Research

Sex differences in training-induced activity of the ubiquitin proteasome system in the dorsal hippocampus and medial prefrontal cortex of male and female mice

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The ubiquitin proteasome system (UPS) is a primary mechanism through which proteins are degraded in cells. UPS activity in the dorsal hippocampus (DH) is necessary for multiple types of memory, including object memory, in male rodents. However, sex differences in DH UPS activation after fear conditioning suggest that other forms of learning may also differentially regulate DH UPS activity in males and females. Here, we examined markers of UPS activity in the synaptic and cytoplasmic fractions of DH and medial prefrontal cortex (mPFC) tissue collected 1 h following object training. In males, training increased phosphorylation of proteasomal subunit Rpt6, 20S proteasome activity, and the amount of PSD-95 in the DH synaptic fraction, as well as proteasome activity in the mPFC synaptic fraction. In females, training did not affect measures of UPS or synaptic activity in the DH synaptic fraction or in either mPFC fraction but increased Rpt6 phosphorylation in the DH cytoplasmic fraction. Overall, training-induced UPS activity was greater in males than in females, greater in the DH than in the mPFC, and greater in synaptic fractions than in cytosol. These data suggest that object training drives sex-specific alterations in UPS activity across brain regions and subcellular compartments important for memory.

[Supplemental material is available for this article.]

The formation of long-term memories requires dynamic regulation of the synaptic proteome. The local regulation of synaptic proteins requires a delicate balance between protein synthesis and degradation in order to permit stable changes in synaptic strength (Giandomenico et al. 2022). The ubiquitin proteasome system (UPS) is a primary pathway regulating the degradation of misfolded or damaged proteins in the brain (Glickman and Ciechanover 2002; Hegde 2017). In this system, proteins are targeted for degradation by a network of signaling molecules and protein complexes that covalently attach the small protein modifier ubiquitin to substrate proteins. After the first ubiquitin molecule becomes bound to the substrate protein, another ubiquitin is attached to an internal lysine residue on the preceding ubiquitin, thereby forming a polyubiquitin chain. Substrate proteins can acquire several different types of polyubiquitin "tags;" however, those at lysine 48 (K48) become targets for degradation by the 26S proteasome complex (Musaus et al. 2020). The 26S proteasome is composed of one or two 19S regulatory caps and a catalytic 20S core. K48 polyubiquitinated proteins initially bind to the 19S outer cap, whereas phosphorylation of the 19S regulatory particle triple-ATPase 6 (Rpt6) subunit initiates unfolding and translocation of the substrate protein into the 20S core (Tanaka 2009). Proteasome activity and K48 polyubiquitination are increased following activation of NMDA receptors (NMDARs) (Colledge et al. 2003; Jarome et al. 2011). Rpt6 can be phosphorylated by cell signaling proteins including calcium calmodulin kinase II (CaMKII) and protein kinase A (PKA), both of which have well-documented roles regulating synaptic plasticity and long-term memory (Upadhya et al. 2006; Zhang et al. 2007; Bingol et al. 2010; Djakovic et al. 2012; Jarome et al. 2013; Devulapalli et al. 2019). As such, the proteasome itself and substrate polyubiquitination are regulated in an activity-dependent manner.

Protein degradation mediated by the UPS is essential for synaptic growth, transmission, and plasticity (Hegde 2017). For example, UPS-mediated protein degradation is necessary for hippocampal long-term potentiation (LTP) in rodents, where inhibition of proteasome-dependent protein degradation in male rat hippocampal slices blocks late-phase LTP (Fonseca et al. 2006; Karpova et al. 2006). In the nucleus, LTP-induced proteasome activity degrades transcriptional repressors following synaptic activity to permit de novo transcription of genes critical for learning and memory (Upadhya et al. 2004; Dong et al. 2008 2014; Smith et al. 2020). However, the UPS appears to play a particularly important role in regulating the protein composition of synapses. Up-regulating synaptic activity in cultured rat hippocampal neurons increases protein polyubiquitination within the postsynaptic density and increases the trafficking of proteasomes to dendritic spines in an NMDA-dependent manner (Ehlers 2003; Bingol and Schuman 2006). This activity-dependent translocation of proteasomes to dendritic spines regulates hippocampal spine outgrowth via CaMKII-dependent phosphorylation of the Rpt6 subunit of the proteasome (Djakovic et al. 2009, 2012; Bingol et al. 2010; Hamilton et al. 2012). The proteasome regulates hippocampal synaptic

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plasticity by targeting the degradation of structural scaffolding proteins to permit the internalization of glutamate receptors within the postsynaptic density (Sheng and Pak 2000; Sheng and Kim 2002; Ehlers 2003; Patrick et al. 2003). For example, the structural postsynaptic density protein 95 (PSD-95) becomes rapidly ubiquitinated following NMDAR stimulation in cultured rat hippocampal neurons (Colledge et al. 2003). Accordingly, the mechanisms that regulate proteasome activity in cultured hippocampal synapses are functionally relevant for memory formation (Jarome and Helmstetter 2013, 2014). Proteasome inhibition around the time of learning impairs memory consolidation in multiple aversively motivated tasks including inhibitory avoidance, conditioned taste aversion, potentiated startle, and contextual fear in male rodents (Lopez-Salon et al. 2001; Yeh et al. 2006; Jarome et al. 2011; Rodriguez-Ortiz et al. 2011; Reis et al. 2013). Of these, learning-induced changes in UPS activity have been most extensively documented in response to contextual fear learning, which increases K48 polyubiquitination, Rpt6 phosphorylation, and 20S proteasome activity in the amygdala of male rats in a manner dependent on activation of NMDARs and CaMKII, but not PKA, activity (Jarome et al. 2011, 2013). Although proteasome activity is also required for the consolidation of spatial and object recognition memories in male rodents (Artinian et al. 2008; Choi et al. 2010; Figueiredo et al. 2015), it is unclear whether nonaversive tasks, such as object placement and object recognition, activate the same UPS signaling mechanisms and proteasome subunits as aversive learning tasks.

In addition, the extent to which nonaversive learning similarly activates UPS-mediated protein degradation in males and females is completely unknown. This information is important in light of recent work showing that UPS activity is regulated in a sex-specific manner across brain regions and subcellular compartments following fear memory formation (Devulapalli et al. 2019, 2021; Dulka et al. 2021; Farrell et al. 2021; Martin et al. 2021). Initial findings demonstrated that male and female rats differentially use CaMKII and PKA to regulate proteasome activity across nuclear, cytosolic, and synaptic compartments and brain regions (hippocampus and basolateral amygdala) following contextual fear conditioning (Devulapalli et al. 2019). These findings not only showed a sex-specific role in the mechanisms that regulate proteasome activity following fear learning but also revealed that PKA plays a more prominent role in regulating proteasome activity across distinct subcellular compartments than previously thought (Devulapalli et al. 2019). Assessing UPS activity across subcellular compartments has also revealed that although training increased proteasome activity and protein polyubiquitination in nuclear basolateral amygdala extracts in male, but not female, rats, UPS activity was required for both sexes in the consolidation of contextual fear memories (Devulapalli et al. 2021). Interestingly, the sex-specific activation of the UPS by contextual fear conditioning differs strikingly in nuclear dorsal hippocampus (DH) extracts, where female, but not male, rats required UPS activity for successful consolidation of contextual fear memories (Martin et al. 2021). These findings collectively demonstrate that males and females differ in their requirement for, and engagement of, UPS activity following learning, and this activity differs across brain regions and cellular compartments in the fear circuit. As such, the involvement of DH UPS activity in regulating the formation of other types of memories could differ substantially between the sexes.

No studies to date have assessed the extent to which the UPS is activated in males and females following the object training approach used to induce memory consolidation in the commonly used object recognition and object placement tasks. As such, the purpose of this study was to examine the extent to which object training alters UPS function in the synaptic and cytoplasmic fractions of DH and medial prefrontal cortex (mPFC) tissue in male and female mice during the consolidation window of long-term memory formation. We were interested in these brain regions because we previously demonstrated that individual and concurrent chemogenetic inactivation of the DH and mPFC impairs spatial and object recognition memory consolidation in female mice (Tuscher et al. 2018), further providing support for the notion that activity of, and interactions between, these brain regions are required for consolidation of object memories (Preston and Eichenbaum 2013; Eichenbaum 2017). Our results suggest that object training increases proteasome subunit phosphorylation and catalytic activity in a manner that differs across brain regions and subcellular compartments and between sexes. We demonstrate that object training in males, but not females, increases proteasome function in the DH and mPFC synaptic fraction, whereas in females, it up-regulates proteasome activity only in the DH cytoplasmic fraction. These results highlight the need for future studies to assess whether females, in addition to males, require proteasome activity for object placement and object recognition paradigms.

Results

Experimental design and training data: males and females were similarly engaged during object training

Male and female C57BL/6 mice were assigned to naïve or trained groups. Naïve mice remained in their home cages, whereas trained mice were allowed to explore two identical objects placed near the corners of an open square arena (Fig. 1A; see the Materials and Methods for details). One hour after object training, DH and mPFC tissues were collected and later fractionated for isolation of crude synaptosomal and cytoplasmic extracts (Fig. 1B) to be used to quantify levels of UPS-related proteins via Western blot and 20S proteasome activity via an ELISA-based activity assay. This time point was selected because of previous reports that multiple aspects of UPS activity are activated in the hippocampus and other brain regions 1 h after contextual fear conditioning in male and female rats (Jarome et al. 2011, 2013; Devulapalli et al. 2019, 2021; Dulka et al. 2021; Martin et al. 2021). For their tissue to be included in Western blot and 20S activity assays, mice were required to accumulate 30 sec of exploration time with the objects during a 20-min training session. During this session, males and females did not differ in the total distance traveled ($t_{(14)} = 0.64$, $P = 0.53$)

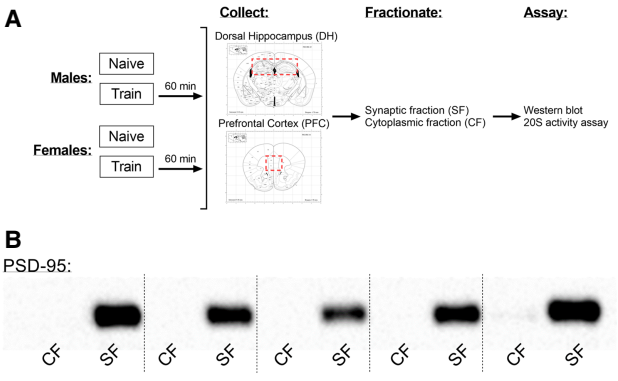

Figure 1. Experimental design for object training, tissue collection, and sample preparation. (*A*) Synaptic and cytoplasmic fractions were collected from male and female mice 1 h following successful completion of object training. (*B*) Representative Western blot image demonstrating successful fractionation. Here, the postsynaptic protein PSD-95 protein was found in synaptic (SF), but not cytoplasmic (CF), fractions of dorsal hippocampus tissue.

(Supplemental Fig. S1A) or in their total number of visits to the objects ($t_{(14)} = 1.73$, $P = 0.11$) (Supplemental Fig. S1B). However, unpaired *t*-tests indicated that females on average tended to complete their training sooner than males ($t_{(14)} = 2.09$, $P = 0.055$) (Supplemental Fig. S1C) and moved around the testing box significantly faster than males ($t_{(14)} = 2.92$, $P = 0.01$) (Supplemental Fig. S1D). These data suggest that males and females had similar engagement with objects during the task, as indicated by distance traveled and total number of object visits, but females were faster and tended to complete the task sooner than males, which is consistent with previous work showing that female mice move faster in an open field than male mice (Frick et al. 2000).

DH synaptic fraction: training increased Rpt6 phosphorylation and proteasome activity in males only

We first determined the extent to which object training alters UPS activity 1 h following object training in synaptic fractions of DH tissue. We quantified three measures of UPS activity: phosphorylation levels of the 19S proteasome subunit Rpt6 at serine 120 (referred to here as pRpt6), 20S chymotrypsin-like proteasome activity, and levels of K48 polyubiquitination (Fig. 2). For pRpt6 levels, we found a significant main effect of training ($F_{(1,33)} = 8.86$, $P = 0.01$) and a trend for a main effect of sex ($F_{(1,33)} = 3.58$, $P = 0.07$) in the absence of a training × sex interaction ($F_{(1,33)} = 0.03$, $P = 0.86$) (Fig. 2A). Although pRpt6 levels were increased by training in both sexes, the effect was greater in males, as suggested by significant within-sex analyses showing differences relative to naïve controls in males ($t_{(15)} = 2.29$, $P = 0.04$) but not females ($t_{(18)} = 1.22$, $P = 0.24$). We then measured 20S proteasome activity in DH synaptic fraction lysates using an in vitro proteasome activity assay (Jarome et al. 2013; Devulapalli et al. 2019; Orsi et al. 2019). We found that chymotrypsin-like activity, the main form of proteasome activity, was significantly affected by training ($F_{(1,34)} = 4.10$, $P = 0.05$), although this effect was primarily driven by males, as suggested by trends for a training × sex interaction ($F_{(1,34)} = 3.09$, $P = 0.09$) (Fig. 2B) and post-hoc difference between trained and naïve males ($P = 0.07$). These trends were consistent with analyses showing increased chymotrypsin-like proteasome activity in trained males ($t_{(16)} = 3.67$, $P = 0.002$), but not females ($t_{(18)} = 0.1649$, $P = 0.87$), relative to naïve controls. The lack of a main effect of sex ($F_{(1,34)} = 0.08$, $P = 0.79$) indicated no overall sex differences in proteasome activity irrespective of training, although it is interesting to note that means of both female groups were intermediate between the two male groups. Finally, we examined whether object training altered levels of K48 polyubiquitination and found no significant main effects of training ($F_{(1,33)} = 0.69$, $P = 0.41$) or sex ($F_{(1,33)} = 0.06$, $P = 0.81$), or a training × sex interaction ($F_{(1,33)} = 0.52$, $P = 0.48$) (Fig. 2C). Consistent with these findings, object training did not alter K48 polyubiquitination in males ($t_{(15)} = 1.16$, $P = 0.26$) or females ($t_{(18)} = 0.09$, $P = 0.93$) relative to naïve controls. Collectively, these findings suggest increased proteasome function in DH synapses among males, but not females, 1 h after object training.

Given the sex-specific increase in proteolytic activity at DH synapses, we next determined the extent to which object training-induced alterations in proteasome function in males influenced levels of plasticity-related proteins, including activity-regulated cytoskeleton protein/activity-related gene 3.1 (Arc/Arg3.1) and postsynaptic density-95 protein (PSD-95) at DH synapses (Fig. 3). We chose to assess Arc and PSD-95 levels because proteasome activity at synapses promotes synaptic remodeling following periods of neural activity (Colledge et al. 2003; Ehlers 2003; Patrick et al. 2003; Jarome et al. 2011), and as such, we expected levels of both proteins to be increased by training. With respect to Arc protein levels at DH synapses, the main effect of

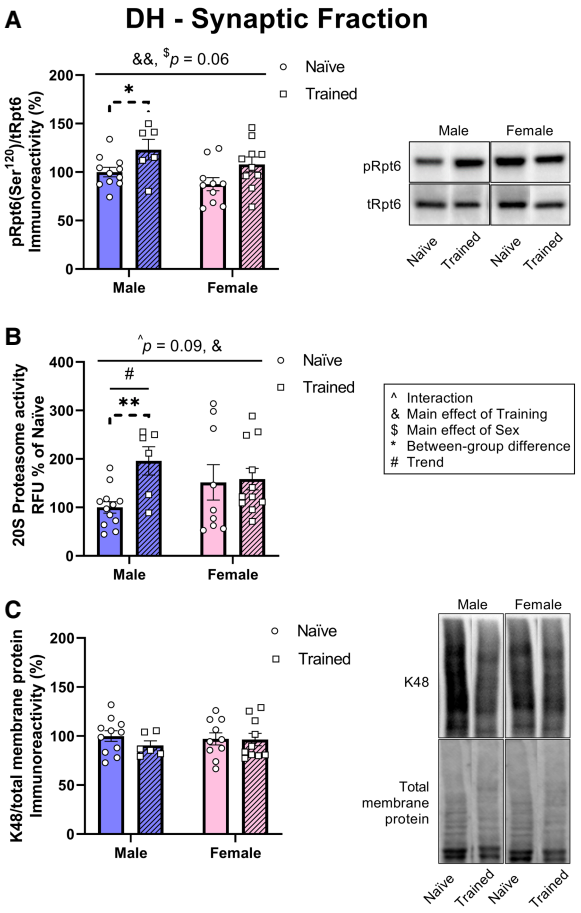

Figure 2. Object training increased proteasome function in the DH synaptic fraction of males only. (*A*) Rpt6 phosphorylation was significantly increased at DH synapses 1 h following object training ([&&] $P < 0.01$), and this trend was particularly apparent in males ([$] $P = 0.06$). Object training alone significantly increased Rpt6 phosphorylation in males ([*] $P < 0.05$, *t*-test) at DH synapses. (*B*) 20S chymotrypsin-like activity was increased following object training at DH synapses ([&] $P < 0.05$, [^] $P = 0.09$), which was driven largely by an increase in trained males relative to naïve males ([#] $P = 0.07$, post-hoc; [**] $P < 0.01$, *t*-test). (*C*) K48 polyubiquitination levels were unaffected by training status or sex. Bars represent the mean ± standard error of the mean (SEM).

training was significant ($F_{(1,32)} = 4.37$, $P = 0.05$) (Fig. 3A) in the absence of a main effect of sex ($F_{(1,32)} = 0.08$, $P = 0.78$) and a training × sex interaction ($F_{(1,32)} = 0.64$, $P = 0.43$), suggesting that object training increases neuronal activity at DH synapses. However, this effect was particularly evident in males, as within-sex analyses indicated that object training increased Arc protein in trained males ($t_{(15)} = 2.80$, $P = 0.01$), but not females ($t_{(17)} = 1.26$, $P = 0.23$), relative to naïve controls. We next examined PSD-95 levels and found a significant main effect of training ($F_{(1,30)} = 5.96$, $P = 0.02$) (Fig. 3B) and sex × training interaction ($F_{(1,30)} = 7.71$, $P = 0.01$), such that PSD-95 levels were higher in trained males than in naïve males ($P = 0.01$). PSD-95 protein was increased in trained males ($t_{(13)} = 3.74$, $P = 0.003$), but not females ($t_{(16)} = 0.47$, $P = 0.65$), relative to naïve controls. The lack of a main effect of sex ($F_{(1,30)} = 0.25$, $P = 0.62$) indicated no overall sex differences in PSD-95 levels irrespective of training. To get a sense of whether training-induced activation of UPS activity is associated with Arc and PSD-95 levels, we performed correlation and linear regression analyses between pRpt6 and Arc, as well as pRpt6 and PSD-95. When all mice (both sexes and both training groups) (Supplemental Fig. S2A,B)

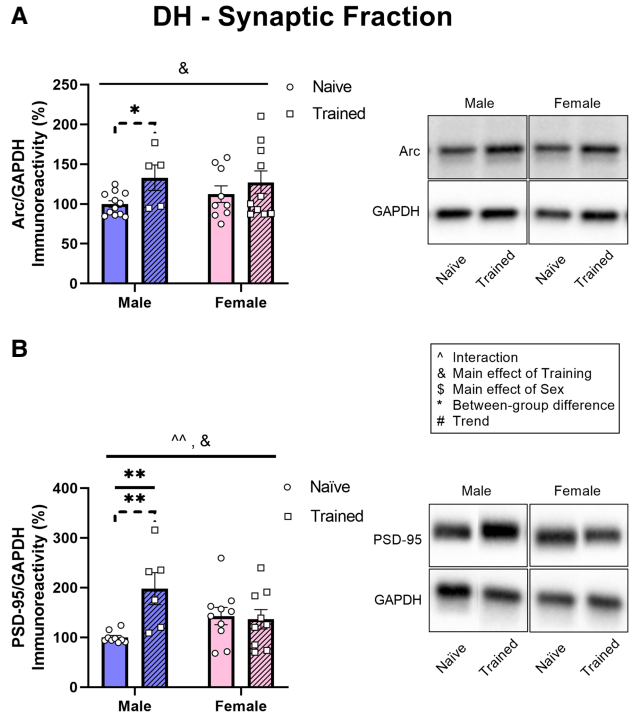

Figure 3. Object training increased Arc protein expression similarly in both sexes but increased PSD-95 protein expression in the DH synaptic fraction of males only. (*A*) Arc protein levels were significantly increased at DH synapses of both sexes 1 h following object training ([&] $P<0.05$). This was particularly true for trained males, who had higher levels of Arc protein than naïve males, as assessed by a within-sex *t*-test ([*] $P<0.05$). (*B*) PSD-95 protein levels were significantly increased following object training in DH synapses ([&] $P<0.05$, [^^] $P<0.01$) and was significantly increased in trained males relative to naive males ([**] $P<0.01$, post-hoc and *t*-test). Bars represent the mean ± SEM.

were included in the analyses, pRpt6 levels were positively correlated with both Arc ($r=0.363$, $F_{(1,31)}=4.69$, $P=0.038$) and PSD-95 ($r=0.535$, $F_{(1,31)}=12.42$, $P=0.0013$) levels. When we limited the analyses to males only (Supplemental Fig. S2C,D), PSD-95 ($r=0.7328$, $F_{(1,13)}=15.07$, $P=0.0019$), but not Arc ($r=0.4059$, $F_{(1,14)}=2.76$, $P=0.119$), remained significant. Similar effects were seen in an analysis of females only (PSD-95: $r=0.5502$, $F_{(1,16)}=6.95$, $P=0.018$; Arc: $r=0.343$, $F_{(1,15)}=2.009$, $P=0.177$) (Supplemental Fig. S2E,F). These data suggest that pRpt6 in the DH synaptic fraction is positively corelated with levels of Arc and PSD-95; however, this relationship remains significant within each sex only for PSD-95. The scatter plots in Supplemental Figure S2 suggest an effect of training, such that naïve mice tend to have lower levels of pRpt6 and Arc/PSD-95, but this effect is most evident in males, particularly for the relationship between pRpt6 and PSD-95. Together, these findings indicate that training in males simultaneously up-regulates proteasomal protein degradation and markers of synaptic activity, which suggests for the first time that protein degradation and structural plasticity are regulated in a sex-specific manner at DH synapses following object training.

Because CaMKII or PKA phosphorylate Rpt6, thereby activating the proteasome, we also examined whether alterations in Rpt6 phosphorylation and proteasomal activity coincided with alterations in CaMKII or PKA phosphorylation at DH synapses. Although previous work has shown that CaMKII and PKA activity regulates proteasome activity in both sexes 1 h following contextual fear conditioning (Devulapalli et al. 2019), object training did not influence CaMKII phosphorylation at this time point in the DH synaptic fraction (Supplemental Fig. S3A). PKA phosphorylation was slightly but significantly reduced in the DH synaptic fraction of females relative to males ($F_{(1,32)}=4.04$, $P=0.05$) (Supplemental Fig. S3B) in the absence of a main effect of training ($F_{(1,32)}=1.48$, $P=0.23$) or a training × sex interaction ($F_{(1,32)}=0.91$, $P=0.35$). Overall, these data indicate no effect on pCaMKII or pPKA levels in DH synapses 1 h after training.

DH cytoplasmic fraction: training increased Rpt6 phosphorylation in females

Recent work has revealed that contextual fear learning differentially regulates UPS activity across subcellular compartments and brain regions in male and female rats (Devulapalli et al. 2019, 2021; Martin et al. 2021). As such, we were also interested in determining the extent to which object training alters UPS activity in the cytoplasmic fraction of DH lysates (Fig. 4). In contrast to our findings in the

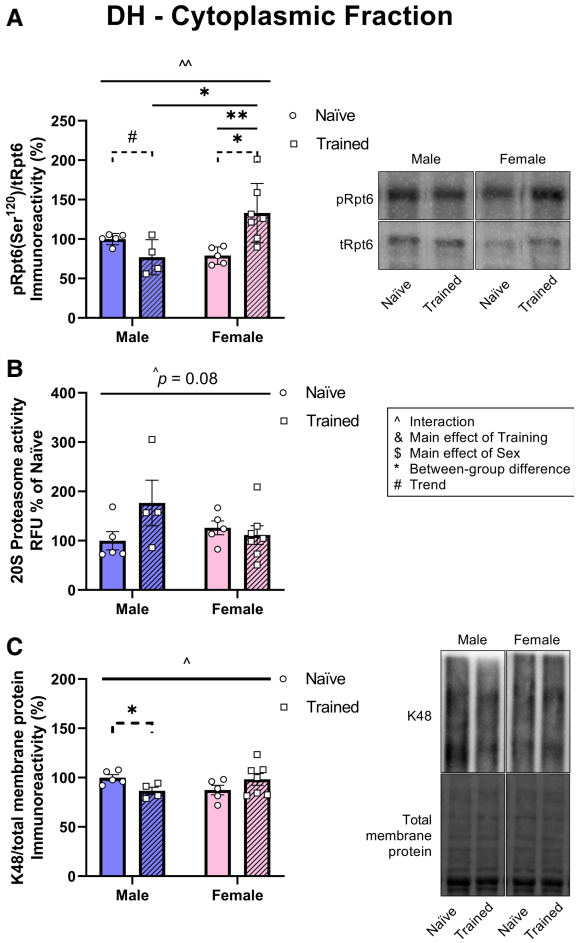

Figure 4. The effects of training on UPS activity in the DH cytoplasmic fraction were limited to Rpt6 phosphorylation in females. (*A*) Rpt6 phosphorylation was significantly increased ([^^] $P<0.01$) in the DH cytoplasmic fraction of trained females relative to both trained males ([*] $P<0.05$) and naïve females ([**] $P<0.01$). Trained males tended to have reduced Rpt6 phosphorylation relative to naïve males ([#] $P=0.06$, *t*-test), whereas trained females had significantly increased Rpt6 phosphorylation relative to naïve females ([*] $P<0.05$, *t*-test). (*B*) 20S chymotrypsin-like proteasome activity tended to be increased in trained males ([^] $P=0.08$) in the DH cytoplasmic fraction. (*C*) Object training tended to decrease K48 polyubiquitination levels in trained males ([^] $P<0.05$), and significantly decreased K48 polyubiquitination in trained males relative to naïve males ([*] $P<0.05$, *t*-test). Bars represent the mean ± SEM.

DH synaptic fraction, we found that UPS activity was reduced by training in DH cytosolic fractions from males and somewhat increased in females. For pRpt6 levels, there was a significant training × sex interaction ($F_{(1,17)} = 12.00$, $P = 0.003$) in the absence of significant main effects of training ($F_{(1,17)} = 1.93$, $P = 0.18$) and sex ($F_{(1,17)} = 2.50$, $P = 0.13$) (Fig. 4A). Post-hoc analysis indicated that pRpt6 levels were increased in trained females relative to both trained males ($P = 0.01$) and naïve females ($P = 0.01$). Furthermore, *t*-tests indicated that trained females had significantly increased pRpt6 levels relative to naïve females ($t_{(10)} = 3.09$, $P = 0.01$). Interestingly, trained males tended to have decreased pRpt6 levels relative to naïve males ($t_{(7)} = 2.21$, $P = 0.06$), which is the opposite of findings in the DH synaptic fraction. With respect to 20S chymotrypsin-like proteasome activity (Fig. 4B), neither main effect was significant (training: $F_{(1,17)} = 1.59$, $P = 0.22$; sex: $F_{(1,17)} = 0.63$, $P = 0.44$), but there was a trend for a training × sex interaction ($F_{(1,17)} = 3.45$, $P = 0.08$), perhaps driven by increased chymotrypsin-like activity in trained males. Nevertheless, within-sex analyses supported no effect of object training on chymotrypsin-like activity in males ($t_{(7)} = 1.68$, $P = 0.14$) or females ($t_{(10)} = 0.57$, $P = 0.58$). As with proteasome activity, neither main effect was significant for K48 polyubiquitination levels (training: $F_{(1,17)} = 0.06$, $P = 0.81$; sex: $F_{(1,17)} = 0.01$, $P = 0.92$), but there was a significant training × sex interaction ($F_{(1,17)} = 5.40$, $P = 0.03$). Consistent with pRpt6 levels, trained males had significantly decreased levels of K48 polyubiquitination relative to naïve males ($t_{(7)} = 2.84$, $P = 0.03$) (Fig. 4C). Female groups did not differ ($t_{(10)} = 1.28$, $P = 0.23$).

Similar to the DH synaptic fraction, there were no significant differences in CaMKII phosphorylation following object training in males or females (Supplemental Fig. S3C). pPKA levels (Supplemental Fig. S3D) were not affected by training ($F_{(1,17)} = 0.18$, $P = 0.67$) or sex ($F_{(1,17)} = 2.76$, $P = 0.12$). However, a significant training × sex interaction ($F_{(1,17)} = 5.62$, $P = 0.03$) and post-hoc analysis revealed that pPKA levels were increased in naïve females relative to naïve males ($P = 0.05$), suggesting higher baseline levels of PKA phosphorylation in the DH cytoplasmic fraction of females.

In sum, data from the DH cytosolic fraction reveal that object training decreased pRpt6 and K48 polyubiquitination levels in males and increased pRpt6 levels in females, without any significant effect on proteasome activity in either sex. These data suggest distinct patterns of sex-dependent effects on UPS activity in the DH cytosolic and synaptic fractions 1 h after training.

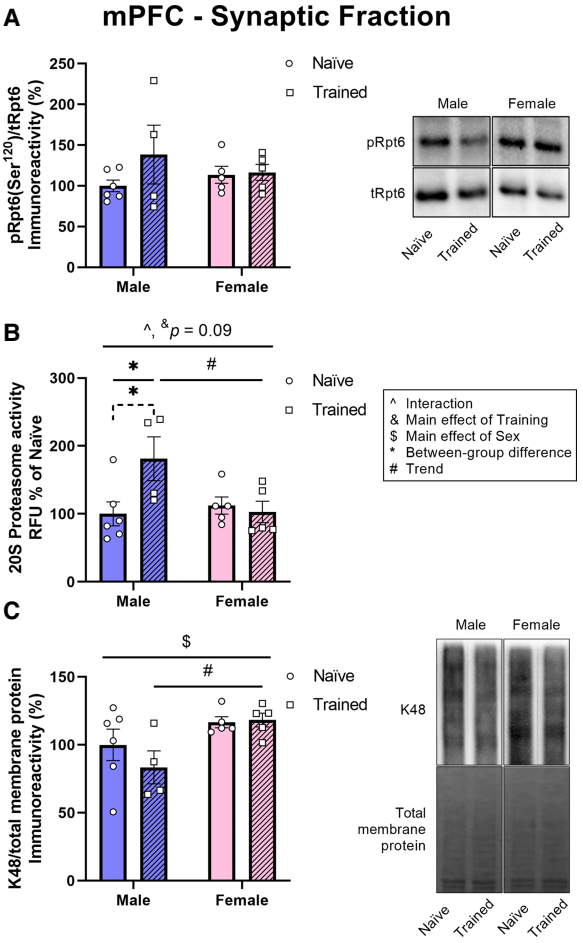

Figure 5. Training increased proteasome activity in mPFC synaptic fractions in males only. (*A*) Rpt6 phosphorylation was unaffected by training or sex. (*B*) Object training significantly increased chymotrypsin-like activity in trained males ([^] $P < 0.05$. [&] $P = 0.09$, [*] $P < 0.05$ vs. naïve males). Trained males also tended to have higher levels than trained females ([#] $P = 0.07$). (*C*) K48 levels were significantly increased in females ([$] $P < 0.05$), and trained females tended to have higher K48 polyubiquitination levels relative to trained males ([#] $P = 0.09$). Bars represent the mean ± SEM.

mPFC synaptic fraction: training increased proteasome activity in males only

We next sought to determine the extent to which object training alters aspects of UPS function at mPFC synapses (Fig. 5). As in the DH synaptic fractions, markers of UPS activity tended to be increased by training in males only. pRpt6 levels were not affected by training ($F_{(1,16)} = 1.58$, $P = 0.23$), sex ($F_{(1,16)} = 0.06$, $P = 0.81$), or their interaction ($F_{(1,16)} = 1.17$, $P = 0.30$) (Fig. 5A). Consistent with these null findings, there was no effect of object training on Rpt6 phosphorylation within males ($t_{(8)} = 1.29$, $P = 0.23$) or females ($t_{(8)} = 0.207$, $P = 0.84$), although the mean was highest in trained males. Consistent with this pattern, 20S chymotrypsin-like proteasome activity was significantly increased by training in males (Fig. 5B), yielding a significant training × sex interaction ($F_{(1,16)} = 5.37$, $P = 0.03$) and a nearly significant main effect of training ($F_{(1,16)} = 3.37$, $P = 0.09$) without a main effect of sex ($F_{(1,16)} = 2.86$, $P = 0.11$). Chymotrypsin-like activity was significantly increased in trained males relative to naïve males (post-hoc $P = 0.05$; $t_{(8)} = 2.41$, $P = 0.04$) but not females ($t_{(8)} = 0.47$, $P = 0.65$). This sex difference is supported by a trend for increased chymotrypsin-like activity in trained males relative to trained females ($P = 0.07$). K48 polyubiquitination levels (Fig. 5C) were not affected by training in either sex, as illustrated by null effects of training ($F_{(1,16)} = 0.65$, $P = 0.43$) and the training × sex interaction ($F_{(1,16)} = 0.98$, $P = 0.34$), as well as within-sex *t*-tests (males: $t_{(8)} = 0.95$, $P = 0.37$; females: $t_{(8)} = 0.29$, $P = 0.78$). However, K48 polyubiquitination levels were affected by sex ($F_{(1,16)} = 7.8$, $P = 0.01$), such that there was a trend for increased K48 polyubiquitination in trained females relative to trained males ($P = 0.09$).

As in the DH, we then assessed synaptic engagement following object training by assessing levels of Arc and PSD-95 protein at mPFC synapses (Fig. 6). We could not perform correlation or regression analyses for these data with the DH synaptic fraction because each sample included just two or three mice due to tissue pooling. With respect to Arc levels, we found a trend for a main effect of training ($F_{(1,16)} = 3.36$, $P = 0.09$) (Fig. 6A) in the absence of a main effect of sex ($F_{(1,16)} = 0.04$, $P = 0.86$) and a training × sex interaction ($F_{(1,16)} = 0.48$, $P = 0.50$). The modest training-induced increase in Arc levels was not apparent in within-sex *t*-test analyses for either sex (males: $t_{(8)} = 1.73$, $P = 0.12$; females: $t_{(8)} = 0.83$, $P = 0.43$). Consistent with the Arc data, analysis of PSD-95 levels revealed a trend for a main effect of training ($F_{(1,16)} = 3.39$, $P = 0.08$) (Fig. 6B) in the absence of a main effect of sex ($F_{(1,16)} = 0.31$, $P =$

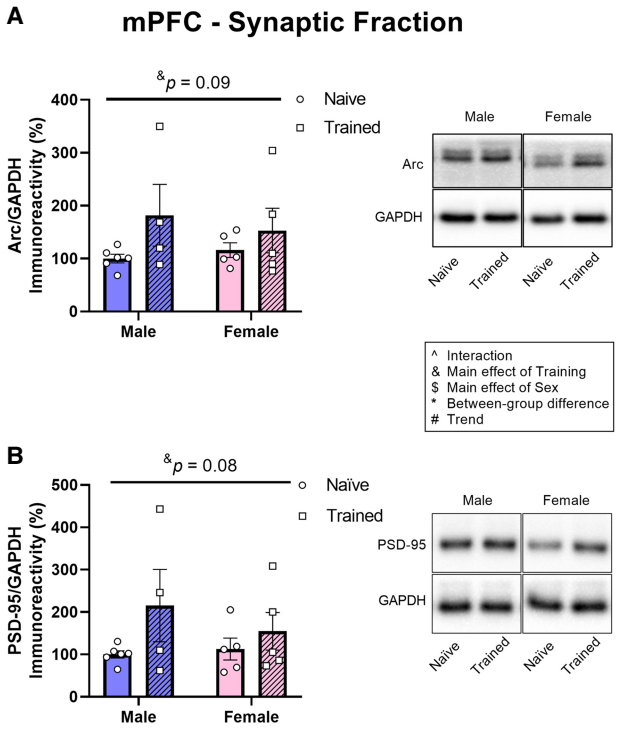

Figure 6. Training modestly increased levels of Arc and PSD-95 protein in mPFC synaptic fractions of both sexes. Object training tended to increase Arc ([&] $P = 0.09$) (*A*) and PSD-95 ([&] $P = 0.08$) (*B*) levels in both sexes. Bars represent the mean ± SEM.

0.58) or a training × sex interaction ($F_{(1,16)} = 0.74$, $P = 0.40$). Moreover, there were also no effects of object training on PSD-95 levels within either sex (males: $t_{(8)} = 1.69$, $P = 0.13$; females: $t_{(8)} = 0.81$, $P = 0.44$) at mPFC synapses. Object training did not alter CaMKII or PKA phosphorylation levels in mPFC synaptic fractions (Supplemental Fig. S4A,B).

Collectively, these findings suggest that although some training-induced up-regulation of proteasome activity is evident in males at this time point, mPFC synapses appear to be less active than DH synapses 1 h following object exploration.

mPFC cytoplasmic fraction: training had no effect on UPS activity in either sex

Rpt6 phosphorylation was affected by sex rather than training (Fig. 7A), as the main effect of sex ($F_{(1,16)} = 10.32$, $P = 0.01$) was significant in the absence of a significant effect of training ($F_{(1,16)} = 2.35$, $P = 0.16$), training × sex interaction ($F_{(1,16)} = 1.11$, $P = 0.31$), or within-sex *t*-tests (male: $t_{(8)} = 1.71$, $P = 0.13$; female: $t_{(8)} = 0.36$, $P = 0.73$). Trained males had significantly lower pRpt6 levels than both naïve ($P = 0.03$) and trained ($P = 0.05$) females. Chymotrypsin-like proteasome activity (Fig. 7B) was not affected by training ($F_{(1,15)} = 0.41$, $P = 0.53$), sex ($F_{(1,15)} = 2.21$, $P = 0.16$), or their interaction ($F_{(1,15)} = 2.0$, $P = 0.18$). Similar null effects were observed for K48 polyubiquitin levels (training: $F_{(1,16)} = 0.002$, $P = 0.96$; sex: $F_{(1,16)} = 0.01$, $P = 0.91$; interaction: $F_{(1,16)} = 0.3$, $P = 0.59$) (Fig. 7C). CaMKII phosphorylation levels were unaffected by training ($F_{(1,16)} = 1.29$, $P = 0.27$), sex ($F_{(1,16)} = 1.12$, $P = 0.31$), or their interaction ($F_{(1,16)} = 1.29$, $P = 0.27$). However, within-sex *t*-test analyses reveal that CaMKII phosphorylation was significantly decreased in trained females ($t_{(8)} = 2.79$, $P = 0.02$), but not males ($t_{(8)} = 0.00$, $P = 1.00$), relative to same-sex naïve controls (Supplemental Fig. S4C). Although PKA phosphorylation levels were unaffected by training ($F_{(1,16)} = 0.15$, $P = 0.70$) and a training × sex interaction ($F_{(1,16)} = 0.3$, $P = 0.59$), there was a main effect of sex ($F_{(1,16)} = 5.45$, $P = 0.03$), indicating that females, irrespective of training status, had lower levels of PKA phosphorylation than males (Supplemental Fig. S4D).

Discussion

Although numerous studies have examined UPS activity following fear learning (Jarome et al. 2011, 2013, 2016; Reis et al. 2013; Devulapalli et al. 2019, 2021; Dulka et al. 2021; Farrell et al. 2021; Martin et al. 2021), no studies to date have compared UPS activity following nonaversive learning in males and females. Here, we assessed the extent to which object training alters UPS function in the synaptic and cytoplasmic fractions of DH and mPFC tissue collected from male and female mice 1 h later. At this time point, we found that object training in males increased Rpt6 phosphorylation and 20S chymotrypsin-like proteasome activity in the DH synaptic fraction, whereas object training in females increased Rpt6 phosphorylation in the DH cytoplasmic fraction. Despite the lack of training-induced alterations in K48 polyubiquitination

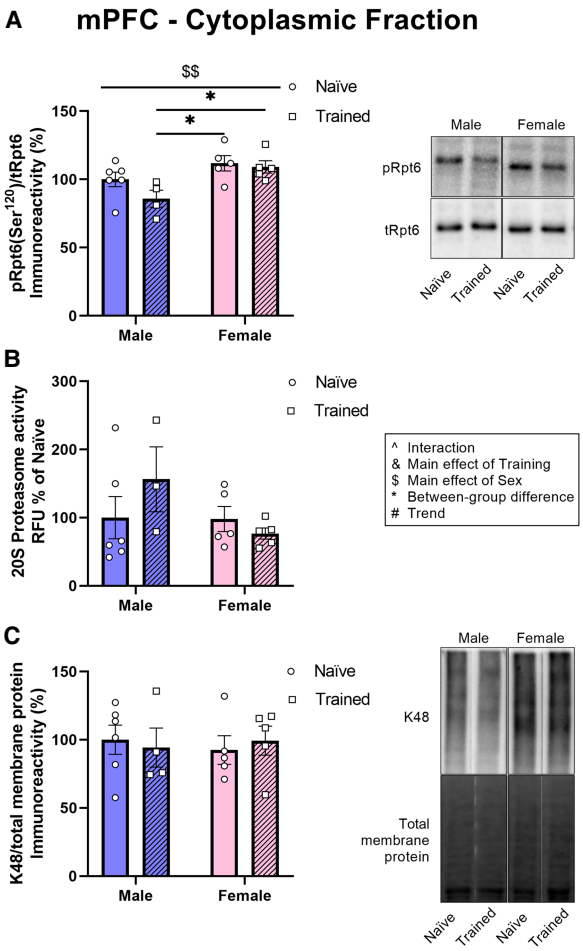

Figure 7. Object training did not affect UPS activity in the mPFC cytoplasmic fraction in either sex. (*A*) Rpt6 phosphorylation levels were significantly increased in females ([$$] $P < 0.01$), such that trained females had higher Rpt6 phosphorylation levels relative to trained males ([*] $P < 0.05$). (*B*,*C*) Chymotrypsin-like proteasome activity and K48 polyubiquitination levels were not affected by training status or sex in the mPFC cytoplasmic fraction. Bars represent the mean ± SEM.

at DH synapses for either sex, object training in males decreased levels of K48 polyubiquitination in the DH cytoplasmic fraction. Object training in males increased only proteasome activity in the mPFC synaptic fraction, and no effects of object training on UPS activity were observed in either sex in the mPFC cytoplasmic fraction. mPFC data tended to be more variable than DH data, perhaps because we pooled tissue between the prelimbic and infralimbic cortices, as well as across mice. Although it may seem curious that Rpt6 phosphorylation, 20S proteasome activity, and K48 polyubiquitination were not regulated in a similar manner within a subcellular fraction, it is important to note that these measures are regulated through multiple different mechanisms, and their overall activity patterns are influenced by time, sample preparation, and brain area, as well as the intensity and duration of training experience. Nevertheless, these findings collectively suggest that multiple measures of UPS activity were increased at DH and mPFC synapses in males 1 h after object training, whereas training-induced activation of the UPS in females was limited to Rpt6 phosphorylation in the DH cytosol. In general, object training induced greater UPS activation in males relative to females, in the DH relative to the mPFC, and in the synaptic fraction relative to the cytosol. These data provide the first evidence to suggest that object training drives sex-specific alterations in UPS activity across brain regions and subcellular compartments.

Object training in males increased Rpt6 phosphorylation and chymotrypsin-like proteasome activity in the DH synaptic fraction, as well as proteasome activity in the mPFC synaptic fraction. These findings, in combination with the lack of training-induced effects seen in male DH and mPFC cytoplasmic fractions, suggest that males up-regulate proteasome activity predominantly at synapses in both brain regions. This synapse-specific increase in proteasome function among trained males is consistent with previous fear-learning work demonstrating that Rpt6 phosphorylation and chymotrypsin-like activity are increased at male rat DH synapses up to 30 min following exposure to a novel context in the absence of a footshock (Cullen et al. 2017). These data suggest that nonaversive contextual learning in male rats promotes protein degradation at synapses in a rapid manner following exposure to a novel environment. Interestingly, however, our training-induced increase in male proteasome activity at synapses is in contrast to other work demonstrating that proteasome activity in DH synaptic, cytosolic, or nuclear compartments is not altered in male rats 1 h after contextual fear conditioning (Martin et al. 2021). These discrepant findings may reflect key differences among these studies, including in the nature of the training, motivational stimuli (aversive vs. nonaversive) used, and differences in engagement of the DH in fear and object memory consolidation.

Although object training did not influence UPS activity among females in synaptic fractions of either brain region, training increased Rpt6 phosphorylation levels in cytoplasmic fractions of DH tissue. These findings are the first to demonstrate that object training up-regulates proteasome subunit phosphorylation in the DH cytoplasmic fraction of females. An important limitation of this study, however, is that we did not assess nuclear UPS activity following training in either sex. Although no study has shown that females up-regulate cytosolic proteasome phosphorylation in an activity-dependent manner, one recent study reported that proteasome activity is increased predominantly in the nuclear, but not cytoplasmic or synaptic, fractions of female, but not male, DH tissue 1 h after contextual fear conditioning (Martin et al. 2021). Although these findings are consistent with our lack of training-induced effects in synaptic fraction, it remains unknown whether nuclear UPS activity is altered following object training in either sex. It should also be noted that although we did not observe increased chymotrypsin-like proteasome activity at this time point, learning in females may regulate other forms of proteolytic activity, such as trypsin-hydrolyzing and peptidyl-glutamyl-hydrolyzing proteasome activities. Previous work demonstrated that males and females regulate all three forms of proteasome activity in a highly sex- and subcellular compartment-specific manner (Devulapalli et al. 2019). For example, PKA inhibition decreases trypsin activity in the DH cytoplasmic fraction of females following contextual fear conditioning (Devulapalli et al. 2019), suggesting that learning-induced activation of PKA in females up-regulates trypsin-like activity in the DH cytoplasmic fraction. These data lend support to the idea that other forms of proteasome activity may be regulated by learning in female DH cytoplasmic fractions, which should be tested in future studies.

With respect to functional correlates of UPS activity, we examined Arc and PSD-95 protein levels because both are associated with increased learning-induced synaptic remodeling and plasticity, which may be possible due to UPS-mediated proteolytic activity. Interestingly, object training increased Arc protein levels in DH synapses of both sexes, whereas levels of PSD-95 protein were increased only in males. Our findings in males are consistent with previous work demonstrating that levels of Arc and PSD-95 protein increase in whole-cell homogenates from the male rat dentate gyrus 1 h following object recognition training (Soulé et al. 2008). Why might PSD-95 protein be increased in male DH synapses at the same time that proteasome activity is up-regulated? Some clues come from NMDAR stimulation in cultured rat hippocampal neurons, which causes PSD-95 to become ubiquitinated within 10 min and produces a persistent loss of PSD-95 protein for 30 min (Colledge et al. 2003). These data indicate that synaptic activity rapidly targets scaffolding proteins for proteasomal degradation, which may then permit structural remodeling in synapses (Schnell et al. 2002; Ehlers 2003; Patrick et al. 2003; Jarome et al. 2011; Ferrara et al. 2019). With respect to temporal regulation of the proteasome following learning, it has been proposed that protein degradation precedes protein synthesis (Jarome and Helmstetter 2013, 2014), and thus an initial wave of proteasome activity that causes protein degradation and synapse destabilization may be followed by a subsequent wave of protein synthesis and synapse establishment. Because we observed changes reflecting both protein degradation and synthesis, we may have observed the end of the initial wave of protein degradation and the early stages of protein synthesis and synapse stabilization. This possibility is supported by previous work demonstrating that object recognition memory consolidation in male rats was disrupted when the proteasome inhibitor lactacystin was infused immediately and 3 h, but not 1.5 or 6 h, into the CA1 area of the DH (Figueiredo et al. 2015). These data suggest multiple waves of protein degradation during consolidation of object recognition memories that peak immediately and 3 h following training. Importantly, these waves may provide crucial opportunities for synaptic proteins like PSD-95 to be degraded in some synapses and synthesized in others to achieve the plasticity needed to encode a memory. Therefore, it will be important in future studies to assess UPS activity and synaptic proteins at multiple time points to test this hypothesis.

Based on previous findings, we hypothesized that training-induced increases in proteasome function would drive increases in K48 polyubiquitination. However, despite training-induced increases in proteasome activity in male DH and mPFC synapses, as well as in the female DH cytoplasmic fraction, no effects of object training were observed on K48 polyubiquitination in either sex. These findings are inconsistent with previous reports demonstrating that inhibitory avoidance training increased proteasome activity and K48 polyubiquitination levels after 4 h in the CA1 region of the male rat hippocampus (Lopez-Salon et al. 2001), as well as other findings from the male rat amygdala that K48 polyubiquitination levels are increased 1 h, and proteasome activity is increased at 4 h, after auditory fear conditioning (Jarome et al. 2013). The latter findings

indicate that training-induced increases in K48 polyubiquitination precede increases in proteasome activity, suggesting that we may have missed the time point at which peak K48 polyubiquitination occurred following object training. Alternatively, it is also possible that training-induced increases in K48 polyubiquitination do not occur on a global level but rather are limited to select proteins within the synapse and cytoplasm that may have been masked by the broad Western blotting technique used in this study. As such, future work should sample shorter time points and assay ubiquitination status of candidate proteins to potentially capture the putative training-induced increase in K48 polyubiquitination.

As with K48 polyubiquitination, we did not observe training-induced alterations in CaMKII or PKA phosphorylation in males or females that overlapped with training-induced changes in proteasome function in either brain region or subcellular compartment. These results are perhaps not surprising given the longer time point assessed and the manner in which CaMKII and PKA become rapidly activated within minutes following synaptic plasticity and learning (Lisman Schulman and Cline 2002; Abel and Nguyen 2008). Thus, we may have observed increases in activation of these kinases had we measured at a shorter time point. It remains unclear whether phosphorylation levels of CaMKII and PKA overlap with training-induced increases in proteasome function. Although a number of in vitro studies in cultured hippocampal neurons showed that CaMKII and PKA can phosphorylate Rpt6 in an activity-dependent manner (Bingol and Schuman 2006; Zhang et al. 2007; Djakovic et al. 2009, 2012; Bingol et al. 2010), in vivo experiments to date have assessed the individual contributions of CaMKII and PKA activity on proteasome function by broadly inhibiting their activities rather than assessing phosphorylation states of these kinases (Jarome et al. 2013, 2016; Devulapalli et al. 2019; Orsi et al. 2019). As such, the timing of CaMKII and PKA phosphorylation relative to training-induced increases in Rpt6 phosphorylation is unclear. As with K48 polyubiquitination, examination of earlier time points will help clarify this issue.

In conclusion, this study adds to the growing body of literature demonstrating that training can have differential effects on UPS activity in males and females. Our findings broadly suggest that object training up-regulates proteasome activity in male DH and mPFC synapses, whereas it up-regulates proteasome activity only in the DH cytoplasmic fraction of females. It is important to note that this conclusion applies only to this single time point, as different aspects of UPS activity may be altered by object training at other time points. Thus, the time course of object training-induced UPS activation should be fully examined in both sexes in future work. Moreover, the mechanisms underlying the observed sex-, brain region-, and compartment-specific differences remain unclear at the present time, as is the relative importance of UPS activity in mediating object recognition and object placement memory consolidation in females and males. Although previous reports demonstrate that proteasome activity in the hippocampus is required for consolidation and reconsolidation of spatial and object recognition memories in male rodents (Artinian et al. 2008; Choi et al. 2010; Figueiredo et al. 2015), it is unknown whether females similarly require proteasome activity for the successful consolidation of object memories. Thus, future work should assess whether the observed brain region- and compartment-specific alterations in UPS activity have functional consequences for the formation of object memories in females.

Materials and Methods

Subjects

Gonadally intact male ($n = 24$) and female ($n = 24$) C57BL/6 mice were obtained from Taconic Biosciences at 10 wk of age and housed individually in shoebox cages in a room (22°C–23°C) with a 12/12-h light–dark cycle. Food and water were provided ad libitum. All procedures were conducted from 10:00 to 17:00 h in a dimly lit quiet room. All procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee following the National Institutes of Health Guide for the Care and Use of Animals.

Behavioral training

To examine the effects of object training on UPS activity, gonadally intact male and female mice were allowed to explore objects in a white open field box (width, 60 cm; length, 60 cm; height, 47 cm). Mice were first assigned to naïve or trained groups ($n = 12$/group/sex). Before training, all mice were handled for 1 min/d for three consecutive days to acclimate them to experimenter manipulation. A small Lego Duplo block was placed in the home cage on the second day of handling to acclimate mice in the trained group to objects. Trained mice were habituated to the open field box for 5 min/day for two consecutive days, during which time they were allowed to move freely in the box without objects present. Twenty-four hours after the second habituation session, mice were rehabituated to the box for 2 min without objects present, and then placed briefly in a holding cage while two identical objects were placed in the upper right and left corners of the box. Mice were then immediately returned to the box and allowed to explore the objects freely until they accumulated 30 sec of exploration time, with a maximum of 20 min allowed for completion of training. Any mouse who failed to complete the training was given another attempt 24 h later. Experimenters manually scored in real time the duration of object exploration using ANYmaze tracking software (Stoelting). Exploration of objects was scored when the front paws or nose contacted either object.

Tissue collection

Sixty minutes after successful completion of object training, mice were cervically dislocated and decapitated, and DH and mPFC tissues were dissected bilaterally using a mouse brain matrix (Ted Pella, Inc.) on wet ice (Fig. 1A). The 1-h time point was chosen based on previous work documenting increases in UPS activity 60 min following consolidation of auditory and contextual fear memories in male rats (Jarome et al. 2011, 2013). Of the original 24 mice assigned to the trained group, a total of eight males and 11 females completed object training and were used for Western blot and proteasome activity analysis.

Crude preparation of synaptosomal and cytoplasmic fractions

Crude synaptosomal membrane and cytoplasmic extracts were obtained as described previously (Jarome et al. 2011, 2013; Dulka et al. 2021) with minor alterations noted below (Fig. 1B). Tissue samples were homogenized in 0.32 M HEPES–sucrose buffer and centrifuged at 1000*g* for 10 min at 4°C. The resulting supernatant was collected and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant containing the cytoplasmic fraction was collected and the resulting pellet containing the crude synaptosomal fraction was resuspended in a hypotonic lysis buffer containing PMSF and EDTA-free protease inhibitor cocktail (Thermo Fisher Scientific). The resulting synaptosomal fraction contained the presynaptic membrane, which could include mitochondria and synaptic vesicles, as well as the postsynaptic membrane and its associated postsynaptic density proteins (Kamat et al. 2014). Total protein concentrations were measured for both synaptic and cytoplasmic fractions using a Bradford protein assay (Bio-Rad) with bovine serum albumin (BSA) as a standard. Western blotting and the proteasome activity assay both require at least 1.5 µg/µL protein; however, not all DH samples yielded these amounts. Thus, after tissue homogenization, sample sizes for Western and proteasome analyses were reduced for both DH synaptic samples ($n = 11$ naïve male; $n = 6$ trained male; $n = 10$ naïve female; $n = 10$ naïve train) and DH cytoplasmic samples ($n = 5$ naïve male; $n = 4$ trained male; $n = 5$ naïve female; $n = 7$ trained

female). Because mPFC samples (~4 mg of tissue/animal) were considerably smaller than DH tissues (~12 mg of tissue/animal), mPFC samples were pooled ($n = 2$–3/group) to reliably obtain synaptic and cytoplasmic fractions ($n = 6$ naïve male; $n = 4$ trained male; $n = 5$ naïve female; $n = 5$ trained female). Samples were stored at −80°C until preparation of aliquots for Western blot and proteasome activity assays.

Primary antibodies

Rabbit primary antibodies used were as follows: K48 polyubiquitin (1:1000; Cell Signaling Technology 8081), phospho-Rpt6 (Ser120; 1:1000; Signalway Antibody 12880), Rpt6 (1:1000; Cell Signaling Technology 13392), phospho-PKA (Thr197; 1:1000; Cell Signaling Technology 5661), PKA (1:1000; Cell Signaling Technology 4782), phospho-CaMKIIα (Thr286; 1:1000; Cell Signaling Technology 12716), CaMKIIα (1:1000; Cell Signaling Technology 4436), PSD95 (1:1000; Cell Signaling Technology 2507), Arc/Arg3.1 (1:1000; Cell Signaling Technology 65650), and GAPDH (1:1000; Cell Signaling Technology 2118).

Western blotting

DH and mPFC samples were normalized to 10 and 5 µg/µL, respectively, by adding corresponding amounts of sample buffer, lysis buffer, and homogenate. Samples were boiled for 5 min to denature proteins. Proteins were electrophoresed in 10-µL aliquots on 10% TGX stain-free precast gels (Bio-Rad) and transferred to PVDF membranes using a TransBlot Turbo transfer system (Bio-Rad). Membranes were blocked in 5% dried nonfat milk/TTBS and incubated overnight at 4°C with the aforementioned primary antibodies. Blots were incubated the next day for 1 h at room temperature with an HRP-conjugated secondary antibody (anti-rabbit IgG, 1:5000; Cell Signaling Technology 7074). Membranes were then developed using Clarity Max chemiluminescent substrate (Bio-Rad) and imaged using a ChemiDoc MP gel imager (Bio-Rad). Densitometry analysis was performed using Image Lab software (Bio-Rad, Image Lab version 6.0.1). Lanes were manually defined, and bands were detected automatically using Image Lab software.

Proteasome activity assay

Proteasome activity assays were performed as described previously (Jarome et al. 2013; Devulapalli et al. 2019; Orsi et al. 2019) with minor modifications. Cytoplasmic and synaptic fractions were normalized to 10 and 5 µg/µL, respectively. Samples were diluted in dH_2O and mixed with reaction buffer (500 mM HEPES at pH 7.5, 500 mM EDTA, 10% NP-40, 10% SDS, 10 mM ATP). The fluorogenic peptide Suc-leu-leu-val-thy-AMC (Enzo Life Sciences BML-P802-0005) was added to samples to assess chymotrypsin-like activity (10 µM). The reaction was incubated for 2 h at 37°C, and fluorescence was monitored 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. The scan with the peak level of AMC was used for statistical analyses. Data are presented as the percent change in relative fluorescent units (RFUs) relative to the naïve group.

Statistical analyses

All statistical analyses were conducted with GraphPad Prism 9. Outliers, defined as values ± 2 standard deviations from the mean, were removed prior to analysis. We first conducted two-way ANOVAs (training × sex) followed by post-hoc Tukey's multiple comparison tests because of previous reports that males and females have different baseline levels of protein polyubiquitination (Devulapalli et al. 2021). Given our a priori interest in comparing UPS activity within each sex, unpaired *t*-tests were also conducted to compare naïve and trained within a sex for each dependent variable. To determine potential relationships between UPS activation and synaptic activity, we performed Pearson correlation and simple linear regression analyses between pRpt6 as a proxy for UPS activity and both Arc and PSD-95 as measures of synaptic activity. Three separate analyses were conducted for males and females combined, males alone, and females alone. These analyses were limited to the DH because this was the only brain region in which samples were collected from individual mice (mPFC samples were pooled). Statistical significance was set at $P \leq 0.05$ for all statistical tests, and trends were determined by $P \leq 0.10$.

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