Females, but not males, require protein degradation in the hippocampus for contextual fear memory formation

Kiley Martin, Madeline Musaus, Shaghayegh Navabpour, Aspen Gustin, W. Keith Ray, Richard F. Helm, and Timothy J. Jarome

1School of Neuroscience, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA; 2Fralin Biomedical Research Institute, Department of Translational Biology, Medicine, and Health, Virginia Polytechnic Institute and State University, Roanoke, Virginia 24016, USA; 3Department of Animal and Poultry Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA; 4Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA

Strong evidence supports a role for protein degradation in fear memory formation. However, these data have been largely done in only male animals. Here, we found that following contextual fear conditioning, females, but not males, had increased levels of proteasome activity and K48 polyubiquitin protein targeting in the dorsal hippocampus, the latter of which occurred at chaperones or RNA processing proteins. In vivo CRISPR–dCas9-mediated repression of protein degradation in the dorsal hippocampus impaired contextual fear memory in females, but not males. These results suggest a sex-specific role for protein degradation in the hippocampus during the consolidation of a contextual fear memory.

[Supplemental material is available for this article.]
contextual fear conditioning procedure, as previously described by our group (Orsi et al. 2019; Devulapalli et al. 2021). In some cases, the CA1 region of the dorsal hippocampus was dissected, subcellular fractions collected and proteasome activity quantified (Orsi et al. 2019; Devulapalli et al. 2021). In other cases, dorsal hippocampus tissue was dissected and purified with a K48-specific tandem ubiquitin binding entity (TUBE) followed by liquid chromatography mass spectrometry (LC/MS). Prior to behavioral training, some animals received stereotactic injections of CRISPR-dCas9 plasmids into the CA1 region of the dorsal hippocampus using our recently described in vivo procedure (Devulapalli et al. 2021; Jarome et al. 2021). These animals underwent behavioral training 2 wk later and were tested for retention to the training context the following day.

We first tested whether proteasome activity was increased in the dorsal hippocampus of male and female rats 1 h after contextual fear conditioning (Fig. 1A), as this is the time point at which numerous studies have reported the earliest increase in protein degradation following fear conditioning (Jarome et al. 2011, 2013; Reis et al. 2013; Orsi et al. 2019; Devulapalli et al. 2021). Performance during the training session for both male and female rats is shown in Figure 1B. In females, we found increased proteasome activity in the nuclear (t1,3 = 2.676, P = 0.0160) (Fig. 1C), but not cytosolic (t1,3 = 1.843, P = 0.0819) (Fig. 1D) or synaptic (t1,3 = 0.6993, P = 0.4933) (Fig. 1E) fractions. Conversely, in males we did not observe changes in proteasome activity in the nuclear (t1,3 = 0.7876, P = 0.4451) (Fig. 1F), cytosolic (t1,7 = 0.6948, P = 0.4966) (Fig. 1G) or synaptic (t1,3 = 0.2738, P = 0.7877) (Fig. 1H) fractions. These results suggest that proteasome degradation is increased in the dorsal hippocampus of female, but not male, rats following contextual fear conditioning.

To test whether the increase in proteasome activity in females was specific to the context-shock association, we next compared naïve animals with those that underwent an immediate shock procedure in which the context and shock are presented in a nonassociative manner (Orsi et al. 2019). Interestingly, we found that immediate shock animals had increased nuclear proteasome activity relative to naïve controls (t1,3 = 3.031, P = 0.0097) (Fig. 1I). This suggests that in females, the protein degradation process is likely being increased in the dorsal hippocampus due to context exposure alone.

To further examine this potential sex difference in protein degradation increases in the dorsal hippocampus, we next quantified changes in degradation-specific K48 polyubiquitination in this region of male and female rats that underwent contextual fear conditioning. Broad Western blot approaches lack the sensitivity to detect changes in K48-targeting of individual proteins and we previously reported no change in global K48 polyubiquitination levels in the dorsal hippocampus of male and female rats following contextual fear conditioning (Devulapalli et al. 2021). To increase our sensitivity and identify specific targets of the protein degradation process, which could be used to infer function, we purified dorsal hippocampus tissue collected from naïve and fear conditioned male and female rats with a K48-specific TUBE followed by LC/MS. Consistent with our proteasome activity data, we detected increased K48 polyubiquitin targeting of 3 proteins in females, with 1 additional protein showing a significantly lower level of K48 targeting (Fig. 2A). Conversely, we did not identify any positive targets of K48 polyubiquitination in males, although one protein did have reduced K48 targeting as a function of learning (Fig. 2A). In females, ingenuity pathway analysis (IPA) of the protein targets of K48 polyubiquitination indicated that a downstream target of ribosomal RNA processing 12 (RRP12) is ribonucleotide reductase (RNR) (Fig. 2B), which is involved in DNA synthesis, and evidence suggests that loss of RRP12 activity is associated with a reduced function of RNR (Tafforeau et al. 2013). Furthermore, the chaperone heat shock protein 40 (HSP40) is involved in regulation of the tyrosine kinase fibroblast growth factor receptor 1 (FGFR1) (Fig. 2C), suggesting potential functional roles of protein degradation in the DNA damage response and intracellular signaling during contextual fear memory consolidation.

Next, we tested whether males and females required protein degradation in the dorsal hippocampus for the consolidation of a
contextual fear memory. Pharmacological manipulations of the proteasome, while specific, cause an artificial accumulation of ubiquitinated proteins (Jarome et al. 2011) that can disrupt a variety of cellular processes independent of protein degradation (Pavlopoulos et al. 2011; Musaus et al. 2020), making interpretation of behavioral outcomes difficult. To overcome this, we recently developed an in vivo CRISPR-dCas9 (dCas9) procedure that can achieve simultaneous, bidirectional control of proteasome activity and protein ubiquitination levels in the brain (Devulapalli et al. 2021). This procedure multiplexes synthetic guide RNAs (gRNA) against proteasome subunit coding gene Psmd14 and ubiquitin coding gene Uba52 in combination with the transcriptionally repressive dCas9-KRAB-MECP2 fusion, resulting in significant reductions in both degradation-specific K48 polyubiquitination levels and proteasome activity within 2 wk of infusion. Using this procedure (Fig. 3A), we tested whether protein degradation was necessary for the consolidation of a contextual fear memory in male and female rats. Consistent with our molecular data, in females inhibition of protein degradation did not impair performance during training (two-way ANOVA: Time $F_{(4,220)}$ = 23.22, $P < 0.0001$; Group $F_{(1,25)}$ = 4.179, $P = 0.0963$; Interaction $F_{(4,220)}$ = 1.7898, $P = 0.1689$) (Fig. 3B) but resulted in significant behavioral deficits during the retention test ($P_{10}$ = 2.31, $P = 0.0435$) (Fig. 3C). However, in males inhibition of protein degradation did not alter performance during training (two-way ANOVA: Time $F_{(4,250)}$ = 53.74, $P < 0.0001$; Group $F_{(1,11)}$ = 0.1962, $P = 0.6646$; Interaction $F_{(4,250)}$ = 0.4251, $P = 0.7899$) (Fig. 3D) or the retention test ($P_{1}$ = 0.136, $P = 0.8938$) (Fig. 3E). Collectively, these results suggest that females, but not males, require protein degradation in the dorsal hippocampus for the formation of a contextual fear memory.

The UPS-associated protein degradation process has been strongly linked to synaptic plasticity underlying memory formation, however, much of the work previously done has been exclusively in male animals. Previous work found that administration of proteasome inhibitors into the dorsal hippocampus of male rodents does not impair a context fear memory (Lee et al. 2008), but does impair formation of a contextual memory that is acquired independent of an aversive shock stimulus (Cullen et al. 2017). Here, we found that females, but not males, had increased protein degradation in the dorsal hippocampus after training on a contextual fear conditioning task, which was likely driven by processing of the context itself. Despite this, genetic inhibition of protein degradation in the dorsal hippocampus impaired contextual fear memory in female, but not male, rats. Collectively, the results suggest that males and females differ in the requirement for protein degradation in the hippocampus for contextual fear memory formation. Importantly, in females increased proteolysis appears to be necessary to process the contextual information important for establishing the CS-UCS relationship, which is in contrast to males where this same process does not appear to be required for processing of a context-shock relationship learned in a single trial (Lee et al. 2008; Cullen et al. 2017).

Interestingly, numerous studies have reported sex differences on contextual fear conditioning tasks in rodents, often times with conflicting results (Maren et al. 1994; Wiltgen et al. 2001; Graham et al. 2009; Keiser et al. 2017; Colon et al. 2018; Devulapalli et al. 2021). While it is unknown why sex differences exist for contextual fear conditioning in rodent models or what contributes to these conflicting results, it is interesting to speculate that this could be due to differences in engagement of the protein degradation...
importance during testing (Devulapalli et al. 2021). Together, these data strongly suggest that sex differences exist in the requirement for protein degradation across other parts of the fear circuit. Despite strong evidence that protein degradation is involved in the storage of various types of memories across several brain regions, to date, the target substrates of the proteasome have remained elusive. Here, we report the first unbiased proteomic analysis of degradation-specific K48 polyubiquitinated protein targets in the dorsal hippocampus following learning. Interestingly, we found that in females there were only three proteins that gained the K48 polyubiquitin mark following fear conditioning, suggesting that while necessary, the protein degradation process may not be robustly engaged in the hippocampus following learning. Importantly, our data identified downstream protein effectors that have known roles in the regulation of intracellular signaling and the DNA damage response, both of which have been implicated in memory consolidation (Johansen et al. 2011; Madabhushi et al. 2015; Li et al. 2019). While we did not directly test the role of protein degradation in regulating the downstream protein targets and processes, the data presented here are exciting as they provide the first unbiased evidence suggesting a functional role for protein degradation during memory formation. Future studies will want to use this information to directly test the functional significance of ubiquitin-proteasome-mediated protein degradation in the dorsal hippocampus during the consolidation of a contextual fear memory.

One limitation of our study was that the K48-TUBE assay could only detect one type of degradation-specific polyubiquitin chains. While K48 is the primary polyubiquitin that targets proteins for degradation (Chau et al. 1989) and generally the most abundant mark in cells, others, such as K11, can lead to proteolysis as well (Matsumoto et al. 2010). However, quantification of these other degradation chains is largely impossible in brain tissue due to technical limitations (Musaus et al. 2020). This leaves open the possibility that males do engage protein degradation in the hippocampus following contextual fear conditioning, but do so via one of the noncanonical polyubiquitin chains. While we cannot fully rule out this possibility, our data showing that inhibition of protein degradation in the dorsal hippocampus did not impair fear memory in male rats strongly suggest that they do not need this process for memory consolidation.

In conclusion, we report a novel sex difference in the role of protein degradation in contextual fear memory consolidation in the dorsal hippocampus. In combination with our previous work, these data suggest that in the hippocampus the protein degradation process is more important for fear memory formation in females than males, though both sexes have a similar need for this process in the amygdala. Considering that females are twice

---

**Figure 3.** CRISPR–dCas9-mediated down-regulation of protein degradation in the dorsal hippocampus impairs memory for a contextual fear conditioning task in females but not males. (A) Experimental design showing that the fear conditioning took place 2 wk after the dorsal hippocampus CRISPR–dCas9 injection, with testing occurring 24 h after training. Synthetic guide RNAs (gRNAs) targeted ubiquitin (Uba52) and proteasome subunit (Psmd14) coding genes and were combined with a transcriptionally repressive dCas9–KRAB–MECP2 fusion. Male and female cohorts were run at separate times. (B, C) In females, injection of the CRISPR–dCas9 constructs did not impact performance during training (B) but significantly impaired retention during testing (C) relative to controls (n = 6 per group). (D, E) In males, injection of the CRISPR–dCas9 constructs did not impact performance during training (D) or retention during testing (E) relative to controls (n = 8 per group). (*) P < 0.05 from Uba52 + Psmd14-gRNA.
as likely to develop posttraumatic stress disorder (PTSD) and Alzheimer’s diseases (Christiansen and Hansen 2015; Beam et al. 2018), these results could provide important information for the understanding of some differences in fear memory formation or age-related hippocampus-dependent memory loss.

Acknowledgments

This work was supported by National Institutes of Health (NIH) grants MH120498, MH120569, MH122414, and MH123742 (to T.J.).

References

Artinin J, McCauran AM, De Jaeger X, Mouleoudis L, Frances B, Rouillet P. 2008. Protein degradation, as with protein synthesis, is required during not only long-term spatial memory consolidation but also reconsolidation. Eur J Neurosci 27: 3009–3019. doi:10.1111/j.1460-9568.2008.06262.x

Beam CR, Kaneshiro C, Jang JY, Reynolds CA, Pedersen NL, Gatzi M. 2018. Differences between women and men in incidence rates of dementia and Alzheimer’s disease. J Alzheimer’s Dis 64: 1077–1083. doi:10.3233/JAD-180141

Bedford I, Faine S, Sheppard PW, Mayer RJ, Roelofs J. 2017. Activity level controls postsynaptic composition and sex-specific differences in hippocampus and amygdala during retrieval. Neuropsychopharmacology 42: 397–407. doi:10.1038/nnp.2016.174

Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK, Furini CR, Myskiw Jde C, Schmidt BE, Myskiw J. 2011. Molecular mechanisms of fear learning and memory. Cell 147: 509–524. doi:10.1016/j.cell.2011.10.009

Keiser AA, Turnbull LM, Darian MA, Feldman DE, Song I, Tronson NC. 2017. Sex differences in contextual fear generalization after reconsolidation of hippocampus and amygdala during retrieval. Neuropsychopharmacology 42: 397–407. doi:10.1038/nnp.2016.174

Lee SH, Choi JH, Lee N, Lee RH, Kim JI, Yu NK, Choi SL, Kim H, Kang BK. 2008. Synaptic protein degradation underlies desensitization of retrieved fear memory. Science 319: 1253–1256. doi:10.1126/science.1150541

Li X, Marshall PR, Leighton LJ, Zajaczkowski EL, Wang Z, Madugalle SU, Yin J, Breedy TW, Wei W. 2019. The DNA repair-associated protein Gadd45α regulates the temporal coding of immediate early gene expression within the prelimbic prefrontal cortex and is required for the consolidation of associative fear memory. J Neurosci 39: 970–983. doi:10.1523/JNEUROSCI.2014-18-2018

Lopez-Salon M, Alonso M, Vianna MB, Viola H, Mello e Souza T, Izquierdo I, Pasquini JM, Medina JH. 2001. The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. Eur J Neurosci 14: 1820–1826. doi:10.1046/j.1460-9568.2001.01806.x

Maren S, De Oca B, Fanselow MS. 1994. Sex differences in hippocampal protein polyubiquitination in the amygdala during the consolidation of associative fear memory. Neuron 18: 1073–1083. doi:10.1016/0896-6273(94)90157-2

Maren S, De Oca B, Fanselow MS. 1994. Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning. Brain Res 661: 25–34. doi:10.1016/0006-8993(94)11762-6

Matsusoto MT, Wickilffe KB, Long KC, Yu C, Bosancic J, Bustos D, Phu L, Kirkpatrick DS, Hymowitz SG, Rape M, et al. 2010. K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. Mol Cell 39: 477–484. doi:10.1016/j.molcel.2010.07.001

Musau M, Navapour S, Jarome TJ. 2020. The diversity of linkage-specific polyubiquitin chains and their role in synaptic plasticity and memory consolidation. Neuronal Learn Mem 174: 107286. doi:10.1016/j.nlm.2020.107286

Orsi SA, Devulapalli RK, Nelsen JL, McFadden T, Surineni R, Jarome TJ. 2019. Distinct subcellular changes in proteasome activity and linkage-specific polyubiquitination in the amygdala during the consolidation and reconsolidation of a fear memory. Neuronal Learn Mem 157: 1–11. doi:10.1016/j.nlm.2018.11.012

Pavlopoulos E, Trifilieff P, Chevalerey V, Fioriti L, Zairis S, Pagano A, Mallaret G, Kandel ER. 2011. Neuronal1 activates CREB3: a function for nonproteolytic ubiquitin in synaptic plasticity and memory storage. Cell 147: 1369–1383. doi:10.1016/j.cell.2011.09.056

Reis DS, Jarome TJ, Helmstetter FJ. 2013. Memory formation for trace fear conditioning requires ubiquitin-proteasome mediated protein degradation in the prefrontal cortex. Front Behav Neurosci 7: 130. doi:10.3389/fnbeh.2013.00150

Rosenberg T, Elköbi A, Dieterich DC, Rosenblum K. 2016a. NMDAR-dependent proteasome activity in the gustatory cortex is necessary for conditioned taste aversion. Neuronal Learn Mem 130: 7–16. doi:10.1016/j.nlm.2016.01.002

Rosenberg T, Elköbi A, Rosenblum K. 2016b. mACHR-dependent decrease in proteasome activity in the gustatory cortex is necessary for novel taste learning. Neuronal Learn Mem 135: 115–124. doi:10.1016/j.nlm.2016.07.029
Rozeske RR, Valerio S, Chaudun F, Herry C. 2015. Prefrontal neuronal circuits of contextual fear conditioning. *Genes Brain Behav* **14**: 22–36. doi:10.1111/gbb.12181

Rubio MD, Wood K, Haroutunian V, Meador-Woodruff JH. 2013. Dysfunction of the ubiquitin proteasome and ubiquitin-like systems in schizophrenia. *Neuropsychopharmacology* **38**: 1910–1920. doi:10.1038/npp.2013.84

Tafforeau L, Zorbas C, Langhendries JL, Mullineux ST, Stamatopoulou V, Mullier R, Wacheul L, Lafontaine DL. 2013. The complexity of human ribosome biogenesis revealed by systematic nucleolar screening of pre-rRNA processing factors. *Mol Cell* **51**: 539–551. doi:10.1016/j.molcel.2013.08.011

Todd TP, DeAngeli NE, Jiang MY, Bucci DJ. 2017. Retrograde amnesia of contextual fear conditioning: evidence for retrosplenial cortex involvement in configural processing. *Behav Neurosci* **131**: 46–54. doi:10.1037/bne0000183

Trask S, Pullins SE, Ferrara NC, Helmstetter FJ. 2021. The anterior retrosplenial cortex encodes event-related information and the posterior retrosplenial cortex encodes context-related information during memory formation. *Neuropsychopharmacology* **46**: 1386–1392. doi:10.1038/s41386-021-00959-x

Wiltgen BJ, Sanders MJ, Behne NS, Fanselow MS. 2001. Sex differences, context preexposure, and the immediate shock deficit in Pavlovian context conditioning with mice. *Behav Neurosci* **115**: 26–32. doi:10.1037/0735-7044.115.1.26

Zheng Q, Huang T, Zhang L, Zhou Y, Luo H, Xu H, Wang X. 2016. Dysregulation of ubiquitin-proteasome system in neurodegenerative diseases. *Front Aging Neurosci* **8**: 303. doi:10.3389/fnagi.2016.00303

*Received April 14, 2021; accepted in revised form June 7, 2021.*