Brief Communication

Role of proteasome-dependent protein degradation in long-term operant memory in *Aplysia*

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We investigated the in vivo role of protein degradation during intermediate (ITM) and long-term memory (LTM) in *Aplysia* using an operant learning paradigm. The proteasome inhibitor MG-132 inhibited the induction and molecular consolidation of LTM with no effect on ITM. Remarkably, maintenance of steady-state protein levels through inhibition of protein synthesis using either anisomycin or rapamycin in conjunction with proteasome inhibition permitted the formation of robust 24 h LTM. Our studies suggest a primary role for proteasomal activity in facilitation of gene transcription for LTM and raise the possibility that synaptic mechanisms are sufficient to sustain 24 h memory.

Paradoxically, while long-term memory formation requires new gene expression and protein synthesis, it may also require protein degradation. Protein degradation associated with synaptic plasticity or memory formation may be necessary for the activation of persistent kinase signaling pathways, removal of inhibitory signals, synaptic tagging or synaptic remodeling/growth (Fioravante and Byrne 2011; Jarome and Helmstetter 2013, 2014). The ubiquitin–proteasome system (UPS) is highly conserved providing the primary mechanism for cytoplasmic protein degradation (Tai and Schuman 2008; Bach and Hegde 2016). The role of the proteasome in memory formation was initially postulated as necessary for removal of inhibitory constraints based upon research in the marine mollusk *Aplysia californica* (Chain et al. 1995; Hegde et al. 1997). Recent research has provided more specific insight into proteasome function, particularly during late hippocampal long-term potentiation in which proteasome activity affects new gene expression as well as modulating dendritic protein synthesis (Dong et al. 2008, 2014a,b; Hegde et al. 2014). However, despite widespread evidence linking the UPS to synaptic plasticity, questions still remain regarding the physiological functions of the proteasome in vivo during long-term memory (LTM) formation as several in vitro studies have shown that initial inhibition of proteasome activity strengthens synapses and enhances memory (Zhao et al. 2003; Dong et al. 2014a). As the mechanisms underlying memory formation are highly conserved, we investigated the role of proteasome function in vivo using *A. californica*.

Initially much of our understanding of the mechanisms underlying memory formation in *Aplysia* originated from research employing nonassociative learning paradigms either in vivo or in vitro (Byrne and Hawkins 2015), but recent research using associative operant learning paradigms in *Aplysia* has revealed the complexity of memory formation even in simple systems (Hawkins and Byrne 2015). In this study, we investigated the role of the ubiquitin–proteasome system in intermediate (ITM) and long-term memory (LTM) in *Aplysia californica* using the operant conditioning paradigm, learning that food is inedible (LFI). LFI memory represents a complex operant form of memory with plasticity observed in buccal ganglia neurons (Levitan et al. 2008; Michel et al. 2011a,b). Although the neurons involved in the feeding circuitry for appetitive and consummatory behaviors have been well-studied and many of the molecular signaling pathways underlying LFI memory have been identified (Katzoff et al. 2002, 2010; Cohen-Armon et al. 2004; Michel et al. 2011a,b, 2013) the specific neurons involved in LFI memory have not been identified. To further our understanding of the fundamental molecular mechanisms in LFI memory formation, we used a pharmacological approach to determine the role of the protein degradation. The open circulatory system of *Aplysia* allows in vivo behavioral pharmacology targeting all neurons involved in LFI memory including potential presynaptic, postsynaptic, and interneurons in the circuit. During LFI training, animals respond to netted seaweed with appetitive and consummatory feeding behaviors forming an association between the seaweed with failure of the swallowing attempts (Schwarz and Susswein 1986; Schwarz et al. 1991). Wild-caught *A. californica*, maintained in separate boxes within large seawater tanks at 15°C, received LFI training using laver seaweed encased in tulle-mesh that elicited biting and swallowing attempts, but could not be physically swallowed. Training was discontinued starting at 25 min with the seaweed bag gently extracted from the mouth during a protraction cycle of the radula resulting in some minor variation in training times (Michel et al. 2012, 2013). As intermediate and long-term LFI memory are strongly regulated by the circadian clock (Lyons et al. 2005; Michel et al. 2013), training occurred at Zeitgeber Time 3 (3 h after lights on). The same protocol was used for testing continuing until the animal rejected the seaweed from the mouth for three consecutive minutes. Memory is denoted by significantly reduced total response time and the time the seaweed was retained in the mouth compared with naïve animals.

In *Aplysia*, nonassociative long-term facilitation requires the ubiquitin–proteasome system for removal of inhibitory proteins facilitating training-induced gene transcription and permitting the transition between short- and long-term memory (Hegde et al. 1997; Chain et al. 1999a,b). To determine whether associative LTM requires proteasome activity, animals were injected through the foot with the reversible proteasome inhibitor MG-132 (Lee and Goldberg 1996) 30 min prior to LFI training using the 25 min training protocol. MG-132 previously has been used to reversibly inhibit proteasomal activity in *Aplysia* ganglia

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and cell cultures (Chain et al. 1999a; Khabour et al. 2002; Kurosu et al. 2007) as well as in mammalian hippocampal studies (Karpova et al. 2006; Hamilton et al. 2012; Forrest et al. 2013). No significant differences were seen in the training responses between animals injected with MG-132 (EMD BioSciences; 130 µL/100 g body weight of 0.5 mM solution in DMSO for an approximate systemic concentration 1 µM) or vehicle (DMSO; 130 µL/100 g body weight) 30 min prior to training. Systemic drug concentrations were estimated using the proportion of the body weight attributed to the hemolymph, previously approximated as 65 mL/100 g body weight (Levenson et al. 1999; Lyons et al. 2006). MG-132 injected 30 min prior to training completely blocked LTM when animals were tested 24 h after training with response times during testing of treated animals similar to naïve animals (Fig. 1A,B). Vehicle-injected animals displayed robust LTM with significant decreases observed in the total response time and the time the seaweed was retained in the mouth. MG-132 alone had no significant effect on baseline responses as animals injected with the drug and then tested 24 h later in the absence of training displayed responses comparable to naïve animals.

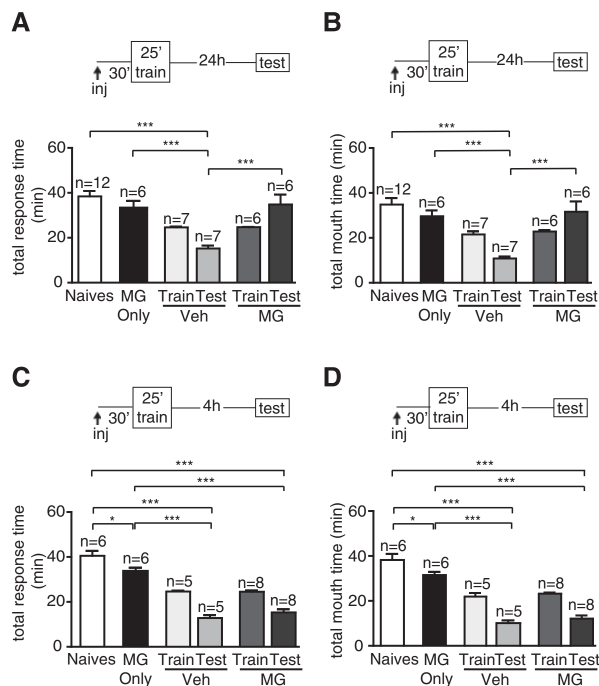

Figure 1. Long-term LFI memory, but not intermediate-term, requires proteasome activity. (*A*,*B*) MG-132 was injected 30 min prior to LFI training to inhibit proteasome activity during the induction of LTM. No differences were observed in the training responses between groups 30 min after drug (Train MG) or vehicle injection (Train Veh). Inhibition of proteasome activity inhibited the induction of LTM with treated animals (Test MG) exhibiting (*A*) total response times ($F_{(5,38)} = 13.61$; $P < 0.0001$) and (*B*) total mouth time ($F_{(5,38)} = 11.10$; $P < 0.0001$) similar to naïve animals. Vehicle-injected animals (Test Veh) displayed robust LTM with significantly decreased responses during testing compared with naïve animals. MG-132 alone in the absence of training had no effect on behavioral responses 24 h after drug injection. Mean and SEM are plotted. Data analysis was performed using ANOVA followed by Bonferroni's multiple comparison test (MCT). Asterisks denote significant differences with (***) $P < 0.001$ and (*) $P < 0.05$. (*C*,*D*). Inhibition of proteasome activity had no effect on ITM with treated animals (Test MG) showing significantly decreased (*C*) total response times ($F_{(5,32)} = 56.53$; $P < 0.0001$) and (*D*) mouth times ($F_{(5,32)} = 47.19$; $P < 0.0001$) compared with naïve and animals injected with MG-132 in the absence of LFI training (MG Only).

These results strongly suggest that the induction of long-term LFI memory requires proteasome activity.

In the past two decades, investigations of proteasome activity have centered on LTM with the role of the proteasome in ITM virtually unknown. Intermediate-term LFI memory extends ~4–6 h after training (Michel et al. 2012); a timeframe similar to the temporal window of other forms of memory including late-LTP that require proteasome activity (Dong et al. 2008, 2014a,b; Hegde et al. 2014) and may be necessary for conversion of early-LTP into late-LTP and stabilization of synaptic strength (Cai et al. 2010). In mice, upregulation of the ubiquitin–proteasome system has been observed 4 h after taste learning (Rosenberg et al. 2016) and 6 h after training during the formation of social recognition memory (Jüch et al. 2009), a similar timeframe to the expression of intermediate-term LFI memory. For intermediate-term LFI memory, persistent PKA activity is required throughout the duration of memory expression (Michel et al. 2012) raising the possibility that proteasomal degradation of PKA regulatory subunits in the cytoplasm may be necessary for ITM. As the same LFI training protocol induces temporally separated and mechanistically distinct intermediate and long-term memory (Michel et al. 2012), we directly compared the role of proteasome activity in ITM with its role in LTM. MG-132 was injected 30 min prior to training with animals tested 4 h later for ITM. No significant differences were observed between groups during training. Approximately 5 h after drug injection, MG-132 alone resulted in a small, but significant, decrease in responses during testing compared with naïve animals. However, drug-injected trained animals demonstrated significantly decreased responses during testing compared with naïve or drug-injected untrained animals with response times comparable to trained vehicle-injected control animals (Fig. 1C,D). These results suggest that the critical role of the proteasome in LFI memory is not the degradation of proteins necessary for persistent cytoplasmic kinase activity or protein synthesis that are necessary for ITM, but instead is linked to processes specific to the formation of LTM such as the induction of new gene expression.

The period of molecular consolidation following training for LTM is characterized by macromolecular synthesis and encompasses a time in which memory is susceptible to disruption (Abel and Lattal 2001; Sweatt 2010). In *Aplysia*, persistent PKA activation is necessary for long-term facilitation involving proteasomal degradation of the type I regulatory subunit of PKA (Hegde et al. 1993; Chain et al. 1995, 1999a,b; Kurosu et al. 2007). During early molecular consolidation, long-term LFI memory also requires persistent PKA activity (Michel et al. 2011b) presumably resulting in its nuclear translocation, subsequent CREB phosphorylation followed by induction of gene transcription of the immediate early gene *ApC/EBP* (Levitan et al. 2008). Consolidation of long-term memory in mammalian models also requires ubiquitin–proteasome-mediated protein degradation (Lopez-Salon et al. 2001; Artinian et al. 2008; Fioravante and Byrne 2011; Jarome et al. 2011; Jarome and Helmstetter 2014). Moreover, distinct windows of proteasome-dependent protein degradation have been found necessary for the consolidation of hippocampal-dependent object recognition memory (Figueiredo et al. 2015). In contrast, the inhibition of proteasome activity post-training enhances the consolidation of LTM in honeybees for olfactory conditioning (Felsenberg et al. 2014). To determine the time frame required for proteasome activity in long-term LFI memory, animals were injected with MG-132 either 1 or 4 h after LFI training. MG-132 treatment 1 h after training inhibited long-term memory with no decreases in response times observed upon testing compared with naïve animals or untrained animals injected with MG-132 (Fig. 2A,B). Thus, proteasomal-dependent protein degradation appears necessary during the period of

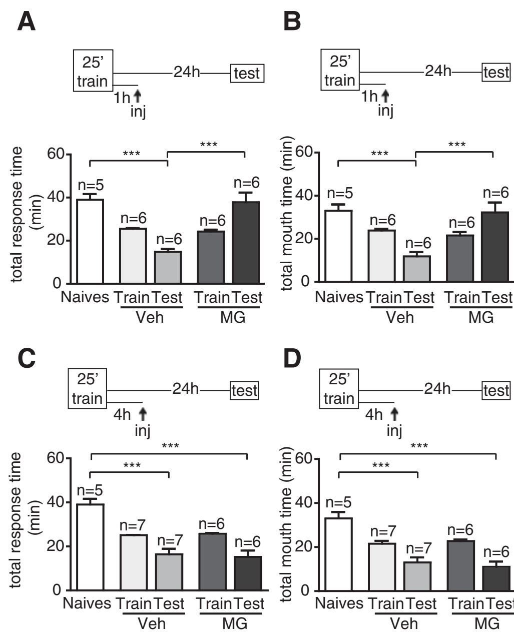

Figure 2. Proteasome activity is necessary post-training for the molecular consolidation of LTM. (*A*,*B*) Injection of MG-132 1 h post-training inhibited LTM with treated animals (Test MG) showing (*A*) total response times ($F_{(4,24)} = 17.04$; $P < 0.0001$) and (*B*) mouth times ($F_{(4,24)} = 10.08$; $P < 0.0001$) similar to naïve animals. Vehicle-treated (Test Veh) animals exhibited LTM with significantly decreased response times during testing compared with naïve animals. Mean and SEM are plotted. Asterisks denote significant differences with (***) $P < 0.001$. (*C*,*D*) Inhibition of proteasome activity 4 h post-training had no effect on LTM. Animals injected with MG-132 (Test MG) displayed robust LTM with significantly decreased (*C*) total response times ($F_{(4,26)} = 19.81$; $P < 0.0001$) and (*D*) mouth times ($F_{(4,26)} = 17.50$; $P < 0.0001$) as compared with naïve animals. No differences were observed between the responses of treated animals with vehicle-injected animals during testing.

macromolecular synthesis for LFI memory. Molecular consolidation of 24 h LFI memory appears to last 3–4 h post-training with gene transcription no longer required 3 h post-training (data not shown) and protein synthesis no longer required 6 h after training (Levitan et al. 2010). Animals injected with MG-132 4 h after training exhibited significantly decreased response times and robust LTM comparable to vehicle-injected animals (Fig. 2C,D). Thus, the timeframe of required proteasome activity for 24 h memory appears to align with the requirements for new gene expression and protein synthesis. Our results are consistent with research investigating the role of the proteasome in late long-term potentiation (late-LTP; 3 h) for which proteasome inhibition hinders CREB-mediated transcription and translation necessary for late-LTP (Dong et al. 2008). However, as later time points were not tested, it remains possible that longer forms of LFI memory, e.g., 48 or 72 h memory, that necessitate additional phases of protein synthesis (Levitan et al. 2010) also require additional periods of proteasome activity.

Intriguingly, while both protein synthesis and protein degradation are necessary for memory formation, the maintenance of steady-state protein levels through the inhibition of protein synthesis and protein degradation permits high-frequency stimulation-induced hippocampal late-LTP (3 h) in vitro (Fonseca et al. 2006). Recent research also showed that metabotropic glutamate receptor-mediated LTD requires protein synthesis to counteract a concurrent increase in proteasome-dependent protein degradation and that LTD can be induced when both translation and proteasome activity are simultaneously inhibited (Klein et al. 2015). To determine whether this type of steady state allows long-term operant memory formation in vivo, we inhibited protein synthesis using the reversible inhibitor anisomycin while simultaneously blocking proteasome activity with MG-132. Dual injection of anisomycin (Sigma-Aldrich; injected 1 mL/100 g body weight of 0.666 mM in artificial seawater for approximate system concentration of 10 μM) and MG-132 did not affect the baseline responses of the animals as treated animals displayed similar responses during training as vehicle-injected animals (dual injections of DMSO and artificial seawater; Fig. 3A,B). Remarkably, concurrent inhibition of protein synthesis and protein degradation permitted robust LTM similar to that observed in trained vehicle-injected animals. Animals injected with anisomycin and MG-132 presented significantly decreased response times in comparison to drug-treated animals that did not receive training (Fig. 3A,B). These results are surprising given the proposed role of proteasome activity in facilitating gene transcription necessary for LTM in multiple models (Hegde et al. 1997; Yamamoto et al. 1999; Dong et al. 2008; Fioravante et al. 2008).

During LTM formation, protein synthesis occurs both at the synapse and in the cell body (Sutton and Schuman 2006; Costa-Mattioli et al. 2009; Wang et al. 2009). Based upon its method of action as an inhibitor of peptidyltransferase and 80S ribosomal activity (Pestka 1972; Barbacid and Vazquez 1974; Gale et al. 1981), anisomycin inhibits synaptic and somatic protein synthesis as observed for facilitation in *Aplysia* cell cultures (Guan and Clark 2006). Local or synaptic protein synthesis appears to comprise a necessary component of LTM across learning paradigms. The mTOR pathway is hypothesized as a primary mechanism through which learning-induced synaptic protein synthesis is regulated across species (Costa-Mattioli et al. 2009; Gkogkas et al. 2010; Graber et al. 2013). Interestingly, proteasome activity may also modulate components of the mTOR pathway (Dong et al. 2014a). In *Aplysia*, the subset of transcripts translated via the TOR pathway comprises a high percentage of the transcripts in synaptic processes regulated by serotonin during facilitation (Casadio et al. 1999; Moccia et al. 2003). Rapamycin as an inhibitor of the TOR pathway inhibits synaptic protein synthesis necessary for long-term synaptic facilitation (Casadio et al. 1999; Moccia et al. 2003; Si et al. 2003; Carroll et al. 2004). Rapamycin-sensitive translation of ribosomal proteins at the synapse has been suggested as a mechanism for synaptic tagging for long-term memory (Moccia et al. 2003). Although rapamycin-dependent protein synthesis clearly plays a critical role in local protein synthesis, a role for somatic rapamycin-dependent protein synthesis in memory cannot be excluded. Rapamycin effectively inhibits intermediate- and long-term LFI memory (Michel et al. 2012) indicating the importance of the mTOR pathway in training-induced protein synthesis for LFI memory. Molecularly, rapamycin also inhibits the persistent phase of MAPK activity associated with intermediate and long-term LFI memory (Michel et al. 2011a, 2012).

Given the importance of local protein synthesis in LTM with an apparent bias toward rapamycin-dependent translation, we investigated whether rapamycin combined with proteasome inhibition permitted LTM. Animals were treated with rapamycin (EMD Biosciences; prepared as a 1 mM DMSO stock solution, diluted to a 3.25 μM working solution with artificial sea water, injected at 1 mL/100 g body weight) and MG-132 prior to training and then tested 24 h later. The dual drug combination had no effect on training responses as no significant differences were observed between the groups. As with the combination of anisomycin and MG-132, dual treatment with rapamycin and MG-132 permitted LTM formation similar to vehicle-injected animals (paired injections of DMSO and ASW; Fig. 3C,D). The prevalence of

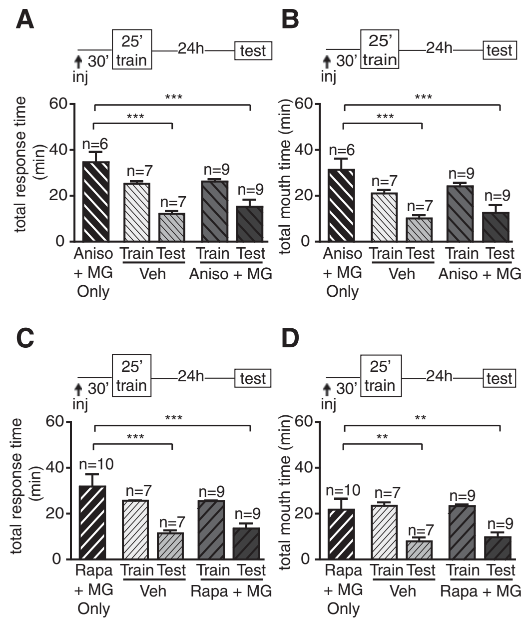

Figure 3. Concurrent inhibition of protein synthesis and protein degradation permits the induction of LTM. (*A*,*B*) To determine whether simultaneous inhibition of protein synthesis and proteasome activity permitted the induction of LTM, animals were injected with anisomycin and MG-132 30 min prior to LFI training. Injection of the two drugs (Train Aniso+MG) did not affect the training responses of the animals compared with vehicle-injected animals (Train Veh). Animals injected with both anisomycin and MG-132 (Test Aniso+MG) demonstrated clear LTM similar to vehicle-injected animals with animals showing significantly decreased (*A*) total response times ($F_{(4,28)} = 14.82$; $P < 0.0001$) and (*B*) mouth times ($F_{(4,28)} = 10.34$; $P < 0.0001$) as compared to drug-treated animals (Aniso+MG) that did not receive training. Data analysis was performed using ANOVA followed by Bonferroni's multiple comparison test (MCT). Asterisks denote significant differences with (***) $P < 0.001$ and (**) $P < 0.01$. (*C*,*D*) Inhibiting protein synthesis using rapamycin along with blocking proteasome activity using MG-132 (approximate systemic concentration 1 μM) 30 min prior to LFI training permitted LTM. Injection of rapamycin with MG-132 did not affect the responses of the animals during training. Treated animals (Test Rapa+MG) displayed robust LTM with significant decreases in (*C*) total response times ($F_{(4,31)} = 8.64$; $P < 0.0001$) and (*D*) time the seaweed was retained in the mouth ($F_{(4,31)} = 7.513$; $P < 0.0001$) as compared with drug-treated (Rapa+MG) naives. Vehicle-injected animals exhibited comparable LTM.

rapamycin-sensitive translation in *Aplysia* processes suggests that maintenance of steady-state protein level at the synapse support 24 h LTM in our experiments, even though proteasome inhibition still persists in the cell body potentially limiting new gene transcription. Moreover, the compensatory mechanisms that permit LFI memory when both protein synthesis and protein degradation are inhibited also appear to bypass the need for persistent MAPK activity as the training-induced increase in MAPK signaling is blocked by rapamycin. One interpretation of these results is that the primary function of persistent MAPK during the molecular consolidation of LFI memory occurs not in the synapse but in the nucleus to facilitate gene transcription. If the inhibition of the proteasome through MG-132 was already limiting transcription, then limitations in transcription due to MAPK inhibition by rapamycin would be occluded. A proposed role for MAPK in facilitating gene transcription in LFI memory is consistent with previous research on long-term sensitization in which the inhibition of MAPK was found to block the training-induced increase in the immediate early gene and transcription factor *Ap*C/EBP (Lyons et al. 2006). MAPK phosphorylation of CREB2 removes an inhibitory constraint for CREB-dependent gene transcription in *Aplysia* (Michael et al. 1998). Furthermore, MAPK phosphorylation stabilizes *Ap*C/EBP limiting its degradation through proteasomal activity (Yamamoto et al. 1999) reinforcing the necessity of MAPK activity for learning-induced gene expression. We hypothesize that a similar situation occurred when animals were treated concurrently with anisomycin and MG-132 with the maintenance of synaptic protein levels providing a mechanism to compensate for limitations in gene transcription following training.

The above results and our interpretation suggest that proteasome activity is necessary for the induction and molecular consolidation of long-term LFI memory. Potentially, proteasome activity also may be required for synaptic growth or remodeling to maintain memory as proteasome activity may shape postsynaptic reorganization and the growth of new dendritic spines as shown in mammalian systems (Bingol and Schuman 2006; Bingol et al. 2010; Bingol and Sheng 2011; Hamilton et al. 2012). However, the limited requirement for proteasome activity following LFI training, e.g., no longer required 4 h after training, is earlier than the timeframe suggested for synaptic growth and the appearance of new varicosities for other learning paradigms in *Aplysia* (Kim et al. 2003; Bailey and Kandel 2008; Bailey et al. 2015). Longer lasting forms of LFI memory, e.g., 48 or 72 h memory, may require additional phases of proteasomal activity for pre- or postsynaptic remodeling. Although in vivo behavioral pharmacology cannot fully explain the necessary functions of the proteasome in long-term operant memory, these studies establish a base for future work at the cellular level and for investigations of longer forms of memory. Moreover, these studies raise the intriguing possibility of using synaptic compensation to strengthen neural circuits in circumstances when gene transcription is compromised thus permitting long-term memory formation and potentially providing insight into future therapies for neural disorders.

Acknowledgments

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