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Article

Sex differences in linear polyubiquitination in the entorhinal cortex during fear memory formation

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SUMMARY

Post-traumatic stress disorder (PTSD) is a major anxiety disorder that has a higher incidence rate in women than men, despite that women do not report experiencing more traumatic events. Understanding the neurobiological mechanisms supporting the formation of fear memories that underlie PTSD and how they vary by sex is important for responding to the etiology of this disorder. We recently reported that linear polyubiquitination, a noncanonical form of ubiquitination that is independent of proteasomal-mediated protein degradation, is increased in the amygdala in a sex-specific manner following contextual fear conditioning in rodents. However, it is unknown if linear polyubiquitination is changed in other parts of the fear circuit following training and if this varies between sexes. Here, we found that contextual fear conditioning results in decreased levels of linear polyubiquitination in the entorhinal cortex (EC) of male, but not female, rats. Further, females had lower resting levels of linear polyubiquitination in the EC, suggesting that sex differences already existed at baseline in this brain region. Conversely, neither sex nor training altered linear polyubiquitination levels in the dorsal hippocampus (DH), prefrontal cortex (PFC) or retrosplenial cortex (RSC). Together, these data provide the first evidence that linear polyubiquitination is altered in a sex and brain region-specific manner during fear memory formation. Our data have important implications for understanding the sex differences that exist in PTSD.

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a severe anxiety disorder with a lifetime prevalence rate of 3.4% to 26.9% in the civilian population (1). This disorder develops following exposure to a traumatic event and current treatment options remain limited. It has been reported that females are 2-3 times more likely than males to develop PTSD (2). However, we do not know the exact reason why females are more prone to this disorder, as they do not report experiencing more traumatic events than males (2). This suggests that sex differences in the prevalence of PTSD may be due to neurobiological differences between males and females.

In order to understand the neurobiology of PTSD sex differences, researchers often use a rodent fear conditioning paradigm, in which an animal (usually rodent) learns to associate a novel stimulus (often an environment) with an aversive stimulus (most often a mild foot shock), as a way to model characteristics (i.e., anxiety, fear) of PTSD in humans (3,4). From this model we know that proteasome-mediated protein degradation is a regulator of the formation of fear memories for traumatic events in the amygdala (5), a brain region involved in the control of fear and anxiety and widely reported to be involved in PTSD (6,7). During proteasome-mediated protein degradation, proteins are degraded by the ubiquitin-proteasome system, where multiple ubiquitin proteins attach to a target substrate and form a lysine-48 (K48) polyubiquitin chain. This K48 polyubiquitin chain is then recognized and degraded by the large proteasome complex (8). Prior evidence suggests that both males and females need proteasome-mediated protein degradation in the amygdala. However, there are potential sex-specific functions for K48 polyubiquitination during fear memory formation as males and females target unique proteins by this ubiquitin modification following the training (fear conditioning) experience (9,10).

In addition to protein degradation, the ubiquitin-proteasome system also can target proteins for other fates. Of the eight unique polyubiquitin modifications that a target substrate can acquire, several of them do not lead to degradation (8). For example, linear polyubiquitination, in which multiple ubiquitin proteins link together at the first methionine of the previous ubiquitin, is a noncanonical form of polyubiquitination that is independent of the proteasome-mediated protein degradation process. Recently, it was found that linear polyubiquitination is also involved in the formation of fear memories in the amygdala (11). Similar to K48 polyubiquitination, while both males and females need linear polyubiquitination in the amygdala, it likely serves unique sex-specific functions during fear memory formation due to the targeting of different proteins across the sexes following training. However, this remains the only study that has examined the proteasome-independent linear polyubiquitination "tag" in the brain. As a result, much remains unknown about how linear polyubiquitination contributes to the formation of fear memories for traumatic events.

In addition to the amygdala, the contextual fear memory (environment associated with foot shock) often studied in these rodent models also requires the dorsal hippocampus (DH), prefrontal cortex (PFC), entorhinal cortex (EC), and retrosplenial cortex (RSC), which together make up the contextual fear circuit of the brain (12-15). Importantly, some of these brain regions are known to be sexually dimorphic,

especially the PFC (16). Despite this, although the need for proteasome-mediated protein degradation in fear memory formation has been explored in the PFC and other parts of the fear circuit, linear polyubiquitination has not been examined outside of the amygdala (13, 17). As a result, it is unknown if linear ubiquitination is altered across the fear circuit in a sex-specific manner during fear memory formation. Based on our previous findings in the amygdala which show that linear polyubiquitination is involved in the formation of fear memories in a sex-specific manner (11), we hypothesized that linear polyubiquitination would be altered differently across the fear circuit in a sex-specific manner during contextual fear memory formation. We found that linear polyubiquitination decreased following fear conditioning in the EC of males but not females, with no training or sex differences observed in the DH, PFC, or RSC. This result is a stark contrast to what we previously observed in the amygdala where linear polyubiquitination increased in both sexes following fear conditioning. Together, these data suggest that linear polyubiquitination is selectively altered across the fear circuit in a sex-specific manner during the formation of fear memories for traumatic events.

RESULTS

Based on previous evidence that linear polyubiquitination is involved in the formation of fear memories in the amygdala in a sex-specific manner, we hypothesized that this atypical polyubiquitin mark would be differentially altered across other parts of the fear circuit in a sex-dependent manner (11). To test this hypothesis, we trained 5 male and 5 female rats in a standard contextual fear conditioning task in which they associate a novel environment (context) with a mild foot shock. These animals were then compared to naive controls that were handled but did not undergo the fear conditioning procedure (n = 5 per sex). Brain tissue was collected 1 hour after the experiment and linear polyubiquitination levels examined using western blotting procedures (**Figure 1A**). During the contextual fear conditioning task, we found significant differences between time points of the training session, which accounts for changes in freezing (fear) behavior across the 5 minute training session ($p < 0.0001$), and between sexes ($p = 0.0042$), which accounts for the overall performance (freezing behavior) of the males and females across the training session. Additionally, there was a Time x Sex interaction ($p = 0.0090$), indicating that the performance, or the freezing (fear) behavior, of the females was lower than that of the males at later, but not earlier, minutes of the training session (**Figure 1B**), which is consistent with prior work from our group (9). Using optical density (OD), or the band/signal intensity, obtained from western blotting, we first examined linear polyubiquitination in the DH because this subcortical brain region is part of the same limbic system that includes the amygdala and is the most studied in context fear memory formation. However, we found that there was not a significant difference in linear polyubiquitination levels between sexes ($p = 0.1367$) or as a result of the contextual fear conditioning training ($p = 0.9686$). Additionally, linear polyubiquitination levels did not differentially change between sexes (i.e., increase in one sex and decrease in another) as a result of the training experience ($p = 0.8119$), meaning that the baseline and training-dependent levels of linear ubiquitination did not vary between the sexes (**Figure 1C**). Overall, we concluded that linear polyubiquitination was not engaged in the DH in either sex following contextual fear conditioning.

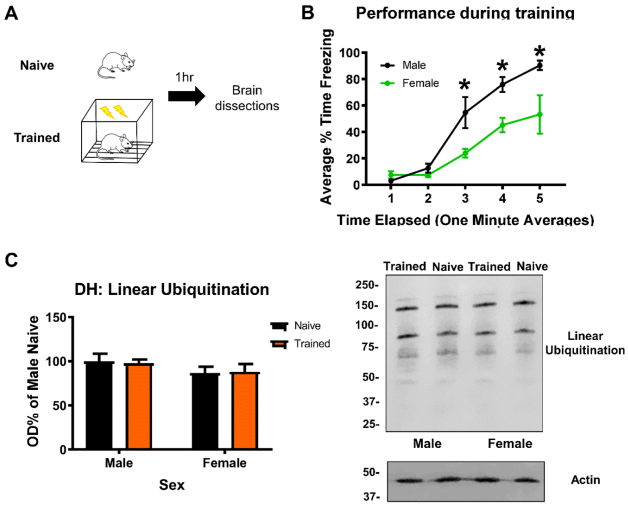

Figure 1: Linear polyubiquitination is not changed in the dorsal hippocampus of male or female rats following contextual fear conditioning. (A) Young adult male and female rats (n = 5 per sex) were trained to a contextual fear conditioning task. One hour later animals were euthanized and brain tissue collected for western blot analysis. Naive animals that were not trained (n = 5 per sex) were used as controls. (B) Performance of male and female rats during the 5-minute fear conditioning procedure. Males displayed higher freezing behavior across the training session. (C) Western blot of linear polyubiquitination in the dorsal hippocampus of naive and fear conditioned male and female rats. Linear polyubiquitination levels were not changed in dorsal hippocampus (DH) of either sex following training, nor were there differences in resting levels between male and female animals. Quantification of western blot image optical density (OD) was performed along the entire molecular standards ladder. The average OD of the naïve male animals was set to 100% and all groups compared to this. *p < 0.05 compared to females.

Both the amygdala and DH are subcortical regions of the brain. As a next step, we also wanted to look at the linear polyubiquitination levels in the cortical regions of the fear circuit such as the EC, PFC, and RSC. In the EC, we found that there was a significant effect for sex ($p = 0.0326$), indicating that in general males and females differed in levels of linear polyubiquitination. Additionally, linear polyubiquitination levels did not change as a result of the fear conditioning experience relative to Naïve controls ($p = 0.6047$). However, we found a significant interaction between sex and training, indicating that linear polyubiquitination levels changed as a result of fear conditioning but in a different direction in males as compared to females ($p = 0.0166$; **Figure 2A**). Together, these data indicate that there was a significant difference in baseline levels of linear polyubiquitination between the sexes, with naive females having lower overall levels than naïve males. This was further altered as a result of training. Importantly, fear conditioning decreased linear polyubiquitination levels in the EC in males (Naive vs. Trained, $P < 0.05$) but not in females. However, in the PFC (**Figure 2B**) and RSC (**Figure 2C**) there were no significant effects for Training (PFC: $p = 0.2928$; RSC: $p = 0.7955$) or Sex (PFC: $p = 0.4949$; RSC: $p = 0.2160$), nor were there significant interactions (PFC: $p = 0.6645$; RSC: $p = 0.5126$), meaning that both sexes had similar levels of baseline linear polyubiquitination and that the training procedure did not alter this in either sex. Overall, only

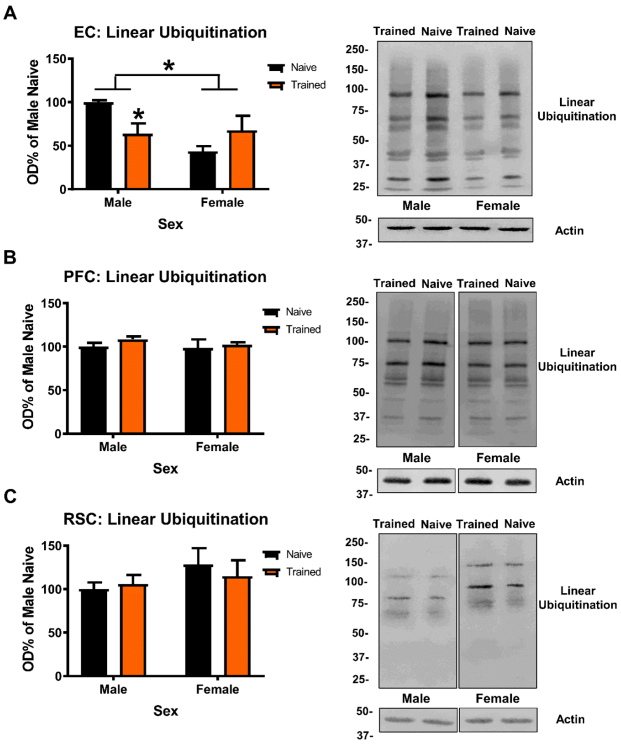

Figure 2: Fear conditioning alters linear polyubiquitination levels in the entorhinal cortex of male but not female rats. (A) Western blot of linear polyubiquitination in the entorhinal cortex (EC) of naive and fear conditioned male and female rats. Fear conditioning decreased linear polyubiquitination levels in the EC of male but not female rats. Conversely, naive female rats had lower resting levels of linear polyubiquitination than naive male rats(n = 4-5 per group). (B-C) Western blot of linear polyubiquitination in the prefrontal cortex (PFC, B) or retrosplenial cortex (RSC, C) of naive and fear conditioned male and female rats. Linear polyubiquitination levels did not differ across sexes nor were altered as a result of fear conditioning in the PFC (B; n = 4-5 per group) or RSC (C; n = 5 per group). Quantification of western blot image optical density (OD) was performed along the entire molecular standards ladder. The average OD of the naive male animals was set to 100% and all groups compared to this. *p < 0.05.

the EC showed a significant change with training or based on sex, suggesting that linear polyubiquitination is selectively altered across the fear circuit in a sex-specific manner.

DISCUSSION

The purpose of this study was to identify whether there were sex differences in the engagement of linear polyubiquitination in the DH, EC, PFC, and RSC during contextual fear memory formation in mice. We found that the EC, but not the DH, PFC, or RSC, showed a reduction with training that was present in males but not females. Females also had a lower baseline level of linear polyubiquitination in the EC compared to males, suggesting that sex differences in this atypical polyubiquitin mark were already present in the EC at rest, prior to any behavioral training. Together, these data expand our understanding of how linear polyubiquitination is likely involved in contextual fear memory formation across a distributed network of brain regions.

Our findings supported our hypothesis that linear polyubiquitination was differentially altered across the fear circuit between the sexes, although this difference was only present in the EC. The surprising finding that linear polyubiquitination decreased in the EC leads to the interesting theory that it may act to promote fear memory formation in the amygdala, as reductions in this region lead to impaired memory, but limit it in the EC in males (11). Conversely, based on our data and prior work showing that loss (reductions) in linear polyubiquitination lead to impaired fear memory in the amygdala, we theorize that linear polyubiquitination may only promote fear memory formation in the amygdala of females (11). This is especially interesting as prior evidence suggests that though the EC is required for forming contextual fear memories, specific molecular changes in the EC act to constrain fear memory formation in male animals (15, 18). However, this has not been tested in females. Additionally, there was a surprising baseline difference in linear polyubiquitination in the EC between sexes. This result relates to our previous work with K48 polyubiquitination where females had higher resting levels in the amygdala compared to males (9). The significance of these baseline differences between sexes remains unknown, but it could be due to hormonal differences between sexes. This question will be of interest in future studies.

The findings presented here provide the first insight into how the levels of linear polyubiquitination vary across the fear circuit as a result of sex and fear conditioning. However, there is still much more left to uncover. One limitation of this study was that the sample size was relatively small, though it was sufficient to detect differences between groups. A larger sample size could have allowed us to find subtle differences between groups in the PFC, RSC, or DH where we observed no effects in the present study. Another limitation is that we did not look at linear polyubiquitination at the individual protein level (10, 11). This limitation could have prevented us from detecting subtle sex and training-related differences in linear polyubiquitination occurring at select proteins, which would have been masked by the global western blot analysis. Furthermore, it is possible that the 1-hour time point was not sufficient to see linear polyubiquitination changes in all brain regions examined, though prior work has shown this time to be sufficient for degradation-specific ubiquitin signaling in the PFC and DH (13, 17). Nonetheless, linear polyubiquitination levels could change sooner or later after behavioral training in the brain regions examined, especially the RSC, where little is known about the temporal dynamics of molecular changes following learning. Future studies should aim to complete a time course experiment examining changes in linear polyubiquitination at different times (1-, 2, -6-hours) after context fear conditioning. Finally, as we did not directly manipulate linear polyubiquitination, we do not know whether it is necessary for fear memory formation in the EC. However, TUBE-LC/MS and direct manipulation approaches of linear polyubiquitination will be of interest in our future studies. Despite these limitations, our data still provide strong evidence that linear polyubiquitination changes across the fear circuit in a sex and brain region-specific manner following contextual fear conditioning.

In conclusion, we presented novel data from a previously unexplored area in neuroscience suggesting that linear polyubiquitination is differentially altered in a sex-specific manner across the fear circuit during fear memory formation in mice. These data have important implications in

understanding the formation of fear memories in both males and females that may underlie PTSD.

MATERIALS AND METHODS

Subjects

These experiments were conducted using 10 male and 10 female 8–9-week-old Sprague Dawley rats that were purchased from a commercial vendor (Envigo, Frederick, MA). Animals were housed with two per cage, and they had access to rat chow and water throughout the experiment. The room they were housed in maintained a cycle of 12 hours of light from 7AM to 7PM and 12 hours of dark, and all experiments were conducted during the light section of the cycle. All procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (IACUC, protocol number 20-233) and conducted in accordance with the ethical guidelines of the National Institutes of Health. Due to university policies that prohibit minors from working with research animals, the animal handling and training procedures described below were performed by K.M. with assistance from T.J.J. Y.G observed these procedures but was not allowed to directly participate.

Apparatus

To train animals to our contextual fear conditioning procedure in which they would learn to associate a novel environment with a mild foot shock, we used two identical Habitest fear conditioning chambers that were developed by Calbourne Instruments (Holliston, MA). This allowed two animals to be trained at a time. Each of these chambers consisted of a steel cage with a grid shock floor, through which the foot shock would be delivered, above a plastic drop pan that was used to collect boli. The chamber was fully enclosed so the animal would not escape, including plexiglass walls on the front and back with steel walls on the sides. A USB camera was located on a steel plate behind the back plexiglass wall and mounted at a 45-degree angle so that it could see the animal at all times, allowing its behavior to be recorded. So that the animal could be observed by the camera, there was an infrared light and a normal house light on the chamber wall, though only the house light was illuminated. Both of these chambers were housed in separate isolation cubicles that had acoustic linings, and a fan was turned on for the duration of the behavioral procedures in order to produce consistent background noise. This was necessary to ensure the animal did not experience extraneous sounds or noises that could influence behavior during the fear conditioning procedure. The foot shock was delivered using FreezeFrame 4 software, which was administered through the grid floor via a Precision Animal Shocker. This software was also used to analyze animal behavior in real-time, scoring the percentage of fear via a freezing response.

Behavioral Procedures

Rats went through a contextual fear conditioning procedure in the Habitest chambers described above. Four days prior to the procedure, the animals underwent handling, which consisted of picking up the animal gently and allowing them to rest on the experimenter's arm for three minutes. This allowed them to be familiar with being moved around and away from their home cage. Handling during the first two days occurred in the animal housing room and the second two days occurred in a separate room where behavioral training was held. For fear conditioning, the rats were placed into the chamber for a one-min baseline and received four unsignaled foot shock presentations (1.0 mA, 1 sec, 59 sec interval between each shock). After a one-min post shock period, the animals were returned to their cages. One hour after training, animals were euthanized using isoflurane, and the brain was removed and flash frozen on dry ice. Naive animals underwent identical handling and brain collection procedures but without undergoing contextual fear conditioning. These animals served as the baseline (unstimulated) control to determine the effects of the training procedure on linear polyubiquitination levels. Males and females went through identical procedures, and the procedures were performed at the same time in a counterbalanced manner (i.e., male, then female, then male, etc.) so they could be directly compared. Thus, all animals had a single 5-min training session during the same day. For training both sexes on the same day, two animals of the same sex were placed in adjacent Habitest chambers for training. Immediately after, the chamber floor and drop pan were removed and washed in a sink before being replaced into the chamber. The chamber walls were wiped with water and 70% isopropanol before the next group of rats, which were of the opposite sex, were trained. This pattern was repeated until all the animals had been trained.

Tissue Collection

Tissue containing the necessary regions (EC, PFC, RSC, and DH) was surgically removed and then blocked in a rat brain matrix (Harvard Apparatus, Holliston, MA) and incubated with dry ice. All brain regions were individually dissected out and frozen at -80ºC until needed. The tissue was then homogenized in buffer intended to extract proteins for identification while preventing loss of linear polyubiquitination from the lysis process (10mM HEPES, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5% IGEPAL, 0.02% SDS, 70mM NEM, 1 µl/ml protease inhibitor cocktail, and 1 µl/ml phosphatase inhibitor cocktail). These samples were then centrifuged for 10 min at 10,000 x g at 4oC to remove debris. The supernatant (liquid layer above the debris pellet) was collected, and protein concentration was determined by using the Bio-Rad (Hercules, CA) DC protein assay. This concentration was used to ensure that equal amounts of protein were loaded on the subsequent western blot assays.

Antibodies

Antibodies used in the western blotting procedure included linear polyubiquitin (1:2500, #AB130, Life Sensors, Malvern, PA) and Actin (1:1000, #4967, Cell Signaling, Danvers, MA).

Western Blot

Western blots were performed to determine the amount of linear polyubiquitination present in our dissected brain tissue. For this, we used 10 µg of normalized protein loaded on 7% Acrylamide gels. The gels were run through SDS-PAGE to separate proteins by molecular weight and transferred to PVDF membranes using a Turbo Transfer System (Biorad). The membranes were incubated in a 50:50 blocking buffer (Li-COR, Lincoln, NE) and TBS + 0.1% Tween-20 (TBSt) for one hour at room temperature, then incubated overnight in primary antibody (linear polyubiquitin, 1:2500) in 50:50 buffer at 4ºC.

Afterwards, membranes were washed 3 times for 10 min with TBSt and incubated in secondary antibody (goat anti-mouse IgB2B, 1:20,000) in 50:50 buffer for 45 min. Membranes were washed twice for 10 min in TBSt, then once in 1X TBS before imaging on an Odyssey Fc (Li-COR). The proteins were then visualized and analyzed using Image Studio Ver 5.2. After the proteins were visualized, membranes were stripped for 10 min with 0.2 M NaOH followed by two 15-min washes in TBSt and blocking buffer for 1 hour. The membranes were then incubated overnight in primary antibody (Actin, 1:1000) in 50:50 buffer at 4ºC. Afterwards, membranes were washed 3 times for 10 min with TBSt and incubated in secondary antibody (goat anti-rabbit, 1:40,000) in 50:50 buffer for 45 min. Membranes were imaged as described above. The proteins were then visualized, and densities were taken using Image Studio software (Li-COR). Linear polyubiquitin densities were taken as a ratio of the Actin density for that sample to account for any possible loading differences.

Statistical Analysis

All data are presented as mean plus standard error. Training data were analyzed with two-way ANOVA (Sex and Time as factors) with Fisher's LSD post hoc tests. Western blot data were analyzed with 2-way ANOVA (Sex and Training as factors) and Fisher's LSD post hoc tests. Statistical outliers were defined as those samples that were two or more standard deviations from the mean and were determined by the outlier function in Prism.

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