Capstone Project Report – Team 6
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Project Background

Post-translational modifications (PTMs) are an essential form of regulation for proteins. One of the main modifications is ubiquitination, which consists of connecting a ubiquitin molecule to a substrate protein. According to the number of molecules connected, it can be mono- or poly-ubiquitination. Molo-polyubiquitination modifies the protein's interaction, localization, or transport. On the other hand, poly-ubiquitination is mostly related to proteasome-dependent degradation, but also protein activity and translocation. The molecules of a poly-ubiquitin chain can be linked to each other in seven different lysine sites. The most common ones are K48-linked chains, which are usually related with proteolysis of the substrates through the ubiquitin-proteasome system, and K63-linked chains, which modulate protein activity, interaction and cellular trafficking of their substrates(Cao et al., 2022).

Regulation of protein activity, expression, and degradation is essential for memory formation. Among other brain regions, the hippocampus plays an essential role in this process. Together with other brain areas, such as the amygdala, it is required in the mechanism of fear memory formation. In fact, the hippocampus requires dynamic bidirectional changes to form long-term contextual fear memories.

Original Study: Decreases in K63 Polyubiquitination in the Hippocampus Promote the Formation of Contextual Fear Memories in Both Males and Females (Preveza et al., 2025)

The research group found that K63 poly-ubiquitination is a necessary PTM in the formation of contextual fear memories in the amygdala of female rats. K63 poly-ubiquitin chains knock down rats in the amygdala showed how K63 is necessary for the formation of fear conditioning memories after behavioral testing (Farrell et al., 2021). This led to two research questions:

- K63 poly-ubiquitination is critical for contextual fear memory consolidation in the hippocampus
- K63 poly-ubiquitination in the hippocampus occurs in a sex-selective manner

To answer these questions, they employ two approaches. In the first experiment, they infuse Sprague-Dawley rats with CRISPR gRNA and dCAS13 editing system in the CA1 hippocampal region. The gRNA targets the K63 codon in ubiquitin, knocking it down. These animals undergo a fear conditioning test to assess their response to contextual fear in the absence of K63 poly-ubiquitin modification within the hippocampus. Infused and control (non-infused) groups had the same number of males and females. Compared to controls, K63 knock-downs show enhanced performance or response to contextual fear. They respond faster to the tests and their memory retention is enhanced, meaning that reduced K63 polyubiquitination in the hippocampus enhances contextual fear memory. This enhancement was observed in a sex-independent manner, since there was no difference between males and females.

For the second experiment assessing the research questions, they train Sprague-Dawley rats in contextual fear conditioning test. They apply foot-shocks in a specific period of time. Control group

received no training. Trained and control groups had the same number of males and females. One hour after the training, the animals are sacrificed and CA1 hippocampal regions are collected. These samples are purified to select the substrates that are marked with K63 poly-ubiquitin chains using tandem ubiquitin binding entity assay. Next, the recovered peptides underwent to liquid chromatography and mass spectrometry, via LC-ESI-MS/MS on an Orbitrap Fusion Lumos Tribid instrument, running each sample in duplicate. The data was analyzed and processed with Proteome Discoverer v. 2.5. The search of a decoy database allowed the determination of a false discovery rate. The protein quantities that they show are the sum of the intensities of the associated peptides. These values were normalized to streptavidin amount and duplicates were averaged. This protein analysis revealed 290 and 285 K63 polyubiquitinated proteins in females and males, respectively, with 207 proteins overlapping.

Team 6 Findings and Comparative Analysis

Our team performed an analysis of the dataset provided, which was divided in two PRIDE entries, one for females and the other for males. The mzID files were converted to PSMs. Decoy hits were assessed, and the result was 0. This result was corroborated by checking the number of proteins without description. The total mount of spectrums observed is 57251 for females and 76701 for males.

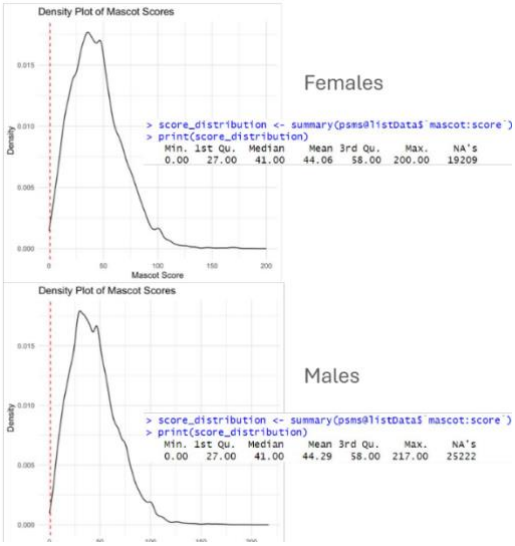

Figure 1 Mascot score distribution

The data provides a Mascot score, which was used to check the distribution of our data (Figure 1). The mascot score is based on the probability that the observed match between the experimental MS/MS spectrum and a theoretical peptide is due to a random chance. A higher score, a more precise matching. The number of scans filtered out are 19093 for females and 25020 for males.

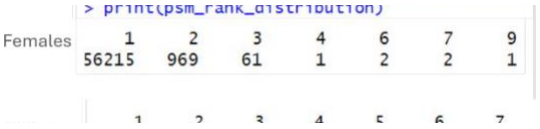

Figure 2 Rank distribution

The rank value indicates the ranking of PSMs per MS/MS spectrum by the search engine, which is typically based on identification score (like Mascot score). If it equals 1 it means that it is the best match for that spectrum and it displays a high identification score. Figure 2 shows the rank distribution. Consequently, our team filtered scans with rank higher to 1, since they are alternative matches for the same spectrum. The number of scans filtered out, hence with tank different to 1, are 1036 for females and 1171 for males.

The team searched for identified proteins in the female dataset, finding a total of 464. The amount of protein components is 363 and the number of protein groups is 40. 1489 unique peptides were identified. Within the male dataset, 662 proteins were identified, 542 protein components and 56 protein groups.

Females

	row_sum
FASFIDK	19
FLEQQNK	17
LAADDFR	16
TLNNKFASFIDK	13
YEDEINK	12
YEDEINKR	12
LLEGEECR	11
KLLEGEECR	11
LALDVEIATYR	10
LASYLDK	9
LEQEIATYR	8
ETMQFLNDR	8
LASYLDKVR	8
FASFIDKVR	8
QNQEYQVLLDVR	8
VTMQNLNDR	7

Males

	row_sum
FASFIDK	23
FLEQQNK	18
YEDEINK	18
YEDEINKR	17
LLEGEECR	15
KLLEGEECR	15
LALDVEIATYR	14
LAADDFR	13
LEQEIATYR	10
LASYLDK	9
TRLEQEIATYR	9
DVDAAYMNK	8
NKYEDEINK	8
NMMAACDPR	8
AEAESWYQTK	8

Figure 3 Hypothetical razor proteins

In both datasets, the group hypothesized that it contains razor peptides even though the analysis shows 0. Since razor peptides correspond to those that are shared by different proteins, it was shared the mount of times peptides are theoretically present in different proteins, at it is shown in Figure 3, which displays hypothetical razor peptides for both datasets.

Table 1 Comparison between original study and Team 6 analysis

	Original study	Team 6
Total proteins female	290	464
Total proteins male	285	662
Unique proteins female	83	28
Unique proteins male	78	42

A differential analysis between the proteins identified in each dataset and the proteins expressed differentially between males and females in the original study was performed. Our team found a smaller amount of differentially expressed proteins between the datasets (Table 1). During the analysis, some of the proteins that were presented as unique in females in the study, were also found in males in our analysis. It is the same case for proteins uniquely expressed in males in the study, several were found in the female dataset after our analysis. Our team believes that these differences may be due to a less strict filtering by which we have a higher number of proteins identified in each dataset. Consequently, it is more complicate for these proteins to be uniquely expressed within female and male groups.

As a discussion and conclusion of the study, which we can corroborate with our findings, is that K63 poly-ubiquitin PTM is sex-independent in the hippocampus. The behavioral tests in the original study confirm that the decrease of this modification within the CA1 area in the hippocampus increases the performance of the animals in contextual fear memory consolidation. However, the proteomic analysis shows protein expression levels that are significantly different between trained animals and controls. These proteins are not shared between males and females, even though the end result, fear memory formation, is the same. This means that for the formation of the same contextual fear memories, the hippocampal area activates different molecular mechanisms between females and males.

In the case of females, the proteins that are marked by K63 poly-ubiquitin chain and show decreased levels after fear conditioning, are characteristic of mitochondrial dysfunction and EIF2 signaling. Mitochondrial dysfunction is related to memory loss in the hippocampus, and EIF2 suppression is linked to increased spatial memory. Their decreased expression in the hippocampal area of female brains can be related to an increased memory formation mechanism. On the other hand, males show lower levels of K63 poly-ubiquitin marked proteins related to MCH class II antigen presentation, which are necessary for the formation of an immune response. Their decrease may play an important role on memory consolidation process.

Bibliography

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