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Protein degradation and memory formation

Diasynou Fioravante¹ and John H. Byrne²

Dept. Neurobiology and Anatomy, W.M. Keck Center for the Neurobiology of Learning and Memory, The University of Texas Medical School at Houston, Houston TX 77030

Abstract

Long-term memories are created when labile short-term memory traces are converted to more enduring forms. This process, called consolidation, is associated with changes in the synthesis of proteins that alter the biophysical properties of neurons and the strength of their synaptic connections. Recently, it has become clear that the consolidation process requires not only protein synthesis but also degradation. Here, we discuss recent findings on the roles of ubiquitination and protein degradation in synaptic plasticity and learning and memory.

Keywords

ubiquitin; neuron; learning; memory; plasticity; proteasome

Introduction

The formation of long-term memories is thought to occur in stages, involving acquisition and consolidation. During acquisition, the animal learns new information, the memory for which is short-lasting (short-term memory). Through the process of consolidation, this short-term memory is converted to a more permanent form, which is available for subsequent retrieval. Historically, long-term memory (LTM) formation was synonymous with increased gene expression and protein synthesis [21,45]. Undoubtedly, gene transcription and translation are necessary for LTM, but recent results indicate that protein degradation may be equally important [3,56]. According to the emerging view, degradation of subsets of proteins may remove inhibitory constraints on memory formation [13]. Together with protein synthesis, protein degradation regulates the availability of synaptic proteins thereby modulating synaptic strength and plasticity, and ultimately memory formation [11].

Regulated degradation of proteins occurs via three main routes: the lysosome, proteases including calpain, thrombin, neurotrypsin and matrix metalloproteinases, and the ubiquitin-proteasome system (UPS)[30,86,90,93,94]. All three routes have been involved in the regulation of cellular signaling and synaptic plasticity, and by extension, in learning and

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²To whom correspondence should be addressed: John H. Byrne, Ph.D., Department of Neurobiology and Anatomy, W.M. Keck Center for the Neurobiology of Learning and Memory, The University of Texas Medical School at Houston, P.O. Box 20708 Houston, TX 77225, Tel: 713 500-5602, Fax: 713 500-0623, John.H.Byrne@uth.tmc.edu.

¹Current address: Dept. Neurobiology, Harvard Medical School, Boston MA 02115

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memory [16,36,55,81,86,90,93,94]. Here, we will focus on the UPS, the major protein degradation pathway involved in the destruction of cytoplasmic targets.

Protein degradation via the ubiquitin-proteasome system

Degradation of proteins via the UPS involves three basic steps: the identification of the target protein, the tagging of the target protein with a ubiquitin chain, and the delivery of the tagged target protein to the 26S proteasome, which degrades ubiquitinated proteins [30] (Fig. 1). Ubiquitination, the covalent addition of ubiquitin molecules on target proteins, plays a role in numerous cellular processes including cell cycle regulation, signal transduction, gene transcription and synaptic plasticity [36,40,71,81,91]. The ubiquitination of target proteins is highly regulated, involving three families of enzymes. First, ubiquitin is activated by a ubiquitin-activating enzyme called E1. Then, activated ubiquitin is transferred to a ubiquitin-conjugating enzyme called E2, which associates with another enzyme, a ubiquitin ligase called E3. The E3 attaches the ubiquitin to a lysine residue in the target protein. Formation of a polyubiquitin chain is initiated with the covalent addition of a second ubiquitin moiety to the first ubiquitin. A large degree of diversity exists among E3s, and some among E2s. The specificity in the ubiquitination process is due to the combinatorial mechanism involving E2s and E3s as well as the physiological state of the target protein (e.g., phosphorylation) [36,100].

Once tagged, the target protein is destined for degradation by the 26S proteasome, a large protein complex located in the nucleus and cytosol of cells. The proteasome is formed by the co-assembly of a core catalytic component with a sedimentation coefficient of 20S and a 19S cap (the regulatory component). The 19S cap is involved in the initial recognition of the polyubiquitin chain on the target protein. Although the target protein is degraded, the polyubiquitin chain is generally not. Deubiquitinating enzymes (DUBs) including ubiquitin C-terminal hydrolases (UCH) and ubiquitin-specific proteases cleave the ubiquitin moieties, which are recycled. Under certain conditions, however, they, too, can be degraded [50,72,77].

The UPS and synaptic plasticity

The UPS is well poised to regulate synaptic transmission and plasticity. Components of the UPS are localized near both pre- and postsynaptic regions of synapses [8,25,68,83]. The postsynaptic localization in particular is thought to be dynamically regulated by synaptic activity, because stimulation with KCl or NMDA induces calcium-calmodulin kinase II (CaMKII)-dependent redistribution of the proteasome from dendritic shafts to spines [8,9]. Furthermore, the levels of several synaptic proteins are regulated by the UPS, including presynaptic regulators of the synaptic vesicle cycle [2,14,26,42,83], scaffold proteins [19,25] and postsynaptic receptors [10,25,60,68] (for a review see [57]). Because of its subcellular localization and its targets, the role of the UPS in synaptic plasticity has been studied extensively in recent years (for reviews see [11,36,86]). We will briefly discuss pharmacological and genetic studies that have implicated the UPS in long-term forms of synaptic plasticity thought to contribute to learning and memory [58,61,84].

The significance of the UPS as a regulator of long-term plasticity is evidenced by its conserved role across phyla. In the invertebrate *Aplysia*, the UPS is necessary for long-term facilitation (LTF) [39,88,101]. In mammals, proteasomal activity is required for long-term potentiation (LTP), although there is disagreement about the role of the proteasome in the two phases of LTP (i.e., early and late LTP). Early LTP (E-LTP) lasts 1-2 h post-induction and involves calcium influx (through NMDA receptors), CamKII and increased surface expression of AMPA receptors [58]. In the hippocampus, pharmacological inhibition of the proteasome has been reported to enhance [24], inhibit [46] or not affect [28] this early phase.

These discrepancies have been attributed to the specificity and concentrations of the inhibitors that have been used [24]. Late LTP (L-LTP) is a temporally and mechanistically distinct phase of LTP that lasts several hours post induction and requires protein synthesis and gene transcription [58]. Blocking proteasomal activity impairs the maintenance of L-LTP [24,28,46]. Intriguingly, the same manipulation alleviates the requirement of protein synthesis for L-LTP [28], suggesting that the expression of L-LTP depends on a balance between protein synthesis and degradation.

In addition to synaptic potentiation, the UPS is important for long-term synaptic depression (LTD) in invertebrates [26] and mammals [17,19,22,41]. In the entorhinal cortex and the hippocampus, pharmacological inhibition of the proteasome blocks NMDA receptor-dependent LTD [19,22] and metabotropic glutamate receptor (mGluR)-dependent LTD [41]. However, two recent studies challenged these results, reporting that in the hippocampus and nucleus accumbens, inhibition of proteasomal function had no effect on NMDA receptor-mediated LTD whereas the same manipulation enhanced mGluR-dependent LTD [17,60]. The reason for these discrepancies is not clear, yet it is intriguing that the UPS may regulate the same form of synaptic plasticity differentially, depending on the induction mechanism.

The involvement of the UPS in synaptic plasticity and the finding that deficits in synaptic plasticity and its underlying mechanisms are implicated in memory impairments [7,67,79] have motivated investigations into the role of the UPS in learning and memory. Here we review recent work supporting this role.

The UPS and memory consolidation

The relationship between the UPS and the various stages of memory has been investigated through pharmacological and genetic studies. Using specific inhibitors of the UPS [62], Lopez-Salon et al. [56] tested the requirement for the UPS in hippocampal-dependent learning and memory. At various times after inhibitory avoidance training, during which rats learned to stay on a platform in order to avoid an electric shock, the proteasome inhibitor lactacystin or proteasome inhibitor I was infused into the dorsal CA1 region of hippocampus. When the inhibitors were administered 1-7 h after training, performance on the task 24 h after training was diminished. In contrast, the inhibitors were ineffective when infused 10 h after training. These results indicate that memory for inhibitory avoidance requires the function of UPS up to 7 h after training and suggested that UPS-mediated proteolysis is important for the consolidation phase of the memory [but see 53], but not its retrieval. Similar conclusions were reached by Artinian et al. [3] using the spatial Morris water maze task (which requires the animal to learn the location of a hidden platform over several training sessions using distal cues, in order to escape the water), although the time window during which UPS function was needed for consolidation varied (immediately but not 3 h after training). Lopez-Salon et al. [56] also found that protein ubiquitination and 26S proteolytic activity were increased in the hippocampus for at least 4 h after training [56], further supporting the requirement for UPS function in the early events of memory consolidation.

Genetic studies have manipulated various components of the UPS in order to determine its role in the formation of LTM. These components include certain ubiquitin ligases [5,43,95,97], ubiquitin ligase-related adaptor proteins [54] and deubiquitinating enzymes [31,76,92] [for review on mouse models of UPS dysfunction see 89]. The E3 ligases (Fig. 1) have been a particular focus of studies using loss-of-function mutations. One such ligase is the UBE3a ubiquitin ligase (a.k.a. E6-AP). Mutations in the *Ube3a* locus give rise to Angelman syndrome (AS), an inherited neurological disorder involving motor dysfunction and intellectual disability [49]. It seems likely that the deficits in AS are due at least in part

to alterations in synaptic plasticity associated with the absence of UBE3a. Specifically, the neocortex of mice that possess a maternal *Ube3a* null mutation shows delayed experience-dependent maturation [97]. Moreover, neocortical LTP and LTD are impaired, as is ocular dominance plasticity [97]. Whereas basal synaptic transmission is normal in the hippocampus of these mutants, hippocampal synaptic plasticity is not. LTP is significantly attenuated for up to 80 min after its induction (E-LTP) [43].

The role of UBE3a and the UPS in long-term memory formation was investigated in the maternal *Ube3a* mutant mice using various behavioral learning tasks [43]. In context fear conditioning, the animal learns to associate their immediate environment (context) with an aversive stimulus (e.g., electric shock) (also see Fig. 3). This type of learning is generally believed to be hippocampus-dependent [48]. In cued fear conditioning, an association is made between a salient cue (e.g., a tone) and the aversive stimulus, and this type of learning is thought to depend primarily on the amygdala [70]. *Ube3a* mutant mice performed poorly compared to controls in the context-dependent conditioning task but performed at control levels in the cue-dependent conditioning task, suggesting that UBE3a function is necessary for memories involving the hippocampus [43]. The lack of an effect on cued-fear conditioning does not agree with the observation that various components of the UPS are upregulated in the amygdala in response to this type of learning [85]. However, as we will discuss below, several studies have found no requirement for the UPS in cued-fear conditioning, questioning the functional relevance of the increases in expression observed by Stork et al. [85]. Indeed, fear-potentiated startle, which involves the amygdala, is facilitated following proteasome inhibition [98].

Work in *Drosophila* corroborates the link between mutations in *Ube3a* and deficient memory formation found in the mammalian studies. Loss-of-function mutations in the *dube3a* gene, which codes for the fly homologue of UBE3a, result in failure to form long-term memory for an olfactory conditioning task [95]. This memory deficit is not due to a failure to learn the association between the odor and the shock used in the task, because immediately after training, mutant animals perform at wild-type control levels [95]. Notably, the mutation does not impair all types of memory in the fly. Mutant animals perform similar to wild types when tested in a different version of the olfactory conditioning task that induces a type of memory not requiring protein synthesis [95].

Progress is being made in identifying proteins whose degradation is regulated by UBE3a. Greer et al. [33] recently identified Arc, a synaptic protein that promotes internalization of AMPA glutamate receptors [15,73,78], as a target for UBE3a. UBE3a ubiquitinates Arc, leading to its degradation through the UPS. In the absence of UBE3a, Arc levels increase in the hippocampus. This loss of control over Arc levels and the consequent reduction in synaptic AMPA receptors impair hippocampal synaptic function [33]. The decrease in AMPA receptors could explain the previously observed deficit in LTP and the failure to form context-dependent fear memories [43], but these hypotheses remain to be tested.

Additional components of the UPS implicated in memory formation include the ubiquitin ligase UBR1 [5] and the adaptor protein CDH1 [54]. UBR1 is part of the N-end rule pathway, which functions to destabilize the N-terminus of target proteins prior to their degradation by the 26S proteasome [51]. When tested in the hidden platform version of the Morris water maze task, mice lacking UBR1 show impaired long-term memory, with no apparent differences in the initial learning of the task [5]. CDH1 is an adaptor protein that brings substrates to the anaphase promoting complex, an important multi-subunit E3 ubiquitin ligase with several pre- and post-mitotic functions [59,80,96]. Brain slices from *Cdh1* heterozygous knockout mice show normal synaptic transmission and E-LTP in the CA1 area of the hippocampus, but show impaired L-LTP [54]. Moreover, *cdh1*

heterozygotes perform poorly in contextual-fear conditioning but at control levels in cued-fear conditioning [54]. This finding agrees well with the observations of Jiang et al. [43] and corroborates the involvement of regulated proteolysis in hippocampus-dependent memory formation.

Tagging of target proteins by ubiquitin ligases is an important aspect of the UPS system, but so is cleavage of the polyubiquitin chain prior to target degradation and recycling of ubiquitin [50]. Several lines of research point to the involvement of DUBs in development, transcription regulation and neurological disorders [47]. With regard to learning and memory, the UCH family of DUBs has attracted particular attention [36,37]. Pioneering studies in *Aplysia* have provided significant insight into the role of the UPS and UCH in memory-related synaptic plasticity. LTF, a form of plasticity thought to underlie long-term memory for sensitization in this animal [18,29], is associated with persistent activation of protein kinase A (PKA) in the absence of persistently increased cAMP levels [32,64]. This persistent activation results from the degradation of the PKA regulatory subunits via the UPS [38]. LTF requires PKA activity and the proteasome [12]. Treatments that induce LTF also increase expression of the *Aplysia* homologue of UCHL1, Ap-UCH [39]. Ap-UCH, which is necessary for LTF, is associated with the proteasome and regulates the rate of degradation by the proteasome [39]. In a simple computational model (Fig. 2), PKA, which is initially activated by elevated cAMP, leads to induction of Ap-UCH, which in turn enhances PKA activity through degradation of its regulatory subunits, thus prolonging the availability of free, active catalytic subunits [69,82]. Persistent PKA activity is likely to help maintain phosphorylation and activation of transcription factors, thereby contributing to the consolidation and persistence of later phases of LTF and long-term memory.

Ap-UCH is involved not only in LTF but also in LTD. In *Aplysia*, LTD requires the proteasome and correlates with increased ubiquitination levels. Treatments that induce LTD also upregulate Ap-UCH expression *via* the transcription factor CREB2 [26]. If Ap-UCH is involved in both LTF and LTD, how is the direction of the plasticity determined? One possible explanation is that the directionality in plasticity comes from the identity of the target proteins, which is determined by the combination of E2s and E3s [99], and probably not by DUBs.

The function of UCHs in plasticity and memory is evolutionarily conserved. In rodents, UCH is upregulated in the hippocampus following passive avoidance training [27]. UCHL1, the mammalian homologue of Ap-UCH, is required for L-LTP in the CA1 region of the hippocampus [76]. Moreover, a specific UCHL1 inhibitor impairs contextual fear conditioning, but not cued-fear conditioning [31]. By varying the time of administration of the inhibitor in relation to training, Gong et al. [31] found that the hydrolytic activity of UCHL1 was required for consolidation of contextual fear memory, but not for its acquisition. However, hippocampal LTP and contextual fear conditioning are intact in mice lacking UCHL3, a related member of the UCH family of DUBs [92], suggesting that different DUBs have selective functions in distinct paradigms of plasticity and memory.

The requirement for UCHL1 in hippocampal-dependent memory formation was corroborated by a later study using UCHL1-deficient gracile axonal dystrophy (*gad*) mice [76]. These mice carry a spontaneous mutation that produces a deletion in exons 7 and 8 of *Uchl1*, resulting in no detectable expression of UCHL1 [75]. To test for memory impairments, *gad* mice and wild type controls were trained in a passive avoidance task, in which animals learn to prefer a brightly lit compartment over a dark one, after the association of the latter with an electrical shock. When tested 2 h after training, *gad* mice performed similar to controls, suggesting that their learning ability was intact. However,

when tested 24 h after training, gad mice performed poorly, suggesting that the absence of UCHL1 reduced the persistence of passive avoidance memory [76].

The UPS and memory reconsolidation

The dynamic nature of memory formation lies at the core of the concept of memory reconsolidation. When a previously acquired memory is retrieved, it becomes labile and susceptible to remodeling [35,65]. The re-establishment of the memory is termed reconsolidation. Although the mechanisms underlying the destabilization and restabilization of the memory after its retrieval are becoming better understood [87], the functional significance of the reconsolidation process remains elusive. Lee [52] recently proposed that reconsolidation, rather than consolidation, is the predominant process taking place whenever more than a single training trial is involved.

The requirement for new protein synthesis in reconsolidation ([1,87] but see [63]) could be explained if reactivated memories were destroyed via the UPS. If so, then inhibiting the UPS during reconsolidation should alleviate the need for new protein synthesis. Lee et al. [53] performed precisely this experiment [see also Fig. 3 and ref. 44]. Mice were trained in a context-fear conditioning paradigm, and 24 h after training the memory was reactivated with a retrieval trial (Retrieval 1). Subsequently, the proteasome inhibitor β -lactone was infused along with the protein synthesis inhibitor anisomycin bilaterally in the hippocampus and memory retrieval was re-tested (Retrieval 2) 24 h later. As expected [e.g., 66], treatment with anisomycin alone impaired performance during Retrieval 2, which suggested deficient reconsolidation. However, co-administration of the UPS inhibitor blocked the amnesic effect of anisomycin, supporting the idea that the UPS destabilizes the reactivated memory and that new protein synthesis is required to restabilize it. The same conclusion was reached by Lee [52], who also found that co-administration of β -lactone mitigated the amnesic effect of anisomycin on reconsolidation of contextual fear conditioning.

Additional evidence for the role of the UPS in reconsolidation was provided through experiments in which the timing of the infusion of the UPS inhibitor (in this case, lactacystin) varied [3]. Hippocampal injection of lactacystin immediately after reactivation of spatial memory for a hidden platform in the Morris water maze impaired retrieval 24 h later, suggesting that reconsolidation of the spatial memory was blocked. However, when lactacystin injection was delayed for 3 h post reactivation, no effect on reconsolidation was observed [3]. These results agree with the observation of Lee et al. [53] that polyubiquitination levels are increased for at least 60 min following memory reactivation, and point to a narrow window following memory reactivation during which UPS function is indispensable. Note, however, that whereas blocking the proteasome impaired reconsolidation in the Artinian et al. [3] study, the same manipulation did not affect reconsolidation in the Lee et al. [53] study. Notably, Lee et al. did not find an effect of proteasome inhibition even on consolidation. The reasons for these discrepancies remain unclear but could involve procedure-specific differences [53,74].

Future directions

An appreciation for the role of protein degradation by the UPS in learning and memory is rapidly gaining momentum. Proposed functions of the UPS include removal of inhibitory constraints on memory formation [4,6,13,24,28], destabilization of synapses to allow for new learning [56], and destabilization of previously learned memories (“unlearning”), implicated in behavioral extinction [53]. However, many fundamental questions remain to be answered. For example, which proteins are targeted by the UPS during learning and memory and what is the functional significance of their degradation? One such protein is the postsynaptic scaffold molecule Shank, which, consistent with previous *in vitro* work [25], is

degraded by the UPS after memory retrieval [53]. The down-regulation of scaffold molecules could destabilize the postsynaptic density and modulate synaptic strength through receptor internalization [19], thus affecting the state of the memory. NCAM, the neural cell adhesion molecule, is also ubiquitinated and down-regulated following passive avoidance training [27], consistent with the hypothesis of synapse destabilization and reorganization during memory consolidation [56].

In addition to identifying the targets of the UPS during learning and memory, future work needs to determine the mechanisms through which proteasomal activity is regulated during consolidation and/or reconsolidation of different types of memory. Some insight into UPS regulation can be gained from recent *in vitro* work suggesting that synaptic activity upregulates proteasomal function in a manner dependent on calcium influx (through NMDA receptors and L-type voltage gated calcium channels) and CamKII [23]. This activity-dependent regulation of the proteasome appears to be bidirectional because proteasomal function is down-regulated when action potentials are blocked with tetrodotoxin [23]. A related question is whether proteasomal activity is differentially regulated for a memory produced by a single training trial vs. a memory that is acquired through repeated training trials (sessions) [52] and if so, how this regulation is achieved. Differential involvement of the UPS in memories acquired through a single training trial (e.g., during contextual fear training) vs. those acquired through repeated trials (e.g., Morris water maze) could perhaps explain discrepancies between studies (e.g., between [3,53]) (see memory reconsolidation section and [74]). The generation of new research tools such as fluorescent reporters targeted by the UPS [20,34] will allow the *in vivo* monitoring of proteasome function and direct assessment of its role during various learning paradigms.

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Figure 1.

The ubiquitin-proteasome system (UPS). Ubiquitin molecules are attached to a target protein via the coordinated action of E1, E2 and E3. Most polyubiquitinated substrates are degraded by the 26S proteasome. Monomeric ubiquitin is recycled by deubiquitinating enzymes (DUBs) such as ubiquitin carboxy-terminal hydrolase. Modified from [37].

Figure 2.

PKA positive feedback loop. Serotonin (5-HT) triggers production of cAMP, which activates PKA and causes dissociation of the regulatory (R) and catalytic (C) subunits. The catalytic subunit translocates to the nucleus, where it activates transcription factors (TF1, TF2) that regulate expression of genes including *Ap-UCH*. Increased *Ap-UCH* contributes to the degradation of the regulatory subunit of PKA, thus forming a positive feedback loop that sustains elevated PKA activity.

Figure 3.

Schematic of a typical experiment to test memory reconsolidation. During fear conditioning training, a conditioned stimulus (CS; e.g., context) is paired with an unconditioned stimulus (US; e.g., electric shock). Twenty four h after training, the CS is presented alone (retrieval 1) and the freezing response of the animal (fear memory) is measured. Subsequently, a drug (e.g., proteasome inhibitor) or vehicle is infused and the animal is retested 24 h later for fear memory (retrieval 2). If the drug interferes with reconsolidation, freezing during retrieval 2 should be impaired compared to vehicle. Modified from [44].