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Cell type-dependent activities of the ubiquitin-proteasome system and selective neurodegeneration

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Abstract

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By Suzanne Tydlacka

A variety of neurological disorders and polyglutamine (polyQ) diseases are caused by misfolded proteins. The common feature of these diseases is late-onset cellular degeneration that selectively affects neurons in distinct brain regions. PolyQ diseases, including Huntington's disease (HD), present a clear case of selective neurodegeneration caused by polyQ expansion-induced protein misfolding, which leads to predominant inclusions in neuronal nuclei. It remains unclear how these ubiquitously expressed disease proteins selectively kill neurons. In HD, mutant huntingtin accumulates in both neurons and astrocytes, but more neuronal cells display huntingtin aggregates. These aggregates colocalize with components of the ubiquitin-proteasome system (UPS), which plays a critical role in clearing misfolded proteins. Using fluorescent reporters that reflect cellular UPS activity, we found that UPS activity in neurons and astrocytes decrease in a time-dependent manner. Neuronal UPS activity is lower than astrocytic UPS activity, which is consistent with the more abundant htt aggregates in neuronal cells. Similarly, in the nucleus and synapses in which mutant htt accumulates, UPS activity is also lower than that in the cell body. Moreover, inhibiting the UPS markedly increases the accumulation of mutant htt in cultured astrocytes. These findings suggest that differential UPS activities are dependent on cell types as well as subcellular localization and account for the preferential accumulation of misfolded proteins in neurons and their selective vulnerability.

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CHAPTER 1

General Introduction

Suzanne Tydlacka wrote the entire content of this chapter and Xiao-Jiang Li edited.

1.1 Huntington's Disease

Huntington's disease (HD) is one of nine polyglutamine (polyQ) neurodegenerative disorders. Most of these disorders are inherited in an autosomal dominant manner, Spinal and Bulbar Muscular Atrophy (SBMA) being the exception, with the CAG expansion that is translated to an expanded glutamine repeat of varying length (Table 1.1). Each of the diseased proteins shows no homology to each other outside of the glutamine repeat (Orr et al., 2007). However, several neuropathological features are common in all. Each polyQ protein is ubiquitously expressed throughout the body yet the mutant protein with an expanded polyQ tract selectively degenerates a subset of neurons in a distinct brain region. In the affected neurons, there is the presence of intranuclear inclusions (NII), which increase with age for all polyQ diseases.

HD is the most characterized polyglutamine neurodegenerative disorder. The gene responsible for HD was discovered in 1993 and was found to be located on the short arm of chromosome 4 (Huntington's Disease Collaborative Research group, 1993). The CAG repeat in the HD gene encodes the polyQ domain that resides in the N-terminal region of huntingtin (htt), a 350-kDa protein. The CAG/glutamine repeat length determines either a diseased or normal state. Unaffected individuals have CAG lengths from 11-35 repeats, and HD patients have CAG repeats from 36-121 (Perutz et al, 1999). Most HD patients have adult onset symptoms that occur in midlife, between the age of 35-50 years, and have 40-50 CAG repeats. The disease progresses over time and becomes fatal within 15-20 years after the onset of symptoms (Ho et al., 20001). CAG/glutamine expansions greater than 50 repeats result in the juvenile form of HD whereas repeat lengths greater than 100

are rare and result in embryonic lethality.

There is an inverse correlation between the age of onset of HD and the number of polyglutamine repeats (polyQ) (Ross et al, 1995). A longer repeat results in an earlier onset of HD. However, this correlation is more significant for patients with repeat lengths greater than 65 polyglutamines whereas the majority of HD patients have between 40 and 55 polyQ repeats. Other modifying genes and environmental factors account for the variance in age of onset and symptoms.

The early symptomatic features of HD patients are subtle difficulties in smooth eye movement, slight uncontrolled movements, and clumsiness (Brandt et al., 1984). As the disease progresses, these motor disturbances worsen such that patients gradually lose their capacity to move and communicate. Bradykinesia and rigidity are common features of late stage HD patients. Most patients suffer from inexplicable muscle wasting and weight loss, despite caloric intake. Death generally occurs as a consequence of heart failure or aspiration pneumonia (Chiu et al, 1982).

One neuropathological hallmark of HD is striatal atrophy. First, the caudate nucleus of the striatum is lost followed by degeneration of the putamen (Vonsattel et al., 1998). The medium spiny neurons are the primary striatal neuron affected in HD. Interestingly, htt immunoreactivity appears to be confined primarily to medium spiny neurons, illustrating a correlation between the expression of htt and the selective vulnerability of medium spiny neurons (Ferrante et al., 1997). As the disease becomes severe, layers III, IV and VI of the cerebral cortex degenerate. In the most severe case, which is classified as grade 4, there can be slight atrophy of the globus pallidus, thalamus, subthalamic nucleus, and substantia nigra.

Another neuropathological hallmark of HD is the presence of intranuclear inclusions (NII) and cytoplasmic inclusions (CI). In an HD mouse model that expresses full-length mutant htt, striatal neurons have the highest concentration of NII followed by cortical neurons. In these HD mice, NII appear before the loss in brain weight which, in turn, precedes the loss of body weight and the onset of neurological symptoms (Gil et al., 2008). It is of interest to note that non-neuronal cells, such as glia, form relatively few aggregates even in the most severe cases of HD mice. Rather, significantly more neurons than glia contain htt aggregates (Shin et al., 2005). It has been found that viral delivery of glial cell line-derived neurotrophic factor (GDNF) into the striatum of a transgenic mouse model of HD decreased the number of neurons containing NII as well as the number of striatal neurons that underwent atrophy (McBride et al., 2006). Understanding the mechanism underlying the accumulation of more htt aggregates in neurons than glial cells would help elucidate the pathogenesis of HD as well as other polyglutamine diseases.

1.2 Misfolded and Aggregated Proteins in Huntington's Disease

Expansion of the polyQ tract in the HD protein (htt) can lead to abnormal protein confirmations, such as β-sheets (Perutz et al., 1994). The abnormal protein confirmations can lead to insoluble aggregates within the cytoplasm and nucleus (Zhou et al., 2003). Accumulation of the insoluble aggregates can produce large inclusions in the nucleus and cytoplasm. Although inclusion bodies are pathological features of polyglutamine diseases, there is great controversy about the role of polyQ aggregates or inclusions in the neuropathology of the diseases. Some studies show that aggregates are toxic while other findings suggest that aggregates are protective (Arrasate et al., 2004; Bowman et al., 2005).

Despite the undefined role of aggregates, it is clear that they result from an accumulation of misfolded proteins and reflect decreased intracellular capacity to clear or remove misfolded proteins (Li et al., 2008).

1.3 **The Ubiquitin-Proteasome System Pathway**

The ubiquitin-proteasome system clears most soluble proteins in the cytoplasm and nucleus. It also plays a key role in degrading short-lived and misfolded proteins. Protein clearance by the UPS involves two sequential reactions, a ubiquitination reaction and a subsequent degradation of the ubiquitinated protein by the proteasome (Ciechanover et al., 2005).

The ubiquitination reaction requires a covalent attachment of ubiquitin, a small and highly conserved peptide (76 residues), to the substrate in three steps (Figure 1.1). First, a ubiquitin monomer is activated by forming an intermolecular thiol ester in its C-terminal Gly with the ubiquitin-activating enzyme (E1) in an ATP-dependent reaction (Ciechanover et al., 1998). Secondly, activated ubiquitin is transferred to a Cys residue in the active site of an ubiquitin-conjugating enzyme (E2). Thirdly, ubiquitin is linked by its C-terminus through an amide isopeptide linkage to the ε-amino group of a Lys residue of the substrate protein. This step is catalyzed by an ubiquitin-protein ligase (E3) that confers specificity to the process by selectively binding to target proteins. Finally, activated ubiquitin molecules are sequentially added at Lys48 to the previously conjugated ubiquitin of the protein, forming a polyubiquitin chain. Proteins tagged with chains of four or more ubiquitins are then recognized and unfolded by the 19S regulatory cap of the 26S proteasome. The unfolded protein can then enter the 20S catalytic core of the proteasome for degradation

(Chau et al., 1989). Ubiquitin monomers are released after proteasome degradation or are actively removed by the ubiquitin carboxyl-terminal hydrolases (Mayer and Wilkinson, 1989).

The 26S proteasome is known as an energy-dependent multicatalytic protease localized both in the nucleus and cytoplasm that degrades polyubiquitylated proteins. It is composed of three major subunits: one 20S catalytic core flanked by two 19S regulatory caps (Figure 1.1). The catalytic core is a barrel shaped compartment made up of four stacked rings of seven different $\alpha_{1\text{-}7}$ (the outer two rings) and $\beta_{1\text{-}7}$ subunits (the inner two rings). Three inner subunits ($\beta_{1,2,5}$) bear the proteolytic activities. The β1 subunit is the site of postglutamyl activity, the β2 subunit is the site for trypsin-like activity and the β5 subunit is the site for chymotrysin-like activity (Layfield et al., 2003). The outer α subunits stabilize the holoenzyme (Wolf et al., 2004). The 19S subunits participate in substrate recognition, untagging, and unfolding. They also induce conformational modifications toward an open state of the α ring and provide the force that drives the substrate into the catalytic core (Pickart et al., 2004).

1.4 **Ubiquitin-Proteasome System in Polyglutamine Diseases**

Two models have been proposed to account for the UPS impairment observed in polyQ diseases. The first model was based on immunocytochemistry staining of HD patients, HD transgenic mouse models, and cell cultures expressing mutant HD in which there was colocalization of NII with ubiquitin and proteasome subunits (DiFiglia et al., 1997; Cummings et al., 1998). The co-labeling studies suggested that the proteasome subunits were sequestered into inclusions thus impairing its normal function. However, the initial

idea that aggregate proteins can have a toxic effect on UPS activity by sequestering some of their components no longer stands, because the amount of UPS elements trapped in the aggregates is a relatively small percent of the total (Bennett et al., 2005). Additionally, cell lines stably expressing the proteasome reporter, GFPu, with wild type or mutant htt illustrated that the proteasome was inhibited by the presence of an expanded polyQ repeat (Bence et al., 2001). This finding leads to the second model that proteins containing expanded polyQ repeats may block the proteasome, thus preventing the entry of substrates and their subsequent degradation.

Evidence supporting this theory is the finding that polyQ protein-mediated impairment of UPS occurrs in the absence of detectable aggregates on 26S proteasome (Bennett et al., 2005). In SCA-1, another polyQ disease, proteasomal degradation of the disease protein, ataxin-1, was delayed by the presence of an expanded polyQ repeat, though ubiquitination was not affected (Cummings et al., 1999). Likewise, another group found that proteasomal function was reduced due to the expression of N-terminal mutant htt with an expanded polyQ tract (Jana et al., 2001). In line with this evidence, our lab has found that the accumulation of N-terminal htt fragments increases with age while proteasome activity decreases (Zhou et al., 2003). The most dramatic decrease in proteasome activity occurred in the striatum followed by the cerebral cortex. Consistently, the striatal region accumulates abundant NII.

In light of this evidence, a working model for age-induced accumulation of NII suggests that aging plays an important role in decreasing UPS activity (Figure 1-2). Aging could generate a variety of cellular changes including oxidative stress, DNA damage, and mitochondrial impairment, which can affect UPS activity. With decreased proteasome

function, it is expected that accumulation of misfolded proteins and formation of aggregates or inclusions occur in cells. The fact that mutant htt forms more aggregates in striatal neurons than cortical neurons or accumulates more in neurons than glial cells suggests that there is cell-type dependent UPS activity. Such differential UPS activity could contribute to the preferential accumulation of mutant polyQ proteins in neuronal cells and selective neuropathology. This is an interesting possibility that is addressed in the present dissertation.

1.5 Aging of the Ubiquitin-Proteasome System

Of the two major steps for UPS dependent degradation, ubiquitylation and degradation, the former does not seem to be affected by aging. Studies of mouse liver or human fibroblasts have revealed no changes with age in levels of Ub, Ub mRNA, and of E1, E2 or different E3s (Carrard et al., 2002). Consequently, the accumulation of Ub-conjugated substrates, common in most aged tissue and in different age-related disorders, is likely to result from a decrease in their removal by the proteasome.

Aging has been found to decrease the content of regulatory proteins in the 20S subunit (Ferrington et al., 2005). More strikingly, age increases the oxidative state of proteasome subunits and is likely to result in changes in UPS regulation (Carrard et al., 2003). When a 16-month-old rat was treated with the antioxidant, melatonin, it was found that melatonin levels decreased carbonyl levels in the hippocampus (Mohsen et al, 2005). Immunohistochemical studies of Alzheimer patients' brains illustrated that carbonyl groups are localized primarily in neurons with tangles rather than in glia (Smith et al., 1996). This deficit of regulatory subunits due to oxidative damage is intriguing and suggests that

neurons tend to be more susceptible to oxidative damage than glia. This could also be the underlying reason as to why more polyQ aggregates form in neurons than in glia. It is possible that neurons are more sensitive to age-induced oxidative stress that can reduce proteasome function thereby affects the degradation of mutant proteins, leading to an accumulation of aggregates and the neuropathology of many neurodegenerative diseases.

1.6 Available Tools to Study Ubiquitin-Proteasome System

Although it is likely that age-dependent UPS impairment contributes to the accumulation of mutant htt in neurons and the late onset of HD, this possibility has not been rigorously explored. The complex of the proteasome, which is a large multicatalytic protease complex consisting of 20S (~700 kDa) and 26S (~2000 kDa) particles (Coux et al., 1996), makes it difficult to investigate which subunit is more important for degradation of mutant htt or is impaired in HD. However, the function of this system can be easily monitored with standard biochemical assays that measure chymotrypsin-like, trypsin-like, and postglutamyl peptidase activity (Table 1-2). Previous studies have assayed the proteolysis of small fluorogenic substrates specific for each of the three catalytic activities of the 20S proteasome in homogenates from HD cell models (Ding et al., 2002) or from whole brain cell extracts from HD mice (Bett et al., 2005). A drawback of these assays is that small fluorogenic substrates are degraded by the 20S proteasome in a ubiquitination-independent manner. As such, these assays are unable to detect alterations at free ubiquitin, polyubiquitination, recognition by the 19S proteasome and unfolding to the 20S proteasome. At best, fluorogenic substrate assay is able to determine which catalytic activity is being hindered. The most significant drawback of this biochemical assay is that

it cannot examine cell-type specific UPS activity, as this assay needs to use tissue homogenates (Hernández et al., 2004). Thus, if UPS impairment only occurs in a specific type of cells in the brain, a fluorogenic substrate assay may not be able to detect this alteration. Additionally, 20S proteasome in tissue homogenate can be in either a latent form or an activated form, depending on the content of proteasome activators and whether detergents or hydrophobic peptides are present during the procedure.

Due to the limits mentioned above for the fluorogenic substrate assay, other methods have been developed to study UPS function. Of particular interest is the use of a Ub^{G76V}-GFP, which has been genetically expressed in transgenic mice to examine proteasome function *in vivo* (Lindsten et al., 2003). The reporter Ub^{G76V}-GFP carries an ubiquitin fusion degradation signal consisting of an N-terminal linked ubiquitin molecule that serves as an acceptor for polyubiquitin chains. The G76V substitution leads to efficient ubiquitination and proteasome degradation of the Ub^{G76V}-GFP reporter under normal conditions. Expression of Ub^{G76V}-GFP was confirmed by RT-PCR in all analyzed tissues; however, upon intraperitoneal administration of the proteasome inhibitor, epoxomycin, there was little detectable fluorescence in the brain. Rather, only primary cultured neurons derived from Ub^{G76V}-GFP reporter mice show fluoresce signals after exposure to epoxomycin. Another limitation in Ub^{G76V}-GFP reporter mice is that proteins with an ubiquitin fusion degradation signal can only be ubiquitinated by specific subsets of E2 and E3 ubiquitin ligases. Thus, the reporter system might fail to reflect UPS impairment if other E2 and E3 enzymes are involved or if the defect is in an early recognition step.

Another proteasome reporter, GFPu, is used to quantify changes in proteasomal

activity (Bence et al., 2001). This reporter consists of GFP tagged with a CL-1 degron sequence specific for E3 ligase recognition and subsequent degradation by the proteasome, resulting in a relatively short half-life (30 minutes) of GFP. Upon proteasome inhibition, the expression of GFPu reporter is increased to reflect proteasome impairment. Although this assay indirectly measures proteasomal function, it provides several advantages. First, it offers a sensitive tool to quantitatively measure proteasomal function, as fluorescent signals can be measured by fluorescence microscopy or fluorometry. The signals of GFPu can also be measured by Western blots with anti-GFP followed by densitometry. Second, it allows one to examine the relationship between the accumulation of misfolded proteins and the decrease in proteasome function in a single cell by analyzing GFPu signals using fluorescent microscopy. Finally, this reporter can be expressed in the brain to examine how cell-type or tissue specific factors regulate proteasome function and protein misfolding *in vivo*. Given the limitations and advantages of each assay, it is important to combine these techniques to examine proteasomal activity in the brain.

1.7 Mouse Models of Huntington's Disease

Transgenic mouse models of HD will be used in this thesis to examine changes in UPS activity in HD. There are different HD mouse models, which provided insight into the pathogenesis of HD. Each mouse model mimics the pathological changes of HD in very different ways.

The first HD mouse model generated is a R6/2 transgenic mouse that has exon 1 of the human htt gene inserted into its genome (Mangiarini et al., 1996). In R6/2 mice,

approximately 144 CAG repeats are present and are driven by the human huntingtin promoter. This mouse model presents striking neuropathological changes characterized by htt inclusions in various brain regions as well as brain atrophy. R6/2 mice have an aggressive behavioral phenotype with symptoms appearing as early as 4 weeks of age with death at 10-13 weeks. Due to its severe phenotype and short lifespan, these mice are useful for studying severe neuropathological changes caused by small N-terminal mutant htt.

The N171-82Q transgenic mouse model was created by inserting the first 171 amino acids of the N-terminal of human htt gene into the mouse genome (Schilling et al., 1999). Expression of transgenic htt is under the mouse prion promoter, which drives transgene expression largely in neurons. N171-82Q mice also have striatal and cortical inclusions and show approximately 25% loss of striatal neurons at 16 weeks of age. In comparison to the R6/2 mouse, N171-82Q has a later onset of behavioral symptoms thus making it a good model for studying presymptomatic therapies. Because mutant htt is also expressed in astrocytes (Shin et al., 2005), N171-82Q mice in which htt expression is not found in astrocytes do not adequately model HD.

The YAC transgenic mouse model used an yeast artificial chromosome (YAC) to express the entire human gene under control of the human huntingtin promoter (Ramaswamy et al., 2007). YAC mouse strains carry 72 or 128 CAG repeats and both strains have a reduction in striatal neurons; however, YAC mice expressing 128 CAG repeats show more abundant striatal NII. These mice show translocation of N-terminal fragments into the nucleus, providing *in vivo* evidence of htt cleavage and the toxic gain of function of these fragments (Hodgson et al., 1999). Additionally, YAC transgenic mice can live more than one year, thereby making them a better model to examine long-term

therapeutic studies.

The above transgenic mice contain multiple copies of the huntingtin gene. This is not representative of the genetics of the disease as it appears in humans who usually have one normal copy of the huntingtin gene and one mutated copy. Secondly, random insertion of the human huntingtin gene in the mouse genome may interfere with the normal function of other genes not related to HD. Finally, the htt protein is driven by an artificial promoter, which may alter the temporal and spatial expression of transgenic htt.

In an attempt to have a more faithful genetic model of HD, HD knock-in (KI) mice were generated. A portion of the mouse huntingtin gene was replaced with a mutant human htt DNA containing 140 CAG repeats such that mutant htt is expressed under the mouse endogenous promoter (Menalled et al., 2003). KI mice should be the most faithful HD mouse models because they carry the mutation in the appropriate genomic and protein context. In KI mice, striatal NII appear first followed by cortical NIIs in layers II and III. The temporal formation of NII in the striatum followed by NII in the cortex correlates with the early and preferential striatal pathology seen in HD patients. Additionally, as the animal ages, the number of NII increases (Zhou et al., 2003). These mice can live up to 2 years of age and have a progressive onset of pathology, making it an ideal model to study the effects of aging on HD pathogenesis.

Comparing KI mice with transgenic mouse models, which express small N-terminal htt fragments and show severe neurological phenotypes and earlier accumulation of N-terminal mutant htt, clearly indicates the toxicity of N-terminal mutant htt (Wang et al., 2008). N-terminal mutant htt fragments cause both nuclear toxicity and cytoplasmic toxicity. Nuclear toxicity of an N-terminal htt fragment containing an expanded polyQ

repeat negatively affects gene transcription. Cytoplasmic toxicity caused by a mutant N-terminal htt fragment can increase oxidative stress, induce mitochondrial dysfunction, and reduce intracellular trafficking (Li and Li, 2007). Thus, KI mice provide a useful HD model to study how N-terminal htt fragments are generated and accumulate in the brain when mutant htt is expressed at the endogenous level. Accordingly, I will primarily use KI mice to examine the relationship between UPS activity and the accumulation of mutant htt fragments.

1.8 Hypothesis

In light of previous findings that HD 150Q knock-in mice have an increase in NII (Zhou et al., 2003) and proteasome enzymatic activity decreases (Wang et al., 2008) with age, we hypothesize that age-dependent decrease in proteasome activity contributes to the accumulation of misfolded proteins in the brain. In addition, mutant htt may further enhance age-induced dysfunction of the UPS (Figure 1-3). We believe that mutant htt can be cleaved and then translocated into the nucleus where it aggregates to form NII. Likewise, cytoplasmic mutant htt can aggregate, creating cytoplasmic inclusions (CI). Since the nucleus has significantly more inclusions than the cytoplasm, we also hypothesize that nuclear UPS activity is more impaired than cytoplasmic UPS by aging. Additionally, the brain striatal region that shows more htt aggregates than other brain regions such as the cortical region may have lower UPS activity.

CHAPTER 2

Use of proteasome reporters to measure ubiquitin-proteasome system activity

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2.1 Abstract

All nine of the polyglutamine (polyQ) neurodegenerative disorders have polyQ inclusions that are positively labeled by antibodies against ubiquitin and proteasome subunits (Cummings et al., 1998; Davies et al., 1997). Proteasome components and ubiquitin have been found to associate with polyglutamine aggregates that are mainly comprised of truncated N-terminal htt fragments containing expanded polyglutamine tracts. *In vitro* experiments also provide biochemical evidence that expanded polyglutamine proteins are degraded by proteasomes yet the rate of degradation is inversely proportional to repeat length (Venkatraman et al., 2004). More recently, subsequent studies offered more evidence for the role of UPS in HD by FRET analysis, which determined a stable association between the proteasome and large repeat polyglutamine (82Q)-containing proteins (Holmberg et al., 2004).

Since the ubiquitin proteasome system (UPS) plays an essential role in degrading damaged or misfolded proteins in the cytoplasm and the nucleus of the cells, these findings raise the possibility that the UPS may be dysregulated and thus less able to clear misfolded proteins. To investigate the specific relation between protein aggregation and UPS activity as well as pathogenesis, we generated the proteasome reporter, GFPu, based on the original publication by Bence et al., 2001 and cloned it into an adenoviral vector. The proteasome reporter, GFPu, contains a constitutively active degradation signal (CL-1) at its C-terminal sequence to sensitively monitor the activity of UPS. The present study validates the use GFPu to measure UPS activity in the cell body, nucleus, cytoplasm, and synapse. These results suggest that GFPu is an effective and promising measurement method to monitor UPS activity.

2.2 Introduction

The ubiquitin-proteasome system (UPS) removes damaged or misfolded proteins by ubiquitinating them via ubiquitin ligases, then targeting these ubiquitinated proteins to the proteasome for degradation (Ciechanover, 2005; Demartino and Gillette, 2007). Normal UPS function is important for preventing diseases that are caused by misfolded proteins. Misfolded polyQ proteins predominantly accumulate in neurons, despite the fact that polyQ disease proteins are widely expressed throughout the brain and body.

To investigate the relationship between protein aggregation and the function of the UPS, we constructed a proteasome reporter consisting of a short degron, CL1, fused to the COOH terminus of a green fluorescent protein (GFPu). This reporter was invented by Bence et al., 2001 to detect UPS activity in cultured cells. Under normal UPS activity, there should be little to no GFP fluorescence. When the proteasome is inhibited there should be an increase in the GFP fluorescence illustrating the inability of the UPS to degrade GFPu. Use of GFPu in cultured cells revealed that protein aggregation directly impaired the function of UPS. Cells containing larger inclusions had stronger GFPu fluorescent intensity and less UPS activity compared to those containing smaller inclusions. Thus the inhibition of UPS activity correlates with inclusion sizes and most importantly, GFPu was able to effectively measure this phenomenon (Bence et al., 2001).

GFPu undergoes the N-end rule pathway (Figure 2-1). The N-end rule defines the stability of proteins according to the nature of their N-terminal residues. Under the N-end rule pathway, the destabilizing residues of the CL1 degron (Arg, Lys, His) of GFPu are recognized by the E3 ligase N-recognin that contains distinct binding sites for CL1 residues (Mogk et al., 2007). N-recognin associates with an ubiquitin-conjugating enzyme (E2) and targets an internal Lys (K) residue of the GFPu for ubiquitylation by E2. The multiubiquitylated GFPu is recognized by the 19S cap

complex of the proteasome and is subsequently unfolded and translocated into the 20S core for proteolysis.

In the present study, we tagged GFP with the short degron, CLI, as described previously (Bence et al., 2001). To express this reporter in neuronal cells, we inserted the newly constructed GFPu into an adenoviral vector (Ad-GFPu). It is critical to have an internal control that can eliminate expression variations caused by viral infection. Thus, another adenoviral vector expressing RFP, without an attached degron, (Ad-RFP) was used as an internal control for co-expression with Ad-GFPu. The ratio of Ad-GFPu to Ad-RFP reflects the relative activity of UPS. In other words, the lower UPS activity would result in the higher ratio of GFPu to RFP.

In order to measure UPS activity in the nucleus or cytoplasm, respectively, GFPu and RFP were tagged with a nuclear localizing sequence (NLS) or nuclear exporting sequence (NES). Additionally, to target these reporters to the presynaptic terminal or postsynaptic terminals, GFPu and RFP were tagged with the presynaptic protein SNAP25 and postsynaptic protein PSD95, respectively. A proteasome inhibitor, MG132 (10μM), was used to treat cells expressing these reporters. As expected, GFPu expression significantly increased whereas RFP expression remained constant after inhibiting UPS by MG132. The increase in GFPu expression was further confirmed by Western blotting by examining the ratio of GFPu to RFP. Thus, the reporters GFPu/RFP generated in this study can effectively reflect UPS activity in different cell types and different subcellular localizations.

2.3 Materials and Methods

GFPu/RFP Viral Vectors: GFPu construct was generated according to the previously described study (Bence et al., 2001). An oligonucleotide encoding ACKNWFSSLSHFVIHL was ligated into the GFP-C1 plasmid (Clontech) to create the proteasome reporter, GFPu. cDNA sequences for DsRED (BD Biosciences) were used to replace GFPu to generate PRK-RFP construct. The GFPu and RFP plasmids were inserted into the shuttle vector of the AdEasy vector system (Qbiogene). Adenovirus amplification and purification were performed according to the method used in our previous study (Shin et al., 2005). Viral titer was determined by measuring the number of infected HEK293 cells expressing GFPu or RFP. All viral stocks were adjusted to $1x10^{10}$ VP/ml.

SNAP25-GFPu/RFP and PSD95-GFPu/RFP Viral Vectors: A CL-1 degron sequence (Bence et al., 2001) was added to the C terminus of GFP in the PRK vector to generate PRK-GFPu construct. cDNA sequences for DsRed (BD Biosciences) were used to replace GFPu to generate PRK-RFP construct. RT-PCR using RNAs from mouse brains was performed to isolate full-length cDNAs for mouse SNAP25 and PSD95. SNAP25 cDNA was isolated with a sense primer 5′-acatcgatATGGCCGAAGACGCAGACATG-3′ and an antisense primer 5′-acacgcgtACCACTTCCCAGCATCTTTG-3′. Full length of PSD95 cDNA was isolated using the sense primer 5′-acatcgatCCAACATGGACTGTCTCTGTATAG-3′ and the antisense primer 5′-atacgcgtGAGTCTCTCTCGGGCTGGGAC-3′. The PCR products were subcloned into the PRK-GFPu and PRK-RFP vectors to express fusion proteins that contain GFPu or RFP at the C terminus of SNAP25 or PSD95. The DNA fragments encoding these fusion proteins were inserted into the shuttle vector of the AdEasy vector system (Qbiogene). The CMV promoter was replaced by human synapsin-1 promoter provided by Dr. S. Kugler (University of Goettingen, Goettingen, Germany). Adenovirus amplification and purification were performed according to the method

used in our previous study (Shin et al., 2005). Viral titer was determined by measuring the number of infected HEK293 cells expressing GFPu or RFP. All viral stocks were adjusted to 10^9 VP/ml.

Neuronal and Glial Cell Cultures and Infection: Neurons were prepared from the cerebral cortex and striatum of rat fetuses at embryonic day 17-18. Viable neurons were plated at 1 x 10^6 cells/ml on poly-D-lysine–coated plastic culture plates (Corning Costar) in B27-supplemented Neurobasal medium (Invitrogen). To reduce the proliferation of glia, cytosine arabinoside was added to the cultures at a final concentration of 5 x 10^6 M 3 days after plating cells. Postnatal day 1 mouse pups (wild-type and homozygous *Hdh*(CAG)150 knock-in) were used for glial culture. Viable glia; cells were plated at 1 x 10^5 cells/ml on plastic culture plates (Corning Costar) in DMEM (Sigma). Mixed glia-neuron co-cultures were plated at 1 x 10^5 cells/ml on poly-D-lysine–coated plastic culture plates (Corning Costar) in DMEM (Sigma).

Cultured neurons and glia that had been cultured for 4-20 days were infected with adenoviral vectors. The adenoviral vectors were incubated with the cultured neurons and glia for 24 h before the medium was removed. After adenoviral infection for 48 h, Western blotting or fluorescent microscopy examined cultured cells.

Cell Line Culture: PC12 cells were cultured in Dulbecco's modified eagle medium supplemented with 5% fetal bovine serum, 10% horse serum, 100 μg/ml penicillin, and 100 μg/ml streptomycine, and incubated at 37°C in a humidified 5% CO_2 atmosphere. Human Embryonic Kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml

streptomycine, and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Antibodies and Reagents: Antibodies against N-terminal human htt (EM48) were generated previously (. Antibodies against an expanded polyQ tract (1C2, 1:5000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:3000were obtained from Millipore (Temecula, CA). Other antibodies used are against the following proteins: γ-tubulin (Sigma, St. Louis, MO; 1:10,000), RFP and GFP (Clontech), NeuN (Millipore), GFAP (Millipore), and ubiquitin (Cell Signaling, Santa Cruz Biotechnology, 1:500). Secondary antibodies were peroxidase-conjugated donkey anti-mouse, -rabbit, -rat, -guinea pig, -goat, or -sheep IgG (H+L) from Jackson Immunoresearch (West Grove, PA; all used at 1:5000).

Proteasome inhibitor treatment with MG132 (A.G. Scientific, Inc), was described previously (Wang et al., 2008). MG132 was added directly to serum-free medium at a final concentration of 10μM MG132 for 12 hours. The negative control only contained DMSO in serum-free medium. After 12 hours, cells were either collected for western blot or they were fixed, stained with the nuclear dye, hoechst (1μg/μl) (Invitrogen), and fluorescent images were taken.

Quantitative Fluorescence Imaging Analysis. Quantitative analysis of captured fluorescent images was performed as described previously (Wang et al., 2008b). Briefly, precise segmentation of bright spots was achieved by setting a lower threshold just above background emission, typically ~23 to 25 gray scale units (gsu), and an upper threshold at the upper limit of the 8-bit gray scale (i.e., 255 gsu). Cell bodies were detected as objects around the nuclei, separated from the neurites by a watershed filter. It starts by running a

"Hi-Pass" filter across the image to enhance edges (replaces each pixel with a value that increases contrast with neighboring pixels) . Cell bodies (7-9/each group) are selected as any objects with a size and signal intensity greater than a user-defined minimum that is above a defined background. We used the Openlab software, which generated a high-content array of measurements that included event number, area, average sum of pixel intensities, and maximum and minimum pixel intensities. The average sum of pixel intensities is selected as the parameter for statistical analysis.

Western Blotting: Cells were plated in 6-well plates at ~80% confluency and infected with 1µl of Ad-GFPu and Ad-RFP (0.5 µl of Ad-GFPu and 0.5 µl of Ad-RFP both with a viral titer of $1x10^{10}$ VP/ml) for each well. Viruses were diluted in serum free medium and incubated for 24 hours. After 24 hours medium (B27-supplemented Neurobasal medium) (Invitrogen) was replaced. After 48 hours cells were collected and resuspended in lysis buffer (10mM tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 10 µM proteinase inhibitor cocktail) and vortexed for 10 seconds. After incubation on ice for 5 minutes lysed cells were centrifuged at 4000 rpm for 5 minutes at 4°C. The pellet was resuspended in NP-40 lysis buffer and protein concentration was determined. Western blotting was performed using either 4-12% or 4-20% polyacrylamide Tris-Glycine gels (Invitrogen). SDS sample buffer was added to protein samples, which were then resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked at room temperature for 1 hour with 5% milk in 1xPBS. Membranes were washed four times in 1xPBS and incubated with primary antibodies in 1xPBS with 3% BSA overnight at 4°C. Membranes were again washed four times in PBS, blocked at room temperature for 1 hour

with 5% milk and incubated in horseradish peroxidase (HRP) conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Immunoreactive bands were visualized using ECL chemiluminescense kits (Amersham Biosciences).

2.4 Results

We examined whether the proteasome reporter, GFPu, inserted into a viral vector under the CMV promoter (Ad-GFPu) can sensitively measure UPS activity (Figure 2-2, a). Ad-RFP lacking a CL1 degron was co-infected in order to serve as an internal control for viral expression. We first infected PC12 cells with both Ad-GFPu and Ad-RFP. Twenty-four hours post-infection, the proteasome inhibitor, MG132 (10μM), was applied to cells (Figure 2-2, b) for 12 hours. Upon MG132 treatment, GFPu expression significantly increased as compared to RFP. An increase in GFPu expression reflects a relative decrease in UPS activity. Western blot analysis provided more quantifiable results of the increase in GFP expression over RFP (Figure 2-3), as MG132 treatment caused an increase in GFPu expression and no change in RFP expression. Consistent with the ability of MG132 to increase the accumulation of ubiquitinated proteins (Leitch et al., 2001), Western blotting analysis also showed increased protein ubiquitination in MG132-treated cells.

To verify that the reporters can sensitively reflect UPS activity in primary neurons, we expressed the reporters in cultured primary neurons from rat cerebral cortex. After MG132 (10μM) treatment, we also observed a significant increase in GFPu signals (Figure 2-4, a). This increase was validated by Western blots, which showed a marked elevation of GFPu compared with RFP following MG132 treatment (Fig. 2-4, b). The level of RFP expression remained constant in both the treated and untreated neurons. Quantifying the

ratio of GFPu to RFP indicated a preferential increase in GFPu after the UPS was inhibited by MG132 ($p<0.001$) (Fig. 2-5). Thus, the ratio of GFPu to RFP represents the relative inhibition of UPS activity and can be used to measure cellular UPS function.

The fact that mutant htt preferentially accumulates in the nuclei of neurons and occasionally in astrocytes of HD mouse brains (Shin et al., 2005; Chou et al., 2008; Wang et al., 2008) also prompted us to use the GFPu/RFP reporters to examine the UPS activity in the nuclei and cytoplasm of cultured cells. To do so, we tagged GFPu/RFP with the nuclear localization sequences (NLS) or nuclear export sequences (NES) (Figure 2-6, a). Thus, NLS-GFPu/RFP is directed to the nucleus to measure nuclear UPS activity, whereas NES-GFPu/RFP remains in the cytoplasm to reflect cytoplasmic UPS activity. The ability of these reporters to detect nuclear or cytoplasmic UPS activity was confirmed by treating transfected astrocytes with MG132 (10μM), which increased GFPu signals in either the nucleus or cytoplasm (Figure 2-6, b & c).

To measure synaptic UPS activity, the GFPu proteasome reporter containing the CL-1 degron is fused with a presynaptic protein, SNAP25, or a postsynaptic protein, PSD95. SNAP25 ensures that GFPu is targeted to the presynaptic terminal while PSD95 ensures that GFPu is targeted to the postsynaptic terminal. To ensure neuronal expression, the fusion proteins are expressed via an adenoviral vector under the control of the neuronal promoter for human synapsin 1, a synaptic vesicle associated protein (Figure 2-7, a). Like GFPu, RFP is also under the control of the neuronal promoter for human synapsin 1 and is tagged with either SNAP25 or PSD95. Western blotting revealed that these reporters were expressed at the correct molecular weights in HEK293 cells (Figure 2-7, b).

Additionally, to confirm that the adenoviral vectors specifically drive transgene

expression in synapses and can monitor UPS activity, we infected cultured cortical neurons with SNAP25-GFPu/RFP or PSD95-GFPu/RFP and treated them with the proteasome inhibitor, MG132 (10μM). As expected, neurons infected with SNAP-25-GFPu/RFP had an increase in synaptic GFPu expression after MG132 treatment (Figure 2-8, a). The localization of SNAP25-GFPu/RFP was verified in synaptic structures, which are characterized by small puncta in neurites. Quantification of the GFPu to RFP ratio in the synapse illustrated the large increase in GFPu expression ($P<0.001$) (Figure 2-8, b). Due to the fact that transgenic reporters are over-expressed in the cell body, there is the presence of diffuse fluorescent signals. However, we did observe a greater ratio of GFPu to RFP in synaptic puncta after MG132 treatment compared to the GFPu to RFP ratio in the cell body ($P<0.05$).

Likewise, we also infected cultured cortical neurons with PSD95-GFPu/RFP and treated them with MG132 (10μM) (Figure 2-8, c). There was an increase in GFPu expression after MG132 treatment in neuronal synapses. The GFPu/RFP ratio illustrated a more significant increase in GFPu expression in synapes than cell bodies (Figure 2-8, D). Thus we were able to confirm that SNAP25-GFPu/RFP and PSD95-GFPu/RFP were able to reflect changes in synaptic UPS activity.

2.5 Discussion

The relevance of the UPS to misfolded protein diseases has generated a demand for methods to monitor the activity of the UPS in cell culture and in organisms (Bence et al., 2005). Numerous methods have been used to assess UPS function in cells, tissues or animals. Yeast two-hybrid assays identify direct interactions of polyglutamine containing

proteins with UPS components, but do not provide functional data. Fluorogenic substrate assays quickly measure chymotrypsin-like, trypsin-like and peptidyl-glutamyl proteasome enzymatic activity. A drawback of these assays is that small fluorogenic substrates are degraded by the 20S proteasome in a ubiquitination-independent manner. More importantly, fluorogenic substrate assays need to use cell homogenates, which limits its use in detecting cell-type specific UPS activity in the brain. As UPS impairment may only occur in a limited number of neurons, it is necessary to develop a method that can sensitively measure UPS activity in different types of cells in vivo.

A proteasome reporter, GFPu, proves useful to quantify changes in proteasome enzymatic activity (Bence et al., 2001). This reporter consists of GFP tagged with a CL-1 degron sequence specific for E3 ligase recognition and subsequent degradation by the proteasome. Upon proteasome inhibition, the expression of GFPu reporter is increased to reflect proteasome impairment. Although this assay indirectly measures proteasomal function, it provides several advantages. First, it offers a sensitive tool to quantitatively measure proteasomal function, as fluorescent signals can be measured by fluorescence microscopy or fluorometry. The signals of GFPu can also be measured by Western blots. Second, it allows one to examine the relationship between the accumulation of misfolded proteins and the decrease in proteasome function in a single cell through the use of fluorescent double labeling of GFPu and protein aggregation. Finally, this reporter can be expressed in the brain to examine how cell-type specific factors regulate proteasome function and protein misfolding *in vivo*.

The present study used an adenoviral vector to express GFPu in neuronal cells and to measure neuronal UPS function. Adenoviral vectors have been recently used to express a

variety of genes in neurons. The advantage of using an adenoviral vector is that it can transduce genes in postmitotic cells. This is especially useful for delivering the proteasome reporter into neurons and for studying how aging affects proteasome function in the brain. Additionally, generating high titer adenovirus is more successful than other commonly used viruses, such as lentivirus. However, the drawback of using an adenoviral vector is that it may induce more immune response following in vivo administration compared with other viral vectors. To overcome this disadvantage we used an adenoviral vector with E1-E3 gene deletions thus creating a replicative deficient viral vector.

GFPu was confirmed as an accurate proteasome reporter by applying the proteasome inhibitor, MG132 (10μM), to PC12 cells and cortical neurons infected with Ad-GFPu/RFP, NLS-GFPu/RFP, NES-GFPu/RFP, SNAP25-GFPu/RFP and PSD95-GFPu/RFP. After MG132 treatment, we observed a significant increase in GFPu expression. This increase was validated by Western blots, which showed a marked elevation of GFPu compared with RFP following MG132 treatment. Quantifying the ratio of GFPu to RFP indicated a preferential increase in GFPu after the UPS was inhibited by MG132. The ratio of GFPu to RFP represented the relative inhibition of UPS activity and as such, GFPu, is a proven tool for measuring cellular UPS activity. Collectively, this provides a rationale for the use of Ad-GFPu to study if there is an age- or aggregate-induced decrease in proteasome activity in primary cell culture and in the mouse brains.

CHAPTER 3

Ubiquitin-proteasome system activity in cultured neurons and astrocytes

This chapter presents work to be published as: Suzanne Tydlacka, Chuan-En Wang, Xuejun Wang, Shi-Hua Li and Xiao-Jiang Li (2008) *J. Neuroscience,* In press. Published work is presented as: Chuan-En Wang, Suzanne Tydlacka, Adam Orr, Shang-Hsun Yang, Rona Graham, Michael Hayden, Shi-Hua Li, Anthony Chan and Xiao-Jiang Li (2008) *Human Molecular Genetics,* 17(17):2738-2751. Suzanne Tydlacka performed all the experiments described in this chapter. Xiao-Jiang Li and Shi-Hua Li aided in the design of experiments and interpretation of results. Xiao-Jiang Li aided in the writing of the result section and editing of this manuscript.

3.1 Abstract

Polyglutamine (polyQ) diseases, including spinocerebellar ataxia (SCA), are caused by misfolded proteins that have the pathological characteristic of aggregating in selective populations of neurons. For example, SCA-1 primarily produces aggregates in Purkinje cells of the cerebellum, SCA-3 leads to aggregates in the thalamus while Huntington's disease (HD) shows abundant aggregates in neurons of the striatum. In all of the polyglutamine diseases, the question remains as to why the aggregation prone polyQ proteins are not efficiently eliminated by the ubiquitin proteasome system (UPS). Since all the polyQ diseases are late-onset neurodegenerative diseases, we examined the effect of age on UPS activity in the brain. Since neurons are the cell types that acquire the most aggregates in HD and astrocytes are the cell types that have the least aggregates, we hypothesized that UPS activity might differ in neurons and astrocytes such that neurons preferentially accumulate more misfolded polyQ proteins.

We found that neurons have a significant decrease in UPS activity compared to astrocytes over culturing time. Neurons had a higher GFPu to RFP ratio as well as less chymotrypsin-like and trypsin-like activity compared to astrocytes. There was no statistically significant effect on UPS activity in astrocytes over culturing time. This finding could potentially explain why more aggregates are present in neurons compared with astrocytes in HD patients. There could be some intrinsic property of neurons that make them more susceptible to age-induced decline of UPS. In addition, striatal UPS activity was lower than cortical UPS activity in both wild-type (WT) and HD150Q knock-in mice (KI). This is of particular interest because in Huntington's disease (HD), striatal neurons have the highest accumulation of aggregates followed by cortical neurons, which

suggests that a less active UPS may be responsible for mutant huntingtin accumulation and aggregation.

3.2 Introduction

HD is caused by a polyglutamine (polyQ) expansion in the NH_2-terminal region of huntingtin (htt). Studies have shown that NH_2-terminal fragments of mutant htt are cytotoxic. For example, transfection of NH_2-terminal mutant htt cause cells to die (Cooper et al. 1998; Li et al., 1999). The toxicity of NH_2-terminal mutant htt is also indicated by its abnormal protein conformation including misfolding, aggregation and formation of inclusions. These misfolded proteins predominantly accumulate in neurons, despite the fact that polyQ disease proteins are widely expressed throughout the body. In fact, a preferential accumulation of NH_2-terminal htt fragments in medium-spiny neurons of the striatum is followed by its accumulation in other types of cells such as cortical neurons. Pathologically, this is reflected in the number of intranuclear inclusions (NII) that are present in striatal and cortical neurons. In contrast, astrocytes have relatively few NII.

PolyQ-containing inclusions are associated with the ubiquitin-proteasome system (UPS) (Lee et al., 1998) and inhibition of proteasome activity increases polyQ protein aggregation and toxicity in cultured cells (Orr et al., 2001). Accumulation of toxic NH_2-terminal htt fragments accumulates in the nucleus and form aggregates in association with the age-dependent decrease in proteasome activity (Zhou et al., 2003). In light of these findings, the present study tests the idea that aging causes impairment in the UPS. We hypothesize that neurons showing age-dependent formation of htt aggregates may have less UPS activity than glial cells during the course of aging. As such, neurons would have a reduced ability to clear toxic NH_2–terminal mutant htt thus promoting accumulation of NII and neuronal disease progression.

In the present study, we found that chymotrypsin-like activity and trypsin-like

activity decreases in WT and HD 150Q KI mice with age. As one might expect, cortical homogenates had a higher degree of proteasome activity than striatal homogenates. There was not a significant difference associated with the presence of mutant htt. Rather the culturing time or aging factor was the primary cause for the decline in proteasome enzymatic activity. When neurons and astrocytes were separately cultured to isolate the cell types in order to measure cell-type specific proteasome activity, neurons had a greater decrease in UPS activity than astrocytes. Finally, we monitored UPS activity in the nucleus and cytoplasm by tagging GFPu with a nuclear localization sequence (NLS) or a nuclear exporting sequence (NES). We found that UPS activity is lower in the nucleus than the cytoplasm, which is consistent with the fact that nucleus favors the formation of large polyQ inclusions. All these findings suggest that decreased UPS activity is strongly associated with the accumulation of misfolded polyQ proteins and the formation of polyQ inclusions.

3.3 Materials and Methods

Animals. *Hdh*(CAG)150 knock-in (HD150Q KI) mice were generated previously and maintained on the SV129/B6 background in the animal facility at Emory University in accordance with institutional guidelines. Genomic DNA was extracted from tails to genotype the mice as WT (7Q/7Q) and KI (150Q/150Q).

Viral Vectors: GFPu construct was generated according to the previously described study (Bence et al., 2001). An oligonucleotide encoding ACKNWFSSLSHFVIHL was ligated into the GFP-C1 plasmid (Clontech) to create the proteasome reporter, GFPu. cDNA sequences for DsRED (BD Biosciences) were used to replace GFPu to generate PRK-RFP construct. The GFPu and RFP plasmids were inserted into the shuttle vector of the AdEasy vector system (Qbiogene). Adenovirus amplification and purification were performed according to the method used in our previous study (Shin et al., 2005). Viral titer was determined by measuring the number of infected HEK293 cells expressing GFPu or RFP. All viral stocks were adjusted to $1x10^{10}$ VP/ml.

Neuronal and Astrocytic Cultures and Infection: Neurons were prepared from the cerebral cortex and striatum of rat fetuses at embryonic day 17-18. Viable neurons were plated at 1×10^6 cells/ml on poly-D-lysine–coated plastic culture plates (Corning Costar) in B27-supplemented Neurobasal medium (Invitrogen). To reduce the proliferation of glia, cytosine arabinoside was added to the cultures at a final concentration of 5×10^6 M 3 days after plating cells. Postnatal day 1 mouse pups (wild-type and homozygous *Hdh*(CAG)150 knock-in) were used for astrocyte culture. Viable astrocytes were plated at 1×10^5 cells/ml on plastic culture plates (Corning Costar) in DMEM (Sigma). Mixed astrocyte-neuron co-

cultures were plated at 1 x 10^5 cells/ml on poly-D-lysine–coated plastic culture plates (Corning Costar) in DMEM (Sigma).

Cultured neurons and astrocytes that had been cultured for 4-20 days were infected with adenoviral vectors. The adenoviral vectors were incubated with the cultured neurons and astrocytes for 24 h before the medium was removed. After adenoviral infection for 48 h, Western blotting or fluorescent microscopy examined cultured cells.

Antibodies and Reagents: Antibodies against N-terminal human htt (EM48) were generated previously (. Antibodies against an expanded polyQ tract (1C2, 1:5000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:3000were obtained from Millipore (Temecula, CA). Other antibodies used are against the following proteins: γ-tubulin (Sigma, St. Louis, MO; 1:10,000), RFP and GFP (Clontech), NeuN (Millipore), GFAP (Millipore), and ubiquitin (Cell Signaling, Santa Cruz Biotechnology, 1:500). Secondary antibodies were peroxidase-conjugated donkey anti-mouse, -rabbit, -rat, -guinea pig, -goat, or -sheep IgG (H+L) from Jackson Immunoresearch (West Grove, PA; all used at 1:5000).

Proteasome inhibitor treatment with MG132 (A.G. Scientific, Inc), was described previously (Wang et al., 2008). MG132 was added directly to serum-free medium at a final concentration of 10μM MG132 for 12 hours. The negative control only contained DMSO in serum-free medium. After 12 hours, cells were either collected for western blot or they were fixed, stained with the nuclear dye, hoechst (1μg/μl) (Invitrogen), and fluorescent images were taken.

Quantitative Fluorescence Imaging Analysis. Quantitative analysis of captured fluorescent images was performed as described previously (Wang et al., 2008b). Briefly,

precise segmentation of bright spots was achieved by setting a lower threshold just above background emission, typically ~23 to 25 gray scale units (gsu), and an upper threshold at the upper limit of the 8-bit gray scale (i.e., 255 gsu). Cell bodies were detected as objects around the nuclei, separated from the neurites by a watershed filter. It starts by running a "Hi-Pass" filter across the image to enhance edges (replaces each pixel with a value that increases contrast with neighboring pixels) . Cell bodies (7-9/each group) are selected as any objects with a size and signal intensity greater than a user-defined minimum that is above a defined background. We used the Openlab software, which generated a high-content array of measurements that included event number, area, average sum of pixel intensities, and maximum and minimum pixel intensities. The average sum of pixel intensities is selected as the parameter for statistical analysis.

Western Blotting: Cells were plated in 6-well plates at ~80% confluency and infected with 1µl of Ad-GFPu and Ad-RFP (0.5 µl of Ad-GFPu and 0.5 µl of Ad-RFP both with a viral titer of $1x10^{10}$ VP/ml) for each well. Viruses were diluted in serum free medium and incubated for 24 hours. After 24 hours medium (B27-supplemented Neurobasal medium (Invitrogen) was replaced. After 48 hours cells were collected and resuspended in lysis buffer (10mM tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 10 µM proteinase inhibitor cocktail) and vortexed for 10 seconds. After incubation on ice for 5 minutes lysed cells were centrifuged at 4000 rpm for 5 minutes at 4°C. The pellet was resuspended in NP-40 lysis buffer and protein concentration was determined. Western blotting was performed using either 4-12% of 4-20% polyacrylamide Tris-Glycine gels (Invitrogen). SDS sample buffer was added to protein samples, which were then resolved

by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked at room temperature for 1 hour with 5% milk in 1xPBS. Membranes were washed four times in 1xPBS and incubated with primary antibodies in 1xPBS with 3% BSA overnight at 4°C. Membranes were again washed four times in PBS, blocked at room temperature for 1 hour with 5% milk and incubated in horseradish peroxidase (HRP) conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Immunoreactive bands were visualized using ECL chemiluminescense kits (Amersham Biosciences).

Immunohistochemistry. Mice were anesthetized and then perfused intracardially with phosphate-buffered saline (PBS, pH 7.2) for 30 s followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.2. Brains were removed, cryoprotected in 30% sucrose at 4°C, and sectioned at 40 µm using a freezing microtome. Free-floating sections were preblocked in 4% normal goat serum (NGS) in PBS, 0.1% Triton-X, and avidin (10 µg/ml), then incubated with EM48 antibody (Gutekunst et al., 1999; Li et al., 2000) at 4°C for 48 h. The rabbit EM48 (1:2000) immunoreactive product was visualized with the avidin–biotin complex kit (Vector ABC Elite, Burlingame, CA). Light micrographs were taken using a Zeiss (Oberkochen, Germany) microscope (Axiovert 200 MOT) equipped with a digital camera (Orca-100; Hamamatsu, Bridgewater, NJ), and the image acquisition software Openlab (Improvision, Lexington, MA). A 20x (LD-Achroplan 20x/0.4 NA) or 63x lens (63x/0.75 NA) was used for light microscopy. Enhanced GFP was imaged using 488-nm excitation and a 500–530-nm band-pass filter, and RFP was imaged using 543-nm excitation and a 565–615-nm band-pass filter. The figures were created using Photoshop 7.0 software (Adobe) and, in some cases where the brightness and contrast of the whole

image needed adjustment, we used the brightness/contrast adjustment function.

Proteasome Activity Assay. For determining proteasome activity, clear whole-cell extracts of neuronal or glial cells were adjusted to 0.5 mg/ml total protein by dilution with homogenization buffer. All assays were done in triplicate and the results were obtained from 2-4 experiments. The chymotrypsin-like activity of 20S β5 was determined using the substrate Suc-LLVY-aminomethylcoumarin (AMC) (Bilmol; 40 µM), and the trypsin-like activity of 20S β2 was determined using the substrate Boc-LRR-AMC (Bilmol; 100 µM). Equal amounts (10 µg) of the extracts were incubated with corresponding substrates in 100 µl of proteasome activity assay buffer [0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA] for 60 min at 37°C. The reactions were stopped by adding 0.8 ml of cold water and placing the reaction mixtures on ice protected from light exposure for at least 10 min. The free AMC fluorescence was quantified by using the CytoFluor multi-well plate reader (FLUOstar, BMG) with excitation and emission wavelengths at 380 and 460 nm, respectively. All readings were standardized using the fluorescence intensity of an equal volume of free 7-amino-4-methyl-coumarin (AMC) solution (40 mM), normalized by the protein concentrations and expressed as nmol/min/mg protein.

Statistical Analysis. Data are expressed as mean ± SEM and were analyzed for statistical significance ($p < 0.05$) with Prism software (version 4, GraphPad Software, San Diego, CA).

3.4 Results

To quantitatively assess the accumulation of N-terminal mutant huntingtin (htt) in HD brains, Western blotting was used to analyze the levels of N-terminal htt fragments in HD150Q knock-in (KI) mice, in which full-length mutant htt is expressed under the control of the endogenous mouse huntingtin gene. We first examined the cerebral cortex of KI mice at 4, 14, and 24 months of age. Western blotting was performed using 1C2, as this antibody specifically detects mutant htt (Figure 3-1, a). Full-length mutant htt was found in the total cell lysates (T), cytosolic fraction (C), and synaptosomal (S) fractions. The minimum level of full-length mutant htt was seen in the nuclear fractions (N) and the highest level in the cytosolic fraction, supporting the idea that full-length htt is predominantly distributed in the cytoplasm. As age increases, more N-terminal htt fragments were found in older KI mice. In addition, more N-terminal htt fragments accumulate in the fractions isolated from KI striatal tissues and this accumulation is age-dependent, and correlates with disease progression (Figure 3-1, b). These findings are consistent with the preferential accumulation of mutant htt in the nuclei of striatal neurons in HD mice expressing N-terminal mutant htt fragments.

The accumulation of more htt fragments in the striatum suggests that striatal neurons may have a decreased capacity to remove toxic htt fragments. As the UPS plays a critical role in clearing polyQ proteins, we compared 20S enzymatic activity (chymotrypsin-like and trypsin-like activity) in the cortex and striatum of WT and KI mice and found there is an age-dependent decrease in proteasome activity (Figure 3-2). We did not observe any significant differences in proteasomal activities between WT and KI mouse brains. Importantly, both chymotrypsin-like and trypsin-like activity is significantly lower in striatal tissue than in cortical tissue from WT or KI mouse brains (Figure 3-2).

However, biochemical assay of brain homogenates derived from mixed populations of cells cannot define UPS activity in neurons. We, therefore, cultured primary neurons from the rat striatum and cortex in order to monitor UPS activity by co-infecting Ad-GFPu and Ad-RFP. Ad-GFPu caries the sequence for UPS-mediated degradation, while Ad-RFP lacks the degradation sequence thus serving as an internal control for viral expression. The cultured striatal and cortical neurons allowed us to simultaneously measure UPS activity in different cell types under the same temporal and culture conditions. Using these proteasome reporters, we found that striatal neurons display decreased UPS activity as compared with cortical neurons (Figure 3-3, a). Quantitative analysis of the ratio of GFPu to RFP verified that striatal neurons show increased GFPu/RFP (1.18 ± 0.09m mean $\pm$ SE, n= 13) compared with cortical neurons (0.59 ± 0.02 mean $\pm$ SE, n=13, $P<0.001$) (Figure 3-3, b). As the increased GFPu/RFP ratio reflects decreased proteasomal activity, these findings suggest that the accumulation of N-terminal htt fragments is determined by cell type-dependent clearance of mutant htt.

The cell-type dependent UPS activity found in striatal and cortical neurons also promoted us to investigate potential differences of UPS activities in neuronal and glial cells. Mutant htt preferentially accumulates in neurons compared with astrocytes (Figure 3-4, a). This phenomenon is evident from the abundant nuclear labeling of neuronal cells in the striatum by EM48, an antibody that preferentially reacts with mutant htt (Gutekunst et al., 1999; Lin et al., 2001). Fewer astrocytes in the white matter of the corpus callosum show EM48 staining. Since N-terminal mutant htt accumulates in the nucleus and forms aggregates (DiFiglia et al., 1997; Gutekunst et al., 1999; Wang et al 2008), we also examined the brains of R6/2 mice that express a small N-terminal (exon1) mutant htt

(Davies et al., 1997). Compared with astrocytes, neuronal cells clearly show more nuclear accumulation of mutant htt and htt aggregates, which are present in both the nuclei and processes (Figure 3-4, b). While the results also show that fewer astrocytes contain nuclear EM48 staining than neuronal cells, which is consistent with our early finding (Shin et al., 2005), comparison of HD KI and R6/2 mice indicates that N-terminal mutant htt accumulates much more readily in neurons than in astrocytes.

Since HD is a late-onset neurodegenerative disorder, astrocytes may be less vulnerable to the age-induced toxic insults compared to neurons. To examine the effects of aging on neurons and astrocytes, we used Ad-GFPu/DsRED, to examine the UPS activity of co-cultured neurons and astrocytes. The time-dependent decline of UPS activity has been noted in a variety of cultured cells (Zhou et al., 2003; Ding et al., 2006), offering us an opportunity to compare the changes in UPS function in cultured neurons and astrocytes. It is clear that neurons (arrows in Figure 3-5) show stronger GFPu signals than astrocytes and that the GFPu level in neuronal cells increases from day 16 to 20. In contrast, astrocytes do not have a significant change in GFPu expression from day 16 to 20.

To clarify further the differences between neurons and astrocytes, we cultured rat cerebral cortical neurons for different days *in vitro* (8, 11, 17 DIV), and then infected them with Ad-GFPu/RFP reporters for 2 days (Figure 3-6, a). As expected, older neuronal cultures displayed a greater number of cells with higher levels of GFPu. High-magnification micrographs indicated that both GFPu and RFP were expressed in the same neurons (Figure 3-6, b). Quantification of the ratio of GFPu to RFP confirmed the time-dependent increase of GFPu in old cultured neurons (Figure 3-7, a). The GFPu/RFP ratio at day 8 was 0.09690 ± 0.01104 (n=11) and significantly increased at day 17 to 0.6948 ±

0.01855 (n=12, $p<0.001$). To verify this fluorescent result, we performed Western blotting and found that GFPu is indeed increased over culturing time, which reflects a decrease in proteasome activity (Figure 3-7, b).

We also isolated astrocytes, the major type of glial cells, from rat cerebral cortex and cultured them for various days *in vitro* (4-20 DIV). Infection of these astrocytes with adenoviral GFPu/RFP reporters yielded a high level of RFP. However, the level of GFPu remained low in astrocytes that had been cultured for different days (Figure 3-8, a). Quantification of the GFPu/RFP ratios in neurons and astrocytes illustrated that there was a slight increase in old cultured astrocytes but a significant increase with time in old cultured neurons ($p<0.01$) (Figure 3-8,b). To verify the results obtained with the fluorescent reporters, we performed biochemical assays to measure the proteasomal chymotrypsin-like and trypsin-like activities in lysates of cultured neurons and astrocytes using specific fluorogenic substrates. As expected, the proteasomal chymotrypsin-like and trypsin-like activities are higher in cultured astrocytes than in neurons (Figure 3-9). In contrast to neurons, cultured astrocytes do not show a significant decrease in proteasomal activity, even after being cultured for 20 days. However, chymotrypsin-like activity in neurons at day 4 (0.9500 ± 0.01414, n=4) was significantly reduced at day 12 (0.3406 ± 0.01511, n=4, $p<0.001$). Similarly, trypsin-like activity in cultured neurons was also decreased from day 4 (2.293 ± 0.01791, n=4) to day 12 (0.8995 ± 0.006551, n=4, $p<0.001$). The differences in UPS activities in cultured neurons versus astrocytes may reflect the intrinsic variation in UPS activities in different cell types.

The fact that mutant htt preferentially accumulates in the nuclei of neurons and astrocytes in HD mouse brains (Shin et al., 2005; Chou et al., 2008; Wang et al., 2008) also

prompted us to use the GFPu/RFP reporters to examine the UPS activity in the nuclei and cytoplasm of cultured cells. To do so, we tagged GFPu/RFP with the nuclear localization sequences (NLS) or nuclear export sequences (NES). Thus, NLS-GFPu/RFP is directed to the nucleus to measure nuclear UPS activity, whereas NES-GFPu/RFP remains in the cytoplasm to reflect cytoplasmic UPS activity.

We then expressed these reporters in cultured cortical neurons and astrocytes. Nuclear NLS-GFPu signal was more intense than cytoplasmic NES-GFPu in cultured neurons (Figure 3-10, a). Similarly, cultured astrocytes also show more intense NLS-GFPu signal in the nucleus than NES-GFPu signal in the cytoplasm (Figure 3-10, b). Quantification of the GFPu/RFP ratio also confirmed that there is more accumulation of NLS-GFPu in the nucleus than NES-GFPu in the cytoplasm of astrocytes and neurons (Figure 3-11). These findings are in agreement with the biochemical results from our previous study showing a lower level of proteasomal activity in the nuclear fraction than in the cytoplasmic fraction of mouse brain tissues (Zhou et al., 2003).

3.5 Discussion

The UPS has two essential functions in eukaryotic cells. It maintains normal cellular function through regulated destruction of critical regulatory proteins such as transcription factors and protein kinases (Hershko et al., 1998). It also recognizes and degrades misfolded and damaged proteins to protect cells against potentially toxic effects of protein aggregation. The latter function of the UPS is of interest in the present study.

It has been shown that loss-of-function mutations in genes encoding UPS components can cause neurodegenerative diseases in humans (Mizuno et al., 2001) and rodents (He et al., 2003) as well as enhancing the cytotoxicity of aggregation-prone proteins linked to dominantly inherited neurodegenerative diseases (Cummings et al., 1999). Others have reported that production or accumulation of intracellular protein aggregates in cells profoundly impairs the functional capacity of the UPS (Bence et al., 2001; Jana et al., 2001; Verhoef et al., 2002). The mechanism by which protein aggregation is linked to UPS impairment remains unclear and thus is the focus of the present study.

We have found that striatal brain homogenates have less chymotrypsin-like and trypsin-like activity compared to cortical brain homogenates. Both striatal and cortical brain UPS activity steadily decreases as the animal ages, but the striatum continuously has lower UPS activity compared to the cortex. However, we did not find any significant difference in UPS activity between WT and HD mouse brains. Rather, it was the effect of aging that created the decreased UPS activity. This finding allowed us to hypothesize that it is age-dependent decline in UPS activity that allows for the progressive and age-dependent accumulation of misfolded polyQ proteins and formation of intranuclear

inclusions (NII).

To further test this hypothesis, we cultured neurons and astrocytes, the major type of glial cells in the brain. Numerous studies on HD have illustrated that NII occur in neurons and relatively few NII are present in astrocytes (Shin et al., 2003; Wang et al., 2008). Due to this fact, it would be of interest to compare the UPS in neurons and astrocytes. Since HD is a late-onset neurodegenerative disorder, astrocytes may be more tolerant of age-induced alterations in UPS activity than neurons. Understanding how neurons and astrocytes degrade mutant polyQ proteins through the UPS is important for elucidating the selective neuropathology in polyQ disorders, as well as developing effective treatments.

Proteasome biochemical assays and the proteasome reporter, GFPu, showed that the UPS in neurons significantly decrease with respect to culturing time. Unlike neurons, astrocytes do not have a significant decrease in UPS activity over time. In order to assess the effect of nuclear and cytoplasmic localized protein aggregation on the UPS, we constructed compartment-specific variants of GFPu. GFPu and RFP constructs were tagged with either a nuclear localizing sequence (NLS) or a nuclear exporting sequence (NES) and transfected into neurons and astrocytes. In both neurons and astrocytes, there was significantly less UPS activity in the nucleus compared to the cytoplasm. This finding is correlated with the preferential accumulation of polyQ proteins in the nucleus and suggests that higher cytoplasmic UPS activity may prevent fewer accumulations of misfolded proteins and subsequent aggregate formation in the cytoplasm of neurons. Taken together, studies presented in this chapter suggest that cell-type and subcellular dependent UPS activities contribute to the preferential accumulation of misfolded polyQ

proteins in neurons and in their nuclei. This idea is further tested using HD mouse models in the studies presented in the following chapters.

CHAPTER 4

UPS activity in neurons and astrocytes in HD knock-in mice brains

This chapter presents work to be published as: Suzanne Tydlacka, Chuan-En Wang, Xuejun Wang, Shi-Hua Li and Xiao-Jiang Li (2008) *J. Neuroscience,* In press. Suzanne Tydlacka performed all the experiments described in this chapter. Xiao-Jiang Li and Shi-Hua Li aided in the design of experiments and interpretation of results. Xiao-Jiang Li aided in the writing of the results section and in editing the manuscript.

4.1 Abstract

A variety of neurodegenerative disorders and polyglutamine (polyQ) diseases are caused by misfolded proteins. The common feature of these diseases is late-onset cellular degeneration that selectively affects neurons in distinct brain regions. PolyQ diseases, including Huntington's disease (HD), present a clear case of selective neurodegeneration caused by polyQ expansion-induced protein misfolding, which also leads to predominant inclusions in neuronal nuclei. It remains unclear how these ubiquitously expressed disease proteins selectively kill neurons. In HD, mutant huntingtin accumulates in both neurons and astrocytes, but more neurons display huntingtin aggregates. These aggregates colocalize with components of the ubiquitin-proteasome system (UPS), which plays a critical role in clearing misfolded proteins (DiFiglia et al., 1997; Ding et al., 2002). By expressing the UPS reporter, GFPu, in astrocytes and neurons in the mouse brain, we observed an age-dependent decrease in UPS activity, which is more pronounced in neurons than astrocytes. Although brain UPS activities were similar between wild-type (WT) and HD 150Q knock-in mice (KI), inhibiting the UPS markedly increases the accumulation of mutant huntingtin in cultured astrocytes. These findings suggest that the lower neuronal UPS activity may account for the preferential accumulation of misfolded proteins in neurons, as well as their selective vulnerability.

4.2 Introduction

In Huntington's disease (HD), selective neurodegeneration preferentially occurs in the striatum and extends to various brain regions as the disease progresses (Vonsattel et al., 1985; Martin and Gusella, 1986). HD is caused by the expansion of a polyglutamine (polyQ) tract in the N-terminal region of huntingtin (htt) (Landles and Bates, 2004; Gusella and Macdonald, 2006), a large 350 kDa protein that is ubiquitously expressed and interacts with a number of proteins (Li and Li 2004). Like other polyQ disease proteins, mutant htt also induces selective neurodegeneration. Understanding the mechanism underlying this selective neurodegeneration will help elucidate the pathogeneses of polyQ diseases and other neurological disorders, such as Alzheimer's and Parkinson's diseases, which also show the selective accumulation of toxic proteins in neurons.

In the brain, glia make up the major population (>90%) of cells and provide neurons with nutrients, growth factors, and other support. Although mutant htt is also expressed in astrocytes, the major cell type of glia (Shin et al., 2005; Chou et al., 2008; Wang et al., 2008), significantly more neurons than astrocytes contain htt aggregates (Shin et al., 2005). As such, neuronal htt toxicity in HD has been better characterized than glial pathology (Li and Li, 2006).

Since neurons are postmitotic cells, their ability to cope with misfolded proteins may be different from that of other cell types, such as astrocytes, which can proliferate and regenerate (Barres and Barde, 2000). Despite the critical role the UPS plays in clearing misfolded proteins in different cell types, little is known about potential differences in UPS activity in neurons versus astrocytes in the brain. Addressing this issue could help explain the mechanisms behind the selective neuropathology in a variety of neurodegenerative disorders that are caused by misfolded proteins. In this study, we focus on UPS activity in neurons and astrocytes in both a WT and in an

HD 150Q knock-in (KI) mouse model of Huntington's disease. We demonstrate that UPS activity decreases in an age-dependent manner and is lower in neurons than astrocytes. However, inhibiting the UPS can increase the accumulation of mutant huntingtin in astrocytes. Our findings suggest that the intrinsically lower UPS activity in neurons is a major contributor to the preferential accumulation of misfolded proteins in neurons seen in various neurodegenerative diseases.

4.3 Materials and Methods

Animals. *Hdh*(CAG)150 knock-in (HD150Q KI) mice were generated previously and maintained on the SV129/B6 background in the animal facility at Emory University in accordance with institutional guidelines. Genomic DNA was extracted from tails to genotype the mice as WT (7Q/7Q) and KI (150Q/150Q).

Antibodies and Reagents. Antibodies against N-terminal human htt (EM48) were generated previously (. Antibodies against an expanded polyQ tract (1C2, 1:5000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:3000were obtained from Millipore (Temecula, CA). Other antibodies used are against the following proteins: γ-tubulin (Sigma, St. Louis, MO; 1:10,000), RFP and GFP (Clontech), NeuN (Millipore), GFAP (Millipore), and ubiquitin (Cell Signaling, Santa Cruz Biotechnology, 1:500). Secondary antibodies were peroxidase-conjugated donkey anti-mouse, -rabbit, -rat, -guinea pig, -goat, or -sheep IgG (H+L) from Jackson Immunoresearch (West Grove, PA; all used at 1:5000).

Adenoviral Vector Construction and Preparation. A CL-1 degron sequence (Bence et al., 2001) was added to the C-terminus of GFP in the PRK vector to generate PRK-GFPu construct. cDNA sequences for red fluorescent protein (RFP) were used to replace GFPu to generate PRK-RFP construct. Generation and purification of adenoviral GFPu and RFP were performed according to the methods used in our previous studies (Dong et al., 2004; Shin et al., 2005). Viral titer was determined by measuring the number of infected HEK293 cells expressing GFPu or RFP. All viral stocks were adjusted to $1x10^{10}$ VP/ml.

Stereotaxic Injection. Experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Emory University Institutional Animal Care and Use Committee. Briefly, mice were anesthetized with intraperitoneal injection of Avertin (250mg/Kg) and positioned in a stereotaxic apparatus. A small incision was made in the scalp, and adenoviral GFPu/RFP at a ratio of 1:1 was injected into different mouse brain regions using stereotaxic coordinates relative to Bregma. Coordinates for the white matter in the corpus callosum were 0.7 mm anterior/posterior, 1.0 mm medial/lateral, and 1.2 mm dorsal/ventral. Coordinates for the cerebral cortex were 0.7 mm anterior/posterior, 1.0 mm medial/lateral, and 0.8 mm dorsal/ventral. Coordinates for the striatum were 0.7 mm anterior/posterior, 1.8 mm medial/lateral, and 2.5 mm dorsal/ventral. A small hole was drilled into the skull, and a 26-gauge needle attached to a 5-μl Hamilton syringe was lowered into the corpus callosum, cerebral cortex, or striatum according to the dorsal/ventral coordinates. A nanoinjector pump (World Precision Instruments) controlled the infusion of 2.0 μl of adenovirus at a rate of 0.4 μl/min, after which the needle was left in place for 5 min to ensure complete diffusion of the viruses. Mice brains were examined using a fluorescent scope at 7 days postinjection.

Proteasome activity assay. For determining proteasome activity, clear whole-cell extracts of neuronal or glial cells were adjusted to 0.5 mg/ml total protein by dilution with homogenization buffer. All assays were done in triplicate and the results were obtained from 4-8 experiments. The chymotrypsin-like activity of 20S β5 was determined using the substrate Suc-LLVY-aminomethylcoumarin (AMC) (Bilmol; 40 μM), and the trypsin-like activity of 20S β2 was determined using the substrate Boc-LRR-AMC (Bilmol; 100 μM).

Equal amounts (10 µg) of the extracts were incubated with corresponding substrates in 100 µl of proteasome activity assay buffer [0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA] for 60 min at 37°C. The reactions were stopped by adding 0.8 ml of cold water and placing the reaction mixtures on ice protected from light exposure for at least 10 min. The free AMC fluorescence was quantified by using the CytoFluor multi-well plate reader (FLUOstar, BMG) with excitation and emission wavelengths at 380 and 460 nm, respectively. All readings were standardized using the fluorescence intensity of an equal volume of free 7-amino-4-methyl-coumarin (AMC) solution (40 mM), normalized by the protein concentrations and expressed as nmol/min/mg protein.

Quantitative Fluorescence Imaging Analysis. Quantitative analysis of captured fluorescent images was performed as described previously (Wang et al., 2008b). Briefly, precise segmentation of bright spots was achieved by setting a lower threshold just above background emission, typically ~23 to 25 gray scale units (gsu), and an upper threshold at the upper limit of the 8-bit gray scale (i.e., 255 gsu). Cell bodies were detected as objects around the nuclei, separated from the neurites by a watershed filter. It starts by running a "Hi-Pass" filter across the image to enhance edges (replaces each pixel with a value that increases contrast with neighboring pixels) . Cell bodies (7-9/each group) are selected as any objects with a size and signal intensity greater than a user-defined minimum that is above a defined background. We used the Openlab software, which generated a high-content array of measurements that included event number, area, average sum of pixel intensities, and maximum and minimum pixel intensities. The average sum of pixel intensities is selected as the parameter for statistical analysis.

Statistical Analysis. Data are expressed as mean ± SEM and were analyzed for statistical significance ($p < 0.05$) with Prism software (version 4, GraphPad Software, San Diego, CA).

4.4 Results

To test whether UPS activity is different in neurons and astrocytes of the brain, we performed stereotaxic injection of adenoviral GFPu/RFP into the neuronal regions (cortex and striatum) and the corpus callosum, which is enriched in astrocytes, the most predominant type of glial cell in the brain. Seven days postinjection, we sectioned the mouse brains and examined GFPu/RFP signals in the injected areas using fluorescent microscopy. Using immunofluorescent double labeling, we verified that stereotaxic injection allowed the transgene to be expressed in neurons and astrocytes (Figure 4-1 a, b, & c). To determine the relationship between aging and UPS activity in the mouse brains, we injected the UPS reporters into the brains of mice at 4, 12, and 24 months of age (Figure 4-2, a). We found that GFPu signals are much weaker than RFP signals in the injected region, which is consistent with an earlier finding that the level of a transgenic fluorescent UPS reporter is barely detectable in mouse brains, perhaps because of its short half-life (Lindsten et al., 2003). Low-magnification micrographs (100X) demonstrate that GFPu signal is slightly higher in the striatum than in the astrocyte-enriched corpus callosum of young mice at 4 months of age (Figure 4-2, a). Importantly, the GFPu signal in the striatum is increased more drastically than in the corpus callosum in 1 and 2 year old mouse brains. In the brain, neuronal nuclei are larger than astrocytic nuclei, and this difference can be viewed by Hoechst dye staining. High-magnification micrographs clearly show that GFPu signal is increased to a greater extent in the neurons of 12-month-old mice compared with astrocytes (Figure 4-2, b).

Quantitative analysis of the ratio of GFPu to RFP verified that striatal neurons have a significantly higher GFPu/RFP ratio than cortical neurons and astrocytes (Figure 4-3),

suggesting that the lowest UPS activity is in striatal neurons. Previous studies using biochemical assays also show lower proteasome activity in the striatum than in the cortex of rat and mouse brain homogenates (Zeng et al., 2005; Wang et al., 2008). Importantly, UPS activity is higher in astrocytes than neurons, as the astrocytic GFP/RFP ratio in the corpus callosum is lower than the neuronal GFPu/RFP ratios in the striatum and cortex (Figure 4-3). Furthermore, the age-dependent increase in GFPu is also greater in neurons than astrocytes.

To examine whether UPS activity in astrocytes and neurons differs between HD and wild-type mice, we also injected adenoviral GFPu/RFP reporters into the striatum and corpus callosum of WT and KI mice at different ages. Quantification of the GFPu/RFP ratio in the corpus callosum did not reveal any significant difference in astrocytes between WT and KI mice at 2, 12 or 24 months of age (Figure 4-4). However, the neuronal GFPu/RFP ratio in the striatal region is slightly higher in KI than in WT mice at 12 months of age, but the difference is not statistically significant. These results are consistent with the *in vitro* finding that neuronal GFPu/RFP ratios are higher than astrocytic GFPu/RFP ratios (Fig. 3-8, b), again suggesting that neuronal UPS activity is lower than astrocytic UPS activity.

The above results also indicate that there is an age-dependent decrease in UPS function in neurons and astrocytes in the brain. If the decreased UPS activity is associated with increased htt aggregation in astrocytes, as well, we should see a greater accumulation of mutant htt in cultured astrocytes after inhibiting the proteasome. Thus, we cultured astrocytes from KI mice and treated them with MG132 (10μM). The inhibition of UPS activity by MG132 markedly increased the accumulation of mutant htt and its degraded

products in cultured astrocytes (Figure 4-5), suggesting that UPS function is important for preventing the accumulation of mutant htt. Taken together, although KI cells do not show significantly different UPS activity compared with WT cells, UPS activity is often higher in astrocytes than neurons; however, suppressing UPS function can increase the accumulation of mutant htt in astrocytes.

4.5 Discussion

Impairment in the UPS in polyQ diseases has long been proposed to play a role in neurodegeneration. For example, isolated ubiquitylated filamentous huntingtin aggregates, extracted from inclusion bodies selectively inhibit the i*n vitro* peptidase activity of the 26S proteasome (Díaz-Hernández et al., 2006). Additionally aggregates produced from expanded polyQ repeats inhibit proteasome degradation (Verhoef et al., 2002). Although there are excellent mouse models for polyQ diseases, assessment of the involvement of the UPS and analysis of its activity *in vivo* remains a major challenge. Only one transgenic mouse expressing the reporter Ub^{G76V}GFP was created in an attempt to monitor UPS activity *in vivo* (Lindsten et al., 2003). In theory, this would have been an ideal mouse model to study UPS activity in brain tissue; however, Ub^{G76V}GFP failed to be adequately expressed in neurons after treatment with the selective proteasome inhibitor, epoxomicin.

In light of this study, we chose to sterotaxically inject the proteasome reporter, GFPu, into the brains of WT and HD150Q knock-in (KI) mice. This method is more invasive than the Ub^{G76V}GFP transgenic mice but it is the only method presently available to monitor UPS activity *in vivo.* As such, we injected Ad-GFPu and Ad-RFP into the cortex, striatum and corpus callosum (an area enriched in astrocytes) of WT and KI mice. This method allowed us to study UPS activity in neurons and astrocytes of WT and KI mice at 4, 12, and 24 months of age. We found that UPS activity was lower in neurons and rapidly declined as the age of the mouse increased. However, UPS activity in astrocytes slowly declined with age but was significantly higher than that in neurons. Interestingly, there was not a significant effect on UPS activity caused by the presence of mutant htt. This would suggest that age-induced decline of neuronal UPS activity is more debilitating

in neurons than in astrocytes. This could explain why neurons have the highest percentage of NII. The number of NII increases as a function of age whereas the activity of UPS decreases. Aging decreases UPS activity in both neurons and astrocytes but neurons appear to be more susceptible to the age-induced toxic insults on UPS and thus accumulate more NII.

In conjunction with the results from cultured cells, which are presented in other Chapters, our results are the first to suggest that there is a cell-type specific difference in age-induced susceptibility to UPS dysfunction. The present study suggests that astrocytes are more tolerant to aging-induced decline of UPS activity than neurons. Aging can induce insults including oxidative stress, mitochondrial dysfunction, altered expression of proteasomal subunits, and DNA damage. These age-dependent changes can impair UPS function. Although the mechanism underlying age-dependent decline in UPS activity remains to be investigated, our findings suggest that improving UPS function can be beneficial for the treatments of HD as well as other diseases caused by misfolded proteins.

CHAPTER 5

Ubiquitin-Proteasome Activity in Neuronal Synapses

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5.1 Abstract

Huntington's disease (HD) is pathologically well characterized by its selective neuronal degeneration, accumulation of aggregates as well as intranuclear (NI) and cytoplasmic inclusions (CI) (DiFiglia et al., 1997; Wang et al., 2008; Gutekunst et al., 1999). The cytoplasmic mutant huntingtin is localized in synapses and can disregulate synaptic function, axonal trafficking, and induce synaptic toxicity (Trushina et al., 2004). Presently, the underlying mechanism of synaptic toxicity caused by the mutant huntingtin is not well defined. Although we have not found a significant difference in UPS activity in total cell lysates from wild type and HD mouse brain tissues, it remains to be investigated whether there is altered synaptic UPS activity in HD. Thus, we targeted the proteasome reporter, GFPu, to presynaptic or postsynaptic terminals HD150Q knock-in (KI) mice. We found that synaptic UPS also decreased in older mice. In addition, the presence of mutant huntingtin is associated with a greater reduction in synaptic UPS activity in old mice. Our findings offer new insight into selective neuronal UPS dysfunction in the synapse of KI mice. This finding could potentially help elucidate the underlying mechanism of synaptic toxicity seen in HD.

5.2 Introduction

The ubiquitin-proteasome system (UPS) plays an essential role in degrading damaged or misfolded proteins (Hershko and Ciechanover, 1998). Recently, the UPS has been examined as a regulator of synaptic plasticity, proper synaptic connectivity during development, growth cones guidance as well as presynaptic and postsynaptic functions (Patrick 2006). In the presynaptic terminal, the UPS regulates presynaptic function through multi-ubiquitination and protein turnover, thereby altering protein activity and vesicle dynamics (Chen et al., 2003). For example, the Ataxia mouse, which displays spontaneous tremors, is defective in the ubiquitin-specific protease Usp14 (Wilson et al., 2002). Usp14 is thought to play a role in the recycling of ubiquitin from multiubiquitinated proteins, thereby maintaining cellular levels of free ubiquitin. Ataxia mice have a 53% decrease in quantal content and hippocampal long-term plasticity which are characteristic of presynaptic deficit and underscore the importance for ubiquitin recycling. In the postsynaptic terminal, the major scaffolding protein (PSD-95) is regulated by the UPS. PSD-95 tethers NMDA- and AMPA-type glutamate receptors to signaling proteins and neuronal cytoskeleton. When NMDA receptors are activated, PSD-95 is ubiquitinated by the E3 ligase Mdm2 and removed from the synapse in a UPS-dependent manner (Colledge et al., 2003).

Recently, increasing evidence has suggested that slight alterations in synaptic function in HD could underlie early symptoms (Smith et al., 2005). It has been found that mice expressing 80 glutamine repeats have reduced synaptic plasticity and impairment of LTP induction (Usdin et al., 1999). Additionally, it is well documented that huntingtin interacts with various cytoskeletal and synaptic vesicle proteins that are essential for

exocytosis and endocytosis. For example, huntingtin interacts with HAP1, which is involved in vesicle trafficking, and HIP1, which is involved in clathrin-mediated endocytosis (Li et al., 2003; Kalchman et al., 1997). Altered interactions of mutant huntingtin with its associated proteins could contribute to abnormal synaptic transmission in HD. For example, proteins that interact with mutant huntingtin could be recruited into inclusions. Under this circumstance, protein levels could be reduced locally at their site of action, even if the total amount of proteins within the cell remains normal. Also, mutant huntingtin could affect the function of a given protein and its normal interaction partners by changing the binding affinity without affecting their expression levels, phosphorylation states, or sequestering them in aggregates. As such, it is of particular interest to elucidate the effect of mutant huntingtin on the UPS in synaptic terminals.

To specifically measure UPS activity in the synapse of wild-type (WT) and HD150Q knock-in (KI) mice, the GFPu proteasome reporter was fused to either SNAP25 or PSD95. SNAP25 is a presynaptic protein and shuttles GFPu to the presynaptic terminal, and PSD95 is a postsynaptic protein and targets GFPu to the postsynaptic terminal. Similar to our previous findings, we found that UPS activity in the synapse of KI mice decreases with age. Additionally, the expression of mutant htt causes increased reduction of synaptic UPS activity in old KI mice. The present findings suggest that the presence of mutant huntingtin in the synapses has a negative effect on UPS function in older mice; however, the primary regulator of UPS activity is age.

5.3 Materials and Methods

HD Mice

HD 150CAG repeat knock-in mice (HdhCAG150) expressing full-length mouse htt with an expanded polyglutamine repeat (150Q) (Lin et al., 2001) were bred and maintained in the animal facility at Emory University as described previously (Yu et al., 2003). Genotyping of transgenic mice was performed using methods described previously (Yu et al., 2003). Heterozygous HD150CAG knock-in mice were used in the study.

Viral Vector Construction and Preparation

A CL-1 degron sequence (Bence et al., 2001) was added to the C terminus of GFP in the PRK vector to generate PRK-GFPu construct. cDNA sequences for DsRed (BD Biosciences) were used to replace GFPu to generate PRK-RFP construct. RT-PCR using RNAs from mouse brains were performed to isolate full-length cDNAs for mouse SNAP25 and PSD95. SNAP25 cDNA was isolated with a sense primer 5′-acatcgatATGGCCGAAGACGCAGACATG-3′ and an antisense primer 5′-acacgcgtACCACTTCCCAGCATCTTTG-3′. Full length of PSD95 cDNA was isolated using the sense primer 5′-acatcgatCCAACATGGACTGTCTCTGTATAG-3′ and the antisense primer 5′-atacgcgtGAGTCTCTCTCGGGCTGGGAC-3′. The PCR products were subcloned into the PRK-GFPu and PRK-RFP vectors to express fusion proteins that contain GFPu or RFP at the C terminus of SNAP25 or PSD95. The DNA fragments encoding these fusion proteins were inserted into the shuttle vector of the AdEasy vector system (Qbiogene). The CMV promoter was replaced by human synapsin-1 promoter provided by Dr. S. Kugler (University of Goettingen, Goettingen, Germany). Adenovirus

amplification and purification were performed according to the method used in our previous study (Shin et al., 2005). Viral titer was determined by measuring the number of infected HEK293 cells expressing GFPu or RFP. All viral stocks were adjusted to 10^9 VP/ml.

Sterotaxic Injection

Fifty HD mice described above and their wild-type littermates were used for this study. All experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of LaboratoryAnimals and the Emory University Institutional Animal Care and Use Committee. In brief, mice were anesthetized with intraperitoneal injections of a combination of ketamine (90 mg/kg) and xylazine (13 mg/kg) and positionedin a stereotaxic apparatus. A small incision was made in the scalp, and the striatum was identified using the following stereotaxic coordinates, relative to Bregma: anterior – posterior +0.8 mm, medial - lateral -1.6 mm, and dorsal - ventral - 3.3 mm. A small hole was drilled into the skull, and a 26-gauge needle attached to a 5- µl Hamilton syringe was lowered into the striatum according to the dorsal – ventral coordinate. A nanoinjector pump (World Precision Instrument) controlled the infusion of 2.0 µl of adenovirus at a rate of 0.4 µl/min, after which the needle was left in place for 5 min to ensure complete diffusion of the viruses. The vectors were localized by immunocytochemistry 7 – 8 days after injection.

Fractionation

Subcellular fractions of mouse brain tissues were prepared using previously

described methods (Phillips et al., 2001). Cortical or striatal tissues from R6/2 or KI mice and control littermates were homogenized in 500 µl homogenization solution (0.32 M sucrose, 15 mM Tris-HCl pH 8.0, 60 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, and 2 mM ATP) at 4°C in an Eppendorf tube. The homogenate was centrifuged at 500 *g* for 2 min to remove unbroken tissue clumps and cells. The supernatant (S1) was centrifuged at 1,300 *g* for 10 min to precipitate nuclei fraction (P1). The (S1) supernatant was transferred to another microfuge tube and centrifuged at 10,000 *g* for 10 min to obtain a mitochondria- and synaptosome-enriched pellet (P2) and the supernatant (S2). The (P2) pellet was resuspended in 500 µl of 0.32 M sucrose and layered onto 750 µl of 0.8 M sucrose in a microfuge tube. The samples were centrifuged at 9,100 *g* for 15 min using a swinging bucket rotor. The 0.8-M sucrose layer and most of the loose pellet containing the synaptosomes were collected and separated from the mitochondrial pellet. The (S2) supernatant was centrifuged at 13,000 *g* for 25 min to yield the supernatant (S3) or cytosolic fraction.

Proteasome Activity Assay

For determining proteasome activity, clear whole-cell extracts or cell fractions were adjusted to 0.5 mg/ml total protein by dilution with homogenization buffer. All assays were done in triplicate. Chymotrypsin-like activity of 20S β5 was determined using the substrate Suc-LLVY-aminomethylcoumarin (AMC) (40 µM; Bilmol), trypsin-like activity of 20S β2 was determined using the substrate Boc-LRR-AMC (Bilmol; 100 µM), and postglutamyl activity of 20S β1 was determined using the substrate Z-LLE-AMC (400 µM; Bilmol). Equal amounts (10 µg) of the extracts were incubated with corresponding substrates in 100

μl proteasome activity assay buffer (0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM ATP, and 1 mM DTT) for 30–60 min at 37°C. The reactions were stopped by adding 0.8 ml of cold water and placing the reaction mixtures on ice for at least 10 min. The free AMC fluorescence was quantified by using the CytoFluor multi-well plate reader (FLUOstar; BMG Labtech) with excitation and emission wavelengths at 380 and 460 nm, respectively. All readings were standardized using the fluorescence intensity of an equal volume of free 7-amino-4-methyl-coumarin (AMC) solution (40 mM), normalized by the protein concentrations and expressed as nmol/min/mg protein.

Quantitative Fluorescence Imaging Analysis

Captured image files were loaded into the Image-Pro Plus (Media Cybernetics, Inc.) environment with the "count/size" function. Precise segmentation of bright spots was achieved by setting a lower threshold just above background emission, typically ~23–25 grayscale units (gsu), and an upper threshold at the upper limit of the 8-bit grayscale (i.e., 255 gsu). Automated image analysis was performed with a custom-built algorithm. The algorithm performs a series of analyses on each image in both the red channel (for RFP) and the green channel (for GFPu) and then calculates quantitative output for fluorescence intensity. The algorithm first corrects background if necessary and attempts to offset background anomalies. Cell bodies were detected as objects around the nuclei, separated from the neurites by a watershed filter. The next step in the algorithm locates synapses. It starts by running a "Hi-Pass" filter across the image to enhance edges (replaces each pixel with a value that increases contrast with neighboring pixels) (Mitchell et al., 2007). Synapses (40–75/each group) and cell bodies (7–9/each group) are selected as any objects

with a size and signal intensity greater than a user-defined minimum that is above a defined background. Image-Pro Plus generated a high-content array of measurements that included event number, area, sum of pixel intensities, and maximum and minimum pixel intensities. The sum of pixel intensities is selected as the parameter for statistical analysis.

Statistical Analysis

Statistical significance ($P < 0.05$) was assessed using the *t* test whenever two groups were compared. When analyzing multiple groups, we used ANOVA with Scheffé's post hoc test to determine statistical significance. Data are presented as mean + SE. Calculations were performed with SigmaPlot 4.11 and Prism (version 4) software.

5.4 Results

Having established the specificity of the fluorescent reporters to reflect synaptic UPS activity (Figure 2-7 and 2-8), we examined synaptic UPS activity in HD150Q knock-in (KI) mice in which the full-length mutant huntingtin is expressed under the control of the endogenous mouse huntingtin promoter and is cleaved to small N-terminal huntingtin fragments. In these mice, N-terminal mutant huntingtin fragments preferentially form aggregates or inclusions in the striatum (Lin et al., 2001). We used 1C2 immunostaining to reveal neuropil aggregates in KI mice (Figure. 5-1, a). Neuropil aggregates are less abundant than nuclear aggregates in KI mice, suggesting that the accumulation of cleaved N-terminal huntingtin fragments in distal neuronal processes requires more time, perhaps because transport of mutant huntingtin to nerve terminals is necessary for this accumulation. However, there is a significant increase of neuropil aggregates from 4 to 12 months (Figure. 5-1, a), allowing us to examine whether there is a correlated change in synaptic UPS activity.

After injection of adenoviral UPS reporters into the striatum of KI mice aged 4, 8, and 12 months, we observed a relative increase in presynaptic and postsynaptic GFPu signals in older mice (Figure. 5-1, b and c). Quantification of the ratio of GFPu/RFP in the synapses of HD mice at different ages also showed a time-dependent increase of this ratio, which likely reflects a decrease in synaptic UPS activity (Figure. 5-2, a and b). To confirm this increase, we used a biochemical assay to examine the chymotrypsin activity of isolated synaptosomes from the cortex and striatum of KI and age-matched wild-type (WT) mice (Figure 5-3). Examining proteasomal activity in synaptosomes did not reveal significant differences between WT and KI mice, except for decreased activity in the striatum of KI

mice at 12 months of age.

Although the biochemical assay for proteasomal activity is less sensitive than the fluorescent reporter at detecting changes in the proteasomal activity in subcellular regions, this assay indicated that cortical synaptosomes from KI mice at 12 months of age had decreased proteasomal activity compared with samples from control littermates. However, the difference in cortical proteasomal activity is not statistically significant. Importantly, a statistically significant decrease in proteasomal activity was seen in synaptosomes from striatal neurons of KI mice and WT littermates at 12 months of age. Thus, synaptic proteasomal activity is inversely correlated with the age-dependent accumulation of N-terminal mutant huntingtin fragments in striatal neurons (Lin et al., 2001).

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5.5 Discussion

We generated adenoviral fluorescent reporters to measure synaptic UPS activity and observed a significant decrease in synaptic UPS activity in KI mice with respect to age. These findings offer important insight into the synaptic dysfunction and selective neuropathology seen in HD. Furthermore, these new reporters can serve as a tool for measuring the function of the synaptic UPS and its regulation under normal physiological or pathological conditions.

Cell models and *in vitro* studies have shown that polyQ-containing proteins can impair the activity of the UPS (Bence et al., 2001; Verhoef et al., 2002; Venkatramen et al., 2004; Bennett et al., 2005). These studies provided valuable information regarding the mechanisms by which polyQ proteins affect UPS function. Early studies suggested that protein aggregation directly impairs the function of the UPS (Bence et al., 2001). It was also shown that the proteasome cannot digest and release polyQ-expanded peptides, which can impair the function of the proteasome (Venkatraman et al., 2004). Recent studies demonstrate that misfolded polyQ proteins can directly affect the function of proteasomes before the formation of inclusions (Bennett et al., 2005; Diaz-Hernandez et al., 2006). Although these studies demonstrate that the mutant huntingtin can directly impair UPS function, they were performed in conditions where the levels of mutant huntingtin exceeded the endogenous level of mutant protein in HD. Thus an important issue is whether the UPS impairment also occurs *in vivo*.

A transgenic approach was used to express a fluorescent UPS reporter ($Ub^{G76V}GFP$) in mice (Lindsten et al., 2003). With this UPS reporter mouse model, it was shown that prion pathology causes impairment of the UPS in the brain (Kristiansen et al., 2007). This

transgenic mouse model led to the discovery that mutant polyQ proteins do not impair UPS activity in the retina of transgenic SCA7 mice (Bowman et al., 2005). In addition, biochemical assays on brain homogenates from HD mice that express exon1 mutant huntingtin did not reveal a reduction in proteasomal activity or the levels of proteasomal subunits LMP2 and LMP7 in HD mice (Díaz-Hernández et al., 2003; Bett et al., 2006). Instead, increased chymotrypsin-like activity was seen in whole brain homogenates of 13-wk-old R6/2 mice (Bett et al., 2006) and in conditional HD (HD94) mice (Díaz-Hernández et al., 2003), which could be the indirect consequence of htt toxicity or cell stress. Nevertheless, measurement of polyubiquitin chains revealed global changes in polyubiquitination in HD mouse brains, suggesting the presence of impaired UPS activity in the HD brain (Bennett et al., 2007). It is possible that the effect of polyQ proteins on UPS activity is dependent on its accumulation and subcellular localization, which cannot be detected by examining whole cell homogenates. Because UPS activity varies in different types of cells and in subcellular regions, it is important to evaluate UPS activity in different subcellular compartments of neurons, especially in synapses in which mutant htt accumulates and affects neurotransmitter release or receptor function in the HD brain (Usdin et al., 2001; Cepeda et al., 2001; Zeron et al., 2002; Smith et al., 2005; Cummings et al., 2006).

Using adenoviral vectors that express synaptic fusion proteins containing fluorescent UPS reporters under the control of a neuronal promoter, we were able to target these reporters to presynaptic or postsynaptic terminals. Targeting of these reporters to defined synaptic structures allows for sensitive detection of changes in UPS activity at synapses. The selective localization of these reporters in small synaptic structures allows

for more reliable quantification of fluorescent signals than quantification of diffuse signals in the cell body. Accordingly, these reporters allowed us to detect for the first time synaptic UPS activity in the brain and also revealed decreased UPS activity in the synapses of KI mouse brains. This decrease was confirmed by biochemical assays of proteasomal activity in isolated synaptosomes. Thus, the reporters we generated are effective in detecting synaptic UPS activity. Since these reporters can differentiate presynaptic and postsynaptic UPS activity, they will be valuable for the investigation of how presynaptic or postsynaptic UPS function is regulated under physiological or pathological conditions.

Using cell homogenates or by expressing GFPu/RFP reporters in the cell bodies, we could not find any significant difference in UPS activity between HD and wild type mouse brains. However, by targeting GFPu/RFP to synapses and by isolating synaptosomal fractions, we uncovered the negative effect of mutant htt on synaptic UPS activity. These findings again suggest that subcellular localization should be taken into account when investigating potential changes in UPS activity.

CHAPTER 6

Conclusions and Future Directions

Written by Suzanne Tydlacka and edited by Xiao-Jiang Li.

6.1 Summary of key findings

Using fluorescent reporters that can detect cellular UPS activities, we found that brain UPS activity declines with age and that this decline correlates with the age-dependent accumulation of mutant htt and late-onset neuropathology. More importantly, we demonstrate for the first time that UPS activity is lower in neurons than in astrocytes in the brain. Because neuronal cells accumulate more mutant htt than glial cells, this finding suggests that decreased cellular UPS activity plays an important role in the accumulation of misfolded proteins. In support of this idea, we also found that striatal neurons have less UPS activity than cortical neurons, which is also consistent with the preferential accumulation of mutant htt in striatal neurons in HD mouse brains.

In addition to cell type-dependent difference in UPS activity, we found that UPS activity is dependent on subcellular localization. PolyQ proteins preferentially accumulate in the nucleus and nerve terminals or synapses. Consistently, nuclear and synaptic UPS activity is also lower than the cytoplasmic UPS activity in the cell body. All these findings suggest that clearance of misfolded proteins by UPS is critical for preventing selective neuropathology in HD.

6.2 Implications

The finding that UPS activity declines in an age-dependent manner has broad implications for age-dependent neurodegenerative diseases, as the lower UPS activity in neurons could account for the preferential accumulation of misfolded or toxic proteins in neurons, which is a prerequisite for mutant proteins to induce selective neurodegeneration.

Because mutant huntingtin is expressed ubiquitously in the brain and body but

selectively kills neurons, cell type-dependent capacity to clear mutant htt is likely to contribute to the selective neuropathology. Previous studies have focused on the expression of mutant polyQ proteins in neurons, with scant attention paid to glial cells, perhaps because glial cells do not contain abundant polyQ aggregates or inclusions. Since glial cells do express polyQ proteins, they may have less capacity to accumulate mutant proteins. Understanding how neuronal and glial cells handle polyQ proteins differently is important for elucidating the pathogenesis of polyQ disorders, as well as developing effective treatments for these diseases.

Because the UPS plays a major role in clearing misfolded proteins, we focused on its function and asked whether differential UPS activities could account for the different accumulations of mutant proteins in neurons and astrocytes. We demonstrated that astrocytic UPS activity is indeed higher than neuronal UPS activity. The preferential increase in GFPu or decrease in UPS activity in brain neurons with age also indicates that the neuronal UPS may be more vulnerable to aging factors than the astrocytic UPS.

By targeting the GFPu/RFP reporters to the nucleus and cytoplasm of neurons and astrocytes, we demonstrated that nuclear UPS activity is intrinsically lower than cytoplasmic UPS activity. The higher cytoplasmic UPS activity may prevent the accumulation of misfolded protein and subsequent aggregate formation in the cytoplasm of the cell body. By targeting the GFPu/RFP reporters to the synapses in mouse brains, we found there is also an age-dependent decrease in synaptic UPS activity in KI mouse brains (Wang et al., 2008). However, in 12-month old mice there is a significant decrease in synaptic UPS activity in the presence of mutant huntingtin. It is important to note that mutant huntingtin did not have an effect on UPS activity in the cell body, perhaps because the normal UPS activity is higher in

the cell body than in synapses such that synaptic UPS is more vulnerable to mutant htt. It seems that targeting the UPS reporters to synapses is necessary to reveal the effects of mutant htt on synaptic UPS activity. The development of UPS reporters to measure synaptic UPS will also help study the role of synaptic UPS in a variety of synaptic functions under different pathological conditions.

The intrinsically lower UPS activity in neurons also has implications for the selective neurodegeneration in other neurological disorders, such as Alzheimer's and Parkinson's diseases, which are caused by misfolded proteins. Given that neurons are also preferentially affected in these age-dependent neurological disorders and that their UPS activity is lower than in the less vulnerable astrocytes, it is possible that boosting UPS function in neurons could alleviate neuropathology. As neuron-astrocyte interactions are critical for the normal function and viability of neurons, reducing the age-dependent decline in UPS activity or maintaining a normal UPS level in astrocytes should also be beneficial for ameliorating the neuropathology caused by misfolded proteins.

6.3 Interpretation

It was hypothesized that mutant htt would further impair the age-induced decrease of UPS activity (Figure 1-3). This hypothesis was strongly based on *in vitro* data. For example, like mutant htt, the disease-associated prion protein PrP^{Sc} has been shown to oligomerize and inhibit the 26S proteasome (Kristiansen et al., 2007). Additionally, the rate of degradation of mutant htt is inversely related to the length of the polyQ repeat (Jana et al., 2001). Finally, aggregates of polyQ proteins inhibit proteasomal degradation in vitro (Verhoef et al., 2002). However, in contrast to these finding, we did not see an obvious

effect of mutant htt on UPS activity in the cell body. The only instance in which mutant htt further inhibited the age-induced decrease in UPS activity was in synapses of 1- year-old KI mice. Two explanations can be offered for the differences in synaptic UPS activity in 1-year-old KI mice. The first explanation is that the UPS in the cell body is less susceptible to the inhibitory effect of mutant htt on UPS function whereas synaptic UPS is affected because of the preferential accumulation of mutant htt in synapses that can also lead to defective intracellular trafficking and reduced ATP production in nerve terminals. The second is that less 26S proteasome is present in the dendrites and axons than in the cell body of neurons such that mutant htt has a greater negative impact on synaptic UPS. Clearly, more studies are needed to determine how mutant htt affects synaptic UPS. Our finding also suggests that aging plays a primary role in decreasing the activity of the proteasome (Figure 6-1). It is possible that aging can cause oxidative damage, decrease in ATP production, and DNA damage, which are insults that affect UPS function. In both neurons and astrocytes there is a significant decrease in UPS activity during aging but neuronal UPS activity is at a lower baseline level compared to astrocytes. This inherent baseline difference in UPS activity between neurons and astrocytes could explain why the effect of age is more detrimental to neurons (Figure 6-1). This theory explains why we observe an increase in NII over time and also why HD is a late onset neurodegenerative disorder.

6.4 Remaining Questions and Future Directions

Based on my results, I envision two major directions in which this work could proceed. The first is to study why neuronal UPS activity is more vulnerable to the insults of aging compared to astrocytic UPS activity. The second is to evaluate and compare the roles of UPS and autophagy in Huntington's disease.

Investigating the mechanism for the preferential decrease of neuronal UPS activity

Aging increases cellular oxidative stress, which could damage the UPS (Farout and Friguet, 2006; Breusing and Grune 2008). Cultured neurons may be more vulnerable to oxidative stress, and this increased vulnerability could contribute to the rapid decline of UPS activity in cultured neurons under *in vitro* conditions. To test if the UPS in neurons is more vulnerable to oxidative stress than astrocytes, one could separately culture neurons and astrocytes and treat each with hydrogen peroxide (H_2O_2). Micromolar concentrations of H_2O_2 are well known to induce oxidative stress in culture. After H_2O_2 treatment, chymotrypsin-like and trypsin-like activity in cultured neurons or glial cells can be measured. Presumably, if astrocytes are less vulnerable to oxidative stress, they should have higher levels of proteasome activity under H_2O_2 treatment.

Aging has also been found to change the 20S core subunit composition. For example, in aged muscles and senescent human fibroblasts, aging caused decreased expression of 20S proteasome resulting from a reduced amount of 19S and 11S subunits (Ferrington et al., 2005; Stratford et al., 2006). The age-related decrease in certain proteasome subunits might be related to the suppression of proteasome activities. Thus, it would interesting to culture neurons and astrocytes and collect the cells at different culturing time points to run a Western blot with antibodies against the 19S cap as well as

other subunits that make up the 20S core. Antibodies to proteasomal core subunits β1i (LMP2), β5i (LMP7), and α5, α7, β1, β5i, β7 (20S) are available. I have done preliminary Western blot analysis on cultured astrocytes and neurons at different culturing time points and found that 20S expression is reduced in neurons as compared with astrocytes. Additional experiments are required to validate these findings.

It is also of interest to note that astrocytes are able to proliferate. This feature may explain why they appear to be less affected than neurons by age-induced insults on UPS activity. To study the effect of proliferation on UPS activity, one could treat each cell type with dbCAMP, which inhibits cell proliferation in culture. After treatment, chymotrypsin-like and trypsin-like enzymatic activities in astrocytes and neurons can be measured and compared.

Aging is known to decrease mitochondrial function (Tifunovic et al., 2004). The activity of the UPS relies on ATP generated by mitochondria. As such, measuring ATP content of neurons and astrocytes after culturing for different times would determine if time-dependent decrease of mitochondrial function in neurons is more pronounced than in astrocytes.

The above experiments using cell cultures will provide us with clues to the preferential decrease of UPS activity in neurons. To validate them or to provide in vivo evidence, adenoviral GFPu/RFP reporters can be expressed via microinjection in neuronal and glial cells in the mouse brains. The injected mice can be treated with oxidative stress inducers. The ratio of GFPu to RFP in neuronal and glial cells can then be examined. If oxidative stress causes a greater reduction of UPS activity in neurons, we should observe the increased ratio of GFPu to RFP in neurons as compared to that in glial cells.

Comparing the roles of UPS and autophagy in Huntington's disease

Studies of the UPS clearly show that inhibiting UPS function increases the levels of both soluble and aggregated forms of mutant huntingtin (Jana et al., 2001; Zhou et al., 2003), suggesting that the UPS plays an important role in clearing misfolded polyglutamine (polyQ) proteins. Subsequent investigation of autophagy in polyQ diseases led to the hypothesis that the UPS may act as a primary route to clear soluble polyQ proteins, whereas autophagy functions to remove aggregation-prone or aggregated proteins (Rubinstein, 2007). There are numerous *in vitro* studies that suggest autophagy can be activated when misfolded proteins are overexpressed in cell culture through transient transfection. If autophagy can truly digest polyQ aggregates or inclusions in cells, we should observe the presence of polyQ aggregates in lysosomes and/or autophagosomes; however, there is still lack of evidence to substantiate this. How autophagy functions when polyQ proteins are expressed at the endogenous level in the brain is an important issue to be resolved. Additionally, it would be of interest to determine whether the UPS or autophagy has a greater role in degrading mutant huntingtin.

This question can be examined by inhibiting autophagy and UPS in neurons cultured from WT and KI mice expressing 150Q. The KI mice express mutant htt and its degraded products at the endogenous level, providing an ideal system to examine the accumulation and degradation of mutant htt. The autophagy inhibitor, BFA, and the proteasome inhibitor, MG132 could be used to treat neuronal and astrocytic cultures. Western blotting to analyze the amount of mutant htt would be more quantitative to reveal which pathway is more likely involved in degrading mutant huntingtin. Likewise,

neuronal and astrocytic cultures from KI mice could be treated with an autophagy activator, rapamycin, and a proteasome activator, PA28gamma (K188E). Rapamycin is known to activate autophagy by suppressing the mammalian target of rapamycin (mTOR) (Wullschleger et al., 2006). PA28gama (K188E) positively influences the ability of the proteasome to cleave polyQ peptides (Pratt & Rechsteiner, 2008). We expect that activation of UPS or autophagy may inhibit the accumulation of mutant N-terminal huntingtin. The different efficacy of the activators and inhibitors of UPS and autophagy will provide important information regarding which pathway is more important in removing mutant htt and other polyQ disease proteins.

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Figure 1-1. Model of the ubiquitin-proteasome system pathway. Conjugation of the ubiquitin to the target protein followed by degradation of the ubiquitinated protein by the 26S proteasome. (1) Activation of ubiquitin by E1. (2) Transfer of activated ubiquitin from E1 to a member of the E2 family. (3) Transfer of activated ubiquitin from E2 to a substrate specifice E3. (4) Formation of a substrate-E3 complex and biosynthesis of a

substrate-anchored polyubiquitin chain. (5) Binding of the polyubiquitinated substrate to the ubiquitin receptor subunit in the 19S complex of the 26S proteasome and degradation of the substrate to short peptides by the 20S complex. (6) Recycling of ubiquitin via the action of isopeptidases.

Figure 1-2. Model for the age-dependent decrease in ubiquitin-proteasome system activity in HD. (A) Under young conditions, misfolded or aggregated mutant htt proteins are efficiently degraded by the proteasome. (B) Under aged conditions, a variety of insults can inhibit normal proteasome function. Oxidative stress, metabolic impairment, mitochondrial instability or dysregulation of ion homeostasis all damage ubiquitin-proteasome system activity resulting in the inability to degrade aggregated proteins. These aggregated proteins will form intranuclear inclusions and the number of intranuclear inclusions will increase as a function of time.

Figure 1-3. Hypothesis. We hypothesize that both aging and misfolded mutant htt impairs the UPS. Proteasomal activity declines with age due to the inhibitory effects of aging-related insults. In addition, (1) misfolded mutant htt or (2) cleaved mutant htt products form cytoplasmic aggregates or inclusions that may impair UPS activity. (3) Degraded mutant htt can also be translocated into the nucleus where it accumulates to form intranuclear inclusions (NII), which may also impair nuclear UPS activity.

Figure 2-1. Model for the N-end rule pathway that governs the proteasome reporter GFPu. CL1 destabilizing residues (Arg, Lys, His) of the substrate are recognized by the E3 ligase N-recognin that contains distinct binding sites for N-end rule substrates, such as GFPu. N-recognin associates with an ubiquitin-conjugating enzyme (E2) and targets internal Lys (K) residue of GFPu for ubiquitylation by E2. The multiubiquitylated GFPu is recognized by the 19S cap complex of the proteasome and AAA+ proteins present in the base of the 19S complex mediate the ATP-driven unfolding and translocation of GFPu into the 20S core for proteolysis. Ubiquitin is spared from degradation through its release from GFPu by deubiquitylating enzymes.

Figure 2-2. Expression of proteasomal reporters in PC12 cells (A) Schematic maps of DNA construct for expressing fluorescent UPS reporters. GFPu is a green fluorescent protein tagged with a CL-1 degron sequence specific for ubiquitination and degradation by the proteasome. RFP lacking a CL-1 degron sequence serves as a control. Both GFPu and RFP were co-expressed by the cytomegalovirus (CMV) promoter in adenoviral vectors. (B) Expression of GFPu/RFP in PC12 cells via adenoviral infection. Inhibiting the proteasome by MG132 (10 μM for 12 h) increased GFPu signals.

Figure 2-3. Western blot of proteasomal reporters in PC12 cells. Western blotting of the infected PC12 cells showing that MG132 treatment increased protein ubiquitination (upper blot) and GFPu compared with RFP and the loading control, tubulin.

Figure 2-4. Expression of proteasomal reporters in neurons. (A) Inhibiting the proteasome by MG132 (10 μM) also increased GFPu signal in cultured primary neurons that were isolated from rat cerebral cortex and had been infected by Ad-GFPu and Ad-RFP for 48 h. (B) Western blotting of cortical neurons infected with each virus shows that MG132 treatment increased GFPu expression compared to RFP.

Figure 2-5. The ratio of GFPu to RFP in cultured cortical neurons. The relative ratio of GFPu to RFP after MG132 treatment. There was a significant increase in GFPu expression with MG132 treatment ($p<0.001$). Thus, the ratio of GFPu to RFP represents the relative inhibition of UPS activity and can be used to measure cellular UPS function.

Figure 2-6. Targeting GFPu/RFP to the nucleus and cytoplasm of cultured astrocytes. (A) Schematic diagram of GFPu/RFP tagged with a nuclear localization sequence (NLS-GFPu/NLS-RFP) or a nuclear exporting sequence (NES-GFPu/NES-RFP). The half-life the NLS-GFPu and NES-GFPu is 60 minutes. (B) Primary astrocytes from wild type mice transfected with NLS-GFPu and NLS-RFP and expressed in the nuclei of transfected cells. MG132 (10μM) treatment for 10 hours increased NLS-GFPu expression thus reflecting a decrease in UPS activity. (C) Primary astrocytes from wild type mice transfected with NES-GFPu and NES-RFP and expressed in the cytoplasm of transfected cells. MG132 (10μM) treatment for 10 hours increased NES-GFPu expression but not NES-RFP. The merged images show the nuclei of cells that were labeled by Hoechst dye. Scale bars: 10μM.

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Figure 2-7. Expression of synaptic UPS reporters. (A) Schematic map of DNA constructs for expressing fluorescent UPS reporters. RFP or GFPu is added to the C-terminus of PSD95 or SNAP25 in an adenoviral vector to express fusion proteins under the control of the human synapsin 1 promoter. (B) Western blots showing the expression of these fusion proteins in HEK 293 cells after adenoviral infection.

Figure 2-8. Presynaptic and postsynaptic UPS reporters reflect UPS activity in cultured neurons. (A) Cortical neurons coinfected with SNAP25-GFPu and SNAP25-RFP and treated with MG132 (10μM). Arrows indicate synapses where puncta were quantified. Bar, 5 μM. (B) The expression ratio of GFPu to RFP in synapses was increased after addition of MG132. (C) Cortical neurons coinfected with PSD95-GFPu and PSD95-RFP and treated with MG132 (10μM). Arrows indicate synapses where puncta were quantified. Bars, 5μM. (D) The expression ratio of GFPu to RFP in postsynaptic terminals was increased after addition of MG132.

Figure 3-1. Accumulation of N-terminal htt fragments in the brains of HD150Q knock-in mice. (A and B) Western blotting of total cell lysates (T), cytosolic (C), synaptosomal (S), and nuclear (N) fractions from cerebral cortex (A) and striatum (B) of HD 150Q KI mice at 4, 14, and 24 months of age. The blots were probed with 1C2 antibody for htt and antibodies for the cytoplasmic protein GAPDH, the synaptic protein syntaxin, or the nuclear protein TBP. As the age of the mice increase so does the presence of N-terminal htt fragments. This is true for both cortical and striatal tissue but is more pronounced in striatal tissue.

Figure 3-2. Decreased proteasomal activity in striatal neurons. Biochemical assays of chymotrypsin-like (top) and trypsin-like (bottom) activity in homogenates from the brain cortex (Ctx) and striatum (Str) of WT and HD150Q KI mice at different ages. There is an age-dependent decrease in both chymotrypsin- and trypsin-like activity in the CTX and STR. There is no significant difference between WT and KI proteasomal activity. The data (mean + SE) were obtained from 4 – 8 mice each group.

Figure 3-3. Decreased striatal UPS activity. (A) Expression of GFPu/RFP in cultured cortical and striatal neurons (DIV 12) from rat brains. The merged images (right panels) show the nuclei of cells that did not express transgenic GFPu/RFP. Scale bar, 10 μm. Striatal neurons expressed more GFPu compared to cortical neurons illustrating a larger dysfunction of UPS than cortical neurons. $P<0.05$; $P<0.01$ as compared to cortical samples. (B) GFPu/RFP ratio illustrates further that striatal neurons have less active UPS compared to cortical neurons.

Figure 3-4. Different accumulations of mutant htt in neuronal versus glial cells in HD mouse brains. (A) EM48 immunohistochemical staining of the striatum (Str) and white matter (WM) of the corpus callosum of wild-type (WT) and HD 150Q knock-in (KI) mice at 15 months of age. Note that EM48 labels more neuronal cells in the striatum than astrocytes in the corpus callosum. (B) EM48 immunohistochemical staining of a R6/2 mouse brain that expresses exon1 mutant htt. Astrocytes in the white matter (WM) of the corpus callosum and in neuronal cells of the striatum (Str) show different extents of mutant htt accumulation. The R6/2 mouse was examined at the age of 12 weeks. Scale bars (A, B): 10 μm.

Figure 3-5. Neurons and astrocytes co-cultured images. Fluorescent images of mixed cultured cells containing neurons (arrows) and astrocytes that had been cultured for 16 or 20 days, then infected by adenoviral GFPu/RFP for 2 days. Note that GFPu signal (green) is higher in neurons than in astrocytes and the neuronal GFPu signal slightly increases with culturing time.

Figure 3-6. Time-dependent increase of GFPu levels in cultured primary neurons. (A) Fluorescent images showing a greater increase in GFPu signal (upper panel) than RFP (lower panel) in cultured cerebral cortical neurons in a time-dependent manner. The cultured neurons at different days (8, 11, and 17 DIV) were infected by adenoviral GFPu/RFP for 2 days before examination. (**B**) High-magnification graphs showing the expression of GFPu and RFP in the same neuron at DIV 17. Scale bars: 10 μm.

Figure 3-7. Quantification of time-dependent increase in GFPu levels in cultured primary neurons. (A**)** Quantification of the ratio (mean + SEM, n=8-17) of GFPu to RFP in cultured primary neurons. ** $p< 0.01$, *** $p<0.001$. (B) Western blot analysis showing the increased level of GFPu in old cultured neurons. The same blot was also probed with antibodies to RFP and tubulin.

Figure 3-8. Expression of GFPu/RFP in cultured astrocytes at different days in culture. (A) Fluorescent images of astrocytes that had been cultured for different days (4, 8, 12, 16, 20) and then infected by adenoviral GFPu/RFP for 2 days. Note that GFPu signals increased only slightly or remained at similar levels in older astrocytes. (B) The ratios (mean + SE, n=18) of GFPu to RFP in cultured neurons or astrocytes at different culturing days. * $p<0.05$, ** $p<0.01$.

Figure 3-9. Quantitative analysis of proteasomal activities in cultured neurons and astrocytes. Biochemical assays of chymotrypsin-like and trypsin-like activities (mean + SEM) of cultured neurons and astrocytes that had been cultured for various days (4-20 days). Chymotrypsin-like activity in neurons at day 4 (0.9500 ± 0.01414, n=4) was significantly reduced at day 12 (0.3406 ± 0.01511, n=4, $p<0.001$). Similarly, trypsin-like activity in cultured neurons was also decreased from day 4 (2.293 ± 0.01791, n=4) to day 12 (0.8995 ± 0.006551, n=4, $p<0.001$). There was no significant difference in chymotrypsin-like and trypsin-like activities of cultured astrocytes from day 8 to day 20.

Figure 3-10. Targeting GFPu/RFP to the nucleus and cytoplasm of cultured cells. (A) NLS-GFPu/RFP or NES-GFPu/RFP were transfected into cultured rat brain cortical neurons at 8 DIV. Note that nuclear NLS-GFPu is more intense than cytoplasmic NES-GFPu. (B) Cultured astrocytes at DIV 20 were infected with adenoviral NLS-GFPu/RFP (upper panel) or NES-GFPu/RFP (lower panel). Note that nuclear NLS-GFPu signal is also more intense than cytoplasmic NES-GFPu. In (A) and (B), merged images show the Hoechst-stained nuclei (blue). Scale bars: 10 μm.

Figure 3-11. Quantification of targeting GFPu/RFP to the nucleus and cytoplasm of cultured cells. Quantification of the ratios (mean + SEM, n=10-16) for NLS-GFPu/RFP in the nucleus and NES-GFPu/RFP in the cytoplasm of astrocytes and neurons. ** $p<0.01$.

Figure 4-1. Fluorescent microscopic examination of adenoviral RFP injected mouse brain regions. Mouse brains injected with adenoviral RFP were subjected to immunolabeling of neurons with anti-NeuN or astrocytes with anti-GFAP. (A) Non-injected brain cortex, (B) adenoviral RFP-injected brain cortex and (C) adenoviral RFP-injected white matter of the corpus callosum were examined. Fluorescent microscopy was performed to reveal adenoviral RFP (red) and NeuN or GFAP (green). The merged images show the nuclei of cells that were labeled by Hoechst dye. Scale bars: 10 μM.

Figure 4-2. Expression of GFPu/RFP reporters in mouse brains. Stereotaxic injection of adenoviral GFPu/RFP into the white matter of the corpus callosum (astrocytes) and the striatum (neurons) to examine their expression in astrocytes and neurons, respectively, in mice at different ages (4, 12, and 24 months). (A) Low-magnification micrographs showing RFP and GFPu signals in the injected regions. Note that the neuronal level of GFPu is higher than the astrocytic GFPu level, suggesting lower UPS activity in neuronal cells. (B) High-magnification micrographs (630X) showing the distribution of GFPu and RFP in the infected neurons (upper panel) and astrocytes (lower panel). Arrows indicate nuclei of neurons or astrocytes that were labeled by Hoechst dye. Scale bars: 10 μm.

Figure 4-3. Differential UPS activities in neurons and astrocytes of mice at different ages. Quantification of the ratios of adenoviral GFPu to RFP in infected neurons and astrocytes in the brains of mice at 4, 12, and 24 months of age. The ratios of GFPu to RFP in astrocytes are lower than neuronal GFPu/RFP in the striatum and cortex at different ages. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4-4. Differential UPS activites in neurons and astrocytes. The ratios of GFPu to RFP in astrocytes and striatal neurons in the adenoviral GFPu/RFP-injected brains of wild-type (WT) and HD 150Q knock-in (KI) mice at the age of 2, 12, and 24 months. No significant difference in the ratios of GFPu to RFP was seen between WT and KI cells, though the ratio was lower in astrocytes than neurons and increased in the old mouse brains.

Figure 4-5. The accumulation of mutant htt in astrocytes from KI mice. Cultured astrocytes (8 DIV) from WT and HD150Q KI mice were treated with MG132 (10 μM) for 12 h. The accumulation of mutant htt and its fragments, which were recognized by an antibody (1C2) specific to expanded polyQ tracts, was increased by using MG132 to inhibit the proteasome. The blots were also probed with anti-tubulin.

Figure 5-1. Neuropil aggregates and synaptic UPS activity in HD knock-in mouse brain. (A) Immunostaining of heterozygous HD knock-in mice (KI) with the 1C2 antibody showing an increase in neuropil aggregates in the striatum with age. Bars, 10μM. (B and C) Expression of presynaptic (SNAP25-GFPu/RFP) (B) and postsynaptic (PSD95-GFPu/RFP) (C) reporters in the striatum of KI mice at 12 months of age 7 days after injection of adenoviral UPS reporters. Note the increase in GFPu signals over time. Bars, 10μM.

Figure 5-2. Quantification of synaptic UPS reporters in the striatum of HD knock-in mice. (A) The ratio of SNAP25-GFPu to SNAP25-RFP in synapses (30-48 in each group) of HD knock-in mice (KI) at 4, 8, and 12 months of age. **, $P<0.01$. (B) The ratio of PSD95-GFPu to PSD95-RFP in synapses (30-48 in each group) of KI mice at 4, 8, and 12 months of age. **, $P<0.01$. Only synaptic puncta in dentrites and synaptic terminals were quantified for both SNAP25-GFPu/RFP (A) and PSD95-GFPu/RFP, respectively (B).

Figure 5-3. Chymotrypsin-like activity in the synaptosomes of wild-type and knock-in mice. Synaptosomes were isolated from the cortex (top panel) and striatum (bottom panel) from wild-type (WT) and knock-in (KI) mice at 4, 8, and 12 months of age. The data were obtained from five experiments and represented as mean + SEM (n=48). *, $P<0.05$ as compared to WT samples.

Figure 6-1. Working model of the UPS activity in HD. Our studies revealed that it is aging that induces a progressive decline of UPS activity in the brain. In young animals, mutant proteins are efficiently degraded by the UPS either in the cytoplasm or in the nucleus. (A & B) In old animals, mutant proteins are not efficiently degraded because the effect of aging has reduced proteasomal function. Aging can dysregulate proteasome function in the cytoplasm resulting in cytoplasmic inclusions (CI). Likewise, aging can also dysregulate proteasome function in the nucleus resulting in intranuclear inclusions (NII). It appears that less UPS activity is in the nucleus than the cytoplasm, accounting for more NII than CI in the HD brain. However, we found that neurons (A) have a lower baseline level of UPS activity compared to astrocytes (B). This cell type-specific difference in baseline UPS activity may account for why neurons are more susceptible to the negative effects of aging compared to astrocytes. In the nerve terminals such as synapses, mutant htt can impair the UPS activity, perhaps because of its inhibitory effect on intracellular transport of mitochondria (Orr et al., 2008), resulting in reduced ATP supply to synaptic UPS.