Identification and Characterization of Multiple Distinct Proteasome Subpopulations in Striatal and Hippocampal Brain Regions

Morgan E. Nelson

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Identification and Characterization of Multiple Distinct Proteasome Subpopulations in Striatal and Hippocampal Brain Regions

by
Morgan E. Nelson

A thesis submitted in partial fulfillment of the requirements for the University Honors Program

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May, 2014
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Abstract

Identification and Characterization of Proteasome Subpopulations in Mouse and Rat Brain Regions

Morgan E. Nelson

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The ubiquitin-proteasome pathway and proteolytic activities of the proteasome complex are responsible for multiple essential cellular processes. Modulation of proteasomal activities can alter the function of various brain regions, and possibly influence the neurodegenerative diseases in a brain region-dependent manner. The proteasome heterogeneity that exists in different brain regions could be a contributing factor to the different sensitivities of these regions reported in neurodegenerative diseases. Utilizing in vitro and in vivo approaches, we provide direct evidence of distinct proteasome subpopulations in different brain regions. Results obtained from this study suggest that proteasome subpopulations differ in their molecular compositions, and in the proteolytic activities excised from different brain regions. Moreover, the proteasome subpopulations identified in brain regions are different from the proteasome subpopulations studied in other tissues. Utilizing iodixanol gradient and fluorescent assay protocols, we found that there are two distinct forms of proteasome complexes with different levels of catalytic activities. Importantly, proteasome subpopulations display different levels of responses to the proteasome inhibitors. An extensive Western blot analysis suggested that the distinct proteasome assembly may be a contributing factor to variations in proteolytic activities. Besides molecular composition, post-translational modifications and specific protein partners may contribute to these differences. This data suggests the possibility of two proteasome subpopulations. The identification of different proteasome subpopulations in brain regions provides mechanistic insights for the temporal and spatial responses of proteasome in various regions of brain. This study identifies proteasome components that may serve as novel therapeutic targets in neurodegenerative diseases.

Keywords: Ubiquitin, Proteasome, Ubiquitin proteasome pathway, Iodixanol gradient, 26s proteasome, Enolase 2, Eno2, Striatum, Hippocampus
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CHAPTER ONE

Background and Introduction

1.1 Ubiquitin Proteasome Pathway

1.1.1 General Purpose

The ubiquitin proteasome pathway is a large ATP-dependent multicatalytic protease system that selectively degrades proteins within the cell. It is the major nonlysosomal proteolytic system in all eukaryotes (Scheffner et al., 1998). The system works by tagging a protein ready for degradation with ubiquitin which is recognized by the proteasome. Abnormally folded and assembled proteins as well as short-lived proteins such as transcription factors, cell cycle regulators and signal transducers, are targeted for ubiquitin proteasome degradation (Scheffner et al., 1998). The polyubiquitinated chain is removed by the proteasome and the protein is unfolded and pushed through the barrel chamber of the proteasome where active sites cleave the protein at particular sites. The ubiquitin proteasome pathway is found mostly in the cytoplasm with about ten to fifteen percent of proteasomes found in the nucleus. This pathway is not found in the membrane-bound organelles except for mitochondria. Mitochondrial proteasomes are much different in their substrate specificities and function so they will not be covered within this paper. (Ding and Keller, 2001)

1.1.2 Proteasome

The 26S proteasome is the ATP dependent protease portion of the ubiquitin proteasome pathway. A protease is an enzyme that breaks down peptide bonds within a
protein. There are two pieces of the 26S proteasome: the 20S proteasome that is ubiquitous in all eukaryotes and the 19S regulatory complex that regulates entry into the proteasome. The 20S proteasome is a barrel shaped structure with a small channel running through it that is ATP independent, and cleaves unfolded proteins. The 19S regulatory complex acts as a gate to the 20S proteasome. It uses ATP to detect ubiquitin, and unfold ubiquitinated proteins. Because the regulatory complex detects ubiquitin, the proteasome will preferentially degrade ubiquitinated proteins. When an ubiquitinated protein approaches the 26S proteasome, the ubiquitin portion docks to the ubiquitin receptors on the regulatory complex. The protein is then unfolded, and fed into the center of the 20S proteasome. Here it is not broken down all the way to amino acids, but into small, nonfunctional peptides. (Finley, 2009) A quantitative analysis of tandem-affinity purified cross-linked (x) protein complexes (QTAX) mass spectrometry approach revealed a protein interaction network with 471 proteins interacting with the yeast 26S proteasome. These proteasomal partners have different functions. (Guerrero, 2008) Some of these partners are accessory proteins that are bound to the outside of the 20S to help direct the proteasome activities (Ding and Keller, 2001).

Proteasomes are able to change in order to fit a cell’s needs. They can change their physical arrangement or their composition to address specific proteolytic needs. There is evidence of this in the difference in proteasomes in different areas of the body. Proteasomes in the nervous system have a unique structure and composition. They contain many proteins of direct importance in neurophysiological and neuropathological processes, and it seems that their functions are regulated in a brain region dependent manner. (Ding and Keller, 2001)
1.1.3 Ubiquitin

Ubiquitin is a protein with a mixed alpha and beta structure with a hydrophobic core. It is comprised of five beta strands and a 3.5 turn alpha helix that lies across a beta sheet. Ubiquitin is thought to be the most conserved protein through evolution with only three amino acid differences between mammals and yeast. This probably has to do with being under high selective pressure due to its involvement in ribosome biogenesis and cell surface recognition. (Pickart, 1998) Its main function in eukaryotes is to tag proteins for degradation by the proteasome, by forming polyubiquitin chains that bind to faulty proteins. Polyubiquitin chains are formed by bonds between lysine residues on the ubiquitin surface. There are seven viable lysine residue spots where bonds can form. These chains bind to proteins by isopeptide linkages between the epsilon-amino groups of lysine residues on the protein and the c-terminal glycine residue on the ubiquitin (Hershko, 1998). The ubiquitin is removed by the regulatory complex on the proteasome, and recycled to tag other faulty proteins.

Fig. 1: Ubiquitin Cascade. Ubiquitin tags a substrate to be degraded by the proteasome and is removed as the substrate enters the proteasome. (Adapted from Nature, Volume 458, Issue 26, 2009)
1.1.4 E1, E2, E3 System

To ubiquitinate a protein for degradation in the ubiquitin proteasome pathway, there are three different proteins involved to insure specificity and selectivity (Scheffner et al., 1998). These proteins are referred to as: E1, E2, and E3.

The E1 protein is the activating enzyme. It requires ATP hydrolysis to activate the c-terminal glycine residue of the ubiquitin protein just before ligation. E1 begins a two-step reaction sequence. The first step is forming ubiquitin adenylate and then the activated ubiquitin is transferred to the thiol site of E1. Contrary to several E2 and E3 protein, there are only two E1 enzymes. (Hershko, 1998) UBE1 is known as the human ubiquitin-activating enzyme (E1) and the human ubiquitin-activating enzyme referred to as UBE1L2. UBE1L2 is a novel E1 enzyme specific for ubiquitin. (Pelzer et al., 2007)

The E2 protein is also known as the ubiquitin conjugating enzyme or the carrier protein. It first takes activated ubiquitin from E1 by transacylation, then using a ubiquitin-protein ligase reaction, it transfers the ubiquitin to a protein substrate (Hershko, 1998). E2 proteins have the potential to transfer ubiquitin directly to substrates, but in vivo, they may need accessory factors for recognition. There are thirteen different known E2 proteins. (Scheffner et al., 1998)

The E3 protein is also known as the ubiquitin-protein ligase or ubiquitin recognin. It helps with substrate recognition, although it may need extra specificity factors for assistance. Without the presence of ubiquitin from E2, the E3 does not carry a function. Multiple E2 enzymes can feed a single E3 so it tends to stay more active than the typical E2. The E3 helps bring the E2 and the substrate closer together so the ubiquitin can be transferred directly from E2 to the substrate. Although the mechanism is not entirely
understood, the E3 does not act like a dock for ubiquitin, but does carry some ligase activity. (Scheffner et al., 1998) In mammals, there are several hundred unique E3 enzymes that work to coordinate, with one of two E1 enzymes, ubiquitination of thousands of substrates (Polo, 2012).

Along with the E1, E2, and E3 proteins, there are additional ancillary factors that help ubiquitinate proteins. These proteins function to help ubiquitin turnover between the E’s. They mostly function in specificity, targeting and regulation. They potentially help guild E2 to E3’s proximity. (Scheffner et al., 1998)

Fig. 2: E1, E2, and E3 cycle. The pathway in which these enzymes attach ubiquitin to a substrate to be degraded. (Adapted from Nature, Volume 456, Issue 26, 2009)
1.2 Proteasome Structure

The 26S proteasome is an ATP-dependent protease that degrades folded proteins. It is composed of thirty different proteins and consists of two subcomplexes: the 20S core particle and the 19S regulatory particle.

The 20S core particle is a barrel-shaped structure with dimensions of eleven nanometers across and fifteen nanometers long. It consists of 28 peptides which are encoded by 14 separate genes. The 28 peptides form four stacked rings of seven peptides. The outer rings are composed of seven alpha subunits labeled alpha1 through alpha7. The alpha rings form a barrier between the 19S regulatory particle and the inner rings. The two inner rings are composed of seven beta subunits labeled beta1 through beta7. The proteolytic active sites are contained here; beta1 containing the caspase-like site, beta2 containing the trypsin-like site, and beta5 containing the chymotrypsin-like site. (Tomko and Hochstrasser, 2013) The caspase-like site cleaves peptides after acidic residues, the trypsin-like site cleaves after basic residues, and the chymotrypsin-like site cleaves after large hydrophobic residues. Because there are different active sites, each is affected by and can be targeted by different inhibitors and mutations. The other inactive beta subunits’ functions are unclear. (Lupas and Baumeister, 1998) The 20S core particle can only degrade unfolded proteins and peptides, so it is necessary to have an outer regulatory portion to unfold the proteins (Sledz et al., 2013).

The 19S regulatory particle is composed of 19 subunits and is responsible for substrate recognition and recruitment (Sledz et al., 2013). It binds, deubiquitinates, unfolds, and translocates substrates into the inner core acting as a gatekeeper. There are two complexes to the regulatory particle: the lid and the base. The base sits in direct
contact with the alpha ring of the 20S core particle but is slightly axially offset. It is composed of nine of the 19 subunits, and is responsible for the ATP-driven substrate unfolding and translocation into the 20S core particle. Subunits within the base attach to the ubiquitin of substrates to be degraded. The lid is composed of the remaining ten subunits. It sits on the long edge of the regulatory particle contacting both the base and the 20S core particle a ring. This acts to hold the 20S and 19S particles together. (Tomko and Hochstrasser, 2013) On the distal end of the lid, there are ubiquitin receptors that create an affinity for the proteasome to the substrates (Sledz et al., 2013).

As ubiquitinated substrates are attached and degraded, the 19S regulatory particle and the 20S core particle go through conformational changes to regulate the opening and closing of the proteasome (Sledz et al., 2013). The two components of the proteasome must work together to effectively breakdown proteins.

Fig. 3: 26s Proteasome Complex. The 26S proteasome complex has a 19S regulatory particles which flank one side or both side of a barrel-shaped 20S catalytic core (adapted from Nature, Volume 450, pgs. 973-982, 2007.)
1.3 Proteasome Regulation

1.3.1 Ubiquitin Tagging

Signals, such as non-native conformations or the inability to find natural partners, mark a protein for degradation. Once this happens, a polyubiquitin chain connects to the protein which is recognized by the proteasome. The ubiquitin chain forms a covalent bond with an internal lysine residue of the protein. To be marked for degradation, there must be a polyubiquitin chain. For example, a single ubiquitin can modify the lysine in the internal histone protein, but this only alters the histone’s cellular function, it does not signal for histone degradation. Specificity and selectivity of protein ubiquitination is extremely important so that a protein will not be broken down that is still useful. This is achieved by a variety of signals, a regulation of the availability of the number of signals, or some variation of both. These signals do not necessarily have to come from the protein in question itself, but it could come from accessory factors within the cell that help ubiquitin recognize a faulty protein. Naturally, some signals work better than others but this helps contribute to accurately specifying and selecting the correct protein for polyubiquitination and degradation. Another form of signaling a protein for degradation is phosphorylation of the protein. This can create easier accessibility of the ubiquitin attachment site or alter amino acids to become ubiquitin attachment sites. Phosphorylation can also alter periphery factors to help specify a protein for ubiquitination. (Scheffner et al., 1998)

1.3.2 Proteasome Changes

Once a protein is tagged for degradation by ubiquitin, it must be recognized by the proteasome. The regulatory particle has ubiquitin receptors, RPN10, RPN13 and
RPT5, at the periphery of the particle that recognizes and attaches to the polyubiquitin tail of the faulty protein. The particle is organized in such a way that the functional sites facilitate movement into and through towards the core particle. The sites for recognition, deubiquitylation, unfolding, and translation are positioned in a sequential order towards the core particle. The ubiquitin acts as a dock that anchors the protein to the proteasome as it is unfolded and then translocated into the central channel. The polyubiquitin tail is then removed. (Tomko and Hochstrasser, 2013) Once it is attached, conformational changes within the regulatory particle activate protease activity within the core particle. The alpha ring of the 20S proteasome rotates about four degrees with respect to the underlying beta ring that opens the channel to allow the deubiquitinated, unfolded protein to pass through. (Sledz et al., 2013)

1.3.3 Deubiquitinating Enzymes

The polyubiquitin chain anchors the protein to the proteasome, threading the protein into the proteasome channel. Once the anchor is not needed, it needs to be removed. (Tomko and Hochstrasser, 2013) Enzymes that remove ubiquitin chains and take apart the polyubiquitin chains are referred to deubiquitinating enzymes. Deubiquitination refers to the process of hydrolyzing the peptide bonds between an ubiquitin chain from the protein to be degraded. Deubiquitinating enzymes fit into 5 different families separated into two groups: Thiol Protease and Metalloprotease Deubiquitinating enzymes. The Thiol Protease Deubiquitinating enzymes include: ubiquitin C-terminal hydrolase domain, ubiquitin-specific processing protease domain, ovarian tumor domain and the Josephin domain. Smaller enzymes in the ubiquitin c-terminal hydrolase domain specialize in cleaving the small leaving groups of the c-
terminus of ubiquitin chains while larger enzymes take apart polyubiquitin chains. The largest domain, ubiquitin-specific processing protease domain, interacts with regions of protein sequence directly or with the help of substrate adaptors. Enzymes in the ovarian tumor domain recognize particular ubiquitin chains and may recognize substrates due to specific chains. Josephin domain enzymes distinguish polyubiquitin chains by variance in length. The metalloprotease deubiquitinating enzymes contain the JAB1/MPN +/MOV34 or JAMM domain which cleave polyubiquitin chains at their base. (Eletr & Wilkinson, 2013)

Fig. 4: Deubiquitinating Enzymes. Deubiquitinating enzymes (DUBs) are divided into 5 families (adapted from Cell, Volume 123, Issue 5, 2005.

Deubiquitinating enzymes play a large role in proteasome regulation. These enzymes can affect the rate of ubiquitination, processing, recycling, and remodeling polyubiquitin chains, they act on the level of localization, and help with transport of proteins to different parts of the cell. By doing these processes, they: co-regulate E3 ligases, remodel ubiquitinated proteins, alter the location of target proteins, and act on the proteasome to activate or deactivate proteolysis. (Eletr & Wilkinson, 2013).
1.4 Interaction at Proteasome with Partners

1.4.1 General Purpose

Proteasomes cannot function on their own; they also rely on many protein and complex partners to regulate proteolysis based on cell activity. The proteasome network consists of over 471 known proteins, and every cell process requires at least ten different proteins (Guerrero et al., 2008). There are currently 138 different proteasome subunit interactor sequences known. Each subunit within the proteasome has between zero to twenty-two interactors (4.6 on average) with an interactor being any substrate, cofactor, or regulator. There are two classes of proteasome partners: partners that help with the catalytic activities whether positively or negatively, and proteins that help with the general assembly of the proteasome. (Coux, 2003)

1.4.2 Proteasome Partner Classes

Proteasome partners that help facilitate catalytic activities can be either individual proteins or whole complexes. One of the main functions of proteasome-interacting proteins (PIPs) is to help bind ubiquitin to substrates and then transfer the complex to the proteasome. These are involved in the E1, E2, and E3 ubiquitination system. PIPs also associate with the subunits of the proteasome to help facilitate proteolysis. Each subunit seems to have unique partners. This could be due to unique spatial location or specific functionality. PIPs have diverse functions including: acting as degradation targets, mediating assistantship or protein degradation, and regulating proteasome structure and function. Proteasome interactions with cellular complexes help regulate biological pathways. These biological pathways include: chromatin remodeling, tRNA aminoacylation, metabolism, transport, translation, DNA replication, endocytosis,
and protein folding. It is possible that the proteasome makes physical connections with these cellular complexes to carry out specific processes. One particular process that proteasomes are associated with is translation. The proteasome complex is particularly important for the quality control of protein synthesis. It is thought that almost fifty percent of new proteins need to be degraded due to errors during translation. Thus proteasomes and initiation and elongation factor complexes tend to have a close proximity to each other. (Guerrero et al., 2008)

The other class of proteasome partners are those associated with assembling the proteasome. There are four specific PIPs found that specifically help with assembly: Nas2, Nas6, Rpn14, and Hsm3. These bind to specific base units during formation of the regulatory particle and initiate assembly of the whole 26S proteasome. These PIPs act as specific chaperones that possess a specific step in the regulatory particle base assembly
which follows a step-wise order. Assembly cannot happen without these chaperones. Once the 26S proteasome is constructed, these chaperones are no longer present. It would appear that the base and the lid of the regulatory particle form separately, but the lid assembly mechanisms are still not understood. (Saeki et al., 2009)

1.4.3 Examples of Proteasome Partners

Proteasome partners differ among tissues within the body due to different cellular functions. Some specific examples of proteasome partners within the heart are PP2A and PKA. They reside in the 20S proteasomes in cardiac cells, and both affect phosphorylation rates of active subunits. PP2A is a phosphatase that lowers phosphorylation rates and slowing down activity. It is especially affective on the beta2 subunits. PKA is a kinase that enhances 20S protease activity, especially in the beta1 and beta 5 subunits. (Zong et al., 2006) Compared to muscle cells, there have been 28 associated proteins found that are different in neural cells. There are 12 proteins that are identical despite the functionality differences between the two cell types. Within the brain, proteasome partners differ between glial and synaptic cells. Some examples of synaptic partners include: TAX1BP1, drebrin, and SNAP-25. These regulate proteolysis in a synapse-specific manner. The glial cells contain a partner, ECM29, that helps stabilize the association between the 19S and 20S components of the proteasome. This could explain why there are proteasomes with a single 19S in the synaptic cells and double 19S in glial cells. (Tai et al., 2010)

1.5 Enolase 2

Also known as Neuron-specific enolase (NSE), gamma-enolase, EC 4.2.1.11, 2-phospho-D-glycerate hydro-lyase, neural enolase, and neuron-specific enolase, enolase 2
(Eno2) is a glycolytic isoenzyme that is found in most neuronal tissues. Eno is an enzyme in the glycolytic pathway that hydrolyzes the dehydration reaction of 2-phosphoglycerate (Piast et al., 2005). Eno2 is one of three enolase isoenzymes found in mammals and is composed of two out of three isoenzyme subunits (alpha, beta, and gamma) that form homodimers or heterodimers. It is the alpha/gamma heterodimer and the gamma/gamma homodimer that are neural-specific. The gamma/gamma homodimer is the most common in neurons. The gamma subunit originates from the cytoplasm of neural cells. (Wiener et al., 2013)

In the brain, ENO2 is involved with energy production, neuroprotection, and neurotrophic events. ENO2 binds to the 19S regulatory complex of the proteasome where it is involved with regulation of the proteasome. Upregulation causes cellular defense mechanisms and is a known brain damage marker due to its cellular location and neuronal specificity. (Valastro et al., 2007, Weiner et al., 2013) There is a significant increase of ENO2 in the striatum of Parkinson’s patients (Al-Jarrah and Jamous, 2011) and in the hippocampus of Alzheimer’s disease (Takano et al., 2012).

1.6 Proteasome Inhibitors

Proteasomes are necessary for protein homeostasis within the cell. Protein degradation is important for many cell processes, and an accumulation of protein can lead to several pathological problems. To maintain homeostasis, proteasome inhibitors are necessary to control the rate of protein degradation. Too many or too few proteins within the cell leads to an imbalanced situation in many cellular processes. Proteasome dysfunction caused by over-expression of inhibitors can lead to a significant increase and
accumulation of ubiquitinated proteins within a cell. This is a prerequisite for many neurodegenerative diseases.

1.7 Proteasomes and Neurodegenerative Diseases

1.7.1 Parkinson’s Disease

Parkinson’s disease is an autosomal recessive disease caused by a mutation in the Parkin gene which encodes for an E3 ligase. It affects about one to two percent of people over sixty years of age. Symptoms that accompany Parkinson’s include: resting tremors, rigidity, and bradykinesia from degeneration of dopaminergic neurons. The c-terminus of Parkin interacts with the c-terminus of the alpha4 subunit of the 20S core particle. Although alpha4 is not a Parkin substrate, the interaction seems to affect the activity of both interactors. The degree and mechanisms of the change in activity is still unknown. (Dächsel et al., 2005)

1.7.2 Alzheimer’s Disease

Alzheimer’s disease is characterized by the neurofibrillary tangles of which ubiquitin is the major component, plaques, and an atypical form of ubiquitin known as UBB+1 (Chadwick et al., 2012). It affects twenty-five to fifty percent of the population over eighty-five years of age and is the most common form of dementia. As the disease progresses, there is a buildup of ubiquitinated proteins due to diminished ubiquitin proteasome system activity in the cortex and hippocampus. Although the mechanism for ubiquitin proteasome system impairment is not well understood, there is a difference in the system-related mRNA expression profiles within the brains of patients with Alzheimer disease. (Vrij et al., 2004)
1.7.3 Huntington's Disease

Huntington’s disease is caused by a stretch of CAG repeats in the coding gene for Huntingtin. People with thirty-five to forty repeats have a much higher risk, and those with 40 or more repeats will develop the disease (Dong et al., 2011). The resulting protein is cleaved into fragments that become inclusion bodies. The disease is characterized by changes in personality, motor control, and cognition, and is diagnosed by rigidity, lack of coordination, rapid eye movement, and a decrease in voluntary muscle tone. About six people out of every one hundred thousand over the age of 50 are affected but there are significantly less cases in the Asian and African populations. In Huntington’s disease patients, there is a marked increase of ubiquitin and ubiquitinated mutant Huntingtin protein in brain tissue. Due to the accumulation of ubiquitinated protein, it is thought that the proteasome system is somehow inhibited although the mechanism is still not understood. (Mitra and Finkbeiner, 2008) In patients with the mutant Huntingtin gene, there is an obvious increase in mutant Huntingtin protein aggregates. It is still unclear if this is due to a faulty ubiquitin proteasome system. (Dong et al., 2011)
CHAPTER TWO

Methods and Materials

2.1 Cell Culture

Experiments were conducted on HC2S2 rat hippocampal cells and striatal cells kindly provided by Dr. Hongmin Wang (University of South Dakota). HC2S2 rat hippocampal cells were cultured using Poly-O and Matrigel coated plates in N2 medium. Two days after the addition of tetracycline (1.0 mg/ml) (Hoshimaru et al., 1996), the cells stopped dividing, and started to extend processes which began to interconnect by 3 days.

Conditionally immortalized wild-type STHdhQ7/Q7 striatal neuronal progenitor cells expressing endogenous normal huntintin protein (Htt), referred to as wild-type striatal cells, were derived from the E14 wild type Htt Q7/Q7 knock-in embryos (Coriell Institute for Medical Research). Striatal cells were grown at 33°C in 10% FCS in DMEM, and were differentiated to neuronal cells by incubating for 12 hours in serum-free DMEM containing α-FGF (10 ng/ml), TPA (20 µM), forskolin (48.6 µM) and dopamine (5 µM, Trettel et al., 2000). Cell lysates were prepared from two cell lines using NE-PER Nuclear and Cytoplasmic Extraction kit in the absence of protease inhibitor cocktail. Due to the absence of protease inhibitor cocktail, after harvesting cells all manipulations were performed at 4°C including washing cells, incubation between steps and centrifugation. We did not use the protease inhibitor cocktail to avoid interfering with the proteolytic cleavage of the proteasome complex. Cytoplasmic extracts were subjected to Iodixanol gradient fractions.
2.2 Preparation of Nuclear and Cytosolic Lysates

The NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific) was used for preparing cell lysates because this kit enables step-wise separation and preparation of cytoplasmic and nuclear extracts from HC2S2 rat hippocampal cells and striatal cells. Generally, we had 10% contamination between nuclear and cytoplasmic fractions, which was sufficient purity for our proteasomal catalytic assay.

For the preparation of nuclear and cytoplasmic fractions, cells were mechanically removed from the tissue culture plates with the aid of cell scrapers. The cells were collected by centrifugation at 1200 RPM using the Sorvall Legend X1R Centrifuge (ThermoFisher Scientific) for 2 minutes and washed with iced-cold 1X D-PBS liquid with calcium, magnesium (pH 7.4). After washing twice with ice-cold PBS, cells were next suspended in an amount of lysis buffer CERI depending on the concentration of cells (see Table 1 below) in a microfuge tube. The cells were vortexed on high for 15 seconds then incubated on ice for 10 minutes. After the incubation, an amount of CERII was added depending on the concentration of cells determined earlier (see Table 1 below) and the contents were mixed on a vortex for 5 seconds and then centrifuged for 5 min at 4°C at 16,000g using GS-15R Centrifuge (Beckman). The supernatant was extracted as the “postnuclear fraction” and stored at -20°C. The nuclear pellet was resuspended in an amount of ice-cold NER depending on the cell concentration determined earlier (see Table 1 below), and incubated on ice for 40 minutes with intermittent 15 second vortexing every 10 minutes. The tube was centrifuged for 10 minutes at 16,000g at 4°C and the supernatant (nuclear extract) was stored at -20°C.
<table>
<thead>
<tr>
<th>Packed Cell Volume</th>
<th>CER1 µL</th>
<th>CER2 µL</th>
<th>NER µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100</td>
<td>5.5</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>11</td>
<td>100</td>
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<tr>
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<td>250</td>
</tr>
<tr>
<td>100</td>
<td>1000</td>
<td>55</td>
<td>500</td>
</tr>
</tbody>
</table>

For tissue lysates, a VWR™ Pellet Mixer (VWR International) was used to mechanically breaking apart the tissues to begin the lysis process. Then, the rest of the procedure continued starting at the addition of CER1 as described above.

2.3 BCA Assay

Protein concentrations of cell lysates were found by comparing the samples to a control gradient of bovine serum albumin (BSA). In a clear, bottom rounded 96 well plate, the first three columns were used to make the control gradient in triplicate. To start, 10 µL PBS were added to all wells in the first 7 rows and 10 µL BSA (2mg/mL) was added to the first three wells of the bottom row. An additional 10 µL of BSA were added to the seventh row, mixed with the PBS, and then half of the mix was removed and added to the sixth row. This was repeated until the second row where the remainder half was discarded. The top row remained to be pure PBS. In the remaining wells, 1 µL samples of cell lysate were added in triplicate diluted in 9 µL PBS, skipping wells A4-A6 which remained to be pure PBS. This set up is pictured in Figure 6.
A working reagent was made by mixing 50 parts BCA reagent A with 1 part BCA reagent B from the Pierce™ BCA Protein Assay Kit (Thermo Scientific). 200 µL of the mixture was added to all wells except A4-A6 which are to remain blank. After being incubated at room temperature for 15 minutes on a shaker, the plate was read using an ELx808 spectrophotometer (BioTek).

### 2.4 Iodixanol Gradient Ultra-Centrifugation

We used iodixanol for gradient fractionation because it maintains relatively constant osmolality and viscosity despite changes in the density of the gradient. Because of the mild iso-osmotic conditions, all organelles and endosomes can be fractioned intact, without loss of water, as the density of the gradient increases.
Cell lysates were layered onto pre-formed, pre-cooled discontinuous iodixanol layers. These layers were formed by mixing Working Solution (WS) (20 mL OptiPrep™ (Accurate Chemical & Scientific Corp.) and 4 mL Diluent (8.5g sucrose per 50 mL water plus 6 mL 100 mM EDTA (Na2•2H2O) and 12 mL 500 mM Hepes stock (free acid) adjusted to pH 7.4) and Homogenization Medium (HM) (17g sucrose in 100 mL water plus 2 mL 100mM EDTA (Na2•2H2O) and 4 mL 500 mM Hepes stock (free acid) adjusted to pH 7.4) in amounts listed in Table 2 below to make concentrations of 8, 16, 28, and 38% iodixanol.

<table>
<thead>
<tr>
<th>% Iodixanol</th>
<th>WS (mL)</th>
<th>HM (mL)</th>
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Iodixanol gradients were layered stepwise in centrifuge tubes (Beckman) using a Labconco Auto Densi-flow gradient collector (Labconco Corporation). Equal total protein amounts were added to the top, based on equal protein concentrations assessed by using the BCA assay technique (Pierce). Tubes were centrifuged for 18 h at 28,500 rpm (<100,000 g) using a Beckman SW41i rotor at 4°C. Twenty sequential equal-volume fractions (<500 µl each) were collected from the top of the tube with the Labconco Auto Densi-flow gradient collector. The samples were stored at -20°C for further experiments.
2.5 Aryl Esterase Assay

Aryl esterase activity was measured as an endoplasmic reticulum marker in iodixanol gradient fractions (25 μl) in a 96 well plate. 75 μl of potassium phosphate buffer, KH$_2$PO$_4$ (0.1 M) adjusted to pH 6.8 with KOH was added to each well followed
by 25 µl indoxyl acetate substrate solution (25 mM) (Sigma). The substrate is dissolved in 95% ethanol, then adjusted to 50% ethanol just prior to use. Samples were incubated at room temperature for 20 minutes. Optical densities were measured in ELx808 spectrophotometer (BioTek) at wavelength of 405nm.

2.6 Leucine Aminopeptidase Assay

Leucine aminopeptidase activity was measured as a trans-Golgi marker (Chi and Lodish, 2000) with the fluorogenic peptide substrate I-leucine-4-methyl-7-coumarinylamide hydrochloride (Sigma). Iodixanol gradient fractions (20 µl) were added to 200 µl of 50 mm Tris-HCl buffer, pH 7.5 containing 0.5 mm substrate in dimethyl sulfoxide (0.5% v/v). Samples were incubated at 25°C for 30 min and 90 min. Fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 440 nm using Victor™ X2 2030 Multilabel Reader (PerkinElmer). Measurement of ER marker (Aryl esterase activity) and Golgi marker (Leucine aminopeptidase activity) ensured the integrity of the fractionation technique and provided satisfactory separation of cytoplasmic compartment with minimal overlap.

2.7 Proteasomal Activity Assays and Analysis

Catalytic assays were performed in triplicate by adding 10 µl of each sample to 200 µl 26S proteasome assay buffer (50 mM Tris-HCL, 40 mM KCl2, 5mM MgCl2, pH=7.5. At time of use add 50 µL 100 mM ATP, 10 µL 1 M DTT, 100 µL 50 mg/mL BSA to 10 mL of buffer) then incubated at 37°C for 30 minutes. Wells were then treated with 2µl Suc-LLVY-AMC, Bz-Val-Gly-Arg-AMC, and Z-Leu-Leu-Glu-AMC substrate (Enzo Life Sciences) for chymotrypsin-like, trypsin-like and caspase-like activity respectively. These were read at time points of 5 hours, 24 hours, and 48 hours using
Victor™ X2 2030 Multilabel Reader (PerkinElmer). We conducted all iodixanol gradient fractionation experiments in the presence of ATP (0.5mM) to maintain the functional integrity of the 26S proteasome, as well as to assist with its assembly. We saw a consistent activity increase from the initial peaks over a 48 hour period. In addition, we ran the assays in the presence of proteasome inhibitor PS-341 (Velcade, 150nM). Activity measured in the presence of Velcade was considered non-proteasomal catalytic activity and subtracted from the total measured proteolytic activity.

2.8 Western Blot

Western blotting was performed with a SDS-PAGE Electrophoresis System as described previously. Briefly, protein samples were resuspended in a loading buffer (BioRad), incubated at 70°C for 5 minutes, and then electrophoresed using precast SDS-PAGE (4-20%) gels followed by blotting using the iBlot™ (Invitrogen) to nitrocellulose membranes. Membranes were blocked with 5% milk in TBS for one hour then probed with primary antibodies overnight. A horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies were added, incubated for an hour, and then the secondary antibodies were detected through autoradiography using enhanced chemiluminescence (SuperSignal West Femto Chemiluminescent Substrate, Pierce) for 5 minutes. Each membrane was exposed to blue basic autorad film (8X10 or 5X7) double emulsion (Bioexpress) in a dark room for 30 seconds to 10 minutes and then transferred to the developing machine.

2.8.1 Primary Antibodies Used

Pan-alpha: Used to locate the 20S core particle. (Proteasome 20S α1,2,3,5,6,& 7 subunits, mAb (MCP231), Enzo Life Sciences, 1:1000 dilution)
S8: Used to locate the 19S regulatory particle. (Proteasome 19S ATPase subunit Rpt6, mAb (p45-110), Enzo Life Sciences, 1:1000 dilution)

Eno2: Used to locate Eno2. (Gamma-Enolase (N-14), IgG, Santa Cruz Biotechnology, 1:500 dilution)

2.9 Proteasome Immunoprecipitation

Mouse monoclonal antibody against the S8 ATPase subunit (Enzo Life Science) was immobilized on magnetic beads IgG to immunoprecipitate the 20S and 26S proteasome complex and associated proteins from cells or tissue cytosolic extracts as described previously (Kriegenburg et al., 2008). Briefly, fractions 3-5 (enriched with proteasome complex with the highest peak for chymotrypsin-like activity) were combined and divided equally into two centrifuge tubes. The volume of the pooled fractions was brought to 500 μL with PBS. Then, tubes were incubated for two hours at 4°C with monoclonal anti-proteasomal subunit S8 antibody or mouse pre-immune serum (4μg). Then 50μL magnetic beads IgG (Invitrogen) were added to each tube and left in room temperature for 10 minutes. Immunoprecipitates were washed three times with PBS plus tween 0.01% and incubated for 5 minutes on a shaker between each wash. The supernatants were discarded. Proteins were eluted using glycine PH 2.5 and sent for mass spectrometry analysis.

2.10 Protein Identification using 2D-nano LC-MS/MS analysis

The proteins obtained from proteasome immunoprecipitation were stored in 50ml glycine pH 2.5. The proteins in-solution were then reduced with 50 mM DTT (Sigma) at 65°C for 5 minutes, followed by alkylation with 100 mM Iodoacetamide (Sigma) before being digested with sequencing grade trypsin (Promega, Madison WI) overnight at 37 °C.
The digestion was stopped by the addition of 0.5% acetic acid. The samples were frozen in dry ice and concentrated in a SpeedVac centrifuge (Thermo Savant). The trypsin-digested peptides were dissolved in 100 mM ammonium formate (pH 10), and separated through 2D-nanoLC with dilution using a 2D-nanoAcquity UPLC (Waters, Milford, MA). The eluted ions were analyzed at Q-TOF Synapt G1 HDMS mass spectrometer (Waters, Milford, MA) using a MassLynx 4.1 (Waters, Milford, MA) by selecting one full precursor MS scan (400-1500 m/z) followed by four MS/MS scans of the most abundant ions detected in the precursor MS scan while operating under dynamic exclusion or direct data acquisition system (Dong et.al., 2011, 2012). Mascot server v2.4 and Mascot Daemon Toolbox v2.4 (www.matrix-science.com, UK) in MS/MS ion search mode (local licenses) were applied to conduct peptide matches (peptide masses and sequence tags) and protein searches against NCBInr v20140304 (37425594 sequences; 13257553858 residues) using taxonomy filter Mus musculus (1729883 sequences). As a complementary analysis, the data coming from Mascot search were exported to ProteoIQ v2.7 (Premier Biosoft, Palo Alto, CA), to perform the proteins list organization, and statistical analysis.
CHAPTER THREE

Results

3.1 Partial Purification of Proteasome Complexes and Method Quality Control Tests

The two regions of the brain we decided to test for proteasome activity were the hippocampus and the striatum since both are affected by common neurodegenerative diseases. In order to test for activity, we first had to isolate the proteasomes in both cell lines. To do this, we used the iodixanol gradient centrifugation method. The cytoplasmic lysates were extracted using the Thermo Scientific lysate kit and then put into an iodixanol gradient. The gradient tubes were then put into an ultra-centrifuge overnight, and then 20 fractions were extracted the next morning using an auto densi-flow gradient collector. Once the 20 fractions were collected, they were checked to make sure each fraction was distinct using aryl esterase (ER marker), leucine aminopeptidase (golgi apparatus marker) and BCA assays. This was also helpful in showing relative locations of activity within the cells. Because each fraction had a distinct representation of the two makers and a variance of protein concentration, the iodixanol gradient proves to be a sufficient method to determine which membrane particles and protein complexes are present and where they can be found. Purification of proteasome complexes in this manner allows us to study proteasome subpopulations in their physiological environment while associated with their normal partner proteins. Other methods may disassociate the partners from the proteasomes or lose them entirely during the purification process. This
way, we can have a much clearer understanding of which partners associate with the proteasomes in both the hippocampus and striatum.

Fig. 8: Control Tests. Sedimentation of the aryl esterase (Endoplasmic Reticulum Marker), Leucine aminopeptidase (Golgi Apparatus Marker) and the total protein concentration of collected fractions according to their distinct fraction. The linearity of the density gradient is shown as a line graph.
3.2 *In vitro* Isolation and Characterization of Proteasome Complexes

Since proteasomes can differ between their catalytic activity and their associated partners, we decided to first test the striatum and hippocampus for their specific catalytic activity. With the 20 fractions for both the striatum and hippocampal cell lines, each fraction was tested for its chymotrypsin, trypsin, and caspase activities. To do this, we ran catalytic assays isolating for the three activities, then graphed them to see exactly where activity was taking place as seen in Figure 9. The most activity took place within fractions seven through twelve with slight variance. The peaks between striatum and hippocampal chymotrypsin and caspase activities are very similar with the peak rising to about the same height and within similar fractions. Contrary to this, the trypsin activity between the two regions appears to be significantly different. The activity peak in the striatum is much lower and in different fractions than the activity peak in the hippocampus. To make sure we were seeing proteasome activity in these peaks, we ran western blot tests isolating for pan alpha (20S core particle) and S8 (19S regulatory particle), and found that the strongest bands occurred in the same fractions as where the peaks were found in the activity graphs. Due to the difference in activity, it can be assumed that the hippocampus and striatum have separate proteasome populations present.
Fig. 9: *In vitro* Results. Cytoplasmic lysates of mouse striatum-derived (STHdhQ7/Q7) and rat hippocampus-derived (HC2S2) cell lines were examined for proteasomal catalytic activity (Panel A). Anti-Pan α and S8 ATPase (Rpt6) antibodies confirmed the catalytically active fractions contained proteasome complexes (fractions 7-12, Panel B). Data are representative of two independent experiments.
3.3 *In vivo* Isolation and Characterization of Proteasome Complexes

Since we saw the differences in trypsin catalytic activity between cells cultured from these regions, we decided to move to tissues. We used striatal and hippocampal tissues dissected from C57BL/6J mice. First we lysed them, separating the cytoplasm from the nucleus, and then ran the same tests as we did for the cell lines testing for chymotrypsin, trypsin, and caspase catalytic activities. We also ran the same western blot experiments to make sure catalytic activity was coming from the proteasome, testing for the 20S core particle and the 19S regulatory complex. Our tissue-based activities unfortunately did not match our previous results. As observed in cells, the caspase and chymotrypsin activities were very similar, but so was the trypsin activity in tissues (Figure 10). Although there does seem to be some variance between the two regions for the three activities, none of them are significant enough to say there is a difference in activity. To further test our hypothesis, we needed to look elsewhere.
Fig. 10: In vivo Results. Striatum and hippocampus regions dissected from C57BL/6J mice were subjected to Iodixanol gradient fractionation and proteolytic activity assay (Panel A). WB analyses confirmed the presence of the 20S core and the 19S cap particles of the proteasome in the regions where activity was observed (Panel B). Data are representative of two independent experiments.
3.4 Shared and Unique 26s Proteasome-Associated Partners in Two Brain Regions

The other way proteasome complexes can differ amongst different tissues is by the protein partners that they associate with. Because we used the iodixanol gradient method to isolate the proteasomes, most of the protein partners remained intact with the proteasomes. To find out which partners existed, we mixed the enriched fractions, or fractions with the highest activity, and thus, highest proteasome concentration, and conducted mass spectrometry experiments. The results indicated that, although there are numerous shared proteins between the striatal and hippocampal proteasomes, there are also many distinct partners (indicated in Figure 11). The partner that stood out the most to us was gamma enolase (ENO2) which was only associating with the striatal proteasomes. Further tests showed that ENO2 can also be found in the hippocampus, but did not associate with the proteasomes in this region. This is important since gamma enolase is a known brain damage marker for many neurodegenerative diseases. Further research will see how the association of ENO2 with the striatal proteasomes may or may not change the overall activity of the striatum and striatal proteasomes. The fact that there are separate proteasome partners between the striatal and hippocampal proteasomes is evidence that there are distinct proteasome populations between these two brain regions.
3.5 Verification of Eno2’s association with Striatal Proteasomes

To verify the results obtained in mass spectrometry, we conducted a set of immunoprecipitation (IP) experiments using mouse striatal and hippocampal brain region tissues. Brain tissue lysates were subjected to a BCA assay to calculate the total concentration of protein. Equal amounts of protein were incubated with 50 µL proteasome mouse 20S α2 subunit (MCP21) antibody immobilized on agarose beads or agarose beads with mouse IgG serum. After the required washing steps, pulled down proteins were eluted with loading buffer, and subjected to a 4-20% SDS-PAGE gel followed by a Western blot using anti-γ Enolase (Eno2) antibody (N-14). Figure 12 shows that both the hippocampus and striatum have detectable levels of Eno2 protein (lanes III and VII), while the anti-α2 (MCP21) IP experiment, which is able to pull down
the entire 26S proteasome, showed Eno2 is only immunoprecipitated in the striatum and not the hippocampus (lanes I versus lane V).

Collectively, these last results indicate Eno2 is exclusively associated with proteasome complexes in the striatum. Further work needs to be done to establish whether this association with striatal proteasome complexes can modify the proteasomes’ catalytic functions in response to stress resulting in an accumulation of aggregated proteins such as in Huntington’s disease.

Fig. 12: Differences in association with Eno2. Eno2 only binds the proteasome complex in the striatum and not the hippocampus. Tissue lysates were subjected to BCA assay and equal amount of protein were loaded to agarose beads with anti-a2 antibody or mouse IgG serum. WB analyses confirmed the presence of Eno2 in both brain regions (lanes III and V11). However, Eno2 only showed association with the striatal proteasome and not proteasome complexes in the hippocampus (Lane V vs I).
CHAPTER FOUR

Discussion

Proteasomal partners and chaperones play a critical role in regulating proteasome activity in response to different types of stress. These partner proteins modify proteasomal functions through different mechanisms. They are able to stabilize or enhance specific proteolytic activities in response to internal or external stressors. In addition, proteasomal partners and chaperones aid the recognition and degradation of substrates positively or negatively resulting in an efficient fine-tuning system. An example of a proteasomal partner capable of modifying proteasomal function is HSP90 which contributes to proteasomal regulation in response to oxidative stress (Conconi and Friguet, 1997). Another example is HDJ-1/Heat shock protein 40 (Hsp40) which is able to increase cell resistance to cytotoxicity associated with oxidative stressors and general proteasome inhibitors. In general, heat shock proteins interfere with oxidative stress by enhancing proteasomal function and reducing proteasomal inhibition due to toxicity Ding and Keller, 2001).

While there have been several reported proteasomal partners that are able to utilize different protein-protein interaction methods, the molecular mechanisms by which these chaperone proteins regulate proteasome activity in response to several neurodegenerative diseases, such as Huntington disease, have yet to be determined. This study introduced a modified iodixanol gradient fractionation strategy that can be used as an efficient density gradient method for isolating proteasome subpopulations in their
native forms with their associated protein partners. This method allows proteasomal partners, even those with low affinity, to stay with the proteasome complex during purification. Additionally, the iodixanol gradient fractionation allows satisfactory separation of proteasome complexes associated with the ER and Golgi apparatus.

Despite identical genetic input, we observed differences in proteasomal proteolytic activity patterns in the striatal and hippocampal brain regions. This suggests the existence of tissue-specific proteasome subpopulations. Since the stoichiometry of proteasome subunits are the same in all tissues, the association of the 26S complex with different proteins can be a potential reason for the functional diversity of the proteasome complexes. Our in vitro IP experiments with mass spectrometry and the follow up in vivo IP experiments using mouse striatum and hippocampus tissues show that the proteasome complex in the striatum has a stronger affinity to its known partner, Eno2, in comparison to the proteasome complex in the hippocampus. Understanding the function of Eno2 as a plausible tissue-specific modulator of the proteasome complexes in striatal tissues could explain the altered proteasomal function in neurodegenerative diseases and the age-related impairment of the proteasome.
CHAPTER FIVE

Future Work

This project and the presented data in this thesis is only in the initial stages. Our discovery about the existence of the proteasome complex with different partners may open up new directions for research in the field of neurodegenerative diseases. There are some potential directions to complete this project and some are listed below:

- What would happen to the proteasomal activity in the presence of over-expressed or silenced Eno2 in striatal and hippocampal cell lines?
- A set of IP experiments done in normal mouse brain regions suggested that the binding of Eno2 to proteasome complexes is likely brain region dependent manner. Similar IP experiments should be considered in a mouse model of Huntington’s disease and probably other available neurodegenerative mouse models.
- As with some other examples for proteasomal partners, does Eno2 work as a shuttle for the proteasome for delivery of specific substrates?
- Neuronal cells suffer from stress in neurodegenerative diseases. For example, the accumulation of the trinucleotide repeats (CAG) in Huntington’s disease leads to a cell stress and eventually cell death. Does the affinity of Eno2 to the proteasome complex alter activity (increase or decrease) upon stress? If it does alter activity, is it in a cell dependent manner?
• What are the levels of Eno2 proteins in different regions of the human brain? Are there any difference in terms of the level or location between normal and Huntington’s disease in the human brain?

• The difference Eno2’s affinity to proteasome complexes should be examined in human brain tissues. One of the key experiments is to determine whether Eno2 has more binding affinity to proteasome complexes in human striatal cells versus hippocampal cells in both normal and neurodegenerative diseases particularly Huntington’s disease.

• The binding affinity of Eno2 to the proteasome complex should be examined in other brain regions such as the pre-frontal cortex. Mapping Eno2 and proteasome binding affinity in different brain regions will provide a better understanding of Eno2’s function in different brain regions.

• A mouse knockout model of Eno2, particularly a conditional mouse model, can further explain the functional role of Eno2 in the brain.

• While our *in vivo* data were not matched with data collected in two cell lines, the chymotrypsin-like activities recorded in tissues suggest possible differences. Further experiments are needed to show whether there is a significant difference between these two brain regions.

• A set of immunocytochemistry will be a necessary parallel experiment to verify the binding between Eno2 and proteasome complexes in the striatal and hippocampal cell lines. The immunohistochemistry will need to be done in the presence and the absence of stress.
CHAPTER SIX

Conclusion

The structural assembly and proteolytic activities of proteasome complexes are strictly regulated and fine-tuned by post-translational modifications, regulatory complexes, proteasome interacting proteins (PIPs), and subunit composition (Schmidt et al., 2005; Zhang et al., 2007). Modulation of 20S core activity in a brain region dependent manner by a specific regulatory protein, in this case Eno2 protein, strongly suggests that the proteasome complex is highly dynamic, and capable of adjusting its proteolytic activity depending on the needs of the cell. On the other hand, this difference in regulatory proteins can make the proteasome complex more sensitive to intrinsic and extrinsic changes. Hence, protein-protein interactions, along with post-translational modifications, and proteasome gene regulation, provide additional levels of regulation and sensitivity upon general stress as well as particular stress during the progression of neurodegenerative diseases. Given that neurodegenerative diseases, such as Huntington’s disease, are associated with the accumulation of misfolded proteins and inhibition of proteasome activities, enhancing endogenous proteasome activity can be a novel therapeutic approach in neurodegenerative diseases. An example of this type of strategy is IU1C, an Usp14 inhibitor, which is able to accelerate proteasomal degradation of oxidized proteins (Lee et al., 2010). Targeting a particular partner of the proteasomal complex, such as Eno2, may demonstrate the possibility of developing proteasome
activating reagents for preventing protein aggregation in a particular brain region without interfering with common proteasome functions in other brain regions or tissues.
References


