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Synaptic protein degradation by the ubiquitin proteasome system

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Synaptic plasticity — the modulation of synaptic strength between a presynaptic terminal and a postsynaptic dendrite — is thought to be a mechanism that underlies learning and memory. It has become increasingly clear that regulated protein synthesis is an important mechanism used to regulate the protein content of synapses that results in changes in synaptic strength. Recent experiments have highlighted a role for the opposing process, that is, regulated protein degradation via the ubiquitin–proteasome system, in synaptic plasticity. These recent findings raise exciting questions as to how proteasomal activity can regulate synapses over different temporal and spatial scales.

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Introduction

Most proteins in a cell are degraded by the ubiquitin–proteasome system (UPS). The target protein is marked for degradation by the attachment of a chain of ubiquitin molecules (polyubiquitin chain). This chain is then recognized by a protein complex called the proteasome, which also carries the enzymatic activities that degrade the target protein. The initial attachment of the polyubiquitin chain to the target protein occurs through a series of enzymatic steps that provide substrate specificity. Delivery of the polyubiquitinated proteins to the proteasome and the final degradation of the protein are also highly regulated events. Collectively, the UPS enzymes are known as E1 (ubiquitin-activating enzyme), E2s (ubiquitin-conjugating enzymes) and E3s (ubiquitin ligases). The UPS can be regulated at each enzymatic step, ensuring that the right protein is degraded at the right time. The polyubiquitin chain can be removed or its length can be modulated by deubiquitinating enzymes (DUBs), providing reversibility to the ubiquitination reaction. Readers should refer to recent reviews that detail the enzymatic machinery of the UPS [1–3].

Localization

The proteasome has been studied most extensively in yeast, in which it plays a key role in the control of the cell cycle. In yeast, some of the UPS enzymes have been identified, as have the proteasome subunits and accessory proteins [4]. In neurons, some of the components of the UPS have been observed in dendrites and near synapses [5–7]. The presence of two main components of the UPS, ubiquitin and proteasome subunits, has been demonstrated at synapses and in the postsynaptic density PSD fraction, by direct immunofluorescence and immunoblotting [5,6]. Members of the E3 family of enzymes (Nedd4, Staring, Siah, E6-AP, MDM2, fbx2, parkin, APC), E2 enzymes (bendless) and deubiquitinating enzymes (usp14, fat facets, UCH-L1, UCH-L3, isopeptidase T) have also been linked to synaptic function [8•,9,10•,11–23]. In addition, proteomic approaches using mass spectroscopy have confirmed the presence of UPS components at synapses [24,25].

Both the ubiquitination and the delivery of the target protein to the proteasome are key regulatory points in the decision of whether to degrade a protein or not. In recent years, it has become apparent that the localization of the UPS machinery itself is also regulated. In yeast, for example, green fluorescent protein (GFP)- labeled proteasome subunits are localized mainly at the nuclear envelope and in the rough endoplasmic reticulum (ER). The proteasome is recruited to the sites of DNA double strand breaks within the nucleus [26]. In addition, dramatic shifts in proteasome localization between cytoplasm and nucleus occur during oocyte development and the mitotic cycle [27–29]. This raises an important question: is proteasome localization also regulated in neurons? In cultured hippocampal neurons, for example, under unstimulated conditions the proteasome can only be detected (immunohistochemically) in about 60 percent of the synapses [5]: the non-homogeneous distribution of the proteasome suggests that there is room for the regulated movement of the proteasome in and out of the synapses.

The first demonstration of a physiological role for UPS in synaptic plasticity came from studies of long-term facilitation (LTF) in *Aplysia* [18,30]. LTF requires the persistent activation of a cAMP-dependent protein kinase (PKA): after LTF is induced, there is a loss of regulatory subunits (R) of PKA without any change in the amount of catalytical subunit. Hegde *et al.* [30] have shown that UPS is involved in the degradation of the regulatory subunits of PKA. This was demonstrated by the appearance of higher molecular conjugates of R subunits in extracts of

Aplysia nervous tissue and in reticulocyte lysates. In support of this, the degradation of R subunits is blocked when the proteasome is removed from the lysates, and stimuli that trigger LTF result in degradation of the R subunits in a proteasome-inhibitor sensitive manner [31]. Degradation of PKA R subunits is further enhanced by the induction of expression of an Ap-ubiquitin C-terminal hydrolyase (Ap-uch) during LTF [18]. Ap-uch is a DUB enzyme that recycles free ubiquitin, thereby increasing the ubiquitin pool ready for attachment to substrate proteins. In principle, Ap-uch expression should affect the degradation of all UPS substrates, not only the PKA R subunits, suggesting the presence of other targets of UPS in LTF.

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 [32]. They have shown that chronic proteasome inhibition, starting just after LTF induction, increases the amount of LTF observed 24 h later. This suggests that proteasome activity acts to constrain synaptic plasticity. They have also shown that proteasome inhibition alone is sufficient to increase synaptic transmission even after 1 h of incubation with inhibitors. Proteasome inhibition also causes structural changes: after 24 h 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 five minutes of proteasome inhibition is sufficient to decrease the free ubiquitin pool and accumulate significant levels of ubiquitinated substrates waiting to be degraded [5]. Because ubiquitin is involved in many cellular processes and not just in degradation [33], the effects reported with long proteasome inhibitor incubations might be due to loss of other functions of ubiquitin (e.g. mono-ubiquitin-dependent endocytosis) and not due to loss of proteasome activity.

Presynaptic targets

A few studies have begun to shed light on the function of UPS in presynaptic nerve terminals. Speese *et al*. [34] have shown that components of UPS (E1 and the proteasome) are present in presynaptic boutons at the *Drosophila* neuro-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 with that in controls. This increase was rapid, suggesting a local degradation of proteins by the UPS. This increase in synaptic transmission might be due to increased presynaptic transmitter release, because there was no change in the amplitude and frequency of mEJCs. What are the possible targets of the UPS in the presynaptic terminal? Speese *et al*. [34] have shown that DUNC-13, a protein regulating synaptic vesicle priming [35], might be involved. Moreover, blocking proteasome activity with inhibitors or genetic disruption of active site subunits of the proteasome increased the abundance of DUNC-13 at the presynaptic terminal. This suggests that the increase in synaptic transmission with proteasome inhibition might be due to DUNC-13 accumulation, but the possibility remains that other substrates are involved. Two other presynaptic proteins that are regulated by UPS are syntaxin-1 and synaptophysin [21,22], two synaptic vesicle proteins that are involved in neurotransmitter release. 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 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 terminals in *Drosophila* motor neurons. Loss of function of highwire, an E3 enzyme, has the same phenotype as the faf gain of function mutants, suggesting that the balance between ubiquitination and deubiquitination is important for presynaptic development [14,36]. Highwire has homologs in *C. elegans* (RPM-1) and mammals (Phr and Pam) [36–40]. The highwire homolog RPM-1 has recently been identified as a negative regulator of the p38 MAPK pathway in *C. elegans* [41•]. 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 [36], whereas rpm-1 mutants in *C. elegans* have a reduced number of synapses [39,40,41•]). 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 pos-

sible that at different synapses, different F-BOX proteins target different substrates leading to different phenotypes for RPM-1 and its homologs.

The presynaptic terminal also harbors deubiquitinating enzyme activities. Depolarization decreases the total content of ubiquitinated substrates in the presynaptic terminal within seconds [13]. One deubiquitinating enzyme that regulates synaptic transmission in the presynaptic terminal is Usp14, which is a ubiquitin-specific protease that recycles ubiquitin from multiubiquitinated proteins. An ataxic mouse described by D'Amato and Hicks [42] has defective Usp14 activity [42]. 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 [20].

Postsynaptic targets

The trafficking of ionotropic glutamate receptors — namely AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate) and NMDA (N-methyl-D-aspartate) receptors — has become an intense area of research in recent years as techniques for examining receptor trafficking have offered a glimpse of the dynamics that might underlie some forms of plasticity [43]. The stability of AMPA and NMDA receptors (GluRs and NMDARs) on the postsynaptic membrane is regulated dynamically. For example, GluRs undergo endocytosis in response to agonist treatment or synaptic stimulation. It has been shown that both polyubiquitination [5] and proteasome activity [5,11] are required for the agonist-induced endocytosis of AMPARs. Patrick *et al.* [5] have shown that treatment with proteasome inhibitors 3 min before stimulation is sufficient to block the endocytosis of GluRs, suggesting that UPS machinery must be very close to the site of receptor endocytosis. More importantly in terms of this short-term proteasome inhibition (~3 min), there is no effect on the free ubiquitin pool, suggesting a direct role of proteasome-dependent degradation.

The requirement for proteasome activity in GluR endocytosis suggests the presence of UPS targets that regulate the GluR trafficking at the synapse. In principle, proteins that stabilize the AMPAR at the surface would be perfect candidates for such UPS target proteins. In recent years, 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 [44–46]. 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 through other proteins [11,47].

The involvement of UPS in regulating glutamate receptor abundance is also shown by studies in *C. elegans*. Burbea *et al.* [48] have shown that the synaptic levels of one of the AMPA-type glutamate receptor homologs in *C. elegans*, glr-1, is regulated by the UPS. Glr-1 is ubiquitinated *in vivo*. Blocking the ubiquitination of glr-1 increases the synaptic abundance of the receptor. Conversely, when ubiquitin is expressed exogenously to promote ubiquitination, there is a decrease in glr-1 synaptic levels that requires an intact endocytosis machinery. These results suggest that ubiquitination of the glr-1 receptor regulates its own synaptic abundance through membrane trafficking. 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.

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 for endocytosis.

Recent studies have identified the UPS machinery that targets the regulators of GluR endocytosis. Kaplan and coworkers [9] 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 [49]. Two groups have reported novel roles for APC in postmitotic neurons. Van Roessel *et al.* [8•] 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 is mutated, both spontaneous and evoked junction potentials are 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 [10•]. 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 [8•]. 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.

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 homeostatic plasticity has prevented a simple analysis of the roles of protein synthesis and degradation by using synthesis or degradation inhibitors. Ehlers [6] has shown, however, that the same manipulations that result in homeostatic plasticity give rise to global changes in postsynaptic density protein content. 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 [6]. The regulation of the PSD composition by the UPS has been shown in experiments in which chronic increases in synaptic activity have increased the ubiquitination of PSD proteins, whereas chronic decreases in activity had the opposite effect. 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. anything more than 1 h) can lead to decreases in the free ubiquitin pool [5]. As such, these data cannot distinguish between a role for the UPS and mono-ubiquitin-dependent processes. Nevertheless, these data 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.

Another postsynaptic protein that is a UPS substrate is the spine-associated Rap GTPase activating protein (SPAR) [50]. SPAR degradation 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 [50,51]. Interestingly, SNK expression is upregulated in the soma and not locally in the dendrites, suggesting a global effect of SNK on spine regulation.

Conclusions

In recent years, UPS biology at the synapse has become an area of intense research. The UPS shares features with other better established synaptic regulatory systems, namely phosphorylation and local protein synthesis. Modulating protein function by post-translational modification and changing the protein content can achieved by UPS, through ubiquitination and protein degradation, respectively. The studies described above point to the importance of UPS on both sides of the synapse. However, many questions remain as to the function and regulation of the UPS at the synapse. What is the extent of protein degradation at the synapse? For example, does spine loss involve degradation of most of the proteins at the synapse or just a few master regulators? This raises the question of the identity of ubiquitinated synaptic proteins. How fast is the protein degradation at the synapse? What are the other components of the UPS at the synapse? Are there synapse-specific components of the UPS? How does synaptic activity regulate the localization and activity of the UPS? How are these changes mediated and sustained for long periods of time? Future work will hopefully shed light on these questions and give us a better understanding of synaptic UPS biology.

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