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Roles of ubiquitination at the synapse

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Abstract

The ubiquitin proteasome system (UPS) was first described as a mechanism for protein degradation more than three decades ago, but the critical roles of the UPS in regulating neuronal synapses have only recently begun to be revealed. Targeted ubiquitination of synaptic proteins affects multiple facets of the synapse throughout its life cycle; from synaptogenesis and synapse elimination to activity-dependent synaptic plasticity and remodeling. The recent identification of specific UPS molecular pathways that act locally at the synapse illustrates the exquisite specificity of ubiquitination in regulating synaptic protein trafficking and degradation events. Synaptic activity has also been shown to determine the subcellular distribution and composition of the proteasome, providing additional mechanisms for locally regulating synaptic protein degradation. Together these advances reveal that tight control of protein turnover plays a conserved, central role in establishing and modulating synapses in neural circuits.

Keywords

Ubiquitin; Proteasome; Synaptogenesis; Neurotransmission; Synaptic plasticity; Angelman syndrome

1. Introduction

Proteasome-mediated protein degradation has been known for 30 years [1,2], but the complexity, regulative capacity and importance of this system has only been widely appreciated in the past decade. Indeed, a high percentage of the human genome is devoted to encoding ubiquitin proteasome system (UPS) effector proteins, and a rapidly growing list of diseases are linked to mutations in these genes [3,4]. In particular, the UPS is closely implicated in many diseases that involve or are exclusive to the nervous system, including neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [5], and neurodevelopmental diseases such as autism spectrum disorders and Angelman syndrome [6]. In all of these diseases, synaptic dysfunction is hypothesized to play a prominent role in pathogenesis.

The UPS functions locally at neuronal synapses [7,8], and protein degradation is now recognized alongside protein translation as a primary means for regulating the abundance and trafficking of proteins critical for neurotransmission. The multiple described UPS synaptic roles include feedback control of transcription, coordination of synapse development vs.

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elimination, local maintenance of neurotransmission strength, and mediating activity-dependent modulation of pre- and postsynaptic function [9–15]. Thus, elucidating UPS regulation at the synapse is now understood to be critical to understanding the physiological changes that determine neurotransmission strength during development and plasticity, as well as the pathological synaptic function changes that occur in a growing range of inherited neurological diseases.

There have been a number of relatively recent reviews of UPS regulation of neuronal and synaptic properties [7,15–18]. Therefore, in this review we focus primarily on the most recent published work that provides novel insights into UPS function at the synapse. In particular, we discuss newly-discovered UPS mechanisms that locally regulate protein degradation to control synaptic functions and new data revealing that proteasome trafficking dictates protein turnover locally at the synapse.

2. The ubiquitin proteasome system

2.1. Enzymatic cascade

Ubiquitin is a 76 amino acid protein conserved across eukaryotic cells. The covalent attachment of ubiquitin to a substrate protein tags that protein for intracellular trafficking or proteasomal degradation [19,20]. Tagging is a highly regulated process that can be controlled at multiple points. The first step is activation of ubiquitin by an activating enzyme (E1) that utilizes an ATP-requiring reaction to generate a high-energy thioester intermediate, E1-S~ubiquitin. The thioester attachment induces a conformational change in the E1 that promotes association with an ubiquitin carrier protein (E2). Next, activated ubiquitin is transferred to the E2 via formation of an additional high-energy thiol intermediate, E2-S~ubiquitin, leading to dissociation from the E1 [21]. In the third step, a substrate-specific ubiquitin E3 ligase interacts with the target protein-E2 ubiquitin complex to transfer ubiquitin to the target protein. In this process an isopeptide linkage is formed between the C-terminal carboxyl group of ubiquitin and the ϵ -amino group on a lysine of the target protein. Additional ubiquitin proteins may be attached to the initial ubiquitin via a lysine 48 (Lys48) linkage, or less commonly via one of the other six possible ubiquitin lysine linkages (Lys6, 11, 27, 29, 33, or 63), forming polyubiquitin chains that may be linear or branched [22,23]. In addition, ubiquitin attachment to additional substrate protein lysine residues may produce multi-ubiquitination.

Mono and multiple mono-ubiquitin attachments may serve as signals for endocytosis or endosomal trafficking of membrane proteins [19]. Polyubiquitin chains of at least four lysine 48 ubiquitins are generally thought to be necessary to target a protein to the proteasome for degradation [24,25]. However, in some cases, Lys29 and Lys63 linked polyubiquitin chains have also been shown to be proteasome degradation signals [22,26]. In contrast, forked chains with multiple linkages do not appear to signal proteasomal degradation [22]. Likewise, in other circumstances, Lys63 ubiquitin linkages allow for specific protein–protein interactions important in signal transduction [27], and in DNA repair [28]. In general, the effects of alternate lysine ubiquitin linkages are still poorly understood. The ubiquitination process is reversible through the action of deubiquitinating enzymes, cysteine proteases that remove the ubiquitin chains from substrate proteins to regenerate free ubiquitin [29].

2.2. E3 ubiquitin ligases

In mammals, there are only two E1 ligases [30], but dozens of E2 ligases, and hundreds of E3 ligases. Ubiquitination specificity is determined principally by this large variety of E3 ligases, which generate the vast number of E2/E3 combinations that each target specific groups of protein substrates. The E2/E3 combination has also recently been shown to determine the ubiquitin chain linkages that are possible [22], dictating the location and types of ubiquitin

attachment. There are two primary domain classes of E3 ligases: HECT (*homologous to E6-AP carboxy terminus*) domain and RING (*really interesting new gene*)-finger domain. There are approximately 50 different HECT E3s in humans, of which the proto-typical member is E6AP (papillomavirus *E6-associated protein*), that has since been designated UBE3A. HECT-domain E3s participate directly in the catalytic transfer of ubiquitin to the substrate protein to form an E3-ubiquitin thioester complex at an active-site cysteine residue within the HECT domain [30]. RING-finger E3s number several hundred in humans, and subgroups include those that function as single proteins, such as parkin, and those that function in multi-subunit proteins. One example of a multi-subunit RING-finger E3 is the Skp1/Cullin/F-box (SCF), consisting of a core complex composed of the Skp1 linker, the Cdc43/Cul1 scaffold, the Rbx1/Roc1/Hrt1 RING-finger E3, and one of a suite of F-box proteins that serve as substrate recognition adaptors [32,33]. Another example is the anaphase promoting complex (APC), composed of at least twelve subunits, with the APC11 RING-finger E3, the APC2 Cul1-related scaffold, and either the Cdh1 or the Cdc20 activator protein, together forming the catalytic core of the complex [34,35]. There are also two related RING-finger like domains, the U-box and PHD/LAP (*plant homeodomain and leukemia-associated protein*) that confer E3 ligase activity [36]. Unlike HECT domain ligases, RING-finger E3s do not form ubiquitin thioester complexes, but instead serve primarily as scaffolds, orienting the E2-ubiquitin complex and the target protein for ubiquitin transfer. A more detailed characterization of E3 ligase structure and function can be found in several recent reviews [31–33,37].

2.3. Proteasome complex

Once committed to the degradation pathway, polyubiquitinated proteins are actively targeted to the proteasome, a proteolytic complex consisting of a 20S core catalytic component with two 19S regulatory subunits attached at either end [20]. The 20S proteasome is a 700 KDa complex composed of two each of α 1–7 and β 1–7 subunits. These 28 total subunits are arranged in four axially stacked heptameric rings with the successive layers consisting of α 1–7, β 1–7, β 1–7, α 1–7 [38]. The 19S regulatory complex, also known as PA700, contains 20 subunits, including six distinct AAA-family ATPases (Rpt1–Rpt6) and fourteen non-ATPase subunits (Rpn1–Rpn14), one of which (Rpn10/S5a) contains a ubiquitin interaction motif (UIM) that binds polyubiquitin [39]. Positioned on either end of the catalytic core, the 19S complexes serve as gatekeepers for substrate entry into the proteasome [38].

The 20S proteasome exists in at least two forms, with substitution of inducible subunits: β 1i (LMP2), β 2i (MECL-1), or β 5i (LMP7) for the constitutive β 1, β 2, and β 5 subunits under certain conditions such as after γ -interferon induction [40,41]. There are also multiple different 11S regulatory complexes that can replace the 19S regulator, designated P28 α , P28 β , and P28 γ [42]. These alternate regulators do not have ATPase function and do not bind polyubiquitin chains. Proteasomes with inducible beta subunits or 11S substitutions for 19S regulators have higher levels of proteolytic activity [43,44].

3. UPS regulation of synaptogenesis

3.1. Axon growth and synapse formation

At the tip of outgrowing axons, motile growth cones sense guidance cues and translate this information into dynamic cytoskeletal reorganizations that orient growth in a specific direction [45]. UPS activity is stimulated by some of these guidance cues and, along with local protein synthesis, protein degradation is believed to play a dynamic role in the structural rearrangements that mediate axon outgrowth [46]. The anaphase promoting complex (APC), one of the first ubiquitin ligases to be identified in post-mitotic neurons [47], has been strongly implicated in coordinating axon growth programs. RNAi knockdown of the APC activator subunit, Cdh1, increased axon length and the rate of axon growth in cultured rat cerebellar

granule neurons. Importantly, in cerebellar slice assays, Cdh1 knockdown also revealed axon tract patterning abnormalities, with defasciculation of fiber tracts and axons wandering across inappropriate layers, including across white matter tracts where myelin traditionally prevents axon growth [12,48]. In non-neuronal cells during cell division, the APC acts predominantly in the nucleus to regulate cell cycle transitions by coordinating the UPS-mediated degradation of transcription factors, such as the transcriptional co-repressor SnoN. Similarly, in post-mitotic neurons, the APC was found to predominantly localize to the nucleus where Cdh1-APC ubiquitinates and targets SnoN for degradation [12,48]. Preventing the APC interaction with SnoN completely eliminated the restraint on axon growth, but did not affect axon patterning in the cerebellum *in vivo* [48], suggesting the presence of additional APC targets.

Subsequently, Cdh1-APC was shown to ubiquitinate and downregulate another transcriptional repressor, Id2 (inhibitor of DNA binding 2) in cultured rat cerebellar neurons. This regulation followed a distinct developmental pattern with an increased rate of Id2 degradation during neuronal maturation. In a search for Id2 substrates, gene expression profiling identified the Nogo receptor, an inhibitor of axonal growth on which myelin inhibitory signals act. Intriguingly, expression of an Id2 mutant resistant to Cdh1-APC mediated degradation, allowed cerebellar neurons to extend axons in the presence of a Nogo receptor ligand, myelin-associated glycoprotein [49], supporting a prominent role for Id2 abundance in determining myelin inhibition of axonal growth. Moreover, the increased rate of Id2 degradation during development of neuronal connections suggests that Cdh1-APC may act as a “neuronal state switch” similar to its role in cell cycle transitions, coordinating the timing of transcriptional programs that regulate axon growth and patterning. Proteins involved in axon growth that are regulated downstream of Id2 include Semaphorin-3a, Jagged-2, Unc5a, and Notch1 [49]. Future work should address the upstream activators of Cdh1-APC in neurons to provide additional insight into the processes that coordinate the developmental timing of transcriptional regulation, and assess whether it has a similar role in the many other CNS regions where APC is expressed.

The critical role for the UPS in regulating terminal axon pathfinding and differentiation has been highlighted by exciting results from genetic screens in *C. elegans* and *Drosophila*. In *C. elegans*, screens for regulators of synaptic growth/differentiation of GABAergic and glutamatergic synapses identified the presynaptic E3 ligase, RPM-1 [50,51]. RPM-1 was found to act as a positive regulator of growth, as RPM-1 mutants have a reduced number of synapses. Another genetic screen identified FSN-1 as an RPM-1-interacting protein. FSN-1 contains an F-box domain and functions in a unique SCF ubiquitin ligase complex in which RPM-1 substitutes for the traditional Rbx1 E3 ligase [52]. Further evaluation showed RPM-1 to be a negative regulator of the p38 MAPK (mitogen-activated protein kinase) signaling pathway, mediating the ubiquitination and degradation of the MAPKKK (MAPK kinase kinase) DLK [53] (Fig. 1). In addition, RPM-1 was recently shown to function independent of its ubiquitin ligase activity in a pathway that signals axon termination [54].

Independently, a *Drosophila* screen for neuromuscular junction (NMJ) synaptic overgrowth identified the RPM-1 homologue, Highwire. In contrast to the effects of RPM-1, *highwire* mutants showed a dramatic increase in the number of NMJ synaptic boutons and synaptic branches, and an increase in total synaptic area [55–57]. The signaling pathway driving this synaptic morphology regulation is complex (Fig. 1). Highwire negatively regulates the transforming growth factor β /bone morphogenetic protein (TGF- β /BMP) signaling cascade, by binding to and promoting the degradation of the SMAD transcriptional co-regulator protein, Medea, which acts as a downstream effector in the pathway [58]. Mutations that impair BMP signaling partially suppress the *highwire* synaptic overgrowth phenotype, whereas excess activation of the BMP pathway leads to synaptic overgrowth. The mechanisms through which BMP signaling tunes synaptic growth remain to be elucidated. More recently, Collins et al.

used a forward genetic screen to show Highwire also targets Wallenda, a MAPKKK homologue to aforementioned *C. elegans* DLK [58], suggesting that UPS presynaptic regulation of a MAPK signaling pathway for regulating growth is highly conserved. Downstream of Wallenda, JNK MAPK and Fos transcription factor activity were necessary for expression of the synaptic overgrowth phenotype [59] (Fig. 1). Similar to RPM-1, Highwire has now been shown to participate in a SCF ubiquitin ligase complex with the F-box protein DSfn [60]. Thus, the RPM-1/Highwire E3 ligase plays an essential role in a conserved presynaptic signaling pathway targeting MAPKKK degradation and the encompassing MAPK pathways. Critical components of both the MAPK and TGF- β /BMP signaling cascades are present in the presynaptic terminal, but much work remains to determine whether the principle effects of RPM-1/Highwire mediated degradation of MAPKKK and Smad proteins are local and synapse-specific, or result from more global nuclear regulation of transcriptional programs that affect synaptic growth and patterning.

The work in *C. elegans* and *Drosophila* has led to the discovery that vertebrate orthologues of RPM-1/Highwire also affect axonal differentiation. In zebrafish, mutations in the RPM-1/Highwire orthologue, Esrom, were shown to disrupt fasciculation, targeting, and mapping retinal axons. This showed that this E3 ligase has additional roles in the CNS involved with axon patterning [61]. In mice, knockout of Phr1, a mammalian orthologue to Highwire/RPM-1/Esrom, led to impaired phrenic nerve development and severely disrupted phrenic nerve terminal morphology [62]. In the CNS, Phr1 knockout mice display striking defects in major axon tracts that include the internal capsule and anterior commissure. Mice with knockout of both Phr1 and the Wallenda/DLK MAPKKK homologue maintain the axon tract deficits, showing that this effect on axon patterning must be mediated through a separate signaling pathway than that regulating synaptic development in *C. elegans* and *Drosophila* [63]. These studies illustrate that the function of highwire/RPM-1/Esrom/Phr1 family of E3 ligases is highly conserved in presynaptic neurons, critically modulating regulation of axon growth and synapse differentiation. Despite this conservation, vertebrate studies demonstrate that these proteins have likely acquired additional functions in regulating axon tract patterning that utilize alternate signaling pathways. Further work on this exciting family of SCF E3 ligases is needed, particularly in mammalian systems, to more clearly define their mechanisms for determining axonal growth, differentiation, and patterning. It will be interesting to see if their effects overlap with those of Cdh-1 APC at the level of coordinating transcriptional programs.

Other neuronally expressed E3 ligases may be involved in overlapping mechanisms in growth cones and presynaptic terminals. APC was identified at the *Drosophila* NMJ through immunolabeling and found to negatively regulate synaptic size by initiating degradation of the scaffold protein Liprin- α [64]. It has not been determined whether APC is expressed in mammalian synapses. However, another RING-domain E3 ligase, Rnf6, was identified in cultured rat hippocampal neurons, where it is highly expressed in axonal projections and co-localizes with LIM kinase 1 (LIMK1) in the growth cone [65]. Rnf6 polyubiquitinates LIMK1, targeting it for proteasome-mediated degradation. Overexpression or knockdown of Rnf6 reduces or stimulates axon outgrowth, respectively [65].

The HECT domain E3 ligase, Ube3a, has also recently been shown to be present in the growth cone [66]. A maternally inherited deficiency for UBE3A causes Angelman syndrome (AS), a neurodevelopment disease characterized by mental retardation, impaired expressive language development, frequent jerky limb movements and difficult to control seizures [67]. At the *Drosophila* NMJ, overexpression of the *Drosophila* homologue (Dube3a) causes an increase in the number of synaptic boutons when expressed presynaptically, and a decrease in bouton number when expressed postsynaptically (Lawrence Reiter, personal communication). These changes may be mediated through the Rho-GEF (Rho-guanine exchange factor) Pebble, and its regulation of the actin-binding GTPase Rac2, which function in a pathway that regulates

dynamic reorganization of the actin cytoskeleton. Mutations in *pebble* have been shown to disrupt neuronal outgrowth at the NMJ [68], and the Pebble protein has been identified as a putative Ube3a target in a *Drosophila* proteomic screen. The involvement of the Pebble orthologue, ECT2, in the pathogenesis of AS was validated in Ube3a deficient mice where ECT2 is mislocalized in hippocampus and cerebellum [69]. While this is an intriguing starting point for future work, many questions about the function of Ube3a at the synapse remain, as no definitive synaptic substrate proteins for Ube3a have yet been identified. In particular, it will be interesting to see a detailed analysis of synaptic changes in both *Drosophila* and mouse AS disease models.

The process of synaptogenesis involves both structural and functional specialization of presynaptic and postsynaptic specializations. The sequence of synaptogenic events has been best characterized at the NMJ synapse, in both invertebrates and vertebrates [45,70]. At the *Drosophila* NMJ, Commissureless (Comm) is a postsynaptic membrane protein that must undergo endocytosis for normal synaptogenesis to occur [71]. Recently, Ing et al. reported that the UPS regulates Comm endocytosis through the action of the HECT-domain E3 ligase Nedd4 [11]. Nedd4 ubiquitinates Comm to trigger its endocytosis. Multiple conditions that prevent Comm ubiquitination or the Nedd4-Comm interaction lead to accumulation of Comm on the surface membrane and consequent aberrant innervation [11]. Use of a monoubiquitin-Comm construct that did not allow longer ubiquitin chains to form was sufficient to promote Comm endocytosis during synaptogenesis [11], suggesting that polyubiquitination is not required.

At the mammalian NMJ, the postsynaptic receptor tyrosine kinase MuSK (muscle specific kinase) and the presynaptically secreted proteoglycan Agrin serve as critical mediators of the signaling cascade that regulates juxtaposed clustering of pre-synaptic and postsynaptic elements [45]. Lu et al. identified a RING-domain E3 ligase, PDZRN3 (PDZ domain containing RING finger 3), that is concentrated postsynaptically at the NMJ, interacts with MuSK and promotes MuSK ubiquitination [13]. Using siRNA knockdown or transgenic overexpression of PDZRN3 in cultured myotubes, Lu et al. showed that PDZRN3 regulates MuSK surface expression, and provided multiple lines of evidence that this occurs by MuSK ubiquitination-dependent endocytosis. The process was largely blocked by lysosomal inhibitors, but proteasome inhibitors had no effect [13], suggesting that ubiquitin is involved in protein targeting, not turn-over.

The similar UPS-mediated endocytosis of Comm and MuSK demonstrate how specific synaptic E3 ligases locally regulate the surface expression of critical effectors of synaptogenesis. As the timing of events is paramount during synapse development, the temporal expression of these E3 ligases must presumably be tightly regulated. Future work will need to address how specific UPS pathways are turned on and off to mediate their precisely ordered effects.

3.2. Pruning and synapse elimination

The remodeling and elimination of synaptic connections is an ongoing process that occurs during normal development and neural circuit refinement, and also aberrantly in progressive neurodegenerative disorders. UPS mechanisms are involved in preventing the dying back of an axon distal to the site of injury, in a process known as Wallerian degeneration [72,73]. Similarly, the UPS plays a critical role in initial stages of normal axon pruning in the *Drosophila* Mushroom Body, a primary brain learning and memory center; in a process that closely resembles Wallerian degeneration [74]. Recently, a *Drosophila* screen for loss of dendritic pruning in sensory neurons identified ubcD1 as an E2 conjugating enzyme involved in the degradation of DIAP1, a caspase-antagonizing E3 ligase. This UPS pathway regulates local caspase activation leading to severing of dendrites, with preservation of the sensory neurons [75,76]. Thus, UPS pathways play a prominent role in both axonal and dendritic

pruning during *Drosophila* development. Hopefully, these exciting findings will stimulate further investigation of conserved pathways in mammalian systems.

Synapse elimination also involves local control of targeted protein degradation. Recently, Ding et al. elegantly showed that local control of synapse elimination in *C. elegans* occurs via a UPS-mediated mechanism [9]. An egg laying motor neuron (HSNL) connects to its target vulval muscles via a cluster of finely mapped synapses in the Primary Synapse Region (PSR). At early stages of development, synapses also form in an adjacent vulvar region, termed the Secondary Synapse Region (SSR), but these synapses are subsequently eliminated to give rise to the adult pattern. Ding et al. have shown that local control of synapse elimination is dependent on the interaction of an immuno-adhesion protein, Syg1, which interferes with the binding of the F-box protein SEL-1 with the SCF complex [9]. If this Syg1-SEL1 interaction is prevented, or if SEL-1 is over-expressed, the SCF complex forms a competent ubiquitin ligase complex that targets synaptic organizing proteins for degradation, leading to synapse elimination. These new data demonstrate that through tight spatial and temporal regulation of E3 ligase activity either synapse stabilization or elimination can be favored. It remains to be tested whether similar pathways are involved in mammalian pruning and synapse elimination.

4. UPS regulation of presynaptic neurotransmission

Following UPS regulation of synapse formation, the UPS has a maintained role in regulating neurotransmission strength. We have identified high levels of proteasome expression in *Drosophila* NMJ presynaptic boutons, and found that both pharmacological and genetic inhibition of the proteasome causes rapid strengthening of neurotransmission via a presynaptic mechanism [8]. Blocking the proteasome resulted in a significant increase in transmission strength within minutes. UNC-13 proteins are known to regulate the efficacy of presynaptic neurotransmitter release by mediating a critical step of synaptic vesicle priming [77]. *Drosophila* UNC-13 is ubiquitinated and accumulates in the presynaptic terminal after proteasome inhibition over the same time course as presynaptic strengthening occurs [8,78]. Thus, UPS-mediated turnover of synaptic proteins occurs on a time scale that is rapid enough to drive acute forms of synaptic plasticity, and UNC-13 is one primary target for this UPS-mediated regulation.

Willeumier et al. have recently shown that acute proteasome inhibition increases the size of the recycling pool of synaptic vesicles in cultured hippocampal neurons [14]. Using optical dye imaging to track synaptic vesicle release and uptake, they found a dramatic increase in the size of the recycling vesicle pool after 2 h of proteasome inhibition. This effect was increased by heightened synaptic activity, and decreased by reduced synaptic activity [14], suggesting an important role for the UPS in activity-mediated regulation of presynaptic transmitter release. A similar increase in the size of the recycling pool was produced by forskolin activation of PKA pathways, but there was no additive effect of PKA and proteasome inhibition, suggesting a shared or intersecting pathway [14]. A cAMP-mediated recruitment of reserve pool vesicles to the recycling pool had been previously demonstrated at the *Drosophila* NMJ [79]. The proteasome inhibition-induced rapid increase in levels of dUNC-13 is abolished by PKA antagonists [78], suggesting one attractive mechanism for this convergence.

Of the presynaptic E3 ligases that have been identified, the best characterized is the membrane-localized SCF E3 ligase SCRAPPER. Yao et al. identified SCRAPPER and demonstrated that it ubiquitinates and mediates the proteasomal degradation of RIM-1 (*rab3-interacting molecule 1*), a presynaptic active zone protein involved in priming of synaptic vesicles for release. In a SCRAPPER knock-out mouse, RIM-1 expression was increased together with a 3-fold increase in miniature EPSC frequency [80], supporting the prediction that SCRAPPER regulates presynaptic transmitter release. The SCRAPPER gene also contains a cyclic-AMP response

element, further implicating the presynaptic PKA signaling cascade in UPS regulation of presynaptic release.

Several additional E3 ligases have been identified at the presynaptic terminal. The aforementioned Highwire has been shown to facilitate presynaptic neurotransmission at the *Drosophila* NMJ, through a yet unidentified pathway that is separate from its effects on synaptic growth regulation [56,59]. Other presynaptic E3 ligases include the ZNRF family of RING-finger ligases [81], the RING-finger E3 ligase Staring, shown to ubiquitinate and regulate t-SNARE Syntaxin-1 levels [82], and the RING-finger domain ligases, Siah-1A and Siah-2A, that bind to the integral synaptic vesicle protein Synaptophysin and promote its ubiquitination and degradation [83]. The identification of these presynaptic E3 ligases and several target proteins involved in synaptic vesicle trafficking and release mechanisms provide a starting point, but more work needs to be focused on how UPS regulation of these proteins affects presynaptic neurotransmission.

Many of these recent studies highlight a potential overlap of UPS-mediated degradation pathways and PKA signaling pathways in presynaptic modulation. This connection is not unexpected given that phosphorylation can serve as a trigger for subsequent ubiquitination [84,85]. Conversely, there is a well-characterized role for UPS-mediated protein degradation of the PKA regulatory subunit that enables PKA activation during *Aplysia* long-term facilitation [86]. The bidirectional interaction between protein phosphorylation and ubiquitination pathways provides an attractive mechanism for temporally linking changes in synaptic activity to protein degradation.

5. UPS regulation of postsynaptic neurotransmission

A fundamental mechanism for adjusting postsynaptic strength is regulation of the number of neurotransmitter-gated ion channel receptors. The UPS regulates postsynaptic receptor numbers through multiple mechanisms, including controlling forward receptor trafficking, the number of postsynaptic density (PSD) insertion slots, endocytosis, and trafficking through recycling and lysosomal pathways (Fig. 2). In *C. elegans*, direct ubiquitination and proteasomal degradation of glutamate receptor GluR-1 was demonstrated in a process requiring clathrin-mediated endocytosis [87]. A subsequent study identified Kel-8, a SCF E3 ligase substrate adaptor protein, as being instrumental in this process [88], although direct association with GluR1 was not demonstrated. The UPS also indirectly regulates postsynaptic GluR1 abundance through pathways involving APC [89] and LIN-23 F-box SCF [90] E3 ligases. In cultured rat hippocampal neurons, UPS function was similarly shown to regulate AMPA-type glutamate receptor internalization, as both AMPA-induced and NMDA-induced endocytosis of AMPA receptors was blocked by brief pretreatments with proteasome inhibitors and was dependent on polyubiquitination [91]. The ubiquitin-like modifier, SUMO, has been shown to target postsynaptic GluR6-containing kainate receptors [92]. While structurally similar to ubiquitin, SUMO does not form chains. Treatment of rat hippocampal neurons with kainate increased GluR6 SUMOylation and deSUMOylation prevented kainate-mediated GluR6 endocytosis. In contrast, SUMOylation did not affect NMDA-induced GluR6 endocytosis [92], suggesting that it is not involved in all endocytic mechanisms. These results imply that ubiquitination and SUMOylation-induced regulation of endocytosis are likely to operate through separate mechanisms. Other potential synaptic ubiquitination targets include long splice variants of type I metabotropic glutamate receptors (mGluR1 and mGluR5) that bind to the E3 ligase Siah (Seven in absentia homologue). When expressed in a heterologous system, mGluR1 and mGluR5 were directly polyubiquitinated and rapidly degraded [93]. If these findings can be extended to neurons, regulation of downstream signaling from type 1 mGluRs provides another potential indirect UPS mechanism for regulating ionotropic glutamate receptor abundance.

There are a growing number of examples showing UPS control of the assembly and forward trafficking of postsynaptic receptors. For nicotinic acetylcholine receptors, surface expression was shown to be regulated by UPS-mediated endoplasmic reticulum associated degradation (ERAD) of unassembled receptor subunits [94]. Moreover, binding of the ubiquitin-like protein, PLIC-1/ubiquilin-1, limited the availability of unassembled nicotinic acetylcholine receptor subunits by targeting them to the proteasome [95]. PLIC-1/ubiquilin-1 has also been shown to bind to an intracellular domain of GABA_A receptor α and β subunits [96], where it seems to have the opposing effect of preventing receptor degradation. In hippocampal neurons, activity-dependent ubiquitination of β 3 subunit-containing GABA_A receptors negatively regulates forward trafficking through the ER [97]. In addition, recent work has shown that GABA_A α 1 subunits are ubiquitinated. An autosomal dominant form of juvenile myoclonic epilepsy is caused by a missense α 1 A322D mutation, and this mutant α 1 subunit protein was shown to be misfolded in the ER leading to rapid UPS-mediated ERAD and reduced surface GABA_A receptor expression [98]. This mechanism can also be reversed: NMDA receptors have been shown to be retrotranslocated from the plasma membrane to the ER [99], where the NR1 subunit is ubiquitinated and targeted for degradation (Fig. 2).

Ubiquitination also targets postsynaptic scaffold proteins for degradation, suggesting a coordinate mechanism for regulating linked proteins in the PSD. In a rat hippocampal synaptosomal preparation, Ehlers demonstrated that proteasome inhibition modulated activity-dependent changes in the PSD over a period of 48 h, and that the multivalent scaffold proteins Shank, GKAP and AKAP79/150 were ubiquitination targets. These UPS-mediated changes were accompanied by changes in the signaling properties of the PSD as measured by assays of the phosphorylation states of CREB and ERK-MAPK [100]. NMDA-mediated internalization of GluR2-containing AMPA receptors is UPS-mediated through a pathway involving direct ubiquitination of the PSD-95 scaffold protein by the E3 ligase MDM2 [101], although an independent study was not able to demonstrate direct PSD-95 ubiquitination [102]. In cultured hippocampal neurons, SPAR (*Spine-associated* guanosine triphosphatase activating protein), an actin regulatory protein, becomes an ubiquitination target after phosphorylation. Over 24 h, UPS-mediated SPAR degradation was accompanied by a decrease in the abundance of PSD-95 and a loss of dendritic spines [85]. Recently, CaMKII (calcium/calmodulin-dependent protein kinase II) activation was shown to promote degradation of the synaptic scaffold protein Liprin- α 1, through both proteasome dependent and independent mechanisms [103]. CaMKII function itself is regulated by the HECT E3 ligase Ube3a. In the chronic absence of Ube3a, CaMKII inhibitory autophosphorylation is increased, resulting in reduced CaMKII activity and reduced PSD localization [104]. Strikingly, learning and behavioral deficits in the AS mouse model were rescued by introducing a mutant CaMKII that cannot undergo inhibitory autophosphorylation [105]. These studies show that over the longer time scale of 24–48 h, UPS-regulated turnover of postsynaptic scaffold proteins is a key element for the morphological and functional remodeling of the PSD.

Recently, we showed that the UPS differentially regulates two ionotropic glutamate receptor classes present in the *Drosophila* NMJ postsynaptic density. These two spatially and functionally distinct GluR classes are composed of common GluRIII (IIC), IID and IIE subunits, and variant GluRIIA (A-class) or GluRIIB (B-class) subunits [106–108]. Genetic postsynaptically-targeted proteasome inhibition caused a rapid increase in B-class receptors over the course of a few hours, with little effect on A-class receptor levels (Fig. 3) [109]. The mechanism likely involves ubiquitin-mediated turnover of Discs Large (DLG), a PSD-95 (postsynaptic density-95) scaffold homolog. DLG was shown to be ubiquitinated and accumulated after genetic proteasome inhibition [109] and is a known determinant of B-class receptor localization/maintenance at the *Drosophila* NMJ [110]. Direct ubiquitination of B-class receptors also remains as a possibility that needs to be explored. These results show that UPS regulation is critically important in determining not only the number of postsynaptic

receptors, but also the subclass specification of these receptors, thereby providing for fine-tuning of postsynaptic responsiveness.

Much remains to be learned about the specific E2/E3 combinations that are active in the postsynaptic compartment, their localization signals and the time course over which they function. In the ER, there are transmembrane E3 ligases that work in concert with AAA-ATPases to translocate membrane proteins into the cytoplasm, where they are targeted through ERAD to the proteasome for degradation [111]. Are similar local mechanisms available for transmembrane protein degradation at the plasma membrane, or is the primary mechanism for their degradation monoubiquitin tagging, that can serve as both an endocytosis and lysosomal sorting signal? It is clear that for each postsynaptic receptor type, UPS-mediated regulation at all points in the receptor life-cycle must be understood. For different receptor types there may well be multiple points of UPS regulation, with individual mechanisms more or less active depending on the postsynaptic environment.

6. UPS role in activity-dependent synaptic modulation

6.1. UPS regulation of LTP and LTD

Recent work suggests that the UPS modulates rapidly-induced types of plasticity, including both long-term potentiation (LTP) and depression (LTD). Earlier study had shown that proteasome inhibition markedly reduces hippocampal LTD mediated by degradation of the postsynaptic PSD-95 scaffold protein, regulating the number of glutamate receptor slots [101]. More recently, Fonseca et al. examined the effects of pharmacological proteasome inhibition on LTP, showing no effect on early LTP but a strong impairment of late-phase LTP (L-LTP) [10]. When proteasome inhibition was combined with protein synthesis inhibition, the effect was reversed and L-LTP restored. The authors suggest a model in which an activity-induced increase in proteasome activity leads to the degradation of proteins that are negative regulators of L-LTP. When combined with activity-dependent protein synthesis of positive L-LTP regulators, L-LTP is favored. In the setting of both protein synthesis and proteasome inhibition, the existing balance of positive and negative regulators must favor L-LTP, but if either proteasome activity or protein synthesis is absent, the balance shifts to favor negative regulators, and L-LTP does not occur [10]. Thus, in this model, protein synthesis and degradation mechanisms work in harmony to mediate activity-dependent changes in synaptic efficacy.

6.2. UPS trafficking in synapse regulation

Recent work suggests that gross movement of proteasomes may be an important mechanism for the activity-dependent regulation of protein composition within individual synapses. Exciting new results have shown that proteasomes are transported in and out of synapses in response to synaptic activity [112]. Bingol et al. used a GFP-labeled proteasome subunit in conjunction with restricted photobleaching of individual dendritic spines and shafts to follow proteasome movements. Increasing synaptic activity led to a 1.5-fold increase in the GFP proteasome signal within dendritic spines. Furthermore, with activity, the immobile fraction of spine proteasomes increased significantly, reflected by a 6-fold decrease in their exit rate from the synapse [112]. This observation suggests that proteasomes are anchored or tethered in the postsynaptic compartment in an activity-dependent manner, likely due to proteasome association with the actin cytoskeleton. As polyribosomes are also transported into dendritic spines during heightened synaptic activity [113], this new study reinforces the idea that local protein synthesis and degradation mechanisms are likely working in concert to fine-tune synaptic plasticity.

Shen et al. have recently demonstrated that proteasome inhibition or bicuculline disinhibition in cultured hippocampal neurons caused translocation of the proteasome from nucleus to cytoplasm [114]. We have discovered a similar shift of UPS components from nucleus to cytoplasm upon transgenic proteasome inhibition in *Drosophila*, where ubiquitinated proteins no longer accumulated in the nucleus after acute proteasome inhibition (Fig. 1) [115]. Shen et al. show that a 19S ATPase regulatory proteasome subunit, Mov34, complexes with a subunit of the SCF E3 ligase complex, Cul3, and an immediate early gene, NAC1, that is upregulated after psychostimulant-induced behavioral plasticity. Heightened synaptic activity promotes rapid and persistent NAC1-dependent translocation of the proteasome and Cul3 to the region of postsynaptic dendritic spines [114]. The activity-dependent trafficking of proteasomes and associated UPS machinery provides an exciting mechanism for tuning local protein degradation by coupling synaptic activity levels with proteasome availability. Identifying the upstream signaling pathways that mediate this change will be an important area for future research.

The composition of the proteasome may also change in an activity-dependent manner. Zif268 is an immediate early gene that is upregulated by heightened synaptic activity, and has been shown to be critical for LTP. A recent study by James et al. showed that the potential transcriptional targets of Zif268 include a large number of UPS related genes [116]. Of the genes identified, 4 (psmb9, psme2, SGK, and TAP1) were evaluated further using RT-PCR and their transcription was confirmed to be downregulated by Zif268. The psmb9 gene encodes the inducible proteasome beta subunit, $\beta 2i$, and psme2 encodes for an inducible regulatory 11S proteasome complex that can replace the 19S regulator complex. Thus, the predicted change in proteasome composition with Zif268 activation would be to decrease proteolytic activity and also potentially increase the specificity of the proteasome for binding ubiquitinated substrates. In a follow-up study, Zif268 activation was also shown to decrease the abundance of the APC cdc20 subunit, reducing APC activity [117]. Taken together, these recent studies suggest that synaptic activity regulates proteasome localization and composition, providing mechanisms for very specific temporal and spatial regulation of the turnover of synaptic proteins.

7. Summary and conclusions

Recent work shows that the UPS plays a critical role in both acute and chronic changes in protein abundance throughout the life-cycle of the synapse; ranging from early axonal pathfinding and target recognition, through synaptogenesis and synapse elimination, and continuing to regulate activity-dependent plasticity in both presynaptic and postsynaptic compartments. The recent identification of many E3 ligases that are active at the synapse should open new frontiers into understanding specific UPS pathways. The identification of neuronal APC and SCF E3 ligase complexes is striking, as these ligases were originally characterized in regulation of cell cycle transitions in development. This has led to speculation that neurons undergoing active axonal growth may be in a relatively undifferentiated state compared with neurons whose axons have already reached their targets [49]. As neurogenesis has been shown to persist in some regions in the adult brain, and as neurons frequently undergo sprouting following injury, it will be important to determine whether APC and SCF ligase mediate “neuronal state switches” in these settings. Moreover, localization of many E3 ligases to both nucleus and synapse suggests that there may be interplay between UPS regulation of transcriptional programs that function in synaptic modulation and local synaptic regulation of protein degradation. Further underscoring this potential association is the finding that neuronal activity mediates transport of UPS elements between nucleus and synapse.

The many recent discoveries of UPS regulation of synaptic function have revealed exquisite fine-tuning of protein levels and localization at the synapse. Many major advances have come from elegant studies utilizing the powerful genetic tools in *C. elegans* and *Drosophila* coupled

to increasingly sophisticate synaptic assays in these systems. Remarkable progress has also been made in unraveling UPS regulation in mammalian synapses, primarily through utilizing neuronal culture systems. However, it is clear that we are just now beginning to appreciate the wide-ranging mechanisms by which the UPS controls synapse assembly, maintenance, and function. Given the vast number of E3 ligases, there are likely a great many disparate pathways to be discovered that tune synaptic function. There is also a great need to understand the neuronal and synaptic targets of these ligases and how their effects interrelate at synapses in vivo. These advances are of paramount importance to elucidating the mechanisms of and developing treatments for the many neurodevelopmental and neurodegenerative diseases that are characterized by UPS dysfunction.

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Fig. 1.

UPS presynaptic pathways. UPS pathways regulate presynaptic differentiation and neurotransmission strength. In *Drosophila* and *C. elegans*, the large E3 ligase Highwire/Rpm-1 operates as part of a unique SCF complex that includes the F-box protein FSN-1/DsFn, which targets the MAPKKK Wallenda/DLK and regulates synaptic growth via JNK MAPK or p38 MAPK signaling, respectively. In *C. elegans*, Rpm-1 regulates axon termination through a separate incompletely characterized pathway. In *Drosophila*, Highwire also regulates presynaptic neurotransmitter release, as does targeted ubiquitination of dUNC-13, a synaptic vesicle priming protein. The APC E3 ligase complex operates presynaptically to regulate synaptic growth by targeting Liprin-α.

Fig. 2.

UPS postsynaptic mechanisms regulating neurotransmitter receptors. There are multiple modes of UPS regulation of receptors in the postsynaptic density. 1. ERAD: Receptor subunits are targeted for degradation by the proteasome after ubiquitination and translocation from the ER. 2. Forward trafficking: Ubiquitination regulates the rate and efficiency of forward trafficking from the ER to the plasma membrane. 3. Scaffold control: Polyubiquitination and targeted degradation of postsynaptic density scaffolding proteins determines the number of receptor slots. 4. Membrane recycling: Mono- or polyubiquitination of receptors and/or endocytic machinery triggers endocytosis. 5. Vesicle trafficking: Monoubiquitination serves as a trafficking signal, directing receptors to the late endosome for lysosomal degradation. 6. Retrotranslocation: Reverse trafficking of surface membrane receptors back to the ER can lead to extracellular domain polyubiquitination and translocation from the membrane for proteasome degradation.

Fig. 3. Genetically targeted postsynaptic proteasome inhibition acutely regulates class-specific glutamate receptor expression. *Drosophila* genetic targeting of postsynaptic proteasome inhibition was induced by feeding third instar larvae RU486-containing food for a brief interval (6 h). Confocal imaging of a NMJ synapse was performed in control (–RU486) and proteasome-blocked (+RU486) animals. A, Representative images from synapses co-labeled for A-class (GluRIIA) or B-class (GluRIIB) receptors and synaptic membrane marker (HRP). Smaller images show higher magnification of individual synaptic boutons labeled for GluRIIA or GluRIIB. B, GluR expression intensity was quantified for GluRIIA and GluRIIB relative to

HRP. GluRIIA was decreased and GluRIIB was increased by proteasome blockade. Bars represent mean $\pm$ SEM, * p <0.05, *** p <0.001.