UBIQUITIN-PROTEASOME SYSTEM AT THE SYNAPSE

Thesis by

Baris Bingol

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY
Pasadena, California
2006
(Defended May 19, 2006)

ACKNOWLEDGEMENTS

This work would not have been possible without the guidance of my advisor, Erin M. Schuman. Her passion for pursuing the big questions in science is something that I will value throughout my career. I am grateful for the supportive research environment that she created, allowing me the freedom to design my experiments and work independently.

I would also like to thank my committee members, Kai Zinn, Ray Deshaies, and Mary B. Kennedy for providing constructive criticism and support throughout my graduate career. Our discussions during committee meetings helped me to judge my experiments from new perspectives. My SURF advisor, Paul W. Sternberg, deserves special credit for letting me experience the research environment at Caltech for the first time as an undergraduate in his lab.

I would also like to thank the current and past members of the Schuman Lab, especially Gentry Patrick for teaching me most of the techniques that I use today, and Chin-Yin Tai for being so knowledgeable and answering my endless questions. Thanks to my friend Dan Gold; I wouldn't be able to see so many movies and taste so many different cuisines without them. I would also like to thank my friend Aaron Jue for coming with me to food fairs, tasting Turkish food that I cook, and watching movies.

Finally, I dedicate this work to my family who has supported me since I first decided to pursue biology. While most families want their children to become doctors or lawyers in my culture, they encouraged me to do science with I don't know how many little microscopes, computers, and science gadgets. Without them, I would not be able to see the half-million dollar microscopes that I am "playing" with right now.

ABSTRACT

Each neuron in the mammalian central nervous system makes up to ten thousand synaptic connections with other neurons yet is able to regulate the strength of individual connections locally. Synaptic enhancement or depression induced at one location on the dendritic arbor does not spread through out the entire neuron. This means neurons must be able to regulate the complement and concentration of the synaptic proteins locally, near synapses. The local concentration of synaptic proteins is influenced by many processes, including protein trafficking, buffering and sequestration, and most directly by protein synthesis and degradation. In recent years, it has been shown that neurons can synthesize proteins locally in their dendrites. These studies have suggested that any cellular process that regulates protein availability could be of importance in regulating synaptic function and plasticity. Indeed, the evidence for the contribution of local protein degradation to the regulation of synaptic function and plasticity has started to emerge in recent years.

Here, we show that synapses have the machinery required to degrade proteins and local protein degradation occurs in the dendrites. Furthermore, we demonstrate the requirement for protein degradation for one of the main cellular correlates of synaptic plasticity, namely the trafficking of glutamate receptors. In turn, we demonstrate how neuronal activity regulates protein degradation at synapses, specifically by mobilizing the enzymatic machinery for protein degradation. These data show that the interplay between protein degradation and synaptic activity functions to sculpt the protein composition of the synapses.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

ABBREVIATIONS USED

ABP	AMPAR binding protein
AMPA	α-amino-3-hydroxy-5-methylsoxazole-4-propionate
APC	anaphase promoting complex
APV	2-amino-5-phosphonovaleric acid
BDNF	brain-derived neurotrophic factor
CA1	cornu ammonis 1 (a hippocampal subfield)
CA3	cornu ammonis 3 (a hippocampal subfield)
CamKIIα	calcium/calmodulin-dependent protein kinase α subunit
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
DG	dentate gyrus
DHPG	(RS)-3,5-dihydroxyphenylglycine
dlg	disc large
DSB	double strand breaks
DUB	deubiquitinating enyzme
E6-AP	e6-associated protein
EJP	excitatory junctional potential
E-LTP	early-phase LTP
EPSP	excitatory postsynaptic potential
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FAF	fat facets
FRAP	fluorescence recovery after photobleaching
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GHR	growth hormone receptor
GK	guanylate kinase

GluR	glutamate receptor
GPCR	G protein coupled receptor
GRASP	GRIP1-associated protein
GRIP	glutamate receptor interacting protein
HBS	HEPES-buffered saline
HECT	homologous to E6-associated protein C-terminus
HFS	high frequency stimulation
Hiw	highwire
IRES	internal ribosomal entry site
kDa	kilodaltons
Kir	Inwardly rectifying potassium channel
LAR	Leukocyte common antigen−related
LatA	latrunculin A
L-LTP	late-phase LTP
LPS	local protein synthesis
LTD	long-term depression
LTF	long-term facilitation
LTF	long-term facilitation
LTP	long-term potentiation
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotrophic glutamate receptor
NMDA	N-methyl-D-aspartate
Nnos	neuronal nitric oxide synthase
NSF	N-ethylmaleimide sensitive factor
NT3	neurotrophin-3
PA	proteasome activator
PDZ	PSD-95, discs-large, ZO-1
PICK1	Protein kinase C alpha binding protein
PKA	protein kinase A
PKC	protein kinase C

PP1	protein phosphatase I
PSD	postsynaptic density
RING	really interesting new gene
RNAi	RNA interference
Rpn	regulatory particle non-ATPase
Rpt	regulatory particle ATPase
SAP	synapse-associated protein
SPAR	spine-associated RapGAP
SynGAP	synaptic Ras-GTPase activating protein
TTX	tetrodotoxin
UBP	ubiquitin-specific processing proteases
UCH	ubiquitin C terminal hydrolyses
UIM	ubiquitin interacting motf
UPS	ubiquitin-proteasome system

Chapter I

INTRODUCTION

Synaptic plasticity -the dynamic modification of functional synaptic strength between neurons of the central nervous system- is generally believed to be a physical mechanism underlying learning and memory. Long-term synaptic plasticity was first demonstrated in mammals by Bliss and Lømo (1973). They examined synaptic transmission between perforant path fibers and granule cells in the dentate gyrus of the hippocampus by recording population excitatory postsynaptic potentials (EPSPs), which are the extracellular responses to perforant path stimulation. In these experiments, they demonstrated that brief high frequency stimulation (HFS) of the perforant path fibers resulted in a long-term increase in amplitude of the EPSPs; this increase lasted for hours in anesthetized animals and for days in implanted rabbits. These experiments provided evidence supporting Hebb's postulate (1949): When a synaptic connection is successfully and repeatedly used to generate synaptic responses between two neurons, the strength of that connection is increased. Similar kinds of synaptic enhancement, referred to as long-term potentiation (LTP), were also shown later in the rat hippocampus between Schaffer collateral/commissural synapses in the CA1 region of the hippocampus as well as in the mossy fiber synapses in the CA3 region of hippocampus (Malenka and Nicoll, 1999).

Input specificity of LTP

One interesting property of LTP is that it is input-specific: LTP induced at one set of synapses in a neuron does not spread to the entire dendritic arbor (Andersen et al., 1977). For example, consider two ESPSs evoked in one pyramidal neuron in response to the stimulation of two different Schaffer collateral inputs. Before induction of LTP, two

EPSPs have the same amplitude. When only one of these inputs is tetanized with HFS to induce LTP, only the EPSP of the tetanized input and not the other is potentiated. This means when generated at one set of synapses by repetitive stimulation, LTP does not spread to the other synapses of the same neuron. Such an ability to regulate synaptic inputs independently, as opposed to carrying the same information at all synapses, endows the neuron with the ability to encode greater information.

Protein synthesis requirement for late-phase LTP

The capacity of the neurons to independently modulate their individual synaptic connections suggests that the cellular processes that mediate the enduring changes during synaptic plasticity must act locally. One interesting example of such a cellular process is protein synthesis: Synapses need to make new proteins in order to achieve enduring changes in their synaptic strength (Frey et al., 1988; Otani et al., 1989; Stanton and Sarvey, 1984). When LTP is induced in hippocampal slices in the presence of protein synthesis inhibitors or transcription inhibitors, an early enhancement in synaptic strength (early-phase LTP (E-LTP), lasting 2 - 3 hours depending on the induction protocol used) is followed by a decline back to the baseline levels of synaptic transmission (Frey and Morris, 1997; Schuman, 1997). The gene expression and protein synthesis-dependent phase of LTP (late-phase LTP (L-LTP)) can last as long as the hippocampal slice remains alive (~12 hours) (Reymann et al., 1985). Furthermore, L-LTP recorded *in vivo* lasts for several days to weeks (Bliss and Gardner-Medwin, 1973; Leung and Shen, 1995; Staubli and Lynch, 1987). The requirement for protein synthesis for late-phase LTP suggests that

the newly synthesized proteins are involved in sculpting the synaptic protein composition to modulate synaptic strength. However, when the two properties of LTP, synapse specificity and protein synthesis requirement, are considered, an interesting question arises (Schuman, 1997): How do the newly synthesized proteins target only the activated synapses if one considers the soma as the main site of protein synthesis?

In order to answer this challenging question, different hypotheses were suggested. One of these hypotheses claims that the newly synthesized "synaptic plasticity" proteins in the soma are transported to the dendrites and then captured at the potentiated synapses by a tag that is generated at the synapse in response to the plasticity-inducing stimuli (Frey and Morris, 1997). A more efficient way of getting around the synapse-specific protein distribution problem is to bypass the somatic protein synthesis and subsequent transport to specific synapses steps and directly synthesize the "synaptic plasticity" proteins locally within the dendrites. This is a much simpler solution compared to the "synaptic tag" idea and could, in principle, endow each synapse with its own set of protein synthesis machinery to synthesize its own proteins. According to the local protein synthesis idea, plasticity-inducing stimuli will generate signals that activate the protein synthesis machinery residing next to the synapses, resulting in site-specific production of proteins required for synaptic plasticity.

Local protein synthesis

There is much evidence showing that dendrites have the capacity to synthesize proteins locally: i) The machinery for protein synthesis exists in the dendrites: Polyribosomes and mRNAs are present in the dendrites and preferentially localized next to the synaptic sites (Bodian, 1965; Steward and Levy, 1982; Steward and Schuman 2001). ii) Protein synthesis can occur in reduced synaptic preparations like synaptosomes (Bagni et al., 2000; Yin et al., 2002). iii) In the CA1 region of the hippocampus, patterned synaptic activation results in the incorporation of ^{3}H-leucine to the proteins in dendritic processes and not soma (Feig and Lipton, 1993). These experiments suggest that dendrites have the protein synthesis machinery that could be regulated by synaptic activity. The demonstration of local protein synthesis during synaptic plasticity relied on the observation that neurotrophin application (BNDF or NT-3) causes a rapid and long lasting increase in synaptic strength (Kang and Schuman, 1995) that is attenuated by brief pretreatments of protein synthesis inhibitor (Kang and Schuman, 1996). In this study, the immediate requirement for protein synthesis in synaptic potentiation suggests that proteins must be synthesized close to synapses. Indeed, the protein synthesis dependent synaptic enhancement did not depend on the presence of neuronal soma: when the soma was mechanically dissociated from the synaptic neurophil, a protein synthesis inhibitor-sensitive synaptic enhancement with neurotrophin application was still observed. This strongly suggests that dendrites have the capacity to synthesize proteins locally during synaptic plasticity.

In addition to the experiments presented above, hippocampal culture systems have been used to study local protein synthesis using biochemistry and imaging. Similar to slices, neurites that are mechanically dissociated from their cell bodies in culture can incorporate ^{3}H-leucine and express proteins following transfection of mRNAs (Torre and Steward, 1992; Crino and Ebervine, 1996). Furthermore, Aakulu et al. were the first to visualize dynamic local protein synthesis in the dendrites using a GFP-based fluorescent protein synthesis reporter. This reporter had a myristoylation signal that limits GFP diffusion and allows the membrane incorporation of GFP near the site of synthesis. Using this reporter, Aakulu et al. demonstrated that protein synthesis can occur in transected dendrites and at distinct and stationary "hot-spots" near synapses (Aakulu et al., 2001).

Protein degradation at the synapse

The molecular and cellular mechanisms underlying synaptic plasticity has been an area of intensive research in recent years. Accumulating data suggest that synaptic plasticity is associated with changes in the synaptic protein content both at the level of modification of existing proteins as well as modulation of the abundance of individual synaptic proteins. The data presented above suggest that the locally synthesized proteins during synaptic plasticity may be one mechanism neurons use to locally regulate their synaptic protein content. In addition to protein synthesis, the local concentration of synaptic proteins is also influenced by protein trafficking, buffering and sequestration, as well as protein degradation. Among these cellular processes, protein degradation could

be considered the most direct regulator of protein abundance because newly synthesized proteins may be functionally inactive and require posttranslational modifications in order to be fully functional. On the other hand, protein degradation is direct and irreversible: when the long lasting nature of the synaptic plasticity is considered, protein degradation may provide another mechanism to sculpt protein content for enduring long-term changes in the synaptic efficacy. Thus, it is not surprising that several recent studies have implicated regulated protein degradation in the control of synaptic development and plasticity (Bingol and Schuman, 2005). Most protein degradation occurs through a highly regulated cellular pathway called the ubiquitin-proteasome system (UPS).

In the subsequent chapters, I will first describe the studies that demonstrate some basic mechanisms of synaptic plasticity and how the UPS is involved in different aspects of synaptic function. Second, I will describe my studies that show protein degradation can occur locally in the dendrites. Third, I will describe a role for UPS in one of the cellular mechanisms of synaptic plasticity, namely glutamate receptor trafficking, and will discuss possible targets of UPS at the synapse. Lastly, I will describe how the localization of UPS components may be regulated by synaptic activity.

Chapter II

LITERATURE REVIEW

SYNAPTIC PLASTICITY

There are two extensively studied forms of synaptic plasticity: long-term potentiation (LTP) and long-term depression (LTD). Work in the last 10 years has shown that there is not only one type of LTP or LTD (Malenka and Bear, 2004). Depending on the stimulation protocols, the type of the synapse studied and the age of the animals used, the cellular mechanism for induction and maintenance of the synaptic plasticity differs. The most widely studied forms of LTP and LTD are *induced* by the activation of one class of ionotrophic glutamate receptors found on the postsynaptic membrane, namely N-methyl-D-aspartate receptors (NMDARs). NMDARs act as coincidence detectors during synaptic transmission: For NMDARs to open, two events have to occur simultaneously, glutamate must bind and postsynaptic membrane must depolarize. This property of the NMDARs allows the synapse to detect if the presynaptic and postsynaptic neurons are active at the same time, a prerequisite for Hebbian plasticity. Opening of NMDARs leads to an influx of Ca^{++} in the postsynaptic cell, where the synaptic plasticity inducing signaling cascades are initiated. The reason why NMDARs require postsynaptic depolarization to open is because of the Mg^{++} block of the channel gate at resting membrane potentials (Mayer et al., 1984). The initial postsynaptic depolarization to remove the Mg^{++} block is provided by another class of glutamate receptor on the postsynaptic membrane, namely α-amino-3-hydroxy-5-methylsoxazole-4-propionate receptors (AMPARs). AMPARs are multimeric protein complexes that are composed of combinations of four different subunits (GluR1-4) (Bettler and Mulle, 1995). In most

adult excitatory neurons, combinations of GluR1-GluR2 or GluR2-GluR3 are found at the synapses. GluR4 containing AMPARs are expressed mainly in early postnatal development (Spreafico et al., 1994). All four GluRs have similar topologies: an N-terminal extracellular domain, which is followed by four transmembrane domains and the intracellular C-terminal domain that mediates most of the protein-protein interactions. AMPARs can be classified based on the length of their C-terminal: GluR1 and 4 have long C-terminal tails whereas GluR2 and GluR3 have short tails. The significance of this distinction is the differential binding of these different C-terminal tails to different sets of proteins at the synapse. Thus, in neurons, different combinations of AMPAR subunits form channels with different interacting partners, endowing them with unique properties that may be important for synaptic plasticity.

The hippocampus

Synaptic plasticity is widely studied in the hippocampal slice preparation owing to the laminar organization of the hippocampus (Reymann et al., 1985). The hippocampus is a telencephalic structure that has two crescent-like shaped regions, Ammon's horn and the dentate gyrus. The characteristic shapes of these regions are formed by the cell bodies of the principal neurons: pyramidal neurons and granular neurons in Ammon's horn and dentate gyrus, respectively. The Ammon's horn is subdivided into three regions, CA1, CA2 and CA3 (CA: *c*ornu *a*mmonis). The hippocampus receives inputs mainly from the entorhinal cortex. The entorhinal cortex axons form the perforant path fibers that make connections to the dendrites of the granule cells of dentate gyrus and pyramidal cells of CA3. LTP was first demonstrated in these

connections between perforant path fibres and granule cells in the dentate gyrus (Bliss and Lømo, 1973).

The flow of information in the hippocampus is uni-directional. The axons of granule cells in dentate gyrus (mossy fibers) form the main input of pyramidal cells of CA3. The axons of pyramidal cells in CA3 (Schaffer collateral fibers) contact with the dendrites of pyramidal cells of CA1. These defined routes for information flow and the laminar structure of the hippocampus make it possible to study the specific synaptic connections by selectively stimulating axons and recording EPSPs either from a single cell from the cell body layer or from a group of neurons from the dendritic layer.

Long-term potentiation

Depending on the type of the synapse studied, the mechanism and the site of induction of LTP (presynaptic or postsynaptic) differ (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). For example, both CA1 and dentate gyrus LTP require the activation of NMDARs and influx of calcium in postsynaptic neurons (Collingridge et al., 1983; Coan et al., 1987; Bashir et al., 1990; Lynch et al., 1983). In contrast, LTP at mossy fiber synapses is independent of NMDARs and calcium influx. However, mossy fiber LTP requires calcium influx in presynaptic neuron (Nicoll and Malenka, 1995; Castillo et al., 1994; Zalutsky and Nicoll, 1990). These findings suggest that LTP exhibited at different synapses uses different underlying mechanisms. During NMDAR-dependent LTP, the events that are downstream of calcium entry are not entirely clear. There are many signaling cascades and proteins implicated in the induction of LTP but in

most cases, it is not clear if they are the "mediators" or "modulators": if they are required for LTP or if they alter LTP but are inessential for its occurrence (Sanes and Lichtman, 1999).

One protein, calcium/calmodulin-dependent protein kinase (CamKII) has been shown to be a possible mediator of the NMDAR-dependent LTP induction (Malinow et al., 1989; Malenka et al., 1989): CaMKII is required for LTP induction and LTP induction activates CamKII (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992). Once activated by CaM, CamKII switches from CaM-dependent to partially CaM-independent state via autophosphorylation on Thr^{286} (Miller et al., 1988; Miller et al., 1986; Kennedy et al., 1990). This allows persistent activation of CamKII even though when the Ca^{+2} signal returns to baseline. The importance of CamKII autophosphorylation was demonstrated in experiments where LTP induction triggered a long lasting increase in CaM-independent form of CamKII (Barria et al., 1997; Ouyang et al., 1997; Fukunaga et al., 1993). Furthermore, constitutively active CamKII mimics and occludes LTP (Pettit et al., 1994; Lledo et al., 1995). LTP is absent in CamKII autophosphorylation site mutant mice (Giese et al., 1998). These experiments suggest that CamKII activity is necessary and sufficient for LTP induction. One potential exception to the requirement for CamKII activity in LTP is for LTP exhibited in young tissue. CamKII expression is low during early postnatal stages of the development (Kelly and Vernon, 1985). Furthermore, blocking CamKII activity has no effect on LTP in young rat pup slices (postnatal day 7 -8). In contrast, CamKII inhibitors block LTP in mature slices (>postnatal day 27). Finally, LTP can be induced in young mouse slices prepared from

CamKII autophosphorylation site mutant mice, suggesting that there is a developmental switch for CamKII requirement in LTP (Yasuda et al., 2003).

AMPAR TRAFFICKING

Recent research suggests that a key mechanism for the expression of NMDAR-dependent LTP in postsynaptic neurons is the up regulation of AMPAR function either by posttranslational modifications or enhanced trafficking. An example of the posttranslational modification of AMPARs is the phosphorylation of the GluR1 subunit by CamKII at S831. Phosphorylation of this residue increases the single channel conductance of GluR1 (Derkach et al., 1999). During LTP, the single channel conductance of GluR1 increases along with an increase in the S831 phosphorylation (Barria et al., 1997; Mammen et al., 1997). Furthermore, LTP is reduced in amplitude in mice where S831 is mutated (Lee et al., 2003). This mouse also contained another mutation at S842 of GluR1, which is a protein kinase A site (Roche et al., 1996; Banke et al., 2000). So it is hard to draw conclusions about the specific requirement for S831 phosphorylation by CamKII during LTP.

It is now clear that one major mechanism to regulate synaptic strength is to change the number of AMPARs present on the postsynaptic membrane. Changing the number of AMPARs allows the synapse to modulate its sensitivity and response to a given amount of neurotransmitter (Lynch and Baudry, 1984; Malinow and Malenka, 2002). Indeed, in recent years, it has been demonstrated that AMPARs can undergo rapid

trafficking in and out of the synaptic membrane both during basal transmission and synaptic plasticity. AMPARs are either trafficked to synapses that contain only NMDARs or to synapses that contain both AMPARs and NMDARs (Isaac, 2003). Synapses that lack AMPARs and contain only NMDARs are called silent synapses. Silent synapses are functionally "inactive" at a resting membrane potential as NMDARs require membrane depolarization to open. Thus, AMPAR trafficking has the potential to change both the number and strength of functional synapses.

Constitutive trafficking of AMPARs

The constitutive trafficking of AMPARs was shown by inhibiting protein-protein interactions between AMPARs and AMPAR-interacting proteins. In these experiments, synaptic AMPAR responses were either increased or decreased depending on the protein-protein interaction blocked (Kim et al., 2001; Daw et al., 2000; Luthi et al., 1999; Lüscher et al., 1999). Although the specificity of the inhibition of the protein-protein interactions might be a problem in these experiments, the main conclusion that the AMPARs undergo basal trafficking during synaptic plasticity still holds true (Malenka, 2003).

Regulated trafficking of AMPARs during LTP

The trafficking of AMPARs during synaptic plasticity has also been extensively studied. Some experiments used a form of GluR1 that has different rectification

properties than endogenous receptors. This allows the measurement of synaptically incorporated (functional) AMPARs during synaptic plasticity both *in vitro* and *in vivo*. Using this approach, it has been shown that synaptic delivery of the GluR1 subunit requires neuronal activity, and during LTP, new GluR1-containing AMPARs are inserted into the synapse (Hayashi et al., 2000). The insertion of new AMPARs to the synapse requires NMDA receptor activity, suggesting a further link between AMPAR delivery and NMDAR-dependent LTP. Pointing to the importance of protein interactions with the C-terminal tail of GluR1, overexpression of GluR1 C-terminal tail blocks LTP (Shi et al., 2001). Furthermore, increased activation of CamKII is sufficient to drive AMPARs to the synapse (Hayashi et al., 2000). The increase in the synaptic population of AMPARs during LTP was also confirmed in dissociated neurons or slice culture experiments. Although the similarity of pharmacologically induced LTP (chem-LTP) in culture and LTP induced in slices with electrodes is in question, the experiments in culture revealed that after LTP, more AMPARs accumulate at synapses (Pickard et al., 2001; Lu et al., 2001; Shi et al., 1999).

Regulated trafficking of AMPARs during LTD

Similar to the addition of new AMPARs to the synapse during LTP, there is growing evidence that suggests that AMPARs are removed from the synapse during LTD. The possibility that LTD may involve synaptic removal of AMPARs came from studies that show that AMPARs undergo rapid endocytosis in response to agonist treatment or synaptic stimulation (Lissin et al., 1999). In addition to stimulation of

AMPARs themselves, NMDAR activation, activation of the PKC pathway, insulin receptor or mGluR receptor activation also induce AMPAR internalization (Beattie et al., 2000; Carroll et al., 1999; Ehlers, 2000; Man et al., 2000; Snyder et al., 2001). AMPAR endocytosis is clathrin-dependent and blocked by either GDPßS or a peptide that prevents the interaction between amphiphysin and dynamin (Carroll et al., 1999; Man et al., 2000; Carroll et al., 1999; Man et al., 2000; Wang and Linden, 2000).

The involvement of AMPAR endocytosis in LTD has been studied at different synapses. In hippocampal cultures, low frequency field stimulation decreases miniature excitatory postsynaptic current (mEPSC) frequency and amplitude, which are traditionally regarded as measures of presynaptic and postsynaptic function, respectively. The decrease in mEPSCs was correlated with a reduction in the number of synaptic AMPARs (Carroll et al., 1999). In hippocampal slices, LTD reduces the synaptic pool of AMPARs detected by subcellular fractionation (Heynen et al., 2000). Furthermore, depleting surface AMPARs with treatments like NSF inhibitory peptide or prestimulation with insulin occludes subsequent LTD (Lüscher et al., 1999; Lüthi et al., 1999). Both LTD and AMPAR endocytosis are blocked by blockade of endocytosis with either GDPßS or a peptide that prevents the interaction between amphiphysin and dynamin. Similar results were also obtained from other central synapses like the excitatory synapses on dopamine cells in the ventral tegmental area and parallel fiber-Purkinje cell synapses in the cerebellum (Luscher et al., 1999; Wang and Linden, 2000; Gutlerner et al., 2002). This suggests that the mechanism of LTD at different synapses may be common. However, different signaling cascades are involved at different synapses for

LTD induction. For example, at hippocampal synapses protein phosphatase 1 (PP1) and calcineurin are required for LTD whereas in the cerebellum and the ventral tegmental area PKC and PKA activity are required, respectively (Ehlers, 2000; Beattie et al., 2000; Wang and Linden , 2000; Gutlerner et al., 2002). It is worth mentioning that calcineurin and PP1 activity as well as calcium are both required for NMDA-induced AMPAR endocytosis and hippocampal LTD (Ehlers, 2000; Beattie et al., 2000). The targets of these phosphatases during hippocampal LTD are not known. One possible target might be the AMPAR itself. During LTD, GluR1 gets dephophorylated at S845, which is a PKA site (Lee et al., 2000). Depotentiation of previously potentiated synapses leads to dephosphorylation of CamKII site S831 (Lee et al., 2000). In addition, mice that lack both of these phosphorylation sites lack LTD, and dissociated culture neurons prepared from these mice do not exhibit NMDA-induced AMPAR internalization (Lee et al., 2003). These results would suggest that dephosphorylation of GluR1 itself is required for LTD induction and AMPAR endocytosis requires dephosphorylation of these residues.

Although the experiments described above point to AMPAR endocytosis as a mechanism for LTD, they suffer from the non-specificity of the manipulations performed. For example, blockade of endocytosis with dynamin mutants or pharmacological manipulations does not specifically interfere with AMPAR endocytosis; other endocytosed proteins may be important. Furthermore, as mentioned earlier, AMPAR endocytosis is induced by activation of different neurotransmitter receptors with different biochemical requirements, yet they all lead to AMPAR endocytosis. Although this suggests that LTD can be induced by activation of many different signaling

pathways, it may also mean that AMPAR endocytosis is a "by-product" of these various stimulation protocols, rather than being causally related to synaptic depression. An attractive idea for the regulation of synaptic depression that has not been tested yet is the removal of AMPARs from the synaptic sites by lateral diffusion along the membrane. As detailed in the next section, AMPARs are capable of diffusing along the plane of the membrane (Borgdorff and Choquet, 2002). In theory, removal of the AMPARs away from the synaptic membrane would be sufficient for synaptic depression without a requirement for AMPAR endocytosis. Indeed, the endocytosis machinery at synapses is not situated within the synaptic membrane but adjacent to it (Blanpied et al., 2002; Racz et al., 2004; Petralia et al., 2003). This suggests that AMPAR removal from the synaptic sites has to precede its endocytosis. Thus, LTD-inducing stimuli may regulate the distribution of AMPARs between the synaptic and extrasynaptic surface and not necessarily between synaptic and intracellular pools.

Lateral diffusion of AMPARs

The idea that receptors move laterally on the membrane and accumulate at synaptic sites through "capture" was first established at the neuromuscular junction. According to this idea, the nerve contact regions on the muscle act as a trap for freely diffusing acetylcholine receptors on the muscle membrane (diffusion-trap model) (Young et al., 1983). This shifts the surface receptor distribution from a uniform and low concentration to a local and high concentration. Similar to acetylcholine receptors, AMPARs also move by diffusion along the membrane. This was first demonstrated by

tracking a latex bead attached to a surface AMPAR (Borgdorff and Choquet, 2002). These experiments revealed that AMPARs diffuse along the membrane and alternate between stationary and mobile states within seconds. More interestingly, AMPARs have longer and more frequent stationary periods as the neurons mature. Furthermore, the sites where AMPARs become stationary are spatially correlated with synaptic sites. The stationary states may correspond to the synaptic accumulation of AMPARs via "capture" of the extrasynaptic AMPARs at the synapse.

The bulkiness of the latex beads (~0.5 μm) used in these experiments precludes the direct visualization of synaptic AMPARs. The use of smaller tags, such as nanometer-sized organic fluorophores, allowed the visualization of AMPARs inside synapses. These experiments demonstrated that AMPARs are also mobile inside the synapses (Tardin et al., 2003). However, diffusion characteristics in extrasynaptic and synaptic surfaces were different. AMPARs move freely on the extrasynaptic membrane, traveling long distances in the order of microns. In contrast, AMPARs at the synapse have restricted diffusion: they move transiently and over areas equal to or smaller than the size of the synapse (~0.1 μm^2). This suggests the presence of obstacles inside the synapse for AMPAR diffusion. These obstacles may include scaffolding proteins, membrane microdomains or other transmembrane proteins (Triller and Choquet, 2005).

Although organic fluorophores are small enough to enter the synaptic cleft (~1 nm), they have the disadvantage of poor photostability. Recently, semiconductor nanocrystals, quantum dots, were used to track the movements of AMPARs and glycine

receptors on the neuronal surface (Groce et al., 2004; Tahan et al., 2003). Quantum dots have strong photoresistance allowing longer recordings of the receptor movements (in the order of minutes) but they are 20 - 30 times larger than organic dyes. Thus, organic dyes, in spite of the photoinstability, deliver the best choice for tracking receptor movements at the synapse whereas quantum dots are more suitable to track extrasynaptic receptors.

In addition to AMPAR surface trafficking, lateral mobilities of other glutamate receptors (NMDARs and metabotrophic glutamate receptors (mGluRs)) and glycine receptors were also studied (Groc et al., 2004; Serge et al., 2002; Dahan et al., 2003). These studies revealed that the amount of time each receptor spends at the synapse depends on the receptor type studied and probably reflects different association–dissociation rates of interactions between different receptor types and scaffolding proteins. A comparison of AMPAR and NMDAR lateral motilities (Groc et al., 2004) demonstrated that AMPARs move at least twice as fast as NMDARs in the extrasynaptic membrane. Diffusion within synapses was indistinguishable for AMPARs and NMDARs. AMPAR and NMDAR lateral movements were also differentially regulated by activity. Neuronal activity induced by KCl-depolarization caused a large increase in the mobility of extrasynaptic AMPARs (~6 times) but not of NMDARs (Groc et al., 2004). The increase in the extrasynaptic mobility was mainly due to an increase in the fraction of AMPARs that are mobile at the extrasynaptic membrane. Blocking neuronal activity with tetrodotoxin (TTX) had the opposite effect: mobility of extrasynaptic AMPARs decreased mainly due to a decrease in the fraction of mobile AMPARs. Similar to KCl, TTX had no effect on NMDAR mobility. Interestingly, local changes in calcium had the

opposite effect on AMPAR mobility compared to global changes induced by KCl or TTX: local increase in calcium concentration slowed down AMPAR diffusion (Borgdoff et al., 2002) suggesting that AMPAR mobility is differentially regulated by global and local changes in neuronal activity.

AMPAR PROTEIN-PROTEIN INTERACTIONS

Many proteins that interact with AMPAR subunits have been identified (Malinow and Malenka, 2002). AMPARs lack functional domains that would direct their trafficking so the trafficking of AMPARs during synaptic plasticity must rely on the interaction with AMPAR-interacting proteins during different stages of trafficking. Some of these proteins that are related to my thesis study are discussed below.

A common domain that mediates many of the AMPAR protein-protein interactions is the PDZ (PSD-95, Discs-large, ZO-1) domain. PDZ domains are modular protein-protein interaction domains that are 80 amino acids long (Kornau et al., 1995, Kennedy, 1995; Cho et al., 1992). PDZ domains are present throughout the animal kingdom but similar sequences have been also found in yeast and even in bacterial proteins. The human genome sequence contains >100 PDZ domain-containing proteins. In mammals, the greatest abundance of PDZ-containing proteins are found at the synapse (Kim and Sheng, 2004). PDZ domains usually interact with the C-terminal ends of interacting partners (Kornau et al., 1995; Kim et al, 1995; Niethammer et al., 1996). In addition, some PDZ domains interact with internal sequences or with other PDZ domains

(Sheng and Sala, 2001; Kornau et al., 1997; Craven and Bredt, 1998.) The presence of multiple PDZ domains in synaptic proteins is common and underlies the scaffolding function of PDZ domain-containing proteins to bring multiple partners of a complex together.

PDZ domain proteins interacting with GluR2 subunit of AMPARs

GRIP1/ABP:

Yeast two-hybrid experiments using the C-terminal tail of GluR2/3 identified two highly related PDZ domain-containing proteins, glutamate receptor interacting protein and AMPAR binding protein (GRIP1 and ABP respectively) (Dong et al., 1997; Srivastava and Ziff, 1999). Both GRIP and ABP contain seven PDZ domains, and the PDZ domains 3, 5, 6 were shown to bind to the C-terminal tails of GluR2 and GluR3. Furthermore, GRIP and ABP bind to each other and to themselves through PDZ-PDZ interactions. The last four amino acids of the GluR2 C terminus are involved in the interactions with GRIP/ABP. The importance of PDZ domain interactions for the GluR2 subunit was shown in experiments where these four amino acids were deleted. Although the PDZ binding site-lacking GluR2 AMPARs were trafficked to the surface to the same extent as wild-type receptors, they were not stabilized at the surface, which led to a reduction in the surface AMPARs over time. Further mutagenesis of GluR2 C-terminal tail, rendering it unable to bind to GRIP but not other PDZ containing proteins (e.g., PICK1 – see below), also led to a reduction in the synaptic AMPAR, suggesting that GRIP may play a role in the stabilization of the AMPAR at the synaptic membrane

(Osten et al., 2000). Consistent with this, in slice cultures, expression of GluR2 that lacks the PDZ domain binding site cannot be detected at synapses, suggesting that GluR2-PDZ interactions, including the GluR2-GRIP interaction, are important for surface stabilization of AMPARs (Shi et al., 2001).

GRIP-interacting proteins

GRIP also interacts with other proteins at the synapse, which fits with its scaffolding function. One of these proteins, identified through yeast two-hybrid screens, is liprin-α, which itself interacts with the LAR family of protein tyrosine phophatases (Ko et al., 2003; Wyszynski et al., 2002). The importance of liprin-α in AMPAR trafficking was shown by the expression of dominant negative constructs that disrupt the GRIP-liprin-α interaction, leading to a reduction in surface AMPARs and dispersal of dendritic AMPAR clusters. Liprin-α also interacts with another protein called GIT-1, a multidomain protein that functions as a GTP-ase activator for the ADP-ribosylation family of GTPases. This class of proteins is implicated in the regulation of the actin cytoskeleton and membrane trafficking. Disrupting the interaction between liprin-α and GIT-1 also reduces the surface clustering of AMPARs in the dendrites (Ko et al., 2003).

Another GRIP-interacting protein is GRIP-associated protein-1 (GRASP-1), which is a neuronal RasGEF (Ye et al., 2000). The interaction between GRIP and GRASP-1 occurs via PDZ domains of these proteins. Overexpression of GRASP-1 in

cultured neurons specifically blocks the synaptic targeting of AMPARs, suggesting a role for GRASP-1 in AMPAR trafficking.

The subcellular distribution of GRIP suggests that GRIP may have trafficking roles upstream of the synaptic targeting of AMPARs (Wyszynski et al., 1999). GRIP is present near synapses but also within the dendritic shaft and in axons. Indeed it was shown that GRIP directly interacts with the heavy chain of conventional kinesin (Setou et al., 2002). Thus, GRIP may serve as a link between the microtubule motor proteins and the AMPARs during dendritic AMPAR trafficking. Furthermore, Liprin-α may be indirectly involved in the dendritic trafficking of AMPARs through interactions with a kinesin family member, KIF1 (Shin et al., 2003). Thus, AMPARs travel along microtubules within a complex of AMPAR-interacting proteins and different types of kinesin motor proteins. The involvement of GRIP at different levels of AMPAR trafficking and perhaps in other cellular processes unrelated to AMPARs is suggested by the GRIP1 knock-out mice, which show hemorrhagic blisters and embryonic lethality (Bladt et al., 2002). These blisters were also found at the lateral ventricle of the brain, suggesting GRIP functions both at synaptic junctions and dermo-epidermal junctions.

PICK1

In addition to GRIP, the GluR2 C terminus interacts with the PDZ domain of another protein, Protein kinase C alpha binding protein (PICK1) (Daw et al., 2000; Xia et al., 1999). PICK1, in addition to PKC, also interacts with other signaling proteins (e.g., the Eph family of ephrin receptors) (Torres and Steward, 1998). PICK1 facilitates

the clustering of GluR2 subunits as shown by co-clustering experiments in heterologous cells. It has also been shown that PICK-1 recruits PKC to the synapse following activation of PKC with phorbol esters. Phorbol ester treatment of neurons also causes AMPAR internalization, and PICK1 overexpression reduces the surface amount of AMPARs (Chung et al., 2000; Perez et al., 2001). These data suggest that PICK1 may be involved in the internalization of AMPARs by acting as a scaffold between PKC and AMPARs.

GluR2 PDZ domain interactions during LTD

Both PICK1 and GRIP bind to the C-terminal end of AMPARs with their PDZ domains. As such, it is intriguing to postulate that these two different proteins with opposite effects on AMPAR surface stability might compete for the same binding site. The regulation of binding of GluR2/3 to GRIP and PICK proteins is mediated by the phosphorylation state of the GluR2 C-terminus S880 residue. This PKC phosphorylation site is within the PDZ domain binding region of GluR2/3 and phosphorylated GluR2/3 binds to PICK1 but not GRIP/ABP (Chung et al., 2000; Matsuda et al., 1999; Matsuda et al., 2000; Perez et al., 2001). PKC activation in hippocampal or cerebellar cultures promotes the phosphorylation of this residue. Furthermore, phorbol esters redistribute PICK1 and PKC to synapses, disperse GluR2 clusters and reduce surface levels of AMPARs both in hippocampus and cerebellar cultures (Chung et al., 2000; Matsuda et al., 1999; Matsuda et al., 2000; Perez et al., 2001). These data suggest that PKC-regulated PICK1 binding to GluR2/3 may have a role in the endocytosis/intracellular stabilization of AMPARs during LTD both in the hippocampus and cerebellum.

However, the experiments performed to address the function of GRIP and PICK1 during hippocampal and cerebellar LTD are conflicting. In hippocampal slices, blocking the binding of GluR2 to both GRIP and PICK1 blocks LTD and increases basal transmission in a PKC-dependent manner. However, blocking only the GluR2-PICK1 interaction has no effect on LTD or basal transmission. This suggests that in the hippocampus, GRIP functions to stabilize internalized receptors or destabilize surface receptors whereas PICK1 has no direct role in LTD (Daw et al., 2000; Hirbec et al., 2003). In contrast to these results, Kim et al. found that blocking GluR2-PICK1 interaction does inhibit hippocampal LTD and increases basal synaptic transmission (Kim et al., 2001). Consistent with these results, in cerebellar cultures, interfering with the GluR2-PICK1 interaction blocks LTD (Xia et al., 2000). Furthermore, a mutant form of GluR2 that cannot be phosphorylated on S880 fails to rescue LTD in cerebellar cultures prepared from GluR2-knock-out mice (Chung et al., 2003). These results suggest that PICK1 is involved in the stabilization of AMPARs intracellularly and/or priming them for endocytosis. Indeed, overexpression of PICK1 in cultured hippocampal cultures decreases surface levels of AMPARs (Perez et al., 2001).

The contradictory results described above may reflect cell type differences between hippocampal and cerebellar preparations. Furthermore, the conclusions drawn from culture preparations do not necessarily apply to slice preparations. Finally, GRIP and PICK1 may have multiple roles during induction of LTD in the hippocampus and cerebellum. The palmitoylation of GRIP suggests this might be the case: GRIP undergoes palmitoylation at its N terminus where the palmitoylation site is generated by alternative splicing of the GRIP transcript (Yamazaki et al., 2001). Importantly only the

palmitoylated form of GRIP is localized to synapses. Non-palmitoylated GRIP remains in the cytosol. This suggests that GRIP may function to stabilize AMPARs both at the synapse and intracellularly and the regulatory step for the AMPAR endocytosis may involve palmitoylation of GRIP.

Regulation of synaptic accumulation of AMPARs by PSD-95

Postsynaptic-density Protein-95 (PSD-95)

PSD-95 is one of the most abundant scaffolding proteins found at the postsynaptic density (PSD) (Cho et al., 1992; Kistner et al., 1993; Peng et al., 2004; Hunt et al., 1996). PSD is an electron-dense structure beneath the postsynaptic membrane that contains a high concentration of glutamate receptors and associated regulatory and scaffolding complexes as well as signaling and cytoskeletal proteins (Kennedy, 1997). PSD-95 belongs to a family of cytoskeleton-associated proteins termed MAGUKs (membrane-associated guanylate kinases) that is composed of four members (*PSD-95*/*SAP90* (synapse-associated protein 90), *PSD-93*/*chapsyn-110*, *SAP102* and *SAP97*). The common structure in this family involves three PDZ domains, one Src homology 3 (SH3) domain, and a guanylate kinase-like (GK) domain (Woods and Bryant, 1993). PSD-95 forms multimers at the synapse similar to GRIP. In contrast to GRIP, which forms multimers through interactions between PDZ domains, PSD-95 forms multimers through N-terminal interactions with each other (Hsueh and Sheng, 1999; Christopherson et al., 2003).

The role of PSD-95 as a scaffolding protein was first demonstrated in *D. melanogaster* where mutations in a homolog of PSD-95, discs large (Dlg) led to a dispersal of Shaker K+ channel clusters (Gramates and Budnik, 1999). At mammalian synapses, PSD-95 interacts with the C-terminal tail of NR2 subunit of NMDARs (Kornau et al., 1995). In contrast to Shaker K+ channels, PSD-95 is not required for the clustering of NMDARs themselves (Sprengel et al., 1998; Migaud et al., 1998; Passafaro et al., 1999). However, the signaling pathways downstream of NMDARs are sensitive to the interaction between PSD-95 and NMDARs, suggesting a crucial role for PSD-95 in bringing different components of signaling pathways together at the synapse. For example, nitric oxide synthase (nNOS) is situated next to the intracellular gate of NMDARs via nNOS-PSD-95 interaction. Signaling downstream of nNOS is disrupted if the interaction between NMDARs and PSD-95 is blocked. This suggests a scaffolding role for PSD-95 critical for the formation of nNOS-PSD-95-NMDAR complex and nNOS signaling (Brenman et al., 1996; McGee and Bredt, 2003). Another example of a PSD-95 scaffolding function is the interaction with proline-rich tyrosine kinase 2 (Pyk2), and its downstream effector Src non-receptor tyrosine kinase family (Kalia and Salter, 2003; Tezuka et al., 1999; Huang et al., 2001). Both of these proteins are implicated in synaptic plasticity and PSD-95 may have a role in bringing these signaling complexes to the NMDAR to regulate the tyrosine phophorylation of NMDARs.

In addition to clustering receptor and signaling complexes, PSD-95 is also involved in regulating the activities of membrane receptors. For example, the single channel conductance of inward rectifier K+ channel (Kir 2.3) is decreased by binding to PSD-95 (Nehring et al., 2000). PSD-95 also is involved in synaptic adhesion. At the *D.*

Melanogaster neuromuscular junction, fasciclin II (FasII) interacts with Dlg during development (Packard et al., 2003). At mammalian synapses, PSD-95 interacts with neuroligin, a membrane protein that interacts with β-neurexins on the presynaptic side of the synapse (Irie et al., 1997). The interaction between neuroligin and neurexins induce presynaptic differentiation, suggesting that PSD-95 may also participate in synapse development (Dean et al., 2003).

PSD-95 is also involved in the regulation of the actin cytoskeleton in spines. Kalirin-7, a GEF for RAC1, binds to PSD-95 (Penzes et al., 2001). RAC1 promotes spine formation in neurons and kalirin-7 functions downstream of EphB receptors, which have been implicated in spine development and NMDAR regulation (Penzes et al., 2003; Takasu et al., 2002). As such, PSD-95 may be involved in the regulation of NMDARs by bringing NMDARs and EphB signaling components together.

Another protein that interacts with PSD-95 is SynGAP, which is a synaptic GAP for the Ras and Rap small GTPases (Chen et al., 1998; Kim et al., 1998; Krapivinsky et al., 2004). Overexpression of SynGAP in hippocampal cultures decreases surface AMPARs via decreasing AMPAR insertion into the membrane (Rumbaugh et al., 2006). Cultures prepared from homozygous knock-out SynGAP mice have accelerated synapse and spine development, resulting in bigger spines in the adult neurons (Vazquez et al., 2004). Consistent with these observations, synaptic transmission is increased in neurons from the SynGAP knock-out mice as well as in neuronal cultures treated with SynGAP siRNA (Rumbaugh et al., 2006). Furthermore, SynGAP heterozygous mice have deficits

in spatial memory learning tasks (Zhu et al., 2002; Komiyama et al., 2002; Kim et al., 2003).

SynGAP suppresses ERK signaling; mice that are heterozygous for SynGAP show increased levels of ERK activity, and overexpression of synGAP reduces activation of ERK (Zhu et al., 2002; Rumbaugh et al., 2006). In contrast, P38 mitogen-activated protein kinase (MAPK) signaling is potentiated in SynGAP overexpressing neurons and reduced in SynGAP knock-out mice. SynGAP is a GAP for both Ras and Rap. The regulation of GAP activity towards these effectors may be regulated by phosphorylation via CamKII on multiple sites. (Oh et al., 2004; Krapivinsky et al., 2004).

The guanylate kinase-like (GK) domain of PSD-95 is enzymatically inactive. However, GK domain participates in protein-protein interactions as well: GKAP, guanylate kinase-associated protein, interacts with the GK domain of all PSD-95 family members (Kim et al., 1997). GKAP also interacts with Shank, which is another scaffolding protein at the synapse (Naisbitt et al., 2000).

Finally, SPAR (spine-associated RapGAP), an inhibitory GAP for RAP, binds to PSD-95 and is a target of the ubiquitin-proteasome pathway, which will be discussed below (Pak and Sheng, 2003).

PSD-95 regulates AMPAR abundance at the synapse

Although PSD-95 does not interact with AMPARs directly, it regulates the abundance of AMPARs at the synapse. Overexpression of PSD-95 in hippocampal cultured neurons increases GluR1 puncta size as well as AMPAR EPSCs (Stein et al., 2003; Beique and Andrade, 2003; Ehrlich and Malinow, 2004). Conversely, knockdown of PSD-95 with RNAi decreases AMPAR EPSCs (Kim et al., 2004). These experiments suggest that synaptic PSD-95 modulates the abundance of AMPARs at the synapse. One of the modes of dynamic regulation of PSD-95 at the synapse involves palmitoylation of PSD-95 (Craven and Bredt, 1999). PSD-95 that has mutations at its N-terminal palmitoylation sites cannot accumulate at synapses, and neuronal activity disperses PSD-95 clusters partly by depalmitoylating PSD-95. This also leads to dispersal of AMPARs at the synapses as well (El-Husseini et al., 2002). In addition to palmitoylation, PSD-95 is also regulated by phosphorylation. Cdk5, a serine-threonine kinase, negatively regulates PSD-95 by directly phosphorylating it at its N-terminal region (Morabito et al., 2004). Phosphorylated PSD-95 cannot form multimers, cluster receptors or localize to synapses. So posttranslational modification of PSD-95 though palmitoylation and phosphorylation during synaptic activity may indirectly regulate AMPAR abundance at the synapse.

PSD-95 does not directly interact with AMPARs so its scaffolding role for AMPARs abundance at the synapse may be surprising. Subsequent studies have shown that PSD-95 acts indirectly to cluster AMPAR at the synapse through interaction with a protein called stargazin. Stargazin is the first transmembrane protein identified that interacts with all AMPAR subunits (Tomita et al., 2003; Tomita et al., 2004; Fukata et

al., 2005; Chen et al., 2000). The first clue for a role of stargazin in AMPAR trafficking came from the stargazer mice, which have spontaneous recessive mutations in the stargazin gene. Stargazer mice lack functional surface AMPARs in cerebellar granule cells and suffer from epilepsy and cerebellar ataxia (Chen et al., 1999; Hashimoto et al., 1999). The stargazin gene codes for a four transmembrane-domain membrane protein with a PDZ domain at the intracellular C-terminal tail.

Biochemical and imaging data suggest that stargazin escorts the AMPARs to the synapse at two steps. The first step is to stabilize the AMPARs on the non-synaptic surface either by actively trafficking AMPARs to the plasma membrane or by stabilizing them at the membrane once the AMPARs are delivered through interactions with other proteins. The second step is to stabilize the non-synaptic surface AMPARs at the synaptic sites through interaction of stargazin with the PDZ domain of PSD-95. This two-step mechanism of AMPAR trafficking to the synapse is supported by experiments in which stargazin can rescue both synaptic and non-synaptic AMPAR currents in the stargazer cerebellar granule cells whereas stargazin that cannot bind to PSD-95 rescues the non-synaptic AMPAR currents but not the synaptic ones (Chen et al., 2000). Furthermore, in hippocampal culture and slices, overexpression of PSD-95 specifically increases the synaptic population of AMPARs whereas overexpression of stargazin specifically increases the surface amount of AMPARs with no change in synaptic AMPAR levels. Expression of stargazin that cannot bind to PSD-95 acts as a dominant negative and decreases synaptic levels of AMPARs (Schnell et al., 2002; El-Husseini et al., 2000; Beique et al., 2003; Ehrlich and Malinow, 2004). These results suggest that

stargazin and PSD-95 are the limiting proteins for the surface and synaptic AMPAR levels, respectively.

UBIQUITIN-PROTEASOME SYSTEM

Most proteins in a cell are degraded by the ubiquitin–proteasome system (UPS). The selective degradation of proteins via the UPS involves three steps: recognition of the target protein via specific signals, marking of the target protein with a ubiquitin chain, and delivery of the target protein to the 26S proteasome, a protein holocomplex that degrades the ubiquitinated proteins.

Ubiquitination enzymes

Ubiquitination is a process whereby target proteins can be marked for degradation by the proteasome. It is a multi-step enzymatic process, using three classes of enzymes (E1s, E2s, and E3s), and involves the sequential transfer of ubiquitin from these enzymes to the target protein. First, ubiquitin needs to become activated. This activation is catalyzed by the ubiquitin-activating enzyme (E1) in an ATP-dependent reaction in which the C-terminal Gly residue of ubiquitin binds to the active site Cys of an E1 in a thioester linkage (Ciechanover et al., 1981; Hershko and Ciechanover, 1998).

Note that the eventual specificity of protein ubiquitination is not dependent on the activity of E1s because there is usually a single E1 enzyme that catalyzes the activation of ubiquitin for all of the cellular ubiquitination reactions. Rather, the specificity of the ubiquitination reaction depends on the later steps of the ubiquitination process. There are a significant but limited number of ubiquitin-carrier enzymes (E2s); and a much larger number of ubiquitin ligases (E3s) (discussed below). Thus, the ubiquitination enzymes form a hierarchical cascade, where the substrate specificity of the overall ubiquitination reaction depends on the specific E2s and E3s that pair up to ubiquitinate the substrate. Each E3 recognizes a set of substrates that share one or more signals for ubiquitination, and they pair up with one or a few E2s (Pickart, 2001).

When the compartmentalized structure of synapses is considered, it is quite possible that there is a similar hierarchy in ubiquitination enzymes. There are likely only a handful of E1s to be found at the synapse. Because the ubiquitination of synaptic proteins will certainly be regulated by synaptic events, it is likely that future research will identify a subset of synaptic E2s and E3s. Of course, target proteins may also be ubiquitinated elsewhere and shipped to the synapses but when the rapidly changing composition of synapses is considered, it makes more sense to keep the machinery for regulation of these proteins at or near the synapse.

E2s

There are 13 genes coding for different E2-like proteins in *S. cerevisiae* genome, and certainly more E2 genes in higher organisms (Hochstrasser, 1996). It is estimated that mammals express at least 20 - 30 different E2s. This increase in number reflects both multiple isoforms of the E2s (Jensen et al., 1995; Rajapurohitam et al., 1999) and the evolution of novel E2s (Hauser et al., 1998).

Functionally, E2s work as carriers of ubiquitin from E1 to E3s or to the substrate. Activated ubiquitin is transferred from E1 to an active site Cys of an E2 in a trans-thiolation reaction, again involving the C-terminal Gly of ubiquitin. There is a 14–16-kDa core domain in E2s, which is ~35% conserved among family members. The C-terminal and N-terminal extensions of E2s are more variable, and are involved in interactions with specific E3s (Mathias, 1998) and may also serve as membrane anchors, bringing them near substrates and E3s (Xie and Varshavsky, 1999; Sommer and Jentsch, 1993).

E3s

Ubiquitin is transferred from an E2 to the target protein either directly or indirectly with the aid of a ubiquitin ligase (E3). Ubiquitin is linked by its C terminus in an amide isopeptide linkage to an ε-amino group of the target protein's Lys residue.

There are two classes of E3 enzymes: HECT domain E3s and RING finger E3s. HECT domain E3s accept and form thiolester intermediates with ubiquitin. Thus, in this case, the transfer of ubiquitin to the target protein occurs from the E3 (Huibregtse et al., 1995). Members of the other class, the so-called RING finger E3s, catalyze the direct transfer of ubiquitin from an E2 without the formation of any ubiquitin-E3 intermediate (Joazeiro et al., 2000). In this case, the E3 functions as an adapter, bringing the E2 and the target protein in proximity to one another so that the ubiquitination of the target protein can occur. Many RING finger proteins, such as SCF are subunits of multi-protein complexes and they contain a scaffold protein that is a member of the cullin family of proteins. The cullins form a rod-like scaffold that contains docking sites for a substrate recognition complex (F-box protein-Skp1) and a catalytical complex (Rbx1 and an E2) (Petroski and Deshaies, 2005). The identity of these modular components determines which substrates will be ubiquitinated and ultimately degraded by the proteasome.

Ubiquitin chain formation

The signal that targets the proteins to the proteasome is a polyubiquitin chain, which is formed by the addition of ubiquitin to Lys48 of the previous ubiquitin in the chain. This polyubiquitin chain is recognized by the proteasome subunits and other proteasome binding proteins. Ubiquitin chains may have different branching patterns and different Lys residues other than Lys48 may be used. *In vivo*, K11, K29, K48 and K63 of ubiquitin can all be used to form polyubiquitin chains (Pickart, 2000).

Deubiquitinating enzymes

Similar to the presence of phosphatases that make the phosphorylation reaction reversible, there are deubiquitinating enzymes (DUBs). DUBs are cysteine proteases that generate free usable ubiquitin from a number of sources including ubiquitin-protein conjugates, ubiquitin adducts and ubiquitin precursors. More than 90 DUBs have been identified from different organisms (Chung and Baek, 1999). This makes them the largest family of proteins in the ubiquitin system, pointing to the importance of ubiquitination reversibility.

There are two classes of DUBs: ubiquitin C-terminal hydrolyses (UCHs) and the ubiquitin-specific processing proteases (UBPs). UCHs usually cleave ubiquitin from the C-terminus of small leaving groups and/or extended peptide chains by hydrolyzing the C-terminus amides and esters of ubiquitin. UBP enzymes are responsible for removing ubiquitin from larger proteins and disassembling polyubiquitin chains. UCH enzymes are well-conserved across species; they have no apparent similarity to UBP enzymes. At least 12 UCH sequences have been identified from different organisms. One UCH has been identified in yeast, two in *C.elegans,* and three in humans. UBP enzymes have a 350 amino acid core catalytical domain and varying lengths of N- and C-terminal extensions, as well as some catalytic domain insertions. These N- and C-terminal

extensions are thought to contribute to the substrate specificity and localization of different UBP enzymes. More than 80 full-length UBP sequences have been identified from different organisms. 16 of these are in yeast, 6 of them are in mouse and 13 of them are in humans (Wilkinson, 2000; Chung and Baek, 1999).

The proteasome

The 26S proteasome is formed by the co-assembly of a 20S proteasome (the catalytic component) and 19S cap (the regulatory component). The overall structure of the proteasome in eukaryotes is conserved (Glickman and Ciechanover, 2002; Pickart and Cohen, 2004; Wolf and Hilt, 2004). The 20S proteasome is a self-compartmentalizing complex with a barrel shape that is composed of four stacked heptameric rings. The two outer rings and the two inner rings contain the same set of seven different α and β subunits, respectively. The beta subunits form a central chamber where the active sites reside on three different beta subunits (β1-2-5 and possibly β7 in mammals) (Glickman and Raveh, 2005; Groll et al., 1999). These subunits are expressed with a propeptide that is posttranslationally removed to yield the active protease site (Chen and Hochstrasser, 1996). The N terminal extensions of the α-subunits form a gated channel to the inside of the proteasome on both ends. This channel is too narrow for a folded protein to pass through and access the active sites. This means the target proteins need to be unfolded to enter the proteasome (Groll et al., 2000; Bajorek et al., 2004). The 20S proteasome has no capacity to degrade ubiquitinated proteins by itself. For ubiquitin-dependent protein degradation to occur, the 20S proteasome has to associate with the regulatory 19S cap

particle. The exact subunit composition of the 19S is less clear than 20S and may include some proteasome-interacting proteins like E3s, polyubiquitin binding proteins and deubiquitinating enzymes (Verma et al., 2000; Glickman and Raveh, 2005). The 19S regulatory cap can be dissociated into two sub-complexes with high salt treatment: the lid and the base (Glickman et al., 1998). The base sits on the α-subunits of the 20S and contains six ATPase subunits (Rpt1-6) alongside three non-ATPase subunits (Rpn1, Rpn2 and Rpn10). The lid of the 19S particle contains eight stoichiometric subunits (Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11 and Rpn12). The ATPases may be involved in the unfolding and channeling of the target protein in to the 20S particle. The interaction between the base 19S and the 20S α-subunits rearranges the α-subunit N-terminal extensions and leads to the opening of the 20S gate enabling substrate access to the active sites of the proteasome (Glickman and Ciechanover, 2002; Pickart and Cohen, 2004; Wolf and Hilt, 2004; Whitby et al., 2000).

Proteasome dynamics

The proteasome structure is not static and the complement of proteins interacting with the defined 20S structure is dynamically regulated. This suggests that the proteasome can undergo different plasticity events that modulate its function, localization and activity (Glickman and Raveh, 2005). One classical example of proteasome plasticity is the assembly of proteasomes during immune response with different active site β subunits (immunoproteasomes) (Kloetzel, 2004). Immunoproteasomes generate a different set of peptides from "housekeeping" proteasomes, ultimately influencing the repertoire of the viral peptides that are presented to T cells.

Another level of proteasome plasticity during the immune response is the binding of alternative caps to the ends of 20S proteasome. As mentioned earlier, the 19S regulatory cap enables the 20S proteasome to degrade ubiquitinated proteins. During an immune response, an alternative cap, PA28/11S-REG binds to one or two ends of 20S proteasome (Tanahashi et al., 2000; Hill et al., 2002). PA28/11S-REG is a dome-shaped structure composed of seven α- and β-subunits. The amount of PA28/11S-REG increases in response to γ-interferon suggesting that PA28 modulates proteasome function during immune responses (Rechsteiner and Hill, 2005). Similar to 19S, PA28 stimulates the peptidase activity of the 20S proteasome but does not stimulate the degradation of folded proteins. The crystal structure of a distantly related activator, PA26, shows that binding of PA26 leads to opening of the gated axial channel into the 20S proteasome (Whitby et al., 2000; Forster et al., 2003). In addition to enabling the target peptides to enter the proteasome interior through the axial gate provided by PA28, another function of PA28 may be to act as "flushers" of the proteasome, facilitating the exit of peptide-products from the 20S particle (Glickman and Raveh, 2005).

Different proteasome subunits undergo posttranslational modifications including phosphorylation and o-glycosylation. The importance of phosphorylation in UPS function is suggested by experiments where phosphatase treatment disassembles 26S proteasomes into 19S and 20S particles. Phosphorylation of the 19S subunit Rpt6 may be required for the 26S assembly as Rpt6 is more phosphorylated in 26S than 20S proteasomes (Satoh et al., 2001). This suggests that phosphorylation has a positive role in proteasome activity by stabilizing 26S proteasome. The physiological significance of phosphorylation of the proteasome was shown in experiments where γ-interferon

treatment decreased the phosphorylation of two 20S α-subunits correlating with the disassembly of 26 proteasome into 19S and 20S particles. This suggests that immune response may involve regulation of the phosphorylation status of α-subunits and displacement of 19S with the subsequent binding of PA28/11S-REG (Bose et al., 2004; Rivett et al., 2001). Many of the subunits that are phosphorylated also undergo covalent modifications via O-linked *N*-acetylglucosamine (*O*-GlcNAc) (o-glycosylation) (Sumegi et al., 2003). Both phosphorylation and o-glycosylation targets the same residues, namely serine and threonine side chains. This suggests that these posttranslational modifications may have opposing effects on the proteasome function. Indeed, it was shown that hyperglycosylated proteasomes have slower hydrolysis rates towards some short synthetic peptide substrates (Zhang et al., 2003).

Localization of the UPS components

There is accumulating evidence that the dynamic localization of proteasome substrates between subcellular compartments may regulate their stability. A well-known example is the cyclin-dependent kinase inhibitor p27 (kip1). p27 is exported from the nucleus and degraded in the cytoplasm during G_1 (Carrano et al., 1999), whereas during S-phase, it is degraded in the nucleus (Kamura et al., 2004). There are two different E3s that prime p27 for degradation at different stages of cell cycle and they are differentially compartmentalized between nucleus and the cytoplasm, providing the spatial control of p27 degradation. Similar to p27, cyclin D1 of humans and the yeast mating switch endonuclease, Ho, must be exported from the nucleus in order to be degraded (Diehl et al., 1997; Kaplun et al., 2003). Furthermore, recruitment of Grr1, an F-box receptor, to

the mother-bud neck after disassembly of the mitotic spindles is observed during cytokinesis. Grr1 targets Hof1, whose degradation at the end of mitosis is necessary for the efficient contraction of the actomyosin ring and cell separation during cytokinesis (Blondel et al., 2005). Finally, the generation of local lamellopodia and filopodia formation in fibroblasts may involve compartmentalized localization of UPS components. In these cells, the signaling proteins that regulate the actin cytoskeleton are differentially distributed between the cell body and the leading edge of the cell. For example, RhoA induces a focal-adhesion complex in the cell body and gets excluded from the leading edge, where Cdc42 and Rac1 function to promote filopodia and lamellopodia, respectively. The differential distribution of RhoA between the cell body and the leading edge is established by the localized degradation of RhoA in lamellopodia and filopodia via localized distribution of its E3, Smurf-1 (Wang et al., 2003). The localized distribution of Smurf-1 and subsequent degradation of RhoA is established by atypical protein kinase C zeta (PKCζ), which is an effector of the Cdc42-Rac1-Par6 polarity complex. These data suggest that the substrates and the components of the ubiquitination machinery are dynamically localized to regulate the stability of the target protein locally.

Localization of the proteasome

In addition to the dynamic recruitment of the substrate and ubiquitinating machinery to the sites of degradation, proteasome localization may also be regulated. Dramatic shifts in proteasome localization between the cytoplasm and the nucleus occur during oocyte development and the mitotic cycle (Yanagawa et al., 2002; Lafarga et al., 2002; Kawahara and Yokosawa, 1992). During DNA repair, 19S and 20S subunits are

recruited to double strand breaks (DSBs). This recruitment requires intact homologous recombination or non-homologous end joining DNA repair pathways; the mutation of proteasome subunits results in hypersensitivity when combined with mutations of the DSB repair genes (Krogen et al., 2004). Similarly, stalled RNA polymerase undergoes rapid degradation by the proteasome and this process may involve recruitment of the proteasome to the site of degradation (Takagi et al., 2005).

It has also been shown that proteasome subunits interact with a number of proteins. These proteasome-protein interactions may be involved in the recruitment of the proteasome to different subcellular compartments. For example, membrane receptors including bovine retinal Ran binding protein-2 (RanBP-2), type-1 tumor necrosis factor receptor (TNFR1), and the ryanodine receptor interacts with the rpn2, rpn2 and rpn10 subunits of the 19S proteasome. These interactions may provide a possible link for the recruitment of the proteasome to the membrane-associated protein complexes (Zhu and Craft, 1998; Ferreira et al., 1998; Boldin et al., 1995; Ferrell et al., 2000). Furthermore, the protein-conducting channel formed by the Sec61 complex in the ER membrane interacts with the proteasome. This suggests that the proteasome may be actively recruited to the ER membrane via an interaction with the Sec61 complex to degrade the misfolded proteins (Kalies et al., 2005). In addition to membrane and ER localization, the proteasome may be localized to the mitotic spindle through interactions with dynactin (Kahana et al., 1998). Finally, there is also evidence for the dynamic recruitment of the proteasome to the centrosome (Wigley et al., 1999).

In addition to the proteins mentioned above, proteasomes also interact with ubiquitination enzymes, DUBs and polyubiquitin binding proteins. These interactions may regulate the localization of the proteasome itself by locally recruiting the proteasome to the sites of ubiquitination. Among ubiquitination enzymes, E2s such as Ubc1, Ubc2, Ubc4 and Ubc5, and E3s such as Ubr1, Ufd4, Hul5 and KIAA10 interact with the proteasomes (Leggett et al., 2002; Tongaonkar et al., 2000; Xie and Varshavsky, 2000; You and Pickart, 2001). These interactions suggest that ubiquitination enzymes may deliver substrate proteins to the proteasome as well. In addition to the ubiquitination enzymes, polyubiquitin binding proteins Rad23, Dsk2 and Ddi1, interact with the 19S proteasome (Funakoshi et al, 2002; Verma et al., 2000; Schauber et al., 1998). These proteins may interact with the proteasome transiently to deliver ubiquitination substrates to the proteasome (Hartmann-Petersen and Gordon, 2004). Finally, proteasomes also interact with DUBs (Holzl et al., 2000; Stone et al., 2004; Verma et al., 2000). The DUB-proteasome interaction may indirectly regulate proteasome localization by trimming the polyubiquitin chain that tether the substrate protein to the proteasome.

UBIQUITIN-PROTEASOME SYSTEM AT THE SYNAPSE

Localization of the UPS at the synapse

The proteasome has been studied most extensively in yeast, where it plays a key role in the control of the cell cycle. As discussed earlier, some of the UPS enzymes have been identified in yeast, as have the proteasome subunits and accessory proteins (Leggett

et al., 2005). In neurons, some of the components of the UPS have been observed in dendrites and near synapses (Patrick et al., 2003; Ehlers, 2003; Chapman et al., 1994). The presence of two main components of the UPS, ubiquitin as well as proteasome subunits, has been demonstrated at synapses and in the postsynaptic density PSD fraction, by direct immunofluorescence and immunoblotting (Ehlers, 2003; Patrick et al., 2003). Synapses also have the ubiquitination machinery as synaptic lysates form tagged-ubiquitin conjugates *in vitro* (Ehlers, 2003). Consistent with this observation, members of the E3 family of enzymes (including Nedd4, Staring, Siah, E6-AP, MDM2, fbx2, parkin, APC), E2 enzymes (bendless) and deubiquitinating enzymes (including usp14, fat facets, UCH-L1, UCH-L3, isopeptidase T) have also been linked to synaptic function (van Roessel et al., 2004; Dreier et al., 2005; Juo and Kaplan, 2004; Colledge et al., 2003; Tanaka et al., 2004; Chen et al., 2003; DiAntonio et al., 2001; Kawakami et al., 1999; Muralidhar and Thomas, 1993; Myat et al., 2002; Hegde et al., 1997; Jiang et al., 1998; Wilson et al., 2002; Chin et al., 2002; Wheeler et al., 2002; Kato et al., 2005). In addition, proteomic approaches using mass spectroscopy have confirmed the presence of UPS components at synapses (Jordan et al., 2004; Li et al., 2004). In particular, deubiquitinating enzymes UCH-L1 and USP5, and proteasome 19S subunit ATPase 9 were identified as components of PSD in these studies. These studies suggest that UPS components may reside inside PSD and not as a remote machinery away from the synaptic site.

The proteasome and synaptic plasticity

The first demonstration of the involvement of UPS in synaptic plasticity came from studies of the mollusk *Aplysia californica*. The sensory-to-motor neuron synapses in *Aplysia* undergo a form of plasticity known as facilitation. This facilitation is presynaptic in nature and requires the action of a cAMP-dependent protein kinase (PKA) for both short- (Klein and Kandel, 1980; Siegelbaum et al., 1982) and long-term facilitation (Kandel and Schwartz, 1982). Long-term facilitation (LTF) requires the persistent activation of PKA, which is achieved by altering the relative levels of the catalytic (C) and regulatory (R) subunits. During LTF, the C subunits remain constant but the R subunits are decreased (Greenberg et al., 1987; Bergold et al., 1990), leading to net increase in PKA activity. Hegde et al. determined that the loss of R subunits was achieved by the ATP-dependent degradation of the R subunits by the ubiquitin-proteasome pathway (Hegde et al., 1993). They showed that depletion of the proteasome from either reticulocyte or nerve tissue lysates blocked the degradation of the R subunits. In addition, a series of higher molecular weight and putative ubiquitin conjugates appeared when recombinant R subunits were added to reticulocyte lysates in the presence of ubiquitin, ATP, and hemin (an inhibitor of proteasome activity). Furthermore, LTF produced by repeated serotonin application was completely blocked by inhibitors of the proteasome, further supporting the importance of ubiquitin-mediated proteolysis (Chain et al., 1999). In addition, there was a specific time window during which LTF was blocked by proteasome inhibitors: The application of lactacystin (an irreversible inhibitor of the proteasome) immediately after serotonin treatment blocked LTF, but application 3 - 6 hours later had no effect. Taken together, these data demonstrate that

the activity of the proteasome is required early in LTF. The switch from short- to long-term facilitation requires CREB-mediated transcription and protein synthesis (Dash et al., 1990). Hegde et al. went on to identify an immediate-early gene product essential for LTF in *Aplysia*: a neuron-specific ubiquitin C-terminal hydrolase (Hegde et al., 1997). This protein associates with the proteasome to increase proteasome activity, presumably by de-ubiquitinating proteins prior to their delivery to the proteasome. Injection of antibodies or antisense oligonucleotides against the hydrolase blocked serotonin induced LTF (Hegde et al., 1997). The deubiquitinating activity of the hydrolase may increase the activity of the proteasome in order to ensure the degradation of protein substrates that block the formation of long-term memory storage.

The data described above suggest that synaptic stimulation activates the UPS, resulting in the degradation of proteins that normally inhibit plasticity. According to this idea, one would expect to see blockade of LTF with proteasome inhibitors. More recently, the Martin group has reported just the opposite result (Zhao et al., 2003). They have shown that chronic proteasome inhibition, starting just after LTF induction, increases the amount of LTF observed 24 hours later. This suggests that proteasome activity acts to constrain synaptic plasticity. They have also shown that proteasome inhibition alone is sufficient to increase basal synaptic transmission even after 1 hour of incubation with inhibitors. Proteasome inhibition also causes structural changes: After 24 hours of proteasome inhibition, the number of sensory-motor synaptic contacts was increased. These effects seem to be regulated by UPS on both sides of the synapse: Blocking the proteasome in isolated postsynaptic neurons caused an increase in glutamate-evoked postsynaptic potentials. However, blocking the proteasome in the

isolated presynaptic sensory cells produced increases in neurite length and branching. In the experiments described above, the proteasome is inhibited for periods in which one would expect to see decreases in free ubiquitin pools. In hippocampal neurons, even 5 minutes of proteasome inhibition is sufficient to decrease the free ubiquitin pool and accumulate significant levels of ubiquitinated substrates waiting to be degraded (Patrick et al., 2003). Because ubiquitin is involved in many cellular processes and not just in degradation (Sigismund et al., 2004), it is important to note that the effects reported with long proteasome inhibitor incubations might be due to loss of other functions of ubiquitin (e.g., monoubiquitin-dependent endocytosis) and not due to loss of proteasome activity.

E3s and synaptic plasticity

Studies of E6-associated protein (E6-AP) also support the idea that proteasomal protein degradation is important for both synaptic and behavioral plasticity. E6-AP was the first member of the HECT domain E3 family to be identified; it contains a conserved 350 amino acid region that defines the HECT domain (Huibregtse et al., 1995). E6-AP is required, together with the papillomavirus E6 oncoprotein, for the ubiquitination and degradation of the tumor suppressor p53 (Scheffner et al., 1995; Huibregtse et al., 1993; Scheffner et al., 1993). E6 serves as an adaptor between E6-AP and p53, allowing E6-AP to catalyze the ubiquitination of p53. However, p53 is not the only target of E6-AP, and for its other substrates, E6-AP does not need an adaptor protein like E6 to transfer the ubiquitin to the substrate. Mutations in the E6-AP gene (*Ube3a*) cause Angelman's Syndrome (AS), a human hereditary disease that results in mental retardation, seizures, an abnormal gut, tremor and ataxia (Kishino et al., 1997). This disorder is associated

with a maternally expressed, imprinted locus mapping to chromosome 15q11-13. The molecular defects, due to point mutations, large deletions, complete absence of the gene, or imprinting mutations (Sutcliffe et al., 1994; Horsthemke et al., 1997; Buiting et al., 1995), lead to loss of E6-AP in those cell types where the paternal allele is silenced.

The mouse model that possesses a maternal *Ube3a* null mutation has demonstrated the importance of E6-AP in learning and memory (Jiang et al., 1998). The phenotype of these mice is comparable to the phenotypes of AS patients. For example, the *Ube3a* null mice exhibit impairments in bar-crossing and rotating rod performance, which correlates with the ataxia and motor incoordination in human AS patients. Furthermore, the presence of inducible seizures and defects in context-dependent learning in the mouse model correlate respectively with the high incidence of epilepsy and cognitive impairment in AS patients. Context-dependent learning was examined using the conditioned fear paradigm in which animals are exposed to an electric shock paired with either the context (cage) or a cue (a tone). Following pairings, control animals typically exhibit conditioned freezing responses to either the cage or the tone. Immediately following pairing, the maternal *Ube3a* mutant mice exhibited freezing indicating that they have normal sensory responses to the shock and normal acquisition of the learned response. After 24 h, however, the maternal deficient mice exhibited significantly less conditioned freezing to the context, but normal conditioning to the auditory tone. This context-dependent learning deficit in maternal deficient mice may be due to a deficiency of Ube3a expression in the hippocampus, as the hippocampus has been shown to be important for contextual conditioning (Kim and Fanselow, 1992).

The properties of synaptic transmission and plasticity were also examined in the Ube3a mice. As stated earlier, LTP is considered as a cellular mechanism of learning and memory (Malinow and Malenka, 2002). The mutant mice exhibited normal basal synaptic transmission but reduced levels of LTP measured within an hour after the inducing stimulus of high frequency stimulation. The authors of this study suggest a link between the reduced LTP in the mutant mice and the learning deficits observed in AS patients (Jiang et al., 1998).

The UPS and homeostatic plasticity

In contrast to LTP and LTD, some forms of synaptic plasticity act on a slower time scale. For example, in homeostatic plasticity, the neuron globally changes its sensitivity to stimulation when it experiences chronic changes (e.g., a loss of action potential dependent synaptic transmission) in activity. The slow time course of conventional homeostatic plasticity has prevented a simple analysis of the roles of protein synthesis and degradation by using synthesis or degradation inhibitors. Ehlers has shown, however, that the same manipulations that result in homeostatic plasticity give rise to global changes in postsynaptic density protein content (Ehlers, 2003). An extensive biochemical analysis demonstrated that chronic activation or inhibition of synaptic transmission remodels the synaptic composition bidirectionally and reversibly. An important observation is the co-regulation of glutamate receptors, signaling proteins and scaffolding proteins in response to manipulation of the activity. This co-regulation with changes in activity suggests that proteins at the PSD exist as postsynaptic protein

ensembles rather than as single entities. The UPS regulates many proteins in the PSD but not all of these proteins are ubiquitinated, so it is possible that a few members of the co-regulated proteins act as "master organizing molecules" and UPS targets these proteins to regulate the protein complexes of the PSD (Ehlers, 2003). The regulation of the PSD composition by the UPS has been shown in experiments in which chronic increases in synaptic activity increased the ubiquitination of PSD proteins, whereas chronic decreases in activity had the opposite effect. Furthermore, the co-regulation of synaptic components induced by activity changes was blocked by chronic proteasome inhibition. As noted above, prolonged treatment with proteasome inhibitors (e.g., greater than 1 h) can lead to decreases in the free ubiquitin pool (Patrick et al., 2003). As such, these data cannot distinguish between a role for the UPS and monoubiquitin-dependent processes. Nevertheless, they do suggest that the homeostatic regulation of the synapse occurs, at least in part, through the modulation of the stability of the individual synaptic proteins by the UPS.

UPS and presynaptic function and development

A few studies have begun to shed light on the function of UPS in presynaptic nerve terminals. Speese et al. (2003) demonstrated that components of UPS (E1 and the proteasome) are present in presynaptic boutons at the *Drosophila* neuron-muscular junction (NMJ). They have also shown that the proteasome is active at these boutons by expressing a conditional fluorescent reporter of proteasome activity. Inhibiting the proteasome caused a 50% increase in evoked excitatory junctional current (EJC) amplitude when compared to controls. This increase was rapid, suggesting a local

degradation of proteins by the UPS. This increase in synaptic transmission is likely due increased transmitter release, because there was no change in the amplitude or frequency of mEJCs. What are the possible presynaptic targets of the UPS? Speese et al. have shown that DUNC-13, a protein that regulates synaptic vesicle priming (Aravamudan et al., 1999), might be involved. Blocking proteasome activity with inhibitors or genetic disruption of proteasome active site subunits increased the abundance of DUNC-13 at the presynaptic C-terminal. Two other presynaptic proteins that are regulated by UPS are syntaxin-1 and synaptophysin (Chin et al., 2002; Wheeler et al., 2002). Although the enzymatic machinery that targets them for degradation is known, the physiological consequence of degradation of these proteins is not yet understood.

Studies in the fly and worm have demonstrated that the UPS is also involved in presynaptic development. For example, gain of function mutations of the deubiquitinating enzyme fat facets (faf) cause overgrowth of the presynaptic C-terminals in *Drosophila* motor neurons. Faf is involved in the development of photoreceptors through modulation of the Ras signaling pathway (Wu et al., 1999). The *faf* phenotype can be suppressed by the mutations in the proteasome, suggesting that faf is involved in the deubiquitination of a protein, which is normally degraded by the proteasome and involved at a key step during development (Huang and Fischer-Vize et al., 1996). There is also a *faf* homolog, *fam*, in mouse (Wood et al., 1997). It binds to cell membranes at cell-to-cell contacts, and also binds to AF-6, a downstream target of ras (Taya et al., 1998).

Recently, *faf* has also been shown to be important for synapse development in *Drosophila* (DiAntonio et al., 2001). The neuromuscular junction (NMJ) of the body wall muscles of *Drosophila* is an easily accessible glutamatergic synapse. Similar to plasticity seen in CNS synapses, the *Drosophila* NMJ can undergo plasticity during development and in adult life. To study the mechanisms that regulate synaptic development in *Drosophila* DiAntonio et al. screened for genes whose overexpression leads to synaptic growth abnormalities: They identified two lines in which *faf* is overexpressed.

The endogenous *faf* transcript is widely and strongly expressed in the developing *Drosophila* CNS. Targeted overexpression of *faf* in *Drosophila* has both morphological and physiological consequences: Anatomical analysis reveals that *faf* overexpression leads to increase in synaptic size, synaptic span (the extent of the muscle covered by the synapse) and the number of synaptic branches. These increases are not seen in flies that do not overexpress *faf* or that overexpress a non-functional *faf*. Furthermore, *faf* overexpression has a physiological phenotype: The evoked excitatory junctional potentials (EJP) are markedly decreased despite the increased synaptic size. This is also accompanied by a small decrease in both the amplitude and frequency of miniature EJPs. A large decrease in EJP with a small decrease in mEJP points to a decrease in the quantal content, which is a measure of number of vesicles released by the nerve. The reduction in both quantal content and mEJP frequency suggests that *faf* overexpression leads to a defect in neurotransmitter release.

To test if the *faf* overexpression phenotype is due to a disruption of ubiquitin-dependent protein degradation at the synapse, DiAntonio et al. overexpressed a yeast DUB in the nervous system of *Drosophila*. This yeast DUB has overlapping substrate specificity with FAF (Wu et al., 1999). Its overexpression also leads to marked synaptic overgrowth and a severe reduction in presynaptic transmitter release, similar to *faf* overexpression. This indicates that antagonizing ubiquitin-dependent protein degradation via DUB overexpression leads to defects in synaptic development.

A screen for viable mutations that were lethal in combination with neuronal *faf* overexpression identified *highwire* (*hiw)* alleles that have the same phenotype as *hiw* loss-of-function phenotype. *Hiw* codes for a RING finger type E3 and the *hiw* loss-of-function phenotype is very similar to the *faf* overexpression phenotype (Wan et al., 2000). *faf* loss-of-function mutants have no defects in their synapses (possibly due to redundancy), but *faf* is required to suppress the physiological, but not the anatomical, phenotype of *hiw* loss-of-function mutants (DiAntonio et al., 2001). This suggests that *faf* acts to inhibit neurotransmitter release in an *hiw* loss-of-function background. The fact that the anatomical phenotype of *hiw* loss-of-function cannot be suppressed by the loss-of-function of *faf* indicates that these two phenotypes are mediated by different substrates of *hiw*. These data suggest that both *hiw* and *faf* control synaptic development through ubiquitination and deubiquitination of substrates critical for synaptic function. These substrates remain to be identified.

Highwire also has homologs in *C. elegans* (RPM-1) and mammals (Phr and Pam) (Wan et al., 2000; Burgess et al., 2004; Guo et al., 1998; Schaefer et al., 2000). The highwire homolog RPM-1 has recently been identified as a negative regulator of the p38 MAPK pathway in *C. elegans* (Nakata et al., 2005). RPM-1 targets DLK-1, a mitogen activated protein kinase kinase kinase (MAPKKK) of the p38 pathway, through direct ubiquitination, and negatively regulates the receptor tyrosine kinase anaplastic lymphoma kinase (ALK). Both RPM-1 and DLK-1 are components of the periactive zone. Loss of *rpm-1* function or activation of the DLK-1 pathway affects synaptic architecture and proportion in similar ways. Interestingly, mutations of RPM-1 and its homologs have divergent effects on the presynaptic development depending on the synapse type (e.g., highwire mutants in *Drosophila* have increased terminal branching and bouton number and reduced synaptic transmission (Wan et al., 2000), whereas rpm-1 mutants in *C. elegans* have a reduced number of synapses (Schaefer et al., 2000; Zhen et al., 2000; Nakata et al., 2005). This suggests the possibility of synapse-specific regulation of development, possibly through different repertoires of UPS components at different synapse types. For example, in *C. elegans*, RPM-1 is a component of the SCF (Skp, Cullen, F-box) complex in which FSN-1 functions as an F-BOX protein. It is possible that at different synapses, different F-BOX proteins target different substrates leading to different mutant phenotypes for RPM-1 and its homologs.

In addition to synaptic development, the presynaptic C-terminal also has deubiquitinating enzyme activities that are important for synaptic function in mature neurons. Depolarization decreases the total content of ubiquitinated substrates in the presynaptic C-terminal within seconds (Chen et al., 2003). One deubiquitinating enzyme

that regulates synaptic transmission in the presynaptic C-terminal is Usp14, which is a ubiquitin-specific protease that recycles ubiquitin from polyubiquitinated proteins. An ataxic mouse described by D'Amato and Hicks (1965) has defective Usp14 activity. The neuromuscular synapses of this mouse exhibit decreased quantal content, and decreased frequency and increased amplitude of miniature end plate potentials. Also, the hippocampal short-term but not long-term plasticity is impaired, suggesting that ubiquitin recycling and regulation of UPS is important for neurotransmitter release and plasticity (Wilson et al., 2002).

Ups and postsynaptic function

The ubiquitination of proteins does not always target them for degradation. As indicated above, the outcome of the ubiquitination reaction depends on both the number of ubiquitin moieties attached and the type of linkage between the individual ubiquitin units. If only one ubiquitin is attached to the target protein, this is called monoubiquitination. Monoubiquitination is involved in diverse cellular functions including histone regulation, budding in retroviruses and endocytosis of plasma membrane proteins (Hicke, 2001).

The endocytosis of surface receptors is a common mechanism to downregulate receptor signaling. There are different endogenous internalization signals in the cytoplasmic domain of plasma membrane receptors. In addition to these endogenous signals, ubiquitin can be attached to the receptor to act as an internalization signal. Ubiquitin appears to be the most common internalization signal employed in yeast and higher eukaryotes. In yeast, there are several membrane proteins in which cytoplasmic

domains need to be ubiquitinated for the endocytosis to occur in response to ligand binding (Shaw et al., 2001). In mammalian cells, many receptors including the epidermal growth factor receptor, platelet-derived growth factor receptor, and growth hormone receptor (GHR), are ubiquitinated in response to ligand binding (Bonifacino and Weissman, 1998).

In addition to the receptors, in some cases the proteins that comprise the endocytic machinery are ubiquitinated. For example, the GHR requires the activity of cellular ubiquitinating enzymes in order to be internalized efficiently even though the ubiquitination of the receptor itself is not required (van Kerkhof et al., 2000). This suggests that proteins, other than the receptor itself, are the required targets of ubiquitination.

One candidate ubiquitinated target of the endocytosis machinery is Eps15. By mapping the regions required for monoubiquitination on Eps15 and EpsR15, Polo et al. identified a region called the ubiquitin-interacting-motif (UIM) that is required for the monoubiquitination of these proteins (Polo et al., 2002). Polo et al. also showed that the monoubiquitination of eps15 is catalyzed by Nedd4, an E3 that also polyubiquitinates the epithelial sodium channel. As Eps15 interacts with clathrin-coated pits, it may form the connection between the monoubiquitinated receptors and the clathrin machinery through its UIM domain (Riezman, 2002).

A role for the ubiquitin-dependent protein degradation machinery has been demonstrated in the downregulation of G protein coupled receptors (GPCRs) (Shenoy, 2001). Ligand binding to GPCRs induces a conformational change in the receptor leading to activation of cellular signaling events. This conformational change also induces phosphorylation of the receptor by the G protein receptor kinases. The phosphorylated receptor is recognized by the adapter protein β-arrestin, which uncouples the receptor from the downstream signaling events, leading to receptor desensitization. Furthermore, phosphorylation links the receptor to the endocytic machinery, namely clathrin and the adapter protein 2 (AP2). Once internalized, receptors are either recycled back to the membrane or degraded (Pierce and Lefkowitz, 2001; Luttrell and Lefkowitz, 2002; Miller and Lefkowitz, 2001).

The process of GPCR endocytosis by ubiquitin-dependent protein modifications has been demonstrated (Shenoy et al., 2001). Shenoy et al. showed that one of the GPCRs, the β2-adrenergic receptor (β2-AR), undergoes ubiquitination in response to ligand binding. Inhibition of the proteasome does not lead to an accumulation of the ubiquitinated receptor, indicating that the receptor itself is not the immediate target of the proteasome. β-arrestins are regulators of GPCRs that bind to phosphorylated receptors and functionally uncouple the receptor from G-protein activation. β2-AR β-arrestin, β-arrestin2, also undergoes ubiquitination in response to ligand binding to β2-AR. But β-arrestin2 ubiquitination is more transient when compared to the ubiquitination of the receptor. This is apparently due to its rapid deubiquitination as ubiquitination of β-arrestin2 is only observed in the presence of DUB inhibitors. Ligand-induced

ubiquitination of the β2-AR is dependent on its interaction with β-arrestin2 because β2-AR mutants that cannot bind to β-arrestin2 are not ubiquitinated in response to ligand binding. Furthermore, in cell lines lacking β-arrestin2, β2-AR cannot be ubiquitinated.

The requirement for β-arrestin2 binding for β2-AR ubiquitination suggests that β-arrestin2 acts to recruit the ubiquitination machinery to β2-AR. A yeast hybrid screen for E3s that interact with β-arrestin2 identified Mdm2. Mdm2 is an E3 and oncoprotein that acts as a negative regulator of p53 (Fang et al., 2000; Honda et al., 1997). Mdm2 can ubiquitinate both β2-AR and β-arrestin2 *in vitro*, but is not required for β2-AR ubiquitination *in vivo*. In contrast, β-arrestin2 cannot be ubiquitinated in cells that lack Mdm2. In cells lacking Mdm2, β2-AR internalization in response to ligand binding is markedly reduced, whereas receptor degradation occurs normally. This suggests that β-arrestin ubiquitination is required for receptor internalization and β2-AR can be degraded even though it cannot be internalized (Shenoy et al., 2001).

Although the E3 that ubiquitinates the β2-AR has not been identified, it has been shown that blocking ubiquitination of β2-AR with Lys mutations blocks the degradation of the receptor. This suggests that ubiquitin-dependent protein degradation is involved in the receptor degradation. Furthermore, blocking the activity of the proteasome also blocks receptor degradation, although it is possible that proteasome is involved in the trafficking of the receptor to the lysosomes (Shenoy et al., 2001). Overall these data suggest that ubiquitination machinery acts to regulate β2-AR internalization. This is accomplished by the regulation of ubiquitination of β-arrestin2 and possibly through

other interactions of the receptor. Receptor ubiquitination is not required for its internalization but it is required for its degradation. Rather, the ubiquitination of β-arrestin2 is critical for the internalization of the receptor.

Regulation of glutamate receptors

As discussed above, the number of functional AMPAR in the postsynaptic membrane can control synaptic strength. As a consequence the study of how AMPARs are delivered to the membrane, endocytosed, and recycled has been a major area of interest in the field of synaptic plasticity. In recent years, the involvement of UPS in the regulation of AMPAR trafficking has been demonstrated.

The involvement of UPS in regulating glutamate receptor abundance is shown by studies in *C. elegans, Drosophila* and in mammals. Burbea et al. demonstrated that the synaptic levels of one of the AMPA-type glutamate receptor homologs in *C. elegans*, glr-1, is regulated by the UPS (Burbea et al., 2002). The glr-1 GluRs are localized to the sensory-interneuron synapse and are required for a mechanosensory behavior, nose touch avoidance, in *C. elegans* (Hart et al., 1995; Maricq et al., 1995; Rongo et al., 1998). Glr-1 is ubiquitinated *in vivo*. Mutations of lysine residues in GLR-1 that reduced ubiquitination increased the abundance of GLR-1 at synapses and altered locomotion behavior in a manner consistent with increased synaptic activity. Conversely, when ubiquitin is expressed exogenously to promote ubiquitination, there was a decrease in glr-1 synaptic levels that required an intact endocytosis machinery. The ubiquitination of GLR-1 in *C. elegans* leading to its downregulation from synapses likely represents a

proteasome-in-dependent use of ubiquitin, akin to the monoubiquitin-dependent internalization of receptors observed in yeast (Hicke, 1999). Moreover, blocking ubiquitination of glr-1 increases the locomotion behavior of *C. elegans*, which is a measure of synaptic strength. These data show that the regulation of glr-1 accumulation at the synapse by UPS regulates both synapses and behavior.

Recent studies in *C. elegans* also have identified the UPS machinery that targets the regulators of GluR endocytosis. Kaplan and co-workers (Dreier et al., 2005) showed that lin-23, a subunit of the SCF ubiquitin ligase complex, might regulate the synaptic glr-1 abundance, not through direct receptor ubiquitination, but rather via the modification of other UPS substrates. One such candidate substrate is BAR-1 (B-catenin in mammals), which is an effector of the Wnt pathway. Another ubiquitin ligase complex that regulates postsynaptic receptor abundance is anaphase-promoting complex (APC), an enzyme complex that is well known for its role in cell cycle regulation (Gieffers et al., 1999). Two groups have reported novel roles for APC in postmitotic neurons. Van Roessel et al. (van Roessel et al., 2004) demonstrated that protein degradation regulated by APC has independent presynaptic and postsynaptic functions at the *Drosophila* NMJ. On the postsynaptic side, APC regulates the abundance of the mammalian GluR subunit homolog GluRIIa. When the APC protein was mutated, both spontaneous and evoked junction potentials were increased. This indicates that either the vesicles are filled with more neurotransmitter or the postsynaptic membrane possesses a higher sensitivity for the neurotransmitter. Because electron micrographs show no obvious presynaptic changes in synaptic vesicles, the authors favor the latter possibility. Indeed, loss of APC results in increased GluRIIa immunoreactivity at the postsynaptic density. The role of

APC in regulating glutamate receptor levels on the postsynaptic membrane has also been recently shown in *C. elegans*. Similar to the experiments described above, the Kaplan group has shown that decreasing APC activity leads to an increased abundance of glr-1, leading to an enhancement of synaptic transmission efficiency in sensory-interneuron synapses (Juo and Kaplan, 2004). The increase in glr-1 abundance coincides with an increase in larval locomotion behavior, similar to that observed in the glr-1 ubiquitination mutants described above. These two independent studies suggest a novel postsynaptic function for APC in postmitotic cells. On the presynaptic side, APC negatively regulates the scaffolding protein liprin-α/SYD2 (van Roessel et al., 2004). The effects of APC loss of function on both synaptic transmission and synapse formation are rescued by disruption of liprin-α on the presynaptic side pointing to a role of liprin-α degradation in synaptic development.

As mentioned above, it has been well established that the abundance of PSD-95 at synapses regulates GluR levels via PSD-95 palmitoylation and indirect interactions with GluRs (Ehrlich and Malinow, 2004; El-Husseini et al., 2002; Stein et al., 2003). In addition to these mechanisms, the amount of PSD-95 at the synapse might be regulated by the UPS, directly or indirectly, through degradation of itself or other proteins (Bingol and Schuman, 2004; Colledge et al., 2003). One of the proteins that leads to PSD-95 loss up on its degradation is the spine-associated Rap GTPase activating protein (SPAR). (Pak and Sheng, 2003). SPAR binds to PSD-95 and promotes the growth of dendritic spines. This function depends on SPAR's GAP domain. SPAR degradation by the UPS leads to loss of spines and a decrease in PSD-95 levels. SPAR is degraded by the UPS only when it is phosphorylated. The kinase that phosphorylates SPAR is serum-inducible

kinase (SNK). SNK is upregulated during synaptic activity, suggesting a model for spine loss in which activity increases SPAR degradation through phosphorylation of SPAR by SNK (Kauselmann et al., 1999; Pak and Sheng, 2003). Interestingly, SNK expression is upregulated in the soma and not locally in the dendrites, suggesting a global effect of SNK on spine regulation.

The ubiquitination of mammalian GluRs has recently been shown in rat hippocampal lysates (G Patrick, unpublished). Brief treatment with AMPA increases the ubiquitinated population of GluRs, suggesting a role for GluR ubiquitination in its own endocytosis. This idea has been tested by expressing a ubiquitination-defective form of the GluR1 subunit in cultured hippocampal neurons. The mutant GluR1 subunits accumulate more on the surface than wild type GluR1, similar to the results obtained with the AMPAR homolog in *C. elegans*. Furthermore, blocking the ubiquitination of GluR1 leads to altered AMPA-induced endocytosis of GluRs in both heterologous cells and cultured hippocampal neurons, suggesting that ubiquitination of GluR1 is necessary for this process (G Patrick, unpublished). Because endocytosed GluRs can be detected intracellularly and recycled, these results imply that it is the monoubiquitination of GluRs that is important. Furthermore, AMPAR endocytosis requires proteasome activity, which will be discussed in Chapter 4.

In the subsequent chapters, I will describe my data that demonstrates the interplay between synaptic activity and UPS. I will first present the evidence that demonstrates protein degradation can occur locally in the dendrites. Second, I will describe a role for UPS in one of the cellular mechanisms of synaptic plasticity, namely glutamate receptor

trafficking and discuss possible targets of UPS at the synapse. Lastly, I will describe how the localization of UPS components may be regulated by synaptic activity.

Chapter III

UPS COMPONENTS AT THE SYNAPSE

Ubiquitin and subunits of the proteasome are heterogeneously distributed in dendrites

If ubiquitin-regulated protein degradation is used during synaptic plasticity then the molecular components required, including ubiquitin and the proteasome, should be present in neuronal dendrites near synapses. To address this, I conducted immunofluorescence labeling in cultured hippocampal neurons, using antibodies directed against either the β-subunits of the proteasome or polyubiquitin chain and monoubiquitinated proteins but not free ubiquitin (clone FK2) (Fujimuro and Yokosawa, 1994). A strong signal for ubiquitin was present in both the cell body and throughout the dendrites (Fig. 3.1a). A similar distribution pattern was observed for the proteasome: strong staining was evident in the cell bodies, and punctate high-intensity clusters were observed in the dendrites (Fig. 3.1b). In order to estimate the extent of synaptic localization of ubiquitin and proteasome, I conducted double-immunofluorescence labeling using antibodies against ubiquitin and proteasome as well as a synaptic marker, synaptophysin. The abundance of ubiquitin precludes a meaningful analysis of synaptic localization, but we did determine the overlap between the proteasome and a synaptic marker, synaptophysin (Fig. 3.1c), by examining whether the regions of greatest intensity for proteasome staining corresponded to synaptic sites. We observed examples of colocalization of the two signals, as well as examples where there was no overlap at all between the two signals: 40.7% ± 2.3% of the proteasome puncta overlapped with the

synaptophysin puncta, whereas 57.8% ± 2.5% of the synaptophysin puncta overlapped with the proteasome.

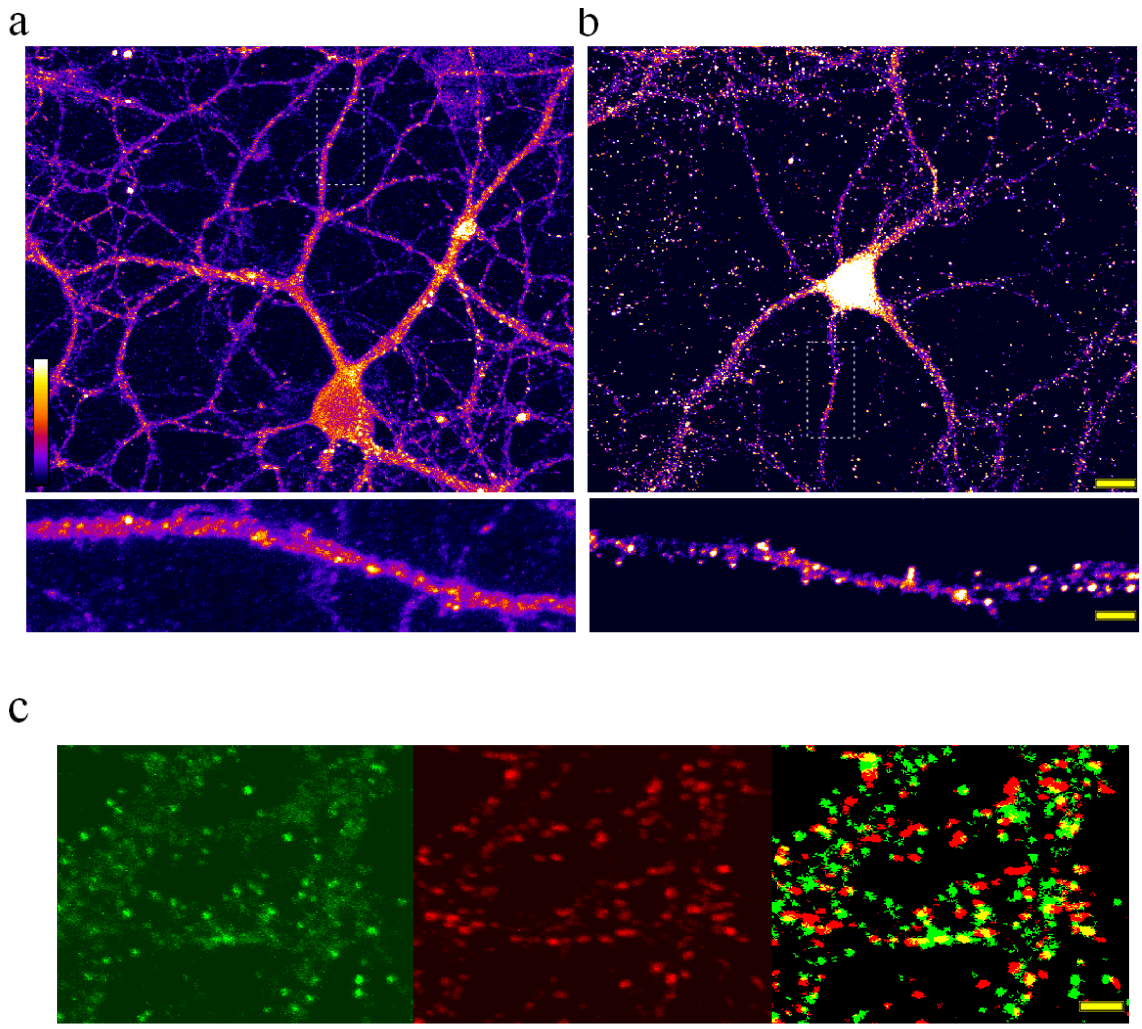

Figure 3.1. Presence of ubiquitin and the proteasome in the soma and dendrites of hippocampal neurons. Shown are dissociated hippocampal neurons immunostained with an antibody directed against ubiquitin (a) or the proteasome (b) (The ubiquitin antibody is directed against polyubiquitin chain and monoubiquitinated proteins but not free ubiquitin (clone FK2). The proteasome antibody is directed against the β-subunits of the proteasome) (Scale bar, 15 μm). The color intensity profile shows the intensity of fluorescence from low (blue-black) to high (white). Images show that both ubiquitin and the proteasome are abundant in both the soma and dendrites of hippocampal neurons. Positive staining is abundant in the dendritic arbor. High-magnification images (below)

show intense immunoreactivity in the dendrites and putative spines. Scale bar, 2.8 μm. (c) Some of the proteasome staining (green) overlaps with that of a synaptic marker, synaptophysin (red). In the image shown, 42.7% of the proteasome puncta overlaps with the synaptophysin puncta, and 56.6% of the synaptophysin puncta overlaps with the proteasome. Scale bar, 4 μm.

Synaptic activity regulates polyubiquitinated protein levels

If synaptic activity regulates the synaptic protein degradation, then ubiquitinated protein levels must be modulated by synaptic activity. In order to examine whether synaptic activity regulates the abundance of ubiquitinated proteins *in situ*, we used an antibody that recognizes the polyubiquitin chain and monoubiquitinated proteins but not free ubiquitin (clone FK2) (Fujimuro and Yokosawa, 1994). Cultured neurons expressing GFP were stimulated (KCl, 60 mM, 1.5 min) and then processed for immunolabeling at different times following stimulation using the FK2 antibody. The staining was performed for both GFP and the ubiquitin, using two different fluorophores. The GFP channel was used as a marker for the dendritic morphology and a reviewer blind to the FK2 signal outlined the "protrusions" from the dendritic surface using GFP template image. A spine mask was then created from these protrusions. The polyubiquitin FK2 signal overlapping with the spine mask was used to determine how much ubiquitin is present in spines (Fig 3.2a). Using the analysis described above, we found that depolarization caused an initial ~67% increase in the ubiquitin level in the spines as well as in the dendritic shaft 10 minutes following stimulation (Fig. 3.2b,c). At 20 and 55 min following stimulation, the amount of ubiquitinated protein in the dendritic spines and the shaft then exhibited a decrease below baseline levels. The decrease in

ubiquitination levels was blocked by a proteasome inhibitor, MG132 (Fig. 3.2b,c). These results suggest that stimulation causes an initial increase in ubiquitination possibly due to activation of the ubiquitination machinery. This increase is followed by the degradation of these ubiquitinated proteins by the proteasome.

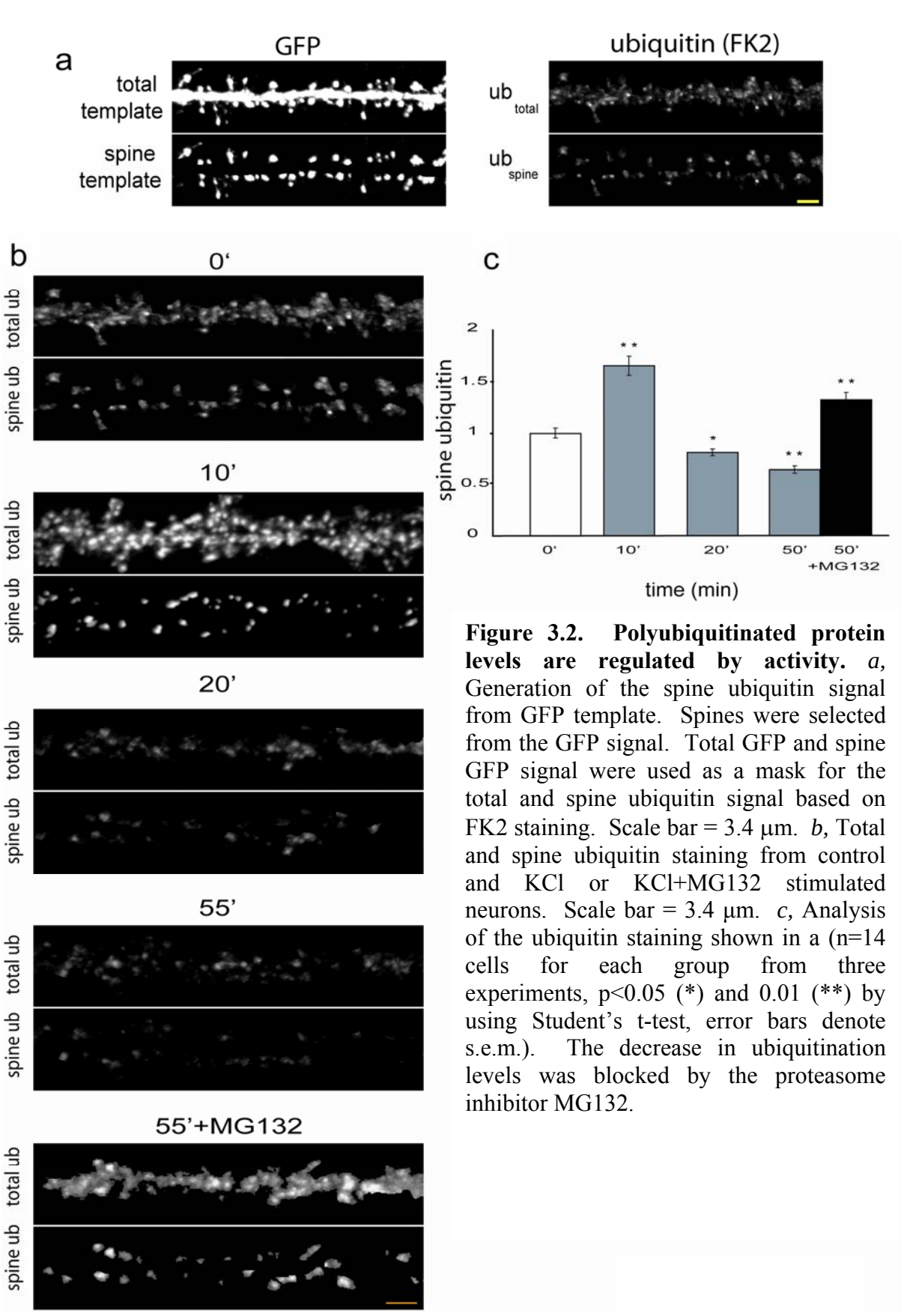

Figure 3.2. Polyubiquitinated protein levels are regulated by activity. *a*, Generation of the spine ubiquitin signal from GFP template. Spines were selected from the GFP signal. Total GFP and spine GFP signal were used as a mask for the total and spine ubiquitin signal based on FK2 staining. Scale bar = 3.4 μm. *b*, Total and spine ubiquitin staining from control and KCl or KCl+MG132 stimulated neurons. Scale bar = 3.4 μm. *c*, Analysis of the ubiquitin staining shown in a (n=14 cells for each group from three experiments, $p<0.05$ (*) and 0.01 (**) by using Student's t-test, error bars denote s.e.m.). The decrease in ubiquitination levels was blocked by the proteasome inhibitor MG132.

Synaptic activity regulates UPS activity

The change in the ubiquitinated protein amount with neuronal stimulation suggests that UPS machinery is regulated by synaptic activity. Immunolabeling for ubiquitin at different time points following stimulation only gives a static picture of the UPS activity. In order to visualize dynamic regulation of UPS by synaptic activity, we used a well-characterized GFP-based proteasomal degradation reporter, Ub^{G76V}-GFP (Dantuma et al., 2000; Goldberg, 2000). Ub^{G76V}-GFP is a ubiquitin-fusion degradation (UFD) pathway substrate. The ubiquitin moiety has a substitution on its last amino acid (G76V), rendering it resistant to cleavage from GFP by DUBs. Furthermore, N-terminal ubiquitin forms the anchor for addition of the polyubiquitin chain to the degradation reporter, which targets Ub^{G76V}-GFP for proteasomal degradation. Ub^{G76V}-GFP undergoes a loss of fluorescence through degradation by the proteasome, allowing the activity of UPS to be dynamically visualized in cells (Neefjes and Dantuma, 2004).

We expressed Ub^{G76V}-GFP in cultured rat hippocampal neurons and conducted time-lapse imaging of dendrites during synaptic depolarization and stimulation. In order to isolate the effects of protein degradation on the reporter fluorescence, all experiments were conducted in the presence of the protein synthesis inhibitor anisomycin. Stimulation of neurons (KCl, 60 mM, 1.5 min) caused a rapid decrease in the reporter signal, indicating that UPS was activated during depolarization (Fig. 3.3a,b). Application of the proteasome inhibitor MG132 blocked the reporter degradation, indicating the specificity of the reporter (Fig. 3.3a,b). In addition, the NMDAR receptor

antagonist APV blocked the depolarization-induced decrease in reporter levels, suggesting that proteasome is activated by NMDA receptor activation during depolarization (Fig. 3.3a,b). Consistent with this, direct activation of NMDA receptors via bath application of NMDA (20 μm, 3 min) was sufficient to decrease the reporter signal (Fig. 3.3b). These results show that UPS is regulated by synaptic activity in a NMDAR-dependent manner.

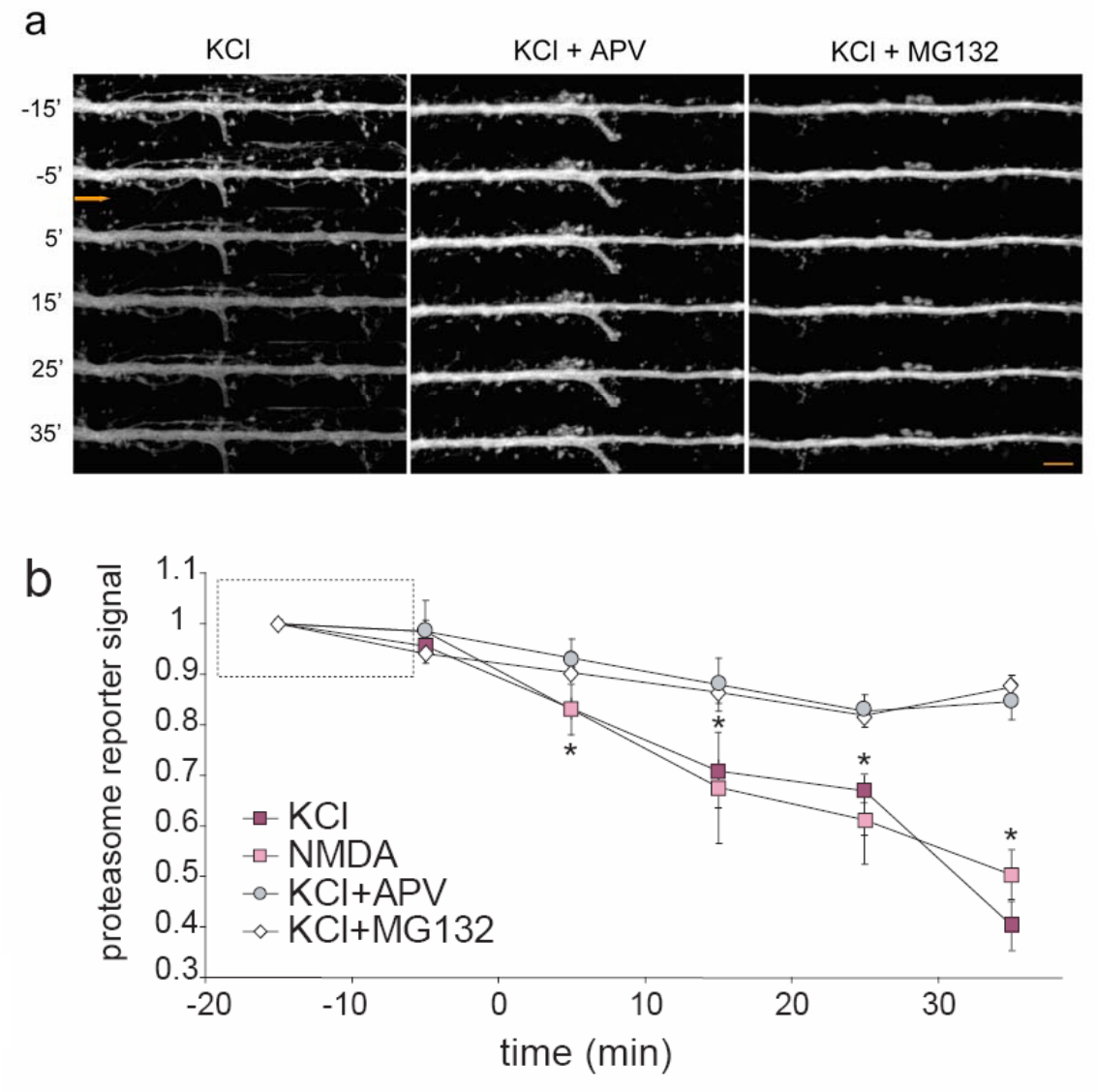

Figure 3.3. KCl stimulation increases proteasome activity. *a,* Time-lapse images of dendrites from neurons expressing UbG76V-GFP. Bath application of KCl (arrow) resulted in NMDAR-dependent loss of fluorescence reflecting reporter degradation. Scale bar = 7.5 μm. *b,* Analysis: addition of either KCl or NMDA caused a significant fluorescence decrease that was prevented by APV or MG132 (n=8 cells for each group from four independent experiments, $p<0.05$ at t=5 min. and thereafter. Error bars denote s.e.m.). Dashed box refers to the region of interest for Fig 3.4.

In order to examine the reporter degradation with higher temporal and spatial resolution, we examined the loss of fluorescence in both the spines and shafts within the first ~150 seconds following stimulation. We found that there was a rapid loss of spine reporter fluorescence within the first ~150 seconds following stimulation (Fig 3.4). The degradation occurred first at spines compared to the rest of the dendrite. This was followed by equal rates of reporter degradation in spines and the shaft, possibly due to diffusion of the reporter from shaft to the spines (where it will be degraded). Another possibility is that the "residual" shaft proteasome is sufficient to degrade some of the shaft reporter.

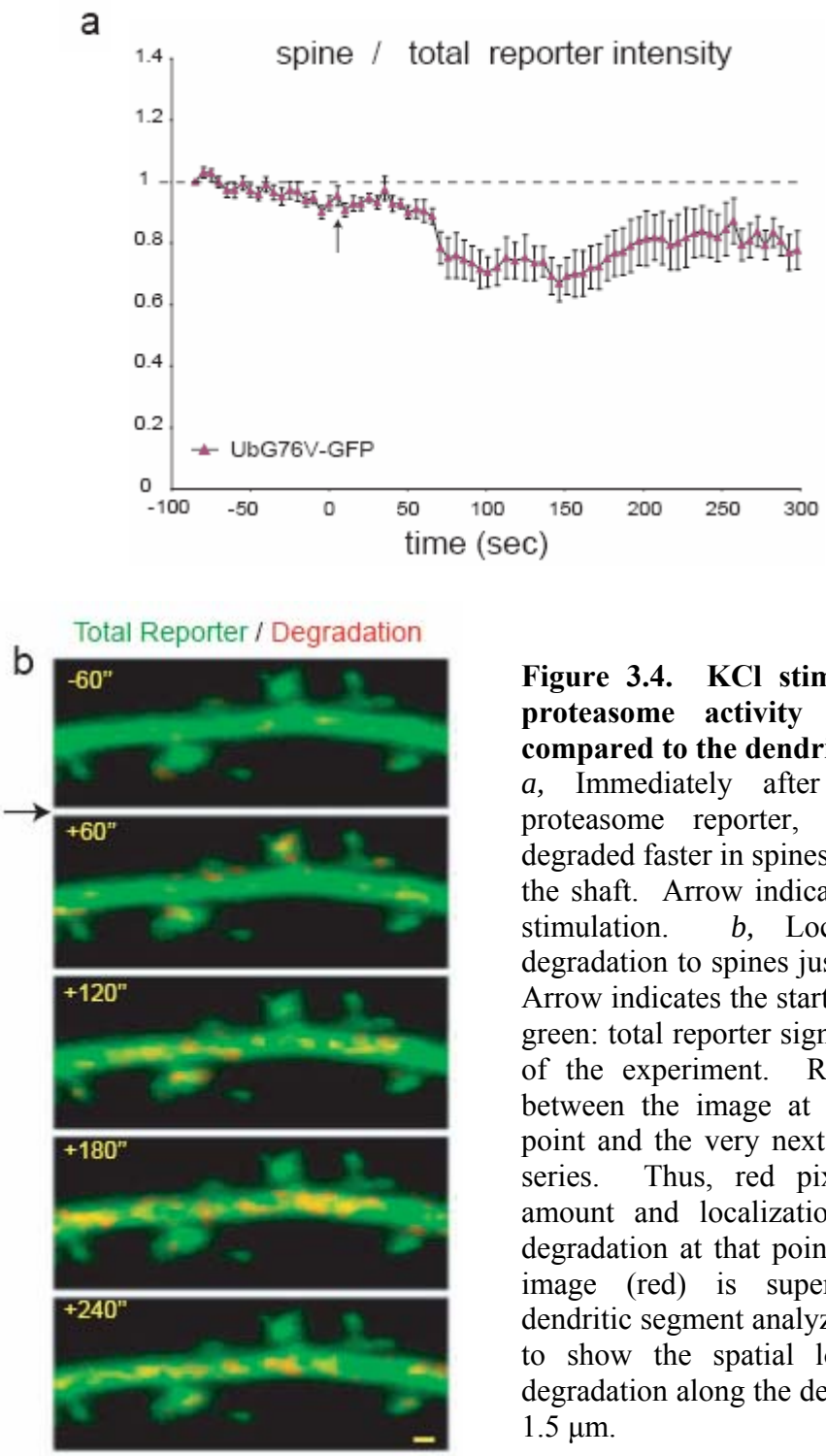

Figure 3.4. KCl stimulation increases proteasome activity more in spines compared to the dendritic shaft.
a, Immediately after stimulation, the proteasome reporter, UbG76V-GFP, is degraded faster in spines when compared to the shaft. Arrow indicates the start of the stimulation. *b,* Localization of the degradation to spines just after stimulation. Arrow indicates the start of the stimulation. green: total reporter signal at the beginning of the experiment. Red: the difference between the image at the indicated time point and the very next image in the time series. Thus, red pixels represent the amount and localization of the reporter degradation at that point. Each difference image (red) is superimposed on the dendritic segment analyzed (green) in order to show the spatial localization of the degradation along the dendrite. Scale bar = 1.5 μm.

Dendrites have the capacity for local protein degradation

As discussed in the introduction, the local regulation of synaptic protein composition endows the neurons with the capacity to regulate their synaptic inputs independently. In order to examine whether the proteasome can be activated locally in the dendrites, we used micropipettes to deliver NMDA locally to dendritic segments and monitored the fluorescent signal of the reporter (Fig. 3.5a,b). We observed a spatially restricted decrease in the intensity of Ub^{G76V}-GFP: the decrease in the reporter signal at the perfused spot was ~41 % of the initial fluorescence whereas the decrease in the areas immediately adjacent to the perfusion spot was ~86 % (Fig. 3.5c,d). For comparison, the reporter signal in the non-perfused dendrites of the same neuron decreased by only 5% (Fig. 3.5c,d). (It is worth noting that the diffusion of intact, fluorescent reporter from regions adjacent to the perfused area likely diminishes the magnitude of the fluorescence decrease we can observe in the perfused area.) In control cells where NMDA was omitted from the perfusion pipette, there were no changes in the GFP signal in the perfused spot or any other dendritic area, indicating that the perfusion procedure does not cause non-specific degradation of the reporter (Fig. 3.5c,d). These results show local protein degradation can occur in the dendrites and can be activated by NMDA receptor activity. These data suggest the capacity for a local response of the ubiquitin proteasome pathway to synaptic events.

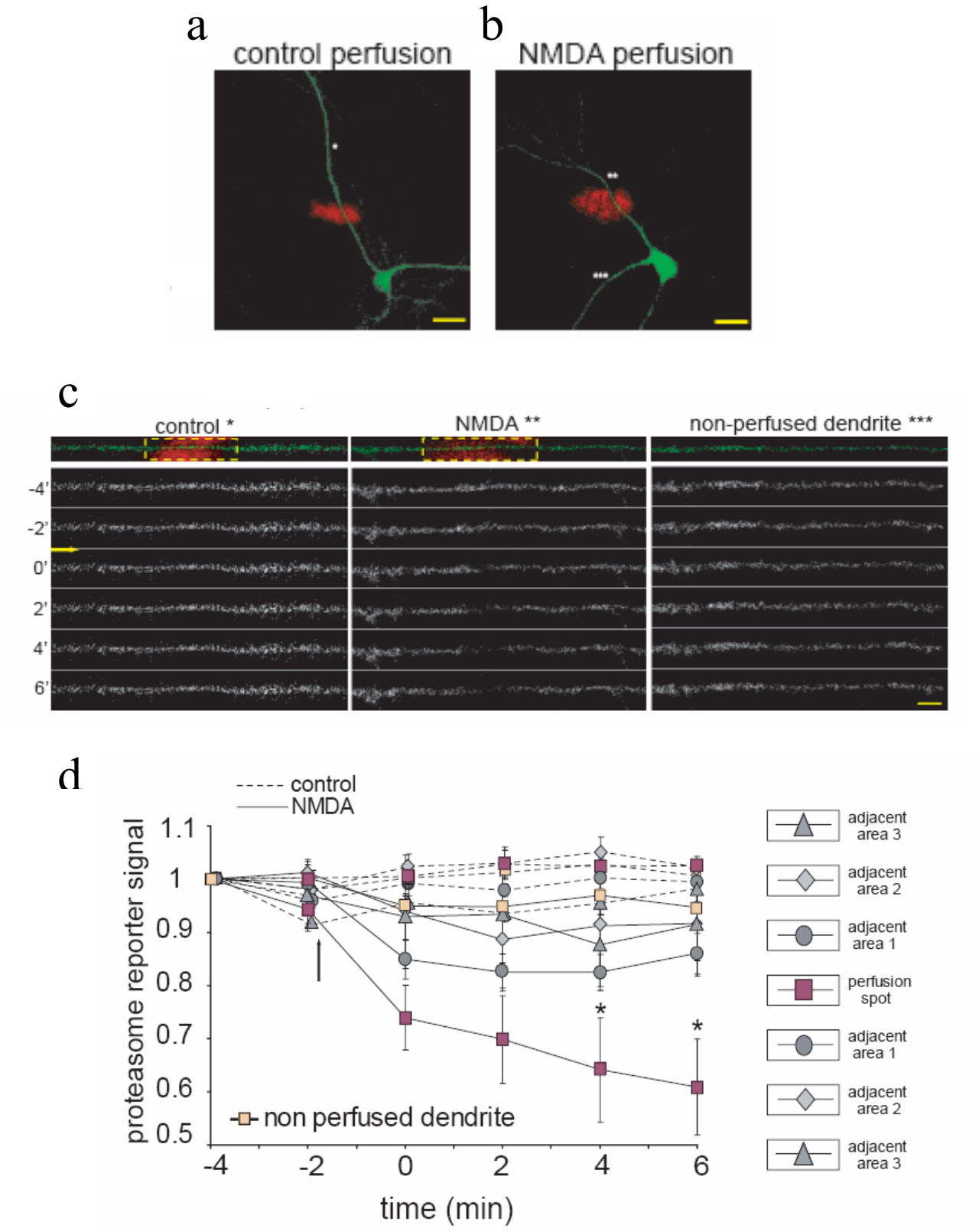

Figure 3.5. Dendrites of hippocampal neurons have the capacity for local protein degradation. *a,b,* UbG76V-GFP expressing cells (in green) and the position of the local

perfusion along the dendrites (in red). Control perfusion is shown in *a* and the NMDA perfusion is shown in *b.* Scale bar = 28 μm. *c,* Higher magnification of the dendrites marked in a and b. (* : control perfusion, **: NMDA perfused dendrite, ***: non-perfused dendrite of the NMDA-perfused neuron). Yellow box indicates the perfusion spot. Yellow arrow indicates the start of the perfusion. Scale bar = 3.5 μm. *d,* Mean change in the reporter signal over time at the perfused spot and areas next to the perfused spot and the non-perfused dendrites. Only NMDA stimulation at the perfusion spot shows significant decrease of the proteasome activity reporter (n = 4 cells for each group from three independent experiments, $p<0.05$ at t=4 min. and thereafter by using ANOVA, error bars denote s.e.m.).

Chapter IV

UPS REGULATES AMPAR TRAFFICKING AT THE SYNAPSE

Proteasome activity is required for agonist-induced endocytosis of GluRs

As discussed above, AMPAR trafficking in and out of the synapse is considered to be one of the main underlying mechanisms of for long-term synaptic plasticity. To address initially the role of protein degradation in the regulation of synaptic function, we asked whether proteasome activity is required for the glutamate-induced internalization of AMPARs. Dissociated hippocampal neurons were live labeled with a primary antibody recognizing an extracellular epitope of either GluR1 or 2 and then treated with the glutamate-receptor agonist AMPA to induce endocytosis of labeled receptors. Internalized receptors were visualized by removing the residual surface antibody followed by fixation, permeabilization and fluorescent secondary antibody labeling. Brief treatment with AMPA resulted in a robust (6- to 8-fold) internalization of either GluR1 or GluR2 (Fig. 4.1a-d). In order to test the role of the proteasome in AMPAR endocytosis, proteasome activity was inhibited before stimulation, using a reversible and an irreversible inhibitor, MG132 and ZL_3VS, respectively. MG132 and ZL_3VS inhibit primarily the chymotrypsin-like activity of the proteasome (Bogyo et al., 1997; Jensen et al., 1995). Brief (20 min) pretreatment with the proteasome inhibitor MG132 or ZL_3VS completely prevented the AMPA-stimulated endocytosis of GluR1 and 2, suggesting that proteasome activity is required for AMPAR endocytosis (Fig. 4.1a-d). Similar inhibition was also observed with a third specific proteasome inhibitor, lactacystin (mean fold internalization of GluR2: control, 1.0 ± 0.38, N = 14; AMPA, 5.88 ± 1.39, N = 28; lactacystin, 0.42 ± 0.24, N = 48). Fig. 4.1c-d show the analysis of somatic immunofluorescence; proteasome inhibition also prevented the AMPA-induced

internalization of dendritic GluRs (mean fold internalization of dendritic GluR1: control, 1.00 ± 0.13, n (dendrites) = 42; AMPA, 6.47 ± 1.86, N = 38; AMPA + MG132, 1.38 ± 0.40, N = 45; mean fold internalization of dendritic GluR2: control, 1.00 ± 0.20, N = 38; AMPA, 7.39 ± 2.87, N = 40; AMPA + MG132, 1.12 ± 0.20, N = 39). The complete inhibition of AMPA-induced internalization of GluR1 and GluR2 observed in the presence of proteasome inhibitors indicates a requirement for protein degradation in AMPA receptor trafficking. Activation of NMDA (N-methyl-D-aspartate) receptors can also induce internalization of GluRs (Beattie et al., 2000). We found that proteasome inhibitors also prevent the NMDA-induced internalization of GluR1 (Fig. 4.1e). Previous studies have suggested different intracellular signals for AMPA versus NMDA-induced internalization of the receptors (e.g., Ehlers, 2000); the blockade of both AMPA- and NMDA-induced internalization observed with proteasome inhibitors indicates that at least part of the internalization mechanism is shared.

The bath application of neurotransmitters to cultured hippocampal neurons likely does not faithfully represent the synaptic release of glutamate, since bath-applied agonists can stimulate both synaptic and extrasynaptic receptors (e.g., Hardinghamet al., 2002). As such, we addressed whether the synaptic release of glutamate, elicited by bicuculline treatment, can also stimulate the internalization of GluR1. We found that a 40 min treatment with bicuculline (50 μM) caused ~3.5-fold increase in internalized GluR1 relative to unstimulated controls (Fig. 4.1f,g). These data indicate that the synaptic release of transmitter can also induce the internalization of the receptors. Moreover, we found that the bicuculline-induced internalization was also blocked by a 20 min pretreatment with MG132 (Fig. 4.1f,g). Taken together, these data suggest that

proteasome activity is required for the internalization of GluRs elicited by either synaptically released or bath-applied agonists.

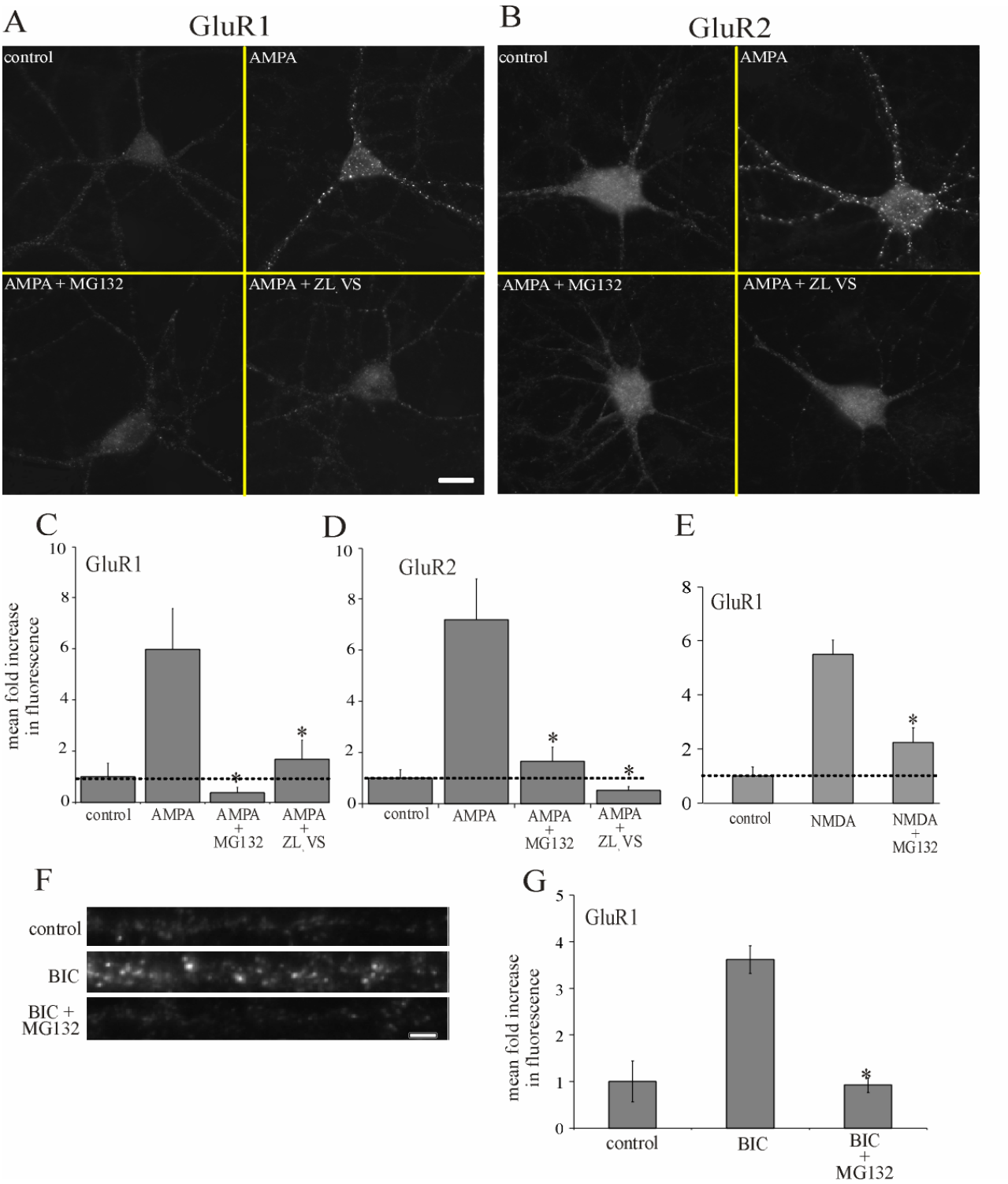

Figure 4.1. AMPA- or Bicuculline-induced internalization of GluR1 or GluR2 is blocked by inhibitors of the proteasome. (A) Shown are representative images of control, AMPA-, AMPA + MG132-, or AMPA + ZL_3VS-treated neurons in which internalization of GluR1 was monitored in dissociated hippocampal neurons. AMPA stimulation increased the pool of internalized GluR1 as measured with fluorescence immunohistochemistry. This agonist-induced internalization was blocked by brief (15–20 min) pretreatment with either of two proteasome inhibitors MG132 or ZL_3VS.(B) Shown are representative images of control, AMPA-, AMPA + MG132-, or AMPA + ZL_3VS-treated neurons in which internalization of GluR2 was monitored in dissociated hippocampal neurons. AMPA stimulation increased the pool of internalized GluR2. This agonist-induced internalization was blocked by brief (15–20 min) pretreatment with either of two proteasome inhibitors MG132 or ZL_3VS. (C) Summary data for GluR1 internalization experiments. AMPA treatment resulted in an average ~6-fold increase in fluorescence; this increase was significantly inhibited ($p \leq 0.01$) by either of two proteasome inhibitors. n (neurons) for each group: control, AMPA, AMPA + MG132, AMPA + ZL_3VS, N = 33, 43, 21, and 25, respectively.(D) Summary data for GluR2 internalization experiments. AMPA treatment resulted in an average ~7.5-fold increase in fluorescence; this increase was significantly inhibited ($p \leq 0.01$) by either of two proteasome inhibitors. n for each group: control, AMPA, AMPA + MG132, AMPA + ZL_3VS, N = 79, 64, 56, and 23, respectively. Scale bar, 10 μm. (E) Summary data for NMDA-induced internalization of GluR1. NMDA treatment resulted in an average ~5.0-fold increase in fluorescence; this increase was significantly inhibited by MG132. n for control, NMDA, and NMDA + MG132 are 47, 27, and 39, respectively. (F) Shown are representative images of control, bicuculline- (BIC), or BIC + MG132-treated neurons in which internalization of GluR1 was monitored in the dendrites of dissociated hippocampal neurons. BIC stimulation increased the pool of internalized GluR1; this internalization was blocked by a 20 min pretreatment with MG132. (G) Summary data for BIC-induced internalization of GluR1. Bicuculline treatment (40 min) resulted in an average ~3.5-fold increase in fluorescence; this increase was significantly inhibited by MG132. n for control, BIC, and BIC + MG132 are 40, 29, and 29, respectively.

Brief inhibition of proteasome activity does not alter the pool of surface GluR1 or GluR2

As agonist-stimulated targets of the proteasome could participate in the endocytosis, exocytosis, and/or insertion of the glutamate receptors, we next examined whether proteasome inhibition alters the pool of surface-expressed AMPA receptors available for endocytosis. Using antibody labeling of live neurons, we compared the

amount of surface GluR1 or GluR2 immunoreactivity following 10 min, 40 min, or 2 hr treatments with MG132. Using the same data acquisition parameters that we used for our internalization experiments, we found that none of the MG132 treatments had a significant effect on the number of either GluR1 or GluR2 receptors detected on the cell surface (Fig. 4.2 a,b). We did observe a substantial loss of surface receptors following AMPA treatment (data not shown). These results suggest that the internalization of receptors stimulated by endogenous glutamate release in our culture system is below the detection threshold of our experiments.

Internalization of the transferrin receptor does not depend on proteasome activity

We addressed the specificity of proteasome action by determining whether ligand-stimulated endocytosis of the transferrin receptor exhibits a similar sensitivity to proteasome inhibition. Application of transferrin to cells is known to induce a receptor-mediated internalization of both transferrin and its receptor (Yamashiro et al., 1984)—this can be quantified by measuring the cytoplasmic accumulation of fluorescently labeled transferrin (Sever et al., 2000). Using this technique (van Kerkhof et al, 2000), we measured the receptor-mediated internalization of transferrin in cultured hippocampal neurons (Fig. 4.2c,d). Pretreatment of neurons with the proteasome inhibitor MG132 did not affect the internalized pool of transferrin (Fig. 4.2c,d). This result indicates that proteasome activity is not required for the ligand-induced internalization of all receptors in neurons.

To address whether proteasome activity is instructive or permissive for GluR internalization, we manipulated the duration of proteasome inhibitor pretreatment prior to AMPA stimulation. We found that a mere 5 min pretreatment with a proteasome inhibitor was sufficient to significantly inhibit AMPA-induced GluR1 internalization (Fig. 4.2e,f). Increasing the duration of proteasome inhibitor pretreatment to 10 or 20 min yielded modest but not significant increases in the inhibition of internalization (Fig. 4.2e,f). That a very brief proteasome pretreatment still inhibits receptor internalization strongly supports the view that AMPA treatment actively stimulates protein degradation, rather than the alternative view that there is constitutive degradation of a protein(s) that is required for GluR internalization.

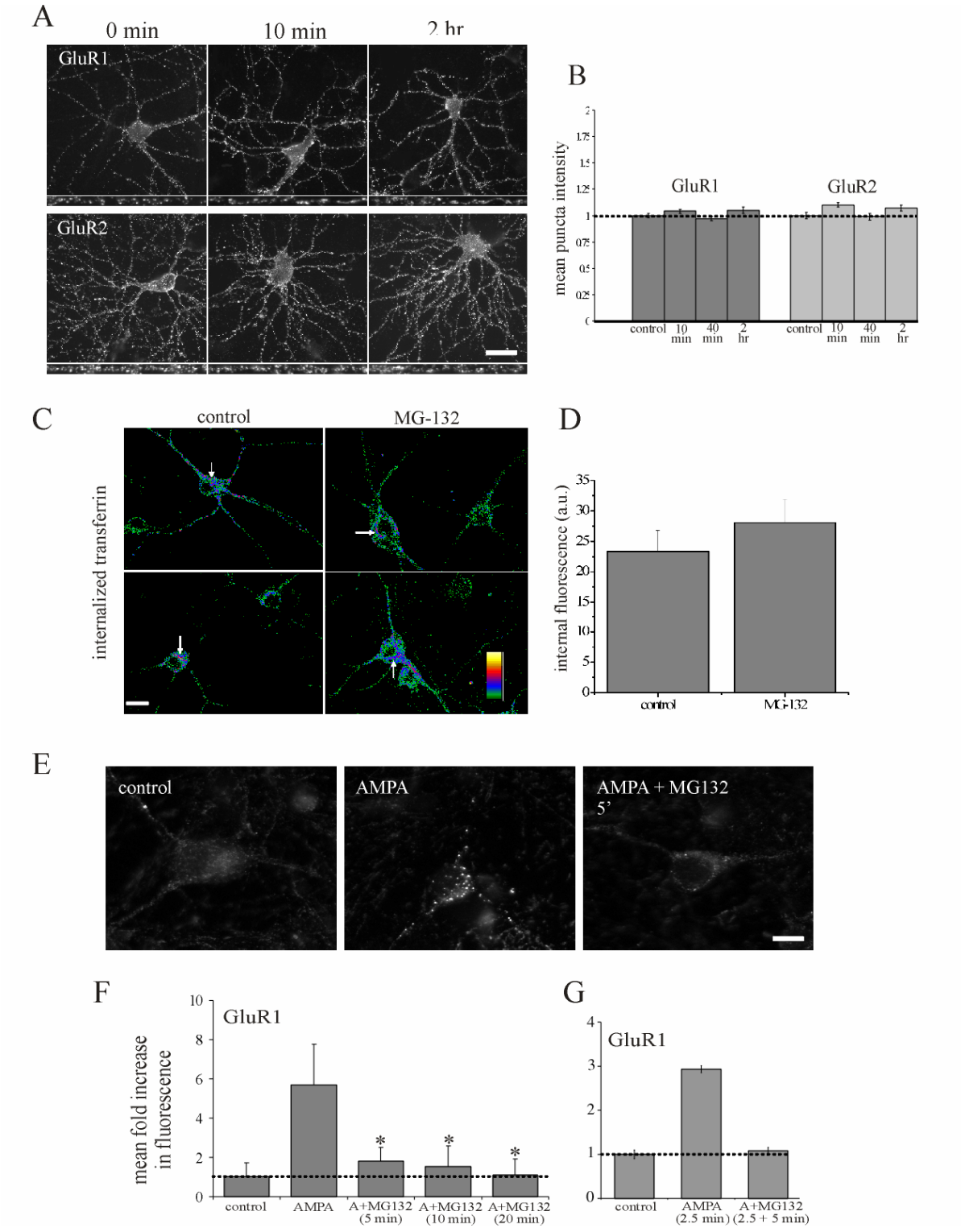

Figure 4.2. Proteasome inhibitors do not alter surface GluRs or block transferrin endocytosis. (A) Representative images for either surface GluR1- or GluR2-labeled neurons treated with vehicle (control), MG132 for 10 min, or MG132 for 2 hr. Scale bar,

10 μm. (B) Summary bar graphs indicating the mean dendritic puncta fluorescence intensity for GluR1 or GluR2 surface receptors. Using the same image acquisition parameters for the detection of internalized GluRs, there was no change in the population of surface GluRs after exposure to MG132. (C) Proteasome inhibitors do not block transferrin receptor endocytosis in hippocampal neurons. Representative images showing similar quantities of internalized transferrin in either control or MG132-treated neurons. Fluorescently labeled transferrin from middle region of confocal z-stacks containing the nucleus, but lacking surface plasma membrane, was analyzed. Arrows note the cytoplasmic and perinuclear accumulation of transferrin. Scale bar, 10 μm. (D) Summary data for tranferrin internalization experiments. There was no significant difference in the amount of internalized transferrin observed in control or MG132-treated neurons. (E) AMPA-induced internalization of GluR1 and GluR2 can be blocked by very brief pretreatment with proteasome inhibitors. Scale bar, 10 μm. (F,G) Summary data for the length of proteasome inhibition pretreatment required to prevent AMPA-induced internalization of GluR1. Pretreatments as brief as 5 min significantly inhibited AMPA-induced internalization of GluR1. n for control, AMPA, and 2.5, 5, 10, and 15 min proteasome inhibitor pretreatment are 23, 21, 18, 21, and 24, respectively.

Polyubiquitination is required for agonist-stimulated internalization of GluR1 and 2

In addition to blocking proteasome activity, proteasome inhibitors can also cause a diminution of the free ubiquitin pool: the inhibition of the proteasome leads to a build-up of polyubiquitinated proteins resulting in decreased availability of free ubiquitin (Schubert et al., 2000). Because single ubiquitin molecules can be used as signals for endocytosis, independent of proteasome activity (Hicke and Riezman, 1996; Hicke 1997; Hicke, 2001), it is important to assess the possible depletion of the ubiquitin pool by proteasome inhibitors. We conducted a biochemical analysis of the free ubiquitin pool in lysates prepared from hippocampal neurons treated with proteasome inhibitors for 5, 20, or 40 min or 24 hr (e.g., (Ehlers, 2003)). We found that treatment with MG132 (50 μM) for 20 min, 40 min, or 24 hr resulted in significant reductions of free ubiquitin (Fig. 4.3 a,b), whereas a 5 min treatment had no significant effect. In figure 4.2, we demonstrated

that a 5 min pretreatment with MG132 (followed by 20 min of AMPA + MG132 treatment) is sufficient to inhibit GluR endocytosis, suggesting that free ubiquitin depletion cannot explain the effects of proteasome inhibitors. To be sure that the effects of MG132 cannot be attributed to free ubiquitin depletion, we conducted an additional experiment in which the total duration of exposure to MG132 was limited to 5 min (2.5 min pretreatment followed by 2.5 min with AMPA). In this experiment, MG132 still inhibited AMPA-stimulated GluR1 endocytosis, indicating a requirement for protein degradation (Fig. 4.2g). In order to examine further the role of polyubiquitination, and hence proteasome activity, in AMPA-induced GluR internalization, we used Sindbis virus to express a ubiquitin chain-elongation mutant (UbK48R) in which lysine at position 48, a site of ubiquitin attachment, is mutated to an arginine (Ward et al, 1995). Expression of this construct thus allows monoubiquitination of proteins (Hicke, 2001) but results in abbreviated ubiquitin chain lengths for polyubiquitinated proteins. We estimated that K48R was expressed at levels 10- to 12-fold higher than endogenous ubiquitin. In control neurons expressing EGFP alone, we observed robust (7- to 12-fold) AMPA-induced internalization of both GluR1 and 2 (Fig. 4.3c-e). In contrast, in neurons expressing K48R (SinK48R-IRES-EGFP) the AMPA-induced GluR internalization was significantly inhibited (Fig. 4.3c-e). Because monoubiquitination is allowed in neurons expressing K48R, these results indicate a requirement for polyubiquitination in the internalization of the glutamate receptors. Taken together with the proteasome inhibition data, these results show that ubiquitin-dependent degradation is essential for the AMPA-induced internalization of GluRs.

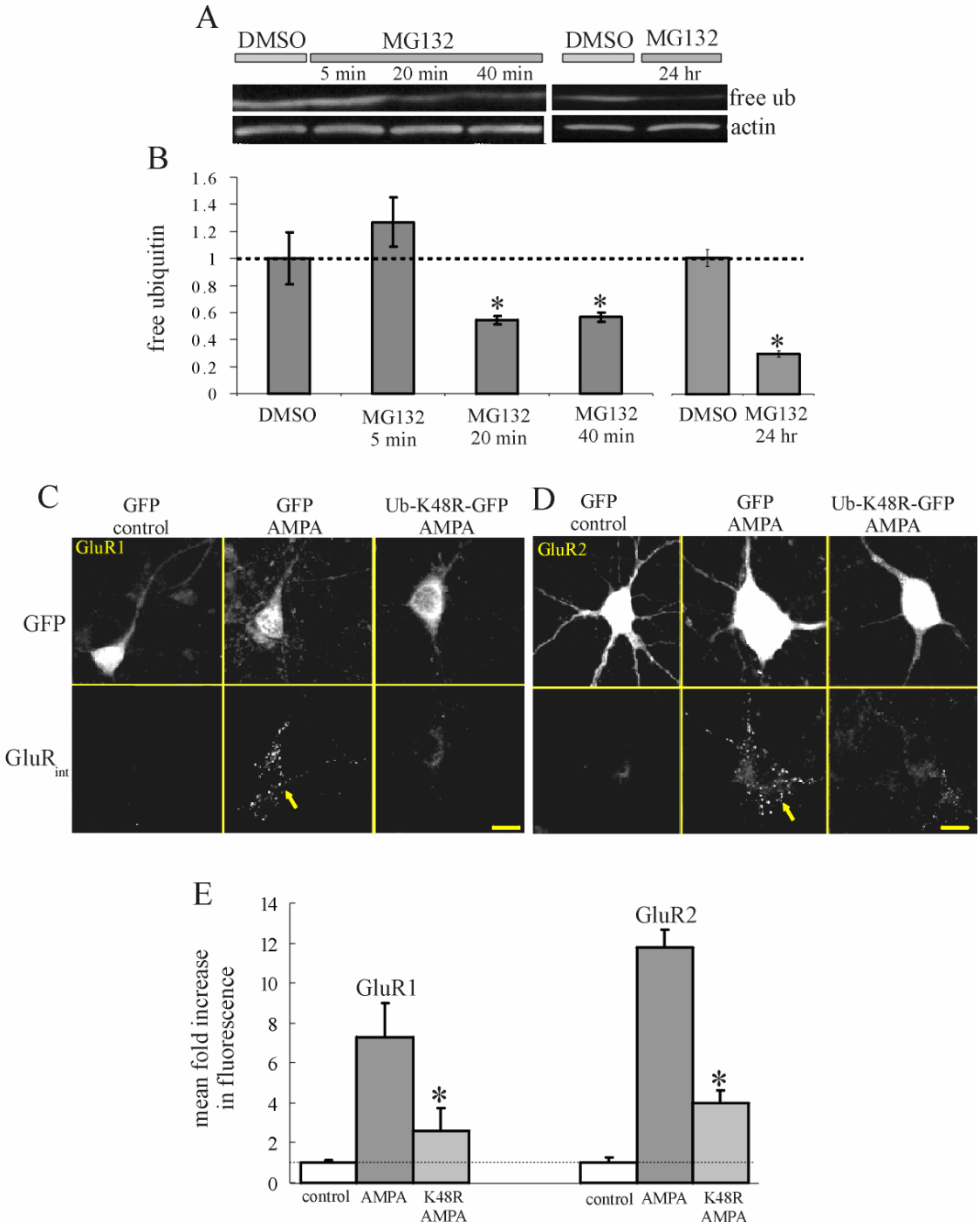

Figure 4.3. AMPA-induced internalization of GluR1 and GluR2 requires polyubiquitination. Brief ≤ 5 min MG132 treatment does not deplete the free ubiquitin pool (A and B). Treatment of hippocampal neurons with MG132 for greater than 5 min results in decreased free ubiquitin as detected with Western blot analysis. Data analyzed in (B) are normalized to actin, same lane, controls. (C and D) GluR1 or GluR2 internalization experiments were performed on Sindbis GFP or Sindbis His_6-myc-

ubiquitin K48R-IRES-GFP-infected neurons. Shown are the GFP signal and the internalized receptor signal for either GluR1 (C) or GluR2 (D). In GFP-expressing neurons, AMPA resulted in a robust internalization of either receptor. In contrast, neurons expressing K48R-IRES-EGFP showed significantly reduced internalization following AMPA treatment. Scale bar, 10 μm. (E) Summary data for experiments in (C) and (D). n for GluR1: GFP, GFP + AMPA, Ub-K48R + AMPA, N = 30, 64, and 67, respectively; GluR2: GFP, GFP + AMPA, Ub-K48R + AMPA, N = 36, 33, and 43, respectively.

Chapter V

POSSIBLE TARGETS OF UPS AT THE SYNAPSE

In order to establish that a protein is a target of UPS at the synapse, the following criteria should be met. First of all, ubiquitinated species of the target protein must be present at synapses. Furthermore, the ubiquitination of the target protein must be regulated by synaptic activity. If these conditions hold true, one would expect to see an activity-dependent and proteasome inhibitor-sensitive decrease in the abundance of the target protein. Finally, if the target protein degradation is involved in AMPAR endocytosis, then specifically inhibiting the target protein degradation must also block AMPAR endocytosis. Blocking the degradation of the target protein can be achieved by deleting the degradation signal or mutating the ubiquitin acceptor lysines within the target protein. As it will be shown below, these are not all trivial undertakings because of the low abundance and instability of the ubiquitinated protein species. Furthermore, the activity-regulated target protein population at the synapse may only be a small fraction of the total target protein in the cell, which would make it hard to detect the decrease in the target protein abundance following stimulation. We have screened the known synaptic proteins to test whether or not they follow the patterns summarized above. Our results suggest that PSD-95 may be an indirect target of UPS.

PSD-95 may be an indirect target of UPS to regulate AMPAR internalization

The data presented so far shows that a brief application of AMPA to cultured hippocampal neurons induces a robust endocytosis of GluRs from the cell surface that requires proteasome activity and polyubiquitination. As discussed in the introduction, PSD-95 may act as a "slot" protein to stabilize the AMPARs at the synapse through its indirect interaction with AMPARs. In order to investigate if PSD-95 levels are coordinately regulated with GluR endocytosis in dissociated hippocampal cultures, we induced GluR internalization with AMPA stimulation and then examined both the internalized pool of GluR1 and PSD-95 using immunocytochemical techniques. If one examines many neurons in the same dish, it is clear that cells exhibit variable levels of internalized GluRs in response to AMPA stimulation (n=4, 3, and 4 cells/dendrites that shows high (194.0 ± 8.9), medium (88.6 ± 4.5) and low (22.6 ± 6.2) level of internalized receptor puncta intensity, respectively) (e.g., Fig. 5.1b). We examined the relationship between the magnitude of internalized GluR and the degree of PSD-95 expression in individual dendrites. We found that cells that robustly internalized GluRs in response to AMPA treatment had significantly reduced levels of PSD-95 puncta (Fig. 5.1a, arrowheads). In contrast, the cells that did not respond to AMPA stimulation by internalizing receptor possessed noticeably higher levels of PSD-95 (Fig. 5.1a, arrows). Indeed, if one examines individual neurons that represent the full range of internalized GluR (no internalized puncta to high levels of internalized puncta) one consistently observes an inverse correlation with PSD levels (Fig. 5.1b,c). These data suggest a link between the absence of PSD-95 and the presence of internalized GluR1. These data are

consistent with the idea that AMPA stimulation leads to a loss of PSD-95 and the internalization of GluR1. It is attractive to propose that AMPA stimulation leads to a degradation of PSD-95 that precedes the internalization of the glutamate receptor. However, the inverse correlation between levels of internalized GluR and PSD-95 (Fig. 5.1c) could also be explained by pre-existing differences in the levels of PSD-95. Suppose, for example, that a subpopulation of neurons begin with less PSD-95 and less surface GluR available for endocytosis. Alternatively, PSD-95 might exert a stabilizing influence on GluRs such that low levels of PSD-95 predispose receptors to internalize.

In order to test for the first possibility, we examined the amount of surface GluR1 present in neurons that have high, medium or low levels of PSD-95 in the absence of stimulation. We did not find a correlation between PSD-95 level and surface GluR1 level (Fig. 5.1d). This observation indicates that the reduced levels of PSD-95 observed in neurons with internalized GluR1 (Fig. 5.1a) is not due to a pre-existing relationship between PSD-95 levels and surface receptors. Furthermore, Schnell et al. (2002) have shown that in organotypic slice cultures, PSD-95-overexpressing cells do not show a change in the response to bath-applied AMPA compared to non-transfected cells. These data thus leave open the possibility that PSD-95 levels are decreased upon AMPA treatment and that this decrease is important for subsequent GluR internalization.

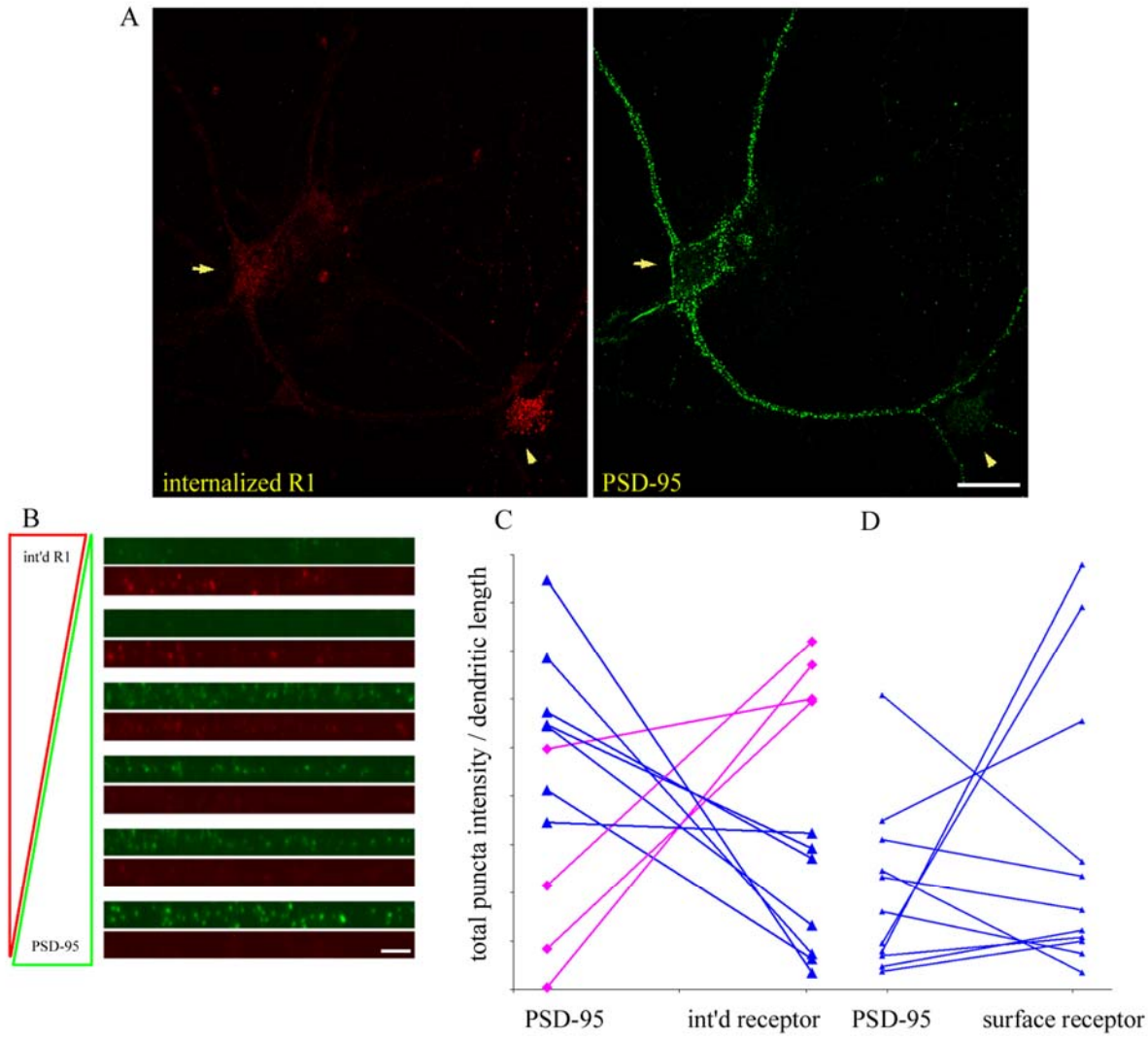

Fig. 5.1. Inverse correlation between internalized GluR and PSD-95. Both the internalized GluR1 pool and PSD-95 were visualized in individual neurons and dendrites following AMPA stimulation. (A) Neurons that responded robustly to AMPA have more internal GluR1 (red) and less PSD-95 (green) puncta (arrowheads) whereas neurons that did not respond to AMPA have diffuse internal GluR1 staining and more PSD-95 puncta (arrows). Internal GluR1 is visualized using the acid stripping technique (see Methods). Scale bar is 10 µm. (B) Examples of imaged dendrites in which there is an inverse correlation between the amount of internalized receptor (red) and PSD-95 levels (green). Scale bar is 3 µm. (C) Quantification of the data shown in B**.** Internalized receptor levels and the corresponding PSD-95 level in each dendrite are plotted as the total intensity of signal per dendritic length (n=11, r^2: −0.62, p<0.01). (D) Absence of correlation between surface GluR1 and PSD-95. Surface receptor levels and the corresponding PSD-95 level in each dendrite are plotted as the total intensity of signal per dendritic length (n=11, r^2: 0.02, p>0.5).

PSD-95 exhibits a proteasome-sensitive downregulation in protein level following AMPA stimulation

As discussed earlier, recent work has shown that the ubiquitin-proteasome pathway plays a major role in regulating synaptic function. In order to test if the AMPA-stimulated PSD-95 decrease observed above is regulated by the ubiquitin–proteasome pathway, we have treated cultured hippocampal neurons with AMPA in the presence and absence of proteasome inhibitor MG132 (Lee and Goldberg, 1998) and monitored PSD-95 levels immunohistochemically. AMPA stimulation caused a significant decrease in both the number and the total intensity of PSD-95 puncta in hippocampal dendrites (Fig. 5.2a, b). The decrease in PSD-95 level was sensitive to a proteasome inhibitor suggesting that the ubiquitin–proteasome pathway—directly or indirectly—participates in this AMPA-induced decrease in PSD-95. In addition, we conducted Western blot analysis from either hippocampal lysates or synaptosomes treated with AMPA. Although an AMPA-stimulated decrease in PSD-95 was not observed in the whole hippocampal lysates (Fig. 5.2c), a proteasome-inhibitor-sensitive decrease in PSD-95 levels was detected in synaptosomes (Fig. 5.2d).

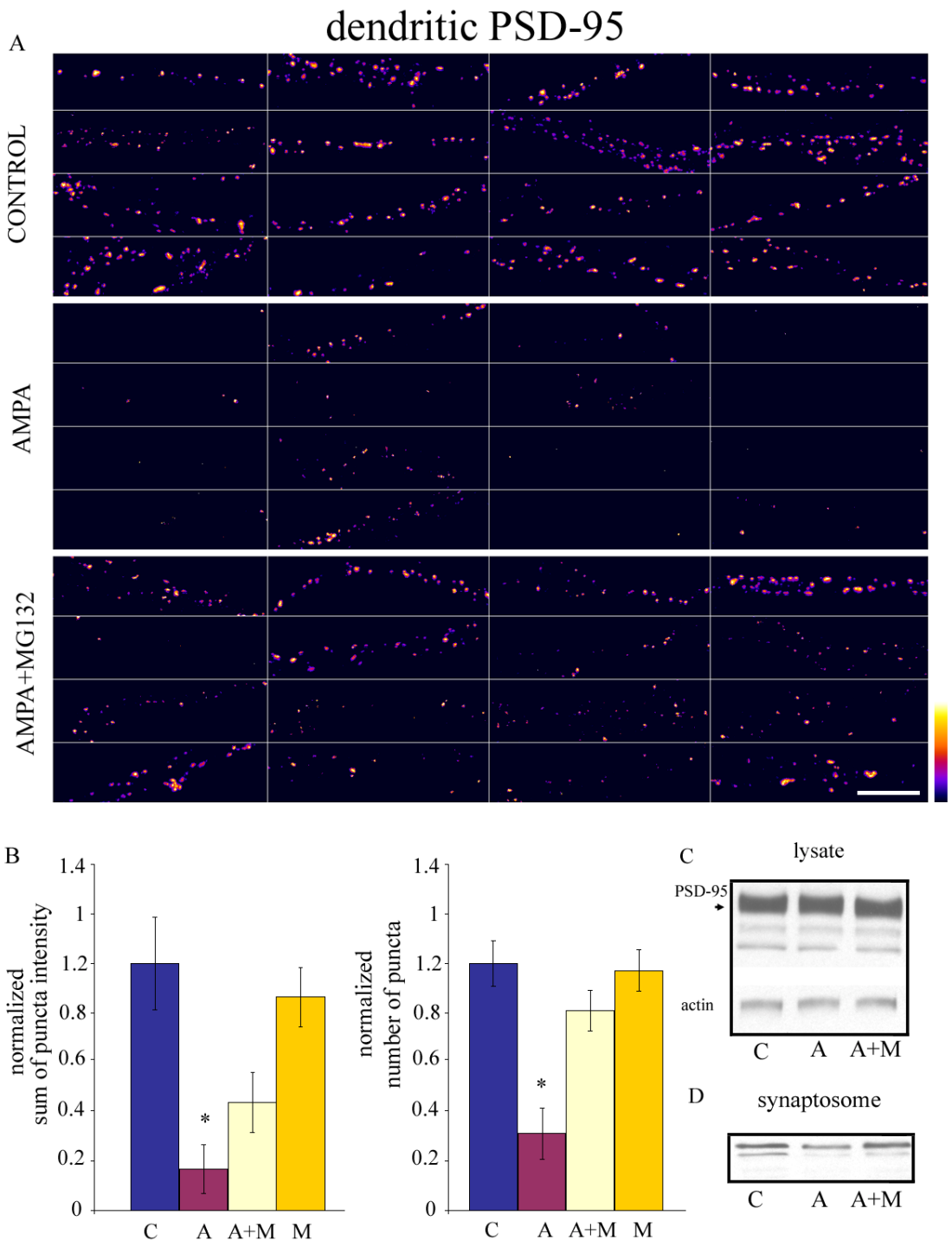

Fig. 5.2. PSD-95 exhibits a proteasome-sensitive downregulation in protein level following AMPA stimulation. (A) PSD-95 immunocytochemistry in dendrites from vehicle treated (top panel), AMPA (middle panel) or AMPA and MG132 treated neurons (bottom panel). AMPA (100 μM) stimulation was for 20 min and MG132 (50 μM) was present 20 min before and during the entire period of AMPA stimulation. (B) Analysis of PSD-95 levels in control, AMPA (A) or AMPA+MG132 (A+M) conditions. AMPA

stimulation decreases the number and total intensity of PSD-95 puncta in an MG132-sensitive manner. Each value is normalized to control (n=16 for the first three conditions and 20 for MG132 alone experiment, p<0.05). (C) Western blot analysis from hippocampal lysates stimulated with AMPA in the presence or absence of MG132 shows no apparent change in PSD-95 protein level. The arrow represents PSD-95 immunoreactivity. Lower blot represents the actin control. (D) Western blot analysis from hippocampal synaptosomes stimulated with AMPA shows a proteasome inhibitor-sensitive decrease in PSD-95 protein levels.

PSD-95 overexpression reduces AMPA-induced GluR1 endocytosis

If PSD-95 is regulated by the ubiquitin–proteasome pathway, overexpression of PSD-95 might saturate the enzymatic machinery required to downregulate PSD-95. Furthermore, if PSD-95 downregulation has a role in GluR1 endocytosis, then overexpression of PSD-95 should also affect GluR endocytosis. In order to investigate these possibilities, we expressed PSD-95-GFP or GFP alone in cultured hippocampal neurons and examined GluR-endocytosis in response to AMPA stimulation. Neurons that expressed GFP alone showed a significant increase in internalized GluR1 following stimulation with AMPA (Fig. 5.3a, b). In contrast, neurons that expressed PSD-95-GFP did not exhibit significant AMPA-stimulated endocytosis of GluR1. Although we cannot rule out the possibility that PSD-95 overexpression results in increased recycling, these results suggest that a limited concentration of PSD-95 is essential for neurons to exhibit AMPA-stimulated GluR1 endocytosis.

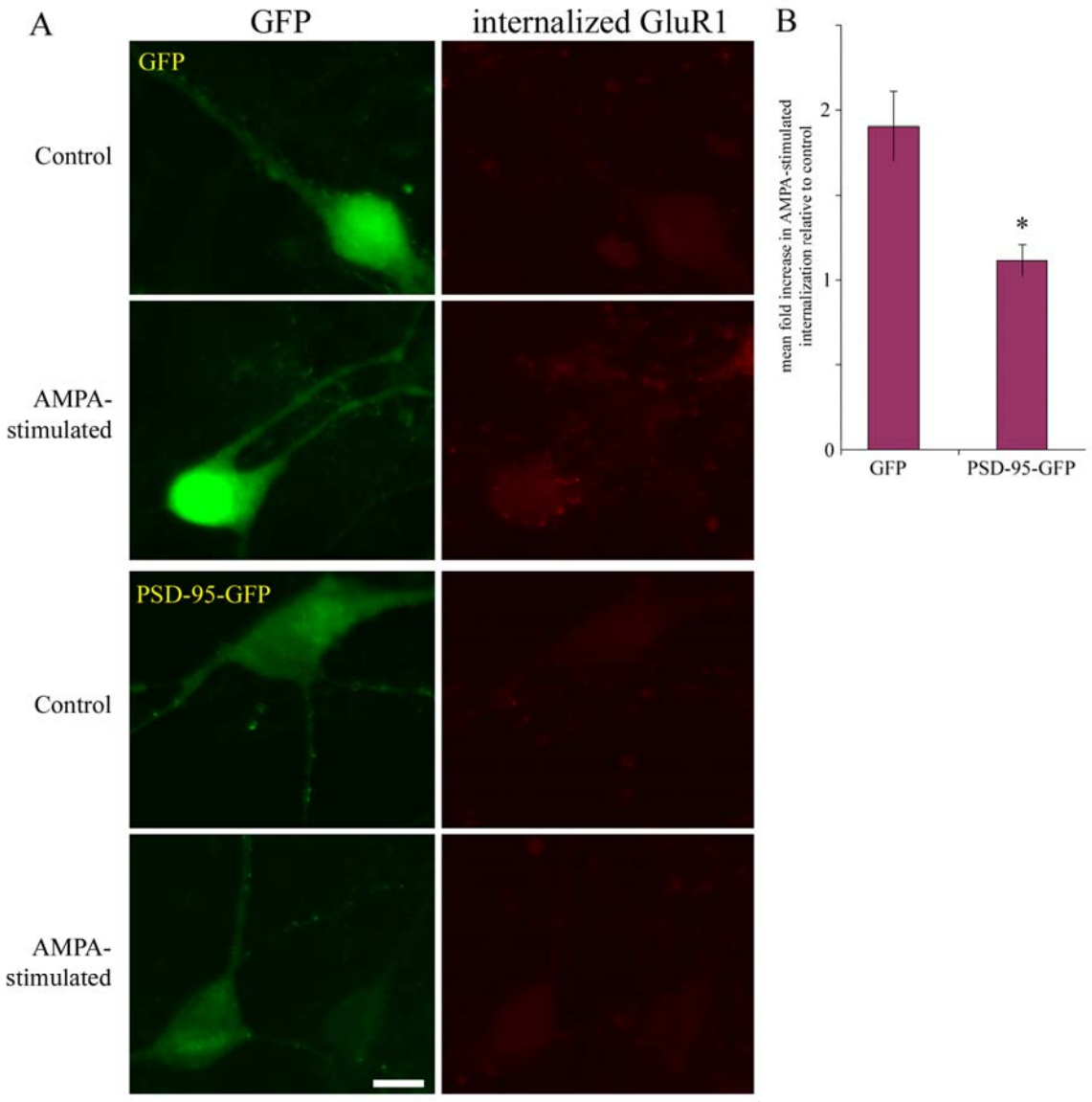

Fig. 5.3. PSD-95 overexpression reduces AMPA-induced GluR1 endocytosis. (A) Shown are the GFP signal (green) and internalized GluR1 signal (red) neurons expressing GFP alone (top four images) or PSD-95-GFP (bottom four images). In GFP-alone-expressing neurons AMPA stimulation led to GluR1 internalization, whereas in PSD-95-expressing neurons AMPA stimulation did not result in GluR1 internalization. Scale bar=5 μm. (B) Analysis showing the AMPA-stimulated internalization of GluR1 in GFP-alone-expressing neurons and the lack of AMPA-stimulated internalization in PSD-95-GFP-expressing neurons (n=30 for each condition, p<0.05).

The ubiquitination of PSD-95 cannot be detected

The ubiquitin–proteasome system marks target proteins to be degraded by the attachment of a polyubiquitin chain. We previously demonstrated that polyubiquitination is required for GluR endocytosis (Patrick et al., 2003) and hypothesized that the target for the proteasome is a protein that interacts directly or indirectly with the GluRs. As such, the target protein should exhibit AMPA-stimulated polyubiquitination. One common way to determine if a protein is ubiquitinated is to pull-down ubiquitinated proteins and then conduct Western blot analysis for the protein of interest (e.g., Ehlers, 2003 and Colledge et al., 2003). We have tried two methods to detect ubiquitinated proteins. The first involves immunoprecipitation (with antibodies to ubiquitin or candidate proteins) and the second is GST-S5a affinity chromatography (Layfield et al., 2001). S5a is a subunit of proteasome that has been shown to bind to polyubiquitin chains (Deveraux et al., 1994). Using either cultured hippocampal neuron lysates (Fig. 5.4) or whole hippocampal lysates (data not shown) we found that we could isolate an abundance of ubiquitinated proteins by either ubiquitin immunoprecipitation followed by Western blot analysis using an anti-ubiquitin antibody (Fig. 5.4a) or GST-S5a chromatography (data not shown). The amount of ubiquitinated proteins that we could isolate was enhanced by a brief (20 min) treatment with MG132 (50 μm) or MG132+NMDA stimulation. We addressed the specificity of our assay by treating the pull-down beads with a deubiquitinating enzyme (DUB) that removes the ubiquitin chains from ubiquitinated proteins. DUB application to the beads from either immunoprecipitation removed all of the polyubiquitin signal (Fig. 5.4a).

Although we have shown that we can pull-down ubiquitinated proteins (Fig. 5.4a), we were unable to observe the ubiquitination of PSD-95 when the same blots were reprobed with an anti-PSD-95 antibody (rabbit polyclonal; Fig. 5.4b). Similar experiments conducted with two additional PSD-95 antibodies also yielded negative results. We were, however, able to detect the polyubiquitination of Shank (Ehlers, 2003) using an anti-Shank antibody after a ubiquitin immunoprecipitation (Fig. 5.4c). We also attempted to demonstrate the ubiquitination of PSD-95 by conducting a reverse immunoprecipitation experiment, in which we first immunoprecipitate PSD-95 using an anti-PSD-95 antibody and then probe the blot with either a PSD-95 or ubiquitin antibody (Fig. 5.4d) (e.g., Colledge et al., 2003). Although we could clearly detect the presence of PSD-95 with the anti-PSD-95 antibody, we did not see any ubiquitin immunoreactivity when the anti-ubiquitin antibody was used. Also, an additional set of immunoprecipitations with different PSD-95 antibodies yielded negative results. These results are consistent with one study in which GST-S5a did not pull-down PSD-95 from cortical culture lysates (Ehlers, 2003). Colledge et al. (2003), however, reported the ubiquitination of PSD-95 from stimulated cultures. They stimulated hippocampal cultures with NMDA (20 μM for 3 min) and observed the polyubiquitination 10 min following NMDA stimulation. We used the same techniques and antibodies as Colledge et al. (2003) (Fig. 5.4d), but did not observe the ubiquitination of PSD-95 from AMPA- (data not shown) or NMDA-stimulated cultures, including experiments in which we looked for ubiquitination at different intervals following NMDA stimulation (Fig. 5.4e). In addition, we considered the possibility that the activity of deubiquitinating enzymes during our assay might remove polyubiquitin chains; to address this we routinely

included DUB inhibitors during pull-downs to increase the pool of ubiquitinated PSD-95 species. Unfortunately, we were still not able to detect ubiquitination of PSD-95. These results suggest that either the ubiquitination of PSD-95 is extremely transient or that PSD-95 may not be a direct target of UPS, instead PSD-95 levels may be regulated by other bona fide and direct proteasomal targets.

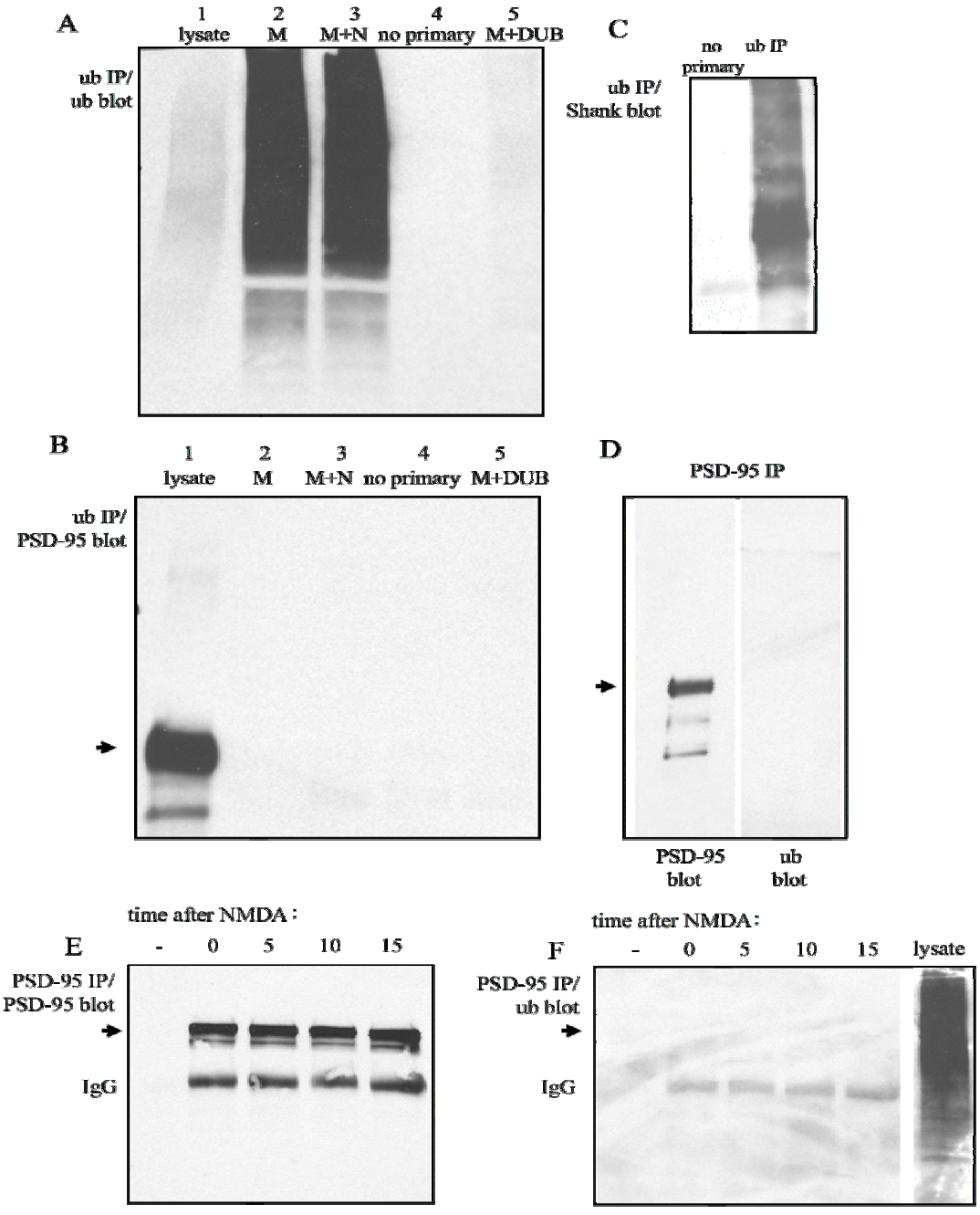

Fig. 5.4. The ubiquitination of PSD-95 cannot be detected. A series of experiments using immunoprecipitation followed by Western blot analysis failed to reveal the ubiquitination of PSD-95. (A) Lysates prepared from cultured hippocampal neurons were subject to immunoprecipitation with an anti-ubiquitin antibody and then probed with anti-ubiquitin antibody. Treatment with MG132 (lane 2) or MG132+NMDA (lane 3) increased the abundance of ubiquitinated proteins relative to untreated lysates. Treatment of the beads with a deubiquitination enzyme abolishes the ubiquitin signal (lane 5). (B) The same blot as shown in A, reprobed with rabbit polyclonal PSD-95

antibody. Although PSD-95 could be detected in the lysate (lane 1), no polyubiquitination of PSD-95 was observed in any of the treatment conditions (lanes 2–5). (C) A blot from ubiquitin IP showing that Shank is ubiquitinated. (D) A reverse immunoprecipitation experiment in which PSD-95 is immunoprecipitated from lysates treated with MG132+NMDA and then Western blot analysis is conducted with either an anti-PSD-95 antibody (left lane) or an anti-ubiquitin antibody. The success of the immunoprecipitation is shown by the presence of PSD-95 in the PSD-95 blot; however, no PSD-95 is detected in the anti-ubiquitin blot. (E) Time course experiment in which the PSD-95 pulldown was conducted at variable intervals (min) following the initiation of NMDA stimulation (3 min total NMDA stimulation). The arrow represents PSD-95 immunoreactivity. IgG is also indicated. (F) The same blot shown in E reprobed using an anti-ubiquitin antibody. No ubiquitinated PSD-95 was detected at any of the intervals examined, although an abundance of ubiquitinated proteins is detected in the lysate (not subjected to PSD-95 IP). The arrow indicates the size of PSD-95 on the gel.

Chapter VI

THE PROTEASOME DYNAMICS AT THE SYNAPSE

Recent studies of UPS regulation have emphasized the importance of substrate recognition and ubiquitination by the enzymes of the conjugation pathway (E1,E2,E3). Downstream of target protein ubiquitination, E3s and other proteins have been proposed to "deliver" the target proteins to the proteasome (Miller and Gordon, 2005). The converse possibility, involving the movement of the proteasome to the target protein is an emerging concept (Glickman and Raveh, 2005), with much less direct experimental support. It is worth noting, however, that biochemical studies have demonstrated the apparent interaction of the proteasome with proteins physically proximal to degradation targets (reviewed in Chapter 2).

The dendrites of neurons are decorated with specialized protrusions called spines, individualized biochemical compartments where excitatory synapses occur. Previous work has shown that ubiquitinated proteins and the components of the UPS can be detected in spines. Furthermore, as discussed in the introduction, UPS functions at the synapse. Here we examine directly the impact of NMDA receptor-dependent neural activity on the localization of the proteasome and find that synaptic activity recruits the proteasome into spines, providing a mechanism for local protein degradation. The proteasome's sequestration is persistent, reflecting an association with the actin-based cytoskeleton.

Activity-dependent proteasome dynamics

The data presented so far suggest that the proteasome locally degrades proteins at synapses. Furthermore, only half of the proteasome is synaptic in hippocampal cultures (Fig. 3.1). Thus, there is room for the regulated delivery of the proteasome to the synapses in response to activity. To examine whether synaptic activity regulates the localization of the proteasome at synapses, we expressed a GFP-tagged 19S proteasome subunit Rpt1 (CIM5/ YKL145W/Yta3) in cultured hippocampal neurons. In order to test the incorporation efficiency of Rpt1-GFP into the endogenous proteasome structure, endogenous proteasome from cultures expressing Rpt1-GFP was immunoprecipitated (with anti-Rpt6 antibody) and blotted for another endogenous subunit, Rpt3, or Rpt1-GFP with a GFP antibody. If all the Rpt1-GFP and Rpt3 are in the same proteasome structure, they should be immunoprecipitated with the same efficiency. Comparison of the lysate:IP ratio on the western blot indicates that the incorporation efficiency of Rpt1-GFP is 77+/-3.4% (from 2 independent experiments) of the endogenous proteasome subunit Rpt3. Furthermore, Rpt1-GFP colocalizes with the endogenous proteasome when the cultures expressing Rpt1-GFP were immunostained with GFP and endogenous proteasome antibodies, suggesting that most of the Rpt1-GFP is within the proteasome structure (Fig. 6.4b). Under basal (non- stimulated) conditions, Rpt1-GFP was diffusely distributed in both dendrites and the spines (Fig. 6.2a). Upon brief depolarization, (KCl 60 mM, 1. 5 min), Rpt1-GFP moved from the dendritic shaft into spines within minutes (Fig. 6.1a-c). Although there was no significant change in the total amount of Rpt1-GFP fluorescence after KCl stimulation, there was, on average, an ~90 % increase in spine

Rpt1-GFP signal (20 min post KCl) (Fig. 6.1c) with individual experiments ranging from no change to an ~350 % increase in spine signal (Fig. 6.1d). The increase in spine proteasome signal was persistent, lasting, on average, for at least an hour following stimulation. The overwhelming majority of the Rpt1-occupied spines exhibited overlap with a presynaptic marker protein, bassoon (Fig. 6.4a), strongly suggesting that these spines are the sites of functional synapses (95.6 ± 1.2 % of the bassoon puncta colocalized with the spines that Rpt1-GFP had entered; 97.8 ± 1.4 % of the Rpt1-GFP occupied spines had bassoon puncta associated with them, n=10 cells).

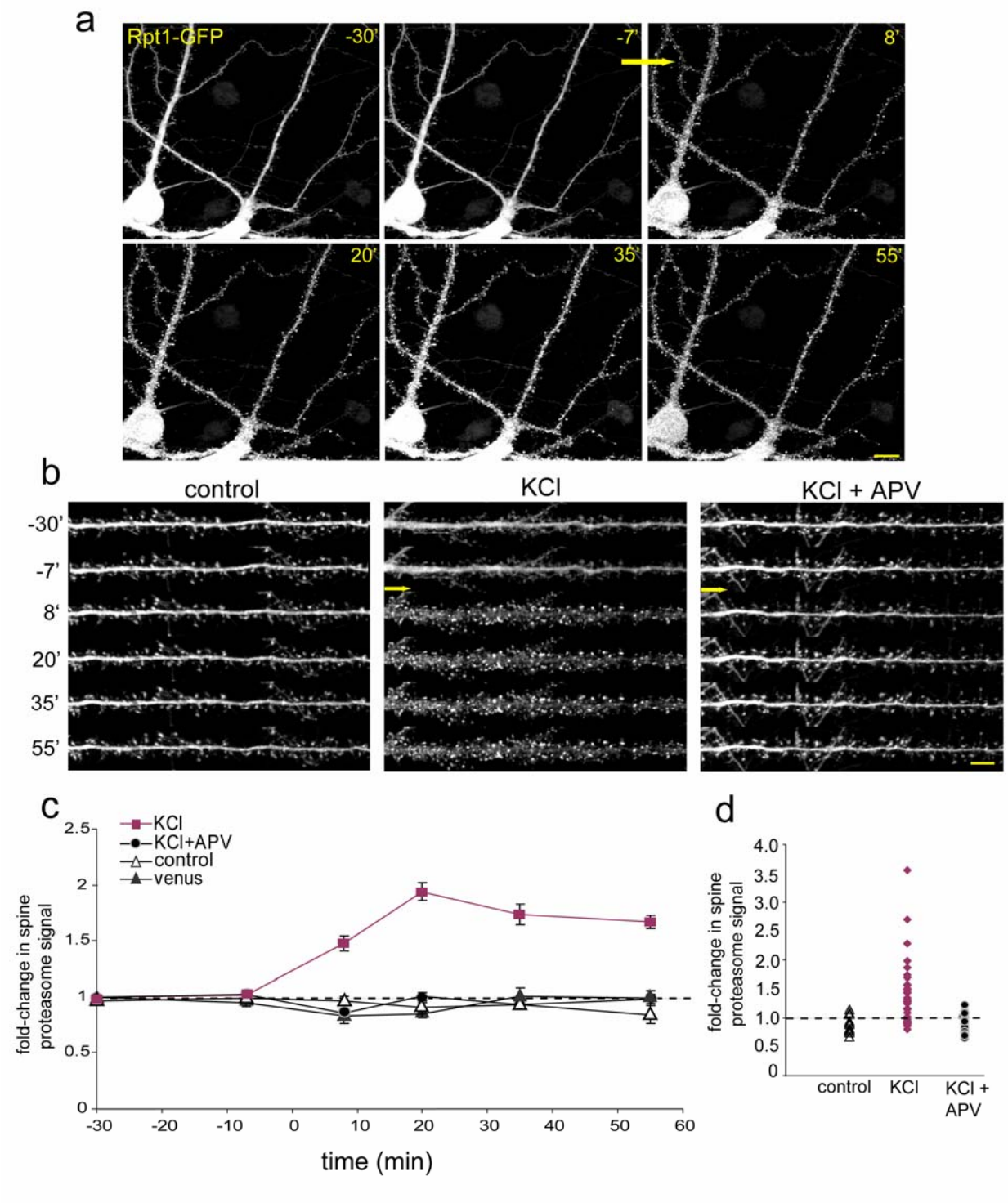
a
Rpt1-GFP
-30'
-7'
8'
20'
35'
55'
b
control
KCl
KCl + APV
-30'
-7'
8'
20'
35'
55'
c
KCl
KCl+APV
control
venus
fold-change in spine proteasome signal
time (min)
d
fold-change in spine proteasome signal
control
KCl
KCl + APV

Figure 6.1. A GFP-labeled subunit of the proteasome, Rpt1-GFP, moves into spines upon depolarization of cultured neurons. *a,* Time-lapse imaging of Rpt1-GFP-labeled neurons. Arrow indicates a brief high KCl stimulation resulting in the rapid accumulation of the proteasome in spines. Scale bar = 11 μm. *b,* Higher resolution time-lapse images of control, KCl or KCl + APV treated dendrites. KCl stimulation occurred at the time indicated by the arrow. Scale bar = 6 μm. *c,* Summary data for all experimental groups. KCl stimulation did not alter the distribution of venus alone. In order to quantify the spine fluorescent signal, all the images in the time series were summed and all the "protrusions" away from the dendritic surface were selected as spines on the summed image. Then, the mean proteasome intensity of the individual spines was quantified for each cell. (From top to bottom, n = 37, 37, 21, 11, and 21 cells for the groups shown from seven independent experiments, KCl group significantly different from all others, at t = 20' and thereafter, p< 0. 01 by ANOVA, error bars denote s.e.m.). *d,* The change in spine signal at 35 min post stimulation for all individual experiments.

The depolarization-induced redistribution of the proteasome does not reflect a bulk movement of proteins into spines as KCl had no effect on the spine fluorescence intensity in neurons expressing either GFP alone (data not shown), venus alone, (Fig. 6.2b, c) or an actin-GFP fusion (Fig. 6.2d, e). The increase in spine proteasome signal can also not be explained by a KCl-induced increase in the number or area of spines (Fig. 6.2b-g). On the other hand, the depolarization-induced redistribution of the proteasome into spines was also observed with a fluorescently-tagged 20S proteasome subunit, α4 (Psma7, C6-I), (Fig. 6.2f, g), indicating that both 19S and 20S subunits undergo activity-dependent trafficking.

The experiments described above suggest that the increase in spine proteasome amount following stimulation is not due to a change in the spine number or morphology. However, these experiments rely on the comparison of the effects of stimulation on different cells; one expressing Rpt1-GFP and others expressing GFP or actin-GFP. In

order to demonstrate that stimulation does not cause spine morphology changes in the same cells that are expressing Rpt1-GFP, we expressed mRFP along with Rpt1-GFP in the same neurons using a Sindbis virus (Rpt1-GFP-IRES-mRFP). Although there was no significant change in the spine number or the spine mRFP signal intensity following KCl stimulation, there was still a ~90% increase in spine Rpt1-GFP signal in the same neurons (20 min post KCl) (Fig. 6.3).

Depolarization with KCl leads to the activation of many voltage-gated ion channels and the release of neurotransmitter. Activation of the NMDA-type glutamate receptor is critical for the initiation of many forms of synaptic plasticity in the CNS (Isaac, 2003). To test if NMDAR activity is required for the proteasome trafficking into spines, we pretreated the neurons with the NMDA receptor antagonist, APV, before and after stimulation. We found that APV blocked the KCl-induced movement of both Rpt1-GFP (Fig. 6.1c,d) and α4-venus (Fig. 6.2g), indicating that NMDAR activity is required for the proteasome to concentrate in spines. In addition, we have observed that direct stimulation with NMDA (20 μM, 3 min) is sufficient to drive the redistribution of Rpt1-GFP into spines (mean fold increase in spine proteasome signal compared to control: 35.6 ± 2.8 %, n = 10 cells for each group, $p<0.05$). Taken together, these data suggest that activation of NMDA receptors specifically recruits the proteasome from dendritic shafts into spines.

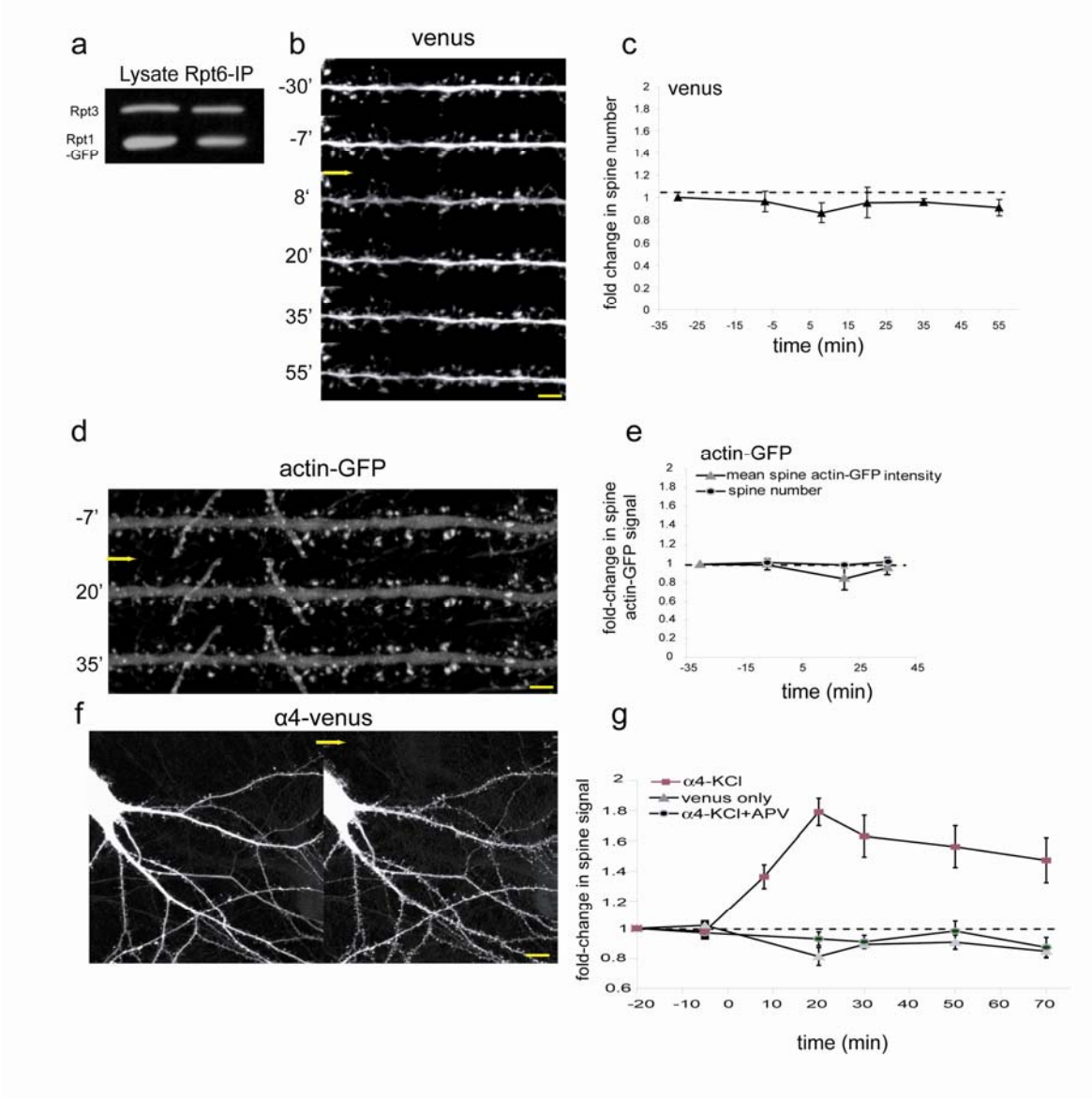

Figure 6.2. Spine morphology is not affected by the neuronal stimulation. *a*, Rpt1-GFP is incorporated in to the proteasome with similar efficiency as endogenous proteasome subunit. Endogenous proteasome from cultures expressing Rpt1-GFP is immunoprecipitated (with anti-Rpt6 antibody) and blotted for another endogenous subunit, Rpt3, or Rpt1-GFP with a GFP antibody. If all the Rpt1-GFP and Rpt3 are in the same proteasome structure, they should be immunoprecipitated with the same efficiency. Comparison of the lysate:IP ratio on the western blot indicates that the incorporation efficiency of Rpt1-GFP is 77+/-3. 4% (from two independent experiments) of the endogenous proteasome subunit Rpt3. (lysate:IP ratio is 1:1.) *b*, Spine number does not change with KCl stimulation (arrow). Scale bar = 4. 5μm. *c*, Analysis of spine number before and after KCl stimulation (n = 20 cells from 3 independent experiments). *d*, Actin-GFP does not shown redistribution in spines with KCl stimulation (arrow). Scale bar = 4 μm *e*, Analysis of actin-GFP signal before and after KCl stimulation for the mean spine intensity and spine number (n = 10 cells from 3 independent experiments). *f,* α4-

venus moves into spines with KCl stimulation. Scale bar = 6 μm. *g*, Group data of α4-venus experiments. APV blocked the KCl-induced trafficking of α4 into spines and spine area does not change with KCl stimulation (n = 21 for each group from four independent experiments, p<0. 05 by using ANOVA at t=20' and thereafter)

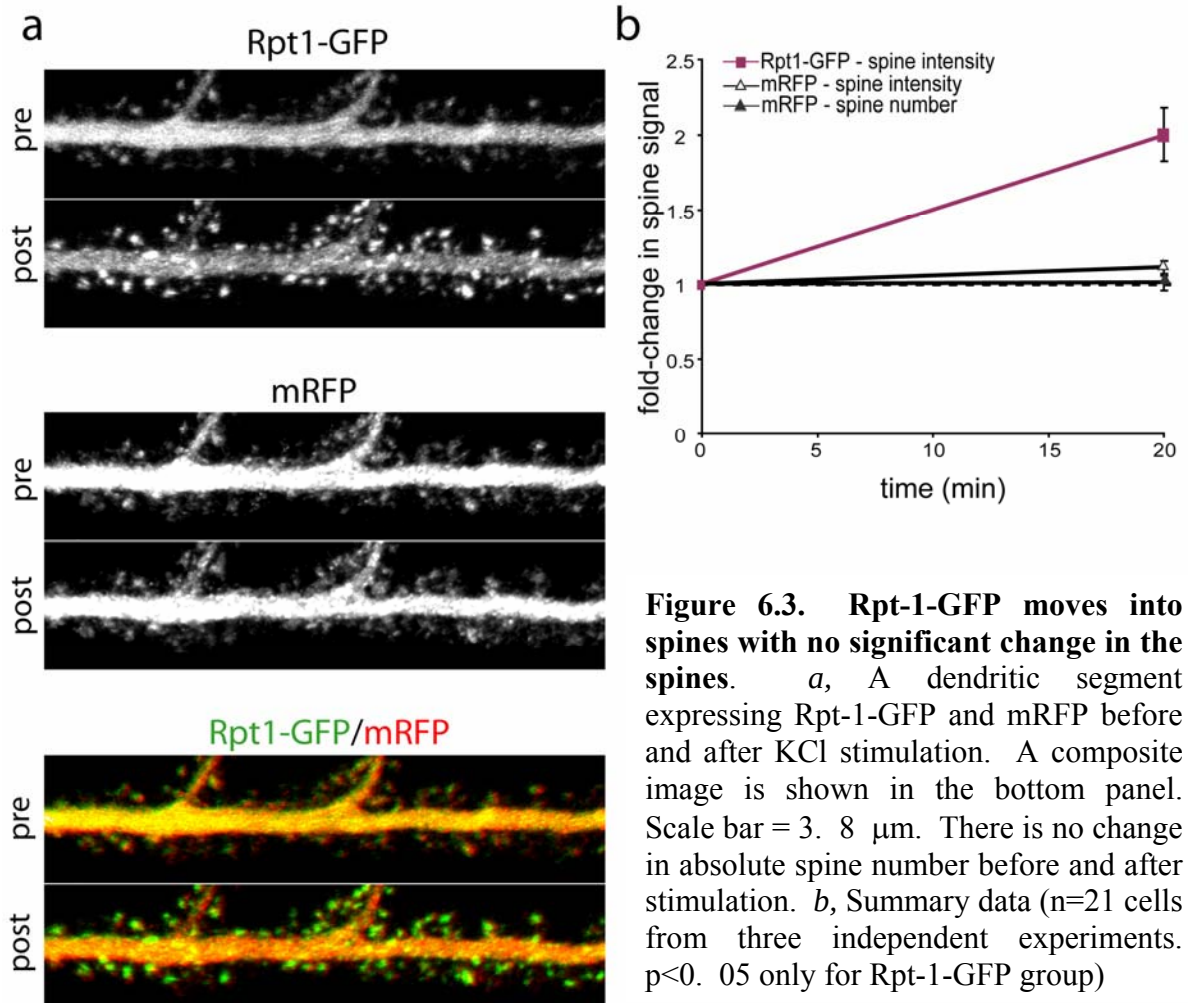

Figure 6.3. Rpt-1-GFP moves into spines with no significant change in the spines. *a*, A dendritic segment expressing Rpt-1-GFP and mRFP before and after KCl stimulation. A composite image is shown in the bottom panel. Scale bar = 3. 8 μm. There is no change in absolute spine number before and after stimulation. *b*, Summary data (n=21 cells from three independent experiments. p<0. 05 only for Rpt-1-GFP group)

Activity-dependent redistribution of the endogenous proteasome

The data described above indicate that synaptic stimulation can promote the movement of both 19S and 20S GFP-tagged proteasome subunits into synaptic areas. We next examined whether the endogenous proteasome shows a similar redistribution. First, we determined the relationship between endogenous proteasome subunits and the Rpt1-GFP signal following stimulation, using retrospective immunostaining. We found that the majority of Rpt1-GFP-inhabited spines were also positively labeled for the core (20S) endogenous proteasome subunits (Fig. 6.4b) as well as actinin (data not shown) (89.7 ± 1.2 of the Rpt1-GFP signal colocalized with the endogenous proteasome and 94.8 ± 1.5 of the endogenous proteasome signal colocalized with Rpt1-GFP signal. 95.6 ± 1.3 % of the actinin puncta colocalized with the spines that Rpt1-GFP had entered and 97.1 ± 1.7 % of the Rpt1-GFP-occupied spines have actinin puncta). We next directly examined whether the endogenous proteasome exhibits a similar redistribution during synaptic activity by comparing its localization in control and stimulated neurons. We conducted fluorescence immunocytochemical labeling using two different proteasome antibodies (Rpt3 or core subunits (Li and Wang, 2002; Verma, 2005) and a presynaptic marker, bassoon (tom Dieck et al., 2005). Stimulation (KCl, 60 mM, 1.5 min) caused a significant increase in the mean intensity of *synaptic* proteasome particles without changing their area or number (data not shown). The total intensity of the proteasome signal, including all shaft and spines, did not change. These data indicate that endogenous proteasomes are also recruited to synaptic sites. The increase in the synaptic

proteasome signal intensity suggests that proteasomes increase their occupancy at synapses that already possess one or more proteasomes (Fig. 6.4c, d).

To address whether a similar movement of the proteasome occurs in native hippocampal circuits, hippocampal slices were stimulated with KCl (60 mM, 6 min) and then synaptosomal lysates were prepared and analyzed by immunoblotting for proteasome subunits. KCl stimulation increased the amount of proteasome detected in stimulated synaptosomes when compared to synaptosomes prepared from unstimulated control slices whereas other synaptic proteins showed no change (Fig. 6.4e, f). These data demonstrate that the endogenous proteasome moves into synapses during stimulation in both cultured hippocampal neurons and hippocampal slices.

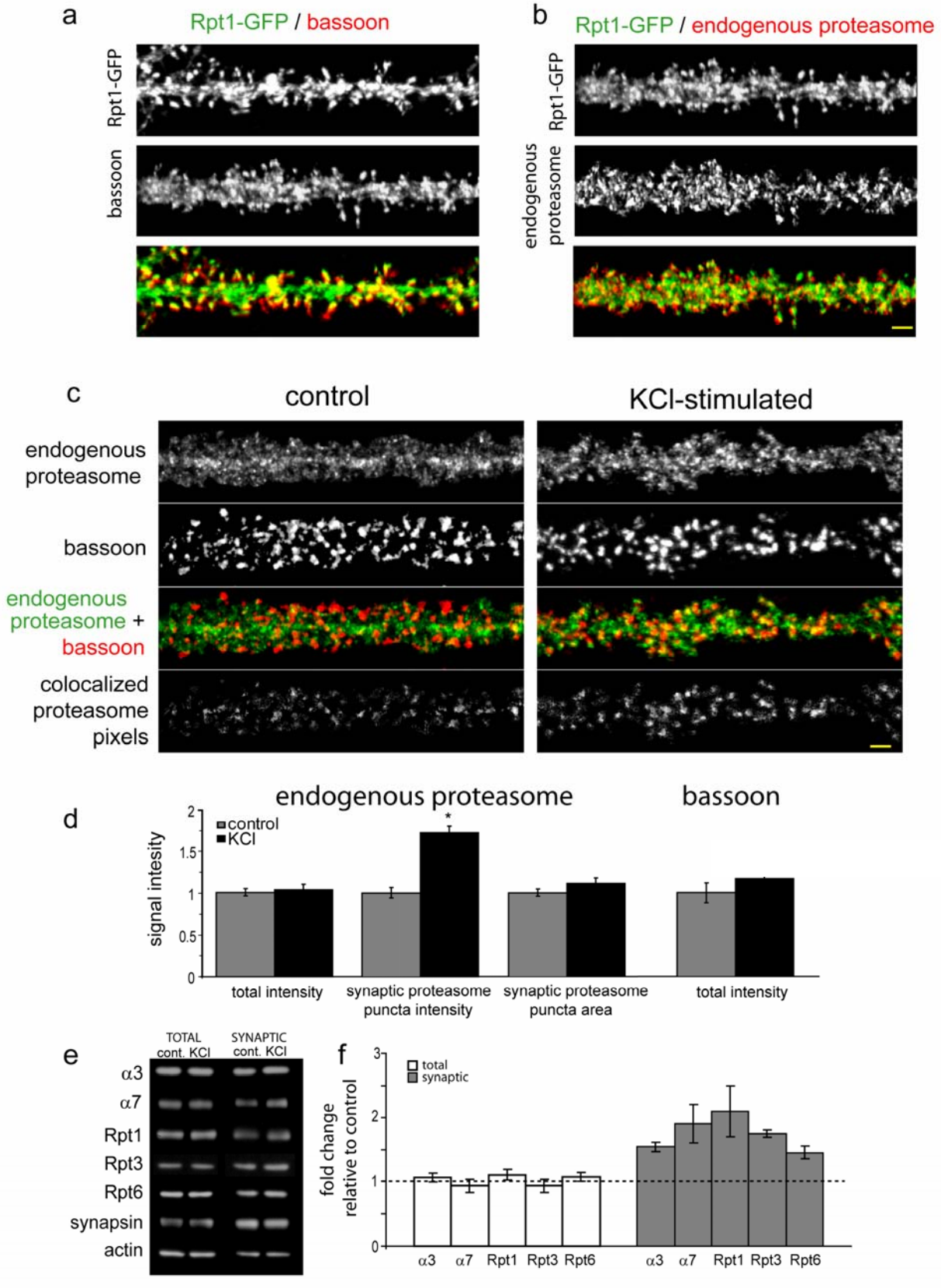
a
Rpt1-GFP / bassoon
Rpt1-GFP
bassoon
b
Rpt1-GFP / endogenous proteasome
Rpt1-GFP
endogenous proteasome
c
control
KCl-stimulated
endogenous proteasome
bassoon
endogenous proteasome + bassoon
colocalized proteasome pixels
d
endogenous proteasome
bassoon
control
KCl
signal intesity
2
1.5
1
0.5
0
*
total intensity
synaptic proteasome puncta intensity
synaptic proteasome puncta area
total intensity
e
TOTAL cont. KCl
SYNAPTIC cont. KCl
α3
α7
Rpt1
Rpt3
Rpt6
synapsin
actin
f
total
synaptic
fold change relative to control
3
2
1
0
α3
α7
Rpt1
Rpt3
Rpt6
α3
α7
Rpt1
Rpt3
Rpt6

Figure 6.4. Endogenous proteasome moves into spines with stimulation. *a and b,* Rpt1-GFP-inhabited spines are associated with presynaptic terminals and endogenous proteasome subunits. Representative images from a time-lapse experiment after KCl stimulation. Retrospective immunostaining for the presynaptic marker bassoon (*a*) indicates that the majority of spines are contacted by presynaptic terminals. Further retrospective immunostaining indicates that Rpt1-GFP puncta are also immunopositive for endogenous core subunits of the proteasome as well as another subunit Rpt3 (data not shown) (b). Scale bar = 2. 7 μm. *c,* KCl stimulation also alters the amount of endogenous proteasome observed at synapses, 10 min after stimulation. Scale bar = 3 μm. *d,* Analysis for endogenous proteasome. (control: n = 17 cells, KCl: n = 24 cells from three independent experiments, $p < 0.05$ for the mean particle intensity). *e,* Enrichment of the endogenous proteasome can be detected in synaptosomes prepared from KCl-stimulated slices. Western blot analysis of the total lysate and synaptic fractions for different proteasome subunits (α3, α7, Rpt1, Rpt3, Rpt6). Synapsin and actin were used as controls. *f,* Group data for the slice experiment shown in *e*. (n=3 independent experiments, $p < 0.05$ by using Student's t-test, error bars denote s.e.m.).

Activity-dependent dynamics of proteasome localization

Many proteins and multi-protein complexes exhibit a continuous shuttling between intracellular compartments or locales. The data presented here indicate that synaptic activity results in the enrichment of the proteasome in spines. In order to understand the dynamics underlying this recruitment, we monitored the dynamics of Rpt1-GFP signals using fluorescent recovery after photobleaching (FRAP). After acquiring baseline images, the Rpt1-GFP signal in individual spines was bleached and the recovery of fluorescence was monitored. Under basal conditions, individual spines exhibited ~ 50-65 % recovery of the spine Rpt1 signal following a single bleaching episode (Fig. 6.5a, c). In control experiments, we observed that a second epoch of bleaching led to an identical rate and level of recovery (Fig. 6.5a). To examine how synaptic stimulation alters dynamics of proteasome localization, we monitored FRAP in

the same individual spines before and after KCl stimulation. Prior to stimulation, spines showed about 65% recovery of fluorescence (Fig. 6.5b, c). After KCl stimulation, which resulted in an increase in the spine Rpt1-signal (Fig. 6.5b), there was a dramatic decrease in the recovery of fluorescence to ~10% (Fig. 6.5b, c). Control spines that were left unbleached showed minimal changes in their fluorescence during the time course of the FRAP experiment. Restating the above numbers, we thus conclude that the immobile fraction of the spine proteasome increases from 35% to 90% with stimulation. These data indicate that the proteasome is actively sequestered in spines upon synaptic stimulation.

In the FRAP experiments described above fluorescent molecules that contribute to the recovery must come from the dendritic shaft, adjacent to the spine. As such, the decreased recovery of the Rpt1 spine signal following stimulation could be due to a decrease in the shaft Rpt1 available for movement into the bleached spine. To assess the likelihood that this could account for the decreased spine FRAP, we deliberately decreased (by bleaching) the shaft signal to a reduced level comparable to that observed following stimulation, monitoring spine FRAP before and after this manipulation on the same spine (Fig. 6. 5d). We found that Rpt1-GFP recovery in the spine before and after the bleaching of the shaft signal was the same, indicating that the decreased recovery of the spine Rpt1-GFP can not be explained by a decrease in the shaft source. This idea is further substantiated by the FLIP results described below.

If stimulation causes the sequestration of the proteasome in the spines then the extent and the rate of the proteasome exit from the spine should also be reduced. In order to test this, we performed fluorescence loss in photobleaching (FLIP) experiments in which an area of the dendritic shaft is repeatedly bleached and the loss of the adjacent spine Rpt1 signal is monitored following each bleach episode, before and after stimulation. Spines that were on a dendritic branch in the same neuron (but not adjacent to a bleached shaft) were also monitored. Prior to stimulation, repeated bleaching of the shaft was associated with a dramatic loss of Rpt1 fluorescence in the spine, presumably owing to the movement of fluorescently-tagged proteasomes out of spines and into the shafts where subsequently undergo bleaching (Fig. 6.5e, f). Following stimulation, there was an increase in the Rpt1-GFP signal in spines that proved to be much more resistant to repeated bleaching of the shaft (Fig. 6.5e, f). Analysis of these data showed that stimulation caused an increase in the immobile fraction of Rpt1 in the spines from ~16% to ~90%. Taken together, the FRAP and FLIP experiments suggest that KCl stimulation causes the sequestration of the proteasome in dendritic spines.

The FRAP experiments reveal that the entry rate of the proteasome to the spines was increased ~1. 5 times with stimulation (FRAP: prestimulation τ: 2.9 ± 2.0 seconds, poststimulation τ: 2.0 ± 0.5 seconds). Conversely, the FLIP experiments reveal that the spine exit rate of the proteasome was dramatically decreased (FLIP: prestimulation τ: 38.3 ± 2.4 seconds, poststimulation: exponential fit not appropriate, data better fit as a straight line). These analyses suggest that the sequestration induced by stimulation is largely due to the decreased rate of proteasome exit from the spines, possibly due to

enhanced protein-protein interactions and to a much lesser extent due to the increased rate of the entry of the proteasome.

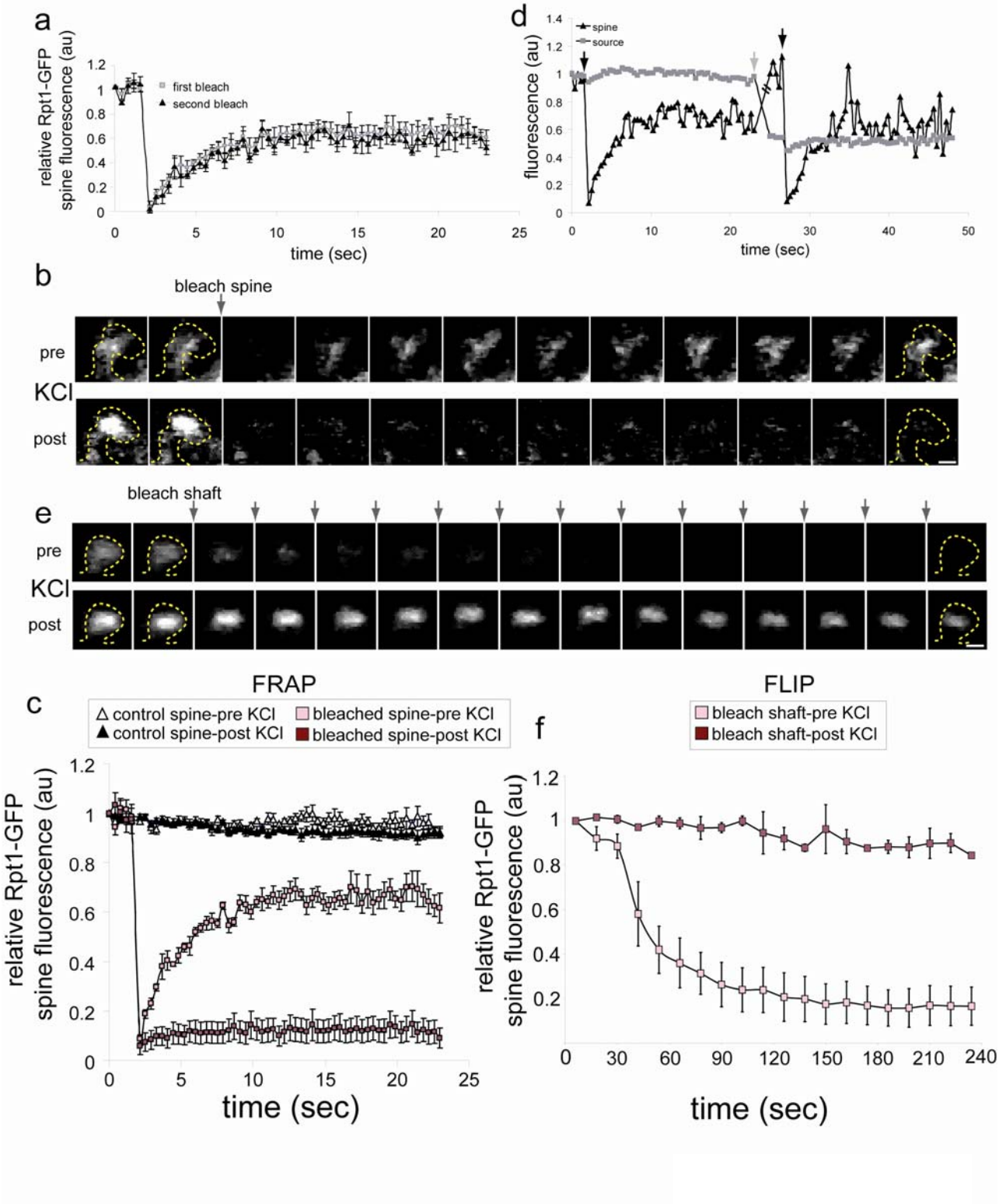

Fig 6.5. Photobleaching of Rpt1-GFP in spines or shafts indicates that the proteasome tightly associates with spines after stimulation. *a,* Analysis of control experiments showing that, in the absence of stimulation, spine fluorescence recovery after photobleaching (FRAP) is similar with repeated bleaching (n = 9 cells from four independent experiments). *b*, Representative FRAP experiment in which a single spine was bleached (arrow) before and after KCl stimulation. Recovery of fluorescence is significant prior to stimulation, but dramatically reduced after stimulation. Scale bar = 0.75 μm. *c*, Analysis of FRAP experiments. The fluorescence signal in each group is normalized to itself at t = 0. Non-bleached control spines did not exhibit significant changes in Rpt1 fluorescence during the experiment. Bleaching of spines was followed by significant recovery of fluorescence prior to stimulation ($\tau = 2.9 \pm 2.0$ sec). FRAP in the same spines was significantly reduced following stimulation, ($\tau = 2.0 \pm 0.5$ sec, n = 9 cells from four independent experiments, $p < 0.01$ by using Student's t-test, error bars denote s.e.m.) *d,* The reduced FRAP observed following stimulation is not due to a reduction in "source" fluorescence in the dendritic shaft. FRAP was monitored in a single spine. The dendritic shaft associated with the spine was bleached to reduce the shaft signal by 50%. A reassessment of FRAP in the spine showed an equivalent level of recovery. (The second round of FRAP is normalized to the spine signal just after bleaching of the shaft which is denoted by a dash). Similar results were obtained in two additional experiments. *e,* Representative FLIP experiment in which the Rpt1 fluorescence of a single spine is monitored after repeated bleaching (arrows) of the associated dendritic shaft. Before KCl stimulation repeated bleaching of the dendritic shaft resulted in dramatic losses of spine fluorescence, indicating the trafficking of Rpt1-GFP into the shaft where it became bleached. After KCl stimulation, (note enhanced Rpt1 signal in spine) there is much less loss of fluorescence in the spine following shaft bleaching, indicating that the Rpt1 signal in the spine is less likely to move. Scale bar = 0.75 μm. *f,* Analysis of FLIP experiments. The fluorescence signal in each group is normalized to itself at t = 0. Repeated bleaching of dendritic shaft lead to a dramatic loss of spine Rpt1 fluorescence prior to KCl stimulation ($\tau = 38.3 \pm 2.4$ sec). After stimulation, the diffusion of Rpt1 from the spine to the shaft was significantly reduced. (The plot is best fit as a straight line. n = 4 cells from three independent experiments, $p<0.01$).

Anchoring of the proteasome to actin cytoskeleton

What is the molecular mechanism by which the proteasome becomes physically concentrated in spines? One obvious mechanism for the sequestration of a protein or organelle in a cellular compartment is attachment, direct or indirect, to the cytoskeleton. In order to study the cytoskeletal association of the proteasome, we have performed detergent extraction experiments in conjunction with immunocytochemistry and Western blot analysis. Detergent extraction has been shown by others to report the cytoskeleton association of glutamate receptors and other postsynaptic proteins (Allison, 1998). Proteins that are resistant to detergent extraction are more tightly associated with the cytoskeleton. We exposed cultured hippocampal neurons to the non-ionic detergent TritonX-100 and then conducted retrospective immunolabeling using antibodies against proteasome subunits and other synaptic proteins. We observed that a large population of the proteasome is not detergent extractable: ~70 - 80% of the proteasome labeling remained after detergent extraction when compared to non-extracted cultures (Fig. 6.6a, b). For comparison, we considered the detergent-resistant fraction of a true cytoskeleton-binding protein, α-actinin, and the prominent synaptic proteins typically less tightly associated with the cytoskeleton, CamKII and synaptophysin (Allison, 1998). Remarkably, the proteasome subunits more closely resembled the bona fide cytoskeletally-associated protein α-actinin than the other synaptic proteins (Fig. 6.6a,b). The partial extractability of the proteasome was also confirmed by Western blot analysis using antibodies to two different proteasome subunits (Rpt1 and α7) (data not shown). In order to estimate the fraction of the actin cytoskeletally-associated proteasome in the Tx-

resistant population, we incubated the hippocampal neurons with actin-disrupting agent latrunculin A (latA) before detergent extraction (Allison, 1998). LatA treatment increased the detergent extractability of the proteasome similar to α-actinin (Fig. 6.6a,b) suggesting that the population of the proteasome that is targeted by detergent extraction is mostly associated with the actin-cytoskeleton. Based on these data, we estimate that ~50% of the proteasome in the dendrites is associated with the actin cytoskeleton. Furthermore, after treatment with latA *alone*, we observed that most of the proteasome puncta disappeared (Fig. 6.6a,b). LatA treatment affected α-actinin similarly, but did not affect CamKII or synaptophysin much (Fig. 6.6a,b). Taken together these data suggests that a substantial fraction (but not all) of the proteasome in the hippocampal neurons is actin cytoskeleton-associated.

In order to visualize the association of the proteasome with the actin cytoskeleton, we conducted double immunolabeling for the proteasome subunits and the actin cytoskeleton in control and detergent extracted cultures. Both Rpt3 and core subunits of the proteasome colocalized with actin at spines (Fig. 6.6c) (42.9 ±1.6% of Rpt3 and 39.5 ±1.0% of core puncta have an actin puncta associated with them. 67.0 ±1.9% and 58.2 ±1.5% of actin puncta have Rpt3 and core puncta associated with them, respectively. n=17 and 23 dendrites for Rpt3/actin and core/actin respectively). The extent of colocalization between the proteasome and the actin is consistent with the detergent extraction and latA experiments described above suggesting that some fraction of the proteasome in the dendrites is associated with the actin cytoskeleton.

In order to test if activity regulates the association of the proteasome with the actin cytoskeleton, we stimulated hippocampal cultures with NMDA (1 min, 20 μM NMDA followed by APV wash). Following stimulation, the cultures were detergent extracted and stained for the proteasome subunits and actin cytoskeleton in order to analyze the actin-associated proteasome population. NMDA stimulation significantly increased the amount of actin-associated proteasome subunits (Fig. 6.6d,e) when compared to control cultures. In order to confirm this result, we immunoblotted the detergent-resistant-proteasome population from stimulated neurons using anti-proteasome antibodies. Stimulation caused a significant increase in the amount of proteasome that is not extracted, (i.e., cytoskeleton-associated), when compared to the unstimulated controls (Fig. 6.6f, g). These data thus show that NMDA stimulation leads to an increase in the proteasome association with the cytoskeleton, suggesting a plausible mechanism for the sequestration of the proteasome in spines with stimulation.

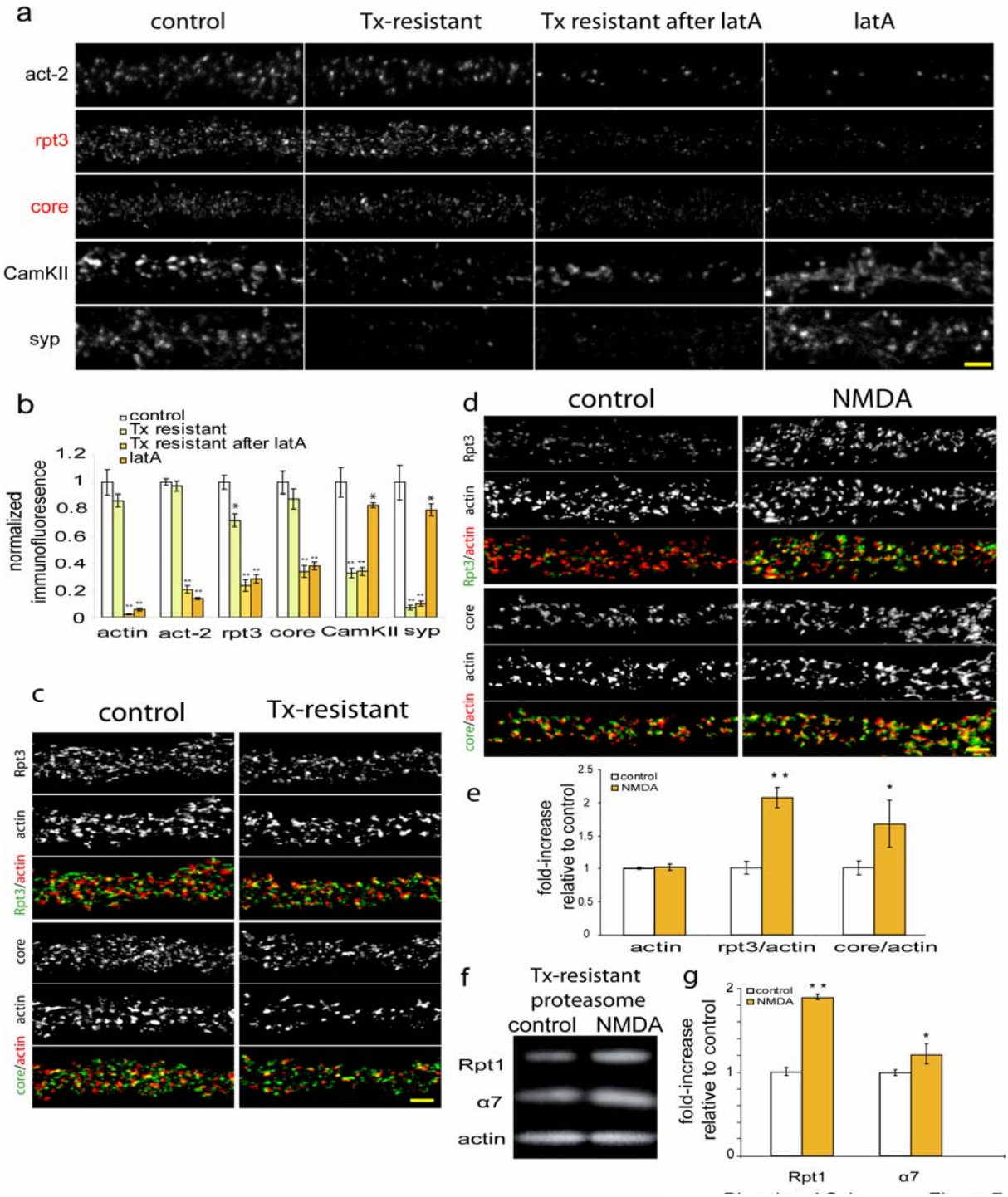

Figure 6.6. The proteasome associates with the actin cytoskeleton in hippocampal neurons. *a,* Immunostaining for the proteasome subunits (Rpt3 and core), α-actinin-2, CamKII, and synaptophysin in control neurons and neurons exposed to TritonX-100 or latrunculin A alone or sequential treatment with latrunculin A followed by TritonX-100.

Scale bar = 4 μm. *b,* The group data for experiments shown in *a*, syp: synaptophysin. (n = 25 cells for each group from three independent experiments, p< 0.05 is denoted by (*) and p < 0. 01 is denoted by (**), error bars denote s.e.m. *c,* Some portion of the proteasome colocalizes with the actin cytoskeleton in control and Tx-extracted neurons. Scale bar = 3 μm. *d,* NMDA stimulation increases the actin bound Rpt3 and core proteasome subunits. Only the proteasome puncta that are associated with an actin puncta are shown. Scale bar = 4 μm. *e,* Quantification of the data shown in d. All the proteasome colocalization values are normalized to the actin staining within each dendrite (n=40 from three independent experiments, p<0. 01 for Rpt3 and p<0.05 for core subunits by using Student's t-test, error bars denote s.e.m.). *g,* NMDA stimulation increases the pool of proteasome that is associated with the actin cytoskeleton. (n=4 independent experiments, p < 0.01 for Rpt1 and p< 0.05 for α7 by using Student's t-test, error bars denote s.e.m.).

The temperature at which the detergent extractions were performed (4°C) suggests that the microtubule-based cytoskeleton is not involved in the proteasome sequestration, as microtubules depolymerize at cold temperatures. To test the role of the microtubule-based network directly, we examined whether depolymerization of the microtubules by vincristine altered the distribution of the endogenous proteasome. After vincristine treatment, individual microtubules disappeared and tubulin paracrystals form throughout the dendrites, which is characteristic of vincristine treatment (Allison et al., 1998; Wolburg and Kurz-Isler, 1978) (Fig. 6.7 a, b) Although vincristine clearly altered the pattern of tubulin staining, it had little effect on the pattern of the proteasome signal (Fig. 6.7 a, c). However, another neuronal protein, GRIP1, which is known to associate with microtubules in neurons (Setou, 2002), clustered with the tubulin paracrystals formed after vincristine treatment (Fig. 6.7 b, c).

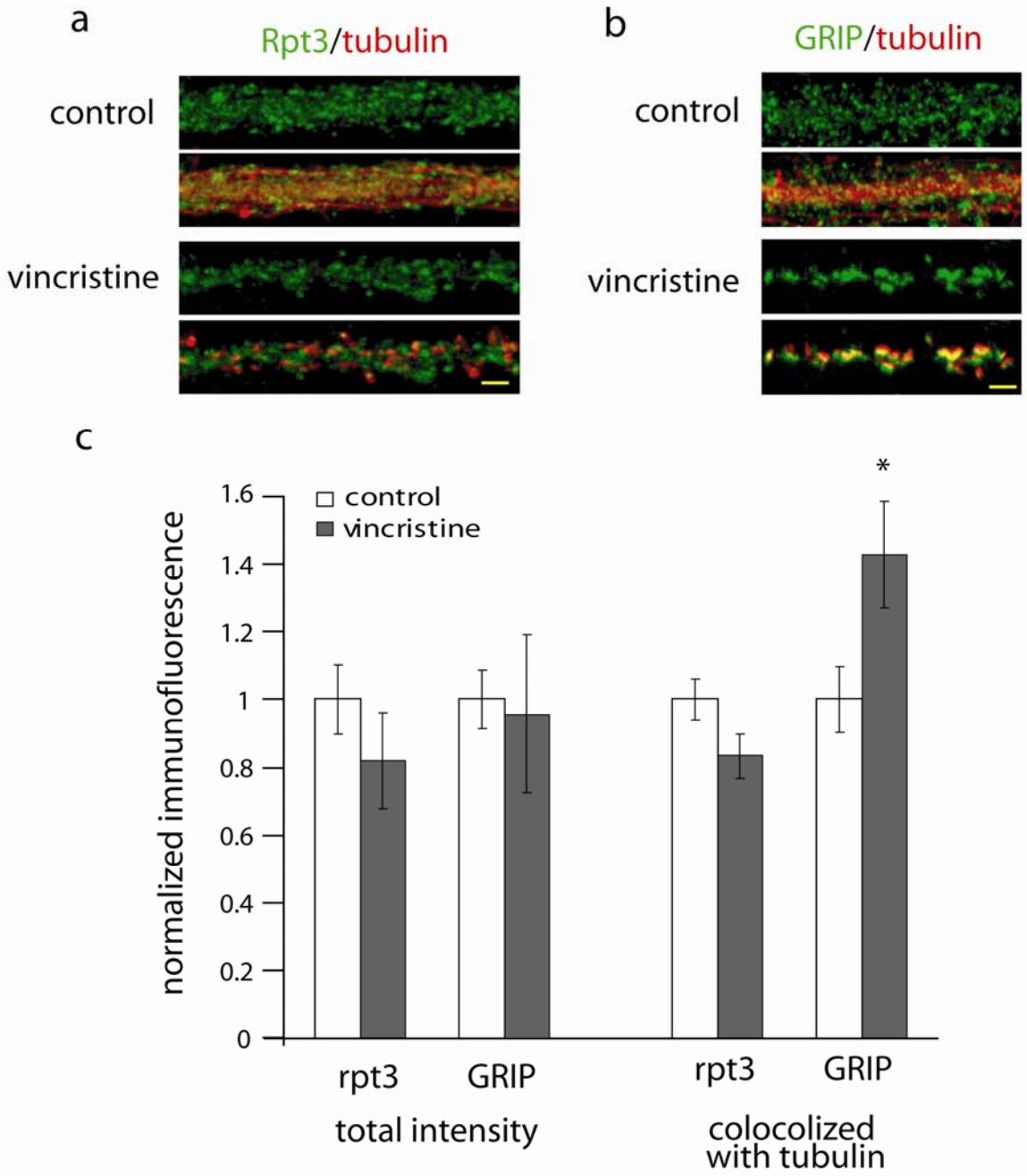

Figure 6.7 Microtubule depolymerization has no significant effect on proteasome distribution. *a*, Microtubule depolymerization with vincristine has no significant effect on proteasome distribution. Scale bar = 5 μm. *b*, GRIP distribution changes up on tubulin paracrystal formation by the vincristine treatment. Scale bar = 4.6 μm. *c*, Group analysis of the vincristine experiment shown in a and b. (n = 18 cells for each group from three independent experiments, $p<0.05$ for the increase in the colocalization between GRIP and tubulin after vincristine treatment).

Chapter VII

DISCUSSION AND FUTURE DIRECTIONS

The ubiquitin-dependent degradation of proteins is important for many different cellular processes, including cell cycle progression, signal transduction, transcriptional regulation, receptor downregulation, and endocytosis (Hershko and Ciechanover, 1998). In recent years it has become clear that the ubiquitin system is also utilized to control neuronal and synaptic function. The UPS regulates diverse synaptic events, including neurotransmitter release, synaptic vesicle recycling in presynaptic terminals and the dynamic behavior of the postsynaptic density and dendritic spines. The work described here contributed to the growing knowledge of synaptic UPS biology by demonstrating that the neuronal activity can regulate the UPS machinery function and localization at the synapse. In addition, I demonstrated that the UPS is an important regulator of glutamate receptor trafficking. Since glutamate receptors are responsible for the vast majority of neurotransmission in the brain, these data have important implications for brain function and plasticity.

Local protein degradation

In Chapter 3 of my thesis work, I have presented data that demonstrate that ubiquitin and the proteasome are abundant in dendrites, often present in or near synapses. These data suggest that the ubiquitination of proteins at synapses could be followed by their degradation via a local proteasome. I have demonstrated the capacity of neurons to perform local protein degradation by locally stimulating the dendrites of hippocampal

neurons and observing a local decrease of a fluorescent proteasome reporter. The capacity to degrade proteins locally near synapses were also suggested by others and my glutamate receptor trafficking experiments: Recently, Ehlers demonstrated ATP-dependent ubiquitin conjugation in both synaptosomal and postsynaptic density preparations (Ehlers, 2003), indicating that ubiquitination can occur in or near synapses. In addition, the components of the proteasome pathway have also been localized to retinal growth cones (Campbell and Holt, 2001) and Drosophila presynaptic terminals where local degradation of a presynaptic protein has recently been observed in Drosophila nerve terminals (Speese et al., 2003). The observation that AMPARs require proteasome activity in order to be internalized suggests that the proteasomal target protein(s) that regulates GluR internalization is degraded in the synaptic compartment, since degradation must precede the internalization of the receptor (Patrick et al., 2003).

Furthermore, the activity-dependent sequestration of the proteasome that we have described in Chapter 6 suggests that the proteasome can actively and locally sculpt the protein composition of the synapse, providing on-site degradation rather than serving as something akin to a remote garbage disposal site.

Considerations about fluorescent proteasome reporter, UbG76V-GFP

UbG76V-GFP is a short-lived fluorescent degradation reporter that allows the in vivo quantification of the ubiquitin/proteasome-dependent proteolysis in mammalian cells (Dantuma et al., 2000). By expressing UbG76V-GFP in hippocampal culture

neurons, I showed synaptic activity causes degradation of this reporter. What is the mechanism of increased degradation of the reporter following stimulation? Does it directly involve in proteasome activation? Or does it suggest the ubiquitination machinery upstream of the proteasome "work" more efficiently after the synaptic activation? UbG76V-GFP is a well characterized reporter: the ubiquitination enzymes that lead to its degradation are well-defined. These enzymes are the components of the UFD (Ubiquitin-Fusion Degradation) pathway. UFD enzymes were first characterized in yeast and they include Ubc4p/5p E2, Ufd4p E3, and Ufd2p E4 (Johnson et al., 1995). Thus, the decrease in the UbG76V-GFP fluorescence with synaptic activity may reflect changes in the activity of any of these UFD pathway enzymes. Alternatively, the decrease in the UbG76V-GFP fluorescence may reflect the changes in the proteasome activity as all the ubiquitinated proteins are degraded via proteasome, irrespective of the ubiquitination machinery involved. There are no studies that demonstrate the presence of UFD pathway components near postsynaptic sites. However, the local decrease of UbG76V-GFP fluorescence after activity suggests that they must be present near synapses. The remote synthesis and ubiquitination of UbG76V-GFP in the cell body followed by diffusion to synaptic sites is unlikely given the short half-life of the reporter (Dantuma et al., 2000). Regardless of the machinery activated (UFD pathway or the proteasome), the local decrease in the reporter fluorescence following NMDAR stimulation is the first evidence demonstrating that dendrites have the capacity to regulate local degradation of proteins dynamically near synapses.

In addition to UbG76V-GFP, other UPS reporters can be utilized to dissect out the mechanisms how synaptic activity regulates the UPS machinery. For example, a recently developed UPS reporter, GFPu, relies on a 16 amino acid degradation signal (CL1) fused to GFP (Bence et al., 2001). The CL1 degradation signal destabilizes other reporters in a Ubc6- and Ubc7-dependent manner (Gilon et al., 1998). Thus, it is possible to study synaptic protein degradation with different reporters that are targets of different ubiquitination cascades.

Implications of faster degradation of UbG76V-GFP in spines compared to the shaft

To test whether UbG76V-GFP degradation occurs faster in spines, I compared reporter degradation in spines versus dendritic shaft just after stimulation. The initial loss of fluorescence occurred mainly in the spines, suggesting that the degradation machinery has to reside near synapses or, alternatively, be mobilized to the synapses following stimulation. The activity-dependent mobilization of the proteasome may be one mechanism how local degradation of the proteins is achieved at the spine level. Furthermore, as discussed below, mobilization of the ubiquitination machinery may be another way to achieve protein degradation specifically in the spines.

The diffusion of the UbG76V-GFP reporter makes it difficult to directly visualize spine-protein degradation: most spine degradation is precluded by the rapid diffusion of the reporter from the shaft to the spine. Future work would employ a similar strategy used in the fluorescent local synthesis reporter where GFP is fused to a myristoylation

signal (Aakulu et al., 2001). This allows membrane insertion of the reporter, which significantly reduces the fluorescent protein diffusion. Furthermore, the degradation reporter can be engineered to be specifically localized to the synapses. GFPu, the CL1 degradation based reporter, has been modified to be localized to or excluded from different cellular compartments, such as the nucleus (Bennett et al., 2005). In this case, the nuclear targeting/excluding signals are well-defined (Boulikas, 1993). There is no such "spine-localization signal" identified so far but one could utilize the protein domains that interact with the postsynaptic proteins or the actin cytoskeleton by fusing such domains to GFP in degradation reporters. Thus, it would be possible to target the degradation reporter specifically to the synapses, which would allow the dynamic visualization of synaptic protein degradation.

The extent of local protein degradation in the dendrites

Analogous to the problem of the extent of local protein synthesis, it is uncertain how local "local protein degradation" is. The system I used to monitor ubiquitin-dependent protein degradation employs a diffusible fluorescent proteasome degradation reporter. The diffusible character of the degradation reporter makes it hard to estimate the extent of local protein degradation: reporter diffusion from the adjacent non-perfused dendritic regions will decrease the "measured" size of the dendritic region where local degradation is observed. In another words, smaller dendritic regions will show local reporter degradation whereas in reality, the degradation takes place on a bigger stretch of the dendrite. As indicated earlier, a myristoylation signal fused to GFP would be used to

limit the diffusion of the degradation reporter, which was utilized in the local synthesis reporter described in chapter 2 (Aakulu et al., 2001).

In addition, the extent of the local protein degradation could be studied in a more physiological context by utilizing the mice that ubiquitously expresses UbG76V-GFP (Lindsten et al., 2003). The hippocampal slices prepared from these mice can be used to study local protein degradation during synaptic plasticity.

AMPAR endocytosis requires proteasome activity

The findings presented in Chapter 4 demonstrate that blocking polyubiquitination or proteasome activity prevents the agonist-induced internalization of AMPARs. These data suggest that the acute activation of GluRs leads to the regulation of the ubiquitin conjugation system and the degradation of a protein(s) required for the internalization of the receptors. In addition to demonstrating a role for UPS in one of the underlying mechanisms of synaptic plasticity, these experiments also showed that UPS acts fast at the synapse. Furthermore, demonstration of the reduction in the free-ubiquitin pool raised questions about assigning roles to the UPS function in experiments where proteasome inhibitors are used.

Acute versus long-term regulation of the proteasome

An important issue to consider is the time scale over which the ubiquitin-proteasome pathway may regulate synaptic function and plasticity. The work presented in Chapter 4 demonstrates an acute activation of the ubiquitin-proteasome system; pretreatment with proteasome inhibitors for 2.5 min was sufficient to block AMPA-induced endocytosis of GluRs. In addition, others have shown that proteasome inhibitors can abolish the netrin-induced turning of growth cones in the time frame of one hour (Campbell and Holt, 2001). A similar rapid action of the proteasome was recently reported at the Drosophila neuromuscular synapse (Speese et al., 2003) or Aplysia sensory-motor synapses (Zhao et al., 2003) where proteasome inhibitors enhanced basal synaptic transmission within an hour of their application. Taken together, our data and these other studies indicate that the ubiquitin-proteasome pathway can acutely regulate synaptic function on a time scale of minutes. Alternatively, Ehlers reported changes in synaptic protein levels that result from global activity changes for 24–48 hr; some of these changes were -sensitive to proteasome inhibitors (Ehlers, 2003). It remains to be determined whether these observations represent a fundamentally different type of regulation by the proteasome, which occurs on a longer time scale, or alternatively, represents the cumulative effects of proteasomal regulation that occurs on shorter time scales, e.g., minutes, as demonstrated in Chapter 4.

Different roles for ubiquitin in modulating GluRs: Mono versus polyubiquitination

The attachment of a ubiquitin molecule to a protein can serve as a signal for endocytosis, whereas the formation of polyubiquitin chains can serve as a recognition signal for proteasomal degradation (Hicke, 1997). Proteasome inhibitors cannot unequivocally establish a role for degradation because prolonged proteasome inhibition can lead to a depletion of the free ubiquitin pool. Indeed, in Chapter 4, we observed inhibitor treatments as short as 20 or 40 min significantly reduced the free ubiquitin pool in hippocampal neurons. These data indicate that effects observed following prolonged treatments with proteasome inhibitors are not sufficient to establish a requirement for proteasome activity. Thus, while assigning a proteasome-dependent role for a synaptic function, it must be well established that the effect seen with proteasome inhibitors is not due to proteasome-independent functions of the monoubiquitination (Hicke, 2001). In our experiments, we found that pretreatment with a proteasome inhibitor for as little as 5 min was sufficient to block AMPA-induced GluR endocytosis but had no effect on the free ubiquitin pool. Moreover, expression of a mutant ubiquitin molecule, K48R, which supports mono but not polyubiquitination, also prevented AMPA-induced GluR endocytosis. In this respect, our data are similar to what has been described for several other ligand-receptor systems in which the polyubiquitination and degradation of proteins are required for the ligand-induced internalization of the receptor (van Kerkhof et al., 2000; Chaturvedi et al., 2001; Yu et al., 2001). In contrast, using genetic manipulations, Burbea et al. favor the idea that ubiquitination of the C. elegans GLR-1 serves as an

endocytosis signal (Burbea et al., 2002). This would suggest that the mode of AMPAR trafficking regulation by UPS may be different between different organisms.

The targets of the UPS at the synapse

In Chapter 4, we provide evidence that activation of GluRs leads to the regulation of the ubiquitin-proteasome system and the degradation of a protein(s) required for the internalization of the receptors. In principle, the target for proteolysis in this process could either be the glutamate receptor itself or a protein(s) that normally prevents the internalization of the receptor. The recognition of internalized GluRs by antibodies as well as the detection of recycled receptors on the cell surface (Lin et al., 2000; Liang and Huganir, 2001; Ehlers, 2000) are not consistent with the idea that the receptor itself is the target. It is more likely that a protein (or proteins) that interacts with the receptors, directly or indirectly, is the proteasomal target. The idea that the abundance of AMPARs at the synapse is regulated by putative "slot" proteins suggests that these "slot" proteins may be the targets of UPS at the synapse during regulation of AMPAR trafficking.

In order to establish that a protein is a UPS target whose degradation is required for AMPAR endocytosis, it must be demonstrated that the target protein is ubiquitinated and its ubiquitination is regulated by activity. Furthermore, neuronal activity must degrade the target protein in a proteasome-dependent manner. Finally, blocking the target protein degradation, either by mutating the ubiquitin acceptor lysines or the degradation motifs of the protein, must block AMPAR endocytosis.

By testing whether or not the known synaptic proteins at the synapse follow the criteria summarized above, we demonstrated that there is a proteasome-dependent downregulation of PSD-95 levels in response to neuronal stimulation. In these experiments, we observed a negative correlation between AMPA-stimulated internalized GluR1 and PSD-95 staining. We also demonstrated a proteasome-dependent AMPA-stimulated decrease in the number and intensity of the PSD-95 puncta. By itself, the observed inverse correlation between PSD-95 and AMPAR does not indicate whether PSD-95 degradation is up or downstream of GluR internalization. The blockade of GluR internalization by PSD-95 overexpression, however, suggests that degradation of PSD-95 is upstream of internalization. These data point to the possibility that PSD-95 may be a target of UPS. If PSD-95 is a direct target of UPS, then it should be polyubiquitinated. Using standard techniques to detect the polyubiquitination of a protein, we were unable to observe a polyubiquitinated PSD-95 species from lysates prepared from either cultured hippocampal neurons or hippocampal slices. It is possible that PSD-95 requires activity to be ubiquitinated as shown by Colledge et al. (2003), but we were not able to detect polyubiquitinated PSD-95 from cultures stimulated with NMDA (the same protocols that Colledge et al. used) or AMPA. It is possible that our failure to observe activity-stimulated PSD-95 ubiquitination reflects differences in culture conditions, or the transient nature of PSD-95 ubiquitination. If the ubiquitination of PSD-95 is transient, however, treatment with a proteasome inhibitor might be expected to stabilize the ubiquitinated population. This was not observed in our experiments. Taken together our data in Chapter 5 suggest that PSD-95 may be an indirect target of UPS regulation. For example, Ehlers (2003) observed other postsynaptic density proteins that are not

ubiquitinated but whose levels can be regulated by activity in a proteasome-dependent manner. In support of this, Pak et al. (2003) have observed the indirect loss of PSD-95 through direct degradation of SPAR by the proteasome. Identifying the bona fide targets of the proteasome and the mechanisms by which these targets regulate proteins like PSD-95 will be the focus of future experiments.

Proteasome dynamics at the synapse

In Chapter 6, we have demonstrated the dynamic redistribution of the proteasome into spines with synaptic activity. The physical concentration of the proteasome in spines is achieved primarily by a decreased rate of proteasomal exit suggesting an active sequestration mechanism. We have demonstrated that the sequestration is mediated by an activity-induced increase in the association of the proteasome with the spine actin cytoskeleton. Taken together, these data indicate that synaptic activity can promote the physical recruitment and sequestration of the proteasome to locally sculpt the protein composition of synapses.

The visualization of the proteasome dynamics using photobleaching shows that in response to activity, spines recruit and compartmentalize the proteasome to the site of action by increasing the proteasome's association with the actin cytoskeleton. This sequestration involves an active "anchoring" of the proteasome within spines, rather than a simple increase in the diffusion rate into the spines. The anchoring of the proteasome

most likely involves the interactions between the actin cytoskeleton associated proteins and individual, as yet unidentified, proteasome subunits.

What is the mechanism of the initial trafficking of proteasomes to spines?

The initial trafficking of the proteasome to the spines may involve either simple diffusion or active transport of the proteasome particles with a motor protein. The main cytoskeleton in spines is the actin cytoskeleton so it is possible that the proteasome moves into spines with actin-based motor proteins. However, recent proteomics studies identified both actin- and microtubule-based motor proteins in the PSD (Walikonis et al., 2000; Jordan et al., 2004; Peng et al., 2004). Thus, we cannot rule out the involvement of the microtubule-based motors as well. Some of these motor proteins were identified in different protein complexes in the dendrites: myosinVI is in a complex with SAP97-GluR1; myosinV and dynein are in a complex with PSD-95 and GKAP (Wu et al., 2002; Naisbitt et al., 2000). Thus, these motor proteins function to deliver proteins to synapses and proteasomes may be one of their cargo proteins. In other systems, proteasomes were shown to interact with a number of motor proteins. For example, in C.elegans proteasome β-Subunit, Pbs-7, interacts with cytoplasmic dynein light chain, dli-1 (Li et al., 2004). The identification of the exact mechanism of proteasome spine targeting will be one of the future topics of proteasome biology at the synapse.

What is the mechanism of the sequestration of the proteasome at the synapse?

Following delivery, proteasomes are "captured" at the spines. This observation is based on the FRAP and FLIP experiments described in chapter 6: The synaptic accumulation of the proteasome following stimulation was mainly due to a decrease in the spine exit rate of the proteasome. The proteasome accumulation at the synapse lasted for two hours following stimulation. This also suggests that proteasomes are actively sequestered at the synapse following stimulation. Multiple models can explain the spine capture of the proteasome with synaptic activity:

a) Cytoskeleton binding of the proteasome: Proteasomes interact with different types of cytoskeleton (Arcangeletti et al., 1997; Galkin et al., 1998; De Conto et al., 1997; Arcangeletti et al., 2000). The main cytoskeletal component in spines is the actin filaments. In hippocampal cultures, detergent extraction has been used to assess the cytoskeletal association of proteins (Allison et al., 1998). Using the same techniques, I demonstrated that both 19S and 20S proteasomes behaved similar to a "true" actin binding protein, actinin-2: most of the dendritic proteasomes were resistant to detergent extractions. Furthermore, the detergent extraction resistant proteasomes were mostly actin cytoskeleton associated as pretreatment with the actin cytoskeleton disrupting drugs increased the detergent extractability of the proteasome. Importantly, NMDAR stimulation increased the actin-bound proteasomes in the spines.

How are proteasomes targeted to the actin cytoskeleton? The strong resemblance of actin-binding properties of the proteasome to actinin-2 suggests the proteasome may directly interact with the actin cytoskeleton. Another possibility is that the actin-binding proteins mediate the interaction between the actin cytoskeleton and the proteasome. Proteasome ATPase-4 subunit interacts directly with the actin in the fly (Giot et al., 2003). 20S proteasome α2 subunit interacts with actin-binding protein, vinculin (Davy et al., 2001). Furthermore, actin-binding protein actinin-1 interacts with the rpn-11 subunit of the 19S proteasome in C.elegans (Davyet al., 2001; Walhout et al., 2000). These interactions were identified in large scale proteomics or yeast-two-hybrid screens and they represent interactions in lower organisms. Thus, they must be validated in the hippocampal synapses. Among these interactions, actinin-proteasome interaction is a good candidate for further study as actinin is also present in PSD (Walikonis et al., 2000). Furthermore, vinculin was identified in NMDAR protein complexes isolated from the brain (Husi et al., 2000).

b) PDZ protein interactions: As introduced in Chapter 2, PDZ domain interactions are the most common theme of protein-protein interactions at the synapse. An exciting possibility is the regulated interaction between proteasome subunits and the scaffolding proteins of the synapse. A search for PDZ-based interaction domains on proteasome subunits reveals that p27 subunit of the 19S proteasome (PSMD9) has a PDZ domain. p27 was purified as a component of 19S proteasomes from bovine erythrocytes and it may be involved in the assembly of the 19S proteasome (Watanabe et al., 1998; DeMartino et al., 1997). However, p27 (NAS2) is non-essential in yeast (Watanabe et

al., 1998) and Nas2p does not co-purify with the yeast 19S proteasomes (Glickman et al., 1998). It is possible that p27 transiently interacts with the 19S proteasome. Interestingly, p27 is also identified as a regulator of insulin gene transcription via E2A family of transcription factors (Thomas et al., 1999). The possibility that the regulation of p27 interaction with the synaptic PDZ domain proteins mediates the spine attachment of the proteasome has to be tested in the hippocampus.

c) Proteasome interacting motifs and the polyubiquitin chain: Recently, several proteins were described that contain ubiquitin-like and ubiquitin-binding domains (Buchberger, 2002). Most of these proteins are linked to the UPS. Some of these proteins are ubiquitin-domain proteins (UDPs): they contain a ubiquitin-like domain (UBL) within their structure but they cannot be conjugated to other proteins. UBL domains endow the UDP proteins with the capacity to interact with the proteasome. Sequence alignment of UBLs from different proteins identified a potential proteasome interacting motif (PIM) (Upadhya and Hegde, 2003). However, the presence or absence of a PIM in a UDP does not always predict its ability to interact with the proteasome.

Some of the UBL containing proteins also contain ubiquitin-associated domains (UBA). UBAs interact with the polyubiquitin chain (Rao and Sastry, 2002; Funakoshi et al., 2002; Wilkinson et al., 2001). As discussed below, UBL/UBA containing proteins function as shuttles for ubiquitinated proteins to the proteasome via their interactions with both the polyubiquitin chain and the proteasomes (Hartmann-Petersen et al., 2003). Thus, the important question is whether the synaptic proteins contain such domains that

would allow them to interact with the proteasome. The proteasome, after reaching the synapses, may interact with one of these proteins, leading to its stabilization at the synapse. According to this idea, the synaptic proteins are acting as the "capture" molecules for the proteasome and they are not necessarily the targets of the proteasome itself. An alternative view is that the polyubiquitin chain of the synaptic proteasome substrates may act to recruit the proteasome (see later).

The possible mechanisms of the synaptic proteasome capture presented above are not mutually exclusive. Proteasomes may tether to the synapse through multiple contacts: while "persistently" attached to the actin cytoskeleton or to other synaptic proteins, they may be tethered to the polyubiquitinated proteins as well.

Proteasome targeting the substrates versus substrates targeting the proteasome

A stable interaction between the proteasome and the substrate is sufficient for substrate degradation by the proteasome (Janse et al., 2004). UPS utilizes different ways to target the substrates to the proteasomes. Initial identification of the Rpn10/Pus1 (S5a) subunit of the 19S proteasome as a polyubiquitin binding protein led to the model where "resident" subunits of the proteasome act as substrate receptors in UPS (Deveraux et al., 1994; Piotrowski et al., 1997). Identification of the polyubiquitin shuttle proteins containing both UBA and UBL domains (Rad23/Rhp23 and Dsk2/Dph1) demonstrated an alternative mode of substrate targeting where the shuttle proteins are not proteasome subunits but they transiently interact with the proteasome (Wilkinson et al., 2000;

Funakoshi et al., 2002; Rao and Sastry, 2002; Lambertson et al., 1999; Saeki et al., 2002). Similar to the role of Rad23/Rhp23 and Dsk2/Dph1 as substrate shuttle proteins, some E3s interact with the proteasome as well, suggesting that ubiquitination machinery also transfers the substrates to the proteasome (Hartmann-Petersen et al., 2003).

The substrate targeting mechanisms presented above suggest that the "mobile" components of the UPS substrate delivery machinery are the shuttle proteins and the substrate. However, some studies suggest that proteasome localization may also be regulated. This idea is based on the interactions of the proteasome subunits with different proteins at different subcellular compartments, such as the plasma membrane, ER membrane, centrosome, and the mitotic spindles (Ferrell et al., 2000). Furthermore, proteasome moves in and out of the nuclear region during the cell cycle (Hirsch and Ploegh, 2000). The movement of the proteasome into spines following synaptic activity is the first demonstration of the dynamic targeting of the proteasome toward its substrates. The dendritic spines gave us a unique opportunity to demonstrate the trafficking of the proteasome as spines are physically well-separated entities compared to other protein degradation sub-compartments in cells, such as the ER membrane.

Why do the neurons localize proteasomes into spines in response to synaptic activation? It is possible that it is more efficient for neurons to localize the proteasome to spines rather than shuttling the synaptic substrate proteins to the proteasome. PSD has a laminar organization where some proteins are buried inside the "core" of the PSD, whereas others remain peripheral (Valtschanoff and Weinberg, 2001). Extracting a few

proteins for targeting to proteasome without disrupting the protein-protein interactions that maintain the PSD integrity may be a difficult task. Thus, the proteasome, either by interacting with the cytoskeleton or other PSD components, may tether to PSD in order to degrade the substrate protein on site.

Finally, local proteasome localization may simply be utilized to regulate the local protein degradation during synaptic plasticity: only the activated synapses that need to degrade proteins in response to synaptic plasticity stimuli recruit the proteasome, thereby achieving local protein degradation. The establishment of a functional link between local protein degradation and local proteasome movement will be the scope of future research.

Other proteins/organelles translocating to the spines

In addition to the proteasome, a few scaffolding proteins, enzymes and organelles were shown to be localized to the synapses or the postsynaptic density following neuronal stimulation. Among the enzymes, CamKII localizes to the postsynaptic density within seconds following glutamate stimulation (Shen and Meyer, 1999). In contrast to the proteasome, stimulation causes the release of CamKII from the actin cytoskeleton followed by the capture at PSD. In PSD, NMDARs may act as the tether for CamKII as the NR2B subunit of NMDARs interacts with CamKII (Bayer et al., 2001). CamKII remains bound to PSD for a few minutes following the drop in Ca++ levels (Shen and Meyer, 1999). Thus, synaptic proteasome localization via activity is more persistent than the CamKII localization. This may reflect the multiple steps required for protein

degradation compared to the enzymatic steps of the phosphorylation of CamKII substrates.

Recently, mitochondria have also been shown to localize to spines following synaptic activity (Li et al., 2004). Localization of the mitochondria to spines requires stronger stimulations (spread over ~1 hour) compared to localization of the proteasomes (~ 2 min). Furthermore, the complete localization of the mitochondria to spines following stimulation takes approximately ~4.5 hours. Mitochondria move along microtubule and actin tracks in axons and the spine trafficking of mitochondria may involve the same motor proteins (Hollenbeck, 2005).

ProfilinII, a small actin-binding protein that regulates actin polymerization at the cell surface was also shown to undergo striking redistribution to spines following stimulation (Ackermann, 2003). Surprisingly, profilinII and proteasome have the same dynamics of distribution: profilinII moves into spines 5-8 minutes following glutamate stimulation and maximal accumulation occurs at 30 minutes. Furthermore, similar to the proteasome trafficking, the distribution was NMDAR-dependent. This raises the possibility that proteasome is escorted to the spines, and perhaps to the actin cytoskeleton, by binding to actin-binding proteins that actively translocate to spines following stimulation. No known interactions between profilinII and proteasome were identified in proteomics studies. Thus, possible involvement of profilinII targeting in proteasome trafficking to spines has to be tested in hippocampal neurons.

Finally, pointing out a role for the translocation of "protein availability machineries" during synaptic plasticity, ribosomes were shown to redistribute to spines following LTP (Ostroff et al., 2002). This was demonstrated by comparing the ribosome distribution (detected by electron microscopy) between slices subjected to either control or tetanic stimulations. LTP increased the percent of ribosomes harboring spines from 12% to %40, measured at 2 hours following LTP induction. It is hard to compare the ribosome dynamics with the proteasome dynamics as the preparations (culture vs. slice) and stimulations (depolarization vs. tetanic stimulation) are quite different between the two experiments.

Future directions

Does the proteasome move into the spines as 26S proteasome or 19S and 20S move separately and assemble into 26S proteasome at the synapse?

In my proteasome trafficking experiments, I cannot distinguish between these two possibilities. The similar kinetics of 19S and 20S movement suggests that the proteasome moves as 26S holoenzyme but this certainly does not exclude the latter possibility. Furthermore, this raises the question whether 19S-20S association is regulated at synapses. For example, it has been demonstrated that the 26S proteasome disassembles into 19S and 20S in yeast in response to starvation in a reversible manner (Bajorek et al., 2003). In mammalian cells, proteasome inhibition causes an increase in the assembly of 26S from 19S and 20S proteasomes (Meiners et al., 2003). It would be

interesting to examine whether such regulated association of 19S and 20S occurs at synapses. This could provide another level of regulation for local protein degradation; local changes in the abundance of 26S proteasome through 19S and 20S proteasome assembly may regulate local ubiquitin-dependent protein degradation.

Are there synapse-type specific components of UPS?

The answers to this question is beginning to emerge: For example, in C. elegans, GABAergic synapses are more sensitive to loss of RPM-1 (E3) function when compared to non-GABAergic synapses, suggesting that the complement of UPS machinery acting on synaptic proteins may be different between different synapse types (Nakata et al., 2005). It is also possible that different synapses have different UPS targets. Furthermore, between organisms the function of individual UPS components may be different. For example, loss of the E3 activities that belong to the same family in fly and worm cause divergent effects on presynaptic differentiation (Wan et al., 2000; Zhen et al., 2000; Schaefer et al., 2000).

What is the functional role of proteasome localization to the spines? Does AMPAR endocytosis require spine proteasome movement?

In my experiments, I have not shown a particular physiological function for proteasome localization to the synapse. Given the many functions of UPS at the synapse,

it can be argued that the proteasome moves into spines because of the increased need for protein degradation following stimulation. In order to show a specific function for the proteasome localization, reagents that specifically inhibit the proteasome localization are necessary. Peptides that block the interaction between the proteasome and its tether protein at the synapse may be one way to accomplish this. If the specific synaptic function studied requires proteasome trafficking, then the effect of blocking the proteasome movement may have the same consequences as blocking the proteasome enzymatic activity. As proteasome activity is required for AMPAR endocytosis, we could speculate that blocking the proteasome movement into spines may also block AMPAR endocytosis. The kinetics of proteasome movement correspond well with the dynamics of AMPAR internalization. Endocytosed AMPARs colocalizes with the proteasomes in the dendrites when stimulated cultures were immunostained for the internalized receptors and the proteasome (Bingol and Schuman, unpublished observations). This suggests that proteasome may be trafficked to the sites where AMPARs are internalized. Thus, future work will focus on the functional role of proteasome movement during AMPAR endocytosis and other synaptic processes.

Is the local degradation of the proteins accompanied by the local translocation of the proteasome? Is the proteasome localization a local event?

In order to answer this question, local stimulations at the level of the spine would be necessary. A proteasome activity reporter and a tagged proteasome subunit can be expressed in the same cells and the local movement of the proteasome and the local

degradation of the proteins can be monitored simultaneously. Furthermore, it is possible to visualize the local proteasome localization in slices in response to synaptic plasticity via a similar technique used for the studies of mitochondria trafficking (Li et al., 2004).

Do the local protein synthesis and local protein degradation function together?

Local protein degradation may regulate the local protein synthesis by different ways. For example, local protein degradation can activate local protein synthesis by degrading the translational inhibitors. An example of this mechanism was recently demonstrated in Drosophila: one of the components of the RNAi pathway was degraded in response to neuronal activity, leading to the synthesis of CamKII near synapses (Ashraf et al., 2006). Thus, local protein degradation can degrade repressors of translation to locally activate protein synthesis. Cytoplasmic polyadenylation element binding protein, CPEB, is another translational repressor that is a target of the UPS (Reverte et al., 2001). RNA binding proteins acting as translational suppressors, such as pumilio, were also linked to learning (Dabnau et al., 2003) and degradation of these repressors through UPS may activate the local protein synthesis. Furthermore, the local protein degradation may work with local protein synthesis in order to fine-tune the synapse specificity by limiting the availability of the newly synthesized proteins.

How does the synapse know how many proteins to synthesize and how many to degrade?

One possible mechanism is that local protein synthesis machinery synthesizes the proteins under control of UPS as mentioned above. Following the insertion of the newly synthesized proteins into the synapse structure, the "excess" unused proteins may be degraded by UPS. For example, there is a protein interaction cascade in PSD composed of PSD-95-GKAP-Shank, where each protein in the cascade interacts with the ones next to it. Importantly, GKAP-Shank complex, if not bound to PSD-95, forms aggregates that are degraded by the proteasome (Romorini et al., 2004). Neither GKAP nor Shank has been shown to be locally synthesized in dendrites. However, they have been found to be ubiquitinated at synapses (Ehlers, 2003). Thus, the molecule numbers may be kept in check by degrading components that cannot find a binding partner. This model implies that there must be "core" components of the synapse that dictates the molecule number by limiting the protein binding sites. The study by Ehlers suggests the presence of "master organizing molecules" at the synapse where the abundance of these molecules dictates the abundance of other proteins (Ehlers, 2003). The molecular organization of the synapse may be regulated by addition/removal of these proteins through local synthesis/degradation to the synaptic protein pool. Thus, the rapid responses to plasticity-inducing stimuli may be mediated by rapid degradation of these master regulators.

What is the molecular mechanism of proteasome movement into the spines?

The molecular modifications that eventually lead to proteasome stabilization at the synapse may occur on the synaptic proteins or on the proteasome itself. Proteasome localization to spines does not require protein synthesis so local synthesis of a proteasome anchor can be ruled out. The un-masking of a UBL domain in a synaptic protein may allow the tethering of the proteasome to the synapse. UBL-UBA domains were shown to participate in the formation of protein dimers (Ryu et al., 2003) and disruption of a UBL-UBA interaction following stimulation may lead to proteasome capture.

As mentioned earlier, the polyubiquitin chain of the substrate proteins may also act as anchors for the proteasome. In this model, the proteasome must leave the synapse once the target protein is degraded. However, the proteasome stays at the synapse upto two hours following stimulation. This means the proteasome, in addition to the polyubiquitin chain, must be utilizing another protein interaction in order to tether to the synapse. Actin-cytoskeleton experiments suggest that this tether must also involve the actin cytoskeleton of the spines. It is possible that the type of stimulation dictates whether proteasome "visits" the synapse transiently or becomes a "persistent" part of the synaptic structure. Future experiments involving synaptic plasticity stimulations may uncover different modes of proteasome trafficking into the synapses.

Identification of the proteasome interacting proteins (PIPs) at the synapse may give hints about the mechanism of proteasome anchoring at the synapse. Using a similar approach to identification of PIPs via mass spectrometric analysis of affinity-purified proteasomes, proteasome populations from stimulated and unstimulated neurons could be compared in order to identify the proteins that bind to the proteasome with activity (Verma et al., 2000).

Are there other dynamic components of UPS at the synapse?

When the interconnected nature of cellular events in the cells is considered, it is not surprising to find that similar or complementary key cellular processes occur simultaneously in the dendrites. For example, if a synapse locally synthesizes a transmembrane protein, it would make sense to find the local machinery for secretory pathway in the dendrites too (Horton and Ehlers, 2003). Following this logic, I believe that close to a full complement of UPS components at the synapse will be identified in the future. Recent work already suggests that E2s, E3s, and DUBs are present in the dendrites in addition to the proteasome (Bingol and Schuman, 2005; Yi and Ehlers, 2005). In addition to the direct executioners of the UPS function at the synapse (e.g., ubiquitination enzymes and the proteasome), modulators of the UPS function that are targets of synaptic activity should also be present near synapses. For example, does neuronal activity regulate the biogenesis of proteasomes near the synapses? Proteasome biogenesis is the process where highly ordered 20S proteasome is formed from the single subunits (Kruger et al., 2001). One intermediate in proteasome biogenesis is the half-

proteasomes that are composed of one α-subunits ring and one β-subunits ring. A protein with a chaperone-like activity, proteasome maturation protein (POMP/Ump1), assists the assembly of 20S proteasomes from two half-proteasomes (Ramos et al., 1998). Interestingly, POMP is present in the dendrites. Furthermore, neuronal activity regulates POMP levels in the dendrites (Bingol and Schuman, unpublished observations). This raises the exciting possibility that local proteasome assembly in the dendrites may be regulated by synaptic activity. Future work will identify how neuronal signaling pathways act on UPS regulators in order to modulate the UPS function locally.

Ubiquitination machinery may also localize dynamically to synaptic sites following stimulation. Nedd4/Rsp5p HECT E3 enzyme is a good candidate for this as it contains an N-terminal C2 domain (Rotin, 2000). C2 domain responds to elevations in intracellular calcium and translocates the C2-harboring protein to the plasma membrane. Nedd4 functions during axon pathfinding as well as regulation of some of the voltage-gated Na+ channels in neurons (Fotia et al., 2004; Myat et al., 2002). Thus, there is a good possibility that Nedd4 localization is regulated in response to stimulation in the dendrites.

Is proteasome mislocalization linked to neurodegenerative diseases?

Accumulation of ubiquitinated proteins is a hallmark of many neurodegenerative diseases, suggesting the malfunction of UPS in these disorders (Mayer, 2003). Most neurodegenerative disorders manifest as a disease of the synapse: cognitive impairment,

synapse loss, and learning deficits were observed in mouse models before the appearance of clinical symptoms associated with such diseases (Hegde, 1994). Given the many functions of UPS at the synapse, it would not be surprising to find out that malfunction of the UPS localization to the synapse is also related to these disorders. The defects of substrate localization to the proteasome may lead to accumulation of toxic ubiquitinated protein aggregates in the neurons. For example, in the pathogenesis of Parkinson's disease, an important player, parkin E3, has an N- terminal UBL domain: parkin associates with the S5a (rpn10) subunit of the proteasome (Sakata et al., 2003). A single mutation in parkin abolishes the binding to the proteasome, suggesting that defects of the substrate targeting to the proteasome may set the events that lead to the clinical symptoms of neurodegenerative diseases. Thus, mislocalization of the proteasome itself to the substrate may also lead to the accumulation of toxic ubiquitinated proteins in the neurons. A clearer picture of the mechanisms underlying the proteasome movement into spines is required in order to establish a role for this process in disease.

The research on UPS biology in other systems continues to unravel new roles for ubiquitin and the proteasome in a wide variety of key cellular processes. However, the question of how our memories are stored at the molecular level in the face of the ongoing protein turnover is still unanswered. The possible molecular correlate of memory, synaptic plasticity, is rapid, local, and persistent. Ubiquitin-dependent protein degradation provides the tools to rapidly sculpt the local protein content of synapses. Future research on the interplay between UPS and synaptic plasticity will hopefully help

us understand how spatial and temporal resolution at the level of synaptic plasticity is achieved and eventually how memories are stored in the brain.

Work Cited

AAKALU, G., SMITH, W. B., NGUYEN, N., JIANG, C. & SCHUMAN, E. M. (2001). Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* **30,** 489-502.

ALLISON, D. W., GELFAND, V. I., SPECTOR, I. & CRAIG, A. M. (1998). Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. *J Neurosci* **18,** 2423-36.

AMSTERDAM, A., PITZER, F. & BAUMEISTER, W. (1993). Changes in intracellular localization of proteasomes in immortalized ovarian granulosa cells during mitosis associated with a role in cell cycle control. *Proc Natl Acad Sci USA* **90,** 99-103.

ARAVAMUDAN, B., FERGESTAD, T., DAVIS, W. S., RODESCH, C. K. & BROADIE, K. (1999). *Drosophila* UNC-13 is essential for synaptic transmission. *Nat Neurosci* **2,** 965-71.

BAGNI, C., MANNUCCI, L., DOTTI, C. G. & AMALDI, F. (2000). Chemical stimulation of synaptosomes modulates alpha -Ca2+/calmodulin-dependent protein kinase II mRNA association to polysomes. *J Neurosci* **20,** RC76.

BAJOREK, M., FINLEY, D. & GLICKMAN, M. H. (2003). Proteasome disassembly and downregulation is correlated with viability during stationary phase. *Curr Biol* **13,** 1140-4.

BANKE, T. G., BOWIE, D., LEE, H., HUGANIR, R. L., SCHOUSBOE, A. & TRAYNELIS, S. F. (2000). Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. *J Neurosci* **20,** 89-102.

BARCO, A., ALARCON, J. M. & KANDEL, E. R. (2002). Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. *Cell* **108,** 689-703.

BARRIA, A., MULLER, D., DERKACH, V., GRIFFITH, L. C. & SODERLING, T. R. (1997). Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. *Science* **276,** 2042-5.

BASHIR, Z. I., TAM, B. & COLLINGRIDGE, G. L. (1990). Activation of the glycine site in the NMDA receptor is necessary for the induction of LTP. *Neurosci Lett* **108,** 261-6.

BEATTIE, E. C., CARROLL, R. C., YU, X., MORISHITA, W., YASUDA, H., VON ZASTROW, M. & MALENKA, R. C. (2000). Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nat Neurosci* **3,** 1291-300.

BEIQUE, J. C. & ANDRADE, R. (2003). PSD-95 regulates synaptic transmission and plasticity in rat cerebral cortex. *J Physiol* **546,** 859-67.

BERGOLD, P. J., SWEATT, J. D., WINICOV, I., WEISS, K. R., KANDEL, E. R. & SCHWARTZ, J. H. (1990). Protein synthesis during acquisition of long-term facilitation is needed for the persistent loss of regulatory subunits of the *Aplysia* cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A* **87,** 3788-91.

BETTLER, B. & MULLE, C. (1995). Review: neurotransmitter receptors. II. AMPA and kainate receptors. *Neuropharmacology* **34,** 123-39.

BINGOL, B. & SCHUMAN, E. M. (2004). A proteasome-sensitive connection between PSD-95 and GluR1 endocytosis. *Neuropharmacology* **47,** 755-63.

BINGOL, B. & SCHUMAN, E. M. (2005). Synaptic protein degradation by the ubiquitin proteasome system. *Curr Opin Neurobiol* **15,** 536-41.

BLADT, F., TAFURI, A., GELKOP, S., LANGILLE, L. & PAWSON, T. (2002). Epidermolysis bullosa and embryonic lethality in mice lacking the multi-PDZ domain protein GRIP1. *Proc Natl Acad Sci U S A* **99,** 6816-21.

BLANPIED, T. A., SCOTT, D. B. & EHLERS, M. D. (2002). Dynamics and regulation of clathrin coats at specialized endocytic zones of dendrites and spines. *Neuron* **36,** 435-49.

BLISS, T. V. & COLLINGRIDGE, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361,** 31-9.

BLISS, T. V. & GARDNER-MEDWIN, A. R. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the unanaestetized rabbit following stimulation of the perforant path. *J Physiol* **232,** 357-74.

BLISS, T. V. & LOMO, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol* **232,** 331-56.

BLONDEL, M., BACH, S., BAMPS, S., DOBBELAERE, J., WIGET, P., LONGARETTI, C., BARRAL, Y., MEIJER, L. & PETER, M. (2005). Degradation of Hof1 by SCF(Grr1) is important for actomyosin contraction during cytokinesis in yeast. *Embo J* **24,** 1440-52.

BODIAN, D. (1965). A Suggestive Relationship of Nerve Cell Rna with Specific Synaptic Sites. *Proc Natl Acad Sci U S A* **53,** 418-25.

BOLDIN, M. P., METT, I. L. & WALLACH, D. (1995). A protein related to a proteasomal subunit binds to the intracellular domain of the p55 TNF receptor upstream to its 'death domain'. *FEBS Lett* **367,** 39-44.

BONIFACINO, J. S. & WEISSMAN, A. M. (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu Rev Cell Dev Biol* **14,** 19-57.

BORGDORFF, A. J. & CHOQUET, D. (2002). Regulation of AMPA receptor lateral movements. *Nature* **417,** 649-53.

BOSE, S., STRATFORD, F. L., BROADFOOT, K. I., MASON, G. G. & RIVETT, A. J. (2004). Phosphorylation of 20S proteasome alpha subunit C8 (alpha7) stabilizes the 26S proteasome and plays a role in the regulation of proteasome complexes by gamma-interferon. *Biochem J* **378,** 177-84.

BRENMAN, J. E., CHAO, D. S., GEE, S. H., MCGEE, A. W., CRAVEN, S. E., SANTILLANO, D. R., WU, Z., HUANG, F., XIA, H., PETERS, M. F., FROEHNER, S. C. & BREDT, D. S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* **84,** 757-67.

BRUNDEL, B. J., AUSMA, J., VAN GELDER, I. C., VAN DER WANT, J. J., VAN GILST, W. H., CRIJNS, H. J. & HENNING, R. H. (2002). Activation of proteolysis by calpains and structural changes in human paroxysmal and persistent atrial fibrillation. *Cardiovasc Res* **54,** 380-9.

BUCHBERGER, A. (2002). From UBA to UBX: new words in the ubiquitin vocabulary. *Trends Cell Biol* **12,** 216-21.

BUITING, K., SAITOH, S., GROSS, S., DITTRICH, B., SCHWARTZ, S., NICHOLLS, R. D. & HORSTHEMKE, B. (1995). Inherited microdeletions in the Angelman and Prader-

Willi syndromes define an imprinting centre on human chromosome 15. *Nat Genet* **9,** 395-400.

BURBEA, M., DREIER, L., DITTMAN, J. S., GRUNWALD, M. E. & KAPLAN, J. M. (2002). Ubiquitin and AP180 regulate the abundance of GLR-1 glutamate receptors at postsynaptic elements in *C. elegans*. *Neuron* **35,** 107-20.

BURGESS, R. W., PETERSON, K. A., JOHNSON, M. J., ROIX, J. J., WELSH, I. C. & O'BRIEN, T. P. (2004). Evidence for a conserved function in synapse formation reveals Phr1 as a candidate gene for respiratory failure in newborn mice. *Mol Cell Biol* **24,** 1096-105.

CAMPBELL, D. S. & HOLT, C. E. (2001). Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron* **32,** 1013-26.

CARRANO, A. C., EYTAN, E., HERSHKO, A. & PAGANO, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol* **1,** 193-9.

CARROLL, R. C., BEATTIE, E. C., XIA, H., LUSCHER, C., ALTSCHULER, Y., NICOLL, R. A., MALENKA, R. C. & VON ZASTROW, M. (1999a). Dynamin-dependent endocytosis of ionotropic glutamate receptors. *Proc Natl Acad Sci USA* **96,** 14112-7.

CARROLL, R. C., LISSIN, D. V., VON ZASTROW, M., NICOLL, R. A. & MALENKA, R. C. (1999b). Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. *Nat Neurosci* **2,** 454-60.

CASTILLO, P. E., WEISSKOPF, M. G. & NICOLL, R. A. (1994). The role of Ca2+ channels in hippocampal mossy fiber synaptic transmission and long-term potentiation. *Neuron* **12,** 261-9.

CAUSEVIC, M., HISLOP, R. G., KERNOHAN, N. M., CAREY, F. A., KAY, R. A., STEELE, R. J. & FULLER-PACE, F. V. (2001). Overexpression and poly-ubiquitylation of the DEAD-box RNA helicase p68 in colorectal tumours. *Oncogene* **20,** 7734-43.

CHAIN, D. G., CASADIO, A., SCHACHER, S., HEGDE, A. N., VALBRUN, M., YAMAMOTO, N., GOLDBERG, A. L., BARTSCH, D., KANDEL, E. R. & SCHWARTZ, J. H. (1999). Mechanisms for generating the autonomous cAMP-dependent protein kinase required for long-term facilitation in *Aplysia*. *Neuron* **22,** 147-56.

CHAPMAN, A. P., SMITH, S. J., RIDER, C. C. & BEESLEY, P. W. (1994). Multiple ubiquitin conjugates are present in rat brain synaptic membranes and postsynaptic densities. *Neurosci Lett* **168,** 238-42.

CHATURVEDI, K., BANDARI, P., CHINEN, N. & HOWELLS, R. D. (2001). Proteasome involvement in agonist-induced down-regulation of mu and delta opioid receptors. *J Biol Chem* **276,** 12345-55.

CHEN, H., POLO, S., DI FIORE, P. P. & DE CAMILLI, P. V. (2003). Rapid Calcium-dependent decrease of protein ubiquitination at synapses. *Proc Natl Acad Sci U S A* **100,** 14908-13.

CHEN, H. J., ROJAS-SOTO, M., OGUNI, A. & KENNEDY, M. B. (1998). A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. *Neuron* **20,** 895-904.

CHEN, L., BAO, S., QIAO, X. & THOMPSON, R. F. (1999). Impaired cerebellar synapse maturation in waggler, a mutant mouse with a disrupted neuronal calcium channel gamma subunit. *Proc Natl Acad Sci U S A* **96,** 12132-7.

CHEN, L., CHETKOVICH, D. M., PETRALIA, R. S., SWEENEY, N. T., KAWASAKI, Y., WENTHOLD, R. J., BREDT, D. S. & NICOLL, R. A. (2000). Stargazin regulates

synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* **408,** 936-43.

CHEN, P. & HOCHSTRASSER, M. (1996). Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. *Cell* **86,** 961-72.

CHEN, Y., SHARP, Z. D. & LEE, W. H. (1997). HEC binds to the seventh regulatory subunit of the 26 S proteasome and modulates the proteolysis of mitotic cyclins. *J Biol Chem* **272,** 24081-7.

CHIN, L. S., VAVALLE, J. P. & LI, L. (2002). Staring, a novel E3 ubiquitin-protein ligase that targets syntaxin 1 for degradation. *J Biol Chem* **277,** 35071-9.

CHO, K. O., HUNT, C. A. & KENNEDY, M. B. (1992). The rat brain postsynaptic density fraction contains a homolog of the *Drosophila* discs-large tumor suppressor protein. *Neuron* **9,** 929-42.

CHRISTOPHERSON, K. S., SWEENEY, N. T., CRAVEN, S. E., KANG, R., EL-HUSSEINI AEL, D. & BREDT, D. S. (2003). Lipid- and protein-mediated multimerization of PSD-95: implications for receptor clustering and assembly of synaptic protein networks. *J Cell Sci* **116,** 3213-9.

CHUNG, C. H. & BAEK, S. H. (1999). Deubiquitinating enzymes: their diversity and emerging roles. *Biochem Biophys Res Commun* **266,** 633-40.

CHUNG, H. J., XIA, J., SCANNEVIN, R. H., ZHANG, X. & HUGANIR, R. L. (2000). Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. *J Neurosci* **20,** 7258-67.

CIECHANOVER, A., HELLER, H., KATZ-ETZION, R. & HERSHKO, A. (1981). Activation of the heat-stable polypeptide of the ATP-dependent proteolytic system. *Proc Natl Acad Sci U S A* **78,** 761-5.

COAN, E. J., SAYWOOD, W. & COLLINGRIDGE, G. L. (1987). MK-801 blocks NMDA receptor-mediated synaptic transmission and long term potentiation in rat hippocampal slices. *Neurosci Lett* **80,** 111-4.

COLLEDGE, M., SNYDER, E. M., CROZIER, R. A., SODERLING, J. A., JIN, Y., LANGEBERG, L. K., LU, H., BEAR, M. F. & SCOTT, J. D. (2003). Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. *Neuron* **40,** 595-607.

COLLINGRIDGE, G. L., KEHL, S. J. & MCLENNAN, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J Physiol* **334,** 33-46.

CRAVEN, S. E. & BREDT, D. S. (1998). PDZ proteins organize synaptic signaling pathways. *Cell* **93,** 495-8.

CRAVEN, S. E., EL-HUSSEINI, A. E. & BREDT, D. S. (1999). Synaptic targeting of the postsynaptic density protein PSD-95 mediated by lipid and protein motifs. *Neuron* **22,** 497-509.

CRINO, P. B. & EBERWINE, J. (1996). Molecular characterization of the dendritic growth cone: regulated mRNA transport and local protein synthesis. *Neuron* **17,** 1173-87.

D'AMATO, C. J. & HICKS, S. P. (1965). Neuropathologic alterations in the ataxia (paralytic) mouse. *Arch Pathol* **80,** 604-12.

D'APUZZO, M., MANDOLESI, G., REIS, G. & SCHUMAN, E. M. (2001). Abundant GFP expression and LTP in hippocampal acute slices by in vivo injection of sindbis virus. *J Neurophysiol* **86,** 1037-42.

DAHAN, M., LEVI, S., LUCCARDINI, C., ROSTAING, P., RIVEAU, B. & TRILLER, A. (2003). Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking. *Science* **302,** 442-5.

DANTUMA, N. P., LINDSTEN, K., GLAS, R., JELLNE, M. & MASUCCI, M. G. (2000). Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat Biotechnol* **18,** 538-43.

DASH, P. K., HOCHNER, B. & KANDEL, E. R. (1990). Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation. *Nature* **345,** 718-21.

DAW, M. I., CHITTAJALLU, R., BORTOLOTTO, Z. A., DEV, K. K., DUPRAT, F., HENLEY, J. M., COLLINGRIDGE, G. L. & ISAAC, J. T. (2000). PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. *Neuron* **28,** 873-86.

DEAN, C., SCHOLL, F. G., CHOIH, J., DEMARIA, S., BERGER, J., ISACOFF, E. & SCHEIFFELE, P. (2003). Neurexin mediates the assembly of presynaptic terminals. *Nat Neurosci* **6,** 708-16.

DERKACH, V., BARRIA, A. & SODERLING, T. R. (1999). Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proc Natl Acad Sci U S A* **96,** 3269-74.

DEV, K. K., NAKANISHI, S. & HENLEY, J. M. (2004). The PDZ domain of PICK1 differentially accepts protein kinase C-alpha and GluR2 as interacting ligands. *J Biol Chem* **279,** 41393-7.

DEVERAUX, Q., USTRELL, V., PICKART, C. & RECHSTEINER, M. (1994). A 26 S protease subunit that binds ubiquitin conjugates. *J Biol Chem* **269,** 7059-61.

DIANTONIO, A., HAGHIGHI, A. P., PORTMAN, S. L., LEE, J. D., AMARANTO, A. M. & GOODMAN, C. S. (2001). Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature* **412,** 449-52.

DIEHL, J. A., ZINDY, F. & SHERR, C. J. (1997). Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev* **11,** 957-72.

DONG, H., O'BRIEN, R. J., FUNG, E. T., LANAHAN, A. A., WORLEY, P. F. & HUGANIR, R. L. (1997). GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature* **386,** 279-84.

DREIER, L., BURBEA, M. & KAPLAN, J. M. (2005). LIN-23-mediated degradation of beta-catenin regulates the abundance of GLR-1 glutamate receptors in the ventral nerve cord of C. elegans. *Neuron* **46,** 51-64.

EHLERS, M. D. (2000). Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* **28,** 511-25.

EHLERS, M. D. (2003). Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat Neurosci* **6,** 231-42.

EHRLICH, I. & MALINOW, R. (2004). Postsynaptic density 95 controls AMPA receptor incorporation during long-term potentiation and experience-driven synaptic plasticity. *J Neurosci* **24,** 916-27.

EL-HUSSEINI, A. E., SCHNELL, E., CHETKOVICH, D. M., NICOLL, R. A. & BREDT, D. S. (2000). PSD-95 involvement in maturation of excitatory synapses. *Science* **290,** 1364-8.

EL-HUSSEINI AEL, D., SCHNELL, E., DAKOJI, S., SWEENEY, N., ZHOU, Q., PRANGE, O., GAUTHIER-CAMPBELL, C., AGUILERA-MORENO, A., NICOLL, R. A. & BREDT, D. S. (2002). Synaptic strength regulated by palmitate cycling on PSD-95. *Cell* **108,** 849-63.

ENENKEL, C., LEHMANN, A. & KLOETZEL, P. M. (1998). Subcellular distribution of proteasomes implicates a major location of protein degradation in the nuclear envelope-ER network in yeast. *Embo J* **17,** 6144-54.

ENGERT, F. & BONHOEFFER, T. (1997). Synapse specificity of long-term potentiation breaks down at short distances. *Nature* **388,** 279-84.

EVERETT, R. D., EARNSHAW, W. C., FINDLAY, J. & LOMONTE, P. (1999). Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. *Embo J* **18,** 1526-38.

FANG, S., JENSEN, J. P., LUDWIG, R. L., VOUSDEN, K. H. & WEISSMAN, A. M. (2000). Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* **275,** 8945-51.

FANG, S. & WEISSMAN, A. M. (2004). A field guide to ubiquitylation. *Cell Mol Life Sci* **61,** 1546-61.

FEIG, S. & LIPTON, P. (1993). Pairing the cholinergic agonist carbachol with patterned Schaffer collateral stimulation initiates protein synthesis in hippocampal CA1 pyramidal cell dendrites via a muscarinic, NMDA-dependent mechanism. *J Neurosci* **13,** 1010-21.

FERREIRA, P. A., YUNFEI, C., SCHICK, D. & ROEPMAN, R. (1998). The cyclophilin-like domain mediates the association of Ran-binding protein 2 with subunits of the 19S regulatory complex of the proteasome. *J Biol Chem* **273,** 24676-82.

FERRELL, K., WILKINSON, C. R., DUBIEL, W. & GORDON, C. (2000). Regulatory subunit interactions of the 26S proteasome, a complex problem. *Trends Biochem Sci* **25,** 83-8.

FISHER, D., COUX, O., BOMPARD-MARECHAL, G. & DOREE, M. (1998). Germinal vesicle material is dispensable for oscillations in cdc2 and MAP kinase activities, cyclin B degradation and synthesis during meiosis in *Xenopus* oocytes. *Biol Cell* **90,** 497-508.

FORSTER, A., WHITBY, F. G. & HILL, C. P. (2003). The pore of activated 20S proteasomes has an ordered 7-fold symmetric conformation. *Embo J* **22,** 4356-64.

FREY, U., KRUG, M., REYMANN, K. G. & MATTHIES, H. (1988). Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. *Brain Res* **452,** 57-65.

FREY, U. & MORRIS, R. G. (1997). Synaptic tagging and long-term potentiation. *Nature* **385,** 533-6.

FUJIMURO, M., SAWADA, H. & YOKOSAWA, H. (1994). Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. *FEBS Lett* **349,** 173-80.

FUKATA, Y., TZINGOUNIS, A. V., TRINIDAD, J. C., FUKATA, M., BURLINGAME, A. L., NICOLL, R. A. & BREDT, D. S. (2005). Molecular constituents of neuronal AMPA receptors. *J Cell Biol* **169,** 399-404.

FUKUNAGA, K., STOPPINI, L., MIYAMOTO, E. & MULLER, D. (1993). Long-term potentiation is associated with an increased activity of Ca2+/calmodulin-dependent protein kinase II. *J Biol Chem* **268,** 7863-7.

FUNAKOSHI, M., SASAKI, T., NISHIMOTO, T. & KOBAYASHI, H. (2002). Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. *Proc Natl Acad Sci U S A* **99,** 745-50.

GIEFFERS, C., PETERS, B. H., KRAMER, E. R., DOTTI, C. G. & PETERS, J. M. (1999). Expression of the CDH1-associated form of the anaphase-promoting complex in postmitotic neurons. *Proc Natl Acad Sci U S A* **96,** 11317-22.

GIESE, K. P., FEDOROV, N. B., FILIPKOWSKI, R. K. & SILVA, A. J. (1998). Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science* **279,** 870-3.

GLICKMAN, M. H. & CIECHANOVER, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82,** 373-428.

GLICKMAN, M. H. & RAVEH, D. (2005). Proteasome plasticity. *FEBS Lett* **579,** 3214-23.

GLICKMAN, M. H., RUBIN, D. M., COUX, O., WEFES, I., PFEIFER, G., CJEKA, Z., BAUMEISTER, W., FRIED, V. A. & FINLEY, D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* **94,** 615-23.

GOLDBERG, A. L. (2000). Probing the proteasome pathway. *Nat Biotechnol* **18,** 494-6.

GORDON, C. (2002). The intracellular localization of the proteasome. *Curr Top Microbiol Immunol* **268,** 175-84.

GRAMATES, L. S. & BUDNIK, V. (1999). Assembly and maturation of the *Drosophila* larval neuromuscular junction. *Int Rev Neurobiol* **43,** 93-117.

GREENBERG, S. M., CASTELLUCCI, V. F., BAYLEY, H. & SCHWARTZ, J. H. (1987). A molecular mechanism for long-term sensitization in *Aplysia*. *Nature* **329,** 62-5.

GROC, L., HEINE, M., COGNET, L., BRICKLEY, K., STEPHENSON, F. A., LOUNIS, B. & CHOQUET, D. (2004). Differential activity-dependent regulation of the lateral mobilities of AMPA and NMDA receptors. *Nat Neurosci* **7,** 695-6.

GROLL, M., BAJOREK, M., KOHLER, A., MORODER, L., RUBIN, D. M., HUBER, R., GLICKMAN, M. H. & FINLEY, D. (2000). A gated channel into the proteasome core particle. *Nat Struct Biol* **7,** 1062-7.

GROLL, M., HEINEMEYER, W., JAGER, S., ULLRICH, T., BOCHTLER, M., WOLF, D. H. & HUBER, R. (1999). The catalytic sites of 20S proteasomes and their role in subunit maturation: a mutational and crystallographic study. *Proc Natl Acad Sci USA* **96,** 10976-83.

GUO, Q., XIE, J., DANG, C. V., LIU, E. T. & BISHOP, J. M. (1998). Identification of a large Myc-binding protein that contains RCC1-like repeats. *Proc Natl Acad Sci USA* **95,** 9172-7.

GUTLERNER, J. L., PENICK, E. C., SNYDER, E. M. & KAUER, J. A. (2002). Novel protein kinase A-dependent long-term depression of excitatory synapses. *Neuron* **36,** 921-31.

HAMES, R. S., CROOKES, R. E., STRAATMAN, K. R., MERDES, A., HAYES, M. J., FARAGHER, A. J. & FRY, A. M. (2005). Dynamic recruitment of Nek2 kinase to the centrosome involves microtubules, PCM-1, and localized proteasomal degradation. *Mol Biol Cell* **16,** 1711-24.

HARDINGHAM, G. E., FUKUNAGA, Y. & BADING, H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* **5,** 405-14.

HART, A. C., SIMS, S. & KAPLAN, J. M. (1995). Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. *Nature* **378,** 82-5.

HARTMANN-PETERSEN, R. & GORDON, C. (2004a). Protein degradation: recognition of ubiquitinylated substrates. *Curr Biol* **14,** R754-6.

HARTMANN-PETERSEN, R. & GORDON, C. (2004b). Proteins interacting with the 26S proteasome. *Cell Mol Life Sci* **61,** 1589-95.

HASHIMOTO, K., FUKAYA, M., QIAO, X., SAKIMURA, K., WATANABE, M. & KANO, M. (1999). Impairment of AMPA receptor function in cerebellar granule cells of ataxic mutant mouse stargazer. *J Neurosci* **19,** 6027-36.

HAUSER, H. P., BARDROFF, M., PYROWOLAKIS, G. & JENTSCH, S. (1998). A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. *J Cell Biol* **141,** 1415-22.

HAYASHI, Y., SHI, S. H., ESTEBAN, J. A., PICCINI, A., PONCER, J. C. & MALINOW, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* **287,** 2262-7.

HEGDE, A. N., GOLDBERG, A. L. & SCHWARTZ, J. H. (1993). Regulatory subunits of cAMP-dependent protein kinases are degraded after conjugation to ubiquitin: a molecular mechanism underlying long-term synaptic plasticity. *Proc Natl Acad Sci U S A* **90,** 7436-40.

HEGDE, A. N., INOKUCHI, K., PEI, W., CASADIO, A., GHIRARDI, M., CHAIN, D. G., MARTIN, K. C., KANDEL, E. R. & SCHWARTZ, J. H. (1997). Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in *Aplysia*. *Cell* **89,** 115-26.

HERSHKO, A. & CIECHANOVER, A. (1998). The ubiquitin system. *Annu Rev Biochem* **67,** 425-79.

HEYNEN, A. J., QUINLAN, E. M., BAE, D. C. & BEAR, M. F. (2000). Bidirectional, activity-dependent regulation of glutamate receptors in the adult hippocampus in vivo. *Neuron* **28,** 527-36.

HICKE, L. (1997). Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *Faseb J* **11,** 1215-26.

HICKE, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol* **9,** 107-12.

HICKE, L. (2001). Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol* **2,** 195-201.

HICKE, L. & RIEZMAN, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* **84,** 277-87.

HILL, C. P., MASTERS, E. I. & WHITBY, F. G. (2002). The 11S regulators of 20S proteasome activity. *Curr Top Microbiol Immunol* **268,** 73-89.

HIRBEC, H., FRANCIS, J. C., LAURI, S. E., BRAITHWAITE, S. P., COUSSEN, F., MULLE, C., DEV, K. K., COUTINHO, V., MEYER, G., ISAAC, J. T., COLLINGRIDGE, G. L. & HENLEY, J. M. (2003). Rapid and differential regulation of AMPA and kainate receptors at hippocampal mossy fiber synapses by PICK1 and GRIP. *Neuron* **37,** 625-38.

HISANAGA, S. & SAITO, T. (2003). The regulation of cyclin-dependent kinase 5 activity through the metabolism of p35 or p39 Cdk5 activator. *Neurosignals* **12,** 221-9.

HOCHSTRASSER, M. (1996). Ubiquitin-dependent protein degradation. *Annu Rev Genet* **30,** 405-39.

HOLZL, H., KAPELARI, B., KELLERMANN, J., SEEMULLER, E., SUMEGI, M., UDVARDY, A., MEDALIA, O., SPERLING, J., MULLER, S. A., ENGEL, A. & BAUMEISTER, W. (2000). The regulatory complex of *Drosophila melanogaster* 26S proteasomes. Subunit composition and localization of a deubiquitylating enzyme. *J Cell Biol* **150,** 119-30.

HONDA, R., TANAKA, H. & YASUDA, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* **420,** 25-7.

HORSTHEMKE, B., DITTRICH, B. & BUITING, K. (1997). Imprinting mutations on human chromosome 15. *Hum Mutat* **10,** 329-37.

HORTON, A. C. & EHLERS, M. D. (2003). Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. *J Neurosci* **23,** 6188-99.

HSUEH, Y. P. & SHENG, M. (1999). Requirement of N-terminal cysteines of PSD-95 for PSD-95 multimerization and ternary complex formation, but not for binding to potassium channel Kv1.4. *J Biol Chem* **274,** 532-6.

HUANG, Y. & FISCHER-VIZE, J. A. (1996). Undifferentiated cells in the developing *Drosophila* eye influence facet assembly and require the Fat facets ubiquitin-specific protease. *Development* **122,** 3207-16.

HUANG, Y., LU, W., ALI, D. W., PELKEY, K. A., PITCHER, G. M., LU, Y. M., AOTO, H., RODER, J. C., SASAKI, T., SALTER, M. W. & MACDONALD, J. F. (2001). CAKbeta/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. *Neuron* **29,** 485-96.

HUIBREGTSE, J. M., SCHEFFNER, M., BEAUDENON, S. & HOWLEY, P. M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci USA* **92,** 2563-7.

HUIBREGTSE, J. M., SCHEFFNER, M. & HOWLEY, P. M. (1993). Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol Cell Biol* **13,** 775-84.

HUNT, C. A., SCHENKER, L. J. & KENNEDY, M. B. (1996). PSD-95 is associated with the postsynaptic density and not with the presynaptic membrane at forebrain synapses. *J Neurosci* **16,** 1380-8.

IRIE, M., HATA, Y., TAKEUCHI, M., ICHTCHENKO, K., TOYODA, A., HIRAO, K., TAKAI, Y., ROSAHL, T. W. & SUDHOF, T. C. (1997). Binding of neuroligins to PSD-95. *Science* **277,** 1511-5.

ISAAC, J. T. (2003). Postsynaptic silent synapses: evidence and mechanisms. *Neuropharmacology* **45,** 450-60.

JENSEN, J. P., BATES, P. W., YANG, M., VIERSTRA, R. D. & WEISSMAN, A. M. (1995). Identification of a family of closely related human ubiquitin conjugating enzymes. *J Biol Chem* **270,** 30408-14.

JIANG, Y. H., ARMSTRONG, D., ALBRECHT, U., ATKINS, C. M., NOEBELS, J. L., EICHELE, G., SWEATT, J. D. & BEAUDET, A. L. (1998). Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* **21,** 799-811.

JOAZEIRO, C. A. & WEISSMAN, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102,** 549-52.

JORDAN, B. A., FERNHOLZ, B. D., BOUSSAC, M., XU, C., GRIGOREAN, G., ZIFF, E. B. & NEUBERT, T. A. (2004). Identification and verification of novel rodent postsynaptic density proteins. *Mol Cell Proteomics* **3,** 857-71.

JUO, P. & KAPLAN, J. M. (2004). The anaphase-promoting complex regulates the abundance of GLR-1 glutamate receptors in the ventral nerve cord of *C. elegans*. *Curr Biol* **14,** 2057-62.

KAHANA, J. A., SCHLENSTEDT, G., EVANCHUK, D. M., GEISER, J. R., HOYT, M. A. & SILVER, P. A. (1998). The yeast dynactin complex is involved in partitioning the mitotic spindle between mother and daughter cells during anaphase B. *Mol Biol Cell* **9,** 1741-56.

KALIA, L. V. & SALTER, M. W. (2003). Interactions between Src family protein tyrosine kinases and PSD-95. *Neuropharmacology* **45,** 720-8.

KALIES, K. U., ALLAN, S., SERGEYENKO, T., KROGER, H. & ROMISCH, K. (2005). The protein translocation channel binds proteasomes to the endoplasmic reticulum membrane. *Embo J* **24,** 2284-93.

KAMURA, T., HARA, T., MATSUMOTO, M., ISHIDA, N., OKUMURA, F., HATAKEYAMA, S., YOSHIDA, M., NAKAYAMA, K. & NAKAYAMA, K. I. (2004). Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. *Nat Cell Biol* **6,** 1229-35.

KANDEL, E. R. & SCHWARTZ, J. H. (1982). Molecular biology of learning: modulation of transmitter release. *Science* **218,** 433-43.

KANG, H. & SCHUMAN, E. M. (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* **267,** 1658-62.

KANG, H. & SCHUMAN, E. M. (1996). A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. *Science* **273,** 1402-6.

KAPLUN, L., IVANTSIV, Y., BAKHRAT, A. & RAVEH, D. (2003). DNA damage response-mediated degradation of Ho endonuclease via the ubiquitin system involves its nuclear export. *J Biol Chem* **278,** 48727-34.

KATO, A., ROUACH, N., NICOLL, R. A. & BREDT, D. S. (2005). Activity-dependent NMDA receptor degradation mediated by retrotranslocation and ubiquitination. *Proc Natl Acad Sci U S A* **102,** 5600-5.

KAUSELMANN, G., WEILER, M., WULFF, P., JESSBERGER, S., KONIETZKO, U., SCAFIDI, J., STAUBLI, U., BEREITER-HAHN, J., STREBHARDT, K. & KUHL, D. (1999). The polo-like protein kinases Fnk and Snk associate with a Ca(2+)- and integrin-binding protein and are regulated dynamically with synaptic plasticity. *Embo J* **18,** 5528-39.

KAWAHARA, H. & YOKOSAWA, H. (1992). Cell cycle-dependent change of proteasome distribution during embryonic development of the ascidian *Halocynthia roretzi*. *Dev Biol* **151,** 27-33.

KAWAKAMI, T., SUZUKI, T., BAEK, S. H., CHUNG, C. H., KAWASAKI, H., HIRANO, H., ICHIYAMA, A., OMATA, M. & TANAKA, K. (1999). Isolation and characterization of cytosolic and membrane-bound deubiquitinylating enzymes from bovine brain. *J Biochem (Tokyo)* **126,** 612-23.

KELLY, P. T. & VERNON, P. (1985). Changes in the subcellular distribution of calmodulin-kinase II during brain development. *Brain Res* **350,** 211-24.

KENNEDY, M. B. (1995). Origin of PDZ (DHR, GLGF) domains. *Trends Biochem Sci* **20,** 350.

KENNEDY, M. B. (1997). The postsynaptic density at glutamatergic synapses. *Trends Neurosci* **20,** 264-8.

KENNEDY, M. B., BENNETT, M. K., BULLEIT, R. F., ERONDU, N. E., JENNINGS, V. R., MILLER, S. G., MOLLOY, S. S., PATTON, B. L. & SCHENKER, L. J. (1990). Structure and regulation of type II calcium/calmodulin-dependent protein kinase in central nervous system neurons. *Cold Spring Harb Symp Quant Biol* **55,** 101-10.

KIM, C. H., CHUNG, H. J., LEE, H. K. & HUGANIR, R. L. (2001). Interaction of the AMPA receptor subunit GluR2/3 with PDZ domains regulates hippocampal long-term depression. *Proc Natl Acad Sci USA* **98,** 11725-30.

KIM, E., NIETHAMMER, M., ROTHSCHILD, A., JAN, Y. N. & SHENG, M. (1995). Clustering of Shaker-type K+ channels by interaction with a family of membrane-associated guanylate kinases. *Nature* **378,** 85-8.

KIM, E. & SHENG, M. (2004). PDZ domain proteins of synapses. *Nat Rev Neurosci* **5,** 771-81.

KIM, J. H., LEE, H. K., TAKAMIYA, K. & HUGANIR, R. L. (2003). The role of synaptic GTPase-activating protein in neuronal development and synaptic plasticity. *J Neurosci* **23,** 1119-24.

KIM, J. H., LIAO, D., LAU, L. F. & HUGANIR, R. L. (1998). SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. *Neuron* **20,** 683-91.

KIM, J. J. & FANSELOW, M. S. (1992). Modality-specific retrograde amnesia of fear. *Science* **256,** 675-7.

KIRKWOOD, A., SILVA, A. & BEAR, M. F. (1997). Age-dependent decrease of synaptic plasticity in the neocortex of alphaCaMKII mutant mice. *Proc Natl Acad Sci USA* **94,** 3380-3.

KISHINO, T., LALANDE, M. & WAGSTAFF, J. (1997). UBE3A/E6-AP mutations cause Angelman syndrome. *Nat Genet* **15,** 70-3.

KISTNER, U., WENZEL, B. M., VEH, R. W., CASES-LANGHOFF, C., GARNER, A. M., APPELTAUER, U., VOSS, B., GUNDELFINGER, E. D. & GARNER, C. C. (1993). SAP90, a rat presynaptic protein related to the product of the *Drosophila* tumor suppressor gene dlg-A. *J Biol Chem* **268,** 4580-3.

KLEIN, M. & KANDEL, E. R. (1980). Mechanism of calcium current modulation underlying presynaptic facilitation and behavioral sensitization in *Aplysia*. *Proc Natl Acad Sci U S A* **77,** 6912-6.

KLOETZEL, P. M. (2004). The proteasome and MHC class I antigen processing. *Biochim Biophys Acta* **1695,** 225-33.

KO, J., KIM, S., VALTSCHANOFF, J. G., SHIN, H., LEE, J. R., SHENG, M., PREMONT, R. T., WEINBERG, R. J. & KIM, E. (2003). Interaction between liprin-alpha and GIT1 is required for AMPA receptor targeting. *J Neurosci* **23,** 1667-77.

KOMIYAMA, N. H., WATABE, A. M., CARLISLE, H. J., PORTER, K., CHARLESWORTH, P., MONTI, J., STRATHDEE, D. J., O'CARROLL, C. M., MARTIN, S. J., MORRIS, R. G., O'DELL, T. J. & GRANT, S. G. (2002). SynGAP regulates ERK/MAPK signaling, synaptic plasticity, and learning in the complex with postsynaptic density 95 and NMDA receptor. *J Neurosci* **22,** 9721-32.

KORNAU, H. C., SCHENKER, L. T., KENNEDY, M. B. & SEEBURG, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269,** 1737-40.

KORNAU, H. C., SEEBURG, P. H. & KENNEDY, M. B. (1997). Interaction of ion channels and receptors with PDZ domain proteins. *Curr Opin Neurobiol* **7,** 368-73.

KRAPIVINSKY, G., MEDINA, I., KRAPIVINSKY, L., GAPON, S. & CLAPHAM, D. E. (2004). SynGAP-MUPP1-CaMKII synaptic complexes regulate p38 MAP kinase activity and NMDA receptor-dependent synaptic AMPA receptor potentiation. *Neuron* **43,** 563-74.

KROGAN, N. J., LAM, M. H., FILLINGHAM, J., KEOGH, M. C., GEBBIA, M., LI, J., DATTA, N., CAGNEY, G., BURATOWSKI, S., EMILI, A. & GREENBLATT, J. F. (2004). Proteasome involvement in the repair of DNA double-strand breaks. *Mol Cell* **16,** 1027-34.

LAFARGA, M., FERNANDEZ, R., MAYO, I., BERCIANO, M. T. & CASTANO, J. G. (2002). Proteasome dynamics during cell cycle in rat Schwann cells. *Glia* **38,** 313-28.

LAVABRE-BERTRAND, T., HENRY, L., GUIRAUD, I., CARILLO, S. & BUREAU, J. P. (2000). [The proteasome and malignant hemopathies]. *Morphologie* **84,** 39-43.

LECKER, S. H., JAGOE, R. T., GILBERT, A., GOMES, M., BARACOS, V., BAILEY, J., PRICE, S. R., MITCH, W. E. & GOLDBERG, A. L. (2004). Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *Faseb J* **18,** 39-51.

LEE, D. H. & GOLDBERG, A. L. (1998). Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* **8,** 397-403.

LEE, H. K., BARBAROSIE, M., KAMEYAMA, K., BEAR, M. F. & HUGANIR, R. L. (2000). Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature* **405,** 955-9.

LEE, H. K., TAKAMIYA, K., HAN, J. S., MAN, H., KIM, C. H., RUMBAUGH, G., YU, S., DING, L., HE, C., PETRALIA, R. S., WENTHOLD, R. J., GALLAGHER, M. & HUGANIR, R. L. (2003). Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* **112,** 631-43.

LEGGETT, D. S., GLICKMAN, M. H. & FINLEY, D. (2005). Purification of proteasomes, proteasome subcomplexes, and proteasome-associated proteins from budding yeast. *Methods Mol Biol* **301,** 57-70.

LEGGETT, D. S., HANNA, J., BORODOVSKY, A., CROSAS, B., SCHMIDT, M., BAKER, R. T., WALZ, T., PLOEGH, H. & FINLEY, D. (2002). Multiple associated proteins regulate proteasome structure and function. *Mol Cell* **10,** 495-507.

LEUNG, L. S. & SHEN, B. (1995). Long-term potentiation at the apical and basal dendritic synapses of CA1 after local stimulation in behaving rats. *J Neurophysiol* **73,** 1938-46.

LI, K. W., HORNSHAW, M. P., VAN DER SCHORS, R. C., WATSON, R., TATE, S., CASETTA, B., JIMENEZ, C. R., GOUWENBERG, Y., GUNDELFINGER, E. D., SMALLA, K. H. & SMIT, A. B. (2004a). Proteomics analysis of rat brain postsynaptic density. Implications of the diverse protein functional groups for the integration of synaptic physiology. *J Biol Chem* **279,** 987-1002.

LI, Z., OKAMOTO, K., HAYASHI, Y. & SHENG, M. (2004b). The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell* **119,** 873-87.

LI, Z. & WANG, C. C. (2002). Functional characterization of the 11 non-ATPase subunit proteins in the trypanosome 19S proteasomal regulatory complex. *J Biol Chem* **277,** 42686-93.

LIANG, F. & HUGANIR, R. L. (2001). Coupling of agonist-induced AMPA receptor internalization with receptor recycling. *J Neurochem* **77,** 1626-31.

LIN, J. W., JU, W., FOSTER, K., LEE, S. H., AHMADIAN, G., WYSZYNSKI, M., WANG, Y. T. & SHENG, M. (2000). Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization. *Nat Neurosci* **3,** 1282-90.

LINDEN, D. J. (2001). The expression of cerebellar LTD in culture is not associated with changes in AMPA-receptor kinetics, agonist affinity, or unitary conductance. *Proc Natl Acad Sci U S A* **98,** 14066-71.

LINDSTEN, K., MENENDEZ-BENITO, V., MASUCCI, M. G. & DANTUMA, N. P. (2003). A transgenic mouse model of the ubiquitin/proteasome system. *Nat Biotechnol* **21,** 897-902.

LISMAN, J., SCHULMAN, H. & CLINE, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci* **3,** 175-90.

LISSIN, D. V., CARROLL, R. C., NICOLL, R. A., MALENKA, R. C. & VON ZASTROW, M. (1999). Rapid, activation-induced redistribution of ionotropic glutamate receptors in cultured hippocampal neurons. *J Neurosci* **19,** 1263-72.

LLEDO, P. M., HJELMSTAD, G. O., MUKHERJI, S., SODERLING, T. R., MALENKA, R. C. & NICOLL, R. A. (1995). Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. *Proc Natl Acad Sci U S A* **92,** 11175-9.

LU, W., MAN, H., JU, W., TRIMBLE, W. S., MACDONALD, J. F. & WANG, Y. T. (2001). Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron* **29,** 243-54.

LUSCHER, C., XIA, H., BEATTIE, E. C., CARROLL, R. C., VON ZASTROW, M., MALENKA, R. C. & NICOLL, R. A. (1999). Role of AMPA receptor cycling in synaptic transmission and plasticity. *Neuron* **24,** 649-58.

LUTHI, A., CHITTAJALLU, R., DUPRAT, F., PALMER, M. J., BENKE, T. A., KIDD, F. L., HENLEY, J. M., ISAAC, J. T. & COLLINGRIDGE, G. L. (1999). Hippocampal LTD expression involves a pool of AMPARs regulated by the NSF-GluR2 interaction. *Neuron* **24,** 389-99.

LUTTRELL, L. M. & LEFKOWITZ, R. J. (2002). The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* **115,** 455-65.

LYNCH, G. & BAUDRY, M. (1984). The biochemistry of memory: a new and specific hypothesis. *Science* **224,** 1057-63.

LYNCH, G., LARSON, J., KELSO, S., BARRIONUEVO, G. & SCHOTTLER, F. (1983). Intracellular injections of EGTA block induction of hippocampal long-term potentiation. *Nature* **305,** 719-21.

MALENKA, R. C. (2003). Synaptic plasticity and AMPA receptor trafficking. *Ann N Y Acad Sci* **1003,** 1-11.

MALENKA, R. C. & BEAR, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* **44,** 5-21.

MALENKA, R. C., KAUER, J. A., PERKEL, D. J., MAUK, M. D., KELLY, P. T., NICOLL, R. A. & WAXHAM, M. N. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature* **340,** 554-7.

MALENKA, R. C. & NICOLL, R. A. (1999). Long-term potentiation-a decade of progress? *Science* **285,** 1870-4.

MALINOW, R. & MALENKA, R. C. (2002). AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* **25,** 103-26.

MALINOW, R., SCHULMAN, H. & TSIEN, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* **245,** 862-6.

MAN, H. Y., LIN, J. W., JU, W. H., AHMADIAN, G., LIU, L., BECKER, L. E., SHENG, M. & WANG, Y. T. (2000). Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. *Neuron* **25,** 649-62.

MANN, F., MIRANDA, E., WEINL, C., HARMER, E. & HOLT, C. E. (2003). B-type Eph receptors and ephrins induce growth cone collapse through distinct intracellular pathways. *J Neurobiol* **57,** 323-36.

MARICQ, A. V., PECKOL, E., DRISCOLL, M. & BARGMANN, C. I. (1995). Mechanosensory signalling in *C. elegans* mediated by the GLR-1 glutamate receptor. *Nature* **378,** 78-81.

MATHIAS, N., STEUSSY, C. N. & GOEBL, M. G. (1998). An essential domain within Cdc34p is required for binding to a complex containing Cdc4p and Cdc53p in *Saccharomyces cerevisiae*. *J Biol Chem* **273,** 4040-5.

MATSUDA, S., LAUNEY, T., MIKAWA, S. & HIRAI, H. (2000). Disruption of AMPA receptor GluR2 clusters following long-term depression induction in cerebellar Purkinje neurons. *Embo J* **19,** 2765-74.

MATSUDA, S., MIKAWA, S. & HIRAI, H. (1999). Phosphorylation of serine-880 in GluR2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein. *J Neurochem* **73,** 1765-8.

MAYER, M. L. (2005). Glutamate receptor ion channels. *Curr Opin Neurobiol* **15,** 282-8.

MAYER, M. L., WESTBROOK, G. L. & GUTHRIE, P. B. (1984). Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones. *Nature* **309,** 261-3.

MCGEE, A. W. & BREDT, D. S. (2003). Assembly and plasticity of the glutamatergic postsynaptic specialization. *Curr Opin Neurobiol* **13,** 111-8.

MEINERS, S., HEYKEN, D., WELLER, A., LUDWIG, A., STANGL, K., KLOETZEL, P. M. & KRUGER, E. (2003). Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of Mammalian proteasomes. *J Biol Chem* **278,** 21517-25.

MIGAUD, M., CHARLESWORTH, P., DEMPSTER, M., WEBSTER, L. C., WATABE, A. M., MAKHINSON, M., HE, Y., RAMSAY, M. F., MORRIS, R. G., MORRISON, J. H., O'DELL, T. J. & GRANT, S. G. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* **396,** 433-9.

MILLER, J. & GORDON, C. (2005). The regulation of proteasome degradation by multi-ubiquitin chain binding proteins. *FEBS Lett* **579,** 3224-30.

MILLER, S. G., PATTON, B. L. & KENNEDY, M. B. (1988). Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca2(+)-independent activity. *Neuron* **1,** 593-604.

MILLER, W. E. & LEFKOWITZ, R. J. (2001). Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Curr Opin Cell Biol* **13,** 139-45.

MORABITO, M. A., SHENG, M. & TSAI, L. H. (2004). Cyclin-dependent kinase 5 phosphorylates the N-terminal domain of the postsynaptic density protein PSD-95 in neurons. *J Neurosci* **24,** 865-76.

MURALIDHAR, M. G. & THOMAS, J. B. (1993). The *Drosophila* bendless gene encodes a neural protein related to ubiquitin-conjugating enzymes. *Neuron* **11,** 253-66.

MURASE, S., MOSSER, E. & SCHUMAN, E. M. (2002). Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron* **35,** 91-105.

NAGAI, T., IBATA, K., PARK, E. S., KUBOTA, M., MIKOSHIBA, K. & MIYAWAKI, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol* **20,** 87-90.

NAKATA, K., ABRAMS, B., GRILL, B., GONCHAROV, A., HUANG, X., CHISHOLM, A. D. & JIN, Y. (2005). Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. *Cell* **120,** 407-20.

NEHRING, R. B., WISCHMEYER, E., DORING, F., VEH, R. W., SHENG, M. & KARSCHIN, A. (2000). Neuronal inwardly rectifying K(+) channels differentially couple to PDZ proteins of the PSD-95/SAP90 family. *J Neurosci* **20,** 156-62.

NICOLL, R. A. & MALENKA, R. C. (1995). Contrasting properties of two forms of long-term potentiation in the hippocampus. *Nature* **377,** 115-8.

NIETHAMMER, M., KIM, E. & SHENG, M. (1996). Interaction between the C terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases. *J Neurosci* **16,** 2157-63.

OH, J. S., MANZERRA, P. & KENNEDY, M. B. (2004). Regulation of the neuron-specific Ras GTPase-activating protein, synGAP, by Ca2+/calmodulin-dependent protein kinase II. *J Biol Chem* **279,** 17980-8.

ORTEGA, J., HEYMANN, J. B., KAJAVA, A. V., USTRELL, V., RECHSTEINER, M. & STEVEN, A. C. (2005). The axial channel of the 20S proteasome opens upon binding of the PA200 activator. *J Mol Biol* **346,** 1221-7.

OSTEN, P., KHATRI, L., PEREZ, J. L., KOHR, G., GIESE, G., DALY, C., SCHULZ, T. W., WENSKY, A., LEE, L. M. & ZIFF, E. B. (2000). Mutagenesis reveals a role for ABP/GRIP binding to GluR2 in synaptic surface accumulation of the AMPA receptor. *Neuron* **27,** 313-25.

OSTEN, P., VALSAMIS, L., HARRIS, A. & SACKTOR, T. C. (1996). Protein synthesis-dependent formation of protein kinase Mzeta in long-term potentiation. *J Neurosci* **16,** 2444-51.

OSTROFF, L. E., FIALA, J. C., ALLWARDT, B. & HARRIS, K. M. (2002). Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices. *Neuron* **35,** 535-45.

OTANI, S., MARSHALL, C. J., TATE, W. P., GODDARD, G. V. & ABRAHAM, W. C. (1989). Maintenance of long-term potentiation in rat dentate gyrus requires protein synthesis but not messenger RNA synthesis immediately post-tetanization. *Neuroscience* **28,** 519-26.

OUYANG, Y., KANTOR, D., HARRIS, K. M., SCHUMAN, E. M. & KENNEDY, M. B. (1997). Visualization of the distribution of autophosphorylated calcium/calmodulin-dependent protein kinase II after tetanic stimulation in the CA1 area of the hippocampus. *J Neurosci* **17,** 5416-27.

PACKARD, M., MATHEW, D. & BUDNIK, V. (2003). FASt remodeling of synapses in *Drosophila*. *Curr Opin Neurobiol* **13,** 527-34.

PAK, D. T. & SHENG, M. (2003). Targeted protein degradation and synapse remodeling by an inducible protein kinase. *Science* **302,** 1368-73.

PALMER, A., MASON, G. G., PARAMIO, J. M., KNECHT, E. & RIVETT, A. J. (1994). Changes in proteasome localization during the cell cycle. *Eur J Cell Biol* **64,** 163-75.

PASSAFARO, M., SALA, C., NIETHAMMER, M. & SHENG, M. (1999). Microtubule binding by CRIPT and its potential role in the synaptic clustering of PSD-95. *Nat Neurosci* **2,** 1063-9.

PASSMORE, L. A. & BARFORD, D. (2004). Getting into position: the catalytic mechanisms of protein ubiquitylation. *Biochem J* **379,** 513-25.

PATRICK, G. N., BINGOL, B., WELD, H. A. & SCHUMAN, E. M. (2003). Ubiquitin-mediated proteasome activity is required for agonist-induced endocytosis of GluRs. *Curr Biol* **13,** 2073-81.

PENG, J., KIM, M. J., CHENG, D., DUONG, D. M., GYGI, S. P. & SHENG, M. (2004). Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. *J Biol Chem* **279,** 21003-11.

PENZES, P., BEESER, A., CHERNOFF, J., SCHILLER, M. R., EIPPER, B. A., MAINS, R. E. & HUGANIR, R. L. (2003). Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. *Neuron* **37,** 263-74.

PENZES, P., JOHNSON, R. C., SATTLER, R., ZHANG, X., HUGANIR, R. L., KAMBAMPATI, V., MAINS, R. E. & EIPPER, B. A. (2001). The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis. *Neuron* **29,** 229-42.

PEREZ, J. L., KHATRI, L., CHANG, C., SRIVASTAVA, S., OSTEN, P. & ZIFF, E. B. (2001). PICK1 targets activated protein kinase Calpha to AMPA receptor clusters in spines of hippocampal neurons and reduces surface levels of the AMPA-type glutamate receptor subunit 2. *J Neurosci* **21,** 5417-28.

PETRALIA, R. S., WANG, Y. X. & WENTHOLD, R. J. (2003). Internalization at glutamatergic synapses during development. *Eur J Neurosci* **18,** 3207-17.

PETROSKI, M. D. & DESHAIES, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* **6,** 9-20.

PETTIT, D. L., PERLMAN, S. & MALINOW, R. (1994). Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. *Science* **266,** 1881-5.

PICKARD, L., NOEL, J., DUCKWORTH, J. K., FITZJOHN, S. M., HENLEY, J. M., COLLINGRIDGE, G. L. & MOLNAR, E. (2001). Transient synaptic activation of NMDA receptors leads to the insertion of native AMPA receptors at hippocampal neuronal plasma membranes. *Neuropharmacology* **41,** 700-13.

PICKART, C. M. (2000). Ubiquitin in chains. *Trends Biochem Sci* **25,** 544-8.

PICKART, C. M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70,** 503-33.

PICKART, C. M. & COHEN, R. E. (2004). Proteasomes and their kin: proteases in the machine age. *Nat Rev Mol Cell Biol* **5,** 177-87.

PIERCE, K. L. & LEFKOWITZ, R. J. (2001). Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci* **2,** 727-33.

POLO, S., SIGISMUND, S., FARETTA, M., GUIDI, M., CAPUA, M. R., BOSSI, G., CHEN, H., DE CAMILLI, P. & DI FIORE, P. P. (2002). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* **416,** 451-5.

RACZ, B., BLANPIED, T. A., EHLERS, M. D. & WEINBERG, R. J. (2004). Lateral organization of endocytic machinery in dendritic spines. *Nat Neurosci* **7,** 917-8.

RAJAPUROHITAM, V., MORALES, C. R., EL-ALFY, M., LEFRANCOIS, S., BEDARD, N. & WING, S. S. (1999). Activation of a UBC4-dependent pathway of ubiquitin conjugation during postnatal development of the rat testis. *Dev Biol* **212,** 217-28.

RECHSTEINER, M. & HILL, C. P. (2005). Mobilizing the proteolytic machine: cell biological roles of proteasome activators and inhibitors. *Trends Cell Biol* **15,** 27-33.

REYMANN, K. G., MALISCH, R., SCHULZECK, K., BRODEMANN, R., OTT, T. & MATTHIES, H. (1985). The duration of long-term potentiation in the CA1 region of the hippocampal slice preparation. *Brain Res Bull* **15,** 249-55.

RIECHMANN, V., GUTIERREZ, G. J., FILARDO, P., NEBREDA, A. R. & EPHRUSSI, A. (2002). Par-1 regulates stability of the posterior determinant Oskar by phosphorylation. *Nat Cell Biol* **4,** 337-42.

RIEZMAN, H. (2002). Cell biology: the ubiquitin connection. *Nature* **416,** 381-3.

RIVETT, A. J. (1993). Characterization of proteasomes isolated from rat liver. *Enzyme Protein* **47,** 210-9.

RIVETT, A. J., BOSE, S., BROOKS, P. & BROADFOOT, K. I. (2001). Regulation of proteasome complexes by gamma-interferon and phosphorylation. *Biochimie* **83,** 363-6.

ROCHE, K. W., O'BRIEN, R. J., MAMMEN, A. L., BERNHARDT, J. & HUGANIR, R. L. (1996). Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* **16,** 1179-88.

RONGO, C., WHITFIELD, C. W., RODAL, A., KIM, S. K. & KAPLAN, J. M. (1998). LIN-10 is a shared component of the polarized protein localization pathways in neurons and epithelia. *Cell* **94,** 751-9.

RUMBAUGH, G., ADAMS, J. P., KIM, J. H. & HUGANIR, R. L. (2006). SynGAP regulates synaptic strength and mitogen-activated protein kinases in cultured neurons. *Proc Natl Acad Sci USA* **103,** 4344-51.

SANES, J. R. & LICHTMAN, J. W. (1999). Can molecules explain long-term potentiation? *Nat Neurosci* **2,** 597-604.

SATOH, K., SASAJIMA, H., NYOUMURA, K. I., YOKOSAWA, H. & SAWADA, H. (2001). Assembly of the 26S proteasome is regulated by phosphorylation of the p45/Rpt6 ATPase subunit. *Biochemistry* **40,** 314-9.

SCHAEFER, A. M., HADWIGER, G. D. & NONET, M. L. (2000). rpm-1, a conserved neuronal gene that regulates targeting and synaptogenesis in *C. elegans*. *Neuron* **26,** 345-56.

SCHAUBER, C., CHEN, L., TONGAONKAR, P., VEGA, I., LAMBERTSON, D., POTTS, W. & MADURA, K. (1998). Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature* **391,** 715-8.

SCHEFFNER, M., HUIBREGTSE, J. M., VIERSTRA, R. D. & HOWLEY, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75,** 495-505.

SCHEFFNER, M., NUBER, U. & HUIBREGTSE, J. M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373,** 81-3.

SCHMIDT, M., HAAS, W., CROSAS, B., SANTAMARIA, P. G., GYGI, S. P., WALZ, T. & FINLEY, D. (2005). The HEAT repeat protein Blm10 regulates the yeast proteasome by capping the core particle. *Nat Struct Mol Biol* **12,** 294-303.

SCHNELL, E., SIZEMORE, M., KARIMZADEGAN, S., CHEN, L., BREDT, D. S. & NICOLL, R. A. (2002). Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. *Proc Natl Acad Sci U S A* **99,** 13902-7.

SCHUBERT, U., OTT, D. E., CHERTOVA, E. N., WELKER, R., TESSMER, U., PRINCIOTTA, M. F., BENNINK, J. R., KRAUSSLICH, H. G. & YEWDELL, J. W. (2000). Proteasome inhibition interferes with gag polyprotein processing, release, and maturation of HIV-1 and HIV-2. *Proc Natl Acad Sci U S A* **97,** 13057-62.

SCHUMAN, E. M. (1997). Synapse specificity and long-term information storage. *Neuron* **18,** 339-42.

SETOU, M., SEOG, D. H., TANAKA, Y., KANAI, Y., TAKEI, Y., KAWAGISHI, M. & HIROKAWA, N. (2002). Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites. *Nature* **417,** 83-7.

SEVER, S., DAMKE, H. & SCHMID, S. L. (2000). Dynamin:GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. *J Cell Biol* **150,** 1137-48.

SHAW, J. D., CUMMINGS, K. B., HUYER, G., MICHAELIS, S. & WENDLAND, B. (2001). Yeast as a model system for studying endocytosis. *Exp Cell Res* **271,** 1-9.

SHENG, M. & SALA, C. (2001). PDZ domains and the organization of supramolecular complexes. *Annu Rev Neurosci* **24,** 1-29.

SHENOY, S. K., MCDONALD, P. H., KOHOUT, T. A. & LEFKOWITZ, R. J. (2001). Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* **294,** 1307-13.

SHI, S., HAYASHI, Y., ESTEBAN, J. A. & MALINOW, R. (2001). Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* **105,** 331-43.

SHI, S. H., HAYASHI, Y., PETRALIA, R. S., ZAMAN, S. H., WENTHOLD, R. J., SVOBODA, K. & MALINOW, R. (1999). Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science* **284,** 1811-6.

SHIN, H., WYSZYNSKI, M., HUH, K. H., VALTSCHANOFF, J. G., LEE, J. R., KO, J., STREULI, M., WEINBERG, R. J., SHENG, M. & KIM, E. (2003). Association of the kinesin motor KIF1A with the multimodular protein liprin-alpha. *J Biol Chem* **278,** 11393-401.

SIEGELBAUM, S. A., CAMARDO, J. S. & KANDEL, E. R. (1982). Serotonin and cyclic AMP close single K+ channels in *Aplysia* sensory neurones. *Nature* **299,** 413-7.

SIGISMUND, S., POLO, S. & DI FIORE, P. P. (2004). Signaling through monoubiquitination. *Curr Top Microbiol Immunol* **286,** 149-85.

SILVA, A. J., STEVENS, C. F., TONEGAWA, S. & WANG, Y. (1992). Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257,** 201-6.

SMITH, W. B., STARCK, S. R., ROBERTS, R. W. & SCHUMAN, E. M. (2005). Dopaminergic stimulation of local protein synthesis enhances surface expression of GluR1 and synaptic transmission in hippocampal neurons. *Neuron* **45,** 765-79.

SNYDER, E. M., PHILPOT, B. D., HUBER, K. M., DONG, X., FALLON, J. R. & BEAR, M. F. (2001). Internalization of ionotropic glutamate receptors in response to mGluR activation. *Nat Neurosci* **4,** 1079-85.

SOMMER, T. & JENTSCH, S. (1993). A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature* **365,** 176-9.

SPEESE, S. D., TROTTA, N., RODESCH, C. K., ARAVAMUDAN, B. & BROADIE, K. (2003). The ubiquitin proteasome system acutely regulates presynaptic protein turnover and synaptic efficacy. *Curr Biol* **13,** 899-910.

SPREAFICO, R., FRASSONI, C., ARCELLI, P., BATTAGLIA, G., WENTHOLD, R. J. & DE BIASI, S. (1994). Distribution of AMPA selective glutamate receptors in the thalamus of adult rats and during postnatal development. A light and ultrastructural immunocytochemical study. *Brain Res Dev Brain Res* **82,** 231-44.

SPRENGEL, R., SUCHANEK, B., AMICO, C., BRUSA, R., BURNASHEV, N., ROZOV, A., HVALBY, O., JENSEN, V., PAULSEN, O., ANDERSEN, P., KIM, J. J., THOMPSON, R. F., SUN, W., WEBSTER, L. C., GRANT, S. G., EILERS, J., KONNERTH, A., LI, J., MCNAMARA, J. O. & SEEBURG, P. H. (1998). Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. *Cell* **92,** 279-89.

SRIVASTAVA, S. & ZIFF, E. B. (1999). ABP: a novel AMPA receptor binding protein. *Ann N Y Acad Sci* **868,** 561-4.

STANTON, P. K. & SARVEY, J. M. (1984). Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. *J Neurosci* **4,** 3080-8.

STAUBLI, U. & LYNCH, G. (1987). Stable hippocampal long-term potentiation elicited by 'theta' pattern stimulation. *Brain Res* **435,** 227-34.

STEIN, V., HOUSE, D. R., BREDT, D. S. & NICOLL, R. A. (2003). Postsynaptic density-95 mimics and occludes hippocampal long-term potentiation and enhances long-term depression. *J Neurosci* **23,** 5503-6.

STEWARD, O. & LEVY, W. B. (1982). Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. *J Neurosci* **2,** 284-91.

STEWARD, O. & SCHUMAN, E. M. (2001). Protein synthesis at synaptic sites on dendrites. *Annu Rev Neurosci* **24,** 299-325.

STEWARD, O. & SCHUMAN, E. M. (2003). Compartmentalized synthesis and degradation of proteins in neurons. *Neuron* **40,** 347-59.

STONE, M., HARTMANN-PETERSEN, R., SEEGER, M., BECH-OTSCHIR, D., WALLACE, M. & GORDON, C. (2004). Uch2/Uch37 is the major deubiquitinating enzyme associated with the 26S proteasome in fission yeast. *J Mol Biol* **344,** 697-706.

SUMEGI, M., HUNYADI-GULYAS, E., MEDZIHRADSZKY, K. F. & UDVARDY, A. (2003). 26S proteasome subunits are O-linked N-acetylglucosamine-modified in *Drosophila melanogaster*. *Biochem Biophys Res Commun* **312,** 1284-9.

SUTCLIFFE, J. S., NAKAO, M., CHRISTIAN, S., ORSTAVIK, K. H., TOMMERUP, N., LEDBETTER, D. H. & BEAUDET, A. L. (1994). Deletions of a differentially methylated CpG island at the SNRPN gene define a putative imprinting control region. *Nat Genet* **8,** 52-8.

TAKAGI, Y., MASUDA, C. A., CHANG, W. H., KOMORI, H., WANG, D., HUNTER, T., JOAZEIRO, C. A. & KORNBERG, R. D. (2005). Ubiquitin ligase activity of TFIIH and the transcriptional response to DNA damage. *Mol Cell* **18,** 237-43.

TAKASU, M. A., DALVA, M. B., ZIGMOND, R. E. & GREENBERG, M. E. (2002). Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. *Science* **295,** 491-5.

TANAHASHI, N., MURAKAMI, Y., MINAMI, Y., SHIMBARA, N., HENDIL, K. B. & TANAKA, K. (2000). Hybrid proteasomes. Induction by interferon-gamma and contribution to ATP-dependent proteolysis. *J Biol Chem* **275,** 14336-45.

TANAKA, K., SUZUKI, T., HATTORI, N. & MIZUNO, Y. (2004). Ubiquitin, proteasome and parkin. *Biochim Biophys Acta* **1695,** 235-47.

TARDIN, C., COGNET, L., BATS, C., LOUNIS, B. & CHOQUET, D. (2003). Direct imaging of lateral movements of AMPA receptors inside synapses. *Embo J* **22,** 4656-65.

TAYA, S., YAMAMOTO, T., KANO, K., KAWANO, Y., IWAMATSU, A., TSUCHIYA, T., TANAKA, K., KANAI-AZUMA, M., WOOD, S. A., MATTICK, J. S. & KAIBUCHI, K. (1998). The Ras target AF-6 is a substrate of the fam deubiquitinating enzyme. *J Cell Biol* **142,** 1053-62.

TEZUKA, T., UMEMORI, H., AKIYAMA, T., NAKANISHI, S. & YAMAMOTO, T. (1999). PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. *Proc Natl Acad Sci U S A* **96,** 435-40.

TOM DIECK, S., ALTROCK, W. D., KESSELS, M. M., QUALMANN, B., REGUS, H., BRAUNER, D., FEJTOVA, A., BRACKO, O., GUNDELFINGER, E. D. & BRANDSTATTER, J. H. (2005). Molecular dissection of the photoreceptor ribbon synapse: physical interaction of Bassoon and RIBEYE is essential for the assembly of the ribbon complex. *J Cell Biol* **168,** 825-36.

TOMITA, S., CHEN, L., KAWASAKI, Y., PETRALIA, R. S., WENTHOLD, R. J., NICOLL, R. A. & BREDT, D. S. (2003). Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. *J Cell Biol* **161,** 805-16.

TOMITA, S., FUKATA, M., NICOLL, R. A. & BREDT, D. S. (2004). Dynamic interaction of stargazin-like TARPs with cycling AMPA receptors at synapses. *Science* **303,** 1508-11.

TONGAONKAR, P., CHEN, L., LAMBERTSON, D., KO, B. & MADURA, K. (2000). Evidence for an interaction between ubiquitin-conjugating enzymes and the 26S proteasome. *Mol Cell Biol* **20,** 4691-8.

TORRE, E. R. & STEWARD, O. (1992). Demonstration of local protein synthesis within dendrites using a new cell culture system that permits the isolation of living axons and dendrites from their cell bodies. *J Neurosci* **12,** 762-72.

TORRES, R., FIRESTEIN, B. L., DONG, H., STAUDINGER, J., OLSON, E. N., HUGANIR, R. L., BREDT, D. S., GALE, N. W. & YANCOPOULOS, G. D. (1998). PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands. *Neuron* **21,** 1453-63.

TRILLER, A. & CHOQUET, D. (2005). Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move! *Trends Neurosci* **28,** 133-9.

TSOKAS, P., GRACE, E. A., CHAN, P., MA, T., SEALFON, S. C., IYENGAR, R., LANDAU, E. M. & BLITZER, R. D. (2005). Local protein synthesis mediates a rapid increase in dendritic elongation factor 1A after induction of late long-term potentiation. *J Neurosci* **25,** 5833-43.

USTRELL, V., HOFFMAN, L., PRATT, G. & RECHSTEINER, M. (2002). PA200, a nuclear proteasome activator involved in DNA repair. *Embo J* **21,** 3516-25.

VAN KERKHOF, P., GOVERS, R., ALVES DOS SANTOS, C. M. & STROUS, G. J. (2000). Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. *J Biol Chem* **275,** 1575-80.

VAN ROESSEL, P., ELLIOTT, D. A., ROBINSON, I. M., PROKOP, A. & BRAND, A. H. (2004). Independent regulation of synaptic size and activity by the anaphase-promoting complex. *Cell* **119,** 707-18.

VARSHAVSKY, A. (2005). Regulated protein degradation. *Trends Biochem Sci* **30,** 283-6.

VAZQUEZ, L. E., CHEN, H. J., SOKOLOVA, I., KNUESEL, I. & KENNEDY, M. B. (2004). SynGAP regulates spine formation. *J Neurosci* **24,** 8862-72.

VERMA, P., CHIERZI, S., CODD, A. M., CAMPBELL, D. S., MEYER, R. L., HOLT, C. E. & FAWCETT, J. W. (2005). Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *J Neurosci* **25,** 331-42.

VERMA, R., CHEN, S., FELDMAN, R., SCHIELTZ, D., YATES, J., DOHMEN, J. & DESHAIES, R. J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. *Mol Biol Cell* **11,** 3425-39.

WALIKONIS, R. S., JENSEN, O. N., MANN, M., PROVANCE, D. W., JR., MERCER, J. A. & KENNEDY, M. B. (2000). Identification of proteins in the postsynaptic density fraction by mass spectrometry. *J Neurosci* **20,** 4069-80.

WAN, H. I., DIANTONIO, A., FETTER, R. D., BERGSTROM, K., STRAUSS, R. & GOODMAN, C. S. (2000). Highwire regulates synaptic growth in *Drosophila*. *Neuron* **26,** 313-29.

WANG, H. R., KANIA, M., BAUMEISTER, W. & NEDERLOF, P. M. (1997). Import of human and Thermoplasma 20S proteasomes into nuclei of HeLa cells requires functional NLS sequences. *Eur J Cell Biol* **73,** 105-13.

WANG, H. R., ZHANG, Y., OZDAMAR, B., OGUNJIMI, A. A., ALEXANDROVA, E., THOMSEN, G. H. & WRANA, J. L. (2003). Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. *Science* **302,** 1775-9.

WANG, Y. T. & LINDEN, D. J. (2000). Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron* **25,** 635-47.

WARD, C. L., OMURA, S. & KOPITO, R. R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* **83,** 121-7.

WEINSTEIN, J. (1997). Cell cycle-regulated expression, phosphorylation, and degradation of p55Cdc. A mammalian homolog of CDC20/Fizzy/slp1. *J Biol Chem* **272,** 28501-11.

WHEELER, T. C., CHIN, L. S., LI, Y., ROUDABUSH, F. L. & LI, L. (2002). Regulation of synaptophysin degradation by mammalian homologues of seven in absentia. *J Biol Chem* **277,** 10273-82.

WHITBY, F. G., MASTERS, E. I., KRAMER, L., KNOWLTON, J. R., YAO, Y., WANG, C. C. & HILL, C. P. (2000). Structural basis for the activation of 20S proteasomes by 11S regulators. *Nature* **408,** 115-20.

WIGLEY, W. C., FABUNMI, R. P., LEE, M. G., MARINO, C. R., MUALLEM, S., DEMARTINO, G. N. & THOMAS, P. J. (1999). Dynamic association of proteasomal machinery with the centrosome. *J Cell Biol* **145,** 481-90.

WILKINSON, C. R., WALLACE, M., MORPHEW, M., PERRY, P., ALLSHIRE, R., JAVERZAT, J. P., MCINTOSH, J. R. & GORDON, C. (1998). Localization of the 26S proteasome during mitosis and meiosis in fission yeast. *Embo J* **17,** 6465-76.

WILKINSON, K. D. (2000). Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. *Semin Cell Dev Biol* **11,** 141-8.

WILSON, S. M., BHATTACHARYYA, B., RACHEL, R. A., COPPOLA, V., TESSAROLLO, L., HOUSEHOLDER, D. B., FLETCHER, C. F., MILLER, R. J., COPELAND, N. G. & JENKINS, N. A. (2002). Synaptic defects in ataxia mice result from a mutation in Usp14, encoding a ubiquitin-specific protease. *Nat Genet* **32,** 420-5.

WOJCIK, C., PAWELETZ, N. & SCHROETER, D. (1995). Localization of proteasomal antigens during different phases of the cell cycle in HeLa cells. *Eur J Cell Biol* **68,** 191-8.

WOLBURG, H. & KURZ-ISLER, G. (1978). Induction of paracrystalline arrays by vincristine in the synaptic formations of the teleost retina. *Cell Tissue Res* **191,** 75-82.

WOLF, D. H. & HILT, W. (2004). The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. *Biochim Biophys Acta* **1695,** 19-31.

WOOD, S. A., PASCOE, W. S., RU, K., YAMADA, T., HIRCHENHAIN, J., KEMLER, R. & MATTICK, J. S. (1997). Cloning and expression analysis of a novel mouse gene with sequence similarity to the *Drosophila* fat facets gene. *Mech Dev* **63,** 29-38.

WOODS, D. F. & BRYANT, P. J. (1993). ZO-1, DlgA and PSD-95/SAP90: homologous proteins in tight, septate and synaptic cell junctions. *Mech Dev* **44,** 85-9.

WU, Z., LI, Q., FORTINI, M. E. & FISCHER, J. A. (1999). Genetic analysis of the role of the *Drosophila* fat facets gene in the ubiquitin pathway. *Dev Genet* **25,** 312-20.

WYSZYNSKI, M., KIM, E., DUNAH, A. W., PASSAFARO, M., VALTSCHANOFF, J. G., SERRA-PAGES, C., STREULI, M., WEINBERG, R. J. & SHENG, M. (2002). Interaction

between GRIP and liprin-alpha/SYD2 is required for AMPA receptor targeting. *Neuron* **34,** 39-52.

WYSZYNSKI, M., KIM, E., YANG, F. C. & SHENG, M. (1998). Biochemical and immunocytochemical characterization of GRIP, a putative AMPA receptor anchoring protein, in rat brain. *Neuropharmacology* **37,** 1335-44.

WYSZYNSKI, M., VALTSCHANOFF, J. G., NAISBITT, S., DUNAH, A. W., KIM, E., STANDAERT, D. G., WEINBERG, R. & SHENG, M. (1999). Association of AMPA receptors with a subset of glutamate receptor-interacting protein in vivo. *J Neurosci* **19,** 6528-37.

XIA, J., CHUNG, H. J., WIHLER, C., HUGANIR, R. L. & LINDEN, D. J. (2000). Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PDZ domain-containing proteins. *Neuron* **28,** 499-510.

XIA, J., ZHANG, X., STAUDINGER, J. & HUGANIR, R. L. (1999). Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. *Neuron* **22,** 179-87.

XIAO, M. Y., ZHOU, Q. & NICOLL, R. A. (2001). Metabotropic glutamate receptor activation causes a rapid redistribution of AMPA receptors. *Neuropharmacology* **41,** 664-71.

XIE, Y. & VARSHAVSKY, A. (1999). The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. *Embo J* **18,** 6832-44.

XIE, Y. & VARSHAVSKY, A. (2000). Physical association of ubiquitin ligases and the 26S proteasome. *Proc Natl Acad Sci U S A* **97,** 2497-502.

YAMASHIRO, D. J., TYCKO, B., FLUSS, S. R. & MAXFIELD, F. R. (1984). Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell* **37,** 789-800.

YAMAZAKI, M., FUKAYA, M., ABE, M., IKENO, K., KAKIZAKI, T., WATANABE, M. & SAKIMURA, K. (2001). Differential palmitoylation of two mouse glutamate receptor interacting protein 1 forms with different N-terminal sequences. *Neurosci Lett* **304,** 81-4.

YANAGAWA, Y., HASEZAWA, S., KUMAGAI, F., OKA, M., FUJIMURO, M., NAITO, T., MAKINO, T., YOKOSAWA, H., TANAKA, K., KOMAMINE, A., HASHIMOTO, J., SATO, T. & NAKAGAWA, H. (2002). Cell-cycle dependent dynamic change of 26S proteasome distribution in tobacco BY-2 cells. *Plant Cell Physiol* **43,** 604-13.

YASUDA, H., BARTH, A. L., STELLWAGEN, D. & MALENKA, R. C. (2003). A developmental switch in the signaling cascades for LTP induction. *Nat Neurosci* **6,** 15-6.

YE, B., LIAO, D., ZHANG, X., ZHANG, P., DONG, H. & HUGANIR, R. L. (2000). GRASP-1: a neuronal RasGEF associated with the AMPA receptor/GRIP complex. *Neuron* **26,** 603-17.

YI, J. J. & EHLERS, M. D. (2005). Ubiquitin and protein turnover in synapse function. *Neuron* **47,** 629-32.

YIN, Y., EDELMAN, G. M. & VANDERKLISH, P. W. (2002). The brain-derived neurotrophic factor enhances synthesis of Arc in synaptoneurosomes. *Proc Natl Acad Sci U S A* **99,** 2368-73.

YOU, J. & PICKART, C. M. (2001). A HECT domain E3 enzyme assembles novel polyubiquitin chains. *J Biol Chem* **276,** 19871-8.

YOUNG, S. H. & POO, M. M. (1983). Rapid lateral diffusion of extrajunctional acetylcholine receptors in the developing muscle membrane of Xenopus tadpole. *J Neurosci* **3,** 225-31.

YU, A. & MALEK, T. R. (2001). The proteasome regulates receptor-mediated endocytosis of interleukin-2. *J Biol Chem* **276,** 381-5.

ZALUTSKY, R. A. & NICOLL, R. A. (1990). Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science* **248,** 1619-24.

ZHANG, F., SU, K., YANG, X., BOWE, D. B., PATERSON, A. J. & KUDLOW, J. E. (2003). O-GlcNAc modification is an endogenous inhibitor of the proteasome. *Cell* **115,** 715-25.

ZHAO, Y., HEGDE, A. N. & MARTIN, K. C. (2003). The ubiquitin proteasome system functions as an inhibitory constraint on synaptic strengthening. *Curr Biol* **13,** 887-98.

ZHEN, M., HUANG, X., BAMBER, B. & JIN, Y. (2000). Regulation of presynaptic terminal organization by *C. elegans* RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. *Neuron* **26,** 331-43.

ZHOU, Q., XIAO, M. & NICOLL, R. A. (2001). Contribution of cytoskeleton to the internalization of AMPA receptors. *Proc Natl Acad Sci U S A* **98,** 1261-6.

ZHU, J. J., QIN, Y., ZHAO, M., VAN AELST, L. & MALINOW, R. (2002). Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* **110,** 443-55.

ZHU, X. & CRAFT, C. M. (1998). Interaction of phosducin and phosducin isoforms with a 26S proteasomal subunit, SUG1. *Mol Vis* **4,** 13.

APPENDIX

MATERIALS AND METHODS

CHAPTER 3 - MATERIALS AND METHODS

Immunostaining:

Dissociated postnatal (P1-2) rat hippocampal neuron cultures were prepared according to standard tissue culture techniques and plated at a density of 230–460 mm^2. In all experiments, neurons were used following 14–21 days *in vitro*. For ubiquitin and proteasome immunohistochemistry, neurons were fixed at 4°C with 4% paraformaldehyde/4% sucrose for 20 min. Fixed cultures were then treated sequentially with methanol, 0.2% TritonX-100, preblock (5% normal goat serum and 0.5% TritonX-100 in Dulbecco's PBS), primary Ab (1:100) in preblock at 4°C overnight, Cy3-conjugated secondary Ab in preblock at room temperature, rinsed with preblock and PBS. Using confocal microscopy, we imaged immunostained specimens in PBS. The extent of colocalization between the proteasome and synaptophysin was determined by thresholding both channels for punctate signal and calculating the percent of the proteasome puncta that overlapped with synaptophysin puncta and vice versa. For the FK2 immunostaining experiments, hippocampal cultures were infected with a Sindbis virus expressing GFP. The staining was performed for both the GFP and the ubiquitin and the GFP channel was used as a marker for the dendritic morphology. A reviewer blind to the FK2 signal outlined the "protrusions" from the dendritic surface using GFP template image. A spine mask was then created from these protrusions. The polyubiquitin FK2 signal overlapping with the spine mask was used for quantification.

Live imaging of cultured neurons:

The degradation reporter, Ub^{G76V}-GFP, developed by another group, consists of GFP carrying a constitutively active degradation signal (Dantuma et al., 2000). The Ub^{G76V}-GFP reporter possesses a ubiquitin fusion degradation signal consisting of an N-terminally linked ubiquitin that can serve as an acceptor for polyubiquitin trees. The G76V mutation prevents the removal of ubiquitin by cellular ubiquitin hydrolases, leading to the ubiquitination and proteasomal degradation of the entire Ub-GFP fusion. The Ub^{G76V}-GFP coding sequence was cloned into pSinRep5 from the EGFP-N1-Ub^{G76V}-GFP vector by standard cloning techniques. Bath stimulation of neurons: During the experiment, cells were continuously bath perfused with HBS/aniso (flow rate =1.5 ml/min) at 37°C. Cells were stimulated with either KCl (1.5 minutes, high KCl-HBS (same as HBS except for 55 mM NaCl and 60 mM KCl)) or NMDA (20 μM, 3 min) by switching the perfusion medium. APV (50 μM, 1h) and MG132 (10 μM, 10 min) were used to antagonize NMDA receptors and proteasome activity, respectively. The cells were imaged with a Zeiss LSM 510 microscope with 40X oil objective and 2X zoom. Z-stacks were acquired at the indicated time points and flattened. In order to quantify the spine fluorescent signal, all the images in the time series were summed and all the "protrusions" away from the dendritic surface were selected as spines on the summed image. Then the percent change in the mean intensity of the spine signal over time is quantified. Local stimulation**:** The local perfusion set-up was as previously described (Aakulu et al., 2001). Before perfusion, the neurons were kept in HBS (37° C) for 1

hour with 40 μM anisomycin to block protein synthesis. In order to visualize the local protein degradation, dendritic segments of cultured hippocampal neurons expressing the Ub^{G76V}-GFP proteasome activity reporter were perfused locally with 50 μM NMDA for 6 minutes. The perfusion pipette contained Alexa-568 dye in order to visualize and quantify the dimensions of the perfusion spot. Cells were also bath perfused with HBS (37° C) during local stimulation. Images were acquired on Olympus IX50 microscope with 40X air objective lens and 2X zoom using FluoView Image acquisition Software. Z-stacks were acquired every 2 minutes (2 baselines + 4 NMDA) with 0.5 μM slice thickness. After the images were flattened, the mean intensity of the GFP signal in the perfusion spot was compared over time and to the adjacent dendritic segments and non-perfused dendrites.

CHAPTER 4 - MATERIALS AND METHODS

Immunostaining and Receptor Internalization Assays:

The sources of the antibodies were as follows: ubiquitin (pAb Calbiochem), α-proteasome (pAb Calbiochem), α-proteasome (pAb Calbiochem), GFP (Clonetech), GluR1 (pAb Oncogene), and GluR2 (mAb Chemicon). In receptor internalization assays, neurons were prelabeled with GluR1 (Oncogene) or GluR2 (Chemicon) antibodies for 15–20 min with either MG132 (50 μM, Peptide International), carboxybenzyl leucyl-leucyl-leucine vinyl sulfone (ZL3VS, 50 μM; gift from Hidde Ploegh), lactacystin

(Calbiochem) proteasome inhibitors, or vehicle control (DMSO) in conditioned media. The proteasome inhibitors are cell permeable substrate analogs that reversibly (MG132) or irreversibly (lactacystin and ZL3VS) inhibit primarily the chymotrypsin-like activity of the proteasome. The neurons were then briefly washed with PBS-MC (PBS + 1 mM MgCl2, 0.1 mM CaCl2). The media was then replaced with conditioned media containing TTX (1 μM) and APV (50 μM) alone or with AMPA (100 μ M) for 2.5 or 20 min (with or without proteasome inhibitors). The neurons were then washed once with cold PBS-MC and incubated on ice with cold 0.5 M NaCl and 0.2 N acetic acid for 4 min to remove all remaining extracellular receptor bound antibodies, washed with PBS-MC, and then fixed for 5 min with 4%paraformaldehyde/4% sucrose solution. The neurons were washed with PBS-MC and then permeabilized and blocked with 0.1% TX-100 in PBS-MC + 2% BSA. Neurons were then labeled with rabbit or mouse Alexa 488 secondary antibodies (Glur1 and GluR2, respectively; Molecular Probes). The internalization of transferrin was conducted with modifications to Sever et al. (2000). In brief, dissociated hippocampal neurons were pretreated with MG132 (50 μM) or DMSO vehicle in conditioned media for 20 min at 37 °C. The cells were then washed 1x with PBS-MC (see above), and the solution was replaced with 20 μg/mL of Alexa 488 transferrin (Molecular Probes) in PBS-MC containing DMSO or MG132 (50 μM) for an additional 20 min at 37 °C. The cells were washed with PBS-MC and fixed for 10 min with 4% paraformaldehyde/4% sucrose and washed with PBS. The cells were then imaged using confocal microscopy and the images were analyzed with NIH Image J analysis software. In the inhibitor pretreatment time course experiments, the period of pretreatment refers to the period of time preceding AMPA stimulation that neurons were exposed to the

inhibitor. As in the above experiments, the inhibitor remained present during AMPA treatment. In the case of Sindbis EGFP or Sindbis His6-myc-ubiquitin K48R IRES EGFP-infected neurons, rabbit or mouse Alexa 568 antibodies (Glur1 and GluR2, respectively; Molecular Probes) were used. As acid stripping treatment eliminated GFP fluorescence, the neurons were labeled with either mouse or rabbit GFP antibodies and Alexa 488 secondary antibodies to detect infected neurons.

Free Ubiquitin Pool:

Free ubiquitin was determined by Western blot analysis. In brief, hippocampal neurons were treated with vehicle (DMSO) or 50 μM MG132 for 5, 20, or 40 min or 24 hr. Cells were lysed in sample loading buffer, boiled for 5 min, run on 15% SDS-PAGE, and transferred to PVDF membrane. The blot, after hydration, was placed in boiling water for 5 min and then blocked in 5% milk in TBST. Immobilized proteins were then detected by the same pAb ubiquitin antibody. Levels of ubiquitin were normalized to actin levels in the same lane.

Image Acquisition and Analysis:

Images of internalized GluR were acquired on an Olympus microscope (40X or 63X immersion objectives) with a Hamamatsu CCD camera. All images were taken from similar focal planes. Images were acquired with Image Pro-Plus acquisition software and

analyzed on NIH Image J version 1.24. In all experiments, the majority of the analyses were conducted blind; as the results from blind analysis did not differ at all from that conducted non-blind, results were pooled. For analysis, a measure of background fluorescence was used to determine threshold values, which was then applied to all sample images in a given experimental set (including controls). The measured fluorescence was then divided by total area to determine mean fluorescence. To avoid signal saturation in the analysis of surface GluR1 and 2, measurements were made from the dendrites. A threshold value above diffuse dendritic fluorescence was used for analysis to determine mean dendritic puncta fluorescence. The same value was applied to both control and experimental samples. Images shown in Figures 1 and 4 were acquired via confocal microscopy (Olympus, with Fluoview acquisition software) with a 60X oil objective lens. Shown are the compressed z-stack images. Internalized perinuclear transferrin was quantified by using the middle z sections (comprising ~4.0 μm, not including surface plasma membrane) in which the nucleus was clearly present. The fluorescent signal in the cell body, not including the surface membrane, was analyzed in control and MG132-treated neurons.

CHAPTER 5 - MATERIALS AND METHODS:

Antibodies:

The following antibodies were used as indicated: for immunocytochemistry: PSD-95 6G6 (ABR) and GluR1 (Oncogene). For PSD-95 immunoprecipitation, either rabbit

polyclonal (gift of Mary Kennedy), Upstate mouse monoclonal K28/43 or PSD-95 6G6 (ABR); for ubiquitin immunoprecipitation, FK2 mouse monoclonal; for PSD-95 immunoblotting, rabbit polyclonal; for ubiquitin immunoblotting, either DAKO rabbit polyclonal, FK2 mouse monoclonal or BD Pharmingen mouse monoclonal 6C1.17; for Shank immunostaining, rabbit polyclonal (gift of Eujoon Kim); for actin immunostaining, Sigma mouse monoclonal AC-40.

Immunocytochemisty:

Dissociated postnatal (P1–2) rat hippocampal neuron cultures prepared as previously described, plated at a density of 230–460 mm^2 (Chapter 4 - Materials and Methods). Two to three week old cultures were used for all the experiments. Receptor internalization assays have been performed as described previously (Chapter 4 - Materials and Methods). Briefly, neurons were kept in the presence or absence of proteasome inhibitor MG132 (50 μM, Peptide International) for 20 min along with GluR1 antibody and they were stimulated with AMPA (100 μm, 20 min). Following acid stripping and fixation, neurons (Patrick et al., 2003) were labeled with PSD-95. Internalized receptors and PSD-95 were visualized by Alexa-568 rabbit and Alexa-488 mouse secondary antibodies (Molecular Probes), respectively. For PSD-95 staining only, live labeling, acid stripping and Alexa-568 rabbit secondary steps were omitted.

Transfection:

In PSD-95 GFP overexpression experiments, neurons were transfected with PSD-95-GFP (a gift of Morgan Sheng) or GFP constructs by Lipofectamine 2000 (Invitrogen). GFP images were acquired before AMPA stimulation. Then, neurons were processed to label internalized receptors as described above.

Image acquisition and analysis:

Images shown in Fig. 5.1 and Fig. 5.2 were acquired via confocal microscopy (Olympus, with Fluoview acquisition software) with a 60X oil objective lens. Shown are the compressed z-stack images. Images shown in Fig. 5.3 were acquired with Olympus microscope (40X immersion objective) with a Hamamatsu CCD camera. All images were taken from similar focal planes. Images were acquired with Image Pro-Plus acquisition software and analyzed on NIH Image J. In both Fig. 5.1 and Fig. 5.2, internalized receptor and PSD-95 puncta from straightened dendrites were thresholded in such a way to maximize the particle number. Thresholds used for individual images were not significantly different between conditions. The thresholded puncta signal was used to quantify the number and total intensity of puncta per dendritic length. In Fig. 5.3, internalized receptor signal is quantified from the cell bodies as the fluorescence intensity per unit area.

PSD-95 Western blotting and synaptosome stimulation:

Two to three week old hippocampal cultures were lysed in RIPA buffer after incubation in MG132 (50 μM, 20 min) before and during AMPA stimulation (100 μM, 20 min). Equal amount of proteins from all conditions were run on a 4–15% gradient gel (Bio-Rad). After gel transfer, membranes were probed for PSD-95 and actin.

Synaptosomes were prepared from ~30 days old rat hippocampus as described in Bagni et al. (2000). Stimulation was performed in buffer containing 10 mM Tris, pH 7.5, 2.2 mM $CaCl_2$, 0.5 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, and 80 mM NaCl. Stimulation was performed at 37 °C with or without MG132 (50 μM, 15 min) pre-treatment followed by AMPA treatment (100 μM, 20 min) in the absence or presence of MG132.

Immunoprecipitation and GST-S5a chromatography:

For immunoprecipitation and GST-S5a chromatography experiments, lysates from either two to three week old hippocampal cultures or whole hippocampus from Sprague–Dawley rats were used. GST-S5a affinity chromatography was performed as described earlier (Ehlers, 2003). Immunoprecipitations were performed as described in Colledge et al. (2003). Cultures were kept in MG132 (50 μM, 20 min) before stimulation. Stimulation was performed either with NMDA (20 μM, 3 min followed by 10 min incubation (Fig. 5.4A–D) or for the "time course experiment" (Fig. 5.4E and F), 3 min stimulation followed by 0, 2, 7, 12 min incubation in conditioned media (Colledge

et al., 2003) or AMPA (100 μM, 20 min). For both GST-S5a chromatography and immunoprecipitations, lysis buffer contained 50 μM MG132 and 1 μM ubiquitin aldehyde (Calbiochem). Deubiquitinating enzyme application has been performed by applying 5 μg each of isopeptidase-T (Calbiochem) and UCH-L3 (Affiniti Research, Exeter, UK) to the beads for 15 min at room temperature.

CHAPTER 6 - MATERIALS AND METHODS

Sindbis Virus Constructs:

Rpt1-GFP, a tagged ATPase-type subunit of the regulatory 19S cap complex, (yeast) coding sequence was amplified from the pBS-Rpt1-GFPHA-HU plasmid with PCR and subcloned in to pcDNA3.1 and subsequently to pSinRep5 vector (Invitrogen) (Enenkel et al., 1998). To generate the α4-venus Sindbis virus , the α4 (MGC# 2581897) cDNA clone in pSPORT6 and venus clone in pCS2-venus were used to amplify α4 and venus PCR products, respectively. Then, venus was cloned in to pcDNA3.1 (venus-pcDNA3.1), followed by the cloning of α4 in to the upstream of the venus coding sequence. The α4-venus was then subcloned into the pDNR-1 vector followed by recombination into pSinRep5-loxP acceptor vector using the cre recombinase (BD Biosciences). In order to test the incorporation efficiency of Rpt1-GFP in to the endogenous proteasome structure, endogenous proteasome from cultures expressing Rpt1-GFP was immunoprecipitated with a subunit-specific antibody against the Rpt6 subunit. The immunoprecipitates were analyzed for the Rpt1-GFP and another

endogenous proteasome subunit, Rpt3. Rpt1-GFP-IRES-mRFP was generated from the pSinRep5-Rpt1-GFP clone by inserting the IRES (pLP-IRES2-EGFP) and mRFP sequences downstream of Rpt1-GFP sequence. The Sindbis virus was produced according the instructions provided by the manufacturer (Invitrogen). Ambion SP6 mMessage Kit was used to produce the RNA for virus production.

Cultured hippocampal neurons:

Neurons cultured for 18-21 DIV were used in all experiments. Neurons were infected by washing them twice with HEPES-buffered solution (HBS) (110 mM NaCl, 5. 4 mM KCl, 1. 8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES-NaOH (pH 7.4)) (37° C), followed by 30 min incubation with the diluted Sindbis virus in conditioned growth media. After infection, cells were washed twice with HBS (37° C) and were further incubated for 12 hours (for GFP- and venus-tagged proteasome subunit expression).

Live imaging of cultured neurons:

Bath stimulation of neurons. To isolate the dynamics of existing proteasome subunits, neurons were kept in anisomycin prior to and during the live imaging of GFP-tagged proteasome subunits. During the experiment, cells were continuously bath perfused with HBS/aniso (flow rate =1.5 ml/min) at 37° C. The live imaging set-up was described in Chapter 3 – Materials and Methods.

FRAP/FLIP:

All of the FRAP/FLIP experiments were performed at 37°C with a Zeiss LSM 510 using a 40X oil objective and 4X zoom. The cultured neurons expressing Rpt1-GFP were imaged before stimulation and 3 - 4 spines with a long neck were chosen to be monitored. For the FRAP experiments, single z-slice images were taken in order to be able to capture the bleaching of the Rpt1-GFP. After taking 5 baseline images, each spine was bleached one time for 150 msec using a laser intensity 200 times greater than that used for image acquisition. After bleaching, recovery of the fluorescence was measured as a function of time. For the FLIP experiments, after taking 3 baseline images, ~ 10 μm of the dendritic shaft was bleached repeatedly for 45 msec before taking the next image in the time series. The loss of the fluorescence from the spines adjacent to the bleached shaft region was monitored with time. After acquiring the pre-stimulation data as described above, neurons were stimulated with KCl, and, and a second round of bleaching (same parameters) was performed on the same spines for the FRAP and the same dendritic shaft segment for the FLIP experiments.

The FRAP data were fit to a one-phase exponential function by using the (Bottom to (Span +Bottom) analysis. ($Y= Span*(1 - \exp(-X/\tau)) + Bottom$) in the GraphPad Prism 4.3 software. Similarly, the FLIP data were fit to a one-phase exponential decay function ($Y=Span*\exp(-X/\tau) + Plateau$) for the prestimulation curve. For the FLIP poststimulation experiments, the data was better fit by a linear function. The time

constant (τ) and the immobile fraction were calculated from the exponential function equations.

Immunocytochemistry:

The antibodies that were used for immunocytochemistry were as follows: Chicken anti-GFP (Aves Labs), mouse anti-actinin-2 (clone EA-53, Sigma), mouse anti-bassoon (StressGen), rabbit core-proteasome (α5, α7, β1, β5i, and β7) (Calbiochem), rabbit core-proteasome (BIOMOL), rabbit anti-Rpt3 (BIOMOL), mouse anti-ubiquitin (FK2 clone, Affiniti), rabbit anti-GRIP CT (Upstate). The actin cytoskeleton was stained with Rhodamine-Phalloidin (Molecular Probes). The secondary antibodies that were used for immunocytochemistry were as follows: Alexa 488 anti-Chicken, Alexa 647 anti-mouse, Alexa 488 anti-Rabbit, Alexa 546 anti-Mouse (all of the secondary antibodies were from Molecular Probes). All the image acquisition was performed with Zeiss LSM 510 Microscope with 40X oil objective and 2-3X zoom. To analyze immunocytochemistry images, LSM files were processed by NIH ImageJ and custom macros were used to quantify colocalization. All of the channels were processed for thresholding and watershed segmentation in 3D, and the total and colocalizing particles were quantified for the mean number, area, and the intensity. For the cytoskeleton experiments, Latrunculin A (5 μM, 24 h, Molecular Probes) and vincristine (5 μM, 5 h, Sigma) were used to disrupt the microfilaments and the microtubules, respectively. For the endogenous proteasome experiments, cultured neurons were stimulated with KCl (1.5 - 3 mins, 60 mM) in conditioned medium and further incubated at 37°C for 12 min. After

stimulation, the neurons were transferred quickly on to ice and processed for immunocytochemistry. Detergent extraction experiments were performed with 0.5 % TritonX-100 as described earlier (Allison et al., 1998). Cultured neurons were stimulated with 20 μM NMDA for 1 minute followed by immediate 50 μM APV wash in order to decrease the neurotoxic effects of NMDA. After NMDA stimulation cultures were incubated for another 19 minutes in the presence of APV. Control dishes received the APV incubation as well, but not the NMDA stimulation. After this stimulation protocol, dishes were processed for fixation with or without prior detergent extraction.

Slice stimulation, synaptosome preparation and Western blot analysis:

Before stimulation, slices were submerged in a chamber and continuously perfused with 32°C artificial cerebral spinal fluid (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11.0 mM D-glucose). Stimulation was performed with ACSF containing 60 mM KCl for 6 minutes. 30 minutes after KCl stimulation, slices were immediately transferred into cold ACSF and processed for synaptosome preparation. Synaptosomes were lysed in 0.2% SDS followed by 1% TritonX-100 containing lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, protease inhibitor mix (Roche)) and equal amounts of proteins were resolved on 10% SDS-PAGE. The antibodies used for Western blotting analysis were as follows: chicken anti-GFP (Aves Labs), mouse anti-α7 (BIOMOL), mouse anti-α3 (BIOMOL), mouse anti-Rpt1 (BIOMOL), mouse anti-Rpt6 (mouse), mouse anti-Rpt3 (BIOMOL), rabbit anti-Rpt3 (BIOMOL), rabbit synapsinI (Chemicon). Appropriate HRP-conjugated secondary

antibodies (HRP-conjugated anti-mouse IgG ab, HRP-conjugated anti-rabbit IgG ab (Jackson ImmunoResearch Laboratories), and HRP-conjugated anti-chicken IgY ab (The Aves Labs)) were used for the detection with ECL Chemiluminescence Reagent (Amersham Biosciences).

Statistical Analysis:

All of the error bars indicate the standard error of the mean. A non-paired two-way Student's t-test was used where indicated. For multiple groups, a one-way ANOVA was performed. All of the data were tested for normal distribution by the Anderson-Darling Test. GraphPad Prism v4.3 as used to compute the p values.