The ubiquitin-specific protease 14 (USP14) is a critical regulator of long-term memory formation

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Numerous studies have suggested a role for ubiquitin–proteasome-mediated protein degradation in learning-dependent synaptic plasticity; however, very little is known about how protein degradation is regulated at the level of the proteasome during memory formation. The ubiquitin-specific protease 14 (USP14) is a proteasomal deubiquitinating enzyme that is thought to regulate protein degradation in neurons; however, it is unknown if USP14 is involved in learning-dependent synaptic plasticity. We found that infusion of a USP14 inhibitor into the amygdala impaired long-term memory for a fear conditioning task, suggesting that USP14 is a critical regulator of long-term memory formation in the amygdala.
several distinct features relative to Context A, including infrared house lamps, textured floor, and 5% acetic acid odor.

Five minutes prior to fear conditioning, rats received bilateral infusions (0.5 μL/side) into the amygdala, as described previously (Jarome et al. 2012). The specific USP14 inhibitor IU1 (100 μM or 200 μM per side; Sigma) was dissolved in DMSO. Clasto-lactacytisin β-lactone (βlac, 150 μM; Sigma) was dissolved in 2% DMSO in distilled H2O. Animals underwent a two-trial auditory fear conditioning paradigm as described previously (Parsons et al. 2010). The context test consisted of placing the animals in the training context for 8 min in the absence of shock. For the auditory CS test, animals were placed into novel Context B and after a 60-s baseline, received eight white noise (72-dB, 30-sec) presentations (60-sec ITI) in the absence of shock. On the third day the animals were brought back to Context B and given a second CS test.

The activity of each rat was recorded on digital video and the amount of movement determined by frame-by-frame changes in pixels using FreezeScan 2.0 software (CleverSys). Statistical outliers were determined as those samples that fell two or more standard deviations above/below the group mean. Data was analyzed using analysis of variance (ANOVA). Fisher least significant difference (LSD) post hoc tests were used where appropriate.

For in vitro proteasome activity assays, animals (n = 4) were overdosed on isoflurane and the amygdala dissected and prepared as described previously (Jarome et al. 2011, 2012). Samples (10 μg) were then diluted in double-distilled H2O and mixed with reaction buffer (250 mM HEPES, pH 7.5, 5 mM EDTA, 0.5% NP-40, 0.01% SDS, 5 mM ATP). Vehicle (DMSO), IU1, βlac, or IU1 + βlac were added to the reactions and incubated for 30 min at 37°C. Fluorogenic peptide Suc-LLVY-AMC (Millipore) was then added to the samples to assess proteasome activity (10 μM) as described previously (Jarome et al. 2013). The reaction was incubated at 37°C for 2 h and fluorescence monitored every 30 min at 360 (excitation)/460 (emission) on a monochromatic plate reader (Synergy H1, Biotek). Protein-free blanks were used and an AMC standard curve was produced.

In our first experiment, we wanted to test if USP14 activity was critical for long-term memory formation in the amygdala (Fig. 1A,B). Animals were given infusions of IU1 or vehicle (DMSO) prior to fear conditioning. During the training session, all animals acquired the task at a similar rate (Fig. 1C). A mixed variable ANOVA revealed a significant effect for time (F(2,27) = 100.269, P < 0.001) but not drug (F(2,27) = 1.210, P = 0.314), and there was not a time by drug interaction (F(2,27) = 2.004, P = 0.154), suggesting that IU1 did not alter the ability of the animals to acquire the task.

The next day, all animals were tested for their responses to the contextual and auditory cues. We found a main effect for drug on the context test (F(2,24) = 6.626, P = 0.005) (Fig. 2A). Fisher LSD post hoc tests revealed significant impairments in retention for the training context in animals that received either 100 μM (P = 0.026) or 200 μM (P = 0.002) concentrations of IU1 relative to DMSO controls, suggesting that IU1 dose-dependently impaired long-term memory for the training context. Interestingly, we did not find a significant effect of the drug on the auditory CS test (F(2,27) = 0.280, P = 0.758) (Fig. 2B), suggesting that USP14 activity was not critical for the consolidation of this type of fear memory in the amygdala. To confirm this, we gave the animals a second CS test the following day (Fig. 2C). Consistent with our previous result, we did not observe any effect of the drug on the second CS test (F(2,27) = 0.045, P = 0.956). Collectively, these results suggest that USP14 activity is critical for the long-term consolidation of contextual, but not auditory, fear memories in the amygdala.

Since USP14 is a regulatory subunit of the proteasome, we next wanted to know if IU1-induced memory impairments were due to altered proteasome activity in the amygdala (Fig. 3). To test this, we inhibited USP14 activity in amygdala lysates and measured proteasome activity using an in vitro proteasome activity assay. As a control, we also incubated samples with βlac, a selective inhibitor of the catalytic subunits of the proteasome. We found main effects for time (F(1,21) = 20.795, P < 0.001) and drug (F(2,21) = 3.053, P = 0.026) and a time by drug interaction (F(2,21) = 2.678, P = 0.043). Fisher LSD post hoc tests revealed a significant reduction in proteasome activity in all βlac groups (all Ps < 0.05) from minutes 30 to 120 (scans 2–5); however, the IU1 groups never differed from DMSO controls at any time throughout the incubation. These results suggest that while blocking USP14 activity with IU1 impaired long-term memory formation in the amygdala, this effect was largely independent of changes in the catalytic activity of the proteasome.

To summarize, we found that transient inhibition of the proteasome de-ubiquitinating enzyme USP14 in the amygdala impairs long-term memory for contextual, but not auditory, fear memories. Additionally, USP14 inhibition did not alter proteasome catalytic activity in the amygdala, suggesting that USP14 likely regulates memory consolidation through a mechanism independent of proteasome activity. Collectively, these results suggest that USP14 activity is critical for long-term memory formation in the amygdala.

We found that inhibiting the activity of the proteasome regulatory subunit USP14 in the amygdala impaired long-term memory formation. Inhibiting USP14 with IU1 enhances the degradation of proteasome substrates both in vitro and in vivo, which is likely through
a mechanism independent of changes in proteasome activity (Koulich et al. 2008; Lee et al. 2010); however, it has never been tested if IU1 alters proteasome activity in vivo. Additionally, USP14 can alter 20S proteasome activity by changing the gating of the 20S proteasome, but it is unknown if IU1 stimulates gate opening in vivo (Peth et al. 2009). Finally, inhibiting proteasome catalytic activity can alter the activity of USP14 on the proteasome, suggesting a coordinated regulation between these two functions on the proteasomes (Borodovsky et al. 2001). Thus it is possible that IU1-induced memory impairments could be due to altered proteasome activity in the amygdala which resulted in a weaker memory trace. To test if IU1 altered the catalytic activity of the 20S proteasome, we inhibited USP14 activity with IU1 and measured proteasome activity in the amygdala. We found that inhibiting USP14 activity in the amygdala with concentrations of IU1 that impaired long-term memory formation did not alter proteasome catalytic activity, even when the concentration was 2–4 times higher than that needed to impair memory retention. Since USP14 inhibition did not alter proteasome activity in the amygdala, this suggests that it is unlikely that the impairments in long-term memory from IU1 infusions were due to altered proteasome catalytic activity. However, USP14 can regulate substrate turnover by the proteasome, suggesting that IU1 altered the degradation of a specific number of substrates. While we could not test changes in the degradation of specific proteasome substrates in the present study, these results do support a critical role for USP14 activity in memory formation in mammals.

Although evidence suggests that inhibition of proteasome activity impairs long-term memory (Lopez-Salon et al. 2001; Artinian et al. 2008; Jarome et al. 2011), other studies have found that proteasome inhibitors enhance (Yeh et al. 2006; Felsenberg et al. 2012) or have no effect (Lee et al. 2008) on memory. Additionally, similar conflicting results have been reported with the proposed cellular analog of memory, long-term potentiation (Fonseca et al. 2006; Dong et al. 2008). Collectively, these results suggest that while ubiquitin–proteasome-mediated proteolysis is critical for synaptic plasticity and long-term memory formation, the exact function of this process can vary depending on brain region and the substrates being targeted for degradation by the proteasome. Here, we add to this growing literature by showing that the proteasome deubiquitinating enzyme USP14 is critical for memory formation. Considering that USP14 is one of the proteins in the ubiquitin–proteasome system that controls substrate degradation by the proteasome, determining what brain regions and behavioral tasks USP14 is involved in will be important to improve our understanding of the role of protein degradation in synaptic plasticity and memory formation.

Very little is known about how protein degradation is regulated during the memory consolidation period. Previously, it has been shown that deubiquitinating enzyme Ap-Uch is critical for the induction of long-term facilitation in Aplysia (Hegde et al. 1997), suggesting that deubiquitinating activity is critical for synaptic plasticity (Chain et al. 1995). However, genetic loss of the deubiquitinating enzyme UchL3 in mice did not alter long-term memory for contextual or auditory fear conditioning, nor did it impair long-term potentiation (LTP) in the hippocampus (Wood et al. 2005), suggesting that deubiquitinating activity may not be necessary for fear memory formation in the brain. Consistent with this, while a knockout of UCH-L1 results in a variety of developmental impairments in mice, it does not alter synaptic plasticity in the hippocampus (Walters et al. 2008), though other studies have suggested a potential role for UCH-L1 in hippocampal LTP (Kabuta et al. 2008). In the present study, we found that transiently inhibiting the deubiquitinating enzyme USP14 impaired long-term memory for a fear conditioning task. In combination with previous studies demonstrating a role for USP14 in synaptic plasticity (Wilson et al. 2002; Walters et al. 2008), these results suggest that USP14 may be a critical deubiquitinating enzyme regulating synaptic plasticity and long-term memory formation in the mammalian brain.

One interesting finding from our study is that IU1 infusions selectively impaired long-term memory for the contextual but not the auditory cue. Generally, pharmacological manipulations of the amygdala result in long-term memory impairments for both auditory and contextual cues (Jarome et al. 2011), suggesting that postsynaptic plasticity in the amygdala underlies the long-term storage of both auditory and contextual fear memories (Johansen et al. 2011; Jarome and Helmstetter 2013). Although it is unclear why contextual fear memories were selectively affected by IU1 in the present experiment, one potential explanation is that USP14 acts to regulate presynaptic plasticity between the amygdala and hippocampus. For example, mice deficient in USP14 have a loss of paired pulse facilitation but normal LTP in the hippocampus (Wilson et al. 2002). Consistent with this, USP14 has been shown to be required to maintain synaptic vesicle pools to sustain synaptic transmission (Bhattacharyya et al. 2012). Thus it is possible that USP14 regulates the consolidation of contextual fear memory through altered plasticity at amygdala inputs to the hippocampus. Indeed, the amygdala has been shown to

![Figure 2](image-url) Inhibition of USP14 activity in the amygdala impairs the consolidation of contextual but not auditory fear memories. (A) Pre-training IU1 infusions dose-dependently impaired long-term memory retention for the training context relative to DMSO controls. (B,C) Pre-training IU1 infusions did not impair long-term memory for the auditory CS when tested 1 d (B) and 2 d (C) after the training. (*) P < 0.05.

![Figure 3](image-url) Inhibition of USP14 activity does not alter proteasome catalytic activity in the amygdala. In vitro proteasome activity was measured in amygdala lysates collected from naive animals (n = 4). Samples were incubated in DMSO, IU1 (100 μM, 200 μM, and 400 μM), iBac (150 μM), and IU1 + iBac (200 μM + 150 μM) and 400 μM + 150 μM) solutions for 30 min prior to the addition of the fluorogenic substrate. IU1 infusions did not alter the time-dependent degradation of the fluorogenic substrate. (*) P < 0.05.
regulate plasticity in the hippocampus during the consolidation of a contextual fear memory (Huff et al. 2006), suggesting that USP14 could potentially regulate this systems level consolidation process between the amygdala and hippocampus. This interpretation is consistent with our result showing that the IU1-induced memory impairments occurred independently of alterations in proteasome catalytic activity since if proteasome activity was altered by USP14 inhibition, we would have expected impairments in memory retention for both the auditory and contextual cues (Jarome et al. 2011). Additionally, since USP14 may have substrate specificity (Hu et al. 2005; Lee et al. 2010), it is possible that USP14 is acting on a limited number of substrates during the memory consolidation process. This would suggest that blocking USP14 activity would only partially replicate effects seen by proteasome inhibition and could explain the selective context effect. Also, since IU1 is likely accelerating substrate degradation, it is possible that the memory impairments observed from IU1 infusion are due to the enhanced degradation of specific proteins that would normally not be degraded by the proteasome during memory formation. Furthermore, it is possible that IU1 is disrupting memory through a process that is not linked to protein degradation. Consistent with this, ubiquitin signaling can regulate learning-dependent synaptic plasticity independent of protein degradation (Pavlopoulos et al. 2011). Since USP14 regulates the length of the ubiquitin chain on a substrate, it is possible that USP14 regulates memory formation by trimming ubiquitin chains and targeting substrates for other nonproteolytic functions. Future studies should aim to more precisely dissect these potential functions of USP14 in the regulation of learning-dependent synaptic plasticity in the amygdala.

In conclusion, our data suggest that USP14 specifically regulates the consolidation of contextual, but not auditory, fear memories. Additionally, USP14-dependent memory formation is independent of changes in proteasome catalytic activity, suggesting that USP14 regulates memory formation through a process independent of changes in proteasome activity. These results show for the first time that USP14 is critical for long-term memory formation in mammals and add to the growing body of evidence implicating the ubiquitin–proteasome system in long-term memory formation.

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