CHARACTERIZATION OF THE BRAIN PROTEASOME AND ITS INTERACTING PROTEINS AND THEIR REGULATION BY NEURONAL ACTIVITY

Thesis by

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You know when you know,
and when you do not know, you do not know,
such is true knowledge.

Confucius
Ancient Chinese educator

Never doubt that a small group of thoughtful,
committed citizens can change the world.
Indeed, it is the only thing that ever has.

Margaret Mead
American anthropologist

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As I reflect back on being a graduate student at Caltech, my greatest satisfaction lies not in how much I have learned about research, or the discoveries I have personally made, although these are integral parts of my training, but in how I feel more curious about the world and more enthusiastic to explore its hidden rules than when I started, or for that matter when I was a schoolboy.

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ABSTRACT

Despite the importance of proteasome-mediated proteolysis in synaptic plasticity, protein quality control, and cell regulation, little is known about proteasome composition and regulation in the brain. This thesis represents the first detailed study of mammalian brain proteasomes. Using a new affinity purification method, 26S proteasomes were isolated from the cytosolic and the synaptic compartments of the rat cortex. The proteins associated with the 26S proteasome were purified and analyzed by tandem mass spectrometry. A total of 30 proteasome-interacting proteins were identified in the brain. Several differences were seen in the spectrum of proteasome-associated proteins in the cytosol and the synaptosome. For example, the proteasome-associated protein ECM29 was found only in the cytosolic 26S proteasome, and the ubiquitin-binding factor TAX1BP1 only in the synaptic 26S proteasome. These findings allowed for further investigations into the interplay between proteasome regulation and synaptic plasticity.

Neuronal exposure to the neurotransmitter NMDA caused the degradation of 19S particles, resulting in lower levels of 26S proteasomes. The levels of ubiquitin conjugates also decreased, as did two proteasome-bound ubiquitin ligases, UBE3A/E6-AP and HUWE1/ARF-BP1, both of which have been linked to neurogenetic disorders associated with mental retardation. Thus, in the brain, proteasomes have a characteristic set of associated proteins that may serve as regulators or cofactors. Moreover, the content and pattern of associated proteins can vary with synaptic activity, in a manner likely to influence synaptic plasticity.

TABLE OF CONTENTS

List of Figures

List of Tables

ABBREVIATIONS

AD	Alzheimer’s disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionate
APS	ammonium persulfate
ATP	adenosine triphosphate
CNS	central nervous system
DNA	deoxyribonucleic acid
DLB	dementia with Lewy bodies
DTT	dithiothreitol
DUB	deubiquitinating enzyme
ER	endoplasmic reticulum
GABA	gamma-aminobutyric acid
GST	glutathione S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IBMPFD	inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia
kDa	kiloDalton
LTD	long-term depression
LTF	long-term facilitation
LTP	long-term potentiation
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
NMDA	N-methyl-D-aspartate

NTA	nitrilotriacetic acid
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PD	Parkinson’s disease
PolyQ	polyglutamine
PSD	postsynaptic density
PBS	phosphate buffered saline
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulfate
SEM	standard error of mean
TEMED	tetramethylethylenediamine
TDP-43	TAR DNA binding protein (43 kiloDalton)
Tris	tris(hydroxymethyl)-aminomethane
UBL	ubiquitin-like domain
UIM	ubiquitin-interacting motif
UPP	ubiquitin-proteasome pathway

CHAPTER 1

Introduction

Neurons have highly polarized morphologies and the connection properties between neurons are constantly modified through mechanisms called synaptic plasticity. In the central nervous system of higher animals, each neuron is postmitotic and basically irreplaceable during the life span of the animal. As we investigate these unique properties of neurons, it is becoming clear that the proteome of a neuron is also very interesting to study. At the level of individual synapses, the local proteome is dynamic and modifiable. Protein synthesis and degradation work hand-in-hand to remodel synaptic properties to allow for synaptic plasticity. At the whole-cell level, the neuron faces the challenge that it cannot get rid of aberrant proteins through cell division, but has to deal with them

internally. The degradation of aberrant and toxic proteins is crucial to the long-term viability of the neuron.

The major site of protein degradation in the eukaryotic cell is the proteasome complex. This thesis represents the first detailed study on neuronal proteasomes, and reveals that they contain many additional previously unknown components. By observing compositional differences in neuronal proteasomes compared to other mammalian cells, it appears that the proteasome complex has evolved differently in neurons to meet their specialized proteolytic demands. Moreover, neuronal proteasomes also show dynamic changes associated with changes in neuronal activity. Characterizing the basic machinery of protein degradation is an important first step toward understanding the crucial role of proteolysis in neuronal function and dysfunction.

1.1. Protein degradation is a fundamental cellular process

It the history of biological sciences, our understanding of catabolic processes often lags behind that of corresponding anabolic processes. Before we understood the basic chemical properties of living organisms, scientists assigned the vital forces that perform chemical transformations to mysterious agents called enzymes. Later it was discovered that enzymes are made of proteins, or polypeptides. The quest to understand the synthesis of proteins and their link to the genetic material eventually led to the discovery of the "central dogma" of molecular biology [1]. Around that time, little attention was paid to the ultimate fate of proteins. It was often assumed that proteins are stable entities that do not turn over, because polypeptides do not undergo spontaneous hydrolysis at physiological conditions.

The first indication that proteins are not stable entities but undergo constant turnover came from isotope labeling studies in the late 1930s [2]. In the 1950s, protein degradation was shown to be adenosine triphosphate (ATP)-dependent [3]. The result appeared surprising because the hydrolysis of the peptide bond is thermodynamically favorable. However, these studies received little attention at the time because the function and mechanism of protein degradation were poorly understood. It was not until the 1980s that we finally uncovered the major proteolytic sites of the eukaryotic cell: the proteasome and the lysosome. The proteasome is a large protein complex and its proteolytic activity is confined to its interior. The lysosome is a membrane-bound organelle with enzymes to breakdown proteins and other macromolecules. The confinement of proteolytic activities within these structures help prevent non-specific proteolysis [4]. The energy expenditure of degradation is related to the selection and delivery of substrates to the proteolytic machinery [5]. In many cases, proteins destined for degradation are tagged by ubiquitin, a 76 amino acid protein [6, 7].

Today, it is well-accepted that the precise regulation of proteolysis underlies many fundamental cellular processes, such as cell division, protein quality control, transcription, DNA repair, and so on [4, 6, 8]. The average protein in yeast has a half-life of <2 hours [9], and as much as one third of the pool of newly synthesized proteins is instantly degraded for quality control purposes, mostly carried out by the proteasome [10]. There are several fundamental reasons to degrade proteins inside the cell: when they are no longer required or need to be actively removed, when they are abnormal (misfolded or chemically damaged), and when a balance between degradation and synthesis is required

to maintain desired protein levels. It is therefore not surprising that protein degradation regulates most aspects of cell biology.

1.2. Neurons have unique protein degradation demands

If all cells require regulated protein degradation to function properly, why are nerve cells of particular interest? The unique morphology of neurons—with specialized zones for presynaptic neurotransmitter release and postsynaptic receptor activation, and the plasticity of synapses—tightly coupled to changes in the synaptic proteome, impose special challenges on the cellular machinery for both protein synthesis and degradation [11, 12]. Within this context, we aim to understand how degradative pathways are tailored to support the physiology of neurons and facilitate key brain functions such as learning and memory [13, 14]. Due to its morphology, the neuron has very high surface-area-to-volume ratios. Thus, membrane protein turnover is an important issue for the neuron. Because the space within the neuron is highly compartmentalized, there is also a need for compartment-specific regulation of protein degradation to modulate synaptic function during learning, memory, and development [14-17].

Since neurons are postmitotic cells, they cannot get rid of aberrant proteins through asymmetric cell division [18]. Throughout the lifespan of a higher animal, each neuron must vigilantly protect itself against protein misfolding and oxidation. Most neurodegenerative disorders in humans are characterized by the presence of pathological protein aggregates, indicative of a failure in protein quality control. It is therefore important to understand protein quality control mechanisms in normal neurons and how they are affected during neurodegenerative conditions.

In sum, due to their unique morphologies, requirements for synaptic plasticity, internal compartmentalization, and the postmitotic life cycle, neurons have very unique protein degradation demands. It is of great interest to understand how neurons have evolved to meet these specialized demands. In particular, this thesis will investigate the unique properties of neuronal proteasome composition and regulation.

CHAPTER 2

Review of the literature

Compared to protein synthesis, the molecular mechanism of protein degradation is much less understood. While there are two major proteolytic sites in the cell, the proteasome and the lysosome, specific details about each protein's termination are in most cases unclear. It is often difficult to determine what triggers the degradation of a particular protein, and how it is delivered and handled by the proteolytic machinery. This chapter will briefly describe the basic principles of the proteasomal and lysosomal proteolytic pathways. An overview will be given on the emerging evidence on the importance proteasome-mediated degradation in synaptic plasticity, and the link between protein degradation and neurodegeneration. Moreover, it will discuss how proteasome

function is dynamically regulated in the cell, especially through a network of proteasome-interacting proteins.

2.1 Degradation by the UPP and the lysosome

Most cellular proteins are degraded by the proteasome and/or the lysosome. We can further classify proteolytic processes into several major pathways (Table 2-1). The most important and versatile of all proteolytic pathways is the ubiquitin-proteasome pathway (UPP), which degrades the majority of intracellular and endoplasmic reticulum (ER) proteins. It is also the focus of this study.

Table 2-1. Major proteolytic pathways in the eukaryotic cell

Proteolytic pathway	Substrates	Substrate ubiquitination
26S proteasome	intracellular proteins, ER proteins, outer mitochondrial membrane proteins	mostly
20S proteasome	partially unfolded proteins? oxidized proteins?	no
endosome-lysosome	plasma membrane proteins, endocytosed proteins	sometimes
autophagy	intracellular proteins, proteins in bulk	sometimes?

The UPP

Ubiquitin is a small protein of 76 amino acids. Ubiquitination (or ubiquitylation) is a posttranslational modification that forms an isopeptide bond between a lysine residue on the protein and the carboxyl terminus of ubiquitin. The system to ubiquitinate a protein consists of four different classes of enzymes: E1–E4. First, ubiquitin is covalently conjugated to the E1 (ubiquitin activating enzyme) in an ATP-dependent reaction, followed by transfer to the E2 (ubiquitin conjugating enzyme). The E3 (ubiquitin-protein ligase) transfers the ubiquitin from the E2 to the substrate protein [8]. After the first ubiquitin is attached (monoubiquitination), the E3 can elongate the ubiquitin chain by creating ubiquitin-ubiquitin isopeptide bonds. The E4 (chain elongation factor) is a class of E3-like enzymes that only catalyzes chain extension [19]. Ubiquitin has seven lysines (K6, K11, K27, K29, K33, K48, and K63), all of which are available and indeed used in vivo for chain extension [20]. The process of ubiquitination is schematically presented in Figure 2-1. The significance of complex ubiquitination patterns is only partially understood: K48 and K11 chains are the most common signals for degradation by the 26S proteasome, while monoubiquitination and K63 chains do not generally specify degradation but have other biological functions including endocytosis [19, 21].

The E3 is largely responsible for target recognition in this system via physical interactions with the substrate. The large number of E3 genes in eukaryotic genomes [22] (see Table 2-2) reflects the highly specific nature of substrate recognition in UPP-mediated degradation [23]. Several classes of UPP factors are involved in the presentation of substrates to the proteasome: ubiquitinating enzymes (E1–E4), deubiquitinating enzymes (DUB), shuttling factors and chaperones. The organization of

these factors may differ for each substrate. Figure 2-1 shows an example of how a protein's ubiquitination pattern can be dynamically edited by E3s, E4s, and DUBs. When K48-polyubiquitinated, the protein can reach the proteasome by diffusion or with the assistance of chaperones and shuttling factors [24]. In some cases, the proteasome can also move toward the substrate [25]. After substrate-proteasome association, DUB and ATP-dependent unfoldase activities help the substrate to enter the proteolytic lumen of the proteasome[26]. The structure and heterogeneity of proteasomes are illustrated in Fig. 2-2.

Table 2-2. Ubiquitination-related proteins encoded in four eukaryotic genomes

Class	*Homo sapiens*	*Mus musculus*	*D. melanogaster*	*S. cerevisiae*
E1	16	16	11	8
E2	53	61	32	15
E3	527	442	189	68
DUB	74	78	27	20

(compiled from ref. [22])

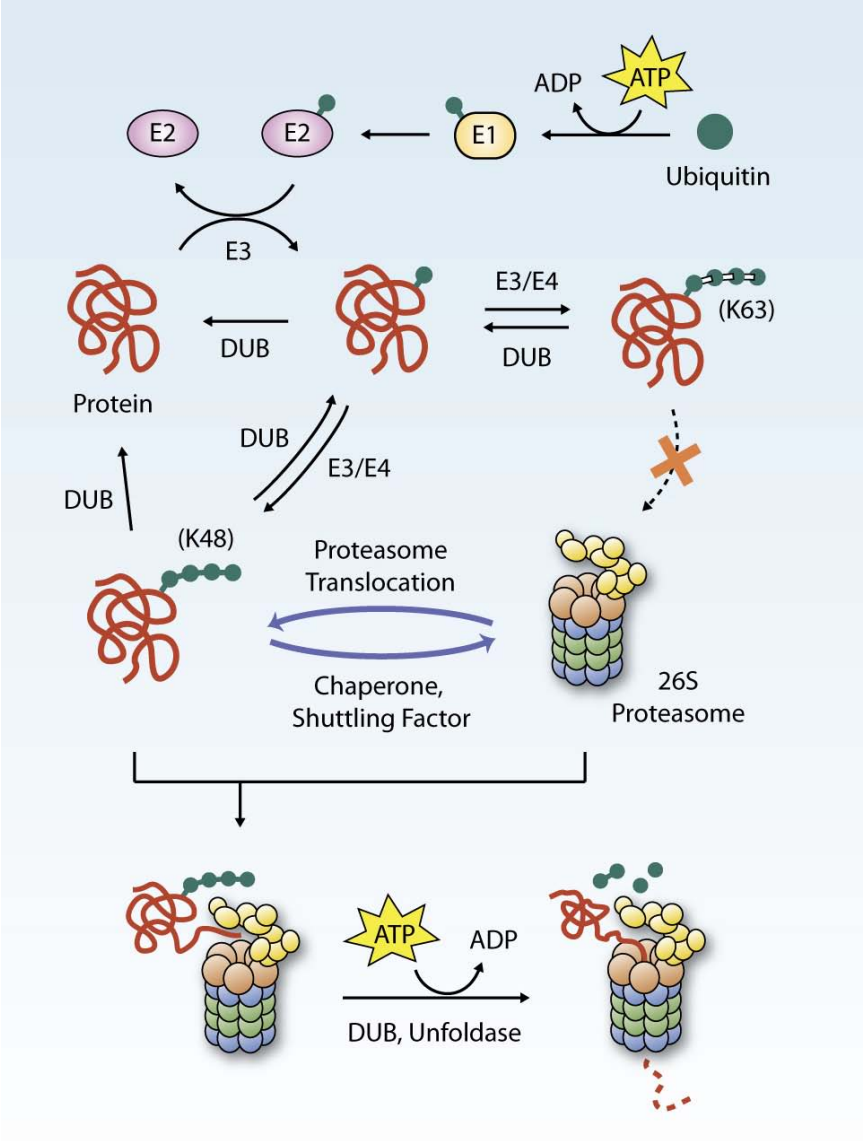


Figure 2-1. A schematic representation of the UPP. Ubiquitination is carried out by a cascade of enzymes (E1, E2, E3). The ubiquitin chain can be further elongated and edited by E3/E4 enzymes and DUBs. K48 chains target substrates to 26S proteasomes for degradation. Substrates are deubiquitinated and unfolded at the proteasome before entering the proteolytic lumen of the 20S particle (see Fig. 2-2 for proteasome structure). ATP is consumed by E1 to activate ubiquitin and by the 19S Rpt subunits to unfold the substrate. K63 chains generally do not lead to proteasomal degradation.

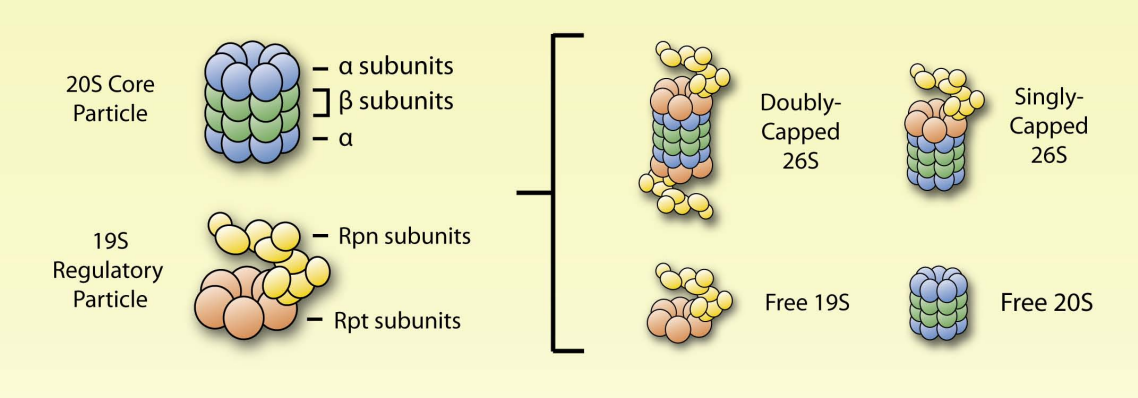


Figure 2-2. Structure of the proteasome. The proteasome is a large, multi-subunit protease complex [26]. The 20S core complex is composed of four heptameric rings of α1-7 and β1-7 subunits. The proteolytic activities are conferred by three of the β subunits in the interior of the barrel. The 19S regulatory complex, or PA700, contains a hexameric ring of six AAA-ATPase subunits (Rpt1–6), and about a dozen non-ATPase (Rpn) subunits. The 19S receives the polyubiquitinated substrate, removes the ubiquitin and, using ATPase activity, unfolds it for translocation into the 20S chamber [26]. Due to experimental difficulties in characterizing proteasome heterogeneity, the nomenclature of proteasome subtypes is not yet standardized in the literature. Different proteasome subtypes and their nomenclature used in this thesis are presented in the figure. Here 26S proteasome will denote both singly- and doubly-capped species, since they both degrade polyubiquitinated proteins. In addition, many interacting proteins can associate stably or transiently with the proteasome. For instance, another regulatory complex of the 20S, called 11S/REG/PA28, is a heptameric ring of PA28 subunits (α, β, γ) and highly abundant in mammalian cells. This complex is thought to activate the 20S by widening the pore [27]. The mix-and-match of 20S, 19S and 11S gives rise additional types of proteasomes [28].

Lysosome and autophagy

Lysosomes are organelles containing acid hydrolases to break down biopolymers and other biomolecules [29] (see Fig. 2-3). To breakdown plasma membrane proteins and endocytosed proteins, substrates are first endocytosed and eventually enter the proteolytic organelles including late endosomes and lysosomes. Many types of signals can regulate endocytosis and sorting, including monoubiquitination and K63-polyubquitination [7, 30]. Endocytosed proteins first arrive at early endosomes, and subsequently get sorted into recycling endosomes (for return to the surface by exocytosis) or multivesicular bodies [31] (for transport to late endosomes or lysosomes). The acid hydrolases in the lumen of lysosomes (pH 4-5) and late endosomes (pH 5-6) are highly active in acidic environments, but lose their activities in the cytosol (pH ~7.2). The confinement and the pH-dependence of hydrolases provide dual safeguards against accidental damage [29, 32]. Intracellular proteins can also enter lysosomes by several autophagy mechanisms [33]. In macroautophagy, large amounts of cytosolic materials or even organelles are surrounded by a double-membrane structure (autophagosome) which fuses with lysosomes. In microautophagy, a small amount of the cytoplasm is internalized through lysosomal invagination. In chaperone-mediated autophagy, proteins unfolded by the chaperone translocate into the lysosome through interactions with lysosome-associated membrane protein 2 (LAMP-2) [34]. During cellular starvation, cellular proteins can be non-specifically degraded through macroautophagy. But little is known about how specific proteins are marked for autophagy [35]. Recent studies implicate that p62/sequestosome 1 may facilitate the macroautophagy of ubiquitinated proteins [36].

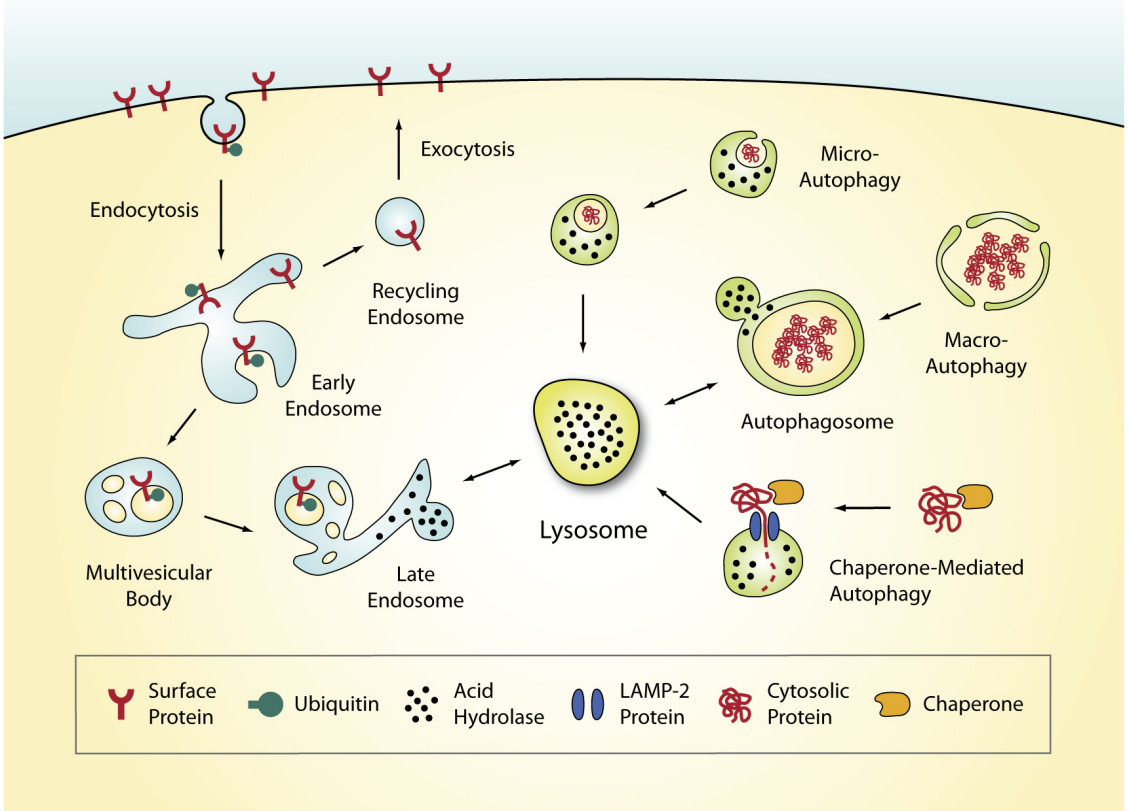


Figure 2-3. Lysosome and autophagy. Lysosomes are organelles containing acid hydrolases to break down biopolymers and other biomolecules, including proteins. Lysosomes can also fuse with other organelles such as late endosomes and autophagosomes to degrade their internal contents. Endocytosed membrane proteins may be recycled back to the surface or degraded in late endosomes or lysosomes. Many types of signals can regulate endocytosis and sorting, including monoubiquitination (shown here) and K63-polyubquitination. Intracellular proteins can enter lysosomes by macroautophagy, microautophagy, or chaperone-mediated autophagy.

2.2 Synaptic plasticity requires protein degradation

As early as 1949, Hebb postulated that “some growth process or metabolic change” may underlie activity-dependent modifications of neuronal connectivity [37]. Since proteins are the molecular machines that mediate signal transduction, it is no surprise that protein synthesis and degradation are important for plasticity and memory [11]. Memory impairment caused by protein synthesis inhibitors was first demonstrated in the 1960s, and subsequently confirmed by many studies [38]. The repression of translation by anisomycin, cycloheximide, or puromycin do not affect learning but the retention of memory when the animal is tested hours or days later. Parallel findings using inhibitors of protein degradation were recently reported. Retrograde amnesia in rats was observed in one-trial inhibitory avoidance learning, when proteasome inhibitor lactacystin was injected into the CA1 region of the hippocampus at 1, 4, or 7 hours post-training, but not 10 hours [39]. In another study, impaired fear learning by injection of anisomycin into the mouse hippocampus was rescued by lactacystin co-injection [40]. These findings implicate that protein synthesis and degradation work in an orchestrated manner to regulate synaptic functions that underlie learning and memory [11].

Since proteasome inhibitors can affect memory in animal behavior studies, what are the underlying molecular and cellular mechanisms? One of the first links between the UPP and synaptic plasticity came from the study of long-term facilitation (LTF) in *Aplysia*, which is similar to long-term potentiation (LTP) in higher animals, at the sensory-to-motor neuron synapse [41]. LTF and LTD are long lasting synaptic changes with enhanced neurotransmission, and in mammals the opposite effect is termed long-term depression (LTD). During LTF induction, proteasome activity is required in the

soma of the presynaptic cell to degrade the regulatory subunit of cAMP-dependent protein kinase (PKA) [41-43]. It was also shown that synaptic strength can be locally regulated by the synaptic UPP. At the sensory-to-motor synapse, proteasome inhibition can lead to both presynaptic and postsynaptic changes including increases in the number of synaptic contacts, the length of sensory neuron processes, and the amplitude of glutamate-evoked postsynaptic potential [44]. Thus, the UPP can regulate synaptic plasticity by different mechanisms at different subcellular compartments.

Recent studies in mammals suggest that an intricate balance between protein synthesis and degradation underlies long-term plasticity and memory. For example, LTP in rat hippocampal slices can be disrupted by proteasome inhibitor lactacystin [45, 46]. However, this effect is rescued by translational inhibitor anisomycin [45]. Interestingly, anisomycin by itself also disrupts hippocampal LTP [47]. However, how synthesis and degradation are coordinated with respect to synaptic modulation remains currently unknown.

UPP regulates synaptic modifications

Like in *Aplysia*, the UPP regulates synaptic transmission at both pre- and postsynaptic terminals in mammals. At the presynapse of cultured hippocampal neurons, for example, the size of the recycling vesicle pool increases by 76% after 2 hours of proteasomal blockade, an effect that is independent of protein synthesis [48]. In hippocampal slices, proteasome inhibition leads to an increase in the frequency of miniature excitatory postsynaptic currents [49], which is largely abolished in transgenic animals lacking SCRAPPER, an E3 enzyme localized to the presynaptic membrane. A key substrate of

SCRAPPER is RIM1, a Ca^{2+}-dependent vesicle-priming factor. Together, SCRAPPER and RIM1 regulate how vesicle release responds to presynaptic calcium levels [49]. Therefore, at the presynapse the UPP influences the size of the vesicle pool and vesicle release.

The UPP also regulates the abundance of proteins involved in the postsynaptic response, including ionotropic glutamate receptors and proteins comprising the postsynaptic density (PSD) [14, 50]. The PSD is an electron-dense structure adjacent to the postsynaptic membrane, visible in electron micrographs, that contains receptor channels, structural proteins and signal transduction proteins. It was shown that chronic blockade of action potential by tetrodotoxin led to the increase of certain PSD proteins (Shank, NR2B, PP1) and the decrease of some others (PSD-95, NR2A, CaMKIIα) [51]. The blockade of inhibitory neurotransmission by bicuculline induced opposite effects. However, changes in PSD composition were blocked when a proteasome inhibitor was co-applied, demonstrating the importance of proteolysis in restructuring the synapse in response to changes in neural activity. Figure 2-4 illustrates some of the proteolytic processes known to occur at the synapse.

The level of neurotransmitter receptors at the synapse is a major determinant of synaptic efficacy. In worms the ubiquitination of glutamate receptors reduces their levels at the synapse, possibly through endocytic mechanisms [52]. In mammals, the ubiquitination of AMPARs and NMDARs have also been observed [53, 54]. Moreover, the turnover of AMPARs at the synapse involves both proteasomal and lysosomal activities. AMPA-induced, clathrin-dependent endocytosis of AMPARs can be blocked by proteasome inhibition and by expression of dominant negative K48R ubiquitin [55].

The depolarization of neurons causes proteasomes to rapidly redistribute into dendritic spines [25]. It has been suggested that UPP activity downregulates PSD95, and thereby disrupts the anchoring of AMPARs [56, 57]. Endocytic zones are localized laterally to the PSD in the dendritic spine [58], and hence the release of AMPARs from the PSD scaffold may indeed be a prerequisite for endocytosis (Fig. 2-4). After endocytosis, AMPARs reside in early endosomes, and may be sorted into recycling endosomes for surface reinsertion or into late endosomes/lysosomes for degradation, depending on subunit composition and whether NMDARs were simultaneously activated [59, 60]. The complex dynamics [61] of AMPARs' degradation, synthesis, posttranslational modification, and trafficking is without doubt a key mechanism of synaptic plasticity that remains to be fully understood.

In spite of these recent progresses, most of the proteins degraded during synaptic remodeling remain unknown. The molecular mechanisms behind these proteolytic events are also difficult to elucidate. We still do not understand which UPP components are important at the synapses and how they work together, which is one of the focuses of this thesis study.

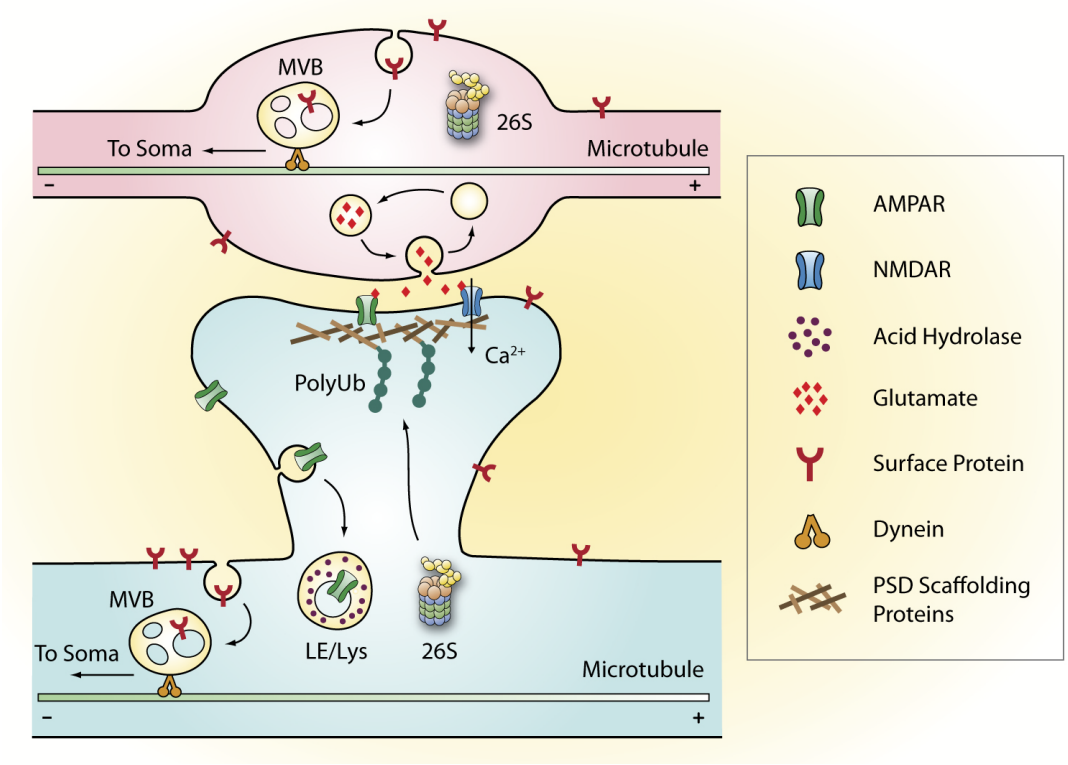


Figure 2-4. Degradation of synaptic proteins. The UPP regulates the recycling and the release of synaptic vesicles at the presynaptic terminal (axonal bouton, top). Released glutamate activates AMPA and NMDA receptors at the postsynaptic terminal (dendritic spine, bottom). This may trigger the polyubiquitination of PSD scaffolding proteins and the translocation of 26S proteasomes into spines. The degradation of PSD scaffolding proteins leads to the de-anchoring of AMPARs, which are endocytosed at the lateral zone of the spine. Endocytosed AMPARs marked for degradation are sorted into late endosomes/lysosomes containing acid hydrolases. In mature hippocampal neurons, endocytosis occurs along the entire dendrite and at the presynaptic terminal but not the shaft of axons [62]. In both cases, many internalized proteins are transported in multivesicular bodies to the soma for breakdown. Retrograde vesicular traffic requires dynein motors that travel toward the minus end of the microtubule. LE/Lys: late endosome/lysosome. MVB: multivesicular body

2.3 Neurodegeneration involves proteolytic dysfunction

A wide array of neurodegenerative disorders are known to occur in humans, and the majority of them are characterized by protein aggregates in the degenerating areas. While different proteins are found in the aggregates of different neurodegenerative disorders (Table 2-3), these proteins share a common feature: they are misfolded into the amyloid fibril structure [63]. The amyloid fibril structure is generally resistant to proteolysis and grows in size by converting normal proteins into their misfolded states. As a result of decreased degradation (not increased synthesis) and the growth of the fibrils, large protein inclusions are formed around or inside degenerating neurons [64]. Collectively, these disorders are now considered protein misfolding diseases, or “proteinopathies” of the central nervous system (CNS), which include Parkinson’s disease (PD), Alzheimer’s disease (AD), polyglutamine (polyQ) expansion diseases, and prion-related disorders [65] (see Table 2-3). In most cases, protein inclusions stain positively with antibodies against ubiquitin [65-67]. Curiously, abnormalities of just four proteins—α-synuclein, amyloid β, tau and TAR DNA binding protein (TDP-43)—appear to be associated with 80–90% of neurodegenerative dementias [68, 69].

Table 2-3. Neurodegenerative protein misfolding diseases

Aggregate protein	Aggregate location	Related disorders
Tau	Intracellular tangles	Alzheimer's disease (AD), frontotemporal lobar dementia, corticobasal degeneration, Pick's disease, progressive supranuclear palsy
α-Synuclein	Intracellular Lewy bodies	Parkinson's disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy
TDP-43	Intracellular	frontotemporal lobar degeneration, amyotrophic lateral sclerosis
PolyQ expansion proteins	Intracellular	Huntington's disease, spinocerebellar ataxias, spinal and bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy
Amyloid β	Extracellular plaques	Alzheimer's disease
Bri2	Extracellular	familial British/Danish dementias

The frequent detection of proteasomes and lysosomes around ubiquitin-positive aggregates in postmortem brains implies that proteins within these aggregates were marked for degradation but were not efficiently removed [65, 70]. Consistent with this model, characterization of Lewy bodies from dementia with Lewy bodies (DLB) and PD patients revealed that the major ubiquitinated protein was α-synuclein [71]. The ubiquitin chain length was between one and three, and K48 linkage was detected. It is known that K48 chains of four ubiquitins or longer is required for efficient targeting to the proteasome [72]. As such, the di- and tri-ubiquitinated α-synuclein species that have been

detected appear to be trapped intermediates that have failed to reach their proteolytic destination. It also implies that some of the misfolded α-synuclein has been successfully degraded by the proteasome, but the rate of proteolysis may not have been fast enough to overcome protein deposition in degenerating neurons.

Furthermore, overall proteasome activity in brain tissue decreases with aging, and further loss is observed in degenerative conditions such as AD and PD [73]. Hence, it has been proposed that neurodegeneration may be linked to degradative dysfunction by several mechanisms [65, 74]. In one possible scenario, the impairment of degradation may be responsible for the decline of neuronal health, while aggregate formation may be a secondary phenomenon. Alternatively, some other factors may impair neuronal health and cause protein aggregation, and in turn these aggregates interfere with normal proteolysis [75, 76] and cause further problems. To this date, it has been difficult to determine if protein aggregation is sufficient to cause neurodegeneration [77], and further studies of proteolytic pathways in the brain may provide insights into why aggregates form and how they impact cellular function.

The UPP and PD

Dysfunction of the UPP [74, 78, 79] and lysosome-autophagy [80, 81] have been implicated in many neurological disorders. For instance, in the UPP, an increasing number of E3 genes are now linked to neurogenetic disorders, including *UBE3A* (*E6-AP*) in Angelman Syndrome, including *UBR1* in Johanson-Blizzard syndrome [82], *NHLRC1* in Lafora's disease [83], as well as *Cul4B* [84] and *HUWE1* [85] in X-linked mental retardation. There is also a group of ~50 degenerative disorders mapped to mutations in

the lysosomal pathway, collectively called lysosomal storage diseases (LSD) because of intralysosomal accumulation of unmetabolized substances [86]. LSDs may affect multiple organs, but most affect the nervous system by compromising neuronal function.

Interesting links between the UPP and neurodegeneration have been found in mutations leading to familial PD. While most PD cases are idiopathic, the identification of monogenic mutations linked to hereditary forms of PD (about eight genes to date) provides new insights into the pathogenesis [87, 88]. One of the more common types of familial PD is caused by mutations in *PARK2* [89], encoding the E3 ligase parkin. Mutations in *PARK2* cause early disease onset with the loss of dopaminergic neurons in the substantia nigra, suggesting that UPP dysfunction may contribute to pathogenesis [79], while Lewy bodies are generally absent [89]. Moreover, recent studies indicate that two additional familial PD genes, *PINK-1 (PARK6)* and *DJ-1 (PARK7)*, encode proteins that form an E3 complex with parkin [90]. The genetic association of UCHL1, a DUB, with familial and sporadic PD is somewhat controversial, but it is found in Lewy bodies [88, 91]. It is also mutated in a form of neurodegenerative ataxia in mice that arises from spontaneous mutations, called gracile axonal dystrophy [92, 93]. These mice exhibit retrograde accumulation of amyloid β aggregates, but not α-synuclein, in gracile tract axons that control the hind limbs. This finding implies that deposits of aggregation-prone proteins may be a secondary effect of global UPP impairment, because UCH-L1 helps maintain the monomeric pool of ubiquitin [94]. As such, about half of the reported PD-causing genes have already been linked to the UPP.

Recent transgenic mouse studies further support the hypothesis that UPP dysfunction can lead to PD and DLB. By inactivating the gene of a 19S proteasome subunit in

specific regions of the brain, the manipulated neurons show decreased 26S levels. 26S proteasome depletion in the cortex and midbrain dopaminergic neurons cause neuronal death and Lewy body (α-synuclein) deposition [95]. In humans, DLB and PD are also characterized by Lewy body accumulation in the cortical and midbrain dopaminergic neurons, respectively, and many patients eventually develop both. This also represents the first established murine model of DLB. Furthermore, in neuroblastoma cell lines, the overexpression of a mutant proteasome subunit also causes α-synuclein aggregation [96]. Therefore, UPP dysfunction is likely to contribute to both sporadic and hereditary forms of PD [97].

2.4 Proteasome function is a regulated by multiple mechanisms

Since each protein is a unique three-dimensional object with a distinct folded structure and chemical properties, it is remarkable that the proteasome can adequately handle thousands of such objects. Although the recognition of proteolytic substrates is largely attributed to polyubiquitination, the proteasome still has to physically interact with each substrate and unfold it for degradation. We do not fully understand how this can be achieved, or why the UPP fails to remove aggregate-prone proteins like TDP-43 or tau in degenerating neurons.

Recent studies have shown that many properties of the proteasome can be regulated by the cell, including its cellular levels, localization, the complement of its interacting proteins, and posttranslational modification states [98, 99]. In the neuron, it has been shown that proteasome levels can decrease with aging [100]. Proteasomes also rapidly redistribute into dendritic spines in an activity-dependent manner [25]. Compared to other

cell types, neurons express much lower levels of proteasome activator PA28β [101], which is further decreased during LTP [102]. Increased neuronal activity also triggers the phosphorylation of proteasomes by calcium/calmodulin dependent kinase II [103]. These studies suggest neuronal proteasomes are regulated in very specific manners, and there may be many additional regulatory points remaining to be discovered.

Originally, the proteasome composition was investigated by column chromatography purifications, which tend to strip away the more loosely associated proteins. What remained were the core subunits of the proteasome complex. More recently, using affinity purifications under gentle conditions, many more interacting proteins have been identified on the proteasome. We may think of proteasome subunits as proteins strongly bound to the proteasome in stoichiometric amounts, and proteasome-interacting proteins as loosely bound proteins in sub-stoichiometric amounts. The subunits of the 20S proteasomes include α1–α7 (outer ring) and β1–β7 (inner ring). The 19S consists of as ATPase-subunits (Rpt1–6) and non-ATPase subunits (Rpn1–3, 5–12) (see Fig. 2-2).

Several recent studies have characterized 26S proteasome interactors from different cell types using affinity purification and tandem mass spectrometry [104-106]. The surprising finding is that the complement of proteasome interactors differs very significantly from one cell type to another. This implies that the each type of cell has a different proteome, and thus requires different proteasome interactors to facilitate different proteolytic tasks. For instance, muscle proteasomes are associated with many myofibrillar proteins and metabolic enzymes. This may be related to the role of proteasome in rapidly degrading muscle fibers during starvation [104]. This prompts us to investigate whether the spectrum of proteasome interactors in neurons is also different

from other cells, and how neuronal proteasomes may be regulated to meet the neuron's specialized proteolytic demands.

CHAPTER 3

Composition of neuronal proteasomes

3.1 Introduction

A cell may contain several forms of proteasomal complexes, including doubly and singly capped 26S, free 20S, and free 19S (Fig. 2-2). The first goal of this study is to understand the distribution of these proteasome species in the brain. Moreover, each of these proteasomes species may be associated with a significant number of proteins. Because 26S proteasomes are responsible for degrading ubiquitinated proteins, this study is focused on characterizing their interacting proteins and regulation. Recently, Besche et al. have described an affinity purification method that allows for the gentle, rapid isolation of 26S proteasomes together with associated proteins [104]. This approach is based upon the high affinity of 26S proteasomes for the UBL domain of Rad23B [107]

and a competitive elution strategy (Fig. 3-1). Through a collaboration with the Goldberg group at Harvard Medical School, I have utilized this newly developed method to purify 26S proteasomes from the adult rat cortex. In particular, 26S proteasomes were successfully purified from biochemically isolated cytosolic and synaptosomal fractions of the cortex. The spectrum of 26S interactors in the cytosol and the synapse were determined by tandem mass spectrometry, and interesting differences were observed. Altogether, brain 26S interactors significantly differ from those found in other mammalian cells [104, 105].

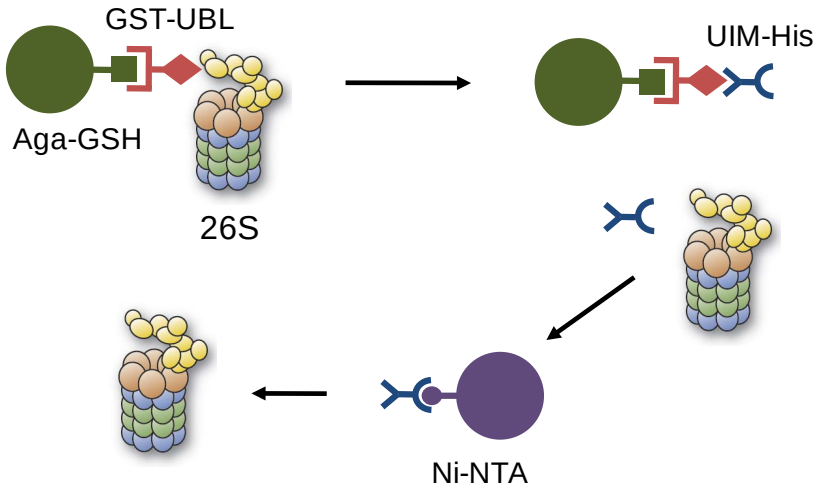
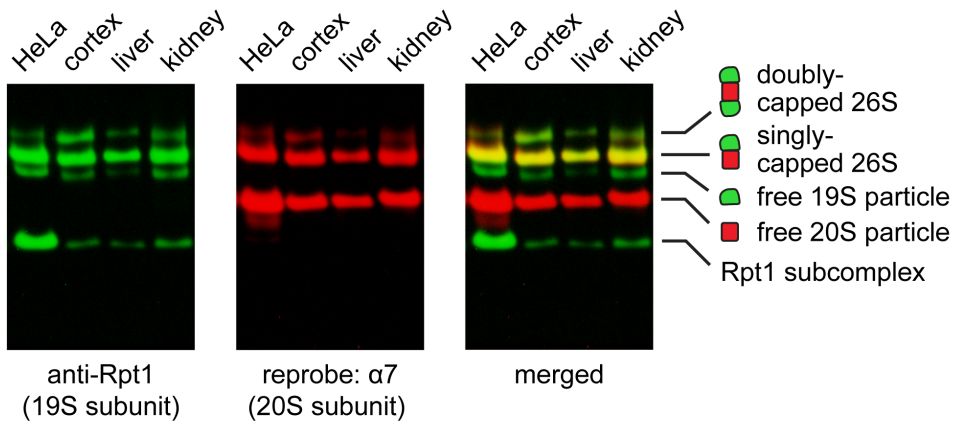


Figure 3-1. Affinity purification of 26S proteasomes. The proteasome is captured using the ubiquitin-like (UBL) domain of Rad23B fused to glutathione-S-transferase (GST) over a matrix of glutathione-agarose (GSH-Aga). Proteasomes are eluted with the ubiquitin-interacting motif (UIM) tagged with polyhistidine. The UIM is taken from the Rpn10 subunit of the proteasome and competes for the UBL. His-UIM can be subsequently removed by absorption to Ni^{2+}-nitrilotriacetic (Ni-NTA) resin. This yields highly purified 26S proteasomes devoid of GST-UBL or His-UIM contamination.

3.2 Neurons are enriched in doubly-capped 26S proteasomes

Since very little is known about the proteasome content of the brain, we first investigated what types of proteasomes may exist in the adult rat cortex. Using native gel electrophoresis followed by immunoblotting against 19S and 20S subunits, we found doubly capped 26S, singly capped 26S, and 20S proteasome in rat cortical extracts (Fig. 3-2a); their relative abundance is quantified and summarized in Fig. 3-2b. Interestingly, the ratio of doubly over singly capped 26S proteasome was higher in the cortex than in liver and the kidney. Additionally, we detected free 19S particles in these cell types, as well as a subcomplex containing the 19S subunit Rpt1 (Fig. 3-2a). My collaborators in Harvard have characterized this complex from liver extracts by tandem mass spectrometry (LC-MS/MS) and found S5b, Rpn1, Rpt1, and Rpt2 (Henrike Besche, personal communication), recently shown to be an intermediate of 19S assembly [108-113]. It should be noted that the resolution of the native gel electrophoresis likely underrepresents the actual heterogeneity of various proteasome species in vivo. For instance, some 20S particle might be associated with the PA28 complex (and other proteins), but these interesting proteins may dissociate readily or fail to alter mobility under these gel conditions. Also, it has been previously observed that a single proteasome band in the native gel can in fact contain diverse interacting proteins in substoichiometric amounts [104].

A



B

cell source	doubly-capped 26S	singly-capped 26S	free 20S proteasome	total 20S	total 20S / mg extract protein
HeLa cell	4±1%	35±2%	61±2%	100%	1.00
rat cortex	14±1%	43±1%	43±2%	100%	0.37±0.03
rat liver	8±2%	43±2%	49±2%	100%	0.52±0.04
rat kidney	8±1%	44±3%	48±5%	100%	0.46±0.04

Figure 3-2. (A) Proteasome species found in different cell types. Extracts of HeLa cell (30 μg), rat cortex (60 μg), rat liver (40 μg), and rat kidney (50 μg) were resolved by 2–5% gradient native gel, immunoblotted against 19S subunit Rpt1, and reprobed against 20S subunit α7. Protein bands were pseudo-colored and merged to illustrate the presence of proteasomes and related species (from top to bottom): doubly-capped 26S proteasome, singly-capped 26S proteasome, free 19S particle and free 20S particle. A fast-migrating band corresponding to a subcomplex of the 19S containing Rpt1 protein was also observed. 19S complex is schematically represented by a green semicircle; 20S complex by a red rectangle. (B) The distribution of different proteasome species in (A), quantified by densitometry (mean ± SEM, n=6)

3.3 Proteasome integrity after subcellular fractionation

In order to understand the role of the UPP in synaptic function and plasticity, I investigated the properties of synaptic proteasomes (outlined in Fig 3-3a). Slight modifications of standard protocols for isolating cytosolic and synaptosomal fraction were sufficient to preserve proteasome integrity, as assessed by native gel electrophoresis (Fig. 3-3b) and activity assays (Fig. 3-4). When cytosolic and synaptosomal extracts were incubated with glutathione S-transferase (GST)-UBL and glutathione sepharose, the flow-through fraction was depleted of 19S-subunit Rpt1 protein (Fig. 3-3c). Thus GST-UBL affinity capture provides an efficient method to purify the entire population of 26S proteasomes, without apparent bias towards a specific subset. By contrast, there was a high amount of α7 subunit remaining in the flow through (Fig. 3-3c), which was consistent with the abundance of free 20S particles (which lack affinity for ubiquitin or UBL-proteins) detected in the native gel (Fig. 3-3b). Also, when GST-alone was used, instead of GST-UBL, no proteasome or other complexes were detectable (Fig. 3-5a). These experiments were carried out in the absence of salt to maximize the content of proteasome-associated proteins [104].

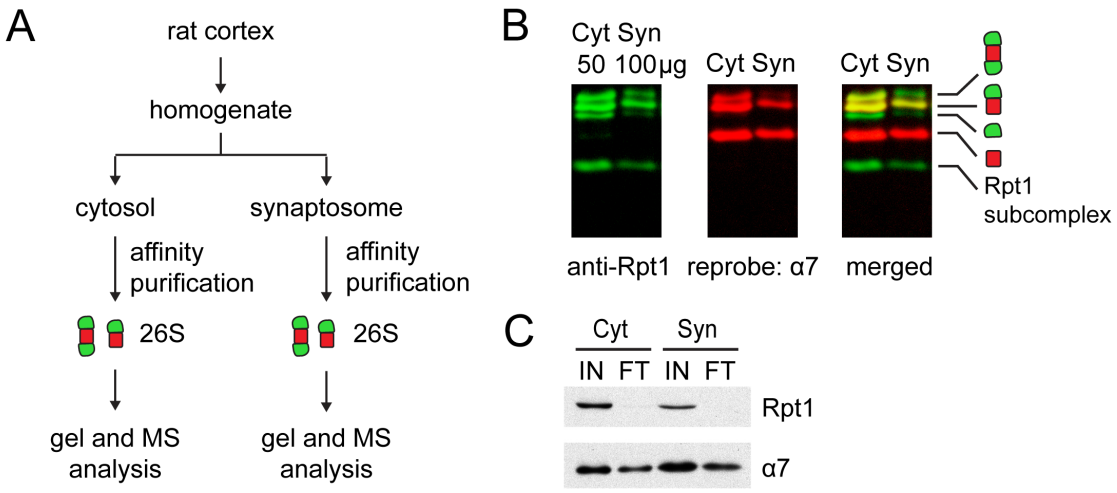


Figure 3-3. (A) Steps used in purification of cytosolic and synaptic 26S proteasomes, and subsequent analysis by gel electrophoresis and mass spectrometry (MS). (B) Proteasome integrity after subcellular fractionation. Cytosolic (Cyt) and synaptic (Syn) extracts prepared from rat cortices were resolved by 2%–5% gradient native gel, immunoblotted against 19S subunit Rpt1, and reprobed for 20S subunit α7. Singly-, doubly-capped 26S, 20S proteasomes and free 19S were detected in both fractions. (C) Efficacy of 26S proteasome isolation by UBL column. Cytosolic (Cyt) and synaptic (Syn) extracts were incubated with GST-UBL and glutathione sepharose. The input (IN) and flow-through (FT) materials were analyzed by SDS-PAGE and immunoblotted against Rpt1 and α7. The depletion of Rpt1 from the flow-through indicated that most 26S proteasomes remained intact during subcellular fractionation and were captured with high efficiency. The high remaining level of α7 subunit in the flow through is consistent with the abundance of free 20S particles detected in (B).

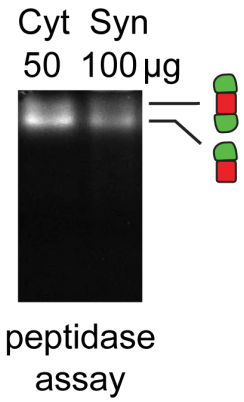


Figure 3-4. Cytosolic and synaptosomal extracts contain active 26S proteasomes. Cytosolic (Cyt) and synaptic (Syn) extracts isolated from rat cortices were resolved by 2%–5% gradient native gel, followed by incubation with Suc-LLVY-AMC, a fluorogenic proteasome substrate. Active 26S proteasomes appear as fluorescent bands.

3.4 Purification of cytosolic and synaptic proteasomes

Using the GST-UBL/His-UIM method, cytosolic and synaptic 26S proteasomes were successfully purified. When resolved by native gel electrophoresis and silver stained, each proteasome sample showed only two prominent bands, corresponding to doubly and singly capped 26S proteasomes (Fig. 3-5a). The identities of proteasome-related bands were confirmed by immunoblotting with antibodies against 19S and 20S subunits (Fig. 3-6). By sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, cytosolic and synaptic 26S proteasomes showed differences in associated proteins, and very few proteins were found in GST controls (Fig. 3-5b). Purified 26S proteasomes from both compartments remained active in hydrolyzing fluorogenic peptide substrates (Fig. 3-5c). Interestingly, the ratio of doubly over singly capped 26S was significantly higher in the cytosol than in the synapse (Fig. 3-5d). Taken together, the data demonstrates that the distribution of proteasome species varies between different cells and tissues as well as between different subcellular compartments of the brain.

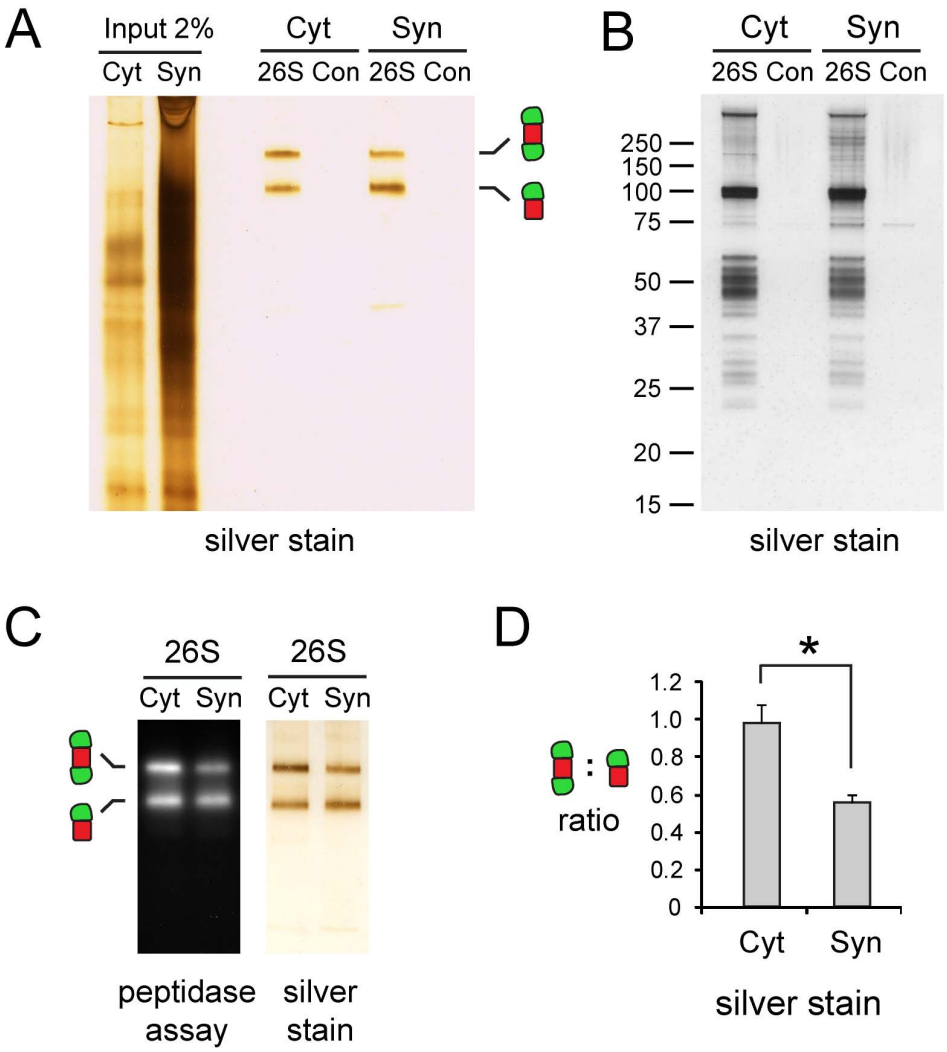


Figure 3-5. (A) Purity of isolated 26S proteasomes. Cytosolic (Cyt) and synaptic (Syn) 26S proteasomes were isolated using GST-UBL/His-UIM. In control (Con) experiments, GST was added instead of GST-UBL. Samples were resolved by 3%-8% gradient native gel and silver-stained. (B) The same samples in (A) analyzed by SDS–PAGE, followed by silver staining. The control experiments showed only a few non-specific protein bands. (C) Activity of isolated proteasomes. Chymotrypsin-like peptidase activity of isolated 26S proteasomes resolved by 3–8% native gel was assayed with Suc-LLVY-AMC. (D) Differences in capped proteasome ratios. Silver-stained 26S proteasome bands in (C) were quantified by densitometry. The ratio of doubly- over singly-capped 26S was significantly higher in the cytosol (Cyt) than the synaptosome (Syn) (mean ± SEM, n=4, *$p<0.05$).

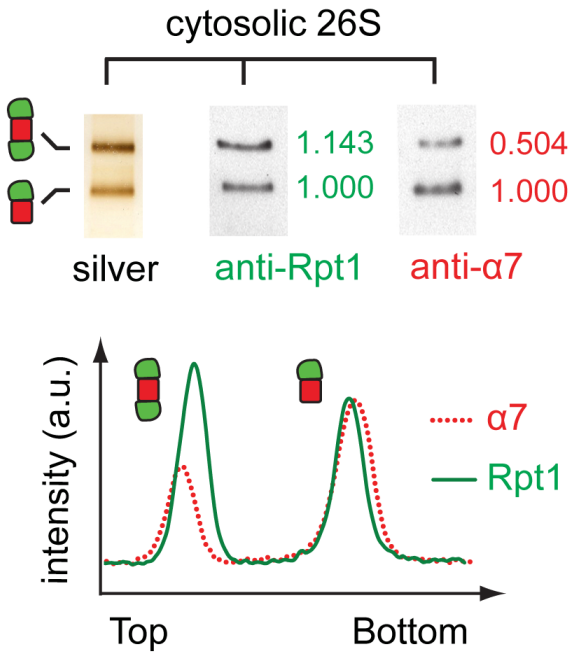


Figure 3-6. Purified 26S proteasomes contain both singly- and doubly-capped species. Isolated cytosolic 26S proteasomes resolved by native gel were silver-stained and immunoblotted against 19S subunit Rpt1 and 20S subunit α7 (top panel). Assuming the 19S:20S ratio in the lower band to be 1:1, band intensities on Western blots are plotted in the lower panel and quantified (see numbers in the top panel). The top band has a 19S:20S ratio close to 2:1 (1.143:0.504). Therefore, the upper band represents 19S-20S-19S (doubly-capped 26S) and the lower band represents 19S-20S (singly-capped 26S).

3.5 Identification of 26S proteasome-interacting proteins

To determine the identity of proteins that co-purified with 26S proteasomes, the samples were digested with trypsin and the resulting peptides were analyzed by mass spectrometry. Protein matches from the GST alone sample, which must represent non-specific contaminants that interact with the matrix, were subtracted from protein matches in the GST-UBL sample. The remaining bound proteins represented either stoichiometric proteasome subunits (α1-7, β1-7, Rpt1-6, Rpn1-3, and Rpn5-12, see Table 3-2) or putative 26S proteasome-interacting proteins (listed in Table 3-1). Included in this latter group were several E3s, DUBs, molecular chaperones, and components of the UPP. During the purification, the majority of ubiquitylated substrates associated with 26S proteasomes were removed because they became bound to His-UIM proteins on Ni-NTA resins, as previously reported [104]. Consequently, ubiquitin or ubiquitin conjugate substrates were not detectable in purified 26S samples. Proteins listed in Table 3-1 therefore most likely represent true proteasome-interacting proteins, not transiently associated proteasome substrates. Many of these proteins have previously also been reported to interact with proteasomes from non-neuronal cells [99, 104-106, 114].

Table 3-1. Mass spectrometric identification of proteins that co-purify with cytosolic and synaptic 26S proteasomes

IPI identifier	Unigene identifier	Gene name	Gene symbol	Cytosolic 26S		Synaptic 26S	
				Unique peptides[a]	Sequence coverage	Unique peptides[a]	Sequence coverage
Substoichiometric proteasome protein							
IPI00367234.3	Rn.154631	Proteasome-associated protein ECM29	Ecm29	6	5%	0	0%
IPI00204510.5	Rn.9320	26S proteasome subunit Rpn13/ ADRM1	Adrm1	3	6%	3	6%
IPI00200601.2	Rn.40430	Thioredoxin-like protein 1/ TRP32	Txnl1	13	56%	14	60%
19S assembly factor							
IPI00194471.1	Rn.13415	26S proteasome non-ATPase subunit 5/ S5b	Psmd5	14	38%	13	37%
IPI00231038.4	Rn.24127	26S proteasome non-ATPase subunit 9/ Bridge-1/ p27	Psmd9	5	24%	5	24%
E3							
IPI00201213.3	Rn.12130	HECT, UBA and WWE domain containing 1/ ARF-BP1	Huwe1	51	21%	54	21%
IPI00365749.3	Rn.198497	Isoform 2 of ubiquitin-protein ligase E3A/ E6-AP	Ube3a	20	27%	16	24%
IPI00768915.1	n/a	Potassium channel modulatory factor 1	Kcmf1	4	27%	4	23%
DUB							
IPI00734588.1	Rn.40424	Ubiquitin carboxyl-terminal hydrolase L5/ UCH37	Uchl5	7	26%	5	23%
IPI00207657.1	Rn.44078	Ubiquitin specific protease 5/ isopeptidase T	Usp5	33	46%	38	48%
IPI00372303.3	Rn.72721	Ubiquitin specific protease 7/ HAUSP	Usp7	8	13%	12	17%
IPI00367362.3	n/a	Ubiquitin specific protease 13	Usp13	9	26%	8	24%
IPI00204532.2	Rn.11790	Ubiquitin specific protease 14	Usp14	20	43%	17	37%

UPP							
IPI00382052.2	Rn.3474	Isoform 2 of TAX1-binding protein 1/ T6BP	Tax1bp1	0	0%	3	6%
IPI00194561.1	Rn.107103	Isoform 1 of sequestosome-1/ p62	Sqstm1	6	28%	5	17%
N-end rule							
IPI00359416.3	Rn.37755	Retinoblastoma protein associated factor p600/ RBAF600/ UBR4	Ubr4	66	18%	74	19%
VCP complex							
IPI00212014.2	Rn.98891	Valosin-containing protein/ p97	Vcp	18	28%	30	41%
IPI00204786.1	Rn.203087	Fas-associated factor 1	Faf1	11	23%	11	21%
Chaperone							
IPI00207355.3	Rn.211303	Heat shock-related 70 kDa protein 2	Hspa2	4	9%	4	9%
Vesicle							
IPI00204644.1	Rn.107689	Isoform B of synaptosomal-associated protein 25	Snap25	0	0%	11	50%
IPI00394488.2	Rn.203179	Glioblastoma amplified sequence/ NIPSNAP2	Gbas	6	31%	6	26%
Cytoskeleton							
IPI00231407.4	Rn.11247	Isoform A of drebrin	Dbn1	0	0%	1	4%
IPI00197579.1	Rn.2458	Isoform 1 of tubulin beta 5	Tubb5	5	17%	4	15%
IPI00195673.1	Rn.98430	Tubulin beta 6	Tubb6	4	13%	3	10%
Calcium binding							
IPI00231771.4	Rn.8937	Protein S100-b	S100b	1	16%	1	16%
Phosphopeptide binding							
IPI00230835.4	Rn.29936	14-3-3 protein gamma	Ywhag	0	0%	1	6%
IPI00324893.4	Rn.1292	14-3-3 protein zeta/delta	Ywhaz	1	6%	1	6%
RASGEF							
IPI00200118.4	Rn.53868	Isoform 2 of GRIP1-associated protein 1/ GRASP-1	Gripap1	0	0%	1	2%
Unknown							
IPI00362402.3	n/a	Alpha/beta hydrolase domain containing 10	Abhd10	0	0%	1	3%

IPI00188755.4	Rn.106046	Caspase recruitment domain-containing protein	C10orf97	4	12%	4	14%
Clathrin complex[b]							
IPI00193983.1	Rn.3589	Clathrin heavy chain	Cltc	0	0%	12	11%
IPI00203346.4	n/a	AP-2 complex subunit alpha-1	Ap2a1	0	0%	1	2%
IPI00471901.3	Rn.34928	AP-2 complex subunit alpha-2	Ap2a2	0	0%	3	6%
IPI00231502.3	Rn.56138	Isoform 2 of AP-2 complex subunit beta-1	Ap2b1	0	0%	2	2%
IPI00196530.1	Rn.3172	AP-2 complex subunit mu-1	Ap2m1	0	0%	2	6%
IPI00358691.2	Rn.162085	Epidermal growth factor receptor pathway substrate 15 isoform B	Eps15	0	0%	3	6%
Mitochondria[c]							
IPI00196750.1	Rn.106922	Single-stranded DNA-binding protein, mitochondrial	Ssbp1	0	0%	4	40%

Note: [a] uniqueness is defined solely by peptide sequence. [b] The clathrin complex appears to directly bind to GST-UBL independent of proteasome. [c] Mitochondrial proteins may be contaminants of synaptosomes.

Table 3-2. 26S proteasome subunits identified by mass spectrometry

IPI identifier	Unigene identifier	Gene name	Gene symbol	Cytosolic 26S		Synaptic 26S	
				Unique peptides	Sequence coverage	Unique peptides	Sequence coverage
20S particle							
IPI00191501.1	Rn.107278	Proteasome subunit alpha type 6 (α1)	Psma6	6	27%	7	31%
IPI00231757.11	n/a	Proteasome subunit alpha type 2 (α2)	Psma2	8	44%	10	50%
IPI00231046.8	Rn.11076	Proteasome subunit alpha type 4 (α3)	Psma4	5	21%	6	16%
IPI00215243.3	Rn.105784	Isoform RC6-IL of proteasome subunit alpha type 7 (α4)	Psma7	11	46%	12	44%
IPI00191502.5	Rn.1276	Proteasome subunit alpha type 5 (α5)	Psma5	7	30%	7	30%
IPI00191748.3	Rn.2668	Proteasome subunit alpha type 1 (α6)	Psma1	13	55%	13	52%
IPI00476178.2	Rn.3997	Proteasome subunit alpha type 3 (α7)	Psma3	6	28%	6	25%
IPI00188686.2	n/a	Proteasome subunit beta type 6 (β1)	Psmb6	4	29%	4	29%
IPI00199980.1	Rn.3846	Proteasome subunit beta type 7 precursor (β2)	Psmb7	4	16%	4	16%
IPI00214889.1	Rn.94551	Proteasome subunit beta type 3 (β3)	Psmb3	5	33%	5	33%
IPI00188584.1	Rn.1981	Proteasome subunit beta type 2 (β4)	Psmb2	10	56%	6	41%
IPI00230992.5	n/a	Proteasome subunit beta type 5 (β5)	Psmb5	12	49%	12	51%
IPI00191749.5	Rn.6016	Proteasome subunit beta type 1 precursor (β6)	Psmb1	7	40%	6	33%
IPI00191505.3	Rn.6169	Proteasome subunit beta type 4 precursor (β7)	Psmb4	8	43%	8	43%

19S particle

IPI00421600.7	Rn.1202	26S proteasome ATPase subunit 2 (Rpt1)	Psmc2	29	55%	28	52%
IPI00211733.1	Rn.10526	26S proteasome ATPase subunit 1 (Rpt2)	Psmc1	24	55%	23	55%
IPI00210158.1	Rn.11341	26S proteasome ATPase subunit 4 (Rpt3)	Psmc4	21	55%	26	70%
IPI00362105.2	Rn.103233	26S proteasome ATPase subunit 6 (Rpt4)	Psmc6	18	53%	18	50%
IPI00190392.3	Rn.11173	26S proteasome ATPase subunit 3 (Rpt5)	Psmc3	27	63%	25	56%
IPI00213587.1	Rn.10972	26S proteasome ATPase subunit 5 (Rpt6)	Psmc5	24	52%	24	55%
IPI00370456.1	Rn.29909	26S proteasome non-ATPase subunit 2 (Rpn1)	Psmd2	40	46%	39	46%
IPI00212512.1	Rn.2891	26S proteasome non-ATPase subunit 1 (Rpn2)	Psmd1	31	37%	29	41%
IPI00370009.1	Rn.101332	26S proteasome non-ATPase subunit 3 (Rpn3)	Psmd3	31	48%	33	54%
IPI00198978.1	Rn.15873	26S proteasome non-ATPase subunit 12 (Rpn5)	Psmd12	27	50%	28	50%
IPI00370382.2	n/a	26S proteasome non-ATPase subunit 11 (Rpn6)	Psmd11	29	55%	27	56%
IPI00189463.1	Rn.103875	26S proteasome non-ATPase subunit 6 (Rpn7)	Psmd6	21	44%	20	39%
IPI00359611.2	Rn.20659	26S proteasome non-ATPase subunit 7 (Rpn8)	Psmd7	12	47%	11	46%
IPI00202283.1	Rn.16918	26S proteasome non-ATPase subunit 13 (Rpn9)	Psmd13	20	56%	21	58%
IPI00372125.3	Rn.161794	26S proteasome non-ATPase subunit 14 (Rpn11)	Psmd14	13	46%	14	53%
IPI00364072.1	Rn.137673	26S proteasome non-ATPase subunit 8 (Rpn12)	Psmd8	7	19%	6	19%

3.6 Confirmation of proteasome-interacting proteins

As it is also possible that a protein can interact directly with the UBL domain of hHR23B (without association with the 26 proteasome), we confirmed the proteasome association of several proteins listed in Table 3-1 by co-sedimentation, which is independent of the GST-UBL interaction. When cytosolic and synaptic extracts were subjected to centrifugation at 100,000x*g* for 6 hr, 20S and 26S proteasomes were largely found in the pellet fraction (Fig. 3-7). Under this condition, proteasome-interacting proteins identified by mass spectrometry should co-sediment with the proteasome. A total of 7 putative proteasome-interacting proteins were examined, including three ubiquitin ligases HUWE1 (HECT, UBA and WWE domain containing 1), E6-AP (E6-associated protein; UBE3A), and KCMF1 (potassium channel modulatory factor 1); USP14 (ubiquitin-specific protease 14, a DUB); PSMD9 (also called p27/Bridge-1), a recently identified 19S assembly factor [108, 109, 113]; ECM29, the only proteasome protein detected exclusively in the cytosol in this study; and 14-3-3γ, which recognizes phosphopeptides and was found only in synaptic proteasomes.

Thus, all proteins tested co-sedimented with the proteasome in the subcellular compartment predicted from the mass spectrometry data (Fig. 3-7). In summary, among the 30 26S-associated proteins identified as listed in Table 1, most were found in both compartments, but only ECM29 was cytosol-specific, and 6 proteins were synaptosome-specific, although four of them could be identified by MS with one unique peptide only.

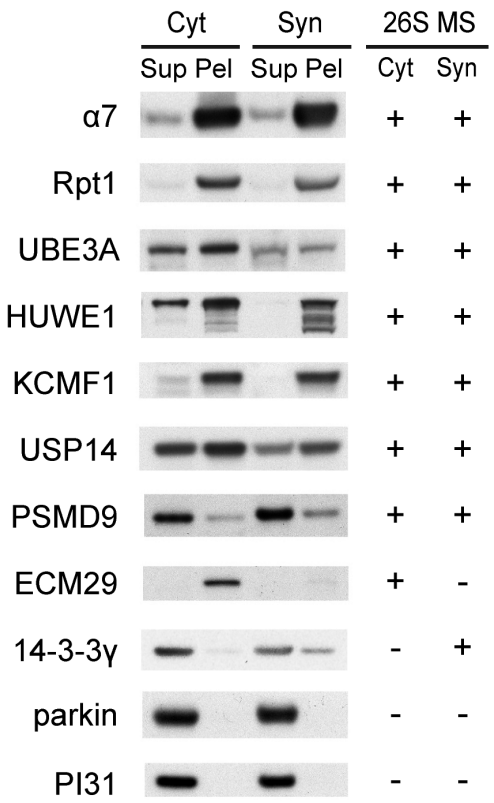


Figure 3-7. Verification of proteasome-interacting proteins. Cytosolic (Cyt) and synaptosomal (Syn) extracts of rat cortices were subjected to centrifugation at 100,000x*g* for 6 hr. The supernatant (Sup) and pellet materials (Pel) were loaded at 1:1 ratio and resolved by SDS-PAGE. The sedimentation of proteasomes was confirmed by the high levels of 20S subunit α7 and 19S subunit Rpt1 in the pellet fraction. Proteins identified by mass spectrometry in 26S proteasomes (26S MS) from the cytosol and the synaptosome are indicated by the positive sign. Agreement between mass spectrometry data and co-sedimentation data was observed for all seven 26S-interacting proteins (UBE3A, HUWE1, KCMF1, USP14, PSMD9, ECM29, 14-3-3γ) probed by specific antibodies (see methods). Parkin and PI31 did not show appreciable association with proteasomes.

In summary, the co-sedimentation data showed very good agreement with the mass spectrometry data to validate our fractionation and purification strategy. It is noteworthy that we could not detect by mass spectrometry parkin (an E3 mutated in autosomal-recessive juvenile Parkinsonism) and PI31 (which may function as an inhibitor of the 20S proteasome), which have been reported as proteasome-interacting proteins in non-neuronal cells [115, 116]. Also, neither co-sedimented with proteasomes (Fig. 3-7).

In addition, clathrin and several of its interactors involved in vesicular trafficking were isolated (Table 3-1). This complex was found to bind the UBL-domain independently of the proteasome in purifications from synaptosomes and microsomes in our preliminary experiments. In this study, ultracentrifugation (1 hr at 100,000x*g*) before UBL-purification efficiently removed this complex; however, traces were still detectable by LC-MS/MS. Whether the clathrin complex can interact with proteasomes remains to be determined.

Another potential concern we had was whether UBL was directly binding to the VCP complex, which contains many factors that can bind to ubiquitin [117]. When proteins captured by GST-UBL affinity capture were immunoblotted against VCP, no signal could be detected (Fig. 3-8). This indicates that, unlike in muscle extracts [104], VCP complexes did not appear to independently bind the UBL domain. We currently cannot explain this difference. Phosphatase inhibitors used in this purification might reduce the binding of VCP complexes to the UBL domain as previously described [104]. In some of the preliminary experiments I performed at the Goldberg lab in Harvard, it appeared that the purification procedure of GST-UBL may also affect its affinity for the VCP complex, but this was not further investigated.

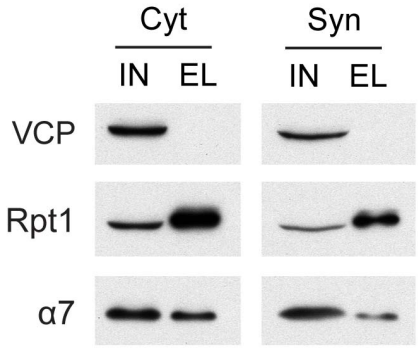


Figure 3-8. VCP is not captured by GST-UBL affinity chromatography. 26S proteasomes from the cytosolic (Cyt) and synaptic (Syn) extracts were purified by the GST-UBL/His-UIM method. The input (IN) material and eluted (EL) fraction containing purified 26S proteasomes were analyzed by SDS-PAGE and immunoblotted against VCP, 19S subunit Rpt1, and 20S subunit α7. The levels of VCP that co-purified with 26S proteasomes were below the limit of Western blot detection.

3.7 Discussions

26S proteasome-interacting proteins have diverse functions

26S proteasome-interacting proteins identified from the rat cortex have diverse cellular roles (Table 3-1). The presence of 3 E3s (KCMF1, HUWE1, and UBE3A) and 5 DUBs (USP5, USP7, USP13, USP14, and UCH37/UCH-L5) is especially significant because they may edit ubiquitin chains or determine substrate selectivity at the proteasome. Some interactors appear to bring substrates to the proteasome by binding to ubiquitylated proteins (Tax1BP1, p62/sequestosome 1) [118, 119] or N-end rule proteins (UBR4/RBAF600) [120]. A heat shock protein 70 family chaperone, HSPA2, may help unfold proteins [121]. The valosin-containing protein (VCP)/p97 complex can bind ubiquitylated proteins and also act as a chaperone [117, 122]. Another interactor, thioredoxin-like 1/TRP32, a disulfide reductase with induced expression upon oxidative stress [123], was recently implicated in transferring nascent chain substrates to the proteasome via a mechanism that involves eEF1A1 [124]. As our purification method utilized RAD23B's UBL domain, RAD23B and related substrate shuttling factors such as ubiquilin 1 [125] were expectedly absent in the interactors we found.

Some of the interactors have been reported as factors that help stabilize or assemble proteasomes, including ECM29 [126, 127], PSMD9 [108, 113, 128], and S5b [110, 112]. The presence of signal transduction proteins may help proteasomes respond to signaling events in the neuron. These include S100B, a small calcium-binding protein [129], and 14-3-3, which recognizes phosphoproteins [130]. Another category of interactors appears

to localize proteasomes to appropriate subcellular structures or locations. These include cytoskeleton proteins (drebrin and tubulin) and the synaptic vesicle protein SNAP-25.

Comparing the spectrum of 26S-interacting proteins from muscle cells, HEK293 cells, and neurons, we find that there are significant differences (smaller than 50% overlap between any two of them). It is not yet understood how proteasome composition is regulated in different cells, which may be an important future direction.

Properties of synaptic proteasomes

There is growing evidence that proteasome-mediated proteolysis plays a critical role in synaptic plasticity (recently reviewed in [12, 14]). We still know relatively little, however, about the proteins targeted for degradation or the molecular mechanisms involved. Although several studies have examined ubiquitin ligases involved in synaptic function [14], a major gap in our knowledge concerns the nature of the proteasome in the central nervous system and in different neuronal compartments. In this study, we used a new isolation method and mass spectrometry to characterize the synaptic and cytosolic 26S proteasomes and their interactors, and on this basis examined activity-induced proteasome changes. Several differences were found between synaptic and cytosolic 26S particles. For example, we found a lower ratio of doubly to singly capped 26S proteasomes in the synapse than in the cytosol. ECM29, which strengthens the association of 19S and 20S particles [126, 127], was present in the cytosolic 26S but not the in the synaptic 26S, which may contribute to the high amount of doubly capped 26S in the cytosol. The functional differences between doubly and singly capped 26S have not been established. Both forms can degrade ubiquitylated proteins [131], and thus it is

possible that the doubly capped form has twice the capacity to bind and degrade substrates. On the other hand, the singly capped 26S has an exposed 20S α-ring that allows it to interact with additional regulators such as PA28 or PA200 [27]. Such interacting proteins may dissociate during native gel electrophoresis. Consequently, the singly-capped species seen here may in fact represent in vivo hybrid particles, 19S-20S-X, where X could be PA28, PA200, or unknown factors.

Several proteasome-interacting proteins identified are unique to the synaptic 26S proteasome (14-3-3γ, TAX1BP1, drebrin, SNAP-25) and provide new insights. 14-3-3γ, which recognizes phosphopeptides [130], may coordinate proteasome function with protein phosphorylation events that regulate synaptic transmission [132]. TAX1BP1 is a ubiquitin-binding protein [118, 133] that may promote substrate-proteasome association at the synapse. It will be interesting to examine which synaptic proteins interact with TAX1BP1. Previous studies suggested that proteasomes are sequestered in dendritic spines by interacting with the actin cytoskeleton [25]. Our data suggests that the interaction may be partially mediated by drebrin, an important actin-binding protein in the spine [134]. At the presynaptic terminal, proteasome-mediated proteolysis can regulate synaptic vesicle release in several ways [48, 49]. We found synaptic proteasomes interact with SNAP-25, a key synaptic vesicle protein [135, 136], which may provide a mechanism for the proteolytic control of vesicle dynamics.

Although cytosolic and synaptic 26S proteasomes interact with many similar proteins (Table 3-1), their properties and functions may differ at the synapse. For example, here we confirm the presence of USP14 on synaptic proteasomes by both affinity co-purification and co-sedimentation. It is noteworthy that the current model for *ataxia* (*ax*^{J})

mice is that the loss of USP14 from synaptic proteasomes may lead to enhanced proteolysis, as occurs in yeast lacking the homologous DUB [114]. *Usp14* has a long and a short splicing isoform in mouse brain, and the latter lacks the N-terminal UBL domain necessary for proteasome binding. In fact, ax^J mice arise from spontaneous mutations in the loss of the long isoform, accompanied by altered synaptic activity and movement defects [137, 138].

Synaptic proteasomes and receptor endocytosis

Regulating the surface level of receptors and channels is a key mechanism in synaptic plasticity [61, 132]. There is increasing evidence that one of the major functions of synaptic proteasomes is to facilitate the endocytosis of receptors and channels (reviewed in [13, 14]), and most likely proteins interacting with synaptic 26S proteasomes contribute to this process.

When neurons are exposed to high concentrations of glutamate or NMDA, glutamate receptors are rapidly endocytosed [59, 60]. Two studies have shown that the endocytosis of glutamate receptors requires proteasome activity [55, 57]. The key proteolytic targets are likely to be the PSD scaffolding proteins that help anchor receptors, including Shank, GKAP, GRIP1, and AKAP79/150 [51, 56, 57, 102]. Glutamate receptors released from the PSD are then internalized at the lateral zone of the spine [58]. As discussed earlier, under these circumstances, 26S proteasomes are recruited into dendritic spines [25], but the ligases that are co-recruited to help determine substrate selectivity are not known. Here we identified multiple E3s and DUBs that associate with both cytosolic and synaptic 26S proteasomes, which may co-migrate with proteasomes to PSD sites. Most,

but not all of these DUBs and ligases are also found on 26S from skeletal muscles [104]. These proteasome-associated DUBs presumably function together with Rpn11 subunit (a different type of DUB) to catalyze the disassembly of the ubiquitin chain to allow the degradation of the protein substrate [139]. Exactly why so many different deubiquitylating activities are required at this step is unclear. Also quite mysterious is why these particular ligases are associated directly with the proteasome since hundreds of other E3s also exist in mammals and the great majority appear to function without association with the 26S. Which postsynaptic proteins are targeted by proteasome-associated E3s and DUBs also remain to be elucidated. The PSD is a large assembly of densely packed proteins [50] and once some PSD scaffolding proteins are ubiquitylated, it is not clear how the proteins dissociate from the PSD for degradation by the proteasome. Among the chaperones we found in synaptic 26S proteasomes, the VCP complex may assist this process, because it is a powerful chaperone and also binds to polyubiquitylated proteins [117].

Some proteasome interactors may help localize the proteasome to endocytic substrates. Sequestosome 1/p62 can bind to the GluR1 subunit of α-amino-3-hydroxy-5-methyl-4-isoazolepropionate (AMPA) receptor, D2 dopamine receptor, type C gamma-aminobutyric acid ($GABA_C$) receptor, and potassium channel subunit Kvβ2 [140]. Similarly, TAX1BP1 has been shown to bind $GABA_C$ receptors [141]. Because p62 and TAX1BP1 also bind to ubiquitylated substrates, we propose that they may recruit the proteasome to facilitate the UPP-dependent endocytosis of receptors and channels.

Based on the proteomic characterization of synaptic 26S proteasomes, we may put forth a tentative model for how 26S interactors facilitate the degradation of PSD proteins

(Fig. 3-9). Currently, the function of most 26S interacting proteins remains uncharacterized, and this model may serve as a starting point for future studies.

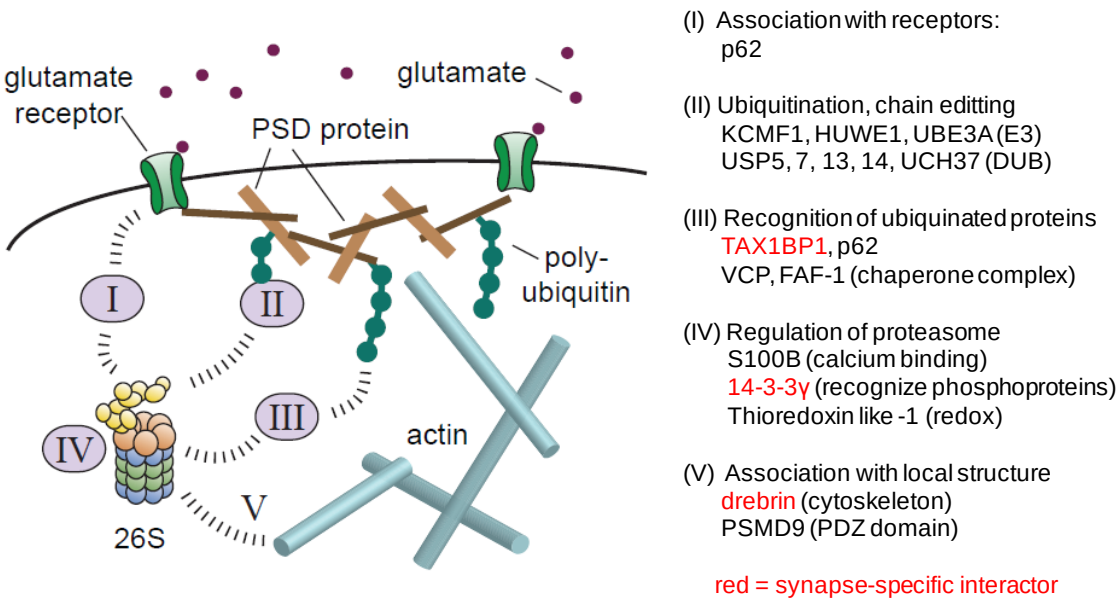


Figure 3-9. A hypothetical model for how proteasome interactors participate in the degradation of PSD proteins. Here, proteasome interactors are divided into functional categories with different roles in proteolysis. However, we know very little about their actual function at the synaptic 26S proteasome.

3.8 Experimental methods

Reagents

Antibodies against the following antigens were purchased commercially: α7 (PW8110), Rpt1 (PW8825), UBE3A (PW0535), ubiquitin conjugates (UG9510), PI31 (PW9710) from Biomol/Enzo (Plymouth Meeting, PA); ECM29 (PA3-035), USP14 (MA1-57109), PA28α (PA1-960) from Affinity Bioreagents/Thermo (Rockford, IL); parkin (MAB5512), ubiquitin monomer (MAB1510) from Chemicon/Millipore (Billerica, MA); PSMD9 (sc-10670), 14-3-3γ (sc-731) from Santa Cruz Biotechnology (Santa Cruz, CA); HUWE1 (A300-486A) from Bethyl (Montgomery, TX); tubulin β3 (T8860) from Sigma (St Louis, MO); KCMF1 (15-288-21213) from Genway (San Diego, CA). Nonidet P-40 (NP-40) was purchased from BDH (Poole, England). Protease inhibitor cocktail (complete, EDTA-free) was purchased from Roche (Indianapolis, IN). Phosphatase inhibitor (cocktail 2) and chloroquine were purchased from Sigma. Epoxomicin was purchased from Biomol and EMD Bioscience (Gibbstown, NJ).

Recombinant protein purification

GST-UBL and *His-UIM* plasmids and their expression in bacteria have been previously described [104]. Cells expressing GST-UBL were lysed by sonication in phosphate buffered saline, and purified using glutathione sepharose 4B (GE Healthcare, Piscataway, NJ). Cells expressing His-UIM were lysed by B-PER extraction reagent (Pierce/Thermo), and purified using Ni-NTA resin (Qiagen, Valencia, CA). Detailed procedures are described below:

pDEST-15-HHR23B^{UBL} plasmid was transformed into BL21AI (Invitrogen) according to manufacturer's instructions. Cells were grown at 37 ^{o}C with ampicillin to an OD_{600} of 0.5 and induced for 3 hr with 1 g/L L-arabinose. Cell pellet from 1 L culture was resuspended in 100 mL ice-cold phosphate buffered saline (PBS, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mL KCl, 137 mM NaCl, pH 7.4) with 2mM DTT, 10 mM $MgCl_2$, 0.2 mg/mL lysozyme and protease inhibitors (complete, Roche). The entire purification procedure was carried out at 4 ^{o}C. Cells were lysed by sonication, and the cell debris was removed by centrifugation at 20,000xg for 30 min. The supernatant was passed through 0.2 μm filters and supplemented with 0.2% Triton X-100, followed by incubation with 20 mL of glutathione sepharose for 2 hr. The resin was transferred to a gravity-flow column, and washed several times in PBS with 0.2% Triton X-100. GST-UBL was eluted by adding 50 mL of elution buffer (75 mM Tris pH 8.8, 150 mM NaCl, 40 mM reduced glutathione), and the collected fractions were combined and dialyzed against buffer B (25 mM HEPES pH 7.5, 10% glycerol, 5 mM $MgCl_2$ 1 mM DTT). Small aliquots of 10 mg/mL GST-UBL were frozen in liquid nitrogen and stored at -80 ^{o}C. The final yield from 1L culture was ~100 mg.

pET26b-S5a^{UIM2} plasmid was transformed into BL21 (DE3) (Promega) according to manufacturer's instructions. Cells were grown at 37 ^{o}C with kanamycin to an OD_{600} of 0.8, followed by induction with 0.5 mM IPTG for 2 hr at 30 ^{o}C. Cell pellet from 1 L culture (~20 mg of His-UIM) was resuspended in 30 mL B-PER extraction reagent (Pierce/Thermo) supplemented with 3 mM 2-mercaptoethanol and DNase I (Sigma). After 15 min of extraction at 25 ^{o}C with mixing, the lysate was cooled to 4 ^{o}C and centrifuged at 20,000xg for 20 min. The supernatant was passed through 0.2 μm filters,

supplemented with 10 mM imidazole, and incubated with 10 mL of Ni-NTA for 1 hr. The resin was transferred to a gravity-flow column and washed first with a 1:1 mixture of B-PER and wash buffer (50 mM Tris pH 7.5, 40 mM imidazole, 300 mM NaCl), and then just wash buffer. 20 mL of elution buffer (50mM Tris pH 7.5, 250 mM imidazole, 300 mM NaCl) was added, and collected fractions containing His-UIM were combined and dialyzed against buffer B with 40 mM KCl. Protein concentration was compared to bovine serum albumin standards (Pierce/Thermo) using two gel-staining methods: Coomassie blue and E-Zinc stain (Pierce/Thermo). Small aliquots of 2 mg/mL His-UIM were frozen in liquid nitrogen and stored at -80 ^{o}C.

Neuronal cultures and tissues

Cortex, liver, and kidney were dissected from 4–6 weeks old male Sprague-Dawley rats. Dissociated hippocampal neurons were prepared and maintained as previously described [142]. Briefly, hippocampi from postnatal day 0 to 2 rats were dissected out and dissociated by papain and plated at a density of 70,000 cells/cm^2 onto poly-D-lysine-coated 60 mm culture dishes (Falcon). Cultures were maintained in neuronal growth medium, which is Neurobasal A medium containing B-27 and Glutamax supplements (Invitrogen), at 37 ^{o}C for 21–28 days before use. Tissue extracts were prepared in buffer A (20 mM HEPES, 0.32 M sucrose, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, protease and phosphatase inhibitors, pH 7.2) with 0.2% NP-40, using motor-driven Potter-Elvehjem homogenizers. Extracts of cultured neurons were prepared in buffer A with 0.5% NP-40, using glass dounce homogenizers. Extracts were cleared by centrifugation at 18,000x*g* for 15 min to remove nuclei and cell debris, and protein concentrations were measured by Coomassie Plus protein assay (Pierce).

Neuronal treatment

Prior to NMDA stimulation, conditioned medium was collected from neuronal culture dishes. Neuronal growth media containing 20 μM NMDA was added to the culture for 3 min, followed by two washes with HEPES buffered saline (20 mM HEPES pH 7.4, 119 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 30 mM glucose). After the second wash, conditioned medium was added to the culture.

Synaptosome isolation

Adult rat cortices rapidly frozen in liquid nitrogen and stored at -80 ^{o}C were used for isolating synaptosomes, based on standard protocols [143] with buffer modifications to stabilize proteasomes. The procedure is schematically represented in Fig. 3-10. Briefly, cortices from 4 rats were homogenized in a motor-driven Potter-Elvehjem homogenizer in 30 mL buffer A (20 mM HEPES, 0.32 M sucrose, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, protease and phosphatase inhibitors, pH 7.2). The homogenate was centrifuged at 1000xg for 5 min, and the supernatant was collected as S1 (if cloudy, centrifuged one more time). S1 was centrifuged at 12,000xg for 20 min, and the pellet (P12) was resuspended in buffer A and centrifuged again at 12,000xg for 20 min. The synaptosome pellet (P12') was resuspended in 11 mL buffer A with 1% NP-40 using a Potter-Elvehjem homogenizer to disrupt the vesicles and extract proteins. This was followed by centrifugation at 18,000xg for 10 min, and then at 100,000xg for 1 hr to sediment clathrin complexes and debris. The supernatant (S100) was collected as synaptosomal extract. To collect the cytosolic extract, supernatant S12 was centrifuged at 100,000xg to remove microsomes. The supernatant was collected as cytosolic extract and supplemented with 1% NP-40. Synaptosomal protein yield from 4 cortices was ~50 mg.

Proteasome capture and sedimentation

GST-UBL was added to cell extracts (50 µg/1 mg of total protein) and incubated with glutathione sepharose to capture and deplete 26S proteasomes. To sediment proteasomes, cell extracts were pre-cleared at 100,000x*g* for 1 hr, followed by centrifugation at 100,000x*g* for 6 hr. Pellets were redissolved in 50 mM Tris pH 7.5, 6M urea, and 1% SDS.

Proteasome activity assay

26S proteasome activity in cell extracts was measured by a proteasome activity kit (Biomol/Enzo) and a fluorometer, based on the hydrolysis of Suc-LLVY-AMC, a fluorogenic substrate for chymotrypsin-like peptidase activity. Native gels containing 26S proteasomes were incubated with reaction buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 50 µM Suc-LLVY-AMC) for 30 min at 37 ^{o}C.

Affinity purification of 26S proteasomes

From cytosolic and synaptosomal extracts (both in buffer A with 1% NP-40), 26S proteasomes were isolated using the GST-UBL/His-UIM method essentially as previously described [104]. The purification was conducted at 4 ^{o}C. First, cytosolic or synaptosomal extracts (20 mg total protein) were incubated for 2 hr with 1 mg GST-UBL and 200 µL glutathione sepharose 4B. After transferring to a gravity-glow column, resins were washed with 20 mL buffer A plus 0.5% NP-40, followed by 30 mL of buffer B plus 1 mM ATP and 5 mM $MgCl_2$. Proteasomes were eluted by a 20 min incubation with 250 µL His-UIM (2 mg/mL, with 2 mM ATP), and repeated one more time. The combined eluate was incubated with 120 µ Ni-NTA for 20 min, and passed through 0.22 µm spin-

filters (ultrafree MC, Millipore). The filtrate contained purified 26S proteasomes. For control samples, 0.7 mg GST was substituted for GST-UBL in the purification procedure.

Immunoblotting and silver staining.

PVDF membranes with transferred proteins were blocked with 5% non-fat milk in 50 mM Tris pH 7.5, 150 mM NaCl, and 0.05% Tween-20. Primary antibodies and HRP-conjugated secondary antibodies were applied in blocking reagent, followed by standard chemiluminescence detection. Band intensities on scanned films were quantified by densitometry using the gel analysis function in ImageJ software. Silver staining was performed with SilverSNAP kit II (Pierce/Thermo).

Native gel electrophoresis

To resolve proteasomes from cell extracts, 2–5% gradient native gels were prepared using the mini-protean 3 system and acrylamide/Bis (37.5:1) from Bio-Rad. The gel formulation was modified from published protocols for discontinuous native gels [144] based on Tris-borate-EDTA buffer (TBE, 90 mM Tris base, 80 mM boric acid, 0.1 mM EDTA, pH 8.3). Each 1.5 mm, 10-well gradient gel was made by mixing 3.3 mL of the upper gel solution (70 mM Tris pH 6.8, 2% acrylamide, 0.06% APS, 0.12% TEMED) and 6.3 mL of lower gel solution (1X TBE, 5% acrylamide, 3.5% sucrose, 1 mM DTT, 1 mM ATP, 5 mM $MgCl_2$, 0.05% APS, 0.1% TEMED). Samples were mixed with 4X loading buffer (100 mM Tris pH 8.0, 20% glycerol) just before loading. Electrophoresis was carried out at 6–10 ^{o}C, in TBE running buffer (with 0.5 mM DTT, 0.5 mM ATP and 2 mM $MgCl_2$). The applied voltage was gradually increased with time (30V for 30 min, 35V for 1 hr, 50 V for 1 hr, 65V for 2–4 hr). To resolve isolated 26S proteasomes, 1.5 mm, 10-well 3–8% Tri-acetate gels (Invitrogen) were used. Gel loading buffer and

running buffer were the same as above. Electrophoresis was carried out at 4 ^{o}C and 150 V for 4 hr.

For immunoblotting, proteins in native gels were transferred to PVDF membranes using Bio-Rad mini-protean 3 transfer system (buffer contains 25 mM Tris base, 192 mM glycine, 0.1% SDS) for 4 hr at 70 V (current limit: 350 mA) in a 4 ^{o}C room. Fluorescent bands around 26S proteasomes were visualized and quantified by standard gel-imaging systems for DNA staining by ethidium bromide.

Mass spectrometry.

Mass spectrometric analysis of purified proteasomes was performed with the generous support of Dr. Wilhelm Haas and Dr. Steven Gygi at Harvard Medical School. Proteasome samples were sent to the Gygi lab, where they were digested with trypsin and the generated peptides were subjected to nanoscale-microcapillary reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS) using an in-house packed C_{18} 125 μm I.D. capillary column and a hybrid linear ion trap/FT-ICR mass spectrometer (LTQ FT, Thermo Electron, Bremen, Germany) as described previously [145]. Using the SEQUEST algorithm, MS/MS data were searched against a concatenated target-decoy database created based on the rat IPI protein database including sequences of known contaminant proteins. Search results were filtered to a protein false discovery rate of less than 1% [146]. Data shown in Table 3-1 and 3-2 were compiled from two independent experiments, and each experiment used four rats to prepare brain homogenates.

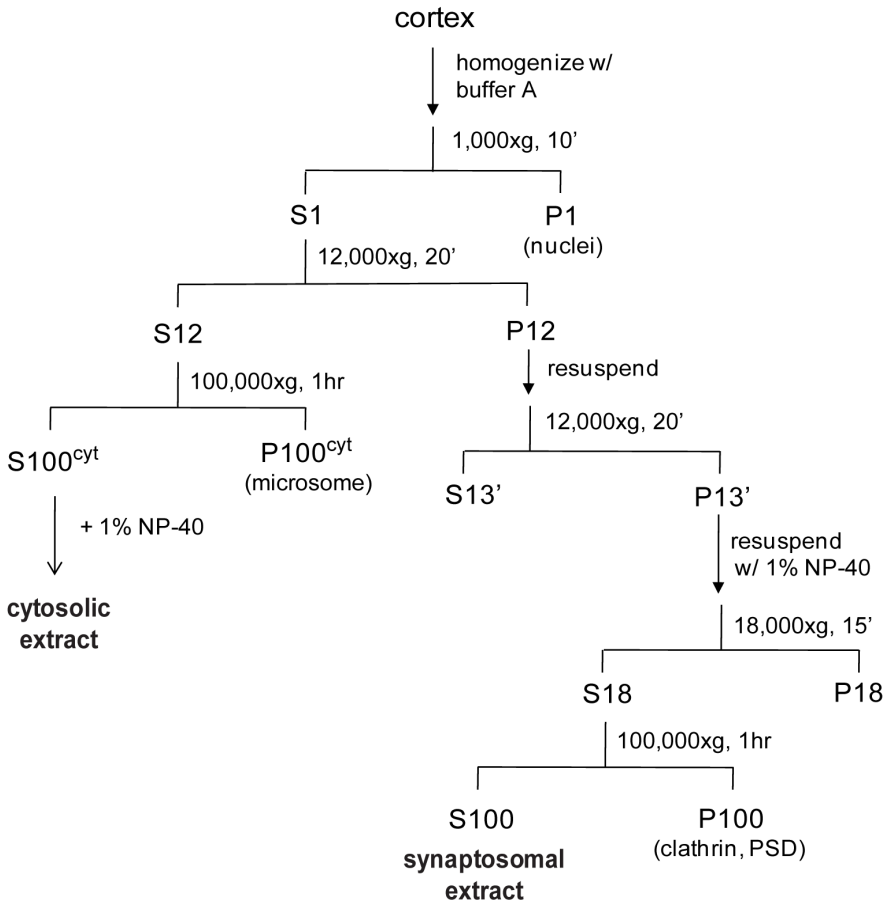


Figure 3-10. A schematic showing the preparation of synaptosomal and cytosolic extracts from rat cortices. We include an additional centrifugation step (1 hr, 100,000xg) after solubilization of synaptosomes with NP-40 and before incubation with the UBL domain. Solubilization leads to binding of an unrelated clathrin complex to the UBL matrix, which complicates the mass spectrometric analysis (data not shown). The respective centrifugation step removes the bulk of this clathrin complex while the proteasome remains fully soluble.

CHAPTER 4

Proteasome composition is regulated by synaptic activity

4.1 Introduction

Studies from non-neuronal cells have suggested that the proteasome complex is highly plastic and subject to many forms of regulation [98]. In neurons, several forms of synaptic plasticity have been shown to require UPP activity [46, 147]. We are therefore interested in understanding how neuronal activity regulates the UPP. Our lab has previously shown that, when neuronal activity is stimulated by depolarization, proteasomes are recruited to dendritic spines to facilitate the endocytosis of glutamate receptors [25, 55, 56]. Hence, protein ubiquitination is not the only aspect of the UPP

regulated by neuronal activity. This chapter further examines whether proteasome levels and compositions are regulated by neuronal activity.

4.2 26S proteasomes decrease after NMDA exposure

A prior study showed that several minutes of neuronal stimulation by KCl or NMDA enhanced the subsequent degradation of a model UPP substrate in dendrites and caused 26S proteasomes to enter dendritic spines within minutes [25]. Therefore we examined whether proteasome levels and proteasome-interacting proteins are regulated by similar activities. In this experiment, cultured rat hippocampal neurons were stimulated with 20 μM NMDA for 3 min, a common protocol for the pharmacological induction of long-term plasticity (chemical LTD) [148]. Due to low synaptosome yields from neuronal cultures, it was not possible to perform subcellular fractionation, and instead we analyzed total extracts at 30 min and 4 hr post-NMDA treatment for changes in proteasome properties.

To our surprise, the greatest changes in neuronal proteasome occurred at 4 hr post-treatment. At this time point, 26S proteasome levels determined by native gel electrophoresis (Fig. 4-1a) showed a significant decrease (25±3%, $p<0.05$). At the same time, levels of the free 19S and the 19S precursor (Rpt1-subcomplex) decreased similarly, and free 20S levels increased by 30±5% ($p<0.05$), while the total 20S level (26S+free 20S) remained constant when compared to untreated controls (Fig. 4-1c). This result is consistent with the disassembly of 26S proteasomes into 20S and 19S particles. However, free 20S particles increased but free 19S particles decreased, suggesting the disassembly of the 19S particle.

Next, we asked if the subunits of the disassembled 19S particles became free proteins or became degraded. At 4 hr post-NMDA, we did not detect free subunits of the 19S in the neuronal extract using the proteasome sedimentation assay. In the sedimentation experiment, free subunits of the 19S should remain in the supernatant instead of precipitating into the pellet, but no 19S protein was detectable in the supernatant (Fig. 4-3a). On the other hand, the total 19S level in the neuronal extract decreased by 33±4% (Fig. 4-1b and 4-1d). Thus, the data suggests that 19S particles dissociated from 20S particles eventually became degraded, resulting in an observed shift from 26S proteasomes to free 20S proteasomes.

To our knowledge, this is the first report of 19S catabolism in mammalian cells and therefore its molecular mechanism remains unclear. To examine if 19S degradation occurs through a UPP- or lysosome-dependent proteolysis, proteasome inhibitor epoxomicin and lysosome inhibitor chloroquine were applied shortly after NMDA was washed out (Fig. 4-2). Surprisingly, neither inhibitor was able to block 19S degradation. There are several possible explanations for this result. First, 19S degradation may occur in a proteasome- and lysosome-independent manner, and additional proteolytic mechanisms may be involved. Secondly, both the proteasome and the lysosome may be able to degrade 19S components and the cell is able to compensate when either pathway is blocked. Third, the inhibitor may be applied too late to affect 19S catabolism. Additional experiments will be required to resolve these possibilities.

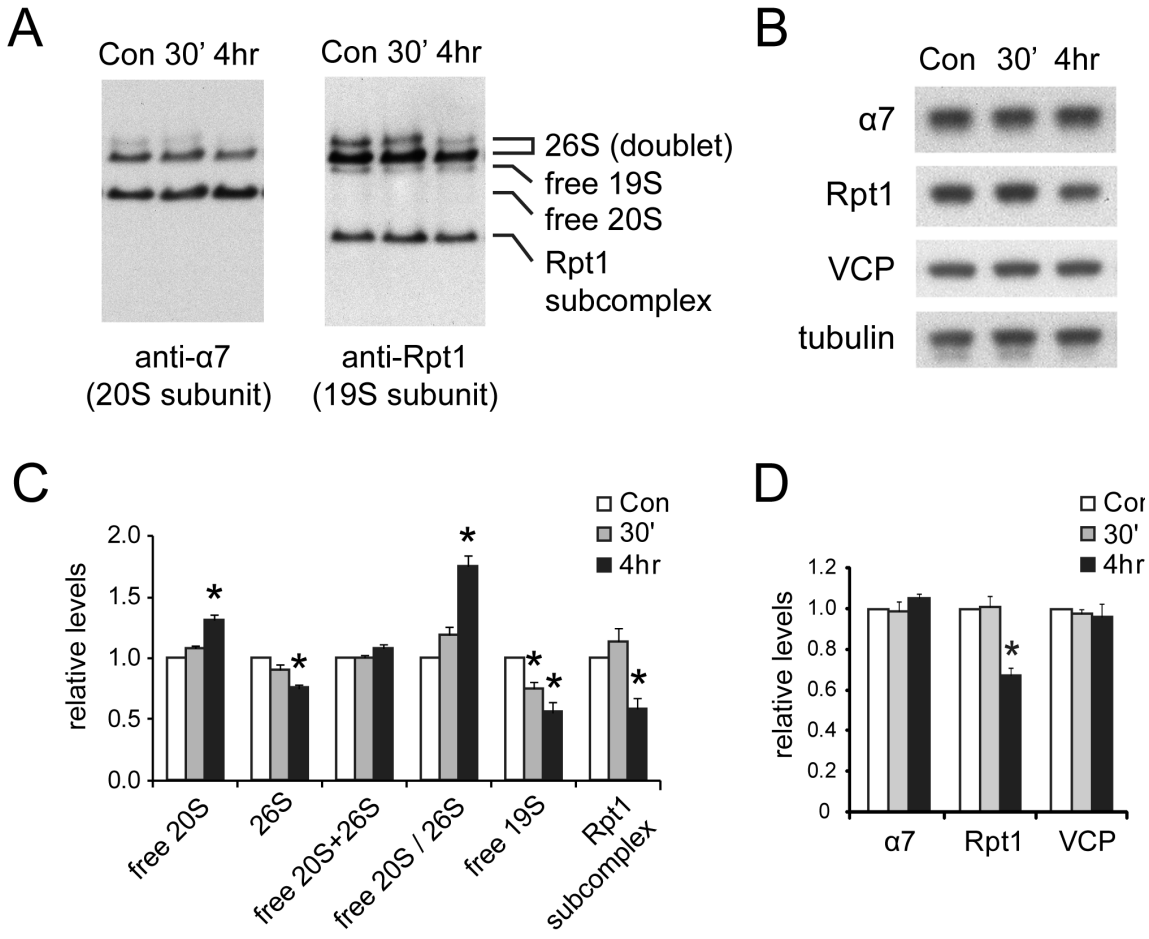


Figure 4-1. (A) Neuronal proteasome profile changes induced by NMDA treatment. Cultured hippocampal neurons were treated with 20 μM NMDA for 3 min, and total cell extracts were prepared at 30 min or 4 hr post-treatment. Untreated control (Con) and treated samples were resolved by 2–5% gradient native gel and immunoblotted against 20S subunit α7 and 19S subunit Rpt1. (B) Extracts from (A) resolved by SDS-PAGE and probed for total levels of α7, Rpt1, and VCP, an abundant UPP protein. Tubulin served as the loading control. (C) Quantification of changes in proteasome profile in (A) by densitometry (n=6, mean ± SEM, *p<0.05). (D) Quantitation of the result in (B) by densitometry. 19S subunit Rpt1 shows significant decrease at 4 hr post-NMDA (n=6, *p<0.05).

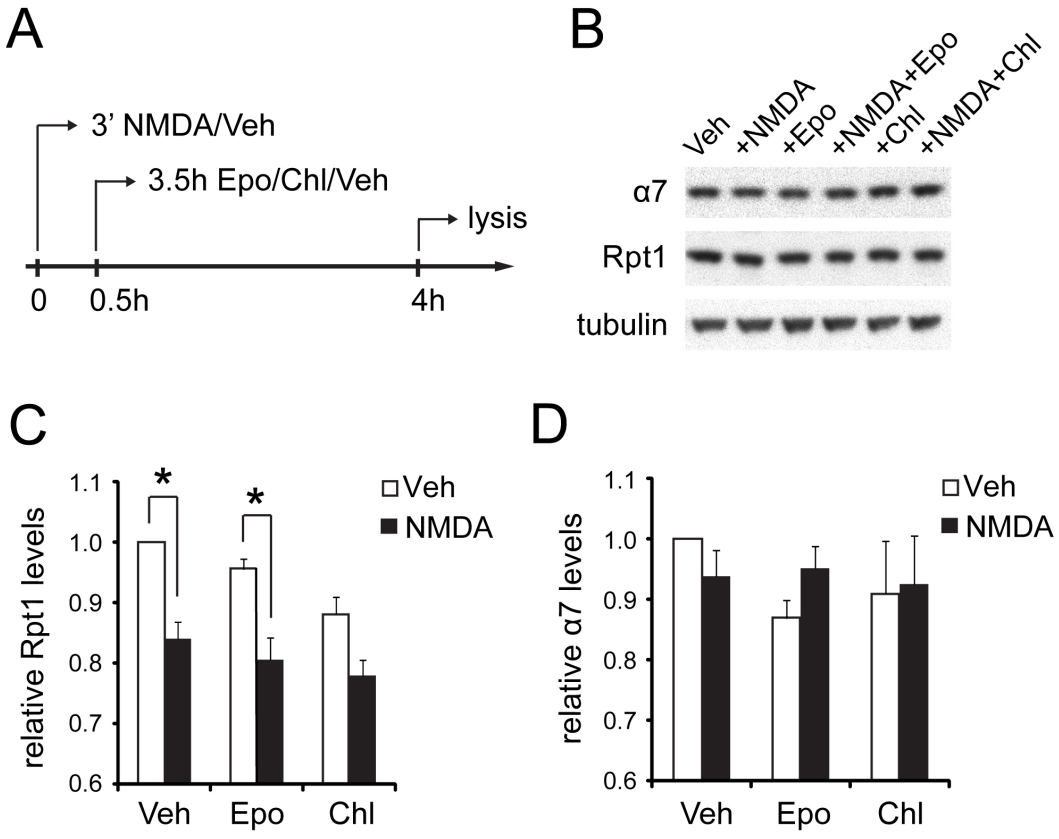


Figure 4-2. 19S degradation is not blocked by proteasome or lysosome inhibitors. (A) Pharmacological treatment of cultured neurons. Cells were treated with 20 μM NMDA, 1 μM proteasome inhibitor epoxomicin (Epo), 0.1 mM lysosome inhibitor chloroquine (Chl), or vehicle (Veh) as indicated. (B) Whole-cell lysates of treated neurons were immunoblotted for 20S subunit α7 and 19S subunit Rpt1. Tubulin served as loading control. (C) Total 19S levels quantified by densitometry. (n=4, mean ± SEM, *p<0.05 by paired *t*-test). Epoxomicin and chloroquine do not prevent NMDA-induced 19S degradation. (D) Total 20S levels quantified by densitometry (mean ± SEM , n=4). NMDA does not induce 20S degradation.

4.3 NMDA exposure affects proteasome-bound E3s

Next, we examined if proteasome-interacting E3s and DUBs were co-degraded with the 19S. While ubiquitin ligases HUWE1 and UBE3A showed significant degradation at 4 hr post-NMDA, the ligase KCMF1 and the DUB enzyme USP14 remained unchanged (Fig. 4-3a and 4-3b). We estimated the degree of HUWE1 and UBE3A's association with proteasomes after NMDA stimulation by co-sedimentation experiments (100,000x*g*, 6 hr) (Fig. 4-3c). At 4 hr post-NMDA, the fraction of HUWE1 and UBE3A in the proteasome pellet was lower than untreated controls (Fig. 4-3d), suggesting that the subpopulation of HUWE1 and UBE3A associated with proteasomes was more sensitive to degradation than the unbound pool. In contrast, neither KCMF1 protein levels nor its association with the proteasome were significantly affected by NMDA exposure (Fig. 4-3b and 4-3d). The data also imply that different E3s may be associated with different subpopulations of proteasomes, because not all E3s are equally affected by 26S disassembly and 19S degradation.

The apparent shift from 26S from 20S proteasomes has two potential outcomes: one is reduced capacity for ubiquitin- and 26S-dependent proteolysis, and the other is increased capacity for ubiquitin-independent, 20S-dependent proteolysis. It has been reported that 20S proteasomes can degrade oxidized proteins independent of ubiquitination [149, 150]. Therefore, we also examined the interactors of 20S proteasomes after NMDA stimulation. After the increase in 20S levels, 20S-interactors such as proteasome activator PA28α and proteasome inhibitor PI31 did not show increased association with 20S proteasomes (Fig. 4-3d). It is still unclear if the newly released 20S proteasomes from the disassembled 26S proteasomes are proteolytically active.

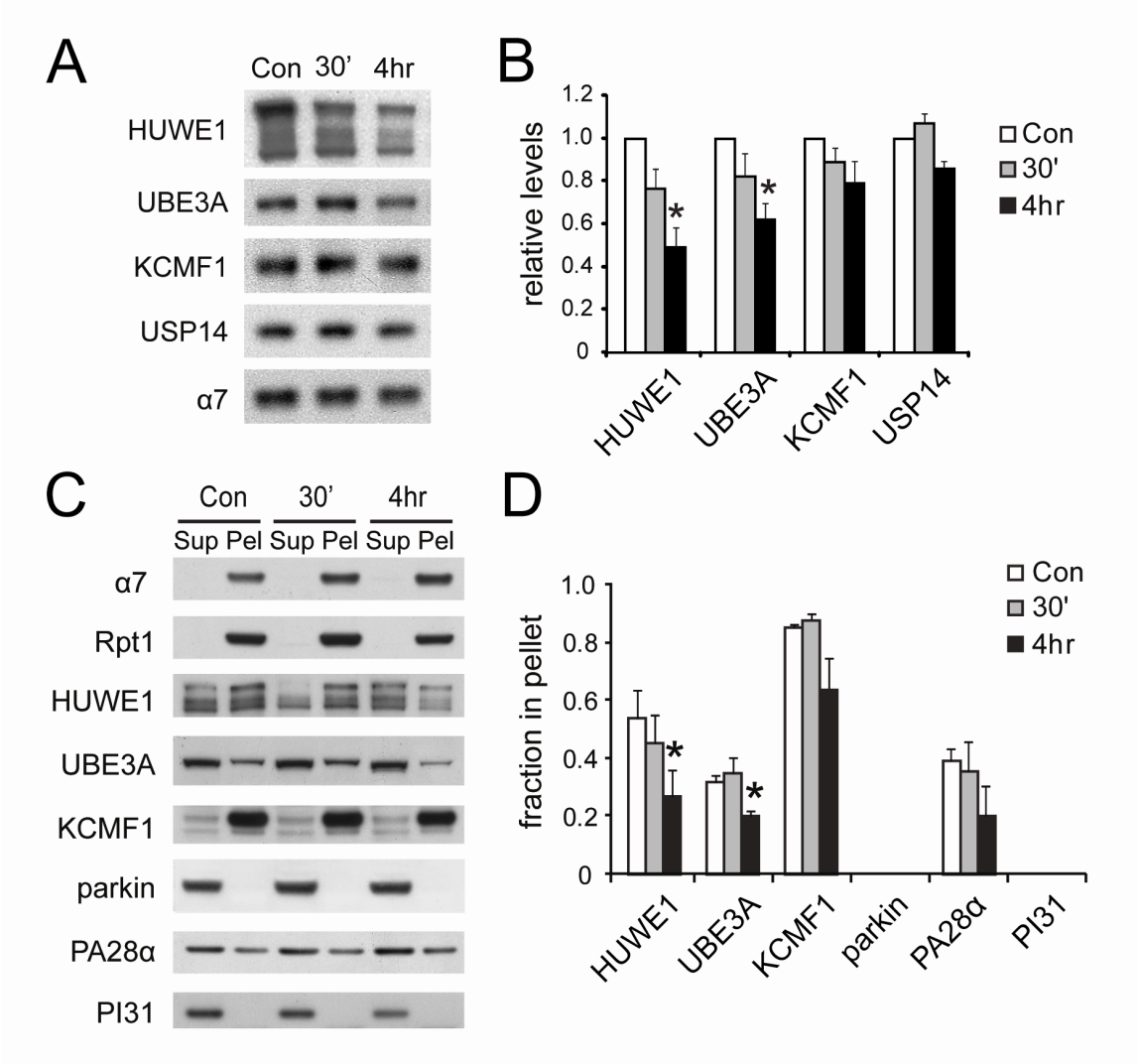


Figure 4-3. (A) NMDA induces the degradation of proteasome-interacting E3s. Cultured hippocampal neurons were treated with 20 μM NMDA for 3 min, and total cell extracts were prepared at 30 min or 4 hr post-treatment. Control (Con) and treated samples were resolved by SDS-PAGE and immunoblotted against proteasome-interacting E3s (HUWE1, UBE3A, KCMF1) and DUB (USP14). (B) Signal intensities in (A) are normalized against 20S subunit α7 and plotted (n=5, mean ± SEM, *p<0.05). (C) Changes in the proteasome-association of E3s. Neuronal extracts from (A) were subjected to ultracentrifugation to sediment proteasomes. Equal amounts of supernatant (Sup) and pellet (Pel) materials were analyzed. The sedimentation property of proteasome-interacting proteins (HUWE1, UBE3A, KCMF1, PA28α) was examined by Western blotting. Parkin and PI31 were not detected in the pellet. (D) Quantitation of the fraction of proteins in (C) that co-sediment with proteasomes (n=4, *p<0.05)

4.4 NMDA exposure alters proteasome activity and ubiquitin levels

Because 26S levels decreased at 4 hr post-NMDA, we also measured if there were corresponding changes in 26S activity based on the hydrolysis of Suc-LLVY-AMC, a proteasome substrate. In a gel-based assay, the activity of the NMDA-treated samples was found to be reduced relative to controls (68±6%, $p<0.05$) (Fig. 4-4a and b). Peptidase assays conducted on neuronal extracts also showed reduced activity in the NMDA-treated samples when compared to controls (73±4%, $p<0.05$) (Fig. 4-4b). The degree of peptidase activity decrease was comparable to the observed decrease in 26S levels (75±3%) (Fig. 4-1b).

Decreases in 26S proteasome activity could lead to the accumulation of ubiquitin-protein conjugates, if there were no global changes in the rate of ubiquitylation and deubiquitylation. While ubiquitin-protein conjugate levels in NMDA-treated neurons showed no change at 30 min post-treatment, a significant decrease was observed at 4 hr (68±10%, $p<0.05$) (Fig. 4-4c and d). The simultaneous and similar decrease in 26S proteasome levels, 26S proteasome activity, and steady-state ubiquitin-conjugate levels at 4 hr post-NMDA suggests a coordinated reduction in the rate of proteolysis mediated by the 26S proteasome, but the fall in ubiquitin conjugates strongly suggest that protein ubiquitylation also decreased.

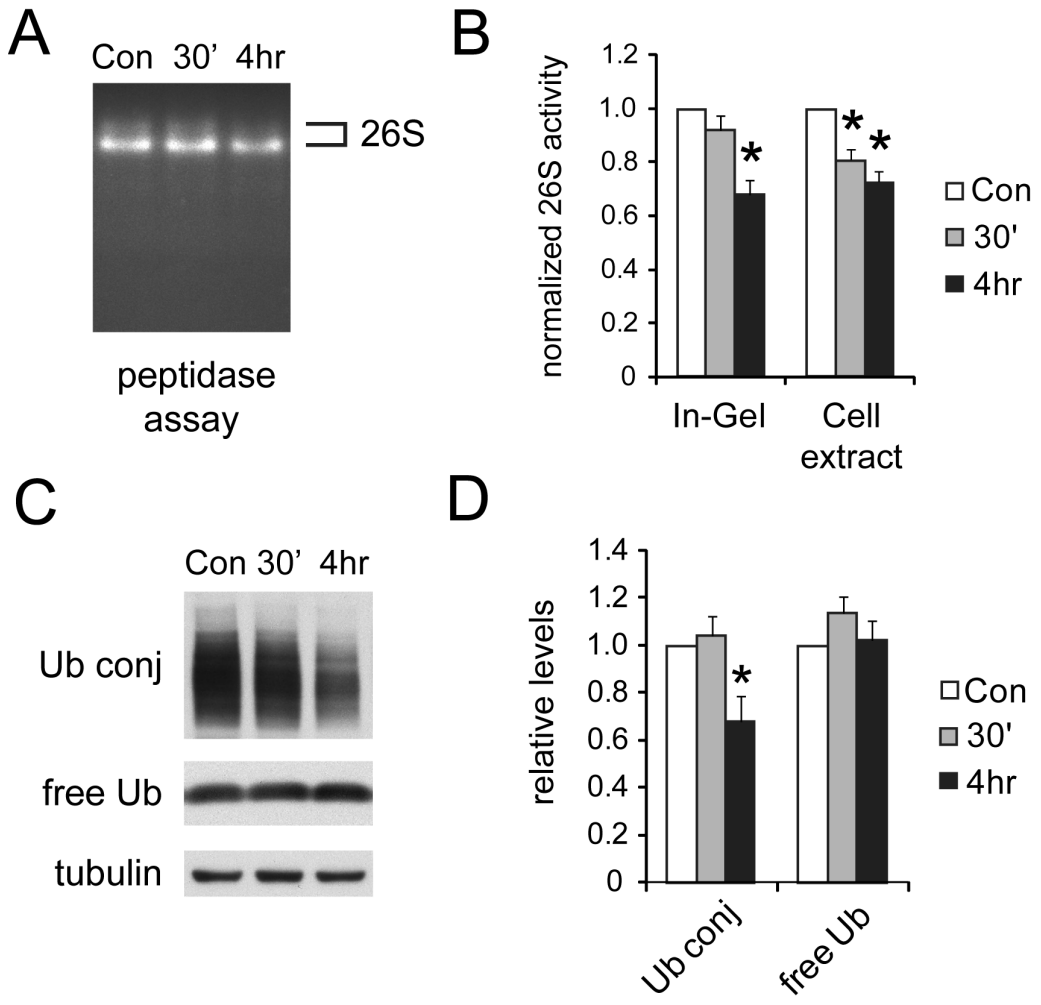


Figure 4-4. (A) Decreases in proteasome activity after NMDA exposure. Neuronal lysates were prepared at 30 min or 4 hr post-NMDA (20 μM, 3 min). Control (Con) and treated samples were resolved by 2–5% gradient native gel. In-gel 26S proteasome activity was detected by the hydrolysis of proteasome substrate Suc-LLVY-AMC. (B) 26S proteasome activity per mg of total protein after NMDA treatment measured by two assays. The first assay measured fluorescent signals shown in (A). In the second assay, Suc-LLVY-AMC was added to the cell extract, and the fluorescence increase was measured over time to calculate 26S proteasome activity (mean ± SEM, n=5, *$p<0.05$). (C) Levels of ubiquitin conjugates after NMDA treatment. Neuronal extracts from (A) were resolved by SDS-PAGE and blotted using antibodies against ubiquitin-protein conjugates (Ub conj) and free monomeric ubiquitin (free Ub). Tubulin served as the loading control. (D) Signals in (C) quantified by densitometry. Ubiquitin-protein conjugates showed significant decreases at 4 hr post-NMDA treatment (n=5, *$p<0.05$).

4.5 Discussions

Long-lasting changes in proteasome levels

In this study, 3 min of neuronal stimulation by 20 μM NMDA application was found to elicit profound changes in the proteasome complex several hours later. How does a brief episode of stimulation create such long-lasting changes in the UPP? It is well established that 3–5 min of 20–50 μM NMDA application causes LTD in CA1 hippocampal neurons and in cultured hippocampal neurons [148, 151] by triggering the internalization of glutamate receptors. This can be considered as a homeostatic mechanism to reduce synaptic transmission after the neuron sense excessive NMDA receptor activity.

Our data suggests that prolonged depression in neuronal activity following LTD induction is accompanied by prolonged decreases in UPP activity. This is consistent with a recent report showing that action potential blockade with tetrodotoxin causes a decrease in dendritic proteasome activity, while prolonged stimulation with bicuculline causes increased proteasome activity, as monitored by the degradation of fluorescent model substrates [103]. It appears that lowering neuronal activity also lowers its requirement for UPP activity.

In yeast, it has been observed that prolonged down-regulation of UPP activity is accompanied by the gradual dissociation of 26S proteasomes into 19S and 20S particles [152]. Our data suggests that, in neurons, the down-regulation of UPP following NMDA-induced LTD also leads to 26S disassembly. This is followed by the degradation of the dissociated 19S particles, as well as the degradation of proteasome-bound E3 ligases,

HUWE1 and UBE3A. This type of delayed response to a brief period of neuronal stimulation may be relevant to the establishment or maintenance of long-term synaptic plasticity and learning. Intriguingly, HUWE1 mutations in humans cause X-linked mental retardation [85], and UBE3A mutations cause Angelman syndrome, characterized by mental retardation, developmental delay, and jerky movement [153]. Most HUWE1 proteins at the synapse, but not the cytosol, appear to be proteasome-bound (Fig. 3-7). Hence, the synaptic function of HUWE1 appears to be mostly proteasome-dependent. UBE3A has been shown to impair LTP and alter spine morphology [154, 155], which may explain why Angelman syndrome patients develop cognitive problems. UBE3A is localized to dendrites and spines [155], and moreover our data suggests that it is partially associated with synaptic proteasomes. Little is known about how HUWE1 and UBE3A contribute to synaptic plasticity and learning, and why their defects lead to mental retardation, and we propose that their interactions with synaptic proteasomes and their synaptic substrates may be important.

The mechanisms of activity-regulated proteasomal changes are largely unclear at this point, and they will need to be addressed by future experiments. First of all, it is not clear why the neuron lowers its 26S levels by 19S degradation after NMDA-induced LTD. It may be that neurons with lower levels of synaptic activity require less UPP activity, and therefore eliminate proteasomes that are in excess. It is also not clear how 19S particles become degraded, and to our knowledge 19S catabolism has never been characterized in eukaryotic cells. Another question is whether the degradation of 19S is triggered by NMDA receptor signaling or by the down-regulation of synaptic activity, or maybe both.

Whether other forms of LTD induction may similarly lead to the reduction of 19S particles should be tested.

Proteasomes and neuronal maintenance

The global decrease in 26S proteasome levels observed after NMDA treatment is in fact very surprising, when we consider the important role of the UPP in neuronal protein quality control. Unlike most cells, neurons cannot dispose of damaged proteins by cell division [156], and therefore must have evolved very stringent mechanisms to protect themselves from abnormal protein. In this context, we may consider how proteasome-interacting proteins may be involved in neuronal protein quality control.

The thirty or so 26S-associated proteins we found in rat cortex differ considerably from those identified by the same approach in rat muscle (62 in total) [104]. In fact, only 13 of the interactors are shared between the two cell types, even though myocytes are postmitotic just like neurons. Protein degradation serves distinct roles in neurons and skeletal muscles, which represent the major amino acid reservoir in mammals. Muscle proteins are mobilized (unlike those in neurons) through accelerated proteolysis in fasting or other stressful conditions [157]. Also, the protein composition in skeletal muscles and neurons is very different, which may necessitate the involvement of different factors to assist proteasome-mediated degradation. Whether such differences in the spectrum of proteasome-associated proteins exist between different brain regions remain to be explored.

The hallmarks of neurodegenerative disorders in general are neuronal inclusions of misfolded proteins, which often contain ubiquitylated proteins and proteasomes [65, 74].

Therefore, factors that help deliver ubiquitylated proteins to proteasomes or facilitate their degradation are likely to be important in preventing protein aggregates or inclusions [12, 67, 97]. In cytosolic 26S proteasomes of the cortex, we identified two factors that seem to be important in reducing the accumulation of such aberrant proteins—the VCP complex and p62. Recently, VCP mutations were discovered in patients with inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) [158]. IBMPFD patients show degenerating neurons with inclusions of ubiquitylated TAR DNA binding protein (TDP-43) [159]. The cause of TDP-43 aggregation is not yet clear, but current models suggest that it is related to a failure in the VCP to facilitate the degradation of TDP-43 [78, 160]. Our isolation of VCP and proteasome together from the cortex is consistent with such a role.

TDP-43 inclusions are also found in sporadic forms of amyotrophic lateral sclerosis and frontotemporal lobar degeneration [161]. It has been proposed that TDP-43, tau, and α-synuclein aggregates in sporadic neurodegenerative disorders are caused by the impairment of UPP-mediated proteolysis or the dysfunction of 26S proteasomes [74, 97]. Testing this hypothesis in humans or animals has proven difficult [77] in the past due to difficulties in isolation of 26S proteasome in their native forms. The affinity purification methods used here should greatly facilitate such studies.

Interestingly, mice deficient in p62 develop neurodegeneration with ubiquitylated, hyperphosphorylated tau inclusions [162]. This is thought to be related to p62’s role in targeting misfolded protein to the proteasome [36] as well as to autophagy [119]. In mice with brain region-specific knockout of a 19S subunit, neurodegeneration is accompanied by accumulation of ubiquitylated α-synuclein inclusions, reminiscent of human PD and

DLB [95]. These mice have decreased neuronal 26S levels with increases in 20S levels. In the present study, we observed a similar decrease in 26S content and increase in neuronal 20S proteasome through 19S dissociation and degradation. These observations highlight that many interesting possibilities for regulating neuronal proteasomes remain to be explored, and such regulations may have important effects on synaptic plasticity and protein quality control.

Substrate delivery pathways to the proteasome

It is very curious that the abnormalities of just there proteins, α-synuclein, tau, and TDP-43, appear to be associated with 80–90% of neurodegenerative dementias [68, 69]. Furthermore, these aggregate proteins often appear together in late-stage dementia patients, a phenomenon termed co-morbidity [163]. Therefore, it might be possible to find a unifying principle or mechanism to explain why these proteins are observed in so many neurodegenerative disorders. Some propose that UPP dysfunction may be a common link between the deposition of different protein inclusions [164]. The data presented in this study appears to support to this idea, by highlighting the potential importance of delivering of ubiquitinated substrates to the proteasome.

It is known that there are multiple mechanisms to bring together the proteasome and its substrates, and our data provides insights into which of these mechanisms may be important for the neuron. Among the cytosolic 26S interactors identified, we found two binding adaptors for polyubiquitinated substrates: p62 and the VCP complex. The 19S also has multiple subunits that bind to ubiquitinated substrates [165]. In addition, binding adaptors carrying UBL domains, including RAD23 and ubiquilin family proteins [125],

are potentially missing in this study because GST-UBL was used to purify proteasomes. As such, p62, VCP, UBL proteins, and the 19S represent different substrate delivery pathways to neuronal proteasome that may work independently or together. Importantly, the genetic inactivation of different delivery pathways is sufficient to cause different pathological inclusions commonly seen in dementia patients (p62→tau, VCP→TDP -43, 19S→α-synuclein, as discussed earlier). This may also explain why degenerating neurons often show co-morbidity of several aggregates species in late-stage dementia. In unhealthy neurons, the UPP dysfunction may be so profound that multiple degradation pathways are impaired and therefore multiple aggregate types appear together. Hence, UPP dysfunction may be one of the unifying mechanisms of protein misfolding problems in neurons.

Although the discussion above implies that UPP dysfunction can be a direct cause of inclusion formation, what contributes to UPP dysfunction remains elusive. Moreover, the UPP involves many genes and its organization and properties can vary between different cell types. For instance, this study suggests that proteasome-interacting proteins can vary significantly. To further understand how UPP function is related to the etiology of neurodegeneration, we will need a much better understanding of how the UPP operates in neurons to degrade different types of proteins.

CHAPTER 5

Conclusion

5.1 General Discussion

The UPP plays two important roles in facilitating the unique functions of the neuron. First, its activity is regulated by synaptic plasticity and participates in the remodeling of synapses. Second, it protects the neuron against aberrant proteins, such as oxidized and misfolded proteins, and especially those known to form inclusions in neurodegenerative disorders. How the UPP accomplishes these tasks in neurons is poorly understood in terms of molecular mechanisms. This thesis study attempts to addresses this issue by characterizing the basic properties of the brain proteasome complex.

This study originated from the simple hypothesis that proteasomes in neurons have different compositions from those in other cells, which was based on the idea that the

unique morphology and physiology of neurons would require specialized proteolytic mechanisms. By combining a novel affinity purification strategy and subcellular fractionation, cytosolic and synaptic 26S proteasomes were successfully isolated from the rat cortex. Proteomic characterization of brain proteasomes by mass spectrometry revealed that the spectrum of interacting proteins is indeed very different from those in muscle or heterologous cells. Proteasome-interacting proteins in the synapse and the cytosol also differ somewhat. This suggests that the proteolytic machinery can be organized differently to meet the proteolytic requirement of a particular cell type and a particular compartment of the cell.

While it is known that neuronal activity can regulate the UPP to facilitate synaptic remodeling, relatively little is understood about its mechanisms. Previous studies have shown that synaptic activity can regulate proteasome activity in dendrites and dendritic spines through posttranslational modification and proteasome redistribution [25, 103]. This study shows that 26S proteasome levels and activity decrease after NMDA stimulation, due to the disassembly of the 26S and the degradation of 19S particles. Moreover, proteasome composition also changes, as observed in the dissociation of interacting proteins such as E3 enzymes HUWE1 and UBE3A.

Previously, it was generally assumed that UPP activity is mostly controlled at the level of ubiquitination, and the proteasome remains relatively unchanged and passively accepts ubiquitinated substrates. This study demonstrates that the neuron also regulates the proteasome complex to modulate UPP activity. In addition, ubiquitin ligases bound to the proteasome are also regulated in a dynamic manner.

Some researchers believe that the gradual reduction of neuronal proteasomes with aging may lead to the build-up of misfolded proteins and hence neurodegeneration [100, 166]. My data suggests that proteasome levels can also decrease quite rapidly after NMDA-induced chemical LTD. Does this decrease compromise the neuron's capacity to handle misfolded proteins? We do not know yet, but this is particularly interesting in the context of Alzheimer's disease. Studies have shown that amyloid β can disrupt glutamate uptake and enhance LTD induction [167]. It is possible that excessive LTD induction can lead to decreased proteasome levels and render the neuron more vulnerable to proteotoxic stresses such as tau aggregation. To understand why degenerating neurons are not properly protected against misfolded proteins, we will need to understand how proteasomes are normally regulated in both healthy and stressed neurons. Although this study is the first to characterize neuronal proteasomes in detail, more questions seem to be raised than answered from such an initial effort.

5.2 Future directions

The study described in this thesis lays an important foundation for future studies of neuronal proteasomes. First, it defines the basic composition of brain proteasomes, which is different from other tissues. Secondly, it shows that neuronal proteasome is dynamically regulated at multiple levels by synaptic activity. However, due to the complexity of the proteasome, our understanding of its actual heterogeneity and dynamics is still very limited.

The actual heterogeneity of neuronal proteasomes cannot be fully revealed by the experimental methods used in this study. It is unlikely for dozens of interacting proteins

to be present on the same proteasome. Proteasome heterogeneity represents a major obstacle when we try to determine how individual substrates are processed by the proteasome, and this needs be addressed in the future. It may be possible to use immunoprecipitation against different proteasome interactors to differentiate between different subpopulations of proteasomes.

Although this study observed a number of interesting proteasome alterations after NMDA treatment, the molecular mechanisms of such changes are unclear. More pharmacological experiments will be needed to dissect the signaling pathway that leads to proteasome alterations. We may expect posttranslational modifications of proteasome subunits and interacting proteins to play important roles in proteasome modulation. In the past, in vitro proteolysis experiments using purified proteasomes have proven to be challenging for many substrates. It would be interesting to see if affinity purified proteasomes containing many more auxiliary factors will be more potent for in vitro proteolysis. The ability to recreate proteasome degradation events in vitro will be very useful for deciphering the molecular steps involved.

While protein aggregation is the most prominent and the most common feature of neurodegeneration, very little is known about how neurons handle toxic proteins liked misfolded or oxidized proteins. In fact, the degradation of misfolded and oxidized proteins is poorly understood in all eukaryotic cells. To understand these processes in the neuron, we must additionally consider the fact that the proteolytic machinery in the neuron differs from other cell types. The brain represents only 2% of the body weight but uses 20% of the oxygen, mostly consumed by neurons. Thus, postmitotic neurons must have evolved efficient mechanisms to deal with protein oxidation. The proteasome is

generally considered as the first line of defense against misfolded and oxidized proteins [149, 150], followed by the lysosome. However, how misfolded proteins and oxidized protein are recognized and delivered to the proteasome remains largely unclear. We may expect at least some of the brain proteasome interacting proteins to serve a function in these processes, but the details remain elusive. The experimental approaches developed in this study may be applied to elucidate proteasome changes when neurons are challenged with misfolded proteins or oxidized proteins. For instance, we can transiently turn on the overexpression of a polyQ protein in mice brain, and purify brain proteasomes to see what interactors have changed due to the overexpression. This may give us some clues to which interacting protein may help proteasomes handle polyQ substrates. The same strategy can be applied to examine the proteasomal adaptations to oxidative stresses applied to neurons. Understanding how neurons handle oxidized and misfolded proteins may be help us elucidate the etiology of neuronal proteinopathies and find better treatments.

The dozens of proteasome interacting proteins identified in this study marks a major advance in our understanding of brain proteasomes. However, most of their functions at the proteasome are not known. This represents a new set of challenges to understand their functions and dynamics. The complexity of the proteasome is daunting because its composition varies from one tissue to another. To further address the heterogeneity of proteasomes may require future advances in proteomic mass spectrometry. The question of individual interactor function may depend on in vitro reconstitution experiments. Moreover, the very fundamental question of how a specific protein is catabolized in a cell has proven very difficult to investigate. Currently, most of the proteolysis inhibitors

available are very broad in their spectrum of action, such as blocking proteasomes, lysosomes, autophagy, or ubiquitination. What will be very useful are specific inhibitors against individual E2s, E3s or proteasome interacting proteins.

All in all, a deeper understanding of neuronal proteasomes is highly relevant to the study of synaptic plasticity and protein misfolding disorders in the brain, and needs to be furthere pursued. The proteasome has a central role in mediating the neuron's proteomic changes and proteomic stability, but we are just beginning to unveil its complexity. Conceptually, enhancing proteasome function may help prevent proteinopathies in the brain. However, no pharmacological agent has been shown to enhance proteasome function in the cell, and this awaits future exploration.

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VITA

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