



Characterization of the brain 26S proteasome and its interacting proteins

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Proteasome-mediated proteolysis is important for synaptic plasticity, neuronal development, protein quality control, and many other processes in neurons. To define proteasome composition in brain, we affinity purified 26S proteasomes from cytosolic and synaptic compartments of the rat cortex. Using tandem mass spectrometry, we identified the standard 26S subunits and a set of 28 proteasome-interacting proteins that associated substoichiometrically and may serve as regulators or cofactors. This set differed from those in other tissues and we also found several proteins that associated only with either the cytosolic or the synaptic proteasome. The latter included the ubiquitin-binding factor TAX1BP1 and synaptic vesicle protein SNAP-25. Native gel electrophoresis revealed a higher proportion of doubly-capped 26S proteasome (19S-20S-19S) in the cortex than in the liver or kidney. To investigate the interplay between proteasome regulation and synaptic plasticity, we exposed cultured neurons to glutamate receptor agonist NMDA. Within 4 h, this agent caused a prolonged decrease in the activity of the ubiquitin-proteasome system as shown by disassembly of 26S proteasomes, decrease in ubiquitin-protein conjugates, and dissociation of the ubiquitin ligases UBE3A (E6-AP) and HUWE1 from the proteasome. Surprisingly, the regulatory 19S particles were rapidly degraded by proteasomal, not lysosomal degradation, and the dissociated E3 enzymes also degraded. Thus the content of proteasomes and their set of associated proteins can be altered by neuronal activity, in a manner likely to influence synaptic plasticity and learning.

Keywords: proteasome, proteasome-interacting protein, ubiquitin ligase, synaptic plasticity, UBE3A, NMDA

INTRODUCTION

In eukaryotic cells, most intracellular proteins are degraded by the ubiquitin-proteasome system (UPS). In this pathway, polyubiquitylation of a protein marks it for degradation by the 26S proteasome (Hershko and Ciechanover, 1998). In neurons, the UPS modulates the function and plasticity of synapses in normal and diseased states (DiAntonio and Hicke, 2004; Yi and Ehlers, 2007; Tai and Schuman, 2008). The UPS has been shown to regulate presynaptic vesicle release (Willeumier et al., 2006; Yao et al., 2007) and the restructuring of the postsynaptic density (PSD) (Ehlers, 2003; Bingol and Schuman, 2004). In fact, the inhibition of proteasome activity can impair synaptic plasticity (Fonseca et al., 2006; Hou et al., 2006; Karpova et al., 2006), and certain types of learning in animals (Lopez-Salon et al., 2001; Lee et al., 2008). Another critical function of the UPS is to protect neurons by clearing damaged and misfolded proteins (Goldberg, 2003). Intraneuronal aggregates of misfolded proteins are the hallmarks of many neurodegenerative disorders, such as α -synuclein in Parkinson's disease and hyperphosphorylated tau in Alzheimer's disease. Frequently, these aggregates contain ubiquitylated proteins and proteasomes, and it has often been suggested that impairment of the UPS underlies neurodegeneration (Sherman and Goldberg, 2001; Rubinsztein, 2006; Bedford et al., 2008b).

There is growing evidence that ubiquitylation is not the only site of regulation of this pathway and that proteasome composition, and presumably function, can be regulated in multiple ways

(Glickman and Raveh, 2005; Schmidt et al., 2005). The proteolytic component of the proteasome is the 20S core particle, which may be associated with several different types of activating particles in addition to the 19S regulatory particle, which binds to ubiquitylated proteins (Rechsteiner and Hill, 2005). In the 26S proteasome, a single 20S particle may associate with one or two 19S particles to form a singly- or doubly-capped 26S proteasome. Several studies have shown that neuron-specific mechanisms of 26S proteasome regulation may underlie synaptic plasticity (DiAntonio and Hicke, 2004; Bingol and Schuman, 2005; Yi and Ehlers, 2007). A striking example is the rapid redistribution of 26S proteasomes into dendritic spines following neuronal stimulation (Bingol and Schuman, 2006). Inside the synapse, proteasome-mediated proteolysis can regulate the internalization of glutamate receptors (Patrick et al., 2003) as well as the abundance of many PSD proteins (Ehlers, 2003; Bingol and Schuman, 2004).

It is also likely that proteasome regulation occurs through changes in the levels of proteasome-interacting proteins. In mammals, USP14 and Rpn13/UCH37, both deubiquitylating enzymes or DUBs, influence substrate degradation through their interaction with the 26S proteasome (Koulich et al., 2008). The spontaneous mutation of Usp14 in mice leads to synaptic ubiquitin defects and altered short-term synaptic plasticity (Wilson et al., 2002; Chen et al., 2009). In addition to 33 different integral subunits, mass-spectrometric analysis of gently-purified proteasomes from

non-neuronal tissues and cells has revealed many proteins that interact with 26S proteasomes (Wang and Huang, 2008; Besche et al., 2009; Scanlon et al., 2009). However, their specific functions and mode of regulation are generally unclear.

In particular, there are no published studies about what proteins interact stoichiometrically with 26S proteasomes in the brain and how they are regulated. Recently, Besche et al. (2009) have described an affinity purification method that allows gentle, rapid isolation of 26S proteasomes together with associated proteins. This approach, which is based upon the high affinity of 26S proteasomes for the ubiquitin-like (UBL) domain of Rad23B (Schauber et al., 1998), has revealed various new proteasome-associated proteins, among them several ubiquitin ligases and DUBs. In this study, we have utilized this newly developed method for purifying 26S proteasomes from the adult rat cortex. We specifically isolated 26S proteasomes from biochemically purified neuronal fractions and compared the interacting proteins found in synaptic and cytosolic fractions by tandem mass spectrometry. We found that the 26S proteasome-interacting proteins differ between the synapse and the cytosol, and even more so between the cortex and other tissues. Moreover, neuronal activity was found to alter the functional capacity of the UPS. Following the exposure of neurons to N-methyl-D-aspartate (NMDA), we identified several novel mechanisms that down-regulate the UPS, including the disassembly of 26S proteasomes, the degradation of proteasome-associated ubiquitin ligases (E3s), and the degradation of 19S regulatory particles in a proteasome-dependent manner.

MATERIALS AND METHODS

REAGENTS

Antibodies against the following antigens were purchased commercially: $\alpha 7$ (PW8110), Rpt1 (PW8825), UBE3A (PW0535), ubiquitin conjugates (UG9510), PI31 (PW9710) from Biomol/Enzo (Plymouth Meeting, PA, USA); ECM29 (PA3-035), USP14 (MA1-57109), PA28 α (PA1-960) from Affinity Bioreagents/Thermo (Rockford, IL, USA); parkin (MAB5512), ubiquitin monomer (MAB1510) from Chemicon/Millipore (Billerica, MA, USA); PSMD9 (sc-10670), 14-3-3 γ (sc-731) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); HUWE1 (A300-486A) from Bethyl (Montgomery, TX, USA); tubulin $\beta 3$ (T8860) from Sigma (St Louis, MO, USA); KCMF1 (15-288-21213) from Genway (San Diego, CA, USA); drebrin (ab12350) and GRASP-1 (ab30576) from Abcam (Cambridge, MA, USA). Nonidet P-40 (NP-40) was purchased from BDH (Poole, England). Protease inhibitor cocktail (complete, EDTA-free) was from Roche (Indianapolis, IN, USA). Phosphatase inhibitor (cocktail 2), NMDA, and chloroquine were from Sigma. Epoxomicin and clasto-lactacystin β -lactone were from Enzo. Tetrodotoxin (TTX), (S)-3,5-dihydroxyphenylglycine (DHPG), 2-amino-5-phosphonopentanoic acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and concanamycin A were from Tocris (Ellisville, MO, USA).

RECOMBINANT PROTEIN PURIFICATION

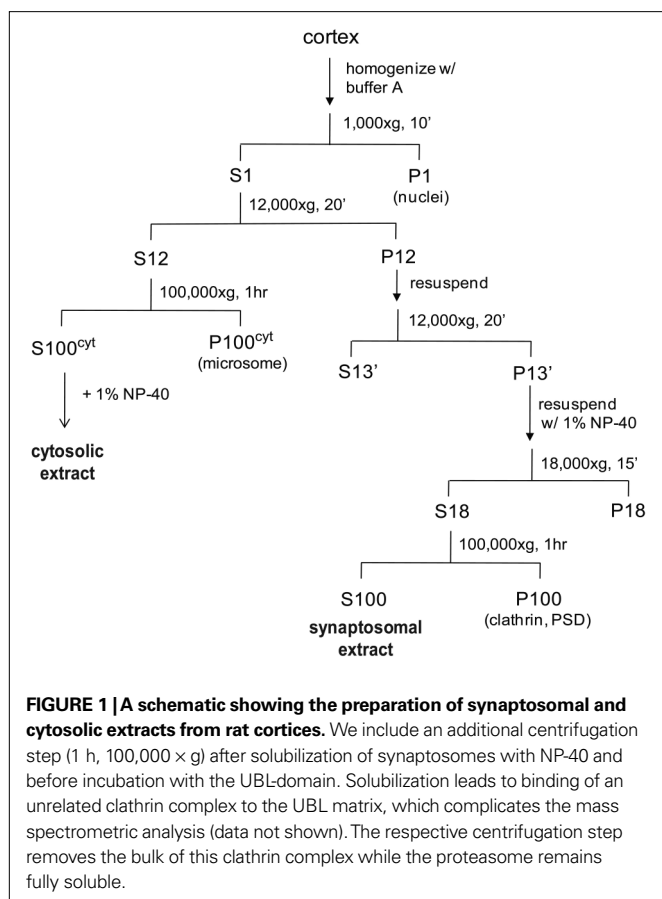
GST-UBL and His₁₀-UIM plasmids and their expression in bacteria have been previously described (Besche et al., 2009). pDEST-15-HHR23B^{UBL} plasmid was transformed into BL21AI (Invitrogen) according to manufacturer's instructions. Cells were grown at

37°C with ampicillin to an OD₆₀₀ of 0.5 and induced for 3 h 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 Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4) with 2 mM DTT, 10 mM MgCl₂, 0.2 mg/mL lysozyme and protease inhibitors (complete, Roche). The entire purification procedure was carried out at 4°C. Cells were lysed by sonication, and the cell debris was removed by centrifugation at 20,000 × g for 30 min. The supernatant was passed through 0.2- μ m filters and supplemented with 0.2% Triton X-100, followed by incubation with 20 mL of glutathione sepharose for 2 h. 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 MgCl₂, 1 mM DTT). Small aliquots of 10 mg/mL GST-UBL were frozen in liquid nitrogen and stored at -80°C. The final yield from 1 L culture was ~100 mg.

pET26b-S5a^{UIM2} plasmid was transformed into BL21 (DE3) (Promega) according to manufacturer's instructions. Cells were grown at 37°C with kanamycin to an OD₆₀₀ of 0.8, followed by induction with 0.5 mM IPTG for 2 h at 30°C. Pellets from 1-L culture (~20 mg of His₁₀-UIM) were 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°C with mixing, the lysate was cooled to 4°C and centrifuged at 20,000 × g for 20 min. The supernatant was passed through 0.2- μ m filters, supplemented with 10 mM imidazole, and incubated with 10 mL of Ni-NTA for 1 h. 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. Twenty milliliters of elution buffer (50 mM Tris pH 7.5, 250 mM imidazole, 300 mM NaCl) was added, and collected fractions containing His₁₀-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 His₁₀-UIM were frozen in liquid nitrogen and stored at -80°C.

SYNAPTOSOME ISOLATION

Adult rat cortices rapidly frozen in liquid nitrogen and stored at -80°C were used for isolating synaptosomes, based on standard protocols (Gordon-Weeks, 1987) with buffer modifications to stabilize proteasomes. The procedure is schematically represented in **Figure 1**. Briefly, cortices from four rats were homogenized in a motor-driven Potter-Elvehjem homogenizer in 30 mL buffer A (20 mM HEPES, 0.32 M sucrose, 5 mM MgCl₂, 2 mM ATP, 2 mM DTT, protease and phosphatase inhibitors, pH 7.2). The homogenate was centrifuged at 1000 × g for 5 min, and the supernatant was collected as S1 (if cloudy, centrifuged one more time). S1 was centrifuged at 12,000 × g for 20 min, and the pellet (P12) was resuspended in buffer A and centrifuged again at 12,000 × g 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 18,000 × g



for 10 min, and then at 100,000 × g for 1 h to sediment clathrin complexes and debris. The supernatant (S100) was collected as synaptosomal extract. To collect the cytosolic extract, supernatant S12 was centrifuged at 100,000 × g to remove microsomes. The supernatant was collected as cytosolic extract and supplemented with 1% NP-40. Synaptosomal protein yield from four cortices was ~50 mg.

PROTEASOME PURIFICATION

The purification of 26S proteasomes was conducted at 4°C. First, cytosolic or synaptosomal extracts (20 mg total protein) were incubated for 2 h 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 MgCl₂. 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.

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

TISSUES AND NEURONAL CULTURES

Cortex, liver and kidney were dissected from 4- to 6-weeks old male Sprague-Dawley rats. Dissociated hippocampal neurons were prepared and maintained as previously described (Aakalu et al., 2001). 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/cm² onto poly-D-lysine-coated 60-mm culture dishes (Falcon). Cultures were maintained in Neurobasal A medium containing B-27 and Glutamax supplements (Invitrogen) at 37°C for 21–28 days before use. Tissue extracts were prepared in buffer A (20 mM HEPES, 0.32 M sucrose, 5 mM MgCl₂, 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 18,000 × g for 15 min to remove nuclei and cell debris, and protein concentrations were measured by Coomassie Plus protein assay (Pierce).

NATIVE GEL ELECTROPHORESIS

To resolve proteasomes in 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 (Dohmen et al., 2005) 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 MgCl₂, 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°C, in TBE running buffer (with 0.5 mM DTT, 0.5 mM ATP and 2 mM MgCl₂). The applied voltage was gradually increased with time (30 V for 30 min, 35 V for 1 h, 50 V for 1 h, 65 V for 2–4 h). 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°C and 150 V for 4 h. 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 h at 70 V (current limit: 350 mA) in a 4°C room.

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).

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 MgCl₂, 1 mM ATP,

1 mM DTT, 50 μ M Suc-LLVY-AMC) for 30 min at 37°C. Fluorescent bands around 26S proteasomes were visualized and quantified by standard gel-imaging systems for DNA staining by ethidium bromide.

MASS SPECTROMETRY

Purified cytosolic and synaptic proteasome samples 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 μ m I.D. capillary column and a hybrid linear ion trap/FT-ICR mass spectrometer (LTQ FT, Thermo Electron, Bremen, Germany) as described previously (Haas et al., 2006). 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. We determined an overall protein false-discovery rate of 1% for the ~100 proteins that made it above the unbiased filter thresholds (Elias and Gygi, 2007). The maximum number of incorrectly assigned proteins in the final list of <50 protein is therefore 1. We believe that this false-discovery rate is hardly matched by other medium-to-large scale analysis methods currently available. Data in **Tables 1 and 2** are compiled from two independent experiments using four rats each.

RESULTS

THE DISTRIBUTION OF PROTEASOME SPECIES IN THE CORTEX

Since very little is known about the proteasome content of the brain, we first investigated what types of proteasomes may exist in the rat cortex. Using native gel electrophoresis followed by

Table 1 | 26S proteasome subunits identified by mass spectrometry.

IPI identifier	Unigene identifier	Gene name	Gene symbol	Cytosolic 26S		Synaptic 26S	
				Unique peptides ^a	Sequence coverage (%)	Unique peptides ^a	Sequence coverage (%)
20S PARTICLE							
IPI00191501.1	Rn.107278	Proteasome subunit alpha type 6 (α 1)	PsmA6	6	27	7	31
IPI00231757.11	n/a	Proteasome subunit alpha type 2 (α 2)	PsmA2	8	44	10	50
IPI00231046.8	Rn.11076	Proteasome subunit alpha type 4 (α 3)	PsmA4	5	21	6	16
IPI00215243.3	Rn.105784	Isoform RC6-IL of proteasome subunit alpha type 7 (α 4)	PsmA7	11	46	12	44
IPI00191502.5	Rn.1276	Proteasome subunit alpha type 5 (α 5)	PsmA5	7	30	7	30
IPI00191748.3	Rn.2668	Proteasome subunit alpha type 1 (α 6)	PsmA1	13	55	13	52
IPI00476178.2	Rn.3997	Proteasome subunit alpha type 3 (α 7)	PsmA3	6	28	6	25
IPI00188686.2	n/a	Proteasome subunit beta type 6 (β 1)	PsmB6	4	29	4	29
IPI00199980.1	Rn.3846	Proteasome subunit beta type 7 precursor (β 2)	PsmB7	4	16	4	16
IPI00214889.1	Rn.94551	Proteasome subunit beta type 3 (β 3)	PsmB3	5	33	5	33
IPI00188584.1	Rn.1981	Proteasome subunit beta type 2 (β 4)	PsmB2	10	56	6	41
IPI00230992.5	n/a	Proteasome subunit beta type 5 (β 5)	PsmB5	12	49	12	51
IPI00191749.5	Rn.6016	Proteasome subunit beta type 1 precursor (β 6)	PsmB1	7	40	6	33
IPI00191505.3	Rn.6169	Proteasome subunit beta type 4 precursor (β 7)	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

Note: ^auniqueness is defined solely by peptide sequence.

Table 2 | 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	Txn1l	13	56	14	60
19S ASSEMBLY FACTOR							
IPI00194471.1	Rn.13415	26S proteasome non-ATPase subunit 5/S5b	Psmc5	14	38	13	37
IPI00231038.4	Rn.24127	26S proteasome non-ATPase subunit 9/Bridge-1/p27	Psmc9	5	24	5	24
E3							
IPI00201213.3	Rn.12130	HECT, UBA and WWF 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	Uch15	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
UPS							
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
PHOSPHOPROTEIN 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							
IPI00188755.4	Rn.106046	Caspase recruitment domain-containing protein	Fam188a	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

^auniqueness is defined solely by peptide sequence.

^bThe clathrin complex appears to directly bind to GST-UBL independent of proteasome.

^cMitochondrial proteins may be contaminants of synaptosomes.

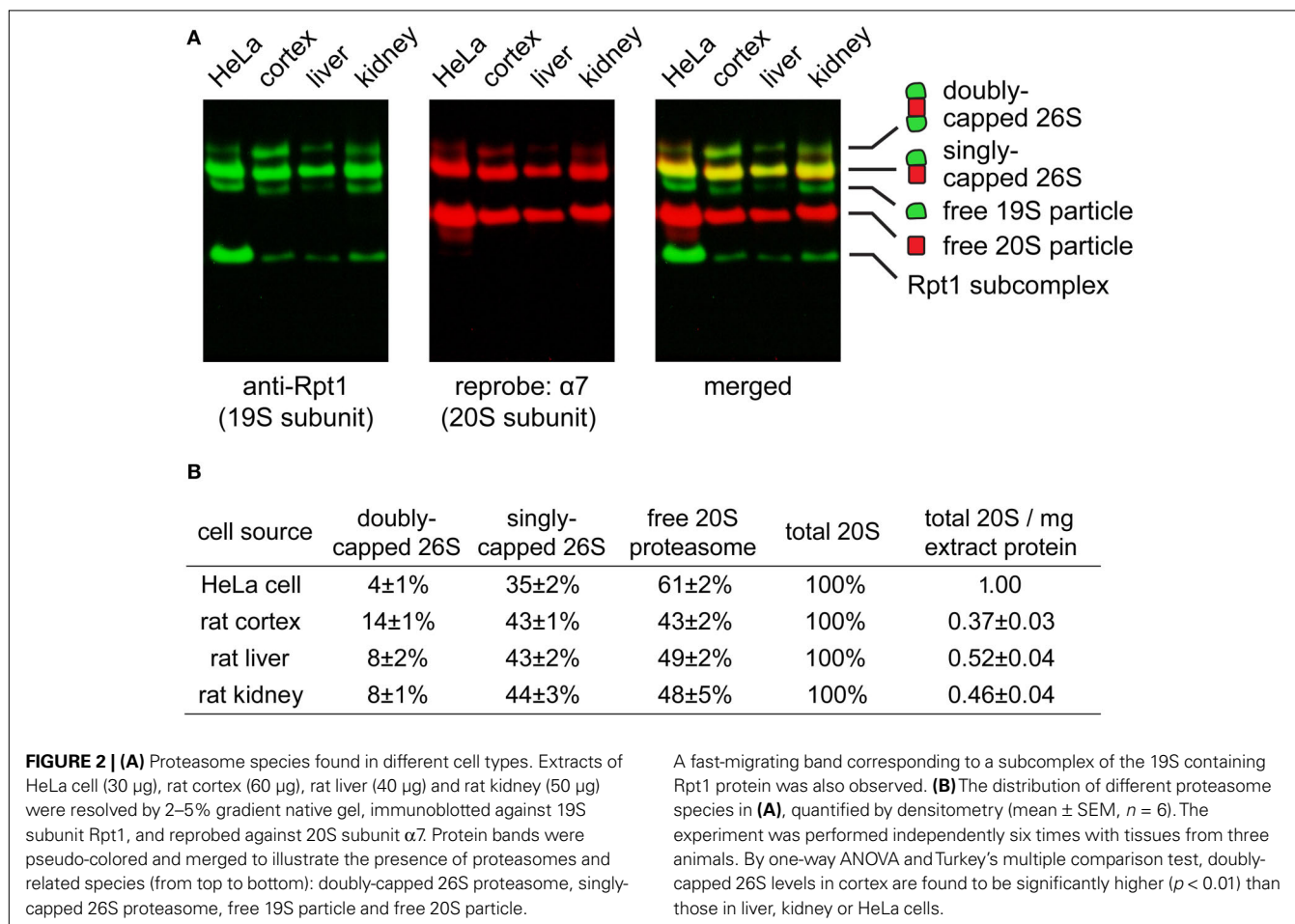
immunoblotting against 19S and 20S subunits, we found doubly-capped 26S, singly-capped 26S, and 20S proteasome in rat cortical extracts (**Figure 2A**). Their relative abundance in cortex, kidney, liver and HeLa cells is quantified and summarized in **Figure 2B**. Interestingly, the level of doubly-capped 26S proteasome was higher in the cortex than in other cell types ($p < 0.01$ by Tukey's test). Additionally, we detected free 19S particles in these cell types, as well as a smaller complex containing the 19S subunit Rpt1 (**Figure 2A**). We characterized this complex from liver extracts by tandem mass spectrometry (LC-MS/MS) and found S5b, Rpn1, Rpt1 and Rpt2. This complex has been recently shown to be an intermediate in 19S assembly (Funakoshi et al., 2009; Kaneko et al., 2009; Le Tallec et al., 2009; Park et al., 2009; Roelofs et al., 2009; Saeki et al., 2009).

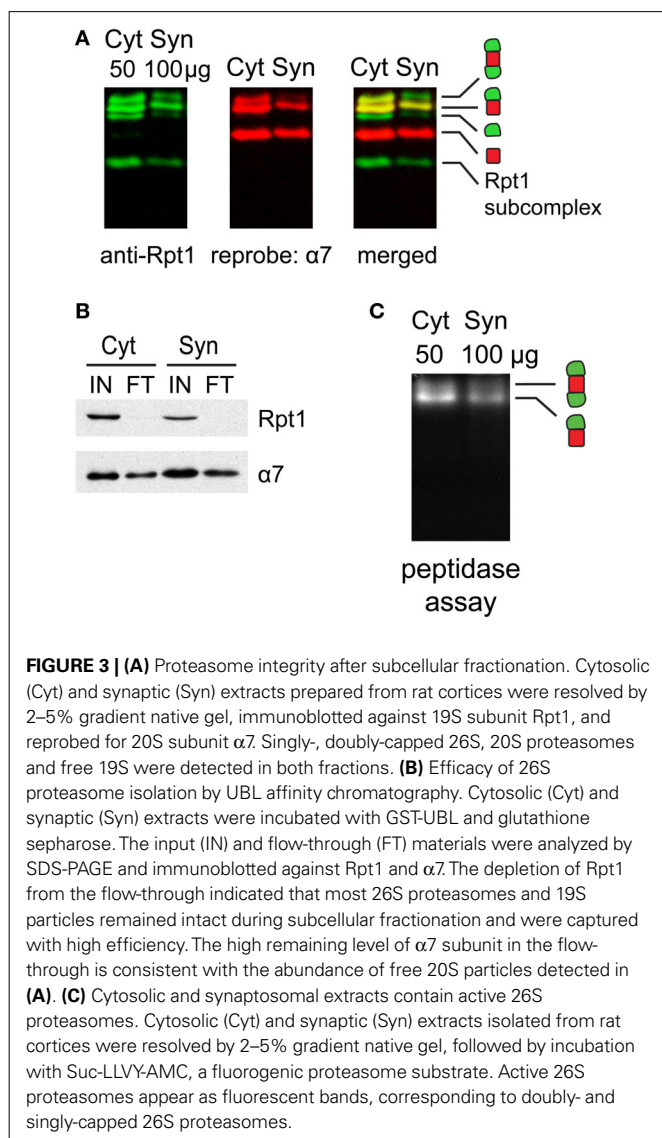
PROTEASOME INTEGRITY AFTER SUBCELLULAR FRACTIONATION

Next, we investigated if proteasomes in different subcellular compartments have different properties by comparing cytosolic and synaptic 26S proteasomes. Slight modifications of standard protocols for isolating cytosolic and synaptosomal fractions (**Figure 1**) were sufficient to preserve proteasome integrity, as assessed by native gel electrophoresis (**Figure 3A**) and enzymatic assays (**Figure 3C**). When cytosolic and synaptosomal extracts were incubated with GST-UBL and glutathione sepharose, the flow-through fraction was

depleted of 19S-subunit Rpt1 protein (**Figure 3B**). 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 $\alpha 7$ subunit remaining in the flow-through (**Figure 3B**), which was consistent with the abundance of free 20S particles (which lack affinity for ubiquitin or UBL-proteins), as detected in the native gel (**Figure 3A**). Also, when GST-alone was used, instead of GST-UBL, no proteasome or other complexes were detectable (**Figure 4A**).

Cytosolic and synaptic 26S proteasomes bound to the GST-UBL matrix were recovered by elution with His-UIM. When resolved by native gel electrophoresis and silver stained, each 26S sample showed only two bands, corresponding to doubly- and singly-capped 26S proteasomes (**Figure 4A**), both of which remain enzymatically active (**Figure 4C**). Interestingly, the ratio of doubly- to singly-capped 26S was significantly higher in the cytosol than in the synapse (**Figure 4D**). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, the band patterns of the cytosolic and synaptic 26S proteasomes showed some differences, and very few proteins were found in GST controls (**Figure 4B**). Taken together, our data demonstrate that the distribution of proteasome species varies between different cells and tissues, as well as between different subcellular compartments of the brain.





The GST-UBL capture method offers several advantages over proteasome-purification strategies based on tandem affinity purification (TAP) tags (Krogan et al., 2004; Guerrero et al., 2006; Wang et al., 2007). TAP tag requires genetic manipulation and the tag may affect proteasome properties, while GST-UBL can efficiently capture endogenous proteasomes from almost any biological source. Instead of tandem affinity chromatography, a one-step elution using His-UIM yields functionally intact, highly-purified proteasomes which can be used for further enzymatic characterizations.

IDENTIFYING PROTEASOME-INTERACTING PROTEINS BY MASS SPECTROMETRY

Because the 26S proteasomes isolated from the cytosol and the synaptosome were highly purified, it was possible to determine their compositions and the nature of the associated proteins by trypsin digestion and LC-MS/MS. These proteasome purifications were carried out in the absence of salt to maximize the content

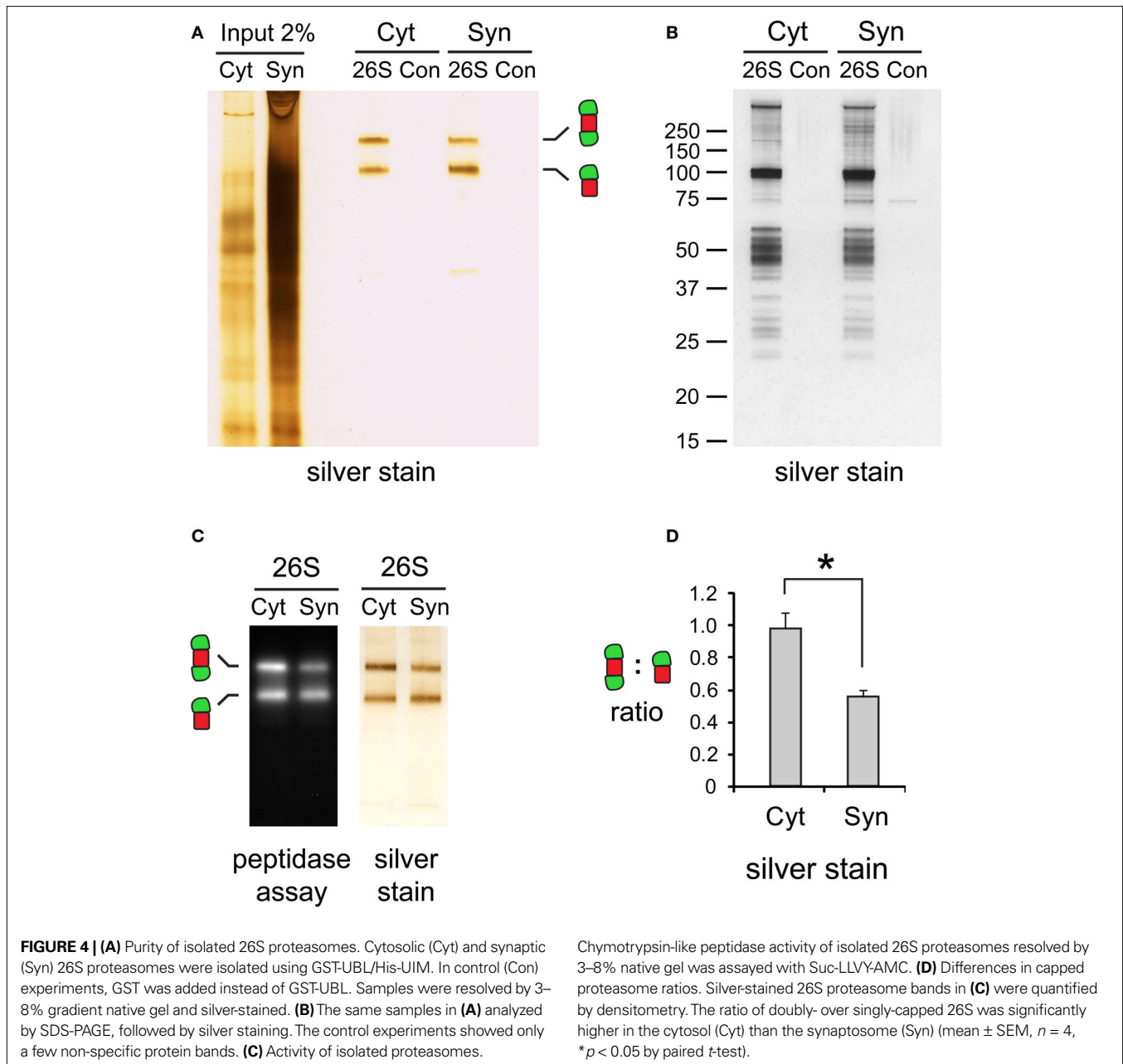
of proteasome-associated proteins (Besche et al., 2009). Proteins bound to the GST-UBL sample that matched proteins bound by GST-alone were non-specific contaminants that interacted with the matrix and were not analyzed further. The remaining identified proteins represented either stoichiometric proteasome subunits (Table 1) or 26S proteasome-interacting proteins (Table 2). There were no discernible differences in proteasome subunits between the cytosol and synaptosome, and no immunoproteasome subunits were detected. Among the ~30 interacting proteins identified, one protein ECM29 was restricted to cytosolic proteasomes, and five proteins (TAX1BP1, SNAP-25, drebrin, GRASP-1, 14-3-3 γ) were found only in synaptic proteasomes. Those interactors shared between the two proteasome populations include several ubiquitin ligases (E3s), DUBs, molecular chaperones, and components of the UPS.

During 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 (Besche et al., 2009). Consequently, neither ubiquitin nor ubiquitin conjugates were detected in purified 26S samples. Proteins listed in Table 2, therefore, most likely represent true proteasome-interacting proteins, not transiently-associated proteasome substrates. Some of these proteins have previously also been reported to interact with proteasomes from non-neuronal cells (Table 3); some appear to be brain-specific, such as the ubiquitin-binding factor TAX1BP1, the E3 enzyme KCMF1, and the synaptic vesicle protein SNAP-25.

VALIDATION OF PROTEASOME-INTERACTIONS

A few proteins (such as drebrin and GRASP-1) listed in Table 2 were identified only by a single matching peptide, and we wanted to validate that they were bona fide interactors using an independent approach. We were able to confirm their presence in purified proteasome preparations by Western blotting using specific antibodies (Figure 5A). In addition, we utilized a co-sedimentation assay to independently assess (in the absence of GST-UBL) if proteins listed in Table 2 are indeed associated with proteasomes. When cytosolic and synaptic extracts were subjected to centrifugation at $100,000 \times g$ for 6 h, 20S and 26S proteasomes were largely found in the pellet fraction (Figure 5C). Under this condition, true proteasome-interacting proteins should also sediment with the proteasome in the pellet, and a total of seven putative interacting proteins from Table 2 were tested. These included three ubiquitin ligases, HUWE1 (HECT, UBA and WWE domain containing 1), UBE3A (E6-associated protein, E6-AP), and KCMF1 (potassium channel modulatory factor 1); USP14 (ubiquitin-specific protease 14, a DUB); PSMD9, a recently identified 19S assembly factor (Funakoshi et al., 2009; Kaneko et al., 2009; Saeki et al., 2009); ECM29, the only proteasome protein detected exclusively in the cytosol; and 14-3-3 γ , which binds phosphoproteins and was found only in synaptic proteasomes. All seven proteins co-sedimented with proteasomes as expected, and their subcellular distribution agrees very well with the mass spectrometry results (Figure 5C). This validates our purification and identification strategy.

In our preliminary experiments, it was observed that the clathrin complex can bind to GST-UBL independently of the proteasome in purifications from synaptosomes and microsomes (data



not shown). In this study, ultracentrifugation for 1 h at $100,000 \times g$ before the UBL-purification efficiently removed most of the clathrin complex from solubilized synaptosomal proteasomes; however, traces were still detectable by LC-MS/MS. Thus, we do not consider clathrin and related vesicular proteins as 26S proteasome interactors (Table 2), although our data cannot formally rule out the possibility of such interactions. The VCP/p97 complex, which is also a key component of the UPS, has been previously observed to bind proteasomes in both muscle and yeast (Verma et al., 2000; Besche et al., 2009). However, it also directly binds to the UBL-domain in complex with associated cofactors in muscle extracts (Besche et al. 2009). We failed to detect by Western blotting any appreciable interaction between VCP and the preparation of GST-UBL used in this study (Figure 5B). The relatively

low levels of VCP and one of its associated factors detected by mass spectrometry in purified 26S proteasomes are thus likely a result of VCP-proteasome interaction in brain, although we cannot formally rule out the potential interaction between VCP and the GST-UBL matrix.

It is also 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 to interact with proteasomes in non-neuronal cells (McCutchen-Maloney et al., 2000; Sakata et al., 2003). Also, neither of these proteins co-sedimented with proteasomes (Figure 5C). These findings further illustrate the specificity of our purification strategy and the differences between proteasome interactors in different cell types.

Table 3 | Comparison of proteasome composition from different mammalian cells determined by proteomic approaches.

Gene symbol ^a	This study		Muscle ^b	HEK293 ^c	HEK293 ^d	Synaptosome (no ATP) ^e
	Cytosol	Synaptosome				
ADRM1	X	X	X	X	X	
DBN1		X				
ECM29	X		X			
FAF1	X	X			X	
FAM188A	X	X				
GBAS	X	X	X			
GRIP1AP1		X				
HSPA2	X	X		X		
HUWE1	X	X	X		X	
KCMF1	X	X				X
PSMD5	X	X				
PSMD9	X	X				
SNAP25		X				X
SQSTM1	X	X	X		X	
TAX1BP1		X				
TXNL1	X	X			X	
TUBB5	X	X				
TUBB6	X	X				
UBE3A	X	X	X	X	X	
UBR4	X	X	X		X	
UCHL5	X	X	X	X	X	
USP13	X	X			X	
USP14	X	X	X	X	X	X
USP5	X	X	X		X	X
USP7	X	X	X			
VCP	X	X	X			
YWHAG		X				
YWHAZ	X	X		X		

^aGene symbols approved by HUGO (Human Genome Organization) gene nomenclature committee.

^bBesche et al. (2009).

^cWang et al. (2007), Wang and Huang (2008).

^dScanlon et al. (2009).

^eBingol et al. (2010).

NMDA TREATMENT SUPPRESSES NEURONAL UPS ACTIVITY

Several forms of synaptic plasticity have been shown to require activity of the UPS (Fonseca et al., 2006; Hou et al., 2006; Karpova et al., 2006), and hence there is a growing interest in understanding how neuronal activity regulates different aspects of the UPS (Ehlers, 2003; Patrick et al., 2003; DiAntonio and Hicke, 2004; Bingol and Schuman, 2006; Bingol et al., 2010). However, the regulation of proteasomes by synaptic activity is hindered by a lack of basic knowledge about neuronal proteasomes. The biochemical characterization of brain proteasomes described above now provides a basis for investigating the interplay between proteasomes and synaptic plasticity. To induce long-term synaptic plasticity, we exposed cultured hippocampal neurons to 20 μ M NMDA for 3 min. NMDA has been shown to induce rapid internalization of glutamate receptors (Li et al., 2004; Delgado et al., 2007), resulting in depressed neurotransmission that lasts for hours, which is also called “chemical long-term depression” (chemical LTD) (Lee et al., 1998). This

is a well-established protocol to investigate synaptic plasticity in cultured hippocampal neurons and hippocampal slices (Lee et al., 1998; Li et al., 2004; Lu and Ziff, 2005; Delgado et al., 2007; Davidson et al., 2009).

Following the exposure of neurons to NMDA, we observed a long-lasting reduction in 26S proteasome activity assayed with fluorogenic peptide substrates (**Figures 6A,B**). The decrease was statistically significant compared to untreated neurons at 4 h post-NMDA treatment. A decrease in 26S proteasome activity should lead to the accumulation of ubiquitin-protein conjugates, as occurs upon treatment with proteasome inhibitors, provided there were no global changes in the rates of ubiquitylation as well as deubiquitylation. Therefore, we measured the levels of ubiquitin-protein conjugates in neurons following NMDA treatment. Surprisingly, the content of ubiquitin-protein conjugates was significantly reduced 4 h after NMDA exposure, while levels of free (monomeric) ubiquitin remained unaffected (**Figures 6C,D**).

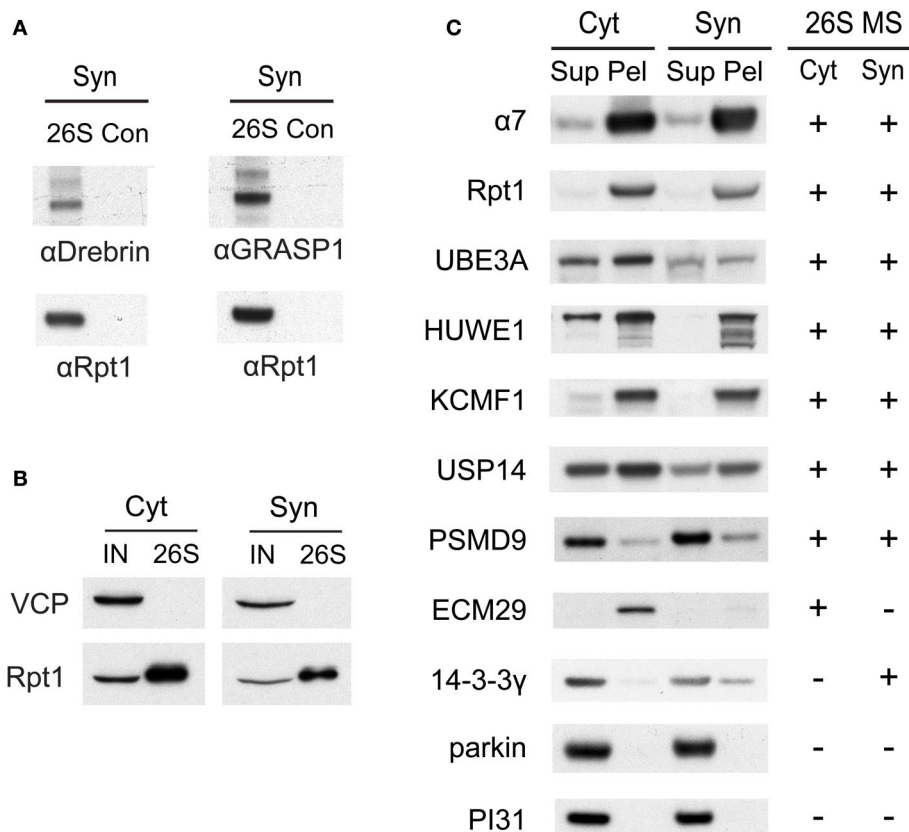


FIGURE 5 | (A) Verification of drebrin and GRASP-1 as interactors of synaptic proteasomes. Both proteins were initially detected in synaptic proteasomes by mass spectrometry, based on a single matching peptide (**Table 2**). These specific interactions are confirmed by SDS-PAGE and Western blotting. Synaptosome (Syn)-derived proteasomes (26S) and control (Con) purifications using only GST are probed with specific antibodies against drebrin, GRASP-1, and 19S subunit Rpt1. **(B)** The VCP complex does not interact appreciably with GST-UBL. 26S proteasomes from the cytosolic (Cyt) and synaptic (Syn) extracts were purified by the GST-UBL/His-UIM method. The input (IN) material and purified 26S proteasomes were immunoblotted against VCP and Rpt1. The amount of VCP captured by the GST-UBL matrix was undetectable by Western blot in three independent experiments. **(C)** Verification of proteasome-interacting

proteins by co-sedimentation. Cytosolic (Cyt) and synaptosomal (Syn) extracts were subjected to centrifugation at $100,000 \times g$ for 6 h. The supernatant (Sup) and pellet (Pel) materials were loaded at 1:1 ratio and resolved by SDS-PAGE. The sedimentation of proteasomes was confirmed by the high levels of 20S subunit $\alpha 7$ 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, 14-3-3 γ) probed by specific antibodies (see Materials and Methods). Parkin and PI31 did not show appreciable association with brain proteasomes. Results shown here are representative of three experiments.

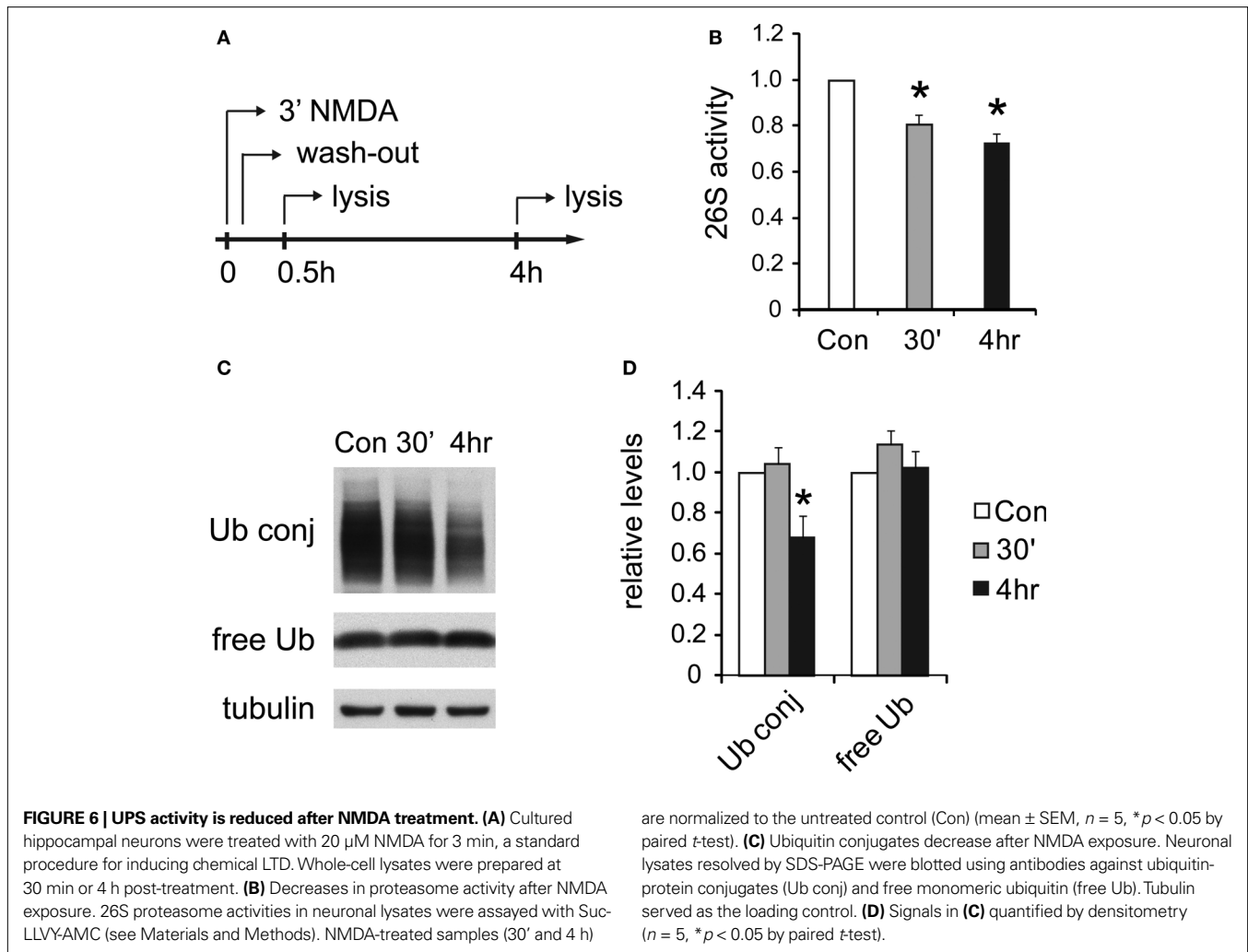
The similar decrease in 26S proteasome activity and ubiquitin-conjugate levels 4 h after NMDA treatment strongly implies that protein ubiquitylation also decreased and suggests a coordinated reduction in the rate of proteolysis mediated by the ubiquitin-proteasome pathway during NMDA-induced plasticity. These results are consistent with earlier reports showing that activity-blockade with TTX leads to decreased proteasome activity (Djakovic et al., 2009) and lower ubiquitin-conjugate levels (Ehlers, 2003).

NMDA TREATMENT CAUSES 26S DISASSEMBLY AND E3 DISSOCIATION

We next investigated the mechanisms responsible for the long-lasting reduction in UPS activity. In yeast, during stationary phase when proteolysis by the UPS appears to be down-regulated, 26S proteasomes gradually disassemble into 19S and 20S particles (Bajorek et al., 2003). Hence, we assessed if similar changes occur in neurons by native gel analysis. As expected,

there was a shift from 26S proteasomes to 20S proteasomes 4 h after NMDA exposure, indicating the disassembly of 26S proteasomes (**Figures 7A,C**).

The above findings suggest that the reduction in 26S levels after NMDA exposure in neurons is coordinated with reduced protein ubiquitylation (**Figures 6C,D**). One possible mechanism is that some of the cell's many ubiquitin ligases may be down-regulated following NMDA treatment. We therefore focused on the three proteasome-bound E3s identified earlier in this study. The degree of association between the E3s and the proteasome was determined by their co-sedimentation properties (at $100,000 \times g$ for 6 h). Two of these E3s, UBE3A and HUWE1, were found to be largely dissociated from proteasomes at 4 h after NMDA exposure, while the ligase KCMF1 was not significantly affected (**Figure 7D**). The loss of specific ligases from the proteasome may potentially decrease proteasome activity and



alter substrate specificity (Kraut et al., 2007). It is noteworthy that UBE3A (E6-AP) was the first E3 to be implicated in synaptic plasticity (Jiang et al., 1998), and it can target synaptic protein Arc for degradation to control glutamate receptor endocytosis (Greer et al., 2010). Our data implies that UBE3A may provide a mechanistic link between NMDA receptor signaling and proteasome activity.

DEGRADATION OF 19S PARTICLES AND PROTEASOME-BOUND E3s

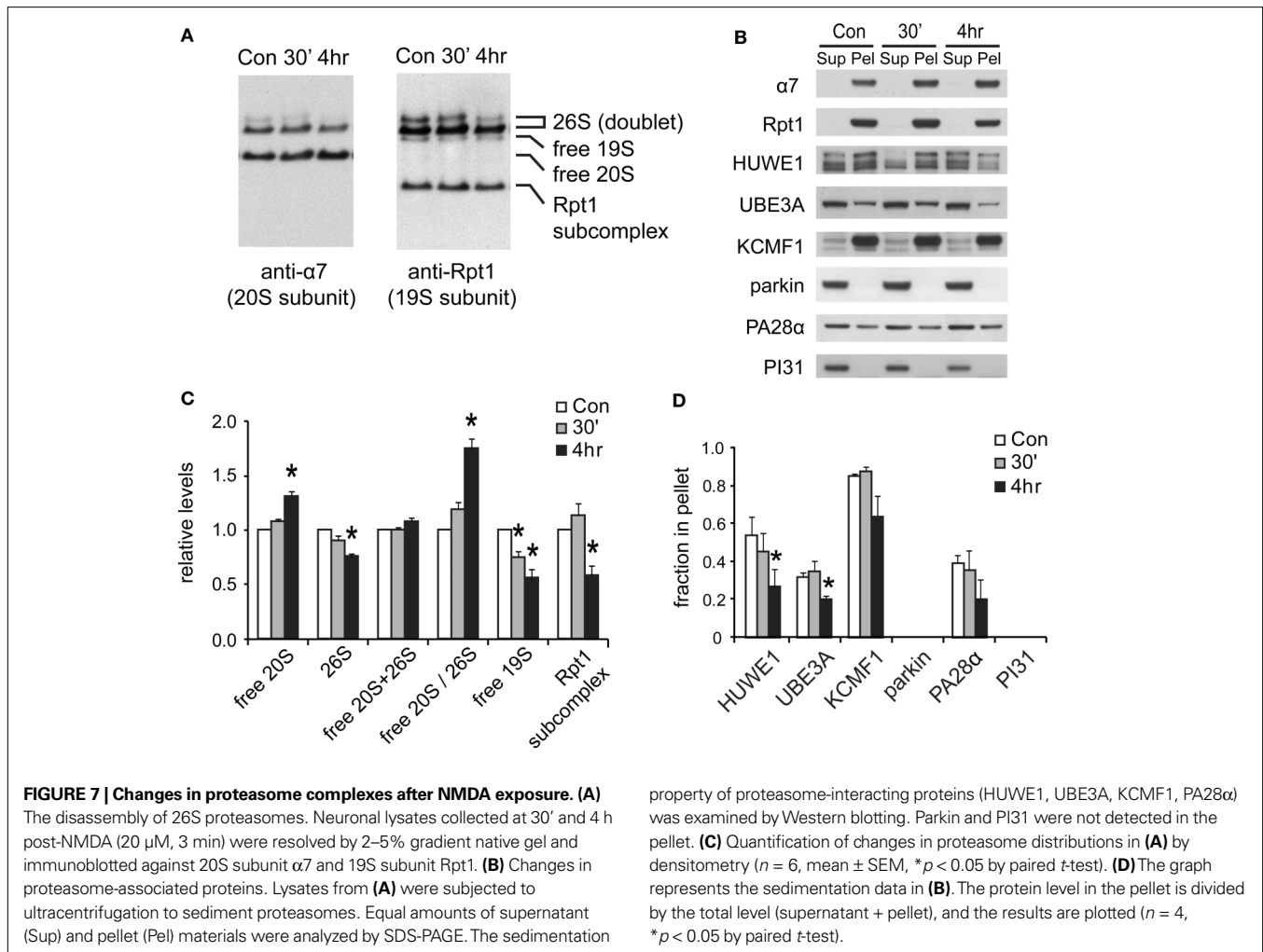
What is the fate of the 19S particles dissociated from 26S proteasomes? In yeast, during stationary phase, free 19S particles appear to remain intact and available for reuse when growth is reinitiated (Bajorek et al., 2003). However, in neurons during NMDA-induced plasticity, we detected a simultaneous decrease in free 19S particles (Figure 7C), suggesting the further disassembly or degradation of the 19S. Therefore, we investigated if the subunits of the disassembled 19S existed as free proteins in the cytosol or were rapidly degraded. Total 19S subunit levels in the lysate were found to decrease significantly (Figures 8A,B), and no free subunits were detectable (Figure 7B). Altogether, these data suggest that 19S particles released from 26S proteasomes were degraded within 4 h of NMDA treatment.

In addition, we investigated the fate of the E3s that dissociated from the proteasome. Like the 19S particle, UBE3A and HUWE1 were degraded by 4 h after NMDA exposure, as indicated by lower protein levels than untreated controls (Figures 8C,D). On the other hand, the E3 KCMF1 did not show either significant degradation or dissociation from the proteasome (Figures 7D and 8D).

In contrast to acute NMDA treatment, which is often associated with a depression of synaptic transmission, other pharmacological manipulations that suppress neuronal activities do not elicit the degradation of 19S particles (Figure 9). These manipulations included adding DHPG to induce metabotropic glutamate receptor (mGluR)-dependent LTD, applying TTX to block action potentials, and TTX + APV + CNQX to block both action potentials and miniature synaptic events. These results suggest that only NMDA receptor signaling may be specifically required for the degradation of 19S particles, in addition to reduced synaptic activity.

19S PARTICLES ARE DEGRADED BY A PROTEASOME-DEPENDENT MECHANISM

Although the proteasome is the major site for protein degradation in the cell and comprises about 2% of cell proteins, surprisingly little is known about the catabolism of the proteasome



itself. A prior study suggested that 20S proteasomes undergo a slow turnover in the liver via lysosomal digestion (Cuervo et al., 1995). To our knowledge, no study has examined the catabolic fate of 19S particles. Thus, we tested if in response to NMDA treatment they are degraded by the proteasome or the lysosomal system.

We tested many different combinations of proteasome and lysosome inhibitors and examined their effects on the degradation of 19S particles induced by NMDA (Figure 10). Proteasome inhibitors including MG132 and epoxomicin + lactacyctsin were the most effective in preventing the NMDA-induced degradation of the 19S. On the other hand, the lysosome inhibitors chloroquine and concanamycin A, which prevent lysosomal acidification, showed no effects on 19S degradation.

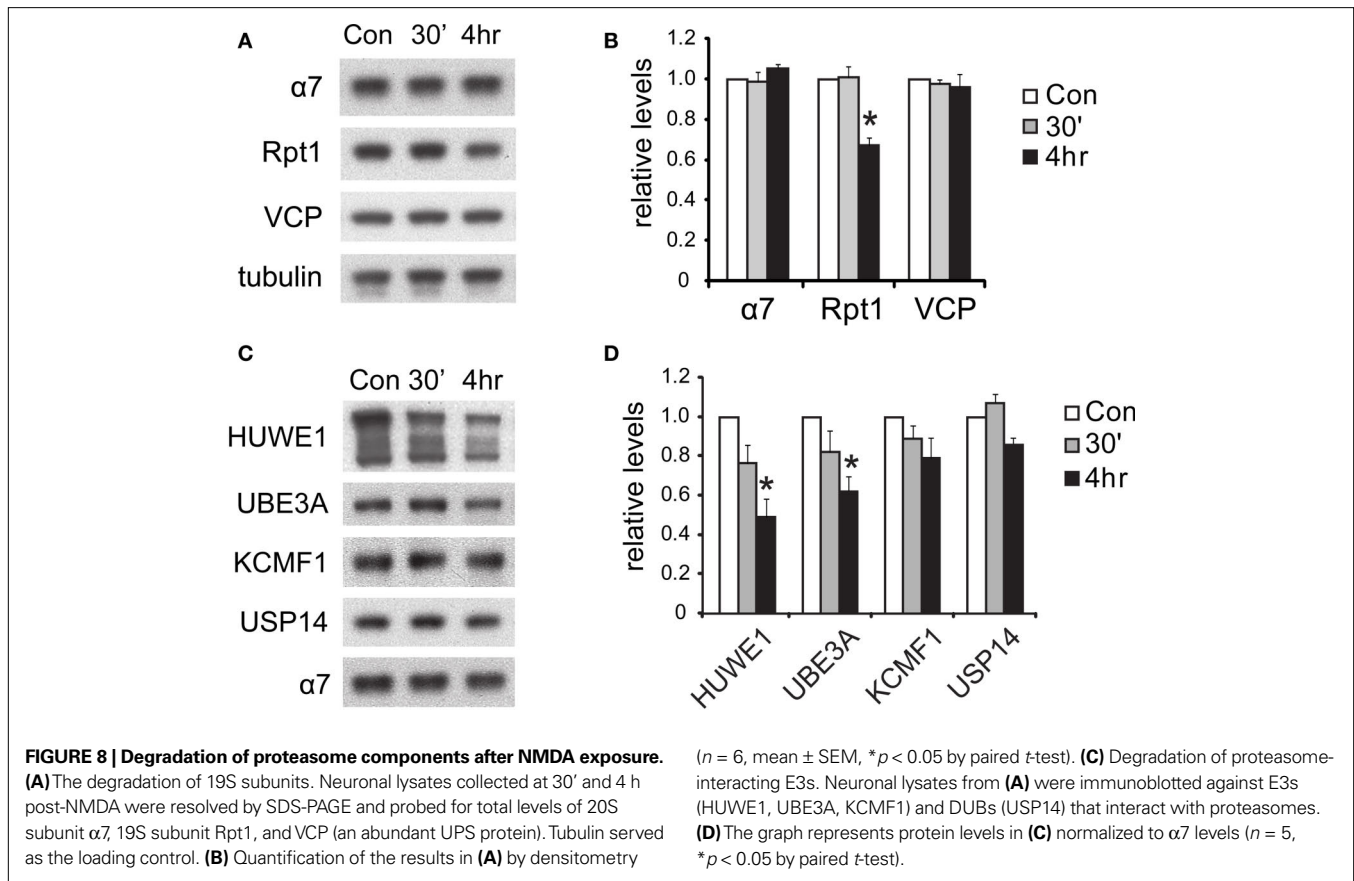
In summary, our data indicate that the reduction in UPS activity after NMDA exposure occurs through several mechanisms, including a reduction of 26S proteasome levels and activities, dissociation of 19S particles from the 26S and their subsequent degradation, decrease of protein–ubiquitin conjugates, and degradation of the E3 enzymes released from the proteasome. The degradation of 19S particles occurs via proteasomal and not lysosomal mechanisms.

property of proteasome-interacting proteins (HUWE1, UBE3A, KCMF1, PA28 α) was examined by Western blotting. Parkin and PI31 were not detected in the pellet. **(C)** Quantification of changes in proteasome distributions in **(A)** by densitometry ($n = 6$, mean \pm SEM, $*p < 0.05$ by paired t -test). **(D)** The graph represents the sedimentation data in **(B)**. The protein level in the pellet is divided by the total level (supernatant + pellet), and the results are plotted ($n = 4$, $*p < 0.05$ by paired t -test).

DISCUSSION

CYTOSOLIC AND SYNAPTIC PROTEASOMES HAVE DIFFERENT COMPOSITIONS

In this study, we isolated and characterized cytosolic and synaptic 26S proteasomes from the rat cortex. One unexpected observation was that the ratio of doubly- to singly-capped 26S proteasomes was higher in the cytosol than in the synapse. Both forms can degrade ubiquitylated proteins (Kriegenburg et al., 2008), but the functional differences between doubly- and singly-capped 26S have not been established. For example, it is unknown whether they differ in rates of degradation or in their substrate affinity or specificity. Singly-capped 26S has an exposed 20S α -ring that allows it to interact with additional regulators such as PA28 or PA200 (Rechsteiner and Hill, 2005). These interacting complexes are likely to dissociate during native gel electrophoresis. Consequently, the singly-capped species observed in our study may in fact represent hybrid 26S (e.g. 19S-20S-PA28) *in vivo*. Unfortunately, the functional roles of these factors on the proteasome are also not well understood. They may facilitate degradation of specific substrates, e.g. PA28 γ has been reported to promote the ubiquitin-independent degradation of the transcriptional coactivator SRC-3 (Li et al., 2006). Thus, it is possible that some singly-capped 26S proteasomes in



association with such cofactors represent more specialized particles that serve distinct functions in different subcellular locations, such as the synapse.

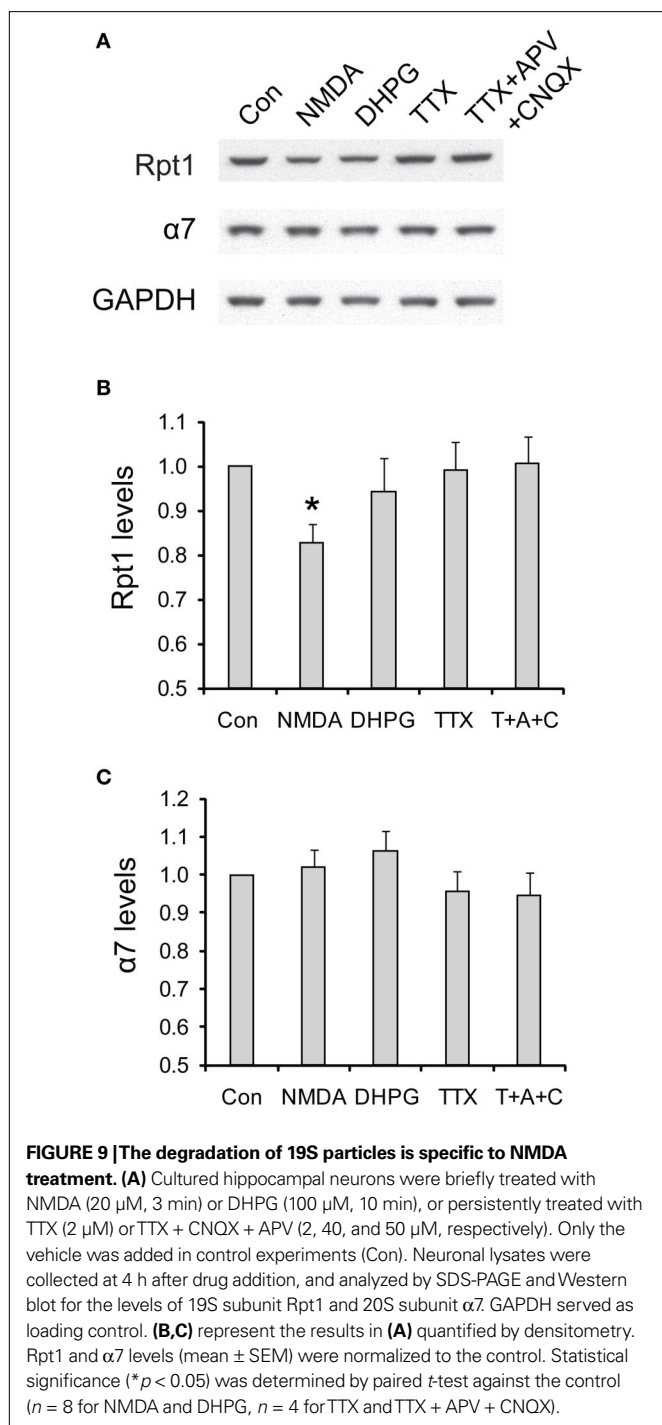
The 28 26S-associated proteins we found in rat cortex differ considerably from those identified by the same approach in rat muscle (Besche et al., 2009). Surprisingly, only 12 are identical, even though myocytes are also postmitotic. Protein degradation however, serves certain very different roles in neurons and skeletal muscle, which represents the major amino acid reservoir in mammals. Unlike proteins in neurons, muscle proteins are mobilized through accelerated proteolysis in fasting or other stressful conditions (Mitch and Goldberg, 1996). Also, the protein composition in skeletal muscles and neurons are very different, which may necessitate the involvement of different factors to assist proteasome-mediated degradation. Whether further differences in the spectrum of proteasome-associated proteins exist between different brain regions remains to be explored.

A surprising finding is that ECM29, which has been reported to stabilize the association of 19S and 20S particles (Leggett et al., 2002; Kleijnen et al., 2007), is only present in the cytosolic 26S but not the synaptic 26S. No factor is known that promotes formation of doubly-capped 26S, but our data suggest ECM29 as a possible candidate.

By mass spectrometry, we identified several proteasome-interacting proteins unique to synaptic 26S proteasomes (14-3-3 γ , TAX1BP1, drebrin, SNAP-25), which may modulate proteolysis in a synapse-specific manner. 14-3-3 γ , which binds many

phosphoproteins in the cytosol (Bridges and Moorhead, 2004), may coordinate proteasome function with the protein phosphorylation events that regulate synaptic transmission (Malenka and Bear, 2004). TAX1BP1 is a ubiquitin-binding protein (Iha et al., 2008; Shembade et al., 2008) 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 (Bingol and Schuman, 2006). Our data suggests that the interaction may be partially mediated by drebrin, an important actin-binding protein in the spine (Takahashi et al., 2009). Moreover, proteasome-mediated proteolysis has been shown to regulate synaptic vesicle release in several ways (Willeumier et al., 2006; Yao et al., 2007). The interaction between synaptic proteasomes and SNAP-25, a key synaptic vesicle protein (DeBello et al., 1995; Montecucco et al., 2005), may provide a mechanism for the proteolytic control of vesicle dynamics.

The various features of the proteasomes in the brain raise many obvious fundamental questions that unfortunately cannot now be answered due to the limits of our knowledge about the UPS. For example, as noted above, the functional differences (if any) between singly-capped and doubly-capped 26S is presently unknown, nor is it clear whether free 20S proteasomes play any significant role in overall intracellular proteolysis by themselves (i.e. when not part of the 26S complex) or in the breakdown of specific proteins although an important role in eliminating inherently structureless proteins has



been proposed (Tsvetkov et al., 2009). Another fundamental issue raised by the present study is the role of the proteasome-associated ubiquitin ligases. Neurons, like other cells, contain many, perhaps hundreds of E3s, and the functional significance of the localization of UBE3A, HUWE1 and KCMF1 on the proteasome is a major mystery. Presumably, the presence of a ubiquitylation system on the proteasome allows more efficient destruction of a substrate than if the substrate is free in the cytosol (or nucleus or synapse) and has to diffuse or be delivered to the 26S for degradation. Interestingly, UBE3A has

recently been implicated in degrading proteins important in synaptic plasticity (Greer et al., 2010). Alternatively, the presence of these E3s on the 26S may indicate a direct role in proteasome function, e.g. in remodeling proteasome-bound ubiquitin chains or enhancing processivity, as has been suggested for the E3, Hul5, which is associated with yeast 26S particle (Crosas et al., 2006). Finally, interpretation of the significance of this spectrum of proteasome-interacting proteins, and differences between synaptic and cytosolic proteasomes, will be facilitated when we learn more about the proteins associated with the 26S in other mammalian tissues. Similar systematic analyses of these proteasomes have not yet been carried out.

NEURONAL ACTIVITY INFLUENCES PROTEASOME DEGRADATION AND INTERACTING E3s

Recent studies have shown that neuronal activity influences the activity of UPS in dendrites and synapses (Bingol and Schuman, 2006; Djakovic et al., 2009). Our findings further demonstrate that the global UPS activity of neurons can also be affected by neuronal activity. Neurons exposed to NMDA showed decreases in the levels of ubiquitin conjugates in whole-cell lysates, which may result from decreased ubiquitylation or increased conjugate degradation. However, after NMDA treatment, the levels of 26S proteasomes decreased due to the degradation of 19S particles. One possible interpretation is that neurons reduce 26S proteasome levels when there are fewer substrates to degrade. We found that 19S degradation is catalyzed by proteasomes and not lysosomes. Although the detailed mechanism is not clear yet, it is likely that the 19S particle dissociates into individual subunits, which are then degraded by the proteasome. Presumably, this NMDA-induced destruction of proteasomes is a mechanism for suppressing overall proteolysis. The proteasome is generally assumed to be a long-lived cell constituent, although the few prior studies (Cuervo et al., 1995) have only investigated turnover of 20S particles. In the NMDA-treated neurons, about a third of the 26S particles and up to 50% of the free 19S were lost in four hours without any concomitant loss of the 20S particle. To our knowledge, this is the first study to observe selective degradation of the 19S particle and the detailed mechanisms will be important to understand in future studies.

The selective degradation of the 19S also resulted in the apparent shift from 26S to 20S proteasomes, which cannot degrade ubiquitylated proteins. The role(s) of free 20S in the cell is not clear since the entry of substrates into its proteolytic chamber is restricted by a gate formed by the N-terminal residues of the α -subunits (Smith et al., 2007; Tanaka, 2009). *In vitro* this gate is continually opening spontaneously and peptides or unfolded proteins can enter the 20S, though at a much lower rate than in the assembled 26S. Degradation of unfolded or inherently structureless proteins by the 20S has often been proposed to occur *in vivo* (e.g. after oxidative damage, or with aging) (Poppek and Grune, 2006; Tsvetkov et al., 2009), but clear evidence for such a role *in vivo* is lacking. On the contrary, there is strong evidence that most unfolded or oxidant-damaged proteins are degraded by a ubiquitin-dependent mechanism requiring the 26S proteasome and VCP/p97 complexes (Medicherla and Goldberg, 2008). Whether having additional 20S proteasomes actually enhances proteolytic functions in neurons is another important issue for further study raised by these data.

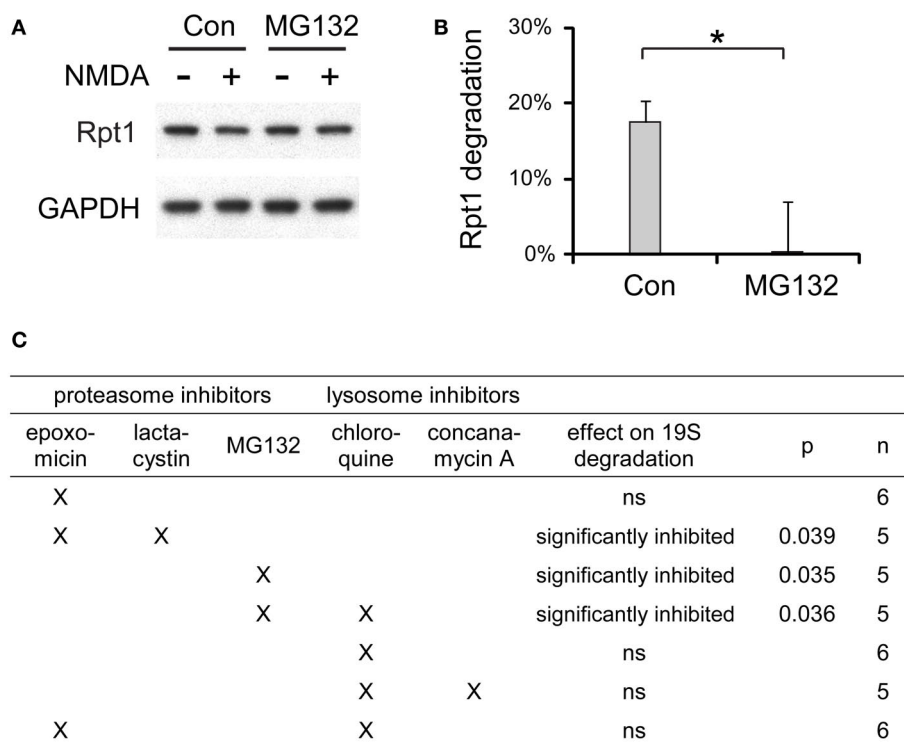


FIGURE 10 | The degradation of 19S particles is proteasome-dependent.

(A) Proteasome inhibitor MG132 (40 μ M) was added to cultured hippocampal neurons 30 min before NMDA stimulation (20 μ M, 3 min). After NMDA washed-out, MG132 was applied again for 4 h before neuronal lysates were collected. In control (Con) experiments, neurons were only treated with NMDA. Rpt1 levels were determined by SDS-PAGE and immunoblotting. GAPDH served as loading control. (B) 19S degradation induced by NMDA in (A) was

quantified by densitometry ($n = 5$, mean \pm SEM). Statistical significance ($*p < 0.05$) was determined by t -test assuming unequal variance. (C) The same experiments as in (A) and (B), performed with different combinations of proteasome inhibitors (2 μ M epoxomicin; 10 μ M lactacystin; 40 μ M MG132) and lysosome inhibitors (150 μ M chloroquine; 100 nM concanamycin A). Statistical significance was determined using t -test (p -value is listed, or ns for not significant, $p > 0.05$).

PROTEASOMES AND SYNAPTIC FUNCTION

Several studies have shown that modifying synaptic strength requires the proteasomal degradation of synaptic proteins (Colledge et al., 2003; Ehlers, 2003; Bingol and Schuman, 2004; Guo and Wang, 2007). In particular, proteasome-mediated proteolysis is required for the endocytosis of glutamate receptors (Colledge et al., 2003; Patrick et al., 2003), after neurons are exposed to high concentrations of glutamate or NMDA (Ehlers, 2000; Lee et al., 2004). Hence, the proteins interacting with synaptic proteasomes identified in this study are likely to be important for synaptic regulation.

We found three E3s (KCMF1, HUWE1, and UBE3A) and five DUBs (USP5, USP7, USP13, USP14, and UCH37) in association with synaptic proteasomes, which may help proteasomes function more efficiently, help determine specificity for certain types of conjugates, or insure the rapid elimination of ubiquitin chains released from the substrate (since free ubiquitin chains can inhibit proteasomal degradation *in vitro*) (Finley, 2009). The proteasome-associated DUBs presumably function together with the Rpn11 subunit (a different type of DUB) to catalyze the disassembly of the ubiquitin chain and the recycling of free ubiquitin (Koulich et al., 2008). In particular, removal of Ubp6 (USP14 homolog) from yeast proteasomes leads to a failure to efficiently remove the ubiquitin chain from substrates and the degradation of some of the attached ubiquitins

(Hanna and Finley, 2007). USP14/Ubp6 also has an important regulatory role in controlling proteasome function (Finley, 2009), for example, together with the ATPases, it regulates gate opening and substrate entry in the 20S (Peth et al., 2009). In fact, there are mice with spontaneous mutations in USP14, called ataxia (*ax^l*) mice, which develop synaptic dysfunction (Wilson et al., 2002; Anderson et al., 2005). Our identification of USP14 on synaptic proteasomes supports the model that reduced synaptic levels of monomeric ubiquitin and ubiquitin conjugates observed in *ax^l* mice (Chen et al., 2009) may be due to altered proteasomes that degrade the substrate-linked ubiquitin and fail to recycle it.

Since hundreds of E3s encoded in the mammalian genome appear to function without apparent association with the proteasome (Semple, 2003), it will be important to learn what is special about the three E3s we identified on synaptic proteasomes. Curiously, two of them have been previously linked to genetic disorders associated with mental retardation in humans. UBE3A mutations cause Angelman syndrome (Lalande and Calciano, 2007), and knockout mice lacking this E3 show defects in synapse morphology, glutamate receptor endocytosis, and LTP (Jiang et al., 1998; Dindot et al., 2008; Greer et al., 2010). HUWE1 mutations are found in X-linked mental retardation (Froyen et al., 2008). Since many mental retardation genes appear to be involved in synaptic dysfunction (Humeau et al.,

2009), our data imply that UBE3A and HUWE1 mutations may cause synaptic defects by altering synaptic proteasome function. Interestingly, UBE3A and HUWE1 dissociate from 26S proteasomes and become degraded after neurons are exposed to NMDA. This simultaneous degradation of 19S particles and these bound E3s represents a novel mechanism of UPS regulation in mammalian cells. It will be important to determine what synaptic protein substrates are normally targeted for degradation by these two E3s, and which are presumably stabilized after NMDA treatment.

PROTEASOMES AND NEURONAL MAINTENANCE

Unlike yeast and other mitotic cells, neurons cannot dispose of damaged proteins by cell division (Aguilaniu et al., 2003). Hence, neuronal proteasomes and their interacting proteins must play important roles in clearing abnormal proteins.

The hallmarks of many neurodegenerative disorders are neuronal aggregates of misfolded proteins, which often contain ubiquitylated proteins and proteasomes (Ross and Poirier, 2004; Rubinsztein, 2006). It is clearly important to identify the factors that help deliver ubiquitylated proteins to proteasomes in neurons, because they may be critical in preventing aggregate formation (Chung et al., 2001; Bedford et al., 2008b; Tai and Schuman, 2008). It is now clear that many ubiquitin conjugates (e.g. misfolded proteins degraded by the ER-associated degradation pathway or oxidatively damaged proteins in yeast) bind first to the VCP/p97 ATPase complex and then are delivered to the 26S complex bound to UBL-proteins (e.g. Rad23 or ubiquilin) (Madura, 2004; Medicherla and Goldberg, 2008; Nakatsukasa et al., 2008; Finley, 2009). Our data show that p62 and the VCP ATPase complex, both of which bind ubiquitylated proteins and assist degradation (Dreveny et al., 2004; Seibenhener et al., 2004), are associated with neuronal proteasomes. It is attractive to conclude that p62 and VCP/p97 function together with UBL-proteins to handle various ubiquitylated substrates at neuronal 26S proteasomes.

Interestingly, genetic studies have shown that the disruption of p62 (Ramesh Babu et al., 2008), VCP (Watts et al., 2004; Neumann et al., 2007), and 19S particle (Bedford et al., 2008a) in the brain leads to ubiquitylated inclusions of tau, TAR-DNA binding protein (TDP-43), and α -synuclein, respectively. In fact, these three types of inclusions are associated with over 80% of sporadic demen-

tias in humans (Cummings, 2004; Bugiani, 2007), which include Alzheimer's disease (tau), dementia with Lewy bodies (α -synuclein), and certain types of frontotemporal dementias (tau or TDP-43). Therefore, protein aggregates found in sporadic dementias may reflect the impairment of different aspects of 26S proteasome function or substrate delivery to it. Severe 26S dysfunction may also contribute to the co-morbidity observed in some dementia cases, characterized by multiple aggregate types (for example, α -synuclein plus TDP-43) (Nakashima-Yasuda et al., 2007; Kovacs et al., 2008). Testing these hypotheses in humans or animals has proven difficult (Lansbury and Lashuel, 2006) in the past due to difficulties in isolation of 26S proteasome in its native forms. The affinity purification methods used here should greatly facilitate such studies of 26S proteasome function in neurodegenerative disorders, studies of how neuronal activity regulates 26S proteasome properties, or the reported progressive decline of proteasome activity with aging (Dahlmann, 2007; Tonoki et al., 2009).

In summary, we found that neuronal proteasomes have a large set of interacting proteins that differs from the set of interacting proteins in muscle (Besche et al., 2009) or yeast (Verma et al., 2000). The proteasome complex varies in different subcellular localizations and changes its content markedly with neuronal activity. Surprisingly, brief activation of NMDA-type glutamate receptors leads to a reduced capacity of the ubiquitin proteasome pathway through rapid disassembly and degradation of the 26S components. This newly discovered level of complexity should be taken into consideration in efforts to understand the molecular mechanisms of synaptic plasticity, the selective degradation of misfolded brain proteins, and the failure to clear such proteins in neurodegenerative diseases.

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