DA (Sai et al., 2008, Watabe and Nakaki, 2008). This would explain the differential effects VMAT2 over-expression has on rotenone and paraquat, as it is unclear if paraquat has a similar effect on VMAT2.

The contribution of VMAT2 to dopaminergic neuron vulnerability is such that some have proposed mice expressing very low (approximately 5% of wild-type) VMAT2 can be used as a model for Parkinson's disease (PD). Gary Miller's group has done extensive work with these mice, termed "VMAT2 LO" mice. VMAT2 LO mice display deficits that follow a degenerative pattern, sometimes not becoming apparent until later in life—12-18 months. These mice display deficits in motor function reminiscent of PD, accompanied by increased indications of oxidative stress and a reduction in striatal dopaminergic neurons (Caudle et al., 2007). They also develop earlier signs of non-motor PD symptoms. At around 5 months old, VMAT2 LO mice have deficits in olfactory discrimination tests. Even earlier, at around 2 months, they have abnormal gastric emptying and circadian activity (Taylor et al., 2009). Even though PD is rarely diagnosed prior to the display of motor symptoms, it is now thought that deficits in olfaction and digestion precede these symptoms, highlighting the potential usefulness of VMAT2 LO mice as an animal model of Parkinson's disease.

While these data don't provide direct evidence that VMAT2 has an inherent neuroprotective role by sequestering dopamine, the implication is very apparent. How VMAT2 modulates dopamine-induced oxidative stress is an ongoing area of research. Furthermore, much

of these data point to the importance of VMAT2 and implicate it in the etiology of diseases and disorders that affect monoamines, especially Parkinson's disease.

1.2.5 Involvement in diseases and disorders

Because of the critical role VMAT2 has in monoaminergic signaling, attention has been paid to its potential role in human diseases and disorders. Investigations have focused mainly on two approaches: genetic analysis and evaluation of VMAT2 levels, often involving *in vivo* ligand binding studies. Dysfunction of the DA and serotonin systems has been implicated in a wide range of disorders, ranging from the psychiatric to the neurodegenerative.

As already hinted at, there is interest in investigating the potential involvement of VMAT2 in PD etiology. The selective vulnerability of monoaminergic neurons to degeneration in this disease has implicated VMAT2—a protein common to all monoaminergic neurons. The contribution of cytosolic dopamine-mediated oxidative stress to this selectivity also implicates VMAT2. It has been suggested that the ratio of dopamine transporter to VMAT2 can impact cell vulnerability in PD—both proteins are key regulators of cytoplasmic dopamine levels (Miller et al., 1999, Gonzalez-Hernandez et al., 2004). For instance, the ratio of DAT to VMAT2 in the putamen is greater than in the caudate, two areas of the striatum. Indeed the putamen has greater cell loss in PD (Miller et al., 1999). Reduced striatal VMAT2 levels and SN mRNA levels have been reported in post-mortem brains of PD patients (Harrington et al., 1996, Miller et al., 1999). VMAT2 is expressed in platelets, and these mRNA levels are also reduced in PD patients, although not correlated with disease severity, age, or treatment status (Zucker et al., 2001b, Sala et al., 2010). Corroborating evidence is obtained from PET imaging using radiolabeled DTBZ (dihydrotetrabenazine). PD patients have a lower number of DTBZ binding sites in striatum and

SN, presumably due to reduced VMAT2 levels (Lee et al., 2000, Bohnen et al., 2006). Interestingly, VMAT2 levels (as assessed both post-mortem and *in vivo*) are reduced when accounting for dopaminergic terminal loss; there is less VMAT2 in the surviving neurons and terminals. Furthermore, isolated vesicles from post-mortem PD patients have decreased VMAT2 uptake, taking into account binding sites (or VMAT2 levels) (Pifl et al., 2014). This data suggests that the function of VMAT2 itself is impaired in PD—not just that there are reduced levels. Further evidence that VMAT2 may be associated with PD comes from the finding that VMAT2 immunoreactivity has been localized to α-synuclein positive Lewy bodies—protein aggregates that are a pathological feature of PD (Yamamoto et al., 2006).

Despite these findings, it is still unclear if VMAT2 is directly involved in the etiology or selective vulnerability of monoaminergic neurons in PD. It is quite possible that the changes seen in VMAT2 are secondary and these changes do not directly affect progression of the disease. In attempt to further differentiate between these possibilities, scientists have looked for genetic differences in PD patients. Results are varied; one research group found polymorphisms in the VMAT2 gene associated with increased risk for PD in an Italian population, while another group did not find the same in a Japanese population (Kariya et al., 2005a, Brighina et al., 2013). It is unclear if any of these polymorphisms have functional consequences; a study using numerous variants for *in vitro* vesicular uptake assays suggests not (Burman et al., 2004). One study found gain-of-function haplotypes that are protective for PD, but only in women (Glatt et al., 2006). The haplotypes found are expected to increase transcriptional activity of VMAT2, indicating increased VMAT2 levels may indeed confer protective qualities. Due to inconsistent results from these studies and small effects, the findings should be replicated before any

conclusions are drawn about genetic vulnerability to PD from VMAT2 mutations or polymorphisms.

While VMAT2 has been investigated in a variety of other disorders, more extensive research has been done in regards to depression, bipolar disorder, and schizophrenia—all of which have evidence of dysfunctional monoaminergic signaling. Monoaminergic, especially serotonergic, dysfunction has long thought to be involved in the etiology of major depressive disorder and bipolar disorder, so investigation into VMAT2 is a logical avenue to follow. Investigation of any potential abnormalities in VMAT2 was also encouraged by the observation that VMAT2 heterozygous knockout mice display signs of a depressive phenotype, which are reversed by administration of antidepressants (Fukui et al., 2007, Taylor et al., 2009). VMAT2 inhibitors also elicit depressive-like symptoms or behavior in both humans and rodents (Preskorn et al., 1984, Frank, 2010, Nunes et al., 2013, Randall et al., 2014). While there is an obvious lack of literature concerning VMAT2 levels (obtained either post-mortem or *via* radiolabeled DTBZ binding) in patients with major depressive disorder, there is evidence of increased VMAT2 DTBZ binding in patients with bipolar disorder, although the difference compared to controls was quite small, possibly due to a low number of subjects (Zubieta et al., 2000, Zubieta et al., 2001). Similar findings were also observed in patients with schizophrenia (Zubieta et al., 2001, Zucker et al., 2002). Although confirmation of these results is needed, VMAT2 haplotypes that occur with increased frequency in patients with schizophrenia and bipolar disorder have been identified (Gutierrez et al., 2007).

It should be noted that genetic or functional changes in VMAT2 alone are unlikely to contribute to the entirety of these disorders, as they can be considered spectrum disorders. That is, the presentation of symptoms vary greatly between individuals and etiology is unlikely to be

one singular cause. Furthermore, there is *in vitro* evidence that selective serotonin reuptake inhibitors (common treatments for major depressive disorder) and *in vivo* evidence that lithium (a common bipolar disorder treatment) affect VMAT2 function (Zucker et al., 2001a, Yasumoto et al., 2009). Treatment status should be taken into consideration when assessing data from human studies, although a low subject number often proves this difficult.

To date, there has only been one VMAT2 mutation definitively linked to a disorder. In 2013, Rilstone and co-authors described a case study of a Saudi Arabian family who displayed Parkinson's disease-like symptoms and global developmental delays. Affected family members had a homozygous mutation corresponding to a proline to leucine mutation in the VMAT2 387 amino acid position. This mutation, located by a transmembrane segment, is thought to have severe structural consequences. Expression of the mutated VMAT2 in cells resulted in greatly reduced VMAT2 uptake activity, about 7% of wild-type. Thought to have autosomal recessive inheritance, this is the only identified VMAT2 mutation in the human population to directly cause major dysfunction (Rilstone et al., 2013).

1.2.6 Regulation

Despite the importance of VMAT2—its critical role in neurotransmission as well as its potential involvement in a wide range of diseases—relatively little is know about its regulation on a molecular level. For instance, few studies have examined the role of post-translational modifications in VMAT2 activity. While residues in the VMAT2 C-terminus have been demonstrated to be constitutively phosphorylated in heterologous cell models, the purpose of these modifications are unclear, as they don't seem to alter baseline uptake activity (Krantz et al., 1997). In another study, phospho-mimetic replacement of two N-terminus residues reduced

uptake activity, although it is unclear if phosphorylation at these sites occurs *in vivo* (Torres and Ruoho, 2014).

There has also been some investigation into protein-protein interactions that regulate VMAT2 activity. Unsurprisingly, several of these efforts have focused on proteins that play a role in the etiology of PD. Mutations in α-synuclein cause familial PD and aggregated α-synuclein is found in Lewy Bodies, a pathological marker of PD. α-Synuclein seems to play a role in vesicular docking prior to fusion and exocytosis, but may also directly impact VMAT (Larsen et al., 2006). *In vitro*, over-expression of wild-type and mutant α-synuclein reduced VMAT2 expression and uptake activity (Lotharius et al., 2002, Guo et al., 2008). While these results implicate α-synuclein in regulating VMAT2 levels, the exact mechanisms of this are unknown. Furthermore, it is unclear if mutant α-synuclein expressed in PD greatly alters VMAT2 function, as both over-expression of mutant and wild-type α-synuclein had essentially the same impact on VMAT2; alternatively, mutant α-synuclein could be a gain-of-function mutation. Another protein implicated in PD, DJ-1, has been shown to modulate VMAT2 activity. A proportion of DJ-1 is localized to synaptic vesicles, but its function is much less clear than α-synuclein's (Usami et al., 2011). DJ-1 overexpression increases VMAT2 mRNA, protein levels, and uptake activity (Ishikawa et al., 2012, Lev et al., 2013). Furthermore, DJ-1 appears to directly bind to VMAT2, perhaps directly impacting its function independent of transcriptional regulation (Ishikawa et al., 2012).

More extensive work, led by Gudrun Ahnert-Hilger, has been done studying the role of G proteins in VMAT regulation. Expression of the heterotrimeric G protein $G\alpha o_2$ down-regulates VMAT1 and VMAT2 activity in cells and in purified vesicle preparations (Ahnert-Hilger et al., 1998, Holtje et al., 2000). Activation of G proteins inhibited VMAT2 uptake activity in

platelets, reducing the V_{max} but increasing the K_m (Holtje et al., 2003). In this paper, the authors also determined that $G\alpha_q$ is responsible for this action in platelets. Furthermore, this regulation appears to depend on vesicular content—regulation did not occur when vesicles were depleted. The authors propose that the first VMAT2 intravesicular loops may act as a 'receptor', sensing vesicular content and mediating this regulatory effect (Brunk et al., 2006).

It should be noted that most of this work has been done in cells and it's not clear if the same findings hold true *in vivo*. Furthermore, many studies have been done with VMAT over-expression, which can result in false positives. Although some look at direct interaction of proteins with VMAT2, some studies do not. Thus, it is not always clear if observed effects on VMAT2 are direct or indirect. In summary, relatively little has been investigated concerning VMAT2 regulation, leaving a major gap in our knowledge of this vital protein.

1.3 DEGRADATION

Degradation and synthesis processes work together to maintain proteostasis. Once thought to be general and unmediated, we now understand that protein degradation is often a highly regulated process. Protein degradation processes are in place to help regulate levels of proteins, remove damaged proteins, and act as a quality control mechanism. The multitude of ways in which proteins can be degraded range from the simple to the very complex. Ultimately, the mechanisms essential for managing degradation in the mammalian neuron can be boiled down to two—the 26S proteasome and the lysosome.

1.3.1 The ubiquitin-proteasome system

The eukaryotic 26S proteasome is a protein complex consisting of an assembly of subunits. Shaped like a hollow cylinder, proteins destined for degradation are fed through and cleaved into small peptides. The core of the proteasome, the 20S particle, contains proteolytic sites necessary for this function. A regulatory 19S particle consists of a base and lid, capping one of the ends of the 20S particle. The 19S particle regulates entry into the 20S particle, as well as the proteolytic activity of the core (Besche et al., 2009, Kim et al., 2011). The 20S core has three main proteolytic sites with trypsin-like, chymotrpypsin-like, or caspase-like proteolyic activity. Theses sites are the main targets for the majority of proteasome inhibitors that have been developed, with most inhibiting one or more of these sites. In addition to being used as research tools, proteasome inhibitors have also been developed for therapeutics, notably cancer treatment (Goldberg, 2007, Kisselev et al., 2012).

The delivery of substrates to the proteasome involves a complicated and highly choreographed series of events. The primary way in which proteins are targeted to the proteasome is via the ubiquitin-proteasome system (UPS)—an all-encompassing term that describes these events, outlined in Figure 4. The most common way proteins are targeted for proteasomal degradation is by ubiquitination (sometimes referred to as ubiquitylation). This post-translational modification involves the formation of an isopeptide bond between the C-terminus of ubiquitin, a small, cytoplasmic protein, and a residue of the substrate protein (Tai and Schuman, 2008, Komander, 2009).

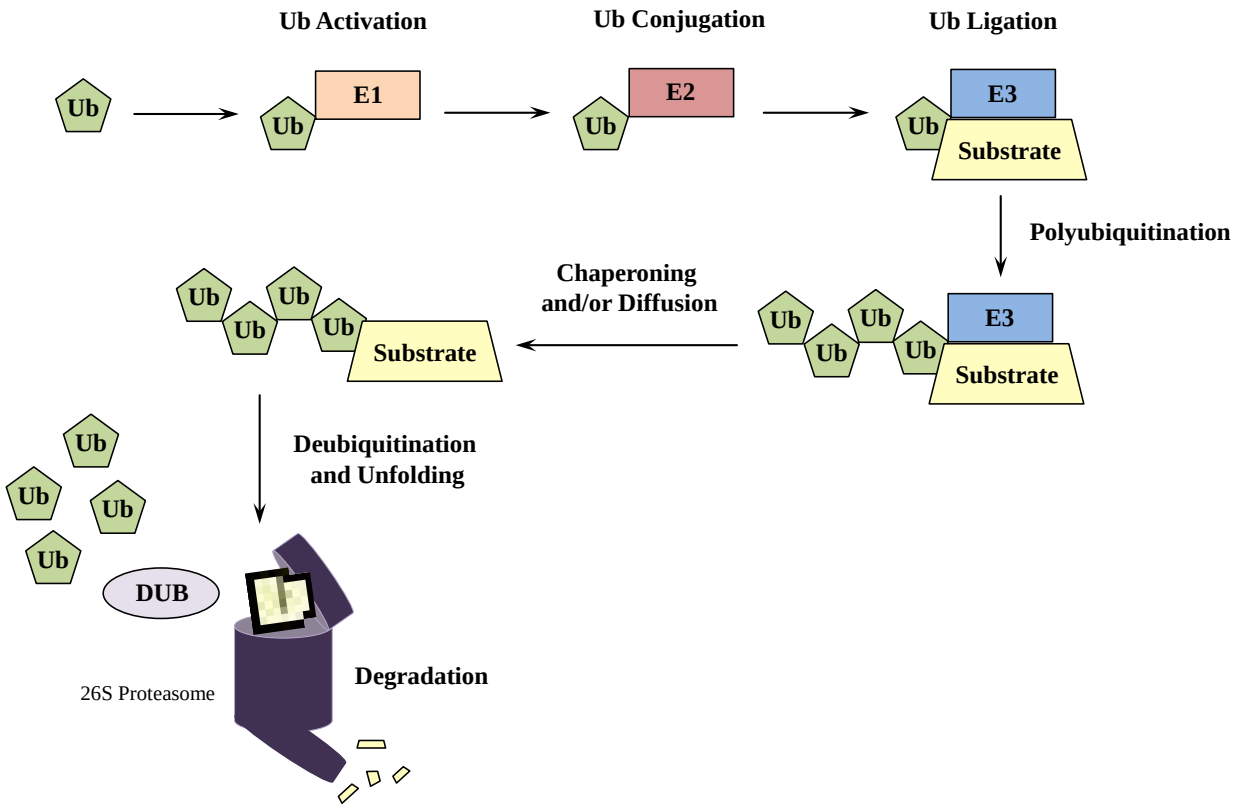


Figure 4. Overview of the UPS. Following ubiquitin activation and conjugation by E1 and E2 enzymes, ubiquitin is then transferred to a substrate by an E3 ubiquitin protein ligase. Further ubiquitin molecules are often added, forming a polyubiquitin chain. The ubiquitinated substrate then diffuses or is chaperoned to the 26S proteasome. At that point, the substrate is then deubiquitinated by enzymes (DUBs) and unfolded prior to degradation by the 26S proteasome.

What sounds like a simple modification in fact requires a series of reactions mediated by several enzymes. Prior to being bound to a substrate, ubiquitin is first 'activated' by being conjugated to an enzyme, E1, in an ATP-dependent reaction. Traditionally, ubiquitin is then transferred to the E2—the ubiquitin conjugating enzyme. Finally, ubiquitin is transferred from the E2 to a substrate by the E3, an ubiquitin protein ligase. While ubiquitin is typically attached to a lysine residue of the substrate protein, there are examples of other residues being ubiquitinated, including the N-terminus, cysteine, serine, or threonine residues (Ben-Saadon et al., 2004, Cadwell and Coscoy, 2005, Wang et al., 2007). There are only a few activating and conjugating enzymes (under 100) identified in the mammalian system, but there exist over 500 E3 ligases (Tai and Schuman, 2008). The cellular localization of the E3 ligase and its ability to interact with substrates is one of the main factors conferring specificity in regulated degradation.

There is an even further degree of regulation built into this system. The modification of a single ubiquitin molecule onto a substrate (monoubiquitination) is just one possible condition. There also exists muli-monoubiquitination, when multiple residues on a substrate are monoubiquitinated (Haglund et al., 2003). Furthermore, polyubiquitination is common, when multiple ubiquitins form a chain off the original ubiquitin bound to the substrate. Adding a layer of even more complexity, ubiquitin itself has multiple residues capable of being ubiquitinated; there are 8 potential polyubiquitin chain types identified. The most common polyubiquitination chain associated with the UPS is K48; the ubiquitin chain is on the lysine 48 residue of the ubiquitin initially bonded to the substrate (Thrower et al., 2000). K11 polyubiquitin chains are also associated with proteasomal degradation, but are less common (Baboshina and Haas, 1996, Xu et al., 2009). Another more common polyubiquitin chain is K63, less often associated with proteasomal degradation. Instead, K63 chains facilitate several other actions, including the

internalization of membrane proteins or lysosomal-mediated degradation, as well as playing a role in DNA repair (Hofmann and Pickart, 1999, Geetha et al., 2005, Duncan et al., 2006, Lauwers et al., 2009, Huang et al., 2013b). Other chains include K6, K27, K29, K33 and Met1 (also called linear, where the chain forms from the N-terminus of ubiquitin). These chains have a variety of other functions (targeting for lysosomal degradation, trafficking, mitophagy, etc.), but have not been as well characterized (Swatek and Komander, 2016). Interestingly, K48 and K63 chains form vastly different structures, with K63 chains having a more open conformation. It is thought that these structural differences are what confer specificity, leading to the specific chains being 'targeted' for different pathways (Jacobson et al., 2009, Komander, 2009). What determines linkage specificity, however, seems to be a combination of which E2 and/or E3 participates in the process (Komander and Rape, 2012, Mattiroli and Sixma, 2014). While the K48 and K63-linked polyubiquitin chains are traditionally segregated in terms of function, there are exceptions. For example, there's evidence that K63-linked polyubiquitin chains are capable of binding with the 26S proteasome, but are subject to deubiquitination more rapidly than K48-linked chains, perhaps limiting proteasomal degradation of K63-linked polyubiquitinated proteins (Jacobson et al., 2009). It has been suggested that there is redundancy within the degradation pathways; that is, degradation machinery accept multiple forms of ubiquitin chains (Xu et al., 2009).

Once the substrate is 'tagged' with ubiquitin, it is either chaperoned or diffuses to proteasomes. As nicely reviewed by Grice and Nathan, once there, accessory proteins work with the 19s regulatory unit of the proteasome to recognize the ubiquitin molecule and aid in deubiquitination (Grice and Nathan, 2016). Prior to entering the 20S core of the proteasome, deubiquitinating enzymes (DUBs) remove the ubiquitin molecule(s). Proteins are then unfolded

before being degraded by the proteolytic sites in the 20S core particle. These steps require a multitude of accessory proteins and enzymes, supplying an additional layer of variability and opportunity for regulation. Many of the involved chaperones and accessory proteins have ubiquitin-interacting motifs, where they bind ubiquitin and in this way, ubiquitin serves as the main cellular signal for proteasome-mediated degradation (Winget and Mayor, 2010, Grice and Nathan, 2016).

The number of accessory proteins involved in the UPS has the ability to confer a great degree of specificity. Because of this, proteasomal-mediated degradation is frequently thought of as being a more targeted approach to degradation, occurring under specific conditions. There are instances in which the proteasome doesn't have a regulatory role, per se, but rather a role in quality control. One of the most studied of these processes is ERAD—endoplasmic reticulum-associated degradation. A brief overview of ERAD is presented in Figure 5. In the ER, proteins are folded and processed prior to being exported to the Golgi. During this, misfolded or damaged proteins are degraded by the process of ERAD, in which they are removed and degraded by cytosolic proteasomes. Misfolded proteins are recognized and then chaperoned to the retrotranslocation machinery by chaperones within the ER—a number of which have been identified (Vembar and Brodsky, 2008, Stolz and Wolf, 2010). The exact mechanisms of retrotranslocation—the movement of substrates across the ER lipid bilayer into the cytoplasm—are not entirely known, although several plausible theories have been proposed (Brodsky, 2012, Christianson and Ye, 2014). It is, however, generally agreed upon that ubiquitination plays a role. E1, E2 and E3 ligases facilitate the ubiquitination of substrates, which again serves as a signal for proteasome-mediated degradation. It is not entirely clear if ubiquitination also serves as a signal for the initiation of retrotranslocation. Retrotranslocation is thought to occur through

a channel, although this has yet to be conclusively identified. Several proteins/complexes have been suggested—the Sec61 complex, members of the Derlin family of proteins, and Hrd1, an E3 ubiquitin ligase (Wiertz et al., 1996, Vashist and Ng, 2004, Carvalho et al., 2006, Scott and Schekman, 2008, Greenblatt et al., 2011, Huang et al., 2013a). Regardless of which of these are mainly involved, it is clear that the ATPase p97/VCP complex is required for the process (Ye et al., 2001, Rabinovich et al., 2002, Richly et al., 2005). This complex not only provides the energy required for retrotranslocation, but also associates with DUBs, ubiquitin ligases, and cofactors with ubiquitin recognition sites. It is thought that retrotranslocation and subsequent degradation by the cytoplasmic proteasome are tightly coupled, although it is unclear if proteasomes are located near the p97/VCP complex or if substrates are shuttled to the proteasome (Christianson and Ye, 2014).

An ongoing field of research, our knowledge of the UPS is constantly changing. One such area of research is the contribution of UPS dysfunction to various diseases and disorders (Chaugule and Walden, 2016). Perhaps some of the most striking examples are the 'proteopathies'—degenerative disorders characterized by protein misfolding and aggregate formation. Included in this group of disorders are devastating neurodegenerative diseases, such as Huntington's, Alzheimer's, and Parkinson's disease. While causal links haven't been definitively determined, the pathologies of proteopathies have been partially attributed to UPS dysfunction. Focusing on PD, there's considerable evidence indicating that the UPS and/or autophagy (discussed in the following section) dysfunction plays a role in the etiology of the disease, including the formation of Lewy bodies—aggregates primarily composed of α-synuclein and ubiquitinated proteins (Rubinsztein, 2006). Patients with sporadic PD have reduced proteasomal activity in the substantia nigra compared to age-matched controls, indicating there

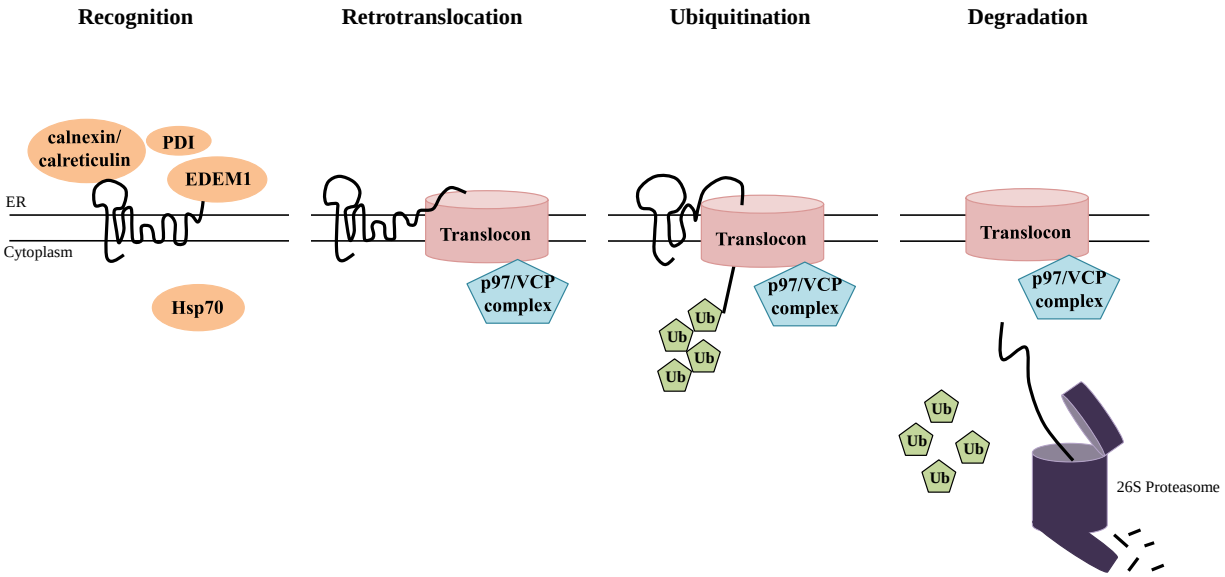


Figure 5. Overview of ERAD. Misfolded or damaged proteins are first recognized by a number of ER and cytosolic chaperones and accessory proteins (a partial list of examples are given). The substrate is then chaperoned to the translocon (Declin, Hrd1, and/or Sec61) where it undergoes retrotranslocation with the aid of the p97/VCP complex. The substrate is then ubiquitinated and targeted to the 26S proteasome for degradation. Note that here, retrotranslocation is presented as an event that occurs prior to ubiquitination. While this may be, it is also possible that the substrate may be ubiquitinated prior to retrotranslocation.

may be a fundamental deficit in the UPS (McNaught et al., 2003). Various rare mutations that cause familial Parkinsonism encode proteins involved in the degradation machinery, such as parkin (an E3 ubiquitin ligase, also involved in mitophagy), UCHL1 (a de-ubiquitinating enzyme), and PINK1 (involved in mitochondrial dynamics, including mitophagy). Additionally, disruptions in ERAD can result in aggregate formation and severe global dysfunction. Just one example of many, mutations in p97/VCP cause inclusion-body myopathy with Paget's disease of bone and frontotemporal dementia (Watts et al., 2004, Weihl et al., 2006).

It should be noted that provided here are brief overviews of the UPS and ERAD. The entire processes and components involved are complex. It is becoming increasingly clear that while the UPS plays a significant role in protein degradation, ubiquitination has roles independent of degradation. There are examples of ubiquitin-independent proteasomal degradation of substrates, further indicating there are aspects of the system we do not yet fully understand (Kravtsova-Ivantsiv and Ciechanover, 2012).

1.3.2 Degradation by the lysosome

The other primary method of protein degradation in eukaryotic cells is by the lysosome. Membrane-bound structures, lysosomes contain specific hydrolases that digest proteins. The pH of lysosomes is acidic, maintained by vacuolar H+-ATPase pumps (Mindell, 2012). While lysosomes contain over 50 known hydroylases, the most well researched are the cathepsins—a group of proteases that function optimally under the acidic lysosomal conditions (Appelqvist et al., 2013). The lysosome has roles in multiple cellular processes, although here I will focus on its role in degradation (Settembre et al., 2013). Unlike the UPS, lysosomes typically degrade membrane proteins, extracellular material, and macromolecules. Once thought to be associated

with unregulated, bulk degradation, it is now clear that targeting to the lysosome for degradation can be just as specific as in the UPS.

A major group of proteins that are degraded by lysosomes are plasma membrane proteins through the endo-lysosomal pathway, represented in Figure 6. Reviewed by Grant and others, essentially, these proteins are internalized through endocytosis and are retained on early endosomes—membrane bound structures that serve as a sorting area (Grant and Donaldson, 2009, Saftig and Klumperman, 2009, Sorkin and von Zastrow, 2009). Recycling endosomes traffic proteins back to the plasma membrane if that is their fate. Early endosomes are then converted into multivesicular bodies (MVBs, also called late endosomes), where further sorting is done. At this point, some proteins are transported back to the trans-Golgi network. If not, these proteins will be degraded by the lysosome when the MVBs fuse with the lysosomal membrane (Grant and Donaldson, 2009, Saftig and Klumperman, 2009, Sorkin and von Zastrow, 2009, Appelqvist et al., 2013).

As with the UPS description, this is a rather simplified view. A multitude of accessory proteins are involved with this process and the maturation of endosomes is complicated, involving pH changes, exchanges of membrane, trafficking of components, and fusion events (Luzio et al., 2007). For example, ubiquitination is believed to play a role in the internalization and targeting of receptors, transporters, and channels to the lysosome for degradation. Several of these rely on ubiquitination—although in some cases the modification seems sufficient, but not necessary for endocytosis, implying there are multiple mechanisms for endocytosis and perhaps, redundancy within the system (Sigismund et al., 2005, Goh et al., 2010, MacGurn et al., 2012). Monoubiquitination and K63-linked polyubiquitin chains have been most associated with lysosomal degradation through this pathway (Haglund et al., 2003, Duncan et al., 2006, Lauwers

et al., 2009, Vina-Vilaseca and Sorkin, 2010, Stringer and Piper, 2011). Accessory proteins, likely members of the epsin family, at the plasma membrane have ubiquitin interacting motifs, which allow them to recognize and sort the ubiquitinated plasma membrane proteins (Polo et al., 2002, Sigismund et al., 2005). A group of proteins called ESCRTs (endosomal sorting complexes required for transport) are thought to play a role in the sorting of these ubiquitinated proteins once they are in endosomes (MacGurn et al., 2012, Hurley, 2015). These ESCRTs also aid in the formation of intraluminal vesicles, vesicles within MVBs containing transmembrane proteins to be degraded. ESCRTs interact with a variety of E3 ligases and DUBs, indicating that degradation signals can be added or removed even at this stage.

Lysosomes are also capable of degrading cytosolic proteins and intracellular components. Many of these substrates come to the lysosome through autophagy, a term that encompasses several different processes. Microautophagy refers to when cytosolic material is engulfed directly at the lysosomal membrane. Chaperone-mediated autophagy is the process by which cytosolic proteins are shuttled to the lysosome for degradation. Finally, macroautophagy, the main autophagy pathway, involves the formation of autophagosomes—membrane-bound structures that form around cytoplasm and/or organelles, engulf them, and then fuse with the lysosome for degradation of the contents.

In chaperone-mediated autophagy, cytosolic proteins are more specifically targeted for degradation by the lysosome. These substrates typically contain a motif (KFERQ, or similar to) that's recognized by chaperones (Dice, 1990). The main chaperone involved in this process is Hsc70, although other accessory chaperones, like Hsp40 and Hsp90, may also be involved (Chiang et al., 1989, Agarraberes and Dice, 2001). Interestingly, Hsc70 has been shown to interact directly with VMAT2 and reduce uptake activity, although the mechanism of this action

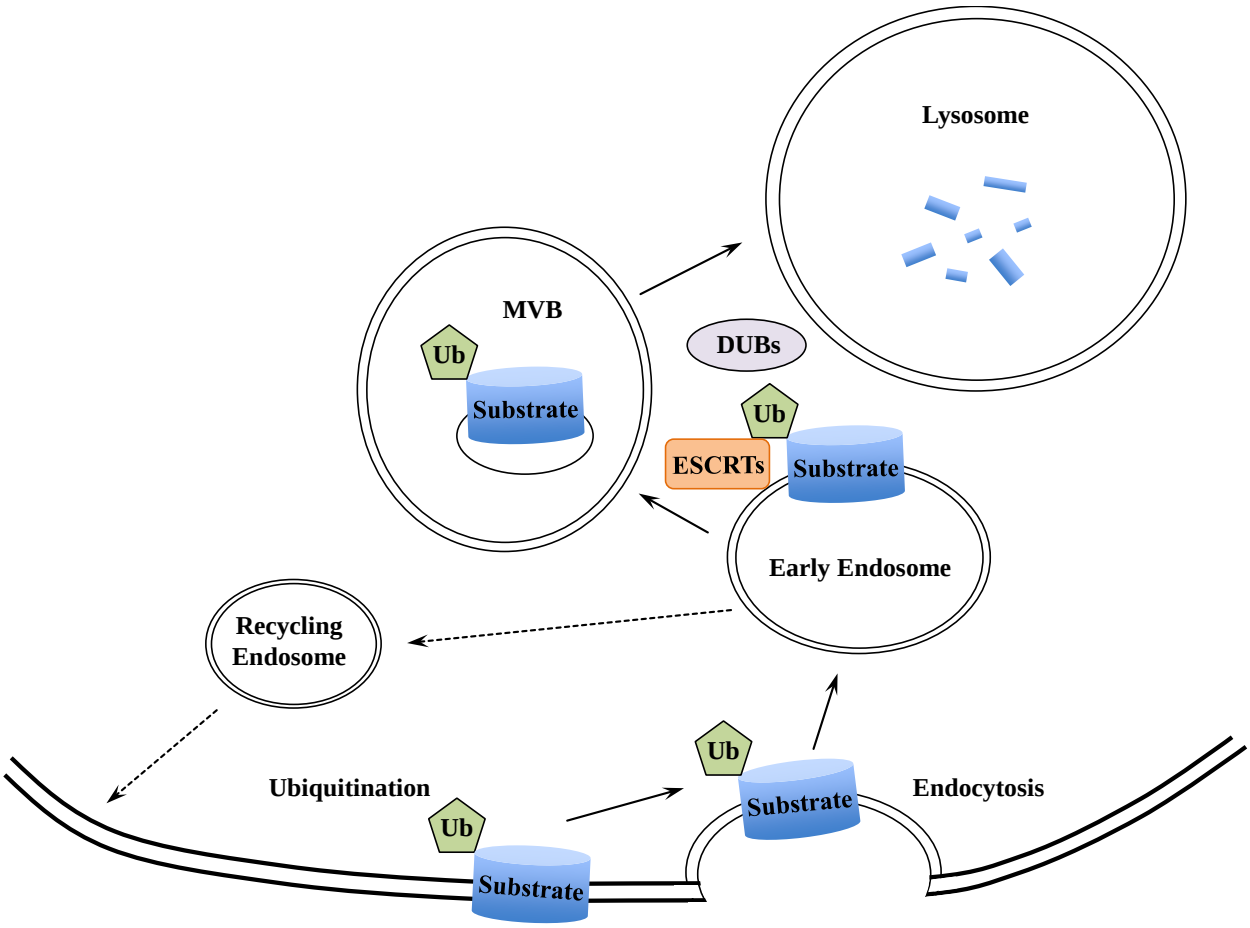


Figure 6. Overview of the endo-lysosomal pathway. Plasma membrane proteins are endocytosed, sometimes (but not always) with ubiquitin acting as an internalization sequence. In early endosomes, proteins are either trafficked to recycling endosomes for transport back to the plasma membrane or trafficked to MVBs, if targeted for degradation. Ultimately, MVBs will fuse with lysosomes, which will degrade the substrate.

is unknown (Requena et al., 2009). Lamp2a, a lysosomal membrane protein, is believed to be the primary receptor for these chaperones/substrates, although there's indication that other receptors undertake this function in the absence of Lamp2a (Cuervo and Dice, 1996, Eskelinen et al., 2004). The process of chaperone-mediated autophagy is important for quality control, but along with macroautophagy, also seems to be an induced pathway in response to stress, including starvation and oxidative stress (Wing et al., 1991, Kiffin et al., 2004).

Unlike chaperone-mediated autophagy, macroautophagy involves an impressive array of signaling pathways and proteins—not only for induction and substrate recognition, but also for autophagosome formation and subsequent fusion with lysosomes. Of note, selective autophagy utilizes the modification of substrates with ubiquitin-like proteins, much as in the UPS system (Mizushima et al., 1998, Welchman et al., 2005, Bento et al., 2016). In fact, there's evidence that ubiquitinated proteins (notably, ubiquitinated, aggregated proteins) can be recognized by autophagosome receptors and degraded through the process of autophagy, highlighting the importance of ubiquitination across all degradation pathways (Komatsu et al., 2007, Kim et al., 2008, Kirkin et al., 2009a, Kirkin et al., 2009b). Macroautophagy is essential as a mechanism of quality control within the cell, acting as a means of degradation for aggregated proteins and damaged organelles. While autophagy is relatively unspecific under certain circumstances (stress, or starvation, for example), it can also be extremely selective. For example, the degradation of damaged organelles is a very selective process. The best characterized is mitophagy, in which damaged mitochondria are identified and selectively targeted for autophagy. Originally believed to play a role in programmed cell death, it's now under debate as to whether autophagy induction causes cell death or is induced as a means to cope with cellular stress. Paradoxically, linked to both cell death and cell survival, the exact role of autophagy

seems to depend on the context and circumstances under which autophagy is induced (Feng et al., 2014, Mrschtik and Ryan, 2015).

As with the UPS, lysosomal dysfunction can cause a number of diseases. There's a large class (over 50) of metabolic disorders categorized as 'lysosomal storage disorders' that are caused by mutations of varying lysosomal proteins (Platt et al., 2012). Depending on the disorder, symptoms vary, but can result in developmental delay or early death. Experimentally, disturbances in autophagy result in severe deficits; neuronal Atg5 and Atg7 (essential components of autophagosome formation) knockout mice develop inclusions, age-related motor-deficits, and reduced vitality (Hara et al., 2006, Komatsu et al., 2006). As with the UPS, deficits in autophagy are thought to play a role in proteopathies. Mutated forms of α-synuclein that cause PD, A30P and A53T, block Lamp2a, which has a role in chaperone mediated autophagy (Cuervo et al., 2004). Under these circumstances, mutant α-synuclein and other substrates are no longer degraded, perhaps contributing to the formation of toxic α-synuclein protofibrils. Notably, patients with Gaucher's disease, a lysosomal storage disorder resulting from glucocerebrosidase mutations, are five times more likely to develop Parkinsonism (Sidransky et al., 2009). Although it is unclear how exactly the two disorders are related, it suggests a link between lysosomal dysfunction and Parkinsonism.

1.3.3 Degradation of synaptic vesicle proteins

There is substantial evidence for the degradation of plasma membrane transmembrane proteins through the endo-lysosomal pathway. It is less clear how synaptic vesicular proteins are degraded; aspects of synaptic vesicle biogenesis and recycling are also less understood. Reviewed extensively by Rizzoli, the classic view of neuronal synaptic vesicle biogenesis is

simplified here. Following synthesis and ER/Golgi processing, synaptic vesicular proteins seem to group in patches within the Golgi (Rizzoli, 2014). "Precursor vesicles" bud from the Golgi and undergo anterograde transport to the presynaptic terminal. These vesicles fuse with the plasma membrane, where synaptic vesicular proteins will diffuse and again sort into distinct patches. Synaptic vesicles form when endocytosis is triggered and at this point, they may fuse to endosomes for further sorting, although this process is poorly understood (Hoopmann et al., 2010). The synaptic vesicles are then filled with neurotransmitter and dock at the active zone prior to exocytosis. Following exocytosis, membrane and synaptic vesicular components are internalized and recycled locally to re-form vesicles through a clathrin-dependent mechanism ('fast' endocytosis) (Holtzman et al., 1971, Heuser and Reese, 1973, Koenig and Ikeda, 1996, de Lange et al., 2003, Saheki and De Camilli, 2012). A second pathway has been proposed, where synaptic vesicles reform from an endosomal intermediate in what has been termed 'bulk' or 'slow' endocytosis (Faundez et al., 1998, Rizzoli et al., 2006, Rizzoli, 2014, Kokotos and Cousin, 2015). For VMAT2, endocytosis depends on the C-terminal motif KEEKMAIL, specifically the isoleucine-leucine pair that may be a recognition site for AP-2, a protein involved in clathrin-mediated endocytosis (Tan et al., 1998). For reference, the KEEKMAIL motif is indicated in Figure 1. Interestingly, there is some evidence indicating that VMAT2 and VGLUT1, the glutamatergic vesicular transporter, have different recycling pathways (Croft et al., 2005, Onoa et al., 2010, Grygoruk et al., 2014). For example, when expressed in primary hippocampal or dopaminergic ventral midbrain neurons, VMAT2 had a slower endocytosis rate than VGLUT1 following stimulation, suggesting the rate differences are the result of the property of the protein and not cell type. The authors suggest VMAT2 may diffuse more within the plasma membrane, but were not able to directly assess this (Onoa et al., 2010).

The existence of proteasomes, but not lysosomes, in the presynaptic terminal has been well established. Because of this, there exist two predominant possibilities for the degradation of synaptic vesicular proteins: (1) individual proteins are degraded at the synapse by the UPS or (2) vesicles or vesicular components are sorted at an endosome intermediate following endocytosis and shuttled via retrograde transport back to the cell body for lysosomal degradation. The importance of UPS function has been implicated in a number of processes at the presynaptic terminal, influencing vesicle dynamics and neurotransmitter release (Speese et al., 2003, Willeumier et al., 2006, Yao et al., 2007, Rinetti and Schweizer, 2010). The potential for its mediation of synaptic vesicular protein degradation, however, has not been fully explored. Both syntaxin and synaptophysin have been proposed to be degraded in a proteasome and ubiquitin-dependent manner (Chin et al., 2002, Wheeler et al., 2002). However, these studies were performed in heterologous expression systems and results have not been confirmed with endogenous protein.

The dislocation of transmembrane, glycosylated proteins necessitates a large energy requirement. Because of this, it seems unlikely that transmembrane vesicular proteins could be degraded by the UPS. There are very few instances of observed post-ER retrotranslocation of proteins. One example is the p97-mediated retrotranslocation of Mfn1 and Mcl1, two outer mitochondrial transmembrane proteins (Xu et al., 2011). Another example comes from work by Peter Espenshade. He and his group have worked to establish the role of a Golgi quality control mechanism for proteins, similar to ERAD. They have established, in yeast, Golgi E3 ligase complexes that participate with Cdc48 (p97 in mammals) in the retrotranslocation of transmembrane substrates (Stewart et al., 2011, Tong et al., 2014, Hwang et al., 2016). However, retrotranslocation of these proteins is not from the plasma membrane or synaptic

vesicular membrane. To this author's knowledge, retrotranslocation of glycosylated, transmembrane proteins from plasma or synaptic vesicular membranes has not been directly demonstrated. Support for lysosome-mediated vesicular protein degradation comes from evidence of retrograde transport back to the cell body. Several researchers have found evidence indicating synaptic vesicles and their components undergo retrograde transport back to the cell body (Tsukita and Ishikawa, 1980, Li et al., 1996, Li and Dahlstrom, 1997). However, none observed lysosomal-dependent degradation directly, rather an assumption was made that the transport back to the cell body was for degradation. The lack of research in the area of synaptic vesicular degradation underlies how difficult this topic is to study. More recently, Patrik Verstreken's group has undertaken this difficult task. Using *Drosophila* as a model system, they have demonstrated that loss of a GTPase activator increases synaptic recycling to endosomes (Uytterhoeven et al., 2011). Loss of dor, an orthologue of the human Vps18 (a member of the HOPS complex that promotes trafficking of vesicles to lysosomes) also results in defective trafficking to lysosomes, providing the identity of potential molecular components involved in vesicular protein degradation (Fernandes et al., 2014). Lysosomal-mediated degradation of VMAT2 has been proposed by researchers, including Annette Fleckenstein's group (German et al., 2015, Sulzer et al., 2016). However, there is no data that directly supports this model of lysosome-mediated VMAT2 degradation.

There is a dearth of information regarding the degradation mechanisms of vesicular proteins, including transporters. Because of this, we sought to identify the mechanisms involved in VMAT2 degradation. To accomplish this, we used pharmacological tools to inhibit degradation processes and then examined VMAT2 levels, cellular localization and ubiquitination. We believe that the regulation of VMAT2 is important to study, as VMAT2

plays an essential role in monoaminergic signaling. The goal of this thesis work was to identify the primary machinery by which VMAT2 is degraded in order to begin to understand the processes involved and contribute knowledge to the field of synaptic vesicular protein degradation.

2.0 MATERIALS AND METHODS

2.1 MODEL SYSTEM

As a model system, I utilized PC12 cells, a rat pheochromocytoma cell line. PC12 cells are used as a model for exocytosis, as they contain vesicles and synthesize dopamine (and sometimes norepinephrine) (Greene and Tischler, 1976, Schweitzer et al., 1995, Kozminski et al., 1998, Pothos et al., 2000). PC12 cells contain small, clear vesicles and large dense core vesicles (or granules) that are comparable to neuronal synaptic vesicles and dense core vesicles, respectively. PC12 cells have endogenous VMAT1 that preferentially localizes to large dense core vesicles (Liu et al., 1994, Weihe et al., 1996). When differentiated with nerve growth factor (NGF), PC12 cells extend neurite-like projections, develop vesicle-rich varicosities along some of these processes, and more closely resemble the phenotype of sympathetic ganglion neurons than when undifferentiated (Westerink and Ewing, 2008).

PC12 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with 5% fetal bovine serum, 10% horse serum, and 1% penicillin/streptomycin at 37°C and 10% carbon dioxide. Where noted, cells were transiently transfected with rat VMAT2 subcloned into the peGFP-N2 vector (VMAT2-GFP, so there's a GFP tag on the C terminus of VMAT2). Transient expression of 10 μg VMAT2-GFP cDNA with Lipofectamine 2000 (Invitrogen) was performed according to

manufacturer's instructions (2.5 μL Lipofectamine per μg of cDNA) and cells were transfected 48 hours prior to use. The majority of experiments were done with PC12 cells stably expressing VMAT2-GFP. Cells were transfected with VMAT2-GFP cDNA, then sorted twice via flow cytometry for GFP. The cell line was then maintained in 50 μg/mL G418 sulfate.

Using crude vesicular preparations, I demonstrated that this VMAT2-GFP construct is capable of serotonin uptake (shown in Appendix A). Even though PC12 cells have endogenous VMAT1, an over-expression system of GFP-tagged VMAT2 was used for a number of reasons. For one, the goal of the project was to investigate the mechanisms of neuronal VMAT degradation. While VMAT1 and VMAT2 sequences are somewhat similar, there are major differences, especially in the cytoplasmic and large intravesicular domains. The GFP tag was used, in part, because it aided in sorting for development of a stable cell line via flow cytometry. Additionally, VMAT2 antibodies can be unreliable, especially for microscopy. While not impossible to perform experiments with untagged VMAT2, the GFP tag provided a means for reliable detection of VMAT2. As demonstrated in Figure 7, a GFP antibody used frequently in these studies detects two main VMAT2 bands—a 'smear' around 120 kDa and a more compact band around 75 kDa. Others have described three un-tagged VMAT2 bands: a mature, glycosylated form at around 75 kDa, a partially glycosylated form at approximately 55 kDa, and a native form at the lowest molecular weight, 45 kDa (Cruz-Muros et al., 2008). I was unable to detect the lowest molecular weight form with the GFP antibody.

EndoH (Endoglycosidase H) digestion was used to confirm the cellular localization of the two bands detected. EndoH is an enzyme that cleaves high-mannose and hybrid oligosaccharides in the core region. EndoH does not cleave complex glycans and therefore does not cleave proteins that have already undergone further processing in the Golgi body. This

enzyme can therefore be used as a way of determining the processing stage of proteins—EndoH digestion will result in a molecular weight shift of N-glycosylated proteins in the endoplasmic reticulum (ER), but not proteins that have exited the ER (Freeze and Kranz, 2010). PC12 cells stably expressing VMAT2-GFP were lysed, equal protein amounts incubated with EndoH and reaction buffer (Promega, catalog #V487A, #V487B) for 4 hours at 37°, and subjected to SDS-PAGE. Membranes were probed with anti-GFP or anti-tubulin and compared to samples not treated with EndoH. As shown in Figure 8, the molecular weight of the VMAT2 smear at 120 kDa remained unchanged following EndoH digestion. The lower, 75 kDa band shifted following EndoH digestion, indicating that it is an immature form of VMAT2 that was still undergoing processing in the ER at the time of lysis. These results confirm what others have demonstrated and concluded about VMAT2 forms and glycosylation states (Yelin et al., 1998, Jassen et al., 2005, Cruz-Muros et al., 2008). For the remainder of this document, the 120 kDa band will be referred to as the 'mature' form of VMAT2 and the 75kDa band as the 'immature' form.

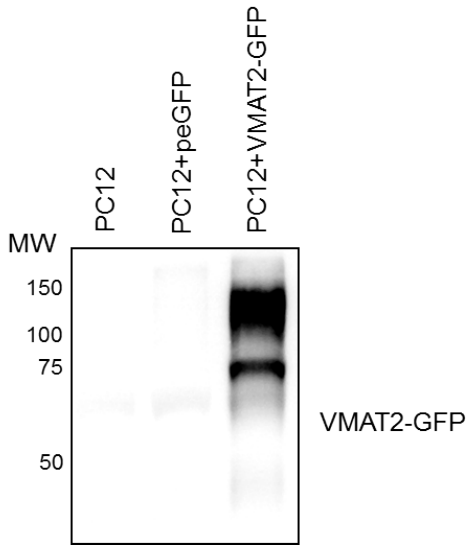


Figure 7. Specificity of GFP antibody. PC12 cells were transfected with 10 μg peGFP-N2 empty vector or 10 μg VMAT2-GFP cloned into peGFP-N2. These cells and untransfected PC12 cells were lysed and subjected to immunoblotting. The membrane was probed with anti-GFP, an antibody frequently used in this project, to detect VMAT2-GFP.

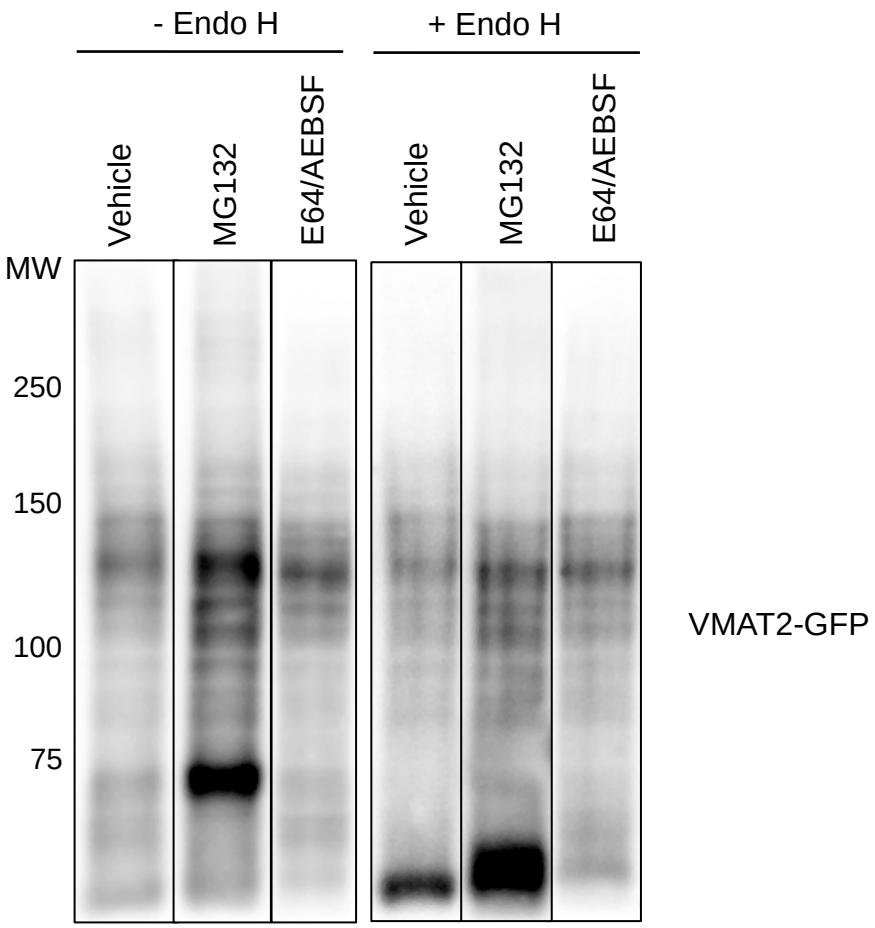


Figure 8. Endo H Digestions. PC12 cells stably expressing VMAT2-GFP were treated with DMSO vehicle, MG132, or E64/AEBSF for 6 hours. Cells were lysed and then digested with Endo H for 4 hours. Note how the molecular weight of the mature form around 120 kD remains the same. The molecular weight of the immature band around 75 kD decreases following Endo H treatment, confirming location in the endoplasmic reticulum and status as the immature VMAT2 band.

2.2 METHODS

2.2.1 Antibodies

For VMAT2 detection, anti-GFP was used at a 1:1000 dilution (Sigma-Aldrich, clone GFP-20, catalog #G6539). Anti-tubulin was used at 1:2000 (Sigma-Aldrich, clone B-5-1-2, catalog #T6074). For ubiquitin detection, anti-ubiquitin was used at 1:500 (Santa Cruz, clone PD41, catalog #sc-8017). Lysine 48-specific ubiquitin antibody (Millipore, clone Apu2, catalog # 05-1307) and lysine 63-specific ubiquitin antibody (Millipore, clone Apu3, catalog #05-1308) were also used at a 1:500 dilution. Anti-parkin was used at a 1:1000 dilution (Santa Cruz, clone PRK8, catalog # sc-32282). For rabbit antibodies, horseradish peroxidase-linked protein A (GE Healthcare, catalog #NA9120V) was used at a 1:4000 dilution as secondary. For mouse antibodies, goat anti-Mouse IgG (H+L)-horseradish peroxidase conjugate (BioRad catalog # 170-6516) was used at 1:5000 as secondary.

In immunocytochemistry experiments, two antibodies for GFP were used: monoclonal anti-GFP from Sigma-Aldrich (used at a 1:1000 dilution, clone GFP-20, catalog #G6539) or rabbit polyclonal anti-GFP from Abcam (used at a 1:2000 dilution, catalog #ab290). Alexa Fluor® 488-conjugated goat anti-rabbit IgG (H&L) (Jackson ImmunoReseach, catalog #111-545-003) and Alexa Fluor® 488-conjugated goat anti-mouse IgG (H&L) (Jackson ImmunoReseach, catalog #115-545-003) were both used as secondary antibodies at a 1:500 dilution for the GFP antibodies. As a marker for the endoplasmic reticulum, I used anti-PDI from Abcam (clone RL90, catalog #ab2792) at a 1:1000 dilution and goat anti-mouse IgG H&L Alexa Fluor® 647 (Abcam, ab150115) at a 1:500 dilution. Two different LAMP1 antibodies were utilized as markers of lysosomes/endosomes: rabbit polyclonal anti-LAMP1 (1:100,

Abcam, catalog #ab24170) or monoclonal anti-LAMP1 (1:50, H4A3, obtained from the Developmental Studies Hybridoma Bank (DSHB), originally deposited by JT August and JEK Hildreth (citation). The secondary antibody utilized for polyclonal anti-LAMP1 was Cy™3-conjugated goat anti-rabbit IgG (H&L) (1:1000, Jackson ImmunoResearch, catalog #111-165-003) and for monoclonal LAMP1, Alexa Fluor® 647 at a 1:1000 dilution.

2.2.2 Drug treatments

Cycloheximide (Sigma-Aldrich), E64 (MP Biochemicals), MG132 (Cayman Chemical), Epoxomicin (Abcam), and Eeyarestatin I (Tocris Bioscience) were dissolved in DMSO while AEBSF (MP Biochemicals) was dissolved in water. Cells were plated in poly-D-lysine-coated plates 24 hours prior to drug addition and then were treated with drugs for up to 18 hours at the following concentrations: 50 μg/mL cycloheximide, 25 μM E64, 200 μM AEBSF, 10 μM MG132, 250 nM Epoxomicin, 10 μM Eeyarestatin I. Following treatments, cells were washed 2 times with ice-cold PBS and lysed for 1 hour at 4°C with rotation in 20 mM HEPES, 125 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 10% glycerol, pH 7.4 in addition to 1% Triton x-100, protease inhibitor cocktail (Millipore, contains AEBSF, aprotinin, E64, EDTA, and leupeptin) and 10 mM N-ethylmaleimide (NEM) (to inhibit de-ubiquitination). Lysates were centrifuged at 10,000 x g for 10 minutes at 4°C and the supernatant was used for subsequent analysis. Protein levels were determined using the BioRad DC protein assay (a colorimetric assay similar to a Lowry assay) and values read on a spectrophotometer at 750 nm.

2.2.3 Immunoblotting

Cell lysates were denatured in western blot sample buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromophenol blue) for 30 minutes at 37°C. Equal amounts of protein were loaded onto 8 or 10% Tris SDS-polyacrylamide gels. Gels were ran in 25 mM Tris, 250 mM glycine, 0.1% SDS running buffer. Transfer onto nitrocellulose membranes were performed at 4°C in 25 mM Tris, 192 mM glycine, 20% methanol transfer buffer. Membranes were then blocked in 5% milk for 1 hour at room temperature with rocking and then incubated overnight in primary antibody (in 2.5% milk) at 4°C with rocking. Membranes were washed 3 times (10 minutes each) with oscillation in TBST (50 mM Tris, 150 mM sodium chloride, 0.2% Tween-20, pH 7.5), incubated in secondary antibody for 1 hour at room temperature with rocking, and then washed another 3 times (10 minutes each) in TBST at room temperature. Chemiluminescent signal was detected using SuperSignal® West Pico kit (Thermo Fisher Scientific).

2.2.4 Immunoprecipitations

PC12 cells stably expressing VMAT2-GFP were plated onto poly-D-lysine-coated coverslips and treated with DMSO vehicle, 10 μM MG132, or 25 μM E64 and 200 μM AEBSF for 6 or 12 hours. Cells were washed twice with ice-cold PBS and lysed for 1 hour at 4°C with rotation in RIPA buffer (50 mM Tris, 150 mM sodium chloride, 0.1% SDS, 0.5% sodium deoxycholate, pH 8.0 with 1% Triton x-100, protease inhibitor cocktail and 10 mM NEM. Lysates were centrifuged at 10,000 rpm for 10 minutes and the supernatant was used for subsequent analysis. Protein levels were determined as described above. 1 mg/mL lysate was incubated with protease

inhibitor cocktail, NEM, and 10 μL anti-VMAT2 (Santa Cruz, C20 clone, catalog #sc-7721) overnight at 4°C with rotation. As an 'IgG' control, goat IgG (Millipore, catalog #PP40) was substituted for VMAT2 antibody. As a 'buffer' control, RIPA buffer was substituted for cell lysate. Following overnight incubation, 80 μL protein G sepharose beads (washed in PBS, then reconstituted 1:1 in RIPA) was added and further incubated for 2 hours at 4°C with rotation. Beads were washed twice in RIPA for 5 minutes each, then one time in PBS for 5 minutes. Beads were then suspended in western blot sample buffer and subjected to immunoblotting as described above.

VMAT2-parkin co-immunoprecipitations were performed largely as described above. PC12 cells were transfected with 10 μg VMAT2-GFP and 10 μg human parkin cloned into a pcDNA33.1 vector using Lipofectamine 2000. 48 hours post-transfection, cells were lysed in 20 mM HEPES, 125 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 10% glycerol, pH 7.4 in addition to 1% Triton x-100 and protease inhibitor cocktail. VMAT2 immunoprecipitation was performed as described in the above paragraph. Following overnight incubation in VMAT2 antibody and 2 hours incubation with 80 μL Protein G sepharose, beads were washed for 5 minutes each in buffer (50 mM Tris, 150 mM sodium chloride, pH 8) plus 1% Triton x-100, buffer alone (just 50 mM Tris, 150 mM sodium chloride, pH 8), and PBS. Immunoblotting was performed as described and membranes were probed with anti-GFP and anti-Parkin.

2.2.5 GST pull downs

VMAT2 cDNA fragments encoding residues 1-18 (the N-terminus), 268-292 (the third cytoplasmic loop), or 466-514 (the C-terminus) were subcloned into the pGEX4T-1 vector and were purified from *E. Coli* using gluthathione-sepharose 4 fast-flow (GE Healthcare).

Approximately equal amounts of GST-VMAT2 fusion proteins comprising either the VMAT2 N-terminus (VMAT2-N), cytosolic loop (VMAT2-L), VMAT2 C-terminus (VMAT2-C), or GST alone, were incubated with 1 mg/mL whole rat brain homogenate overnight at 4°C with rotation. Beads were washed with 50 mM Tris, 150 mM sodium chloride, pH 8 first with, then without 1% Triton tx-100. Following a final wash with PBS, beads were then re-suspended in western blot sample buffer and subjected to immunoblotting as described above. Prior to blocking, membranes were incubated with Ponceau red stain to visualize relative levels of VMAT2-GST fusion proteins. Membranes were then probed with anti-parkin to detect the ability of the GST-VMAT2 fusion proteins to 'pull down' parkin.

2.2.6 Pulse-chase assay

This pulse-chase protocol is modified from a number of other studies, most of which examine half-lives of transmembrane proteins (Zhang et al., 2000, Imai et al., 2001, Chin et al., 2002, Moriyoshi et al., 2004). PC12 cells stably expressing VMAT2-GFP were plated onto poly-D-lysine-coated 60 mm plates. The following day, cells were washed twice with PBS and incubated in cysteine/methionine-free DMEM for 1 hour. When experiments were performed to assess the impact of proteasomal inhibition on VMAT2 half-life, cells were pre-incubated with DMSO vehicle or 5 μM MG132 for two hours and then 'pulsed' with 100 μCi [^{35}S] methionine/cysteine mix (EasyTag™ EXPRESS^{35}S Protein Labeling Mix, Perkin Elmer, catalog #NEG7720) for 1 hour. When experiments were performed to assess the impact of lysosomal inhibition on VMAT2 half-life, cells were 'pulsed' for 24 hours to maximize labeling of mature VMAT2. Following incubation with ^{35}S, plates were washed twice with media and 'chased' in DMEM containing an excess (5 mM) of methionine and cystine. DMSO or 5 μM MG132 was

added to chase media and cells were lysed immediately or after 1, 2, 4, 6, or 8 hours incubation. Alternatively, following a 24 hour 'pulse', cells were washed, incubated in chase media containing DMSO or 25 μM E64 and 200 μM AEBSF, and lysed immediately or after 6, 12, 24, 36, or 48 hours incubation. Cells were lysed in RIPA buffer as described previously. Protein content was determined and a portion of lysate was subjected to SDS-PAGE as described. Membranes were probed with anti-GFP and anti-tubulin to assess VMAT2-GFP levels.

500 μg of the remaining cell lysate was subjected to immunoprecipitation. Lysate was pre-cleared with 40 μL protein G beads (1:1 in RIPA), protein inhibitor cocktail, and 10 mM NEM for 2 hours at 4°C with rotation. Samples were centrifuged and the supernatant was then incubated overnight with 5 μL anti-VMAT2 (Santa Cruz, C20 clone, catalog #sc-7721) at 4°C with rotation. 60 μL protein G beads (1:1 in RIPA) were then added for 1 hour incubation and beads were then washed for 5 minutes once with RIPA and then twice with PBS. Beads were then re-suspended in sample buffer described previously and samples were subjected to SDS-PAGE. A portion of immunoprecipitation elute was used for autoradiography, while a smaller portion was then used for immunoblotting and probed for anti-GFP (to determine immunoprecipitation consistency). For autoradiography, gels were dried and exposed onto Carestream Kodak BioMax MS film (Sigma, catalog #2363022) at -80°C for 2-5 days until developed.

2.2.7 Immunocytochemistry

PC12 cells stably expressing VMAT2-GFP were plated onto poly-D-lysine-coated glass coverslips and maintained in DMEM containing 0.05% fetal bovine serum and 1% penicillin/streptomycin. Cells were differentiated with 100 ng/mL nerve growth factor (NGF,

Promega, catalog #G5141) for 3 days, given every other day. During this time, cells developed neurite-like extensions to other cells and a more neuronal-like morphology. Following differentiation, cells were treated with DMSO vehicle for 6 or 24 hours, 5 μM MG132 for 6 hours, or 25 μM E64/200 μM AEBSF for 24 hours. Staining was performed at room temperature following a protocol established by the Center for Biologic Imaging at University of Pittsburgh. Coverslips were washed 3 times with PBS and fixed in 2% paraformaldehyde for 15 minutes. Cells were then washed another 2 times with PBS and permeabilized with 0.1% Triton x-100 for 15 minutes. Cells were washed 3 times with PBS, a further 5 times with 0.5% BSA in PBS (PBB) and blocked in 5% normal goal serum diluted in PBB for 45 minutes. Samples were then washed 4 times with PBB and incubated in primary antibody diluted in PBB for 1 hour. Non-specific staining by secondary antibodies were assessed by incubating coverslips with PBB instead of primary antibody. Coverslips were washed 4 times in PBB and incubated in secondary antibody diluted in PBB for 45 minutes. Cells were washed with PBB 5 times and incubated in 1 mg/100 mL Hoeschst stain for approximately 30 seconds to stain nuclei. Coverslips were then washed 4 times with PBS and affixed to slides with fluoromount. Slides were stored at 4°C until imaging. 1 micron thick Z stack images were obtained on a Nikon A1R confocal using a 60x oil objective and the NIS Elements Imaging software. Co-localization analysis to determine Manders' Coefficients was performed using ImageJ software (NIH, Bethesda, MD, JACoP plugin) following background subtraction.

2.2.8 Live Cell Imaging

These experiments were performed at the McKnight Brain Institute of the University of Florida (Gainesville, Florida) under the excellent guidance of Dr. Habibeh Khoshbouei and Danielle

Sambo. PC12 cells stably expressing VMAT2-GFP were plated into poly-D-lysine-coated Mat-Tek dishes (#P35G-1.5-14-C) and maintained in phenol-red-free DMEM containing 0.05% fetal bovine serum and 1% penicillin/streptomycin. Cells were differentiated with 100 ng/mL nerve growth factor (NGF, Promega, catalog #G5141) for 3-5 days, given every other day, until cells displayed neuronal-like morphology. Cells were imaged on a Nikon A1RMPsi-STORM4.0 multiphoton/super resolution imaging system with CO_2 and thermal regulation (kept at 5% and 37°C, respectively). Images were obtained using a 60x oil objective and the NIS Elements Imaging software. Z stack sampling was performed every 30 minutes at multiple points in the dishes following the addition of DMSO vehicle, 5 μM MG132, or 25 μM E64/200 μM AEBSF. Whole cell fluorescence intensity analysis was performed using Nikon NIS Elements software after background subtraction. Line analysis was performed using ImageJ, also following background subtraction.

2.2.9 Vesicular Uptake

Crude vesicular preparations were made as described in Parra et al. 2008 (Parra et al., 2008). PC12 cells stably expressing VMAT2-GFP were washed two times with PBS and re-suspended in 150 mM sodium chloride, 10 mM HEPES, 0.5 mM EGTA, 0.1 mM magnesium chloride, 320 mM sucrose, pH 7.4 plus protease inhibitor cocktail. Cells were homogenized by 10 strokes in a glass/teflon hand homogenizer and then passed through a 25 gauge needles 25 times. Homogenate was centrifuged at 4°C for 5 minutes at 1000 x g. The supernatant (S1) was centrifuged at 4°C for 35 minutes at 27000 x g. The resulting supernatant (S2) was further centrifuged at 4°C for 2 hours at 180,000 x g. The subsequent pellet (P3, a crude vesicular-enriched prep) was used in vesicular uptake assays.

Radioactive vesicular uptake assays were performed as described in Requena et al. 2009 and Parra et al. 2008 (Parra et al., 2008, Requena et al., 2009). P3 preps were re-suspended in 10 mM HEPES, 0.5 mM EGTA, 0.1 mM magnesium chloride, 200 mM sucrose, pH 7.4. Uptake buffer consisted of 20 mM HEPES, 100 mM potassium tartrate, pH 7.4 with the addition of 2.5 mM magnesium sulfate and 2 mM ATP. Nonspecific uptake was determined using 100 μM reserpine, a potent VMAT1 and VMAT2 inhibitor. Since PC12 cells also contain VMAT1, VMAT2-specific uptake was determined using 1 μM tetrabenazine (TBZ), a VMAT2-specific inhibitor (Erickson et al., 1996, Holtje et al., 2000). Uptake buffer and 200 μg vesicular prep were incubated at 30°C for 10 minutes. 0.1 μM [^{3}H]-serotonin (Perkin Elmer, catalog #NET4980) was added and samples were incubated at 30°C for 6 minutes. Background was determined under the same conditions, except without the addition of vesicular prep. Uptake was terminated by the addition of cold buffer (20 mM HEPES, 100 mM potassium tartrate, pH 7.4) and filtered through 0.2 μm filter paper (PALL supor 200, catalog #60305) that had been soaked in 0.3% polyethleneimine for at least 30 minutes. Filter paper was washed with cold uptake buffer and [^{3}H]-serotonin on the filter paper was determined using a scintillation counter. Background values were subtracted from total uptake and uptake with reserpine or TBZ. Uptake with reserpine or TBZ are graphed as percentage of total uptake.

2.2.10 MTS Assay

To determine cytotoxicity of drugs utilized, a colorimetric MTS assay was used according to manufacturer's instructions (Abcam). 10-15 x 10^3 cells were plated in poly-D-lysine coated 96 well plates and treated up to 18 hours with the aforementioned drug concentrations or the appropriate DMSO vehicle percentage. Following drug treatment, the MTS solution was added

and cells were incubated for 30 minutes at 37°C. After brief shaking, absorbance was read on a plate reader at 490 nm. Data from drug treated cells were calculated as percentage of vehicle for analysis. For comparison, drug treatments at later times were compared to drug treatment time at 1 hour, as no true '0 hour' timepoint existed due to the 30 minute incubation time with MTS.

2.2.11 Statistics

Western blot bands were quantified using ImageJ (NIH, Bethesda, MD). VMAT2-GFP density values were normalized to tubulin density values prior to further analysis. All graphs were plotted and statistics performed on GraphPad Prism (GraphPad Software, La Jolla, CA, www.graphpad.com). Most graphed data are represented as mean +/- SEM or as individual points with the mean indicated. Unless otherwise indicated, data were analyzed using Kruskal-Wallis non-parametric ANOVAs and Dunn's multiple comparison post-hoc tests. Data from time-lapse live imaging experiments were analyzed using repeated measures ANOVAs, as well as Dunnett's multiple comparison post-hoc tests. An unpaired two-tailed t-test was used to analyze co-localization data.

3.0 RESULTS

3.1 DETERMINATION OF VMAT2 HALF-LIFE

To begin to understand VMAT2 degradation mechanisms, I first examined the half-life of the transporter. To my knowledge, nothing has been published concerning the half-life of VMAT2. Because VMAT2 is a glycosylated, 12 transmembrane domain transporter, it is assumed to be a very stable protein and the half-life is expected to be quite long. 50 μg/mL of cycloheximide, a protein synthesis inhibitor, was used to determine VMAT2 half-life when transiently over-expressed in PC12 cells (Anwar et al., 2011). As demonstrated in Figure 9, the mature form of VMAT2 levels decreased with increasing cycloheximide treatment time, reaching approximately 50% of control (0 hours incubation) by 12 hours incubation. However, this is a relatively long cycloheximide treatment time, as cycloheximide is very toxic at long incubation times. Indeed, I observed reduced protein content at later time points, indicating cell death. Treatment of cells with a reduced concentration of cycloheximide, 10 μg/mL, also resulted in significant cell death at longer incubation times. These observations lead me to believe that the cycloheximide method is a crude estimation of VMAT2 half-life, complicated by compromised cell functioning.

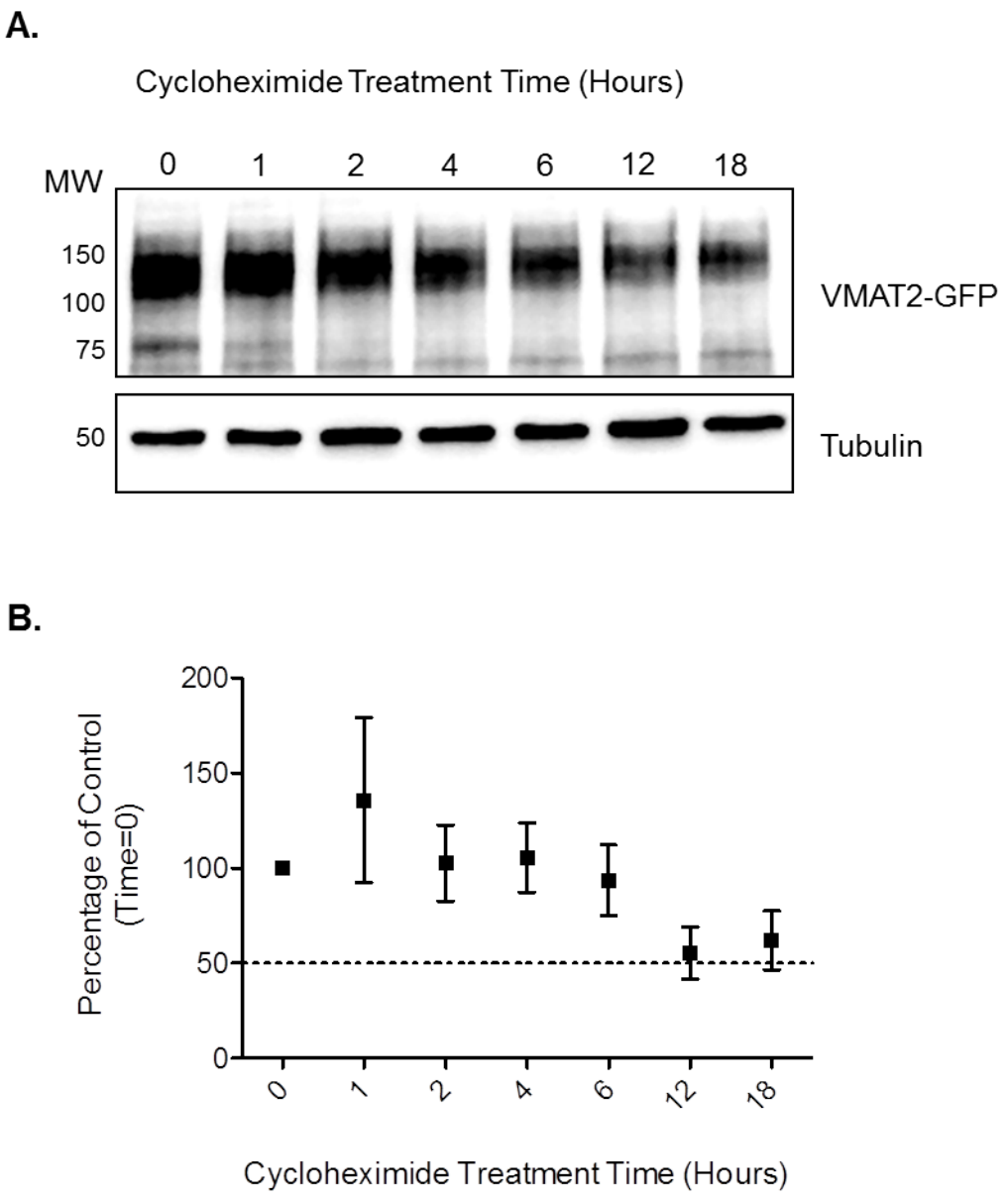


Figure 9. VMAT2 half-life following cycloheximide. PC12 cells transfected with VMAT2-GFP were incubated with 50 μg/mL cycloheximide to inhibit protein synthesis. Cells were lysed at the indicated times and subjected to immunoblotting. (A) Membranes were probed with anti-GFP and anti-tubulin. (B) Mature VMAT2-GFP was quantified using ImageJ and normalized to tubulin levels. Data from 3 independent experiments are expressed as mean +/- SEM.

Due to the limitations of using cycloheximide for longer periods of time, a pulse-chase method was then used to determine VMAT2 half-life in PC12 cells stably expressing VMAT2-GFP. Results from the cycloheximide experiments and previous data concerning other transmembrane proteins indicate that VMAT2 turnover is likely relatively slow. Because of this, cells were 'pulsed' for a long period of time with radiolabeled cysteine/methionine (24 hours) in order to maximize the radiolabeled mature VMAT2 that would be synthesized during that time period. Cells were then 'chased' for an even longer period of time—up to 48 hours. Using this method, the half-life of mature VMAT2 is approximately 35 hours, as shown in Figure 10. There is discrepancy between the VMAT2 half-life value using the cycloheximide method or the pulse-chase method. However, the value obtained using the pulse-chase method more closely resembles what has been previously reported for plasma membrane transporters; to my knowledge the half-life for synaptic vesicular transporters has not been reported (Chu and Doyle, 1985, Vicentic et al., 1999, Kimmel et al., 2001). Furthermore, the pulse-chase methodology does not result in massive cell death, while long cycloheximide incubations do, yielding results less able to interpret. It should be noted that this is an estimation of VMAT2 in an overexpression cell model system; *in vivo* estimations for endogenous protein could be quite different.

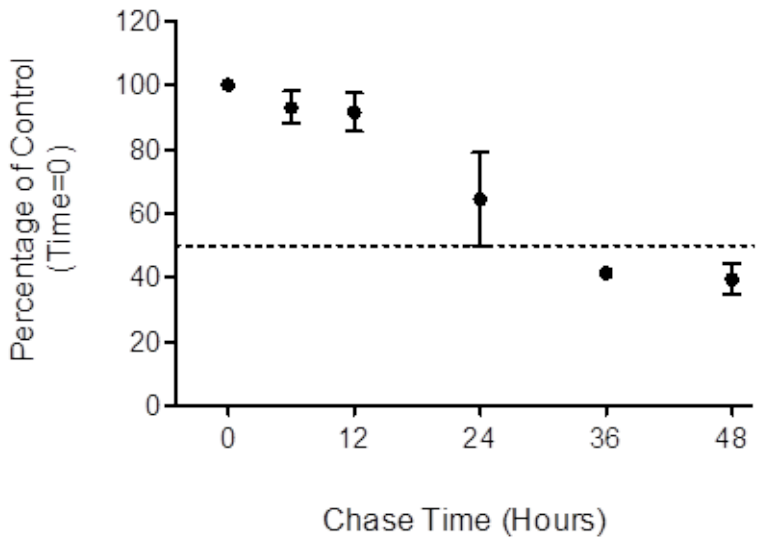


Figure 10. VMAT2 half-life according to the pulse-chase method. PC12 cells stably expressing VMAT2-GFP were 'pulsed' with radiolabeled methionine/cysteine for 24 hours, then 'chased' for up to 48 hours. Samples underwent SDS-PAGE and autoradiography for detection of radiolabeled VMAT2. Mature VMAT2 was quantified using ImageJ. Data from 3 independent experiments are expressed as mean +/- SEM.

3.2 EFFECT OF PROTEASOME OR LYSOSOME INHIBITION ON VMAT2

3.2.1 VMAT2 levels

The goal of this project was to investigate mechanisms by which mature VMAT2 is degraded. Although synaptic vesicular proteins are traditionally thought to be degraded by the lysosome, few researchers have sought to examine the degradation of specific synaptic vesicular proteins. To do this, I primarily utilized pharmacological tools available to inhibit the function of lysosomes or proteasomes. To inhibit lysosomal activity, I used E64 plus AEBSF. E64 is an inhibitor of lysosome-specific cysteine-type cathepsin proteases (Barrett et al., 1982, Ahlberg et

al., 1985). Since other types of proteases are in lysosomes, I also used AEBSF, a common serine protease inhibitor (Kollmann et al., 2009). While AEBSF has been reported to affect the trypsin-like catalytic site of the 26S proteasome, it does not affect the size of degradation products, suggesting its impact on overall proteasomal function is minimal (Kisselev et al., 1999). Other lysosomal inhibitors that disrupt the acidic pH of the lysosome are more commonly used and more effective but were not used here as they could also inadvertently disrupt vesicular pH gradients required for normal VMAT2 function (Ahlberg et al., 1985, Wilcox and Mason, 1992). To inhibit the proteasome, I primarily used the more common MG132, a peptide aldehyde that reversibly inhibits the chymotrypsin-like site of the 26S proteasome (Rock et al., 1994, Lee and Goldberg, 1996, Kisselev et al., 2012). Epoxomicin, a more specific inhibitor, was also used to confirm findings obtained with MG132 treatment. Epoxomicin is an epoxyketone that irreversibly binds to the β subunits in the 20S proteasome core, effectively inhibiting all three major catalytic sites (Meng et al., 1999, Sin et al., 1999, Kisselev et al., 2012).

PC12 cells stably expressing VMAT2-GFP were treated with 25 μM E64 and 200 μM AEBSF for up to 18 hours. I then lysed the cells and ran equal amounts of proteins for western blotting. Membranes were probed with anti-GFP to detect VMAT2 and anti-tubulin (Figure 11A is a representative blot). As shown in Figure 11, at no point during the observed treatment time did lysosomal inhibition significantly affect mature or immature VMAT2 levels. As a control for E64 effectiveness, blots were also probed with anti-ubiquitin (clone PD41). As seen in Figure 12, higher molecular weight (above 100 kD) polyubiquitinated proteins accumulated after E64 treatment for 12 and 18 hours (non-parametric ANOVA $p=0.0198$; Dunn's post-hoc test $p<0.05$ for 12 vs 0 hours and 18 vs 0 hours). These data suggest E64 is entering the cells and having an overall effect, although not on VMAT2 under these conditions.

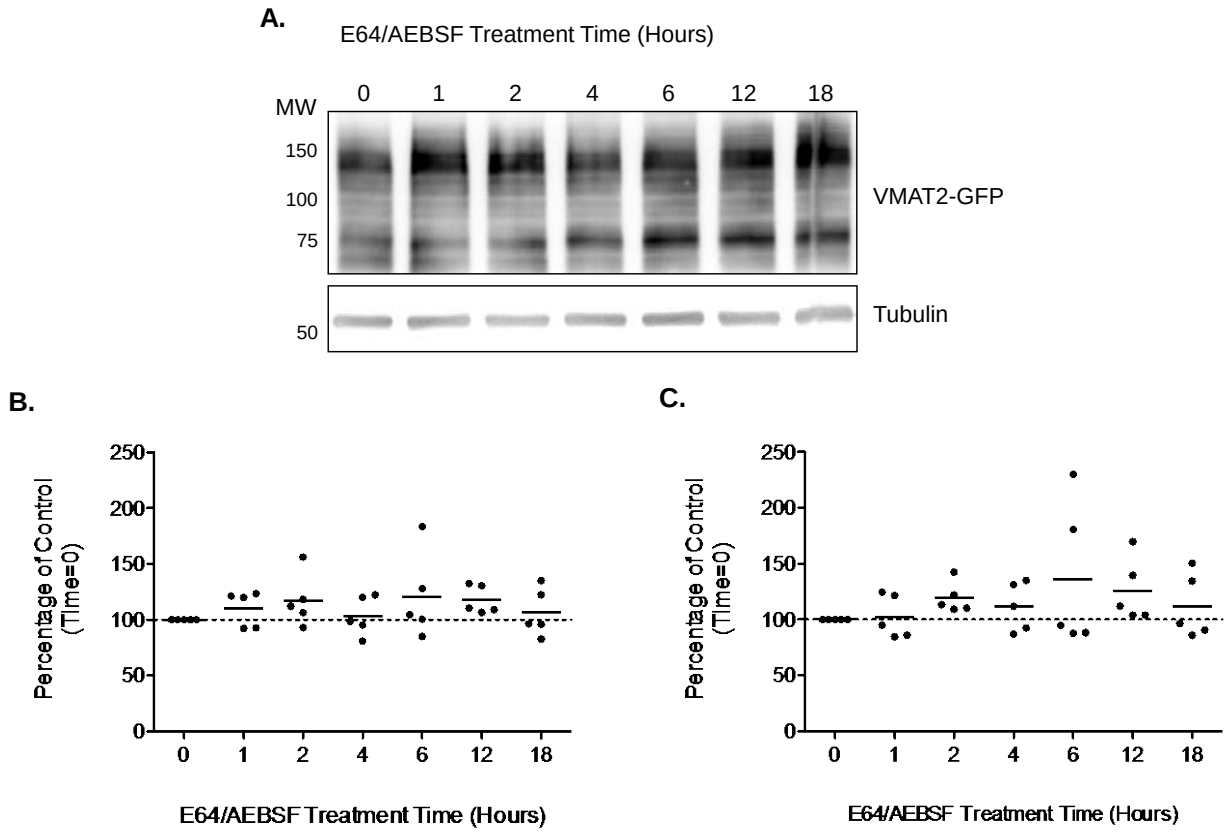


Figure 11. VMAT2 does not accumulate following lysosomal inhibition. PC12 cells stably expressing VMAT2-GFP were incubated with 25 μM E64 and 200 μM AEBSF to inhibit lysosomal function. Cells were lysed at the indicated times and subjected to immunoblotting. (A) Membranes were probed with anti-GFP and anti-tubulin. Mature (B) and immature (C) VMAT2 was quantified using ImageJ. Individual points with the mean indicated are plotted from 5 independent experiments.

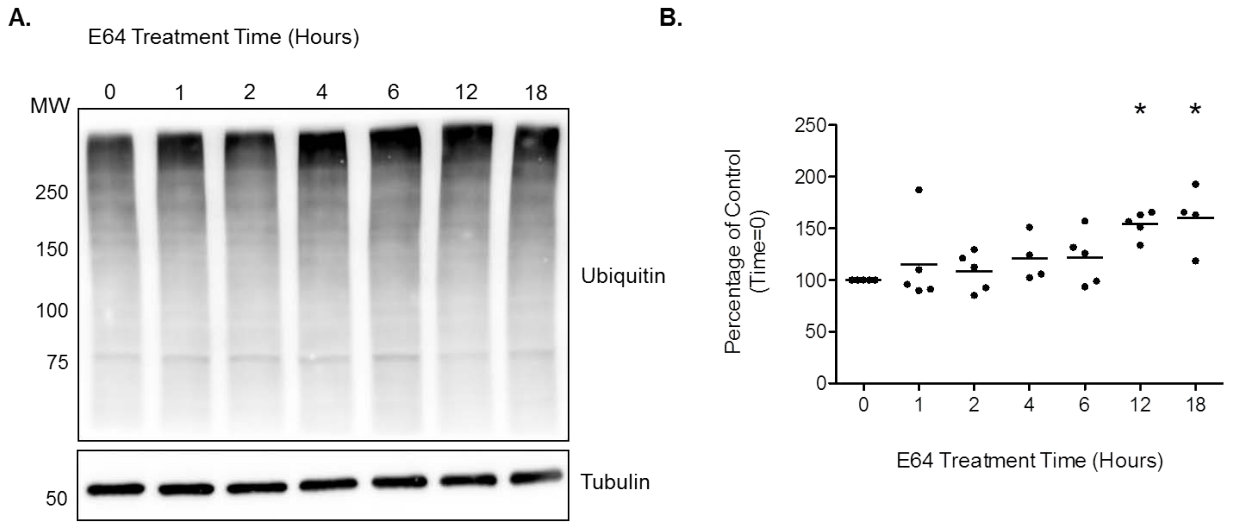


Figure 12. Polyubiquitinated proteins accumulate after lysosomal inhibition. PC12 cells stably expressing VMAT2-GFP were incubated with 25 μM E64 to inhibit lysosomal function. Cells were lysed at the indicated times and subjected to immunoblotting. (A) Membranes were probed with anti-ubiquitin and anti-tubulin. (B) Polyubiquitinated proteins above 100 kD were quantified using ImageJ. Individual points with the mean indicated are plotted from 5 independent experiments.* indicates statistical significant difference from the 0 time point.

PC12 cells stably expressing VMAT2-GFP were similarly treated with 10 μM MG132. In contrast to results obtained with E64/AEBSF, MG132 treatment resulted in accumulation of mature VMAT2, shown in Figure 13. Inhibition of the proteasome also resulted in an even greater accumulation of the immature form of VMAT2. Interestingly, mature VMAT2 began to accumulate at an earlier incubation time than immature VMAT2. Although there was a trend at earlier time points, immature VMAT2 did not significantly increase compared to the 0 time point until 6, 12 and 18 hours of MG132 incubation (non-parametric ANOVA $p<0.0001$; Dunn's post-hoc test $p<0.05$ for 6 vs 0 hours, 12 vs 0 hours and 18 vs 0 hours). Mature VMAT2 did not significantly increase compared to the 0 time point until 12 and 18 hours of MG132 incubation (non-parametric ANOVA $p=0.0332$; Dunn's post-hoc test $p<0.05$ for 12 vs 0 hours and 18 vs 0 hours).

To confirm these results, cells were also treated with 250 nM epoxomicin, a more specific proteasome inhibitor. Peptide aldehydes, but not, to my knowledge, epoxomicin, have been reported to inhibit calpains and cysteine proteases (Sasaki et al., 1990, Rock et al., 1994, Tsubuki et al., 1996). As shown in Figure 14, both mature and immature forms of VMAT2 accumulated following incubation, confirming results observed following MG132 treatment. However, the changes in both mature and immature VMAT2 did not reach statistical significance. The changes are more a trend and likely require increased sample size to reach statistical significance, although I am interpreting these results as indication of biological significance.

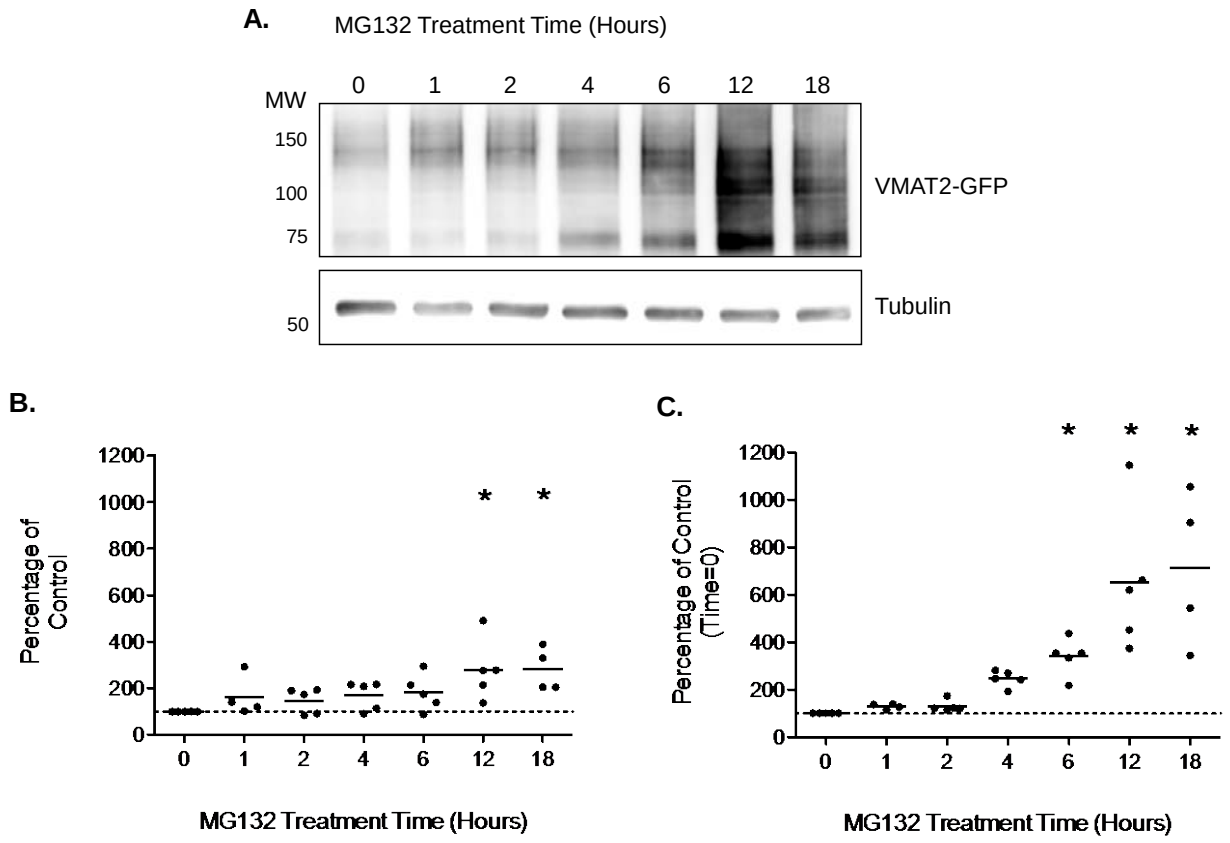


Figure 13. Mature and immature VMAT2 accumulate following proteasomal inhibition with MG132. PC12 cells stably expressing VMAT2-GFP were incubated with 10 μM MG132 to inhibit proteasome function. Cells were lysed at the indicated times and subjected to immunoblotting. (A) Membranes were probed with anti-GFP and anti-tubulin. Mature (B) and immature (C) VMAT2 was quantified using ImageJ. Individual points with the mean indicated are plotted from 5 independent experiments. * indicates statistical significant difference from the 0 time point.

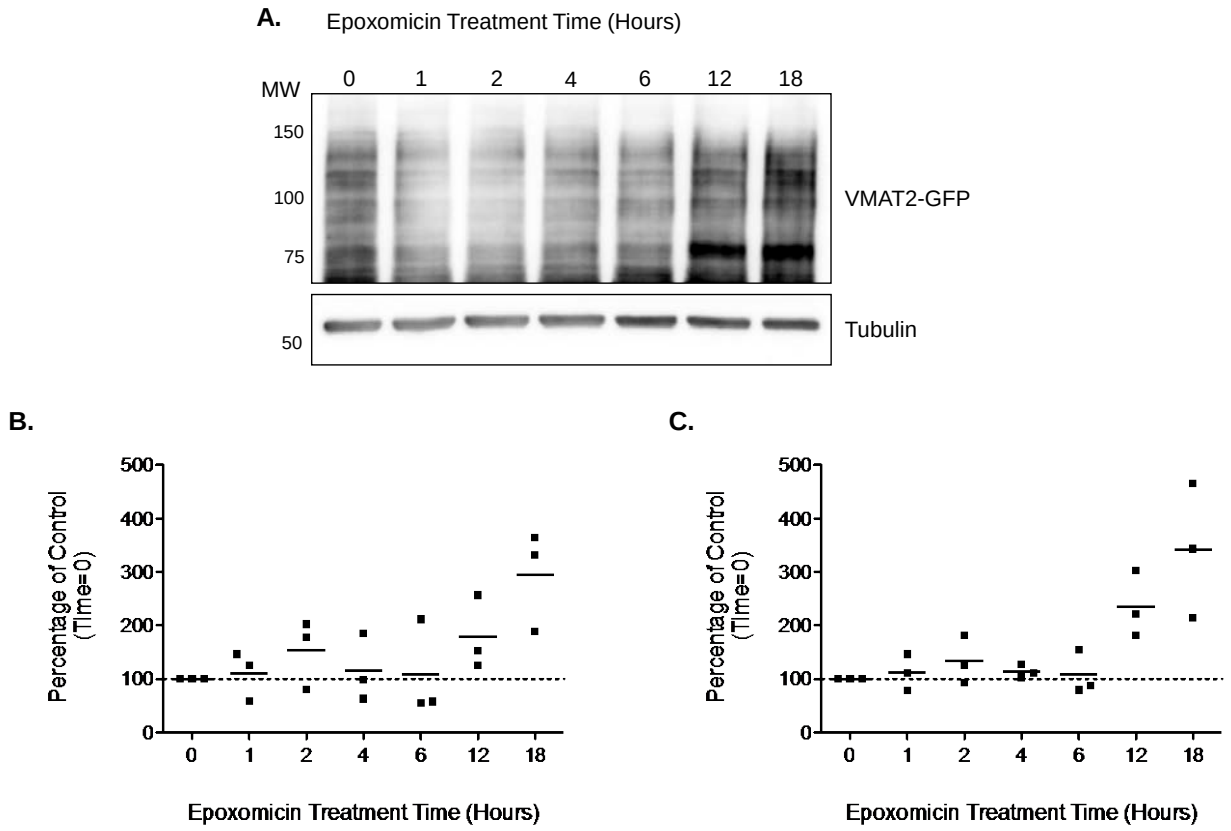


Figure 14. Mature and immature VMAT2 accumulate following proteasomal inhibition with epoxomicin. PC12 cells stably expressing VMAT2-GFP were incubated with 250 nM epoxomicin, a more selective proteasome inhibitor. Cells were lysed at the indicated times and subjected to immunoblotting. (A) Membranes were probed with anti-GFP and anti-tubulin. Mature (B) and immature (C) VMAT2 was quantified using ImageJ. Individual points with the mean indicated are plotted from 3 independent experiments.

Since the process of ERAD depends on proteasomal function, inhibiting the actions of proteasomes also ultimately inhibits the process of ERAD. To determine if the effects on mature VMAT2 following MG132 or epoxomicin treatment were due solely to ERAD inhibition, cells were treated with 10 μM eeyarestatin I, an inhibitor of p97 (and perhaps Sec61) that's specifically targeted to the ER, effectively preventing retrotranslocation (Wang et al., 2008, Cross et al., 2009, Wang et al., 2010). While immature VMAT2 accumulated following eeyarestatin I, mature VMAT2 did not (shown in Figure 15; non-parametric ANOVA for immature VMAT2 $p<0.05$, Dunn's post-hoc test $p<0.05$ for 18 vs 0 hours). In fact, there is a trend toward a reduction in mature VMAT2-GFP, although this was not statistically significant. These data indicate that accumulation of the mature form of VMAT2 after MG132 or epoxomicin is not due to ERAD inhibition whereas the accumulation of immature VMAT2 following MG132 or epoxomicin treatment could be due to ERAD inhibition. However, p97 has also been reported to be involved in ER to Golgi trafficking (Zhang et al., 1994, Roy et al., 2000). Therefore, these results may reflect inhibition of that process by eeyarestatin I and not eliminate the possibility of an effect of ERAD inhibition on mature VMAT2 accumulation following inhibition of the proteasome. Dalal and co-authors have called into question the involvement of p97 in ER to Golgi trafficking, as results from dominant negative expression of mutant p97 did not support this claim (Dalal et al., 2004). However, the authors observed the formation of ER vacuoles following induction of the dominant negative mutant p97, which could then impact not only ER function, but ER to Golgi trafficking. These results could be clarified utilizing a specific ERAD inhibitor that does not directly impact trafficking in the secretory pathway.

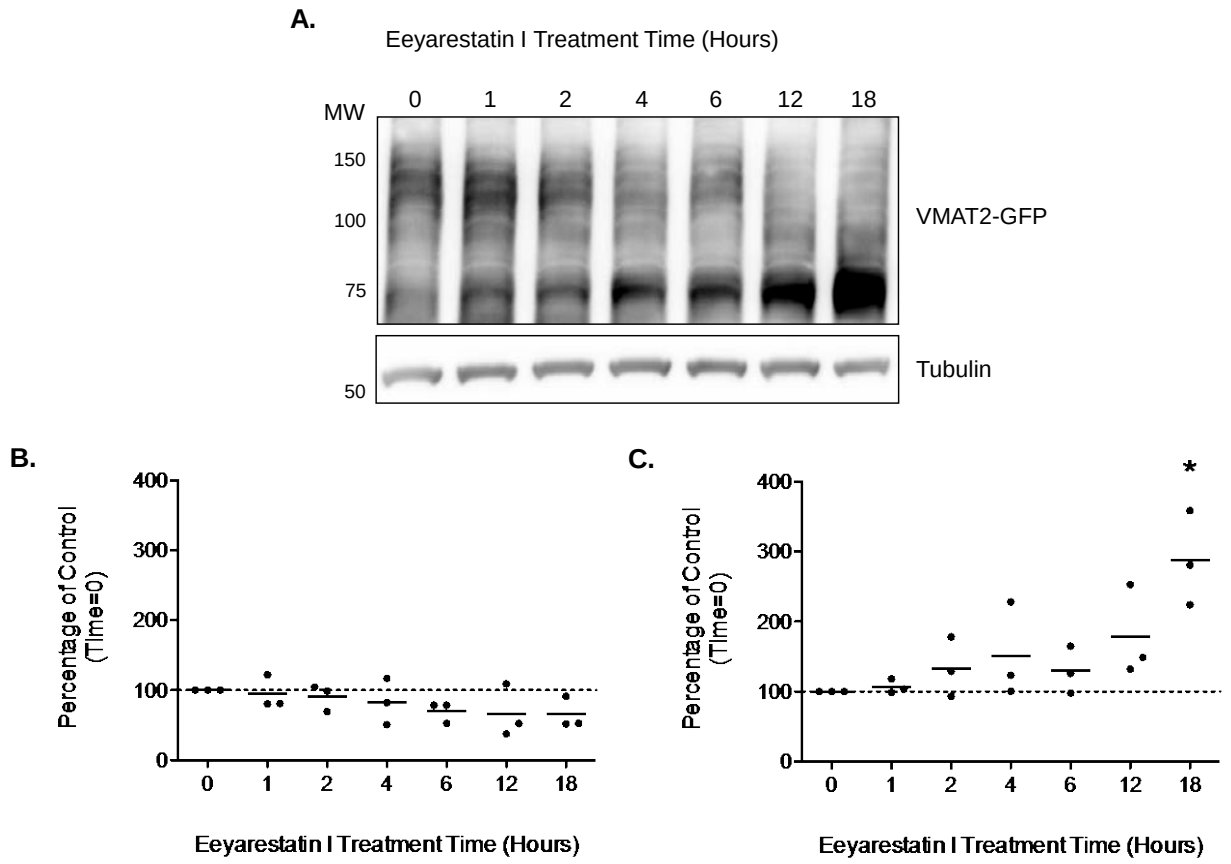


Figure 15. Immature VMAT2 accumulates following ERAD inhibition. PC12 cells stably expressing VMAT2-GFP were incubated with 10 μM eeyarestatin I, an ERAD inhibitor. Cells were lysed at the indicated times and subjected to immunoblotting. (A) Membranes were probed with anti-GFP and anti-tubulin. Mature (B) and immature (C) VMAT2 was quantified using ImageJ. Individual points with the mean indicated are plotted from 3 independent experiments * indicates statistical significant difference from the 0 time point.

Based on the estimated VMAT2 half-life reported here and other reports of long half-life for most transporters, there's reason to believe the turnover time for VMAT2 is fairly long. Because of this, I used relatively long drug incubation times. Inhibiting degradation systems, especially inhibiting proteasomal function, has deleterious consequences on overall cell functioning (Lam and Cadenas, 2008, Wang et al., 2015a). MTS Assays were performed to address the degree of compromised cellular functioning during drug treatments. While there was a slight decrease in cellular viability over time for most drug treatments, as shown in Figure 16, there was not massive cell death at the indicated drug times and concentrations. Data are represented as percentage of DMSO vehicle control, taking into account any reduction in cell viability due to the presence of DMSO, although DMSO itself did not result in significant loss of cell viability (data not shown). There was a statistically significant reduction in cellular function 25 μM E64/200 μM AEBSF treatment after 12 hours of drug treatment (non-parametric ANOVA $p=0.0736$, Dunn's post-hoc test $p<0.05$ for 12 hours vs 1 hour treatment time). Although not reaching statistical significance, there is a consistent observed reduction in cell function following 10 μM MG132 and epoxomicin treatment. These data suggest that caution should be used in interpreting data from long incubation times of compounds inhibiting both the proteasome and lysosome, as cellular function is compromised. However, with any drug treatment, cell function was not reduced more than 15%, indicating massive cell death was not occurring.

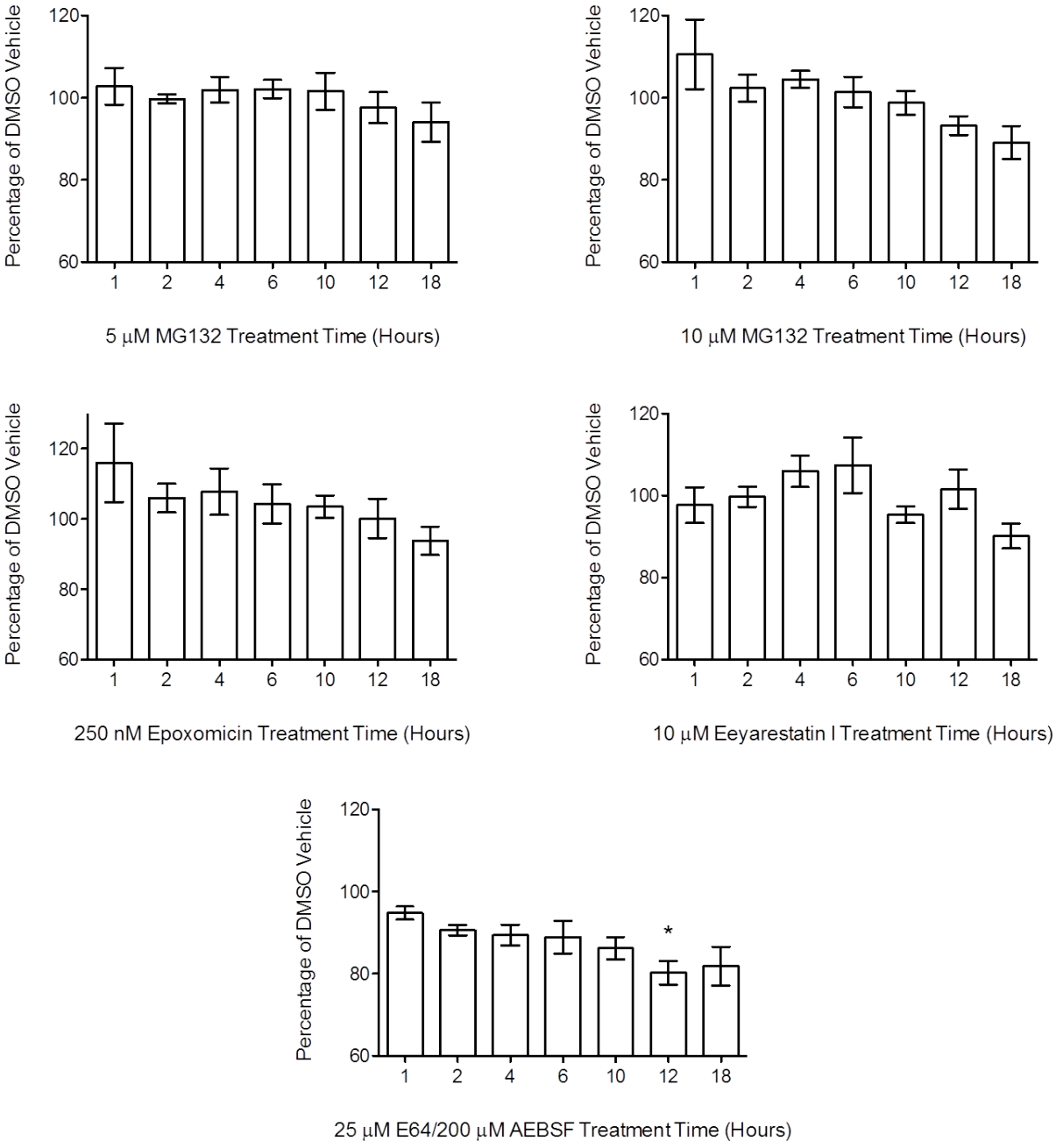


Figure 16. Cell viability following drug treatments. PC12 cells stably expressing VMAT2-GFP were incubated with the indicated drugs for up to 18 hours. Cell viability was assessed using a colorimetric MTS assay. Data expressed as mean +/- SEM are represented as percentage of DMSO vehicle and are from 4 independent experiments. * indicates significant difference from the 1 hour time point.

3.2.2 VMAT2 half-life

Data collected from whole cell lysate is the product of a sum of multiple processes. Hence, total VMAT2 levels following inhibition of degradation are a product of both degradation and synthesis. To more directly examine VMAT2 degradation, I again employed the pulse-chase assay. To measure the effect of inhibiting lysosomal function on VMAT2 half-life, PC12 cells stably expressing VMAT2-GFP were 'pulsed' for 24 hours, and 'chased' for long periods of time—up to 48 hours. During the chase period, cells were treated with DMSO vehicle or 25 μM E64 and 200 μM AEBSF. As seen in Figure 17, inhibition of lysosomal function did not affect the degradation rate or half-life of VMAT2. These data strongly indicate that under basal conditions, VMAT2 degradation is unaffected by lysosomal inhibition.

Due to the cumulative toxic effects of MG132 over long treatment times, it was not feasible to repeat this experiment while inhibiting the proteasome. In lieu of this, pulse-chase assays were performed with a shorter, 1 hour 'pulse' time and shorter 'chase' times, up to 8 hours. Under these circumstances, MG132 had no effect on the half-life of mature VMAT2. There was a trend toward MG132 treatment increasing the half-life of immature VMAT2. As before, control western blots were also performed on lysate and immunoprecipitation samples from the pulse-chase assays. Upon further investigation, as seen in Figure 18E, MG132 seems to affect the degree of VMAT2 immunoprecipitation. While more immature VMAT2 was immunoprecipitated following MG132 treatment, less mature VMAT2 was immunoprecipitated. This may indicate that inhibiting the proteasome may result in accumulation of modified mature VMAT2 that interferes with antibody-protein binding. Importantly, the levels of immunoprecipitated VMAT2 were unaffected by lysosomal inhibition, indicating E64/AEBSF treatment did not interfere with antibody-protein binding (refer to Figure 17E). A variety of

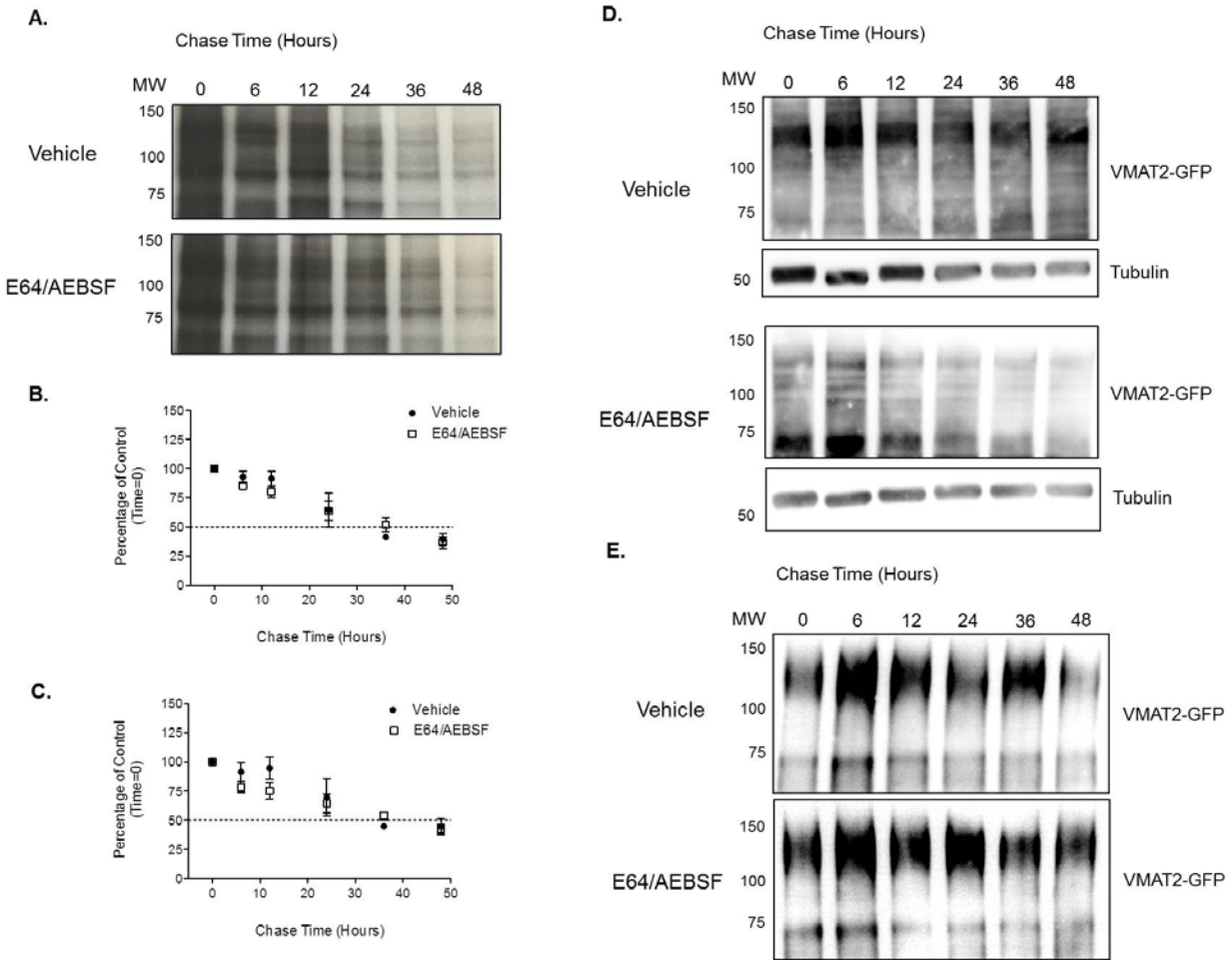


Figure 17. VMAT2 half-life following lysosomal inhibition. PC12 cells stably expressing VMAT2-GFP were 'pulsed' with radiolabeled methionine/cysteine for 24 hours, then 'chased' for up to 48 hours with DMSO vehicle or 25 μM E64 and 200 μM AEBSF. (A) Samples underwent SDS-PAGE and autoradiography for detection of radiolabeled VMAT2. Mature (B) and immature (C) VMAT2 was quantified using ImageJ. Data from 3 independent experiments are expressed as mean +/- SEM. Control immunoblots were ran to determine VMAT2 levels in lysate (D) and to evaluate consistency of the immunoprecipitation (E).

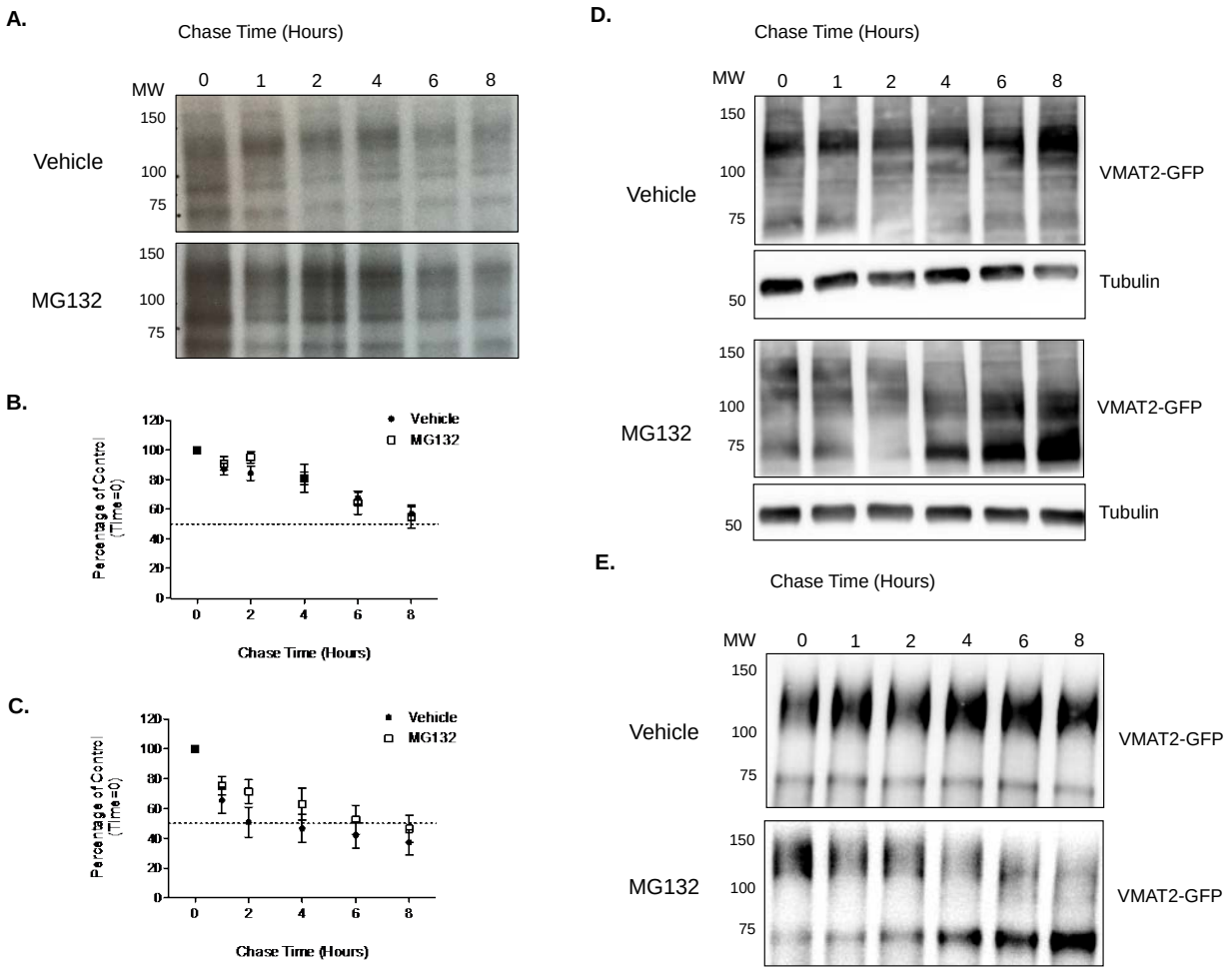


Figure 18. VMAT2 half-life following proteasomal inhibition. PC12 cells stably expressing VMAT2-GFP were 'pulsed' with radiolabeled methionine/cysteine for 1 hour, then 'chased' for up to 8 hours with DMSO vehicle or 5 μM MG132. (A) Samples underwent SDS-PAGE and autoradiography for detection of radiolabeled VMAT2. Mature (B) and immature (C) VMAT2 was quantified using ImageJ. Data from 3 independent experiments are expressed as mean +/- SEM. Control immunoblots were ran to determine VMAT2 levels in lysate (D) and to evaluate consistency of the immunoprecipitation (E). Note the inconsistent immunoprecipitation of VMAT2 following MG132 treatment compared to vehicle treatment.

methods were utilized to equalize the immunoprecipitation of VMAT2 following MG132 treatment, but were unsuccessful. Unfortunately, this issue renders the interpretation of the pulse-chase data difficult, as a reduction in radiolabeled VMAT2 may reflect a reduction in immunoprecipitated VMAT2 from the sample and not an accurate representation of reduced VMAT2.

3.2.3 VMAT2 cellular localization

The data obtained here strongly suggests VMAT2 accumulates following proteasomal, but not lysosomal inhibition. To confirm this and further assess the impacts of proteasomal and lysosomal inhibition on VMAT2 cellular localization, microscopy was used. Time-lapse multi-point microscopy was performed to visualize cells over time after being treated with MG132 or E64. PC12 cells stably expressing VMAT2-GFP were differentiated with nerve growth factor (NGF) for 3-5 days, until the cells began to develop a more neuronal morphology (protruding extensions that make connections with the extensions of other cells). Cells were then imaged for up to 10 hours following addition of DMSO vehicle, 5 μM MG132, or 25 μM E64/200 μM AEBSF. Sampling was performed every 30 minutes at multiple points within a dish. Figure 19 displays example images taken for each drug treatment at 0, 3, 6, and 9 hours. Overall, drug treatments caused many cells to retract their processes and 'ball up' or shrink—features characteristic of PC12 cells when insulted (Walkinshaw and Waters, 1994, Ivins et al., 1999). This was especially true following E64/AEBSF treatment, when many cells retracted processes, clumped together, and eventually perished. A subset of cells also appeared to display a collapse of the nucleus, indicating the function of these cells was severely compromised, sometimes followed by cell death. Although the nucleus was not labeled in these experiments, it is assumed

to be the circular area located in the PC12 cell soma devoid of VMAT2-GFP. The area immediately surrounding this VMAT2-GFP void has slightly increased levels of VMAT2-GFP and is assumed to be the ER and Golgi region; a clear example is shown in the "Vehicle" panel of Figure 18. Confirmation of these cellular locations could be confirmed with nucleus and ER markers used in live imaging studies. Likewise, definitive identity of the plasma membrane (to assess cell size) could also be determined using such tools. Analysis of fluorescent intensity of VMAT2-GFP revealed a significant, although subtle, increase in GFP intensity beginning at 7 hours following addition of MG132 (Figure 20, repeated measures ANOVA $p<0.0001$, Dunnett's post-hoc test $p<0.05$ for 7, 8, 9, and 10 hours treatment vs. 0 hour time point). This increase was not observed following addition of DMSO vehicle. The increase in VMAT2-GFP corroborates the increased VMAT2 protein levels following MG132 treatment seen in cell lysate. While there was a statistically significant overall effect of E64 treatment (repeated measures ANOVA $p=0.01$), post-hoc analysis did not yield significant differences between any treatment time point and the 0 hour time point, perhaps because of a low number of cells analyzed. VMAT2-GFP distribution across the soma was also analyzed following these drug treatments. As shown in Figure 21, MG132 treatment caused an accumulation of VMAT2-GFP near the nucleus, likely in or near the ER. Note the reduced size of what is assumed to be the nucleus (located around 10 microns) following MG132 or E64/AEBSF treatment, indicating collapse of the nucleus, caused by the toxic effects of these compounds.

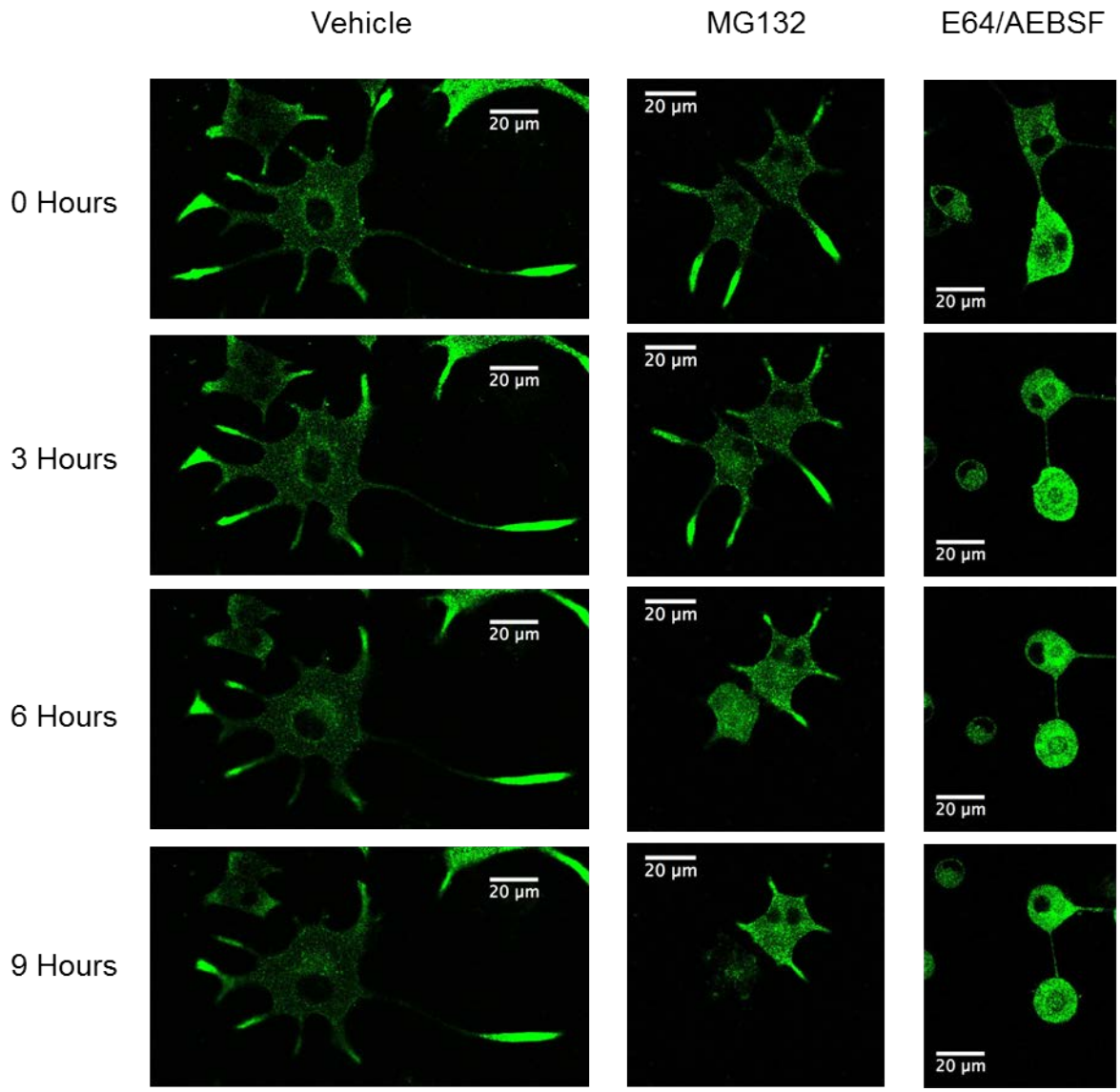


Figure 19. Time-lapse live cell imaging following proteasomal or lysosomal inhibition. PC12 cells were differentiated with NGF and treated for up to 10 hours with DMSO vehicle, 5 µM MG132, or 25 µM E64/200 µM AEBSF. During this time, z stacks were obtained every 30 minutes. Displayed here are example images from 0, 3, 6, or 9 hours drug treatment.

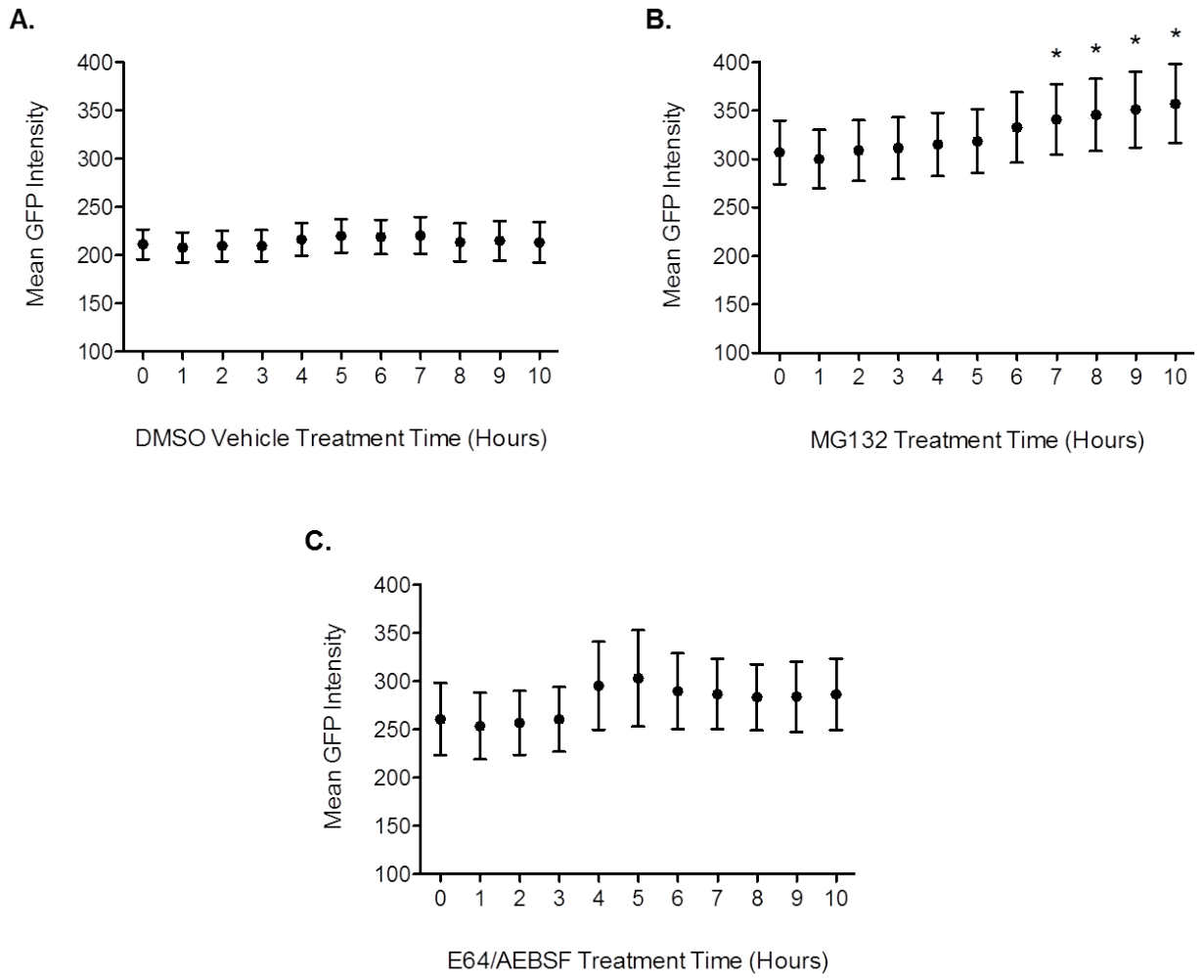


Figure 20. Proteasomal inhibition increases VMAT2-GFP levels during time-lapse live imaging. PC12 cells were differentiated with NGF and treated for up to 10 hours with (A) DMSO vehicle, (B) 5 μM MG132, or (C) 25 μM E64/200 μM AEBSF. Mean GFP fluorescent intensity was measured at each hour and plotted as mean +/- SEM. * indicates $p<0.05$ as compared to the 0 time point. For DMSO, MG132, and E64 treatment, the number of cells analyzed were 27, 57, and 10, respectively.

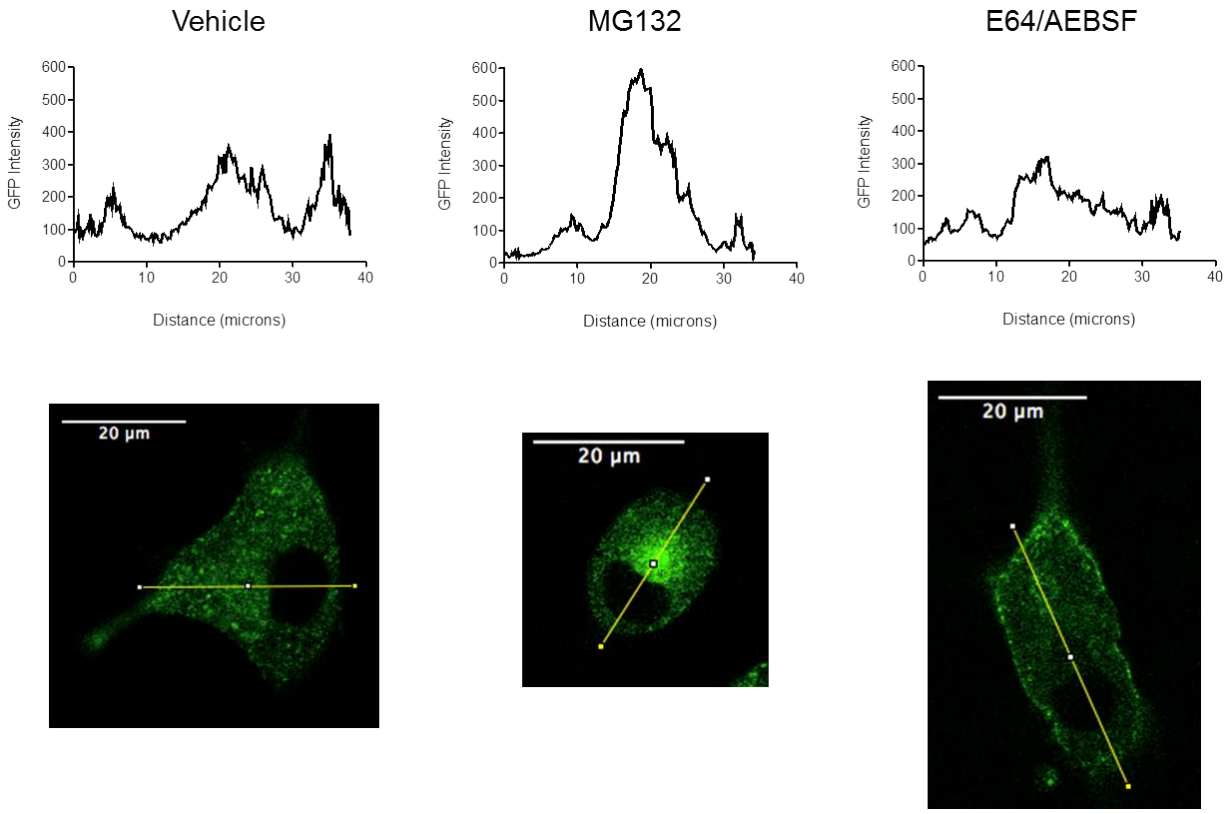


Figure 21. VMAT2-GFP distribution across the soma after 6 hours drug treatment. PC12 cells underwent live cell imaging after being treated with DMSO vehicle, 5 µM MG132, or 25 µM E64/200 µM AEBSF. Graphs are average GFP intensity across somas after 6 hours treatment. VMAT2-GFP intensity is increased and proximity to the nucleus is closer after MG132 treatment. The nucleus is around the 10 micron distance marker in all groups. Data are represented as mean (n=12 cells per treatment group). Example images are shown with yellow lines indicating where analysis was performed.

According to my previous data, immature VMAT2 accumulates to a great extent following proteasomal inhibition. It is probable that this accumulation is due to the inhibition of ERAD, resulting in the build-up of VMAT2 located at the level of the ER. To further confirm this, co-localization analysis was performed between VMAT2-GFP and PDI, an endoplasmic reticulum (ER) marker. PC12 cells stably expressing VMAT2-GFP were differentiated for 3 days with NGF, until cells displayed neurite-like extensions. Cells were then treated for 6 hours with 5 μM MG132 or DMSO vehicle, fixed, and stained to label VMAT2-GFP and PDI. Confocal images, shown in Figure 22, were obtained and co-localization analysis was performed to determine Manders Coefficient—the fraction of VMAT2-GFP that overlaps with PDI (Manders et al., 1993, Dunn et al., 2011). Following MG132 treatment, Manders Coefficient was increased, indicating more VMAT2 is co-localized with the ER following MG132 (t-test, $p<0.0001$). Although the resolution using the method is not high enough to definitively state that PDI and VMAT2 co-localize, this data indicates that immature VMAT2 is accumulating, likely at the ER, after proteasomal inhibition.

Similar co-localization experiments were attempted with VMAT2-GFP and LAMP1, a lysosomal marker, following E64/AEBSF treatment. Cells were treated for 24 hours with DMSO vehicle or 25 μM E64/200 μM AEBSF, fixed, and stained for GFP and LAMP1. Unfortunately, as demonstrated in Appendix B, LAMP1 labeling was quite extensive and appears to be nonspecific, including staining throughout the nucleus, yielding results difficult to interpret. Two different LAMP1 antibodies were used, both displaying similar degrees of non-specificity. A third LAMP1 antibody (Abcam, catalog #ab25630) and a LAMP2 antibody (Novus catalog #NB300-591) were also attempted, although neither resulted in a strong signal over background.

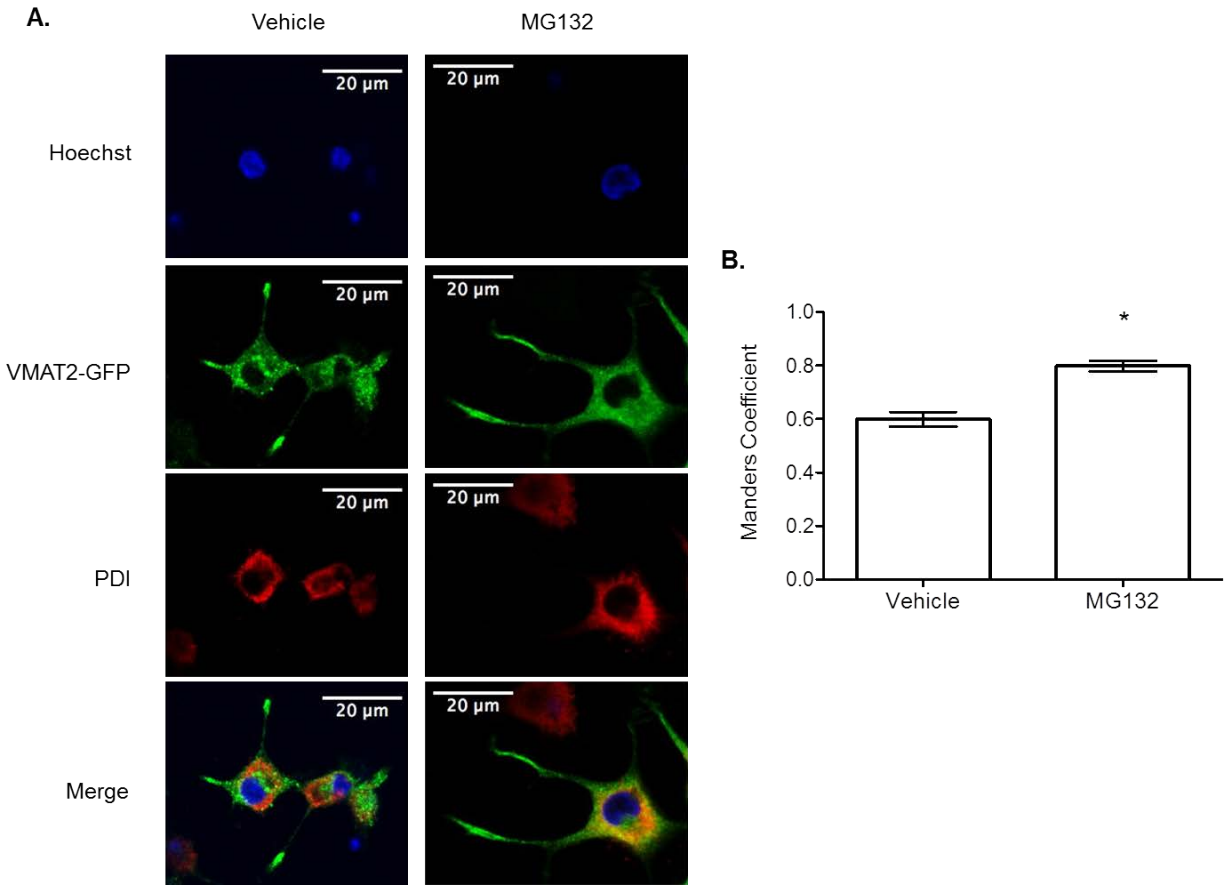


Figure 22. Proteasomal inhibition increases fraction of VMAT2 localized to the ER. PC12 cells were differentiated with NGF and treated for 6 hours with DMSO vehicle or 5 µM MG132. (A) Cells were stained for GFP (green), PDI (a marker of the endoplasmic reticulum, red), or Hoechst stain to label nuclei (blue). (B) Manders coefficient was calculated to assess the fraction of VMAT2-GFP co-localized with PDI and plotted as mean +/- SEM. * indicates $p<0.05$. For vehicle and MG132 groups the number of cells analyzed were 22 and 33, respectively.

3.3 VMAT2 UBIQUITINATION

Ubiquitination is a post-translational modification commonly associated with degradation. While primarily involved as a signal for degradation by the proteasome, ubiquitination can also be involved in trafficking or lysosomal degradation. Because of these roles of ubiquitination, I became interested in investigating the potential involvement of this modification in VMAT2 degradation. From PC12 cell lysate (stably expressing VMAT2-GFP), VMAT2 was immunoprecipitated, samples subjected to immunoblotting, and membranes were probed with anti-ubiquitin. Under basal conditions, I was unable to detect ubiquitinated VMAT2 under these circumstances. There is no indication under what circumstances VMAT2 may be ubiquitinated and it is possible that the level of basal VMAT2 ubiquitination is quite low and beyond my detection capability using this method.

To increase a potential signal, allowing for detection of VMAT2 ubiquitination, cells were treated for 12 hours with 10 μM MG132 or 25 μM E64 and 200 μM AEBSF. Immunoprecipitations and immunoblotting was performed as described and membranes were again probed with anti-ubiquitin. As seen in Figure 23A, MG132, but not E64/AEBSF treatment resulted in the detection of ubiquitinated VMAT2. As 12 hours is a fairly long incubation time, this experiment was repeated with 6 hours incubation time. A slightly decreased, but still present, ubiquitinated VMAT2 was detected after proteasomal, but not lysosomal, inhibition as shown in Figure 23B. These data indicated ubiquitinated VMAT2 accumulates following inhibition of the proteasome, but not lysosome.

To further strengthen this hypothesis that ubiquitinated VMAT2 accumulates following inhibition of the proteasome, these experiments were repeated, probing membranes with K48-specific ubiquitin or K63-specific ubiquitin antibodies. K48-linked polyubiquitin chains are

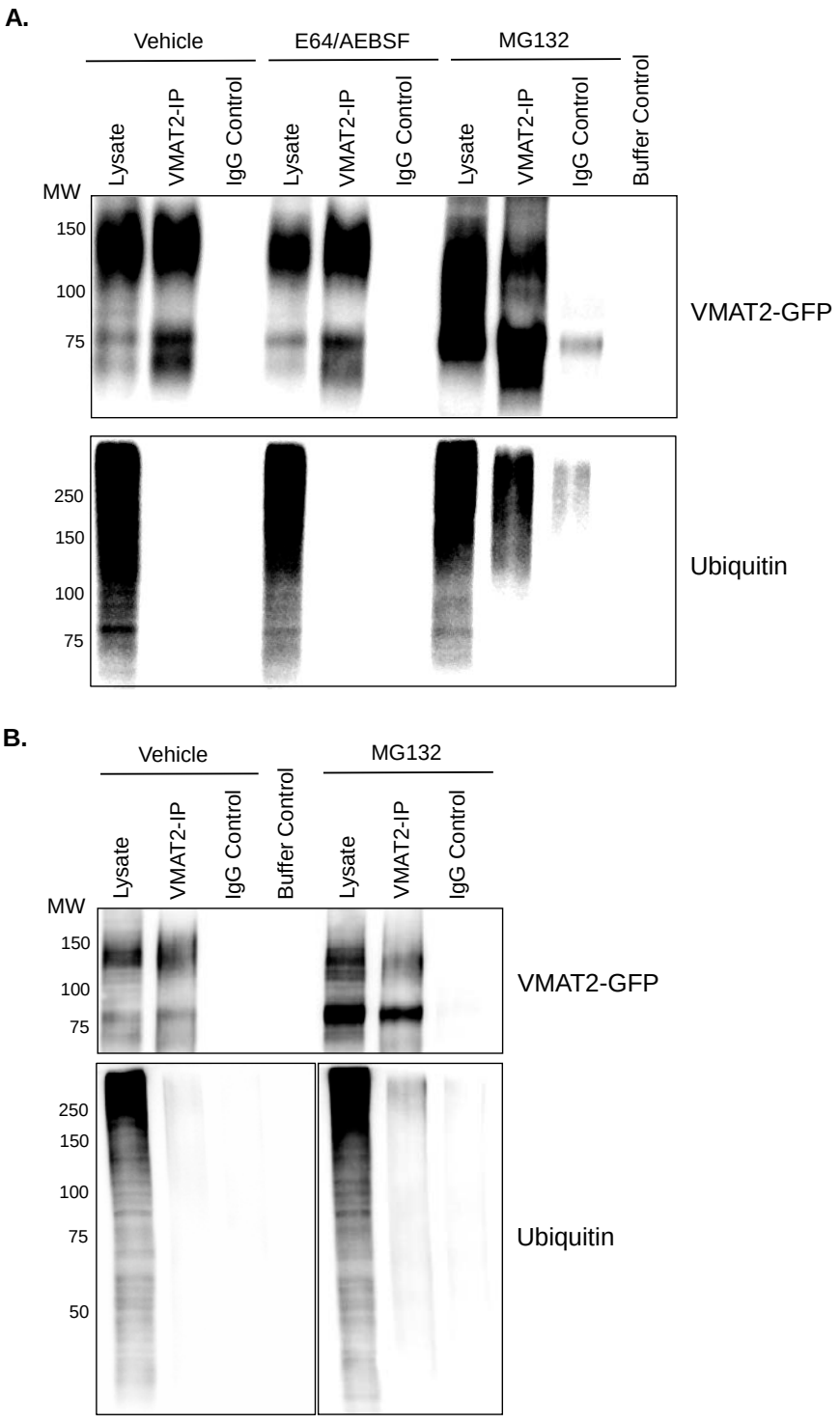


Figure 23. Proteasomal inhibition increases ubiquitinated VMAT2. PC12 cells stably expressing VMAT2-GFP were treated with DMSO vehicle, 25 μM E64/200 μM AMEBSF, or 10 μM MG132 for (A) 12 or (B) 6 hours. VMAT2 was immunoprecipitated from cell lysate using a VMAT2 antibody (C20, Santa Cruz) or goat IgG as a control. Samples were subjected to immunoblotting and membranes were probed with anti-GFP to detect VMAT2-GFP or anti-ubiquitin to detect ubiquitinated VMAT2.

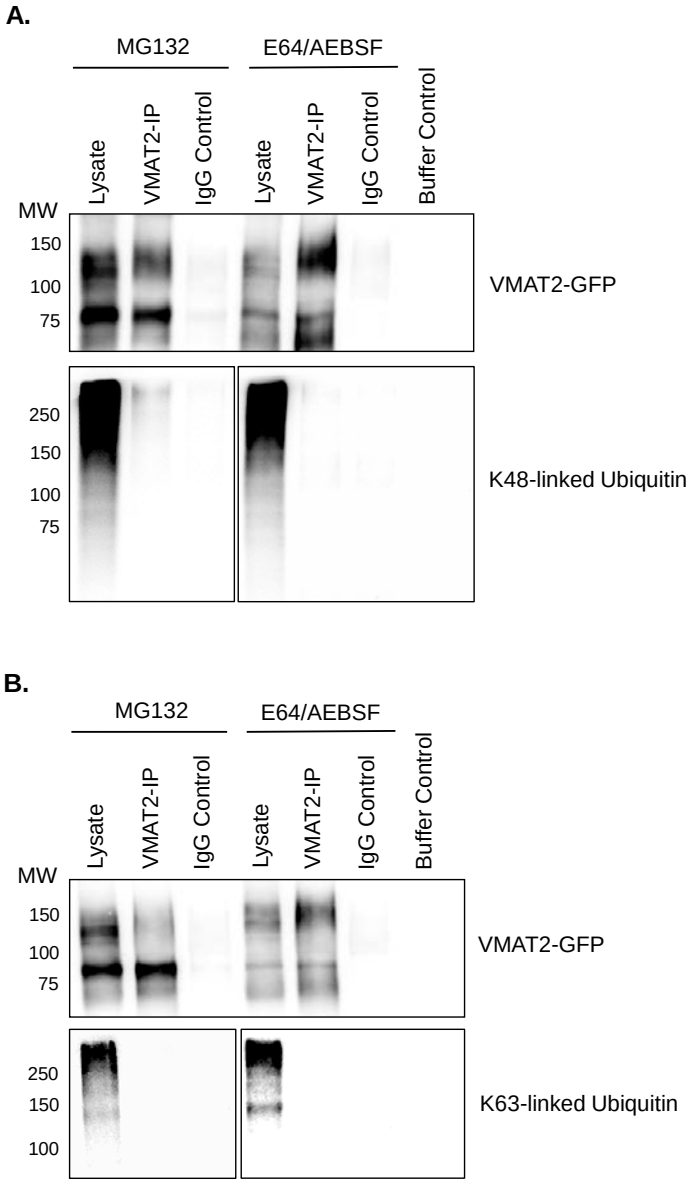


Figure 24. Proteasomal inhibition increases K48-linked polyubiquitinated VMAT2. PC12 cells stably expressing VMAT2-GFP were treated with 10 μM MG132 or 25 μM E64/200 μM AMEBSF for 6 hours. VMAT2 was immunoprecipitated from cell lysate using a VMAT2 antibody (C20, Santa Cruz) or goat IgG as a control. Samples were subjected to immunoblotting and membranes were probed with (A) K48-linked polyubiquitin-specific or (B) K63-linked polyubiquitin-specific antibody.

typically associated with degradation by the proteasome (Thrower et al., 2000). K63-linked polyubiquitin chains are usually associated with lysosomal degradation or acting as a trafficking signal (Geetha et al., 2005, Duncan et al., 2006, Lauwers et al., 2009, Huang et al., 2013b). As demonstrated in Figure 24, K48-specific ubiquitin antibody, but not K63-specific ubiquitin antibody detected ubiquitinated VMAT2 following 6 hours of MG132 treatment. Following 6 hours of E64/AEBSF treatment, the K48-specific ubiquitin antibody very weakly detected ubiquitinated VMAT2, while the K63-specific ubiquitin antibody did not detect any ubiquitinated VMAT2. These data indicate that ubiquitinated VMAT2 may be destined for the proteasome to undergo degradation.

3.4 POTENTIAL ROLE OF PARKIN IN VMAT2 DEGRADATION

The results obtained in this project suggest VMAT2 can be degraded by the proteasome. Degradation by the proteasome is often a specific process, dependent on multiple enzymes. The E3 enzyme most often confers specificity, catalyzing the transfer of ubiquitin to the substrate. Because of this, I began looking into potential E3 ligases that are involved in VMAT2 degradation. Parkin, one such E3 ligase, has several putative substrates and is best known for its Parkinsonism-causing mutations. The most clear role parkin has is in mitophagy, where it is part of a series of events inducing the degradation of damaged mitochondria (Geisler et al., 2010, Cai et al., 2012, Chen and Dorn, 2013). Interestingly, parkin has been localized to synaptic vesicles, although its exact role there is unknown (Kubo et al., 2001, Mouatt-Prigent et al., 2004). Appropriate to its role in Parkinsonism, *Drosophila* expressing human mutant parkin exhibit degenerative dopaminergic cell loss. This effect worsened when VMAT was knocked down and

was ameliorated when VMAT was over-expressed (Sang et al., 2007). Although it was hypothesized that cytosolic dopamine contributes to mutant parkin-induced degeneration, the possibility that parkin, itself, affects VMAT was not ruled out.

We first found that purified GST-VMAT2 N terminus, and to a lesser extent GST-VMAT2 C terminus, pulled down endogenous parkin from whole rat brain homogenate (Figure 25). Furthermore, VMAT2 co-immunoprecipitated parkin from PC12 cells over-expressing both proteins, as shown in Figure 26. These data indicate that VMAT2 and parkin may interact and raise the tantalizing possibility that VMAT2 may be a parkin substrate.

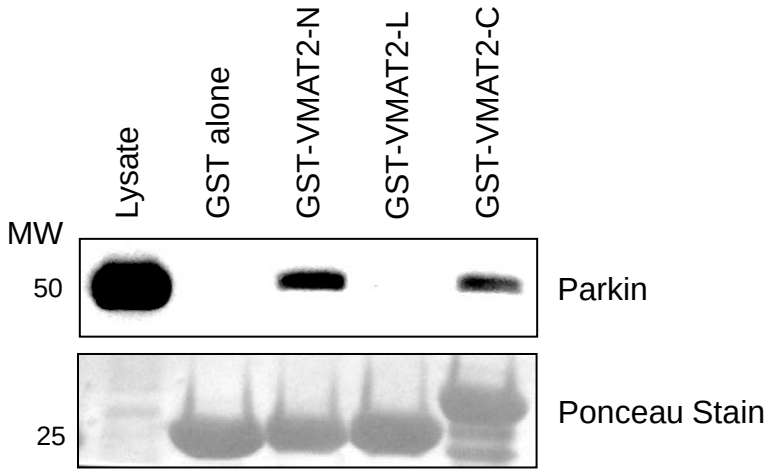


Figure 25. GST-VMAT2 fragments pull down parkin. Purified GST alone, GST-VMAT2-N terminus, GST-VMAT2-cytoplasmic loop, or GST-VMAT2-C terminus were incubated with rat brain homogenate. Samples underwent immunoblotting and membranes were stained with Ponceau or probed with parkin antibody. The N terminus and to a lesser extent the C terminus of VMAT2 were able to pull down endogenous parkin.

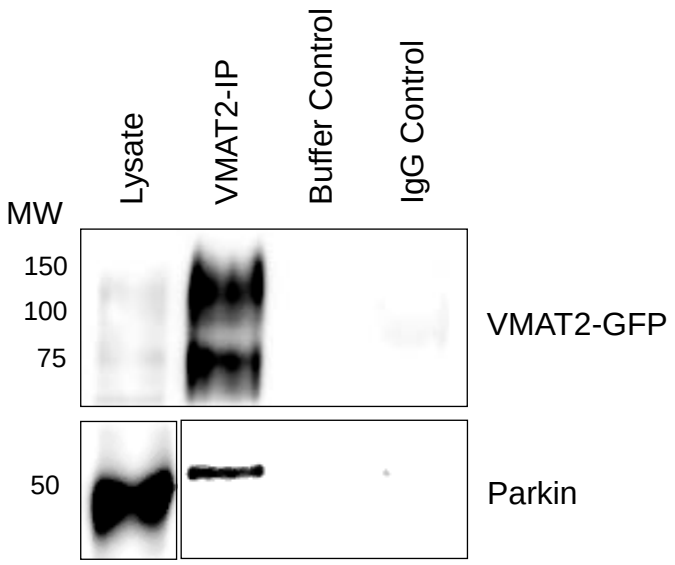


Figure 26. VMAT2 and parkin co-immunoprecipitate. VMAT2 was immunoprecipitated from PC12 cells over-expressing both VMAT2-GFP and parkin. Western blot membranes were probed parkin and with GFP to assess immunoprecipitation of VMAT2. Negative controls include VMAT2 antibody incubated with buffer in lieu of cell lysate and goat IgG instead of VMAT2 antibody incubated with cell lysate.

I then sought to determine if parkin was able to affect VMAT2 degradation. While there was little indication parkin was altering VMAT2 directly, a series of articles were published midway through the project that could explain why. Several groups have now demonstrated that parkin is in a constitutively autoinhibited conformation and requires phosphorylation of the serine 65 residue for activation (Wauer and Komander, 2013, Kazlauskaite et al., 2014, Koyano et al., 2014, Im and Chung, 2015). The strategy was adjusted and I began to clone mutations that would result in constitutively active parkin. Unfortunately, a series of technical challenges ensued, rending me unable to complete this undertaking. A combination of poor transfection efficiency in PC12 cells and technical difficulties with subcloning kept this portion of the project at a standstill.

4.0 DISCUSSION

4.1 SUMMARY OF RESULTS

The purpose of this project was to investigate the mechanisms of VMAT2 degradation under basal conditions. I used several techniques to evaluate VMAT2 levels, half-life, cellular localization, and ubiquitination when degradation machinery was inhibited. In all cases, the data suggest that VMAT2 is degraded through the ubiquitin-proteasome pathway. Although the primary interest was in the degradation of mature VMAT2, immature VMAT2 was also evaluated. Inhibiting the proteasome and the process of ER-associated degradation resulted in an accumulation of immature VMAT2. In addition to inhibiting ERAD, eeyarestatin I may also inhibit ER to Golgi trafficking as previously discussed, perhaps accounting for the accumulation of immature VMAT2. However, VMAT2 co-localized more with the ER after proteasomal inhibition, again indicating that VMAT2 processing and folding in the ER is regulated by ER-associated degradation. While it is accepted that ERAD acts as a quality control mechanism for the majority of transmembrane proteins and these results are perhaps unsurprising, this is first time this process has been implicated for VMAT2.

To follow up on the potential role of ERAD in VMAT2 quality control, one could now examine in further detail what specific machinery is involved. To confirm the role of ERAD in VMAT2 quality assurance, it would be valid to then look further into what ERAD proteins are

involved in immature VMAT2 degradation. Identification of VMAT2 binding partners would be a fine first step in this process. Protein immunoprecipitation followed by mass spectrometry identification of binding partners remains a useful tool for such identification. Genetic tools, such as RNA interference or CRISPR, could be used to knock-down proteins known to be involved in ERAD, such as EDEM1 or Hrd1, or any candidate proteins identified previously by mass spectrometry. The effects on VMAT2 trafficking and function could then be assessed by confocal imaging and uptake assays. ERAD is an important component of protein quality control; its dysfunction has deleterious consequences on the cell and presumably, VMAT2 function. *In vitro,* ERAD inhibition causes ER swelling and formation of vacuoles, impacting overall function (Dalal et al., 2004). There is also some *in vitro* evidence indicating Huntington's disease-causing mutant huntingtin adversely affects ERAD, perhaps contributing to disease pathology (Duennwald and Lindquist, 2008, Yang et al., 2010). Mutations in p97/VCP, impacting ERAD, cause IBMPFD, inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia, highlighting the importance of ERAD (Watts et al., 2004, Weihl et al., 2006). In the PC12 cell model system used in this study, ERAD inhibition with Eeyarestatin I not only resulted in accumulation of immature VMAT2, but also resulted in a decrease (although statistically insignificant) of mature VMAT2. While these results could be due to reduced ER to Golgi trafficking, they might also indicate that ERAD dysfunction could prevent maturation and further processing of VMAT2, affecting its overall function.

Surprisingly, mature VMAT2 accumulated following proteasomal, but not lysosomal inhibition. While other processes may account for this effect, these results imply that the mature form of VMAT2 may also be degraded by the proteasome. However, there are alternative explanations for these results, which must be interpreted with care. Inhibiting the proteasome

influences many cellular processes, which could, in turn affect VMAT2 levels. For example, Longva and colleagues suggest that while the EGF receptor accumulates following proteasomal inhibition, it's not likely the proteasome is a direct target of EGFR degradation (Longva et al., 2002). Rather, proteasomal inhibition could be affecting a factor that's involved with EGFR trafficking or lysosome-mediated degradation. Since I did not observe VMAT2 accumulation following lysosomal inhibition, this interpretation is unlikely. What's more likely is the alternative explanation that the accumulation of mature VMAT2 following proteasomal inhibition is an effect of increased synthesis, trafficking or processing of immature VMAT2. Proteasome inhibition can block degradation of transcription factors that normally limit transcription, thereby increasing total transcription of a protein (Lipford and Deshaies, 2003, Auld et al., 2006). In this case, VMAT2 synthesis should be assessed by qPCR or a related technique under conditions of proteasomal inhibition to confirm or reject this interpretation. Inhibiting the proteasome also induces the heat shock response, which includes the upregulation of both cytosolic and ER molecular chaperones (Bush et al., 1997, Lee and Goldberg, 1998). These ER chaperones could promote folding and processing of proteins in the ER, possibly contributing to the accumulation of mature VMAT2.

The accumulation of K48-linked polyubiquitinated VMAT2 also supports the hypothesis that VMAT2 is degraded in a proteasome-dependent manner, as K48-linked polyubiquitin chains are often associated with targeting for proteasomal degradation. It is unclear using this method if the accumulated VMAT2 is the immature or mature form. As the western blot expresses as a smear of high molecular weight ubiquitinated VMAT2, it is not possible to determine the exact form of the ubiquitinated VMAT2 without further experimentation. This smear, seen frequently in the literature, is probably the result of slower migration due to glycosylation and multiple

ubiquitination states. These data demonstrating the accumulation of K48 polyubiquitinated VMAT2 don't exclude the possible involvement of other polyubiquitin chains or monoubiquitination. Only K48-linked and K63-linked chains were investigated, as these are the most common. Certainly polyubiquitination and monoubiquitination aren't mutually exclusive; VMAT2 is likely capable of having multiple ubiquitination states, depending on the circumstance. Subcellular fractionation to isolate compartments and separate mature from immature VMAT2 could be used in conjunction with mass spectrometry to more definitively identify ubiquitin chain types conjugated to each form of VMAT2. Similar techniques, as well as the utilization of single point mutations could also be used to identify potential residues that are ubiquitinated.

The accumulation of polyubiquitinated VMAT2 following MG132 treatment implies, but does not show definitive proof of proteasomal degradation. Ubiquitination is involved in several cellular processes, not just degradation. For instance, ubiquitination could be an internalization signal for endocytosis, as has been suggested for numerous transporters and receptors (Geetha et al., 2005, Miranda et al., 2007, Eden et al., 2012). When VMAT2 ubiquitination residues are identified, as suggested in the previous paragraph, they could then be mutated and the localization and function of VMAT2 assessed. It is unclear if VMAT2 degradation is a specific, regulated process. If so, perhaps a particular VMAT2 residue is vital for the ubiquitination. Perhaps specific enzymes (namely, E3 enzymes) play a role in VMAT2 degradation. Identification of an E3 enzyme that regulates VMAT2 degradation would provide an avenue of VMAT2 regulation that has remained a black box.

Despite the traditional belief that transmembrane proteins are primarily degraded through the endo-lysosomal pathway, there was little evidence suggesting lysosomal inhibition affected

VMAT2. There could be several reasons for this surprising result. Others have suggested E64 has relatively poor penetration into cells (Wilcox and Mason, 1992). However, as others have also demonstrated, polyubiquitinated proteins accumulated following E64 incubation, indicating E64 was entering cells and being effective (Qiao and Zhang, 2009). As E64 is only inhibiting a subset of proteases located in lysosomes, it's possible that other, non-inhibited proteases are responsible for VMAT2 degradation. While other compounds are more effective at completely blocking lysosomal degradation, they do so by altering the acidic environment within the organelle, often by disrupting the pH gradient (Ahlberg et al., 1985). Using these compounds is not entirely feasible, as they may then disrupt the pH gradient of vesicles, perhaps interfering with VMAT2 in a way independent of degradation. These experiments would yield results equally difficult to interpret.

It is also possible that VMAT2 degradation is lysosome-dependent under specific circumstances. This project focused mainly on degradation under basal conditions, or degradation as a means of regulating VMAT2. As with many receptors and transporters, is it possible that lysosomal-mediated VMAT2 degradation is activity-dependent. That is, following exocytosis, VMAT2 is integrated within the plasma membrane to later undergo endocytosis and sorting (perhaps to the lysosome for degradation). Under these conditions, synaptic activity would be a pre-requisite for VMAT2 degradation. It would be straightforward to investigate this first in an *in vitro* cell model, then potentially in slice preparations. For example, VMAT2 levels, localization, and ubiquitination could be assessed following depolarization.

These experiments were performed in a PC12 cell model system—lysosomal-mediated degradation of VMAT2 may play a more prominent role in neurons. It is likely that VMAT2 is preferentially trafficked to dense core vesicles in PC12 cells (Yao et al., 2004). It has been

proposed that these vesicles, like secretory granules, do not fully collapse with the plasma membrane and recycle locally, but 'return' to the trans-Golgi network (Kelly, 1993, Sulzer et al., 2016). Contrary to this, there is evidence that LDCVs in PC12 cells can undergo local recycling similar to synaptic vesicles, perhaps indicating a difference between LDCVs containing monoamines and DCVs containing peptides or hormones (Bauer et al., 2004a, Bauer et al., 2004b).

In addition to activity-induced degradation, another circumstance under which VMAT could be potentially degraded is following psychostimulant exposure. There is substantial evidence that shortly (1 hour) after high doses of methamphetamine administration *in vivo*, VMAT2 levels and uptake activity are reduced (Brown et al., 2000, Eyerman and Yamamoto, 2007, Chu et al., 2010). It has been suggested that VMAT2 is trafficked out of the synaptic terminal or redistributed from a "cytoplasmic" fraction to a "membrane-bound" fraction (Riddle et al., 2002). However, a satisfying explanation for this reduction or redistribution has not been found. Intriguingly, co-current with this reduction in VMAT2 levels, there was increased VMAT nitrosylation (Eyerman and Yamamoto, 2007). S-nitrosylation is a post-translational modification that has been shown to reduce VMAT2 uptake activity *in vitro* (Wang et al., 2015b). While the exact mechanisms nitrosylation exerts on VMAT2 is unknown, this modification has been implicated in the degradation of some proteins (Gow et al., 1996, Dunlop et al., 2002). It is possible that VMAT2 is targeted for degradation following high methamphetamine doses. Perhaps this reduction in VMAT2 levels further contribute to methamphetamine-induced loss of synaptic terminals, further increasing cytosolic dopamine levels after initially increasing them by inhibiting (or reversing) VMAT2.

Interestingly, preliminary data from our laboratory shows that administration of the potent psychostimulant methylenedioxypyrovalerone (MDPV) in rats results in a significant decrease of VMAT2 protein levels in the striatum (see Appendix C). These results were recapitulated in PC12 cells expressing VMAT2 and the dopamine transporter. Similar to results seem following methamphetamine administration, these findings may identify conditions for VMAT2 degradation and also validate our PC12 cell model to study VMAT2 degradation mechanisms. While methamphetamine is thought to re-distribute dopamine from vesicles to the cytoplasm, it is unknown if the same is true of MDPV (Sulzer and Rayport, 1990, Sulzer et al., 1995). MDPV has been described as a dopamine transporter blocker; it has not been identified as a dopamine transporter substrate and its actions, if any, on VMAT2 or vesicles are unknown (Baumann et al., 2013, Anneken et al., 2015). Pursuit of MDPV (and methamphetamine) mechanisms of action would be a logical and valuable avenue to follow, contributing to our knowledge of interplay between methamphetamine or MDPV and VMAT2.

In summary, these data indicate that VMAT2 is degraded primarily by the proteasome under basal conditions. Under these conditions, there is no evidence indicating VMAT2 is degraded in a lysosome-dependent manner. Further experimentation is required to confirm these findings, as there are multiple interpretations and explanations for the data. However, a model for VMAT2 degradation is emerging, suggesting the proteasome may play a larger role than initially thought. Under the basal conditions tested in this study, VMAT2 degradation may be more dependent on the proteasome, as suggested in Figure 27. Although not tested here, it is possible that VMAT2 degradation is lysosomal-dependent following periods of neuron activity, as suggested in Figure 28. The two degradation pathways are not mutually exclusive and VMAT2 degradation may very well rely on both.

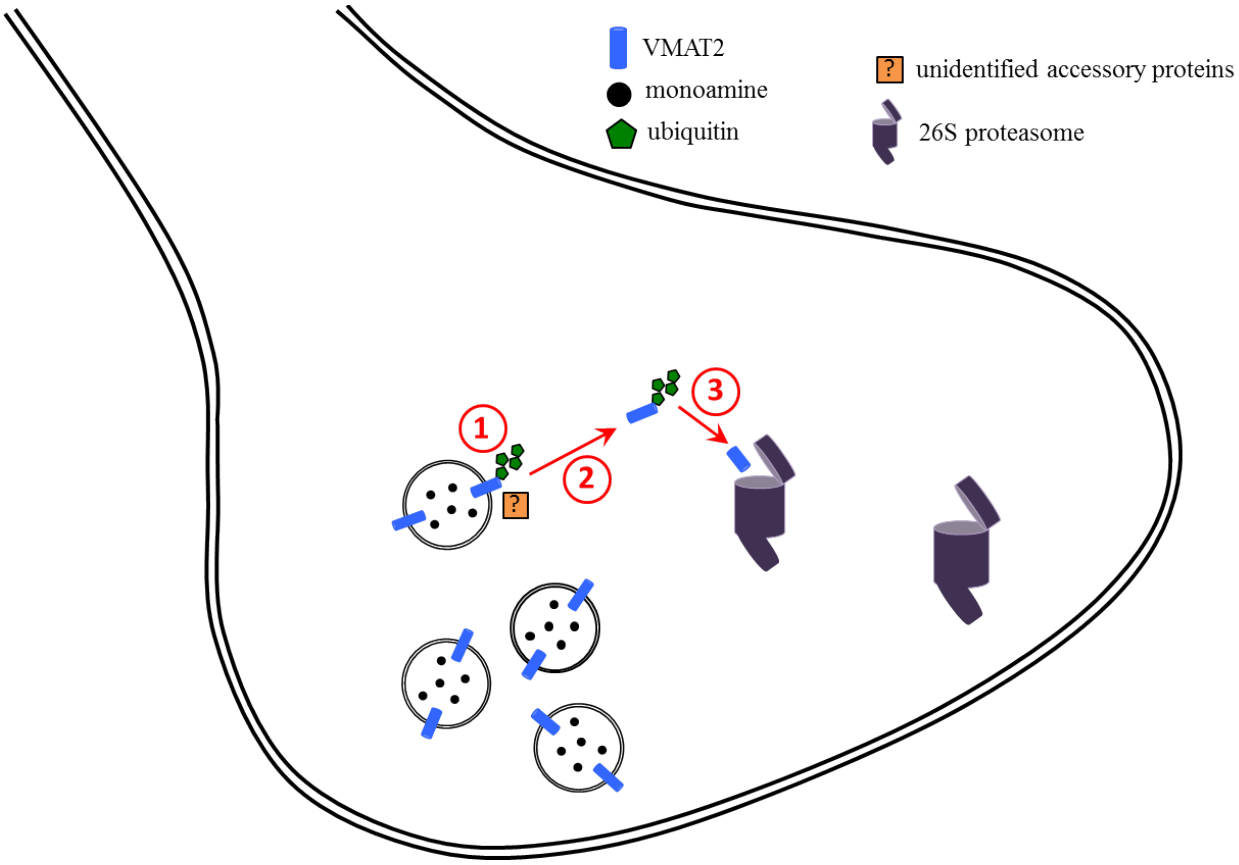


Figure 27. Model of proteasome-mediated VMAT2 degradation. Under basal conditions (or unknown conditions), VMAT2 degradation may be mediated by the proteasome. In a mechanism of fast and local degradation, VMAT2 located in the presynaptic terminal could be ubiquitinated (**1**) and targeted for degradation by the proteasome (**3**). To do so, accessory proteins (so far, unidentified) would have to be involved in the retrotranslocation of VMAT2 from the vesicular membrane (**2**).

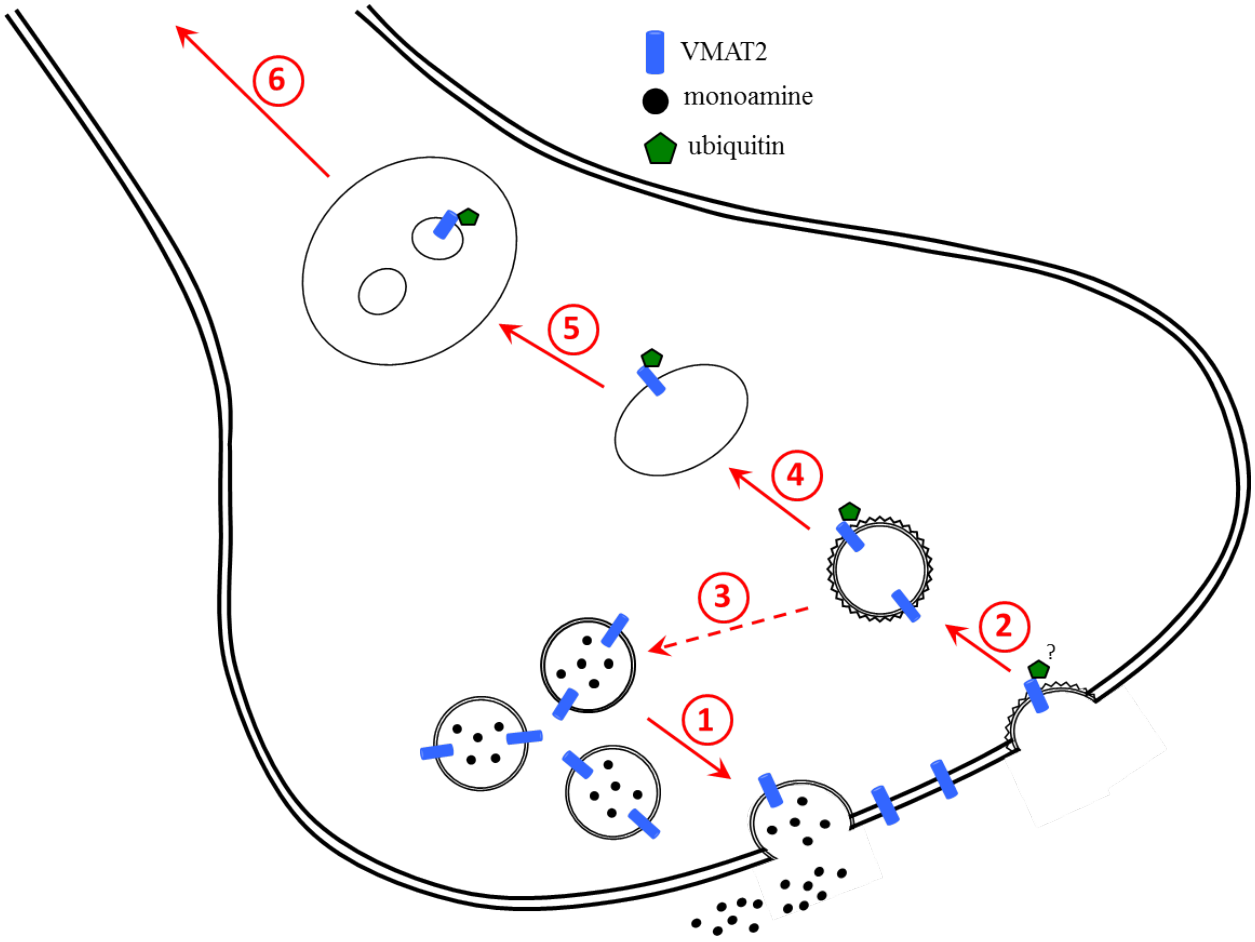


Figure 28. Model of lysosome-mediated VMAT2 degradation. Following exocytosis (**1**), VMAT2 is internalized (possibly with ubiquitination acting as an internalization signal) in a clathrin-dependent manner (**2**) and either undergoes recycling for further use at the presynaptic terminal (**3**) or is sorted into early endosomes (**4**). At this point, VMAT2 targeted for degradation would be further sorted into multivesicular bodies (**5**), which then undergo retrograde transportation back to the cell body for lysosomal degradation (**6**). This pathway of VMAT2 degradation may be dependent on exocytosis and would be a slow process, as it requires retrograde transport to the cell body.

4.2 LIMITATIONS OF STUDY

There are several limitations of this project and caveats to interpreting this data, some already discussed. One has to be cautious when extrapolating information from a cell model system and making inferences about *in vivo* mechanisms. Firstly, it is unclear if VMAT2 is trafficked and regulated in PC12 cells as it would be in neurons. PC12 cells have been used considerably to evaluate catecholamine exocytosis and mechanisms of VMAT2 regulation (Schweitzer et al., 1995, Kozminski et al., 1998, Holtje et al., 2000, Pothos et al., 2000). Despite this, it is not known if PC12 cells contain the full complement of accessory proteins or processes that would regulate VMAT2 in neurons. Additionally, PC12 cells contain endogenous VMAT1 and it is unclear if this would affect results when assessing exogenously added VMAT2 (Liu et al., 1994).

Caution should be taken when interpreting results from an over-expression system. Mechanisms of degradation that occur in an over-expression system may not be the same as occurs with an endogenous protein. Furthermore, there was a GFP tag on the VMAT2 I utilized. GFP is a fairly large tag and could potentially interfere with ubiquitination sites or other binding sites (Yewdell et al., 2011). Unfortunately, there are technical difficulties when investigating proteins like VMAT2, for which reliable antibodies are difficult to find, necessitating an over-expression system using a GFP-tagged VMAT2. Where possible, a neuronal cell model endogenously expressing VMAT2 should be used to replicate these results. The majority of these experiments would be quite difficult to perform in primary dopamine neurons—a gold standard for *in vitro* cell models. The low yield of dopaminergic neurons in most primary neuronal preparations provides technical challenges. However, it would be useful to further explore the feasibility of these experiments in primary dopamine neurons, as these data, regardless of the results, would contribute useful information to the field.

Interpreting data from manipulations of degradation systems is complicated. Inhibition of one system affects the others. For example, there's evidence that long (24 hours) inhibition of the lysosome also inhibits proteasomal function (Qiao and Zhang, 2009). Ubiquitinated proteins accumulate when either lysosomal or proteasomal function are inhibited. Unable to be degraded, these proteins remain ubiquitinated, thereby limiting free ubiquitin and indirectly inhibiting the other degradation process (Swaminathan et al., 1999). However, proteasome inhibition has also been demonstrated to up-regulate autophagy, in part due to ER stress and the subsequent unfolded protein response (Rideout et al., 2004, Iwata et al., 2005, Ding et al., 2007, Pandey et al., 2007, Ding and Yin, 2008, Du et al., 2009). Thought to be merely compensation, it may be that the interplay between the two systems is more complex (Korolchuk et al., 2010, Lilienbaum, 2013). For example, inhibition of autophagy also inhibits the UPS, although this may be an indirect effect. Korolchuk and co-authors propose that autophagy inhibition results in the accumulation of p62, which competes with ubiquitin binding proteins that normally act to shuttle ubiquitinated proteins to the proteasome (Korolchuk et al., 2009a, Korolchuk et al., 2009b). In this way, substrates are unable to get to the proteasome, resulting in an apparent decrease in proteasome activity. Because of this interplay between degradation pathways, it is difficult to isolate results and attribute them to one system. Inhibiting one system can cause a chain reaction that alters the entire homeostasis of the cell. Inhibiting the proteasome affects numerous proteins and these proteins could, in turn, affect numerous other proteins. Therefore, results from this study using proteasome and lysosome inhibitors may be indirect and due to alterations in other processes and/or compensatory mechanisms.

4.3 IMPLICATIONS FOR THE FIELD

It has long been believed that post-ER degradation of plasma membrane and synaptic vesicular transmembrane proteins are primarily mediated by the lysosome, often through the endo-lysosomal pathway, as shown in Figure 28. This theory is logical, as it would require a relatively great amount of effort and appropriate assembly of accessory proteins to remove a glycosylated transmembrane protein from a membrane, allowing it to be degraded by the proteasome. This basic fact has limited research on the possibility of proteasome-mediated degradation of mature transmembrane proteins post-ER and Golgi processing. As previously stated, there exist only a few examples of post-ER retrotranslocation of transmembrane proteins, occurring in the Golgi and mitochondria (Stewart et al., 2011, Xu et al., 2011, Tong et al., 2014, Hwang et al., 2016). Although it seems unlikely that the cellular machinery required to do such a thing exists at the presynaptic terminal for plasma membrane or synaptic vesicular proteins, I urge the scientific community not to close themselves to the possibility. As it stands, lysosomes have not been observed in the presynaptic terminals of dopamine neurons. Therefore, the neuron must spend energy transporting transmembrane proteins back to the cell body for lysosomal degradation. Alternatively, the presynaptic terminal contains a number of proteasomes, which already play an important role in regulating various synaptic proteins. Degradation mediated by the proteasome would provide a fast, local mechanism for regulation of pre-synaptic protein levels. In contrast, degradation mediated by the lysosome can take days, as it requires retrograde transport back to the cell body.

In addition to proteins normally thought to be involved in retrotranslocation (translocon pore, p97 or a related ATPase, etc.), there is another mechanism that may aid in the retrotranslocation of transmembrane proteins. Intramembrane proteases are a group of proteases

that cut transmembrane proteins, facilitating the removal of a portion from the membrane (Sannerud and Annaert, 2009, Avci and Lemberg, 2015, Langosch et al., 2015). Thought to be a specific, targeted process, these proteases are capable of cleaving proteins in a hydrophobic environment (Langosch et al., 2015). There is evidence that they aid in the process of ERAD, but they also exist in a number of other places in the cell including the Golgi, endosomes, and plasma membrane (Friedmann et al., 2006, Boname et al., 2014, Avci and Lemberg, 2015). The products of intramembrane protease cleavage sometimes act as signaling molecules, but can also initiate protein degradation (Alba et al., 2002, Kanehara et al., 2002, Avci and Lemberg, 2015). To my knowledge, intramembrane proteases have not been observed in synaptic vesicles. While speculative, it is an interesting thought that if there, they could possibly aid in the fast removal of synaptic vesicular proteins. This would be a way in which synaptic vesicular proteins could be regulated while still within the synaptic vesicle.

The idea that VMAT2 would be degraded via the endo-lysosomal pathway relies on the full collapse of the vesicle into the plasma membrane during exocytosis, as it seen with traditional synaptic vesicles. Although contested, there is indication that some vesicles undergo "kiss and run", when they release a portion of their contents through a pore instead of fully collapsing (Zhang et al., 2007, Park et al., 2012). In chromaffin cells, the release of DA through this mechanism has been observed (Fulop et al., 2005). It's unclear if this also occurs in dopaminergic neurons, although it's been suggested as a mechanism of release for DCVs in PC12 cells as well, particularly relevant for this project (Omiatek et al., 2010). It is also unknown if and when synaptic vesicles undergoing kiss and run recycle through the endosomal pathway and if so, how they are targeted for degradation. In fact, there is much unknown in the

field regarding SV and DCV recycling and even less about the degradation of vesicular proteins. Any research regarding such topics would contribute knowledge to this field.

The hypothesis that synaptic vesicular proteins, especially transporters, can be degraded in a specific manner is intriguing. Targeted degradation of vesicular transporters would be a mechanism of regulation that would definitively reduce neurotransmission without relying on time-consuming transcriptional methods. Post-translational modifications—including ubiquitination—are a relatively fast way to regulate protein activity. Ubiquitination is most often immediately associated with degradation. It's becoming increasingly clear that it has other roles, including post-Golgi trafficking or acting as an internalization signal for plasma membrane proteins (Komander and Rape, 2012, Yuan et al., 2014, Swatek and Komander, 2016). Its role in the regulation—beyond just degradation—of synaptic vesicular proteins, including VMAT2, should be further investigated.

Another intriguing idea is that synaptic vesicles could themselves, as a whole, be degraded *via* autophagy, similar to mitophagy. Parkin, with the aid of other proteins, targets damaged mitochondria for degradation by the autophagy system. Parkin has also been observed at the membrane of synaptic vesicles; it isn't known exactly why. There is, however, recent evidence that autophagy may be involved with synaptic vesicle degradation. Specifically, autophagy activation by rapamycin reduced the number of presynaptic vesicles and evoked dopamine release (Hernandez et al., 2012). Interestingly, these effects were seen in slices not containing dopaminergic cell bodies. As lysosomes are traditionally not thought to reside in presynaptic terminals, the results imply that autophagy could locally regulate synaptic vesicles without the action of lysosomes. These data strongly indicate autophagy plays a role in synaptic

vesicle degradation; it would be worthwhile to further investigate this mechanism and any potential involvement of parkin.

The involvement of the dopaminergic system in the etiology of several neurological disorders and diseases has made molecular components of this system attractive therapeutic targets. VMAT2 is not excluded from this, and has been proposed as treatment in several instances. Currently, TBZ, the VMAT2 inhibitor is approved for treatment of Huntington's disease and chorea (Frank, 2010). These movement disorders are characterized by excessive movement, thus VMAT2 inhibition is a logical avenue of treatment. While VMAT inhibition has also been proposed as a treatment for psychostimulant abuse, support for this option is varied (Dwoskin and Crooks, 2002, Wilhelm et al., 2008). Lobeline reduces amphetamine-induced dopamine output, suggesting it may then reduce the "rewarding" effect of psychostimulants that enhance dopamine release (Miller et al., 2001). However, the side effects of VMAT inhibition can be quite severe, including depressive-like symptoms, subtle motor deficits, as well as peripheral effects in the gut. Moreover, long-term inhibition of VMAT2 may be detrimental, as evidenced by increased vulnerability to toxic insults and degeneration in models of VMAT2 knockdown or pharmacological inhibition.

While there exists tools to reduce VMAT activity, there are none developed to increase VMAT activity. Such a tool would be useful in diseases of monoaminergic depletion, such as Parkinson's disease. Targets to increase protein activity are more difficult to identify and so are uncommon therapies. Inhibiting VMAT2 degradation to increase VMAT2 levels may be an attractive option for therapeutic development, but hinges on specificity. It is unknown if the degradation of synaptic vesicle proteins can be individually modulated, or occurs *en masse*. It is possible that at least under some circumstances, synaptic vesicle proteins are degraded using

pathways more suggestive of bulk degradation, such as autophagy. Furthermore, the inhibition of ERAD or other protein quality control mechanisms should be avoided. Inhibition of these processes would likely not be beneficial and may be counter-productive, reducing levels of mature and correctly folded VMAT2 that are ultimately trafficked to the vesicle. Unfortunately, too little is known about VMAT2 regulation to even begin identifying viable activating options. Identifying in detail the molecular mechanisms of VMAT2 degradation could open up this door and provide viable therapeutic targets designed to increase VMAT2 levels and treat devastating diseases.

APPENDIX A

VMAT UPTAKE IN PC12 CELLS OVER-EXPRESSING VMAT2-GFP

Radiolabeled serotonin vesicular uptake assays were performed on crude vesicular preparations. Nonspecific uptake was measured by addition of 100 μM reserpine, a potent VMAT1 and VMAT2 inhibitor. VMAT2 uptake was measured by addition of 1 μM TBZ, a VMAT2 inhibitor. Unexpectedly, the addition of reserpine or TBZ reduced uptake similarly, indicating that VMAT2 is contributing to nearly the entire measured uptake. There are multiple explanations for this. For one, the levels of VMAT1 are unknown in this model and it is possible that the over-expression of VMAT2 reduces them, or makes their contribution negligible. The more likely explanation is that TBZ is also inhibiting VMAT1 at the concentration used. However, others have reported little or no effect of 1 μM TBZ on VMAT1 activity in PC12 and other cell lines (Erickson et al., 1996, Holtje et al., 2000). At 1 μM, TBZ is unlikely to effect 100% of VMAT1 activity, as it should affect 100% of VMAT2 activity, thereby leading me to conclude that the majority of measured vesicular uptake is VMAT2-mediated.

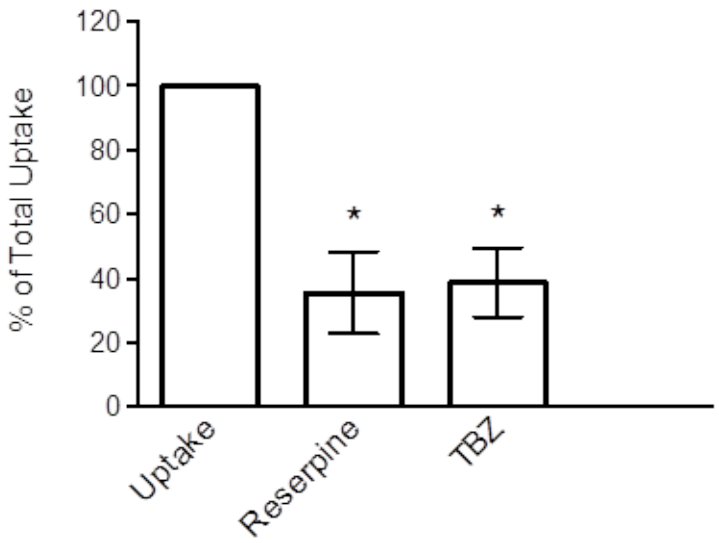
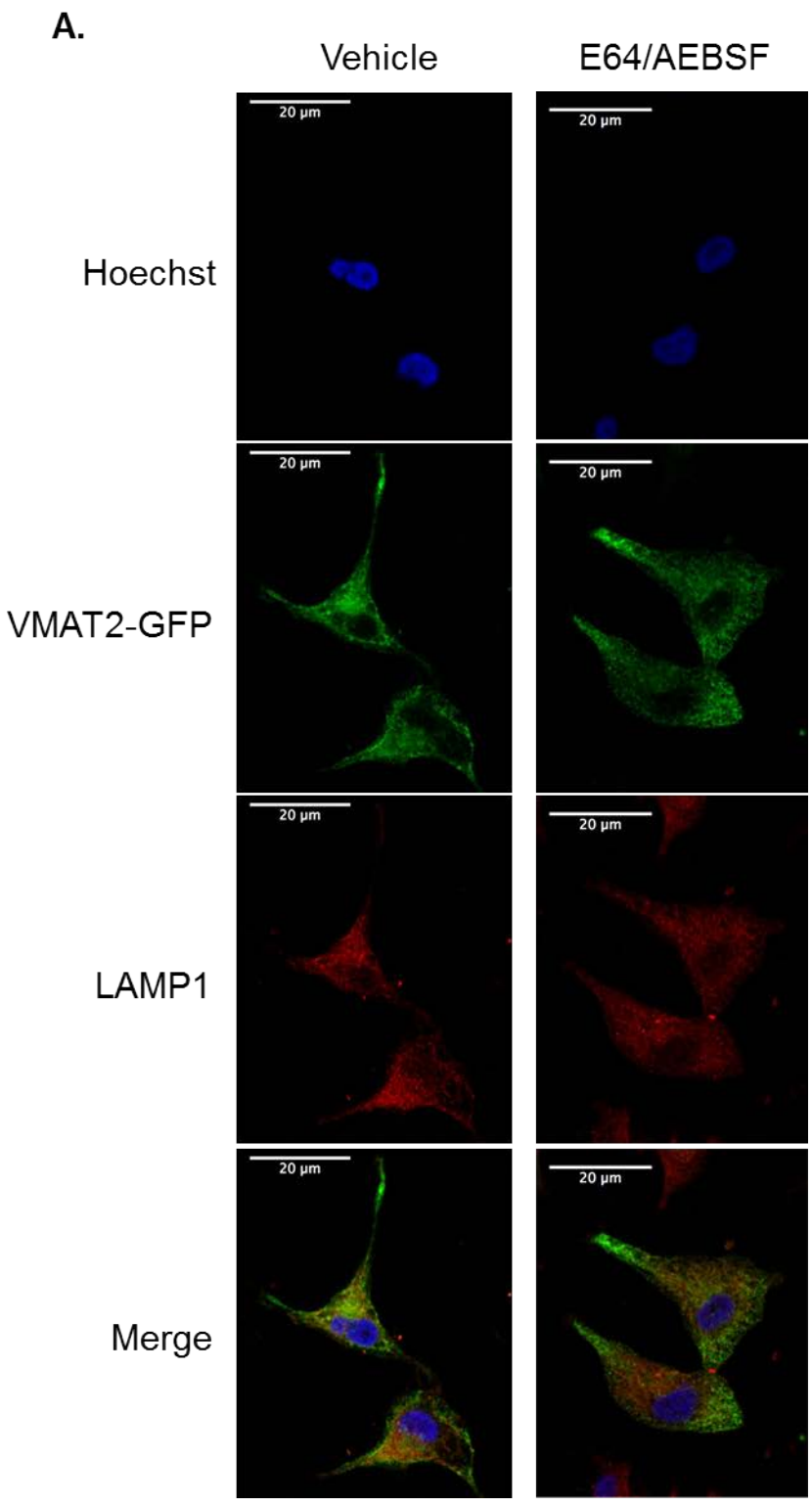


Figure 29. Vesicular Uptake in PC12 Cells Stably Expressing VMAT2-GFP. Radiolabeled serotonin uptake was measured in a crude vesicular preparation. 100 μM reserpine and 1 μM TBZ blocked a portion of total uptake, suggesting the VMAT2-GFP construct is functional. Data represented as mean, reserpine and TBZ data represented as mean +/- SEM (n=4 independent experiments). Non-parametric one-way ANOVA analysis resulted in $p=0.0249$ significant difference between groups. * indicates $p<0.05$ as compared to total uptake from Dunn's post-hoc tests.

APPENDIX B

LAMP1 NONSPECIFIC LABELING

Cells were treated with DMSO vehicle of E64/AEBSF for 24 hours, a lengthy incubation in attempt to maximize any effect E64 may have on VMAT2. Cells were then stained with two different LAMP1 antibodies. Both LAMP1 antibodies displayed what appears to be nonspecific labeling. LAMP1 is expected to label more discretely and display a punctate pattern. Instead, LAMP1 labeling with these antibodies appears throughout the cell in a rather diffuse manner, suggesting a lack of specificity.


A.
Vehicle
E64/AEBSF
Hoechst
VMAT2-GFP
LAMP1
Merge
20 µm

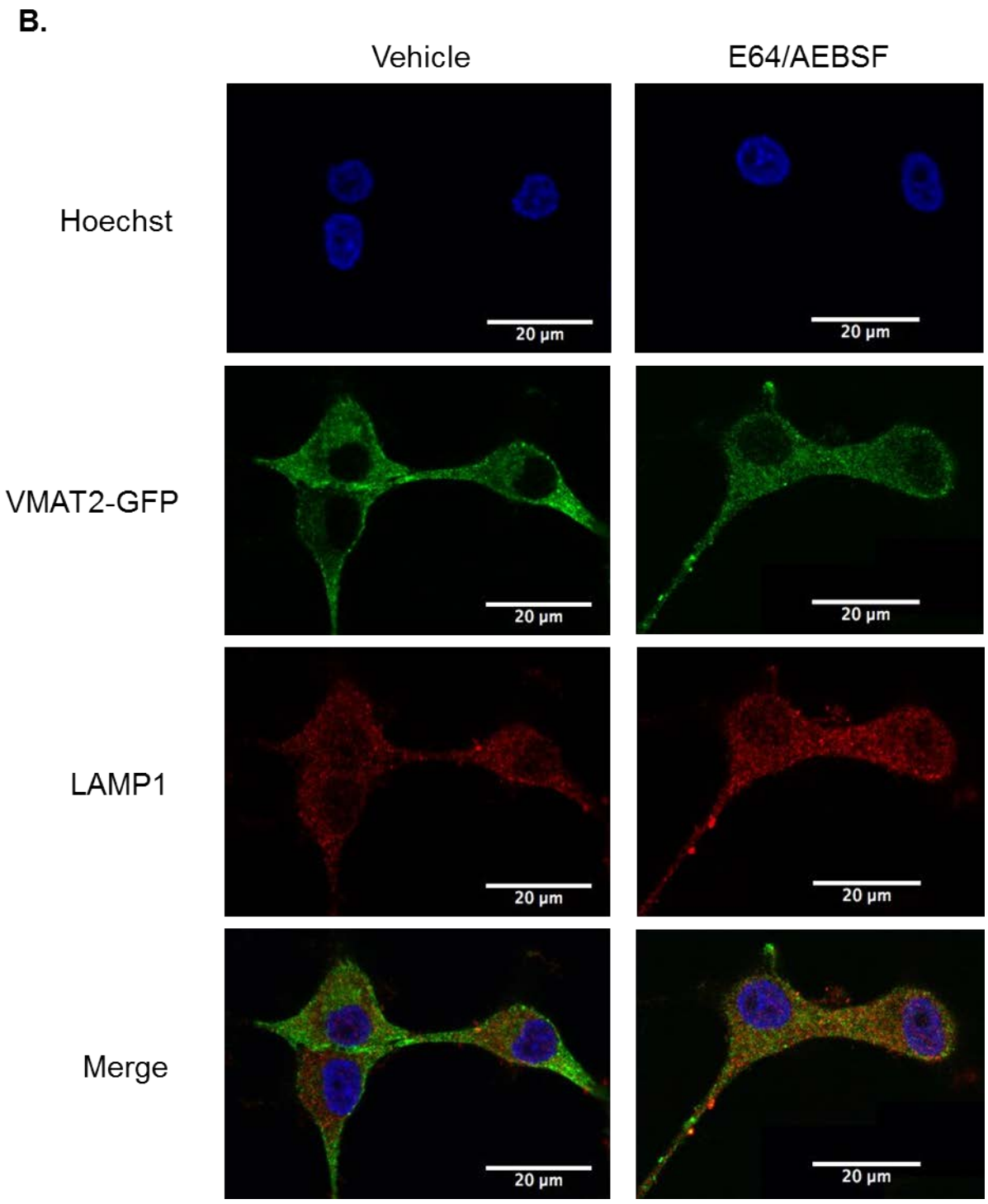


Figure 30. LAMP1 Antibody non-specific staining. PC12 cells were differentiated with NGF and treated for 24 hours with DMSO vehicle or 25 µM E64/200 µM AEBSF. Cells were stained for VMAT2-GFP (green), LAMP1 (a marker of endosomes and lysosomes, red), or Hoechst stain to label nuclei (blue). (A) A rabbit polyclonal LAMP1 antibody from Abcam was used and (B) a monoclonal antibody from the DSHB was also used. Both antibodies appear to display nonspecific labeling.

APPENDIX C

EFFECT OF MDPV ON VMAT2

Rats were injected with 3 mg/kg MDPV and sacrificed 24 h later. The striatum was dissected and VMAT2 levels were examined by western blot. PC12 cells stably expressing VMAT2-GFP with or without transfected DAT (MDPV blocks DAT) were incubated with 1 μM MDPV for the indicated times and again, VMAT2 levels examined by western blot (Baumann et al., 2013, Anneken et al., 2015). In both instances, MDPV administration reduced VMAT2 levels, suggesting a model for VMAT2 degradation. Although preliminary, these result are promising and may provide a means of further investigating mechanisms of VMAT2 degradation.

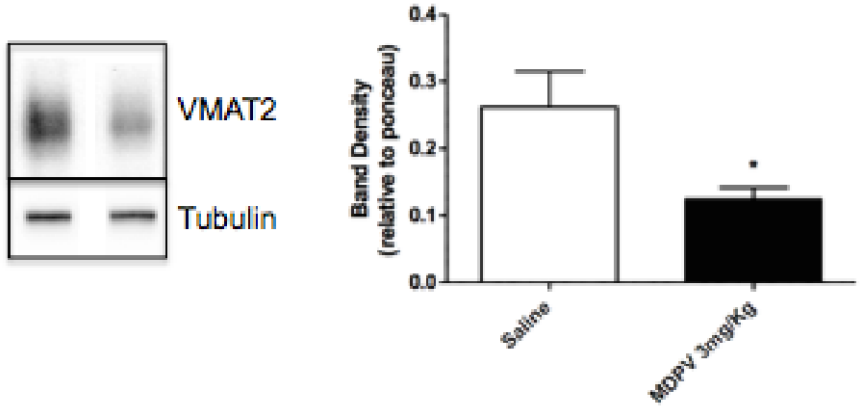


Figure 31. MDPV reduces striatal VMAT2 levels. Rats were given 3 mg/kg MDPV (closed bar) or saline (open bar) and sacrificed 24 hours later. VMAT2 levels were determined by western blots, quantified, and represented as mean + SEM (n=5). MDPV administration significantly reduced VMAT2 levels, indicating increased VMAT2 degradation.

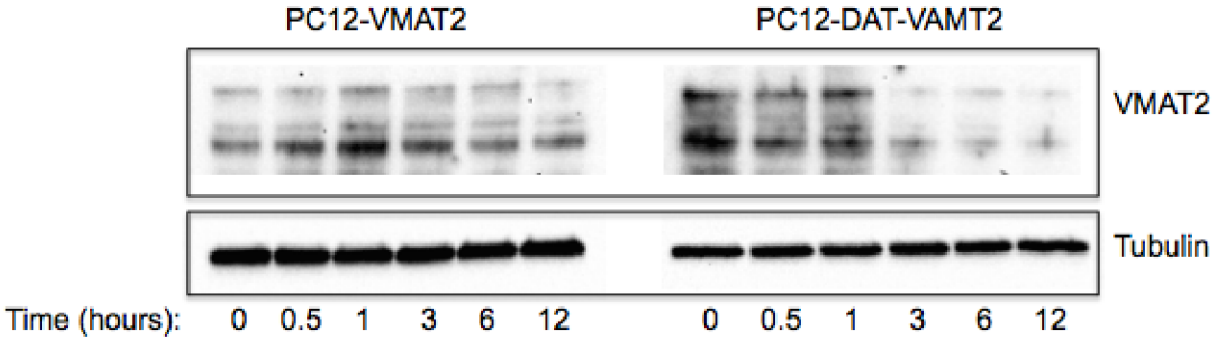


Figure 32. MDPV reduces VMAT2 levels in PC12 cells. PC12 cells stably expressing VMAT2-GFP transfected with or without DAT were treated with 1 μM MDPV for the indicated times. VMAT2 levels were determined by western blots. VMAT2 levels decreased with increasing MDPV treatment time in cells also expressing DAT. These results confirm observations in the previous figure and provide validation for a cell model examining VMAT2 degradation.

BIBLIOGRAPHY

Agarraberes FA, Dice JF (2001) A molecular chaperone complex at the lysosomal membrane is required for protein translocation. Journal of cell science 114:2491-2499.

Ahlberg J, Berkenstam A, Henell F, Glaumann H (1985) Degradation of short and long lived proteins in isolated rat liver lysosomes. Effects of pH, temperature, and proteolytic inhibitors. The Journal of biological chemistry 260:5847-5854.

Ahnert-Hilger G, Nurnberg B, Exner T, Schafer T, Jahn R (1998) The heterotrimeric G protein Go2 regulates catecholamine uptake by secretory vesicles. The EMBO journal 17:406-413.

Alba BM, Leeds JA, Onufryk C, Lu CZ, Gross CA (2002) DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response. Genes & development 16:2156-2168.

Anneken JH, Angoa-Perez M, Kuhn DM (2015) 3,4-Methylenedioxypyrovalerone prevents while methylone enhances methamphetamine-induced damage to dopamine nerve endings: beta-ketoamphetamine modulation of neurotoxicity by the dopamine transporter. Journal of neurochemistry 133:211-222.

Anwar A, Norris DA, Fujita M (2011) Ubiquitin proteasomal pathway mediated degradation of p53 in melanoma. Archives of biochemistry and biophysics 508:198-203.

Appelqvist H, Waster P, Kagedal K, Ollinger K (2013) The lysosome: from waste bag to potential therapeutic target. Journal of molecular cell biology 5:214-226.

Auld KL, Brown CR, Casolari JM, Komili S, Silver PA (2006) Genomic association of the proteasome demonstrates overlapping gene regulatory activity with transcription factor substrates. Molecular cell 21:861-871.

Avci D, Lemberg MK (2015) Clipping or Extracting: Two Ways to Membrane Protein Degradation. Trends in cell biology 25:611-622.

Baboshina OV, Haas AL (1996) Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2EPF and RAD6 are recognized by 26 S proteasome subunit 5. The Journal of biological chemistry 271:2823-2831.

Baldo BA, Kelley AE (2007) Discrete neurochemical coding of distinguishable motivational processes: insights from nucleus accumbens control of feeding. Psychopharmacology 191:439-459.

Barrett AJ, Kembhavi AA, Brown MA, Kirschke H, Knight CG, Tamai M, Hanada K (1982) L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. The Biochemical journal 201:189-198.

Bauer RA, Khera RS, Lieber JL, Angleson JK (2004a) Recycling of intact dense core vesicles in neurites of NGF-treated PC12 cells. FEBS letters 571:107-111.

Bauer RA, Overlease RL, Lieber JL, Angleson JK (2004b) Retention and stimulus-dependent recycling of dense core vesicle content in neuroendocrine cells. Journal of cell science 117:2193-2202.

Baumann MH, Partilla JS, Lehner KR, Thorndike EB, Hoffman AF, Holy M, Rothman RB, Goldberg SR, Lupica CR, Sitte HH, Brandt SD, Tella SR, Cozzi NV, Schindler CW (2013) Powerful cocaine-like actions of 3,4-methylenedioxypyrovalerone (MDPV), a principal constituent of psychoactive 'bath salts' products. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 38:552-562.

Beaulieu JM, Gainetdinov RR (2011) The physiology, signaling, and pharmacology of dopamine receptors. Pharmacological reviews 63:182-217.

Ben-Saadon R, Fajerman I, Ziv T, Hellman U, Schwartz AL, Ciechanover A (2004) The tumor suppressor protein p16(INK4a) and the human papillomavirus oncoprotein-58 E7 are naturally occurring lysine-less proteins that are degraded by the ubiquitin system - Direct evidence for ubiquitination at the N-terminal residue. Journal of Biological Chemistry 279:41414-41421.

Bento CF, Renna M, Ghislat G, Puri C, Ashkenazi A, Vicinanza M, Menzies FM, Rubinsztein DC (2016) Mammalian Autophagy: How Does It Work? Annual review of biochemistry 85:685-713.

Besche HC, Peth A, Goldberg AL (2009) Getting to First Base in Proteasome Assembly. Cell 138:25-28.

Bocchio M, McHugh SB, Bannerman DM, Sharp T, Capogna M (2016) Serotonin, Amygdala and Fear: Assembling the Puzzle. Frontiers in neural circuits 10:24.

Bohnen NI, Albin RL, Koeppe RA, Wernette KA, Kilbourn MR, Minoshima S, Frey KA (2006) Positron emission tomography of monoaminergic vesicular binding in aging and Parkinson disease. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 26:1198-1212.

Boname JM, Bloor S, Wandel MP, Nathan JA, Antrobus R, Dingwell KS, Thurston TL, Smith DL, Smith JC, Randow F, Lehner PJ (2014) Cleavage by signal peptide peptidase is

required for the degradation of selected tail-anchored proteins. The Journal of cell biology 205:847-862.

Brighina L, Riva C, Bertola F, Saracchi E, Fermi S, Goldwurm S, Ferrarese C (2013) Analysis of vesicular monoamine transporter 2 polymorphisms in Parkinson's disease. Neurobiology of aging 34:1712 e1719-1713.

Brodsky JL (2012) Cleaning up: ER-associated degradation to the rescue. Cell 151:1163-1167.

Brown JM, Hanson GR, Fleckenstein AE (2000) Methamphetamine rapidly decreases vesicular dopamine uptake. Journal of neurochemistry 74:2221-2223.

Brunk I, Blex C, Rachakonda S, Holtje M, Winter S, Pahner I, Walther DJ, Ahnert-Hilger G (2006) The first luminal domain of vesicular monoamine transporters mediates G-protein-dependent regulation of transmitter uptake. Journal of Biological Chemistry 281:33373-33385.

Burman J, Tran CH, Glatt C, Freimer NB, Edwards RH (2004) The effect of rare human sequence variants on the function of vesicular monoamine transporter 2. Pharmacogenetics 14:587-594.

Bush KT, Goldberg AL, Nigam SK (1997) Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. The Journal of biological chemistry 272:9086-9092.

Cadet JL, Brannock C (1998) Free radicals and the pathobiology of brain dopamine systems. Neurochemistry international 32:117-131.

Cadwell K, Coscoy L (2005) Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. Science 309:127-130.

Cai Q, Zakaria HM, Simone A, Sheng ZH (2012) Spatial parkin translocation and degradation of damaged mitochondria via mitophagy in live cortical neurons. Current biology : CB 22:545-552.

Cartier EA, Parra LA, Baust TB, Quiroz M, Salazar G, Faundez V, Egana L, Torres GE (2010) A Biochemical and Functional Protein Complex Involving Dopamine Synthesis and Transport into Synaptic Vesicles. Journal of Biological Chemistry 285:1957-1966.

Carvalho P, Goder V, Rapoport TA (2006) Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell 126:361-373.

Caudle WM, Richardson JR, Wang MZ, Taylor TN, Guillot TS, McCormack AL, Colebrooke RE, Di Monte DA, Emson PC, Miller GW (2007) Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. Journal of Neuroscience 27:8138-8148.

Chaugule VK, Walden H (2016) Specificity and disease in the ubiquitin system. Biochemical Society transactions 44:212-227.

Chen Y, Dorn GW, 2nd (2013) PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science 340:471-475.

Chiang HL, Terlecky SR, Plant CP, Dice JF (1989) A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science 246:382-385.

Chin LS, Vavalle JP, Li L (2002) Staring, a novel E3 ubiquitin-protein ligase that targets syntaxin 1for degradation. Journal of Biological Chemistry 277:35071-35079.

Choi HJ, Jang YJ, Kim HJ, Hwang O (2000) Tetrahydrobiopterin is released from and causes preferential death of catecholaminergic cells by oxidative stress. Molecular pharmacology 58:633-640.

Choi HJ, Kim SW, Lee SY, Hwang O (2003) Dopamine-dependent cytotoxicity of tetrahydrobiopterin: a possible mechanism for selective neurodegeneration in Parkinson's disease. Journal of neurochemistry 86:143-152.

Choi HJ, Lee SY, Cho Y, Hwang O (2005) Inhibition of vesicular monoamine transporter enhances vulnerability of dopaminergic cells: relevance to Parkinson's disease. Neurochemistry international 46:329-335.

Christianson JC, Ye Y (2014) Cleaning up in the endoplasmic reticulum: ubiquitin in charge. Nature structural & molecular biology 21:325-335.

Chu FF, Doyle D (1985) Turnover of plasma membrane proteins in rat hepatoma cells and primary cultures of rat hepatocytes. The Journal of biological chemistry 260:3097-3107.

Chu PW, Hadlock GC, Vieira-Brock P, Stout K, Hanson GR, Fleckenstein AE (2010) Methamphetamine alters vesicular monoamine transporter-2 function and potassium-stimulated dopamine release. Journal of neurochemistry 115:325-332.

Clark D, White FJ (1987) D1 dopamine receptor--the search for a function: a critical evaluation of the D1/D2 dopamine receptor classification and its functional implications. Synapse 1:347-388.

Colebrooke RE, Humby T, Lynch PJ, McGowan DP, Xia J, Emson PC (2006) Age-related decline in striatal dopamine content and motor performance occurs in the absence of nigral cell loss in a genetic mouse model of Parkinson's disease. The European journal of neuroscience 24:2622-2630.

Colliver TL, Pyott SJ, Achalabun M, Ewing AG (2000) VMAT-Mediated changes in quantal size and vesicular volume. Journal of Neuroscience 20:5276-5282.

Connett RJ, Kirshner N (1970) Purification and properties of bovine phenylethanolamine N-methyltransferase. The Journal of biological chemistry 245:329-334.

Croft BG, Fortin GD, Corera AT, Edwards RH, Beaudet A, Trudeau LE, Fon EA (2005) Normal biogenesis and cycling of empty synaptic vesicles in dopamine neurons of vesicular monoamine transporter 2 knockout mice. Molecular biology of the cell 16:306-315.

Cross BC, McKibbin C, Callan AC, Roboti P, Piacenti M, Rabu C, Wilson CM, Whitehead R, Flitsch SL, Pool MR, High S, Swanton E (2009) Eeyarestatin I inhibits Sec61-mediated protein translocation at the endoplasmic reticulum. Journal of cell science 122:4393-4400.

Cruz-Muros I, Afonso-Oramas D, Abreu P, Rodriguez M, Gonzalez MC, Gonzalez-Hernandez T (2008) Deglycosylation and subcellular redistribution of VMAT2 in the mesostriatal system during normal aging. Neurobiology of aging 29:1702-1711.

Cubells JF, Rayport S, Rajendran G, Sulzer D (1994) Methamphetamine Neurotoxicity Involves Vacuolation of Endocytic Organelles and Dopamine-Dependent Intracellular Oxidative Stress. Journal of Neuroscience 14:2260-2271.

Cuervo AM, Dice JF (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. Science 273:501-503.

Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D (2004) Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science 305:1292-1295.

Dalal S, Rosser MF, Cyr DM, Hanson PI (2004) Distinct roles for the AAA ATPases NSF and p97 in the secretory pathway. Molecular biology of the cell 15:637-648.

Daniels AJ, Reinhard JF, Jr. (1988) Energy-driven uptake of the neurotoxin 1-methyl-4-phenylpyridinium into chromaffin granules via the catecholamine transporter. The Journal of biological chemistry 263:5034-5036.

DaSilva JN, Carey JE, Sherman PS, Pisani TJ, Kilbourn MR (1994) Characterization of [11C]tetrabenazine as an in vivo radioligand for the vesicular monoamine transporter. Nuclear medicine and biology 21:151-156.

DaSilva JN, Kilbourn MR (1992) In vivo binding of [11C]tetrabenazine to vesicular monoamine transporters in mouse brain. Life sciences 51:593-600.

de Lange RP, de Roos AD, Borst JG (2003) Two modes of vesicle recycling in the rat calyx of Held. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:10164-10173.

Dice JF (1990) Peptide Sequences That Target Cytosolic Proteins for Lysosomal Proteolysis. Trends in biochemical sciences 15:305-309.

Ding WX, Ni HM, Gao W, Yoshimori T, Stolz DB, Ron D, Yin XM (2007) Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. The American journal of pathology 171:513-524.

Ding WX, Yin XM (2008) Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. Autophagy 4:141-150.

Du Y, Yang D, Li L, Luo G, Li T, Fan X, Wang Q, Zhang X, Wang Y, Le W (2009) An insight into the mechanistic role of p53-mediated autophagy induction in response to proteasomal inhibition-induced neurotoxicity. Autophagy 5:663-675.

Duennwald ML, Lindquist S (2008) Impaired ERAD and ER stress are early and specific events in polyglutamine toxicity. Genes & development 22:3308-3319.

Duncan LM, Piper S, Dodd RB, Saville MK, Sanderson CM, Luzio JP, Lehner PJ (2006) Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. The EMBO journal 25:1635-1645.

Dunlop RA, Rodgers KJ, Dean RT (2002) Recent developments in the intracellular degradation of oxidized proteins. Free radical biology & medicine 33:894-906.

Dunn KW, Kamocka MM, McDonald JH (2011) A practical guide to evaluating colocalization in biological microscopy. American journal of physiology Cell physiology 300:C723-742.

Dwoskin LP, Crooks PA (2002) A novel mechanism of action and potential use for lobeline as a treatment for psychostimulant abuse. Biochemical pharmacology 63:89-98.

Eden ER, Huang F, Sorkin A, Futter CE (2012) The role of EGF receptor ubiquitination in regulating its intracellular traffic. Traffic 13:329-337.

Edmondson DE, Mattevi A, Binda C, Li M, Hubalek F (2004) Structure and mechanism of monoamine oxidase. Current medicinal chemistry 11:1983-1993.

Erickson JD, Eiden LE, Hoffman BJ (1992) Expression cloning of a reserpine-sensitive vesicular monoamine transporter. Proceedings of the National Academy of Sciences of the United States of America 89:10993-10997.

Erickson JD, Schafer MK, Bonner TI, Eiden LE, Weihe E (1996) 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 93:5166-5171.

Eskelinen EL, Schmidt CK, Neu S, Willenborg M, Fuertes G, Salvador N, Tanaka Y, Lullmann-Rauch R, Hartmann D, Heeren J, von Figura K, Knecht E, Saftig P (2004) Disturbed cholesterol traffic but normal proteolytic function in LAMP-1/LAMP-2 double-deficient fibroblasts. Molecular biology of the cell 15:3132-3145.

Eyerman DJ, Yamamoto BK (2007) A rapid oxidation and persistent decrease in the vesicular monoamine transporter 2 after methamphetamine. Journal of neurochemistry 103:1219-1227.

Faundez V, Horng JT, Kelly RB (1998) A function for the AP3 coat complex in synaptic vesicle formation from endosomes. Cell 93:423-432.

Feng Y, He D, Yao Z, Klionsky DJ (2014) The machinery of macroautophagy. Cell research 24:24-41.

Fernandes AC, Uytterhoeven V, Kuenen S, Wang YC, Slabbaert JR, Swerts J, Kasprowicz J, Aerts S, Verstreken P (2014) Reduced synaptic vesicle protein degradation at lysosomes curbs TBC1D24/sky-induced neurodegeneration. The Journal of cell biology 207:453-462.

Floresco SB, West AR, Ash B, Moore H, Grace AA (2003) Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. Nature neuroscience 6:968-973.

Fon EA, Pothos EN, Sun BC, Killeen N, Sulzer D, Edwards RH (1997) Vesicular transport regulates monoamine storage and release but is not essential for amphetamine action. Neuron 19:1271-1283.

Frank S (2010) Tetrabenazine: the first approved drug for the treatment of chorea in US patients with Huntington disease. Neuropsychiatric disease and treatment 6:657-665.

Freeze HH, Kranz C (2010) Endoglycosidase and glycoamidase release of N-linked glycans. Current protocols in molecular biology Chapter 17:Unit 17 13A.

Freyberg Z, Sonders MS, Aguilar JI, Hiranita T, Karam CS, Flores J, Pizzo AB, Zhang Y, Farino ZJ, Chen A, Martin CA, Kopajtic TA, Fei H, Hu G, Lin YY, Mosharov EV, McCabe BD, Freyberg R, Wimalasena K, Hsin LW, Sames D, Krantz DE, Katz JL, Sulzer D, Javitch JA (2016) Mechanisms of amphetamine action illuminated through optical monitoring of dopamine synaptic vesicles in Drosophila brain. Nature communications 7:10652.

Friedmann E, Hauben E, Maylandt K, Schleeger S, Vreugde S, Lichtenthaler SF, Kuhn PH, Stauffer D, Rovelli G, Martoglio B (2006) SPPL2a and SPPL2b promote intramembrane proteolysis of TNFalpha in activated dendritic cells to trigger IL-12 production. Nature cell biology 8:843-848.

Fukui M, Rodriguiz RM, Zhou J, Jiang SX, Phillips LE, Caron MG, Wetsel WC (2007) Vmat2 heterozygous mutant mice display a depressive-like phenotype. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:10520-10529.

Fulop T, Radabaugh S, Smith C (2005) Activity-dependent differential transmitter release in mouse adrenal chromaffin cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:7324-7332.

Fumagalli F, Gainetdinov RR, Wang YM, Valenzano KJ, Miller GW, Caron MG (1999) Increased methamphetamine neurotoxicity in heterozygous vesicular monoamine transporter 2 knock-out mice. Journal of Neuroscience 19:2424-2431.

Gainetdinov RR, Fumagalli F, Wang YM, Jones SR, Levey AI, Miller GW, Caron MG (1998) Increased MPTP neurotoxicity in vesicular monoamine transporter 2 heterozygote knockout mice. Journal of neurochemistry 70:1973-1978.

Geetha T, Jiang JX, Wooten MW (2005) Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. Molecular cell 20:301-312.

Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, Springer W (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nature cell biology 12:119-131.

German CL, Baladi MG, McFadden LM, Hanson GR, Fleckenstein AE (2015) Regulation of the Dopamine and Vesicular Monoamine Transporters: Pharmacological Targets and Implications for Disease. Pharmacological reviews 67:1005-1024.

Glatt CE, Wahner AD, White DJ, Ruiz-Linares A, Ritz B (2006) Gain-of-function haplotypes in the vesicular monoamine transporter promoter are protective for Parkinson disease in women. Human molecular genetics 15:299-305.

Goh LK, Huang F, Kim W, Gygi S, Sorkin A (2010) Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. The Journal of cell biology 189:871-883.

Goldberg AL (2007) Functions of the proteasome: from protein degradation and immune surveillance to cancer therapy. Biochemical Society transactions 35:12-17.

Goldstein DS, Kopin IJ, Sharabi Y (2014) Catecholamine autotoxicity. Implications for pharmacology and therapeutics of Parkinson disease and related disorders. Pharmacology & therapeutics 144:268-282.

Gonzalez-Hernandez T, Barroso-Chinea P, Muros ID, Perez-Delgado MD, Rodriguez M (2004) Expression of dopamine and vesicular monoamine transporters and differential vulnerability of mesostriatal dopaminergic neurons. Journal of Comparative Neurology 479:198-215.

Gow AJ, Duran D, Malcolm S, Ischiropoulos H (1996) Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. FEBS letters 385:63-66.

Grace AA, Bunney BS (1984a) The control of firing pattern in nigral dopamine neurons: burst firing. The Journal of neuroscience : the official journal of the Society for Neuroscience 4:2877-2890.

Grace AA, Bunney BS (1984b) The control of firing pattern in nigral dopamine neurons: single spike firing. The Journal of neuroscience : the official journal of the Society for Neuroscience 4:2866-2876.

Graham DG (1978) Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Molecular pharmacology 14:633-643.

Grant BD, Donaldson JG (2009) Pathways and mechanisms of endocytic recycling. Nature reviews Molecular cell biology 10:597-608.

Greenblatt EJ, Olzmann JA, Kopito RR (2011) Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant alpha-1 antitrypsin from the endoplasmic reticulum. Nature structural & molecular biology 18:1147-1152.

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proceedings of the National Academy of Sciences of the United States of America 73:2424-2428.

Grice GL, Nathan JA (2016) The recognition of ubiquitinated proteins by the proteasome. Cellular and molecular life sciences : CMLS 73:3497-3506.

Groenewegen HJ, Wright CI, Beijer AV, Voorn P (1999) Convergence and segregation of ventral striatal inputs and outputs. Annals of the New York Academy of Sciences 877:49-63.

Grygoruk A, Chen A, Martin CA, Lawal HO, Fei H, Gutierrez G, Biedermann T, Najibi R, Hadi R, Chouhan AK, Murphy NP, Schweizer FE, Macleod GT, Maidment NT, Krantz DE (2014) The redistribution of Drosophila vesicular monoamine transporter mutants from synaptic vesicles to large dense-core vesicles impairs amine-dependent behaviors. The Journal of neuroscience : the official journal of the Society for Neuroscience 34:6924-6937.

Guillot TS, Shepherd KR, Richardson JR, Wang MZ, Li YJ, Emson PC, Miller GW (2008) Reduced vesicular storage of dopamine exacerbates methamphetamine-induced neurodegeneration and astrogliosis. Journal of neurochemistry 106:2205-2217.

Guo JT, Chen AQ, Kong Q, Zhu H, Ma CM, Qin C (2008) Inhibition of vesicular monoamine transporter-2 activity in alpha-synuclein stably transfected SH-SY5Y cells. Cellular and molecular neurobiology 28:35-47.

Gutierrez B, Rosa A, Papiol S, Arrufat FJ, Catalan R, Salgado P, Peralta V, Cuesta MJ, Fananas L (2007) Identification of two risk haplotypes for schizophrenia and bipolar disorder in the synaptic vesicle monoamine transporter gene (SVMT). American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 144B:502-507.

Guzman JN, Hernandez A, Galarraga E, Tapia D, Laville A, Vergara R, Aceves J, Bargas J (2003) Dopaminergic modulation of axon collaterals interconnecting spiny neurons of the rat striatum. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:8931-8940.

Haber SN (2014) The place of dopamine in the cortico-basal ganglia circuit. Neuroscience 282:248-257.

Haber SN, Fudge JL, McFarland NR (2000) Striatonigrostriatal pathways in primates form an ascending spiral from the shell to the dorsolateral striatum. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:2369-2382.

Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nature cell biology 5:461-466.

Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441:885-889.

Harrington KA, Augood SJ, Kingsbury AE, Foster OJF, Emson PC (1996) Dopamine transporter (DAT) and synaptic vesicle amine transporter (VMAT2) gene expression in the substantia nigra of control and Parkinson's disease. Molecular Brain Research 36:157-162.

Harvey J, Lacey MG (1997) A postsynaptic interaction between dopamine D1 and NMDA receptors promotes presynaptic inhibition in the rat nucleus accumbens via adenosine release. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:5271-5280.

Hastings TG, Lewis DA, Zigmond MJ (1996) Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. Proceedings of the National Academy of Sciences of the United States of America 93:1956-1961.

Hattori T, McGeer PL, McGeer EG (1979) Dendro axonic neurotransmission. II. Morphological sites for the synthesis, binding and release of neurotransmitters in dopaminergic dendrites in the substantia nigra and cholinergic dendrites in the neostriatum. Brain research 170:71-83.

Hernandez D, Torres CA, Setlik W, Cebrian C, Mosharov EV, Tang GM, Cheng HC, Kholodilov N, Yarygina O, Burke RE, Gershon M, Sulzer D (2012) Regulation of Presynaptic Neurotransmission by Macroautophagy. Neuron 74:277-284.

Heuser JE, Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. The Journal of cell biology 57:315-344.

Hoffman BJ, Hansson SR, Mezey E, Palkovits M (1998) Localization and dynamic regulation of biogenic amine transporters in the mammalian central nervous system. Frontiers in Neuroendocrinology 19:187-231.

Hofmann RM, Pickart CM (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell 96:645-653.

Holtje M, von Jagow B, Pahner I, Lautenschlager M, Hortnagl H, Nurnberg B, Jahn R, Ahnert-Hilger G (2000) The neuronal monoamine transporter VMAT2 is regulated by the trimeric GTPase Go(2). Journal of Neuroscience 20:2131-2141.

Holtje M, Winter S, Walther D, Pahner I, Hortnagl H, Ottersen OP, Bader M, Ahnert-Hilger G (2003) The vesicular monoamine content regulates VMAT2 activity through Galphaq in mouse platelets. Evidence for autoregulation of vesicular transmitter uptake. The Journal of biological chemistry 278:15850-15858.

Holtzman E, Freeman AR, Kashner LA (1971) Stimulation-dependent alterations in peroxidase uptake at lobster neuromuscular junctions. Science 173:733-736.

Hoopmann P, Punge A, Barysch SV, Westphal V, Buckers J, Opazo F, Bethani I, Lauterbach MA, Hell SW, Rizzoli SO (2010) Endosomal sorting of readily releasable synaptic vesicles. Proceedings of the National Academy of Sciences of the United States of America 107:19055-19060.

Huang CH, Hsiao HT, Chu YR, Ye Y, Chen X (2013a) Derlin2 protein facilitates HRD1-mediated retro-translocation of sonic hedgehog at the endoplasmic reticulum. The Journal of biological chemistry 288:25330-25339.

Huang F, Zeng X, Kim W, Balasubramani M, Fortian A, Gygi SP, Yates NA, Sorkin A (2013b) Lysine 63-linked polyubiquitination is required for EGF receptor degradation. Proceedings of the National Academy of Sciences of the United States of America 110:15722-15727.

Hurley JH (2015) ESCRTs are everywhere. The EMBO journal 34:2398-2407.

Hwang J, Ribbens D, Raychaudhuri S, Cairns L, Gu H, Frost A, Urban S, Espenshade PJ (2016) A Golgi rhomboid protease Rbd2 recruits Cdc48 to cleave yeast SREBP. The EMBO journal 35:2332-2349.

Im E, Chung KC (2015) Dyrk1A phosphorylates parkin at Ser-131 and negatively regulates its ubiquitin E3 ligase activity. Journal of neurochemistry 134:756-768.

Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of parkin. Cell 105:891-902.

Ishikawa S, Tanaka Y, Takahashi-Niki K, Niki T, Ariga H, Iguchi-Ariga SMM (2012) Stimulation of vesicular monoamine transporter 2 activity by DJ-1 in SH-SY5Y cells. Biochemical and biophysical research communications 421:813-818.

Ivins KJ, Ivins JK, Sharp JP, Cotman CW (1999) Multiple pathways of apoptosis in PC12 cells. CrmA inhibits apoptosis induced by beta-amyloid. The Journal of biological chemistry 274:2107-2112.

Iwata A, Riley BE, Johnston JA, Kopito RR (2005) HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. The Journal of biological chemistry 280:40282-40292.

Jacobson AD, Zhang NY, Xu P, Han KJ, Noone S, Peng J, Liu CW (2009) The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26 s proteasome. The Journal of biological chemistry 284:35485-35494.

Jassen AK, Brown JM, Panas HN, Miller GM, Xiao D, Madras BK (2005) Variants of the primate vesicular monoamine transporter-2. Brain research Molecular brain research 139:251-257.

Kanehara K, Ito K, Akiyama Y (2002) YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA. Genes & development 16:2147-2155.

Kariya S, Hirano M, Takahashi N, Furiya Y, Ueno S (2005a) Lack of association between polymorphic microsatellites of the VMAT2 gene and Parkinson's disease in Japan. Journal of the neurological sciences 232:91-94.

Kariya S, Takahashi N, Hirano M, Ueno S (2005b) Increased vulnerability to L-DOPA toxicity in dopaminergic neurons from VMAT2 heterozygote knockout mice. Journal of Molecular Neuroscience 27:277-279.

Kazlauskaite A, Kelly V, Johnson C, Baillie C, Hastie CJ, Peggie M, Macartney T, Woodroof HI, Alessi DR, Pedrioli PG, Muqit MM (2014) Phosphorylation of Parkin at Serine65 is essential for activation: elaboration of a Miro1 substrate-based assay of Parkin E3 ligase activity. Open biology 4:130213.

Kelly RB (1993) Storage and release of neurotransmitters. Cell 72 Suppl:43-53.

Kiffin R, Christian C, Knecht E, Cuervo AM (2004) Activation of chaperone-mediated autophagy during oxidative stress. Molecular biology of the cell 15:4829-4840.

Kim HM, Yu YD, Cheng YF (2011) Structure characterization of the 26S proteasome. Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms 1809:67-79.

Kim PK, Hailey DW, Mullen RT, Lippincott-Schwartz J (2008) Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proceedings of the National Academy of Sciences of the United States of America 105:20567-20574.

Kimmel HL, Joyce AR, Carroll FI, Kuhar MJ (2001) Dopamine D1 and D2 receptors influence dopamine transporter synthesis and degradation in the rat. The Journal of pharmacology and experimental therapeutics 298:129-140.

Kirkin V, Lamark T, Sou YS, Bjorkoy G, Nunn JL, Bruun JA, Shvets E, McEwan DG, Clausen TH, Wild P, Bilusic I, Theurillat JP, Overvatn A, Ishii T, Elazar Z, Komatsu M, Dikic I,

Johansen T (2009a) A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Molecular cell 33:505-516.

Kirkin V, McEwan DG, Novak I, Dikic I (2009b) A Role for Ubiquitin in Selective Autophagy. Molecular cell 34:259-269.

Kisselev AF, Akopian TN, Castillo V, Goldberg AL (1999) Proteasome active sites allosterically regulate each other, suggesting a cyclical bite-chew mechanism for protein breakdown. Molecular cell 4:395-402.

Kisselev AF, van der Linden WA, Overkleeft HS (2012) Proteasome Inhibitors: An Expanding Army Attacking a Unique Target. Chemistry & biology 19:99-115.

Knoth J, Zallakian M, Njus D (1981) Stoichiometry of H+-linked dopamine transport in chromaffin granule ghosts. Biochemistry 20:6625-6629.

Koenig JH, Ikeda K (1996) Synaptic vesicles have two distinct recycling pathways. The Journal of cell biology 135:797-808.

Kokotos AC, Cousin MA (2015) Synaptic vesicle generation from central nerve terminal endosomes. Traffic 16:229-240.

Kollmann K, Damme M, Deuschl F, Kahle J, D'Hooge R, Lullmann-Rauch R, Lubke T (2009) Molecular characterization and gene disruption of mouse lysosomal putative serine carboxypeptidase 1. The FEBS journal 276:1356-1369.

Komander D (2009) The emerging complexity of protein ubiquitination. Biochemical Society transactions 37:937-953.

Komander D, Rape M (2012) The ubiquitin code. Annual review of biochemistry 81:203-229.

Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 441:880-884.

Komatsu M, Waguri S, Koike M, Sou Y, Ueno T, Hara T, Mizushima N, Iwata J, Ezaki J, Murata S, Hamazaki J, Nishito Y, Iemura S, Natsume T, Yanagawa T, Uwayama J, Warabi E, Yoshida H, Ishii T, Kobayashi A, Yamamoto M, Yue Z, Uchiyama Y, Kominami E, Tanaka K (2007) Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131:1149-1163.

Korolchuk VI, Mansilla A, Menzies FM, Rubinsztein DC (2009a) Autophagy inhibition compromises degradation of ubiquitin-proteasome pathway substrates. Molecular cell 33:517-527.

Korolchuk VI, Menzies FM, Rubinsztein DC (2009b) A novel link between autophagy and the ubiquitin-proteasome system. Autophagy 5:862-863.

Korolchuk VI, Menzies FM, Rubinsztein DC (2010) Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. FEBS letters 584:1393-1398.

Koyano F, Okatsu K, Kosako H, Tamura Y, Go E, Kimura M, Kimura Y, Tsuchiya H, Yoshihara H, Hirokawa T, Endo T, Fon EA, Trempe JF, Saeki Y, Tanaka K, Matsuda N (2014) Ubiquitin is phosphorylated by PINK1 to activate parkin. Nature 510:162-166.

Kozminski KD, Gutman DA, Davila V, Sulzer D, Ewing AG (1998) Voltammetric and pharmacological characterization of dopamine release from single exocytotic events at rat pheochromocytoma (PC12) cells. Analytical chemistry 70:3123-3130.

Krantz DE, Peter D, Liu Y, Edwards RH (1997) Phosphorylation of a vesicular monoamine transporter by casein kinase II. The Journal of biological chemistry 272:6752-6759.

Krantz DE, Waites C, Oorschot V, Liu Y, Wilson RI, Tan PK, Klumperman J, Edwards RH (2000) A phosphorylation site regulates sorting of the vesicular acetylcholine transporter to dense core vesicles. The Journal of cell biology 149:379-396.

Kravtsova-Ivantsiv Y, Ciechanover A (2012) Non-canonical ubiquitin-based signals for proteasomal degradation. Journal of cell science 125:539-548.

Kubo SI, Kitami T, Noda S, Shimura H, Uchiyama Y, Asakawa S, Minoshima S, Shimizu N, Mizuno Y, Hattori N (2001) Parkin is associated with cellular vesicles. Journal of neurochemistry 78:42-54.

Lachman HM, Papolos DF, Saito T, Yu YM, Szumlanski CL, Weinshilboum RM (1996) Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. Pharmacogenetics 6:243-250.

Lam PY, Cadenas E (2008) Compromised proteasome degradation elevates neuronal nitric oxide synthase levels and induces apoptotic cell death. Archives of biochemistry and biophysics 478:181-186.

Langosch D, Scharnagl C, Steiner H, Lemberg MK (2015) Understanding intramembrane proteolysis: from protein dynamics to reaction kinetics. Trends in biochemical sciences 40:318-327.

Larsen KE, Fon EA, Hastings TG, Edwards RH, Sulzer D (2002) Methamphetamine-induced degeneration of dopaminergic neurons involves autophagy and upregulation of dopamine synthesis. Journal of Neuroscience 22:8951-8960.

Larsen KE, Schmitz Y, Troyer MD, Mosharov E, Dietrich P, Quazi AZ, Savalle M, Nemani V, Chaudhry FA, Edwards RH, Stefanis L, Sulzer D (2006) Alpha-synuclein overexpression in PC12 and chromaffin cells impairs catecholamine release by interfering with a late step in exocytosis. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:11915-11922.

Lauwers E, Jacob C, Andre B (2009) K63-linked ubiquitin chains as a specific signal for protein sorting into the multivesicular body pathway. Journal of Cell Biology 185:493-502.

LaVoie MJ, Hastings TG (1999) Peroxynitrite- and nitrite-induced oxidation of dopamine: Implications for nitric oxide in dopaminergic cell loss. Journal of neurochemistry 73:2546-2554.

Lawal HO, Chang HY, Terrell AN, Brooks ES, Pulido D, Simon AF, Krantz DE (2010) The Drosophila vesicular monoamine transporter reduces pesticide-induced loss of dopaminergic neurons. Neurobiology of disease 40:102-112.

Lee CS, Samii A, Sossi V, Ruth TJ, Schulzer M, Holden JE, Wudel J, Pal PK, de la Fuente-Fernandez R, Calne DB, Stoessl AJ (2000) In vivo positron emission tomographic evidence for compensatory changes in presynaptic dopaminergic nerve terminals in Parkinson's disease. Annals of neurology 47:493-503.

Lee DH, Goldberg AL (1996) Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. The Journal of biological chemistry 271:27280-27284.

Lee DH, Goldberg AL (1998) Proteasome inhibitors cause induction of heat shock proteins and trehalose, which together confer thermotolerance in Saccharomyces cerevisiae. Mol Cell Biol 18:30-38.

Lev N, Barhum Y, Pilosof NS, Ickowicz D, Cohen HY, Melamed E, Offen D (2013) DJ-1 protects against dopamine toxicity: implications for Parkinson's disease and aging. The journals of gerontology Series A, Biological sciences and medical sciences 68:215-225.

Levin EY, Levenberg B, Kaufman S (1960) The enzymatic conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. The Journal of biological chemistry 235:2080-2086.

Li H, Waites CL, Staal RG, Dobryy Y, Park J, Sulzer DL, Edwards RH (2005) Sorting of vesicular monoamine transporter 2 to the regulated secretory pathway confers the somatodendritic exocytosis of monoamines. Neuron 48:619-633.

Li JY, Dahlstrom A (1997) Axonal transport of synaptic vesicle proteins in the rat optic nerve. Journal of neurobiology 32:237-250.

Li JY, Edelmann L, Jahn R, Dahlstrom A (1996) Axonal transport and distribution of synaptobrevin I and II in the rat peripheral nervous system. The Journal of neuroscience : the official journal of the Society for Neuroscience 16:137-147.

Lilienbaum A (2013) Relationship between the proteasomal system and autophagy. International journal of biochemistry and molecular biology 4:1-26.

Linert W, Herlinger E, Jameson RF, Kienzl E, Jellinger K, Youdim MB (1996) Dopamine, 6-hydroxydopamine, iron, and dioxygen--their mutual interactions and possible implication in the development of Parkinson's disease. Biochimica et biophysica acta 1316:160-168.

Lipford JR, Deshaies RJ (2003) Diverse roles for ubiquitin-dependent proteolysis in transcriptional activation. Nature cell biology 5:845-850.

Liu Y, Peter D, Roghani A, Schuldiner S, Prive GG, Eisenberg D, Brecha N, Edwards RH (1992) A cDNA that suppresses MPP+ toxicity encodes a vesicular amine transporter. Cell 70:539-551.

Liu Y, Schweitzer ES, Nirenberg MJ, Pickel VM, Evans CJ, Edwards RH (1994) Preferential localization of a vesicular monoamine transporter to dense core vesicles in PC12 cells. The Journal of cell biology 127:1419-1433.

Lohr KM, Bernstein AI, Stout KA, Dunn AR, Lazo CR, Alter SP, Wang M, Li Y, Fan X, Hess EJ, Yi H, Vecchio LM, Goldstein DS, Guillot TS, Salahpour A, Miller GW (2014) Increased vesicular monoamine transporter enhances dopamine release and opposes Parkinson disease-related neurodegeneration in vivo. Proceedings of the National Academy of Sciences of the United States of America 111:9977-9982.

Lohr KM, Stout KA, Dunn AR, Wang M, Salahpour A, Guillot TS, Miller GW (2015) Increased Vesicular Monoamine Transporter 2 (VMAT2; Slc18a2) Protects against Methamphetamine Toxicity. ACS chemical neuroscience 6:790-799.

Longva KE, Blystad FD, Stang E, Larsen AM, Johannessen LE, Madshus IH (2002) Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. The Journal of cell biology 156:843-854.

Lotharius J, Barg S, Wiekop P, Lundberg C, Raymon HK, Brundin P (2002) Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line. The Journal of biological chemistry 277:38884-38894.

Lotharius J, O'Malley KL (2000) The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity. The Journal of biological chemistry 275:38581-38588.

Lovenberg W, Weissbach H, Udenfriend S (1962) Aromatic L-amino acid decarboxylase. The Journal of biological chemistry 237:89-93.

Luzio JP, Pryor PR, Bright NA (2007) Lysosomes: fusion and function. Nature Reviews Molecular Cell Biology 8:622-632.

MacGurn JA, Hsu PC, Emr SD (2012) Ubiquitin and membrane protein turnover: from cradle to grave. Annual review of biochemistry 81:231-259.

Manders EMM, Verbeek FJ, Aten JA (1993) Measurement of co-localization of objects in dual-colour confocal images. Journal of microscopy 169:375-382.

Maron R, Stern Y, Kanner BI, Schuldiner S (1983) Functional asymmetry of the amine transporter from chromaffin granules. The Journal of biological chemistry 258:11476-11481.

Mattiroli F, Sixma TK (2014) Lysine-targeting specificity in ubiquitin and ubiquitin-like modification pathways. Nature structural & molecular biology 21:308-316.

McNaught KS, Belizaire R, Isacson O, Jenner P, Olanow CW (2003) Altered proteasomal function in sporadic Parkinson's disease. Experimental neurology 179:38-46.

Meng L, Mohan R, Kwok BH, Elofsson M, Sin N, Crews CM (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. Proceedings of the National Academy of Sciences of the United States of America 96:10403-10408.

Merickel A, Edwards RH (1995) Transport of Histamine by Vesicular Monoamine Transporter-2. Neuropharmacology 34:1543-1547.

Miller DK, Crooks PA, Teng L, Witkin JM, Munzar P, Goldberg SR, Acri JB, Dwoskin LP (2001) Lobeline inhibits the neurochemical and behavioral effects of amphetamine. The Journal of pharmacology and experimental therapeutics 296:1023-1034.

Miller GW, Erickson JD, Perez JT, Penland SN, Mash DC, Rye DB, Levey AI (1999) Immunochemical analysis of vesicular monoamine transporter (VMAT2) protein in Parkinson's disease. Experimental neurology 156:138-148.

Mindell JA (2012) Lysosomal acidification mechanisms. Annual review of physiology 74:69-86.

Miranda M, Dionne KR, Sorkina T, Sorkin A (2007) Three ubiquitin conjugation sites in the amino terminus of the dopamine transporter mediate protein kinase C-dependent endocytosis of the transporter. Molecular biology of the cell 18:313-323.

Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M, Ohsumi Y (1998) A protein conjugation system essential for autophagy. Nature 395:395-398.

Moriyoshi K, Iijima K, Fujii H, Ito H, Cho Y, Nakanishi S (2004) Seven in absentia homolog 1A mediates ubiquitination and degradation of group 1 metabotropic glutamate receptors. Proceedings of the National Academy of Sciences of the United States of America 101:8614-8619.

Mosharov EV, Larsen KE, Kanter E, Phillips KA, Wilson K, Schmitz Y, Krantz DE, Kobayashi K, Edwards RH, Sulzer D (2009) Interplay between cytosolic dopamine, calcium, and alpha-synuclein causes selective death of substantia nigra neurons. Neuron 62:218-229.

Mouatt-Prigent A, Muriel MP, Gu WJ, El Hachimi KH, Lucking CB, Brice A, Hirsch EC (2004) Ultrastructural localization of parkin in the rat brainstem, thalamus and basal ganglia. Journal of neural transmission 111:1209-1218.

Mrschtik M, Ryan KM (2015) Lysosomal proteins in cell death and autophagy. The FEBS journal 282:1858-1870.

Mundorf ML, Hochstetler SE, Wightman RM (1999) Amine weak bases disrupt vesicular storage and promote exocytosis in chromaffin cells. Journal of neurochemistry 73:2397-2405.

Nagatsu T, Levitt M, Udenfriend S (1964a) Conversion of L-tyrosine to 3,4-dihydroxyphenylalanine by cell-free preparations of brain and sympathetically innervated tissues. Biochemical and biophysical research communications 14:543-549.

Nagatsu T, Levitt M, Udenfriend S (1964b) Tyrosine Hydroxylase. The Initial Step in Norepinephrine Biosynthesis. The Journal of biological chemistry 239:2910-2917.

Nirenberg MJ, Chan J, Liu Y, Edwards RH, Pickel VM (1996) Ultrastructural localization of the vesicular monoamine transporter-2 in midbrain dopaminergic neurons: potential sites for somatodendritic storage and release of dopamine. Journal of Neuroscience 16:4135-4145.

Nunes EJ, Randall PA, Hart EE, Freeland C, Yohn SE, Baqi Y, Muller CE, Lopez-Cruz L, Correa M, Salamone JD (2013) Effort-related motivational effects of the VMAT-2 inhibitor tetrabenazine: implications for animal models of the motivational symptoms of depression. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:19120-19130.

Omiatek DM, Dong Y, Heien ML, Ewing AG (2010) Only a Fraction of Quantal Content is Released During Exocytosis as Revealed by Electrochemical Cytometry of Secretory Vesicles. ACS chemical neuroscience 1:234-245.

Onoa B, Li HY, Gagnon-Bartsch JA, Elias LAB, Edwards RH (2010) Vesicular Monoamine and Glutamate Transporters Select Distinct Synaptic Vesicle Recycling Pathways. Journal of Neuroscience 30:7917-7927.

Pandey UB, Nie Z, Batlevi Y, McCray BA, Ritson GP, Nedelsky NB, Schwartz SL, DiProspero NA, Knight MA, Schuldiner O, Padmanabhan R, Hild M, Berry DL, Garza D, Hubbert CC, Yao TP, Baehrecke EH, Taylor JP (2007) HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature 447:859-863.

Park H, Li Y, Tsien RW (2012) Influence of synaptic vesicle position on release probability and exocytotic fusion mode. Science 335:1362-1366.

Parra LA, Baust T, El Mestikawy S, Quiroz M, Hoffman B, Haflett JM, Yao JK, Torres GE (2008) The Orphan Transporter Rxt1/NTT4 (SLC6A17) Functions as a Synaptic Vesicle Amino Acid Transporter Selective for Proline, Glycine, Leucine, and Alanine. Molecular pharmacology 74:1521-1532.

Pennartz CM, Dolleman-Van der Weel MJ, Kitai ST, Lopes da Silva FH (1992) Presynaptic dopamine D1 receptors attenuate excitatory and inhibitory limbic inputs to the shell

region of the rat nucleus accumbens studied in vitro. Journal of neurophysiology 67:1325-1334.

Peter D, Jimenez J, Liu Y, Kim J, Edwards RH (1994) The chromaffin granule and synaptic vesicle amine transporters differ in substrate recognition and sensitivity to inhibitors. The Journal of biological chemistry 269:7231-7237.

Peter D, Liu Y, Sternini C, de Giorgio R, Brecha N, Edwards RH (1995) Differential expression of two vesicular monoamine transporters. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:6179-6188.

Peter D, Vu T, Edwards RH (1996) Chimeric vesicular monoamine transporters identify structural domains that influence substrate affinity and sensitivity to tetrabenazine. The Journal of biological chemistry 271:2979-2986.

Pettibone DJ, Totaro JA, Pflueger AB (1984) Tetrabenazine-induced depletion of brain monoamines: characterization and interaction with selected antidepressants. European journal of pharmacology 102:425-430.

Pifl C, Drobny H, Reither H, Hornykiewicz O, Singer EA (1995) Mechanism of the dopamine-releasing actions of amphetamine and cocaine: plasmalemmal dopamine transporter versus vesicular monoamine transporter. Molecular pharmacology 47:368-373.

Pifl C, Rajput A, Reither H, Blesa J, Cavada C, Obeso JA, Rajput AH, Hornykiewicz O (2014) Is Parkinson's disease a vesicular dopamine storage disorder? Evidence from a study in isolated synaptic vesicles of human and nonhuman primate striatum. The Journal of neuroscience : the official journal of the Society for Neuroscience 34:8210-8218.

Platt FM, Boland B, van der Spoel AC (2012) The cell biology of disease: lysosomal storage disorders: the cellular impact of lysosomal dysfunction. The Journal of cell biology 199:723-734.

Polo S, Sigismund S, Faretta M, Guidi M, Capua MR, Bossi G, Chen H, De Camilli P, Di Fiore PP (2002) A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature 416:451-455.

Pothos EN, Larsen KE, Krantz DE, Liu Y, Haycock JW, Setlik W, Gershon MD, Edwards RH, Sulzer D (2000) Synaptic vesicle transporter expression regulates vesicle phenotype and quantal size. Journal of Neuroscience 20:7297-7306.

Preskorn SH, Kent TA, Glotzbach RK, Irwin GH, Solnick JV (1984) Cerebromicrocirculatory defects in animal model of depression. Psychopharmacology 84:196-199.

Puig MV, Gulledge AT (2011) Serotonin and prefrontal cortex function: neurons, networks, and circuits. Molecular neurobiology 44:449-464.

Qiao L, Zhang J (2009) Inhibition of lysosomal functions reduces proteasomal activity. Neuroscience letters 456:15-19.

Rabinovich E, Kerem A, Frohlich KU, Diamant N, Bar-Nun S (2002) AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol Cell Biol 22:626-634.

Randall PA, Lee CA, Nunes EJ, Yohn SE, Nowak V, Khan B, Shah P, Pandit S, Vemuri VK, Makriyannis A, Baqi Y, Muller CE, Correa M, Salamone JD (2014) The VMAT-2 inhibitor tetrabenazine affects effort-related decision making in a progressive ratio/chow feeding choice task: reversal with antidepressant drugs. PloS one 9:e99320.

Requena DF, Parra LA, Baust TB, Quiroz M, Leak RK, Garcia-Olivares J, Torres GE (2009) The molecular chaperone Hsc70 interacts with the vesicular monoamine transporter-2. Journal of neurochemistry 110:581-594.

Reveron ME, Savelieva KV, Tillerson JL, McCormack AL, Di Monte DA, Miller GW (2002) L-DOPA does not cause neurotoxicity in VMAT2 heterozygote knockout mice. Neurotoxicology 23:611-619.

Richly H, Rape M, Braun S, Rumpf S, Hoege C, Jentsch S (2005) A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell 120:73-84.

Riddle EL, Topham MK, Haycock JW, Hanson GR, Fleckenstein AE (2002) Differential trafficking of the vesicular monoamine transporter-2 by methamphetamine and cocaine. European journal of pharmacology 449:71-74.

Rideout HJ, Lang-Rollin I, Stefanis L (2004) Involvement of macroautophagy in the dissolution of neuronal inclusions. The international journal of biochemistry & cell biology 36:2551-2562.

Rilstone JJ, Alkhater RA, Minassian BA (2013) Brain Dopamine-Serotonin Vesicular Transport Disease and Its Treatment. New Engl J Med 368:543-550.

Rinetti GV, Schweizer FE (2010) Ubiquitination acutely regulates presynaptic neurotransmitter release in mammalian neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:3157-3166.

Rizzoli SO (2014) Synaptic vesicle recycling: steps and principles. The EMBO journal 33:788-822.

Rizzoli SO, Bethani I, Zwilling D, Wenzel D, Siddiqui TJ, Brandhorst D, Jahn R (2006) Evidence for early endosome-like fusion of recently endocytosed synaptic vesicles. Traffic 7:1163-1176.

Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg AL (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78:761-771.

Roy L, Bergeron JJ, Lavoie C, Hendriks R, Gushue J, Fazel A, Pelletier A, Morre DJ, Subramaniam VN, Hong W, Paiement J (2000) Role of p97 and syntaxin 5 in the assembly of transitional endoplasmic reticulum. Molecular biology of the cell 11:2529-2542.

Rubinsztein DC (2006) The roles of intracellular protein-degradation pathways in neurodegeneration. Nature 443:780-786.

Saftig P, Klumperman J (2009) Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. Nature Reviews Molecular Cell Biology 10:623-635.

Saheki Y, De Camilli P (2012) Synaptic vesicle endocytosis. Cold Spring Harbor perspectives in biology 4:a005645.

Sai Y, Wu Q, Le W, Ye F, Li Y, Dong Z (2008) Rotenone-induced PC12 cell toxicity is caused by oxidative stress resulting from altered dopamine metabolism. Toxicology in vitro : an international journal published in association with BIBRA 22:1461-1468.

Sala G, Brighina L, Saracchi E, Fermi S, Riva C, Carrozza V, Pirovano M, Ferrarese C (2010) Vesicular monoamine transporter 2 mRNA levels are reduced in platelets from patients with Parkinson's disease. Journal of neural transmission 117:1093-1098.

Salamone JD (1994) The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. Behavioural brain research 61:117-133.

Sang TK, Chang HY, Lawless GM, Ratnaparkhi A, Mee L, Ackerson LC, Maidment NT, Krantz DE, Jackson GR (2007) A Drosophila model of mutant human parkin-induced toxicity demonstrates selective loss of dopaminergic neurons and dependence on cellular dopamine. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:981-992.

Sannerud R, Annaert W (2009) Trafficking, a key player in regulated intramembrane proteolysis. Seminars in cell & developmental biology 20:183-190.

Sasaki T, Kishi M, Saito M, Tanaka T, Higuchi N, Kominami E, Katunuma N, Murachi T (1990) Inhibitory effect of di- and tripeptidyl aldehydes on calpains and cathepsins. Journal of enzyme inhibition 3:195-201.

Scherman D, Henry JP (1984) Reserpine binding to bovine chromaffin granule membranes. Characterization and comparison with dihydrotetrabenazine binding. Molecular pharmacology 25:113-122.

Schweitzer ES, Sanderson MJ, Wasterlain CG (1995) Inhibition of regulated catecholamine secretion from PC12 cells by the Ca2+/calmodulin kinase II inhibitor KN-62. Journal of cell science 108 (Pt 7):2619-2628.

Scott DC, Schekman R (2008) Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins. The Journal of cell biology 181:1095-1105.

Seeman P, Van Tol HH (1994) Dopamine receptor pharmacology. Trends in pharmacological sciences 15:264-270.

Segura-Aguilar J, Paris I, Munoz P, Ferrari E, Zecca L, Zucca FA (2014) Protective and toxic roles of dopamine in Parkinson's disease. Journal of neurochemistry 129:898-915.

Sesack SR, Aoki C, Pickel VM (1994) Ultrastructural localization of D2 receptor-like immunoreactivity in midbrain dopamine neurons and their striatal targets. The Journal of neuroscience : the official journal of the Society for Neuroscience 14:88-106.

Settembre C, Fraldi A, Medina DL, Ballabio A (2013) Signals from the lysosome: a control centre for cellular clearance and energy metabolism. Nature reviews Molecular cell biology 14:283-296.

Sidransky E, Nalls MA, Aasly JO, Aharon-Peretz J, Annesi G, Barbosa ER, Bar-Shira A, Berg D, Bras J, Brice A, Chen CM, Clark LN, Condroyer C, De Marco EV, Durr A, Eblan MJ, Fahn S, Farrer MJ, Fung HC, Gan-Or Z, Gasser T, Gershoni-Baruch R, Giladi N, Griffith A, Gurevich T, Januario C, Kropp P, Lang AE, Lee-Chen GJ, Lesage S, Marder K, Mata IF, Mirelman A, Mitsui J, Mizuta I, Nicoletti G, Oliveira C, Ottman R, Orr-Urtreger A, Pereira LV, Quattrone A, Rogaeva E, Rolfs A, Rosenbaum H, Rozenberg R, Samii A, Samaddar T, Schulte C, Sharma M, Singleton A, Spitz M, Tan EK, Tayebi N, Toda T, Troiano AR, Tsuji S, Wittstock M, Wolfsberg TG, Wu YR, Zabetian CP, Zhao Y, Ziegler SG (2009) Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. The New England journal of medicine 361:1651-1661.

Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, Di Fiore PP, Polo S (2005) Clathrin-independent endocytosis of ubiquitinated cargos. Proceedings of the National Academy of Sciences of the United States of America 102:2760-2765.

Sin N, Kim KB, Elofsson M, Meng L, Auth H, Kwok BH, Crews CM (1999) Total synthesis of the potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology. Bioorganic & medicinal chemistry letters 9:2283-2288.

Sorkin A, von Zastrow M (2009) Endocytosis and signalling: intertwining molecular networks. Nature reviews Molecular cell biology 10:609-622.

Speese SD, Trotta N, Rodesch CK, Aravamudan B, Broadie K (2003) The ubiquitin proteasome system acutely regulates presynaptic protein turnover and synaptic efficacy. Current biology : CB 13:899-910.

Stewart EV, Nwosu CC, Tong Z, Roguev A, Cummins TD, Kim DU, Hayles J, Park HO, Hoe KL, Powell DW, Krogan NJ, Espenshade PJ (2011) Yeast SREBP cleavage activation requires the Golgi Dsc E3 ligase complex. Molecular cell 42:160-171.

Stolz A, Wolf DH (2010) Endoplasmic reticulum associated protein degradation: A chaperone assisted journey to hell. Biochimica Et Biophysica Acta-Molecular Cell Research 1803:694-705.

Stoof JC, Kebabian JW (1981) Opposing roles for D-1 and D-2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. Nature 294:366-368.

Stringer DK, Piper RC (2011) A single ubiquitin is sufficient for cargo protein entry into MVBs in the absence of ESCRT ubiquitination. The Journal of cell biology 192:229-242.

Sulzer D, Chen TK, Lau YY, Kristensen H, Rayport S, Ewing A (1995) Amphetamine Redistributes Dopamine from Synaptic Vesicles to the Cytosol and Promotes Reverse Transport. Journal of Neuroscience 15:4102-4108.

Sulzer D, Cragg SJ, Rice ME (2016) Striatal dopamine neurotransmission: regulation of release and uptake. Basal ganglia 6:123-148.

Sulzer D, Joyce MP, Lin L, Geldwert D, Haber SN, Hattori T, Rayport S (1998) Dopamine neurons make glutamatergic synapses in vitro. The Journal of neuroscience : the official journal of the Society for Neuroscience 18:4588-4602.

Sulzer D, Rayport S (1990) Amphetamine and other psychostimulants reduce pH gradients in midbrain dopaminergic neurons and chromaffin granules: a mechanism of action. Neuron 5:797-808.

Sulzer D, Zecca L (2000) Intraneuronal dopamine-quinone synthesis: a review. Neurotoxicity research 1:181-195.

Surratt CK, Persico AM, Yang XD, Edgar SR, Bird GS, Hawkins AL, Griffin CA, Li X, Jabs EW, Uhl GR (1993) A human synaptic vesicle monoamine transporter cDNA predicts posttranslational modifications, reveals chromosome 10 gene localization and identifies TaqI RFLPs. FEBS letters 318:325-330.

Sutton MA, Beninger RJ (1999) Psychopharmacology of conditioned reward: evidence for a rewarding signal at D1-like dopamine receptors. Psychopharmacology 144:95-110.

Swaminathan S, Amerik AY, Hochstrasser M (1999) The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. Molecular biology of the cell 10:2583-2594.

Swatek KN, Komander D (2016) Ubiquitin modifications. Cell research 26:399-422.

Tai HC, Schuman EM (2008) Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. Nature reviews Neuroscience 9:826-838.

Takahashi N, Miner LL, Sora I, Ujike H, Revay RS, Kostic V, JacksonLewis V, Przedborski S, Uhl GR (1997) VMAT2 knockout mice: Heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. Proceedings of the National Academy of Sciences of the United States of America 94:9938-9943.

Takahashi N, Uhl G (1997) Murine vesicular monoamine transporter 2: molecular cloning and genomic structure. Brain research Molecular brain research 49:7-14.

Tan PK, Waites C, Liu YJ, Krantz DE, Edwards RH (1998) A leucine-based motif mediates the endocytosis of vesicular monoamine and acetylcholine transporters. Journal of Biological Chemistry 273:17351-17360.

Taylor TN, Caudle WM, Shepherd KR, Noorian A, Jackson CR, Iuvone PM, Weinshenker D, Greene JG, Miller GW (2009) Nonmotor Symptoms of Parkinson's Disease Revealed in an Animal Model with Reduced Monoamine Storage Capacity. Journal of Neuroscience 29:8103-8113.

Tecuapetla F, Koos T, Tepper JM, Kabbani N, Yeckel MF (2009) Differential dopaminergic modulation of neostriatal synaptic connections of striatopallidal axon collaterals. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:8977-8990.

Tecuapetla F, Patel JC, Xenias H, English D, Tadros I, Shah F, Berlin J, Deisseroth K, Rice ME, Tepper JM, Koos T (2010) Glutamatergic signaling by mesolimbic dopamine neurons in the nucleus accumbens. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7105-7110.

Teng L, Crooks PA, Dwoskin LP (1998) Lobeline displaces [3H]dihydrotetrabenazine binding and releases [3H]dopamine from rat striatal synaptic vesicles: comparison with d-amphetamine. Journal of neurochemistry 71:258-265.

Teng L, Crooks PA, Sonsalla PK, Dwoskin LP (1997) Lobeline and nicotine evoke [3H]overflow from rat striatal slices preloaded with [3H]dopamine: differential inhibition of synaptosomal and vesicular [3H]dopamine uptake. The Journal of pharmacology and experimental therapeutics 280:1432-1444.

Thrower JS, Hoffman L, Rechsteiner M, Pickart CM (2000) Recognition of the polyubiquitin proteolytic signal. The EMBO journal 19:94-102.

Tong Z, Kim MS, Pandey A, Espenshade PJ (2014) Identification of candidate substrates for the Golgi Tul1 E3 ligase using quantitative diGly proteomics in yeast. Molecular & cellular proteomics : MCP 13:2871-2882.

Törk I (1990) Anatomy of the serotonergic system. Annals of the New York Academy of Sciences 600:9-34; discussion 34-35.

Torres B, Ruoho AE (2014) N-terminus regulation of VMAT2 mediates methamphetamine-stimulated efflux. Neuroscience 259:194-202.

Tritsch NX, Ding JB, Sabatini BL (2012) Dopaminergic neurons inhibit striatal output through non-canonical release of GABA. Nature 490:262-266.

Tritsch NX, Sabatini BL (2012) Dopaminergic modulation of synaptic transmission in cortex and striatum. Neuron 76:33-50.

Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S (1996) Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. Journal of biochemistry 119:572-576.

Tsukita S, Ishikawa H (1980) The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles. The Journal of cell biology 84:513-530.

Ungerstedt U (1971) Stereotaxic mapping of the monoamine pathways in the rat brain. Acta physiologica Scandinavica Supplementum 367:1-48.

Usami Y, Hatano T, Imai S, Kubo S, Sato S, Saiki S, Fujioka Y, Ohba Y, Sato F, Funayama M, Eguchi H, Shiba K, Ariga H, Shen J, Hattori N (2011) DJ-1 associates with synaptic membranes. Neurobiology of disease 43:651-662.

Usiello A, Baik JH, Rouge-Pont F, Picetti R, Dierich A, LeMeur M, Piazza PV, Borrelli E (2000) Distinct functions of the two isoforms of dopamine D2 receptors. Nature 408:199-203.

Uytterhoeven V, Kuenen S, Kasprowicz J, Miskiewicz K, Verstreken P (2011) Loss of skywalker reveals synaptic endosomes as sorting stations for synaptic vesicle proteins. Cell 145:117-132.

Vander Borght TM, Kilbourn MR, Koeppe RA, DaSilva JN, Carey JE, Kuhl DE, Frey KA (1995) In vivo imaging of the brain vesicular monoamine transporter. Journal of nuclear medicine : official publication, Society of Nuclear Medicine 36:2252-2260.

Varoqui H, Erickson JD (1998) The cytoplasmic tail of the vesicular acetylcholine transporter contains a synaptic vesicle targeting signal. The Journal of biological chemistry 273:9094-9098.

Vashist S, Ng DT (2004) Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. The Journal of cell biology 165:41-52.

Vembar SS, Brodsky JL (2008) One step at a time: endoplasmic reticulum-associated degradation. Nature Reviews Molecular Cell Biology 9:944-U930.

Verheij MMM, Cools AR (2008) Twenty years of dopamine research: Individual differences in the response of accumbal dopamine to environmental and pharmacological challenges. European journal of pharmacology 585:228-244.

Versteeg RI, Serlie MJ, Kalsbeek A, la Fleur SE (2015) Serotonin, a possible intermediate between disturbed circadian rhythms and metabolic disease. Neuroscience 301:155-167.

Vicentic A, Battaglia G, Carroll FI, Kuhar MJ (1999) Serotonin transporter production and degradation rates: studies with RTI-76. Brain research 841:1-10.

Vina-Vilaseca A, Sorkin A (2010) Lysine 63-linked Polyubiquitination of the Dopamine Transporter Requires WW3 and WW4 Domains of Nedd4-2 and UBE2D Ubiquitin-conjugating Enzymes. Journal of Biological Chemistry 285:7645-7656.

Waites CL, Mehta A, Tan PK, Thomas G, Edwards RH, Krantz DE (2001) An acidic motif retains vesicular monoamine transporter 2 on large dense core vesicles. Journal of Cell Biology 152:1159-1168.

Walkinshaw G, Waters CM (1994) Neurotoxin-induced cell death in neuronal PC12 cells is mediated by induction of apoptosis. Neuroscience 63:975-987.

Wang Q, Li L, Ye Y (2008) Inhibition of p97-dependent protein degradation by Eeyarestatin I. The Journal of biological chemistry 283:7445-7454.

Wang Q, Shinkre BA, Lee JG, Weniger MA, Liu Y, Chen W, Wiestner A, Trenkle WC, Ye Y (2010) The ERAD inhibitor Eeyarestatin I is a bifunctional compound with a membrane-binding domain and a p97/VCP inhibitory group. PloS one 5:e15479.

Wang R, Zhao J, Zhang J, Liu W, Zhao M, Li J, Lv J, Li Y (2015a) Effect of lysosomal and ubiquitin-proteasome system dysfunction on the abnormal aggregation of alpha-synuclein in PC12 cells. Experimental and therapeutic medicine 9:2088-2094.

Wang XL, Herr RA, Chua WJ, Lybarger L, Wiertz EJHJ, Hansen TH (2007) Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. Journal of Cell Biology 177:613-624.

Wang Y, Zhou Z, Leylek T, Tan H, Sun Y, Parkinson FE, Wang JF (2015b) Protein cysteine S-nitrosylation inhibits vesicular uptake of neurotransmitters. Neuroscience 311:374-381.

Wang YM, Gainetdinov RR, Fumagalli F, Xu F, Jones SR, Bock CB, Miller GW, Wightman RM, Caron MG (1997) Knockout of the vesicular monoamine transporter 2 gene results in neonatal death and supersensitivity to cocaine and amphetamine. Neuron 19:1285-1296.

Watabe M, Nakaki T (2008) Mitochondrial complex I inhibitor rotenone inhibits and redistributes vesicular monoamine transporter 2 via nitration in human dopaminergic SH-SY5Y cells. Molecular pharmacology 74:933-940.

Watts GD, Wymer J, Kovach MJ, Mehta SG, Mumm S, Darvish D, Pestronk A, Whyte MP, Kimonis VE (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. Nature genetics 36:377-381.

Wauer T, Komander D (2013) Structure of the human Parkin ligase domain in an autoinhibited state. The EMBO journal 32:2099-2112.

Weihe E, Tao-Cheng JH, Schafer MK, Erickson JD, Eiden LE (1996) Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a

specific population of small synaptic vesicles. Proceedings of the National Academy of Sciences of the United States of America 93:3547-3552.

Weihl CC, Dalal S, Pestronk A, Hanson PI (2006) Inclusion body myopathy-associated mutations in p97/VCP impair endoplasmic reticulum-associated degradation. Human molecular genetics 15:189-199.

Weingarten P, Zhou QY (2001) Protection of intracellular dopamine cytotoxicity by dopamine disposition and metabolism factors. Journal of neurochemistry 77:776-785.

Welchman RL, Gordon C, Mayer RJ (2005) Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nature Reviews Molecular Cell Biology 6:599-609.

Westerink RH, Ewing AG (2008) The PC12 cell as model for neurosecretion. Acta physiologica 192:273-285.

Wheeler TC, Chin LS, Li YK, Roudabush FL, Li LA (2002) Regulation of synaptophysin degradation by mammalian homologues of Seven in Absentia. Journal of Biological Chemistry 277:10273-10282.

Wiertz EJ, Tortorella D, Bogyo M, Yu J, Mothes W, Jones TR, Rapoport TA, Ploegh HL (1996) Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 384:432-438.

Wilcox D, Mason RW (1992) Inhibition of cysteine proteinases in lysosomes and whole cells. The Biochemical journal 285 (Pt 2):495-502.

Wilhelm CJ, Johnson RA, Eshleman AJ, Janowsky A (2008) Lobeline effects on tonic and methamphetamine-induced dopamine release. Biochemical pharmacology 75:1411-1415.

Willeumier K, Pulst SM, Schweizer FE (2006) Proteasome inhibition triggers activity-dependent increase in the size of the recycling vesicle pool in cultured hippocampal neurons. Journal of Neuroscience 26:11333-11341.

Wing SS, Chiang HL, Goldberg AL, Dice JF (1991) Proteins containing peptide sequences related to Lys-Phe-Glu-Arg-Gln are selectively depleted in liver and heart, but not skeletal muscle, of fasted rats. The Biochemical journal 275 (Pt 1):165-169.

Winget JM, Mayor T (2010) The Diversity of Ubiquitin Recognition: Hot Spots and Varied Specificity. Molecular cell 38:627-635.

Wise RA (2009) Roles for nigrostriatal--not just mesocorticolimbic--dopamine in reward and addiction. Trends in neurosciences 32:517-524.

Xu P, Duong DM, Seyfried NT, Cheng D, Xie Y, Robert J, Rush J, Hochstrasser M, Finley D, Peng J (2009) Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. Cell 137:133-145.

Xu S, Peng G, Wang Y, Fang S, Karbowski M (2011) The AAA-ATPase p97 is essential for outer mitochondrial membrane protein turnover. Molecular biology of the cell 22:291-300.

Yaffe D, Radestock S, Shuster Y, Forrest LR, Schuldiner S (2013) Identification of molecular hinge points mediating alternating access in the vesicular monoamine transporter VMAT2. Proceedings of the National Academy of Sciences of the United States of America 110:E1332-1341.

Yamamoto S, Fukae J, Mori H, Mizuno Y, Hattori N (2006) Positive immunoreactivity for vesicular monoamine transporter 2 in Lewy bodies and Lewy neurites in substantia nigra. Neuroscience letters 396:187-191.

Yang H, Liu C, Zhong Y, Luo S, Monteiro MJ, Fang S (2010) Huntingtin interacts with the cue domain of gp78 and inhibits gp78 binding to ubiquitin and p97/VCP. PloS one 5:e8905.

Yao I, Takagi H, Ageta H, Kahyo T, Sato S, Hatanaka K, Fukuda Y, Chiba T, Morone N, Yuasa S, Inokuchi K, Ohtsuka T, MacGregor GR, Tanaka K, Setou M (2007) SCRAPPER-dependent ubiquitination of active zone protein RIM1 regulates synaptic vesicle release. Cell 130:943-957.

Yao J, Erickson JD, Hersh LB (2004) Protein kinase A affects trafficking of the vesicular monoamine transporters in PC12 cells. Traffic 5:1006-1016.

Yao J, Hersh LB (2007) The vesicular monoamine transporter 2 contains trafficking signals in both its N-glycosylation and C-terminal domains. Journal of neurochemistry 100:1387-1396.

Yasumoto S, Tamura K, Karasawa J, Hasegawa R, Ikeda K, Yamamoto T, Yamamoto H (2009) Inhibitory effect of selective serotonin reuptake inhibitors on the vesicular monoamine transporter 2. Neuroscience letters 454:229-232.

Ye Y, Meyer HH, Rapoport TA (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature 414:652-656.

Yelin R, Steiner-Mordoch S, Aroeti B, Schuldiner S (1998) Glycosylation of a vesicular monoamine transporter: a mutation in a conserved proline residue affects the activity, glycosylation, and localization of the transporter. Journal of neurochemistry 71:2518-2527.

Yewdell JW, Lacsina JR, Rechsteiner MC, Nicchitta CV (2011) Out with the old, in with the new? Comparing methods for measuring protein degradation. Cell biology international 35:457-462.

Yuan WC, Lee YR, Lin SY, Chang LY, Tan YP, Hung CC, Kuo JC, Liu CH, Lin MY, Xu M, Chen ZJ, Chen RH (2014) K33-Linked Polyubiquitination of Coronin 7 by Cul3-KLHL20 Ubiquitin E3 Ligase Regulates Protein Trafficking. Molecular cell 54:586-600.

Yung KK, Bolam JP, Smith AD, Hersch SM, Ciliax BJ, Levey AI (1995) Immunocytochemical localization of D1 and D2 dopamine receptors in the basal ganglia of the rat: light and electron microscopy. Neuroscience 65:709-730.

Zhang G, Stackman RW, Jr. (2015) The role of serotonin 5-HT2A receptors in memory and cognition. Frontiers in pharmacology 6:225.

Zhang L, Ashendel CL, Becker GW, Morre DJ (1994) Isolation and characterization of the principal ATPase associated with transitional endoplasmic reticulum of rat liver. The Journal of cell biology 127:1871-1883.

Zhang Q, Cao YQ, Tsien RW (2007) Quantum dots provide an optical signal specific to full collapse fusion of synaptic vesicles. Proceedings of the National Academy of Sciences of the United States of America 104:17843-17848.

Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM (2000) Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proceedings of the National Academy of Sciences of the United States of America 97:13354-13359.

Zubieta JK, Huguelet P, Ohl LE, Koeppe RA, Kilbourn MR, Carr JM, Giordani BJ, Frey KA (2000) High vesicular monoamine transporter binding in asymptomatic bipolar I disorder: sex differences and cognitive correlates. The American journal of psychiatry 157:1619-1628.

Zubieta JK, Taylor SF, Huguelet P, Koeppe RA, Kilbourn MR, Frey KA (2001) Vesicular monoamine transporter concentrations in bipolar disorder type I, schizophrenia, and healthy subjects. Biological psychiatry 49:110-116.

Zucker M, Valevski A, Weizman A, Rehavi M (2002) Increased platelet vesicular monoamine transporter density in adult schizophrenia patients. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 12:343-347.

Zucker M, Weizman A, Harel D, Rehavi M (2001a) Changes in vesicular monoamine transporter (VMAT2) and synaptophysin in rat Substantia nigra and prefrontal cortex induced by psychotropic drugs. Neuropsychobiology 44:187-191.

Zucker M, Weizman A, Rehavi M (2001b) Characterization of high-affinity [3H]TBZOH binding to the human platelet vesicular monoamine transporter. Life sciences 69:2311-2317.