*Experiment 1: Genes in the BLA Implicated in Second-Order Fear Conditioning*

In our first experiment, we sought to determine genes within the BLA that might be implicated in second order fear conditioning. Rats in the second order fear group underwent two stages of training: S1-shock followed by S2-S1 pairings (Second Order). Two control groups were used. The first (First Order) was exposed to S1-shock pairings in stage 1 and the context alone in stage 2. The second group (Context) was exposed to the context alone in both stages 1 and 2. Second Order and First Order rats learned to fear either S1 (First Order; Figure 1A) or both S1 and S2 (Second Order; Figure 1B and C). The context-only group did not show any fear to the context (data not shown).

Figure 1. Fear learning in Second and First Order groups

Tissue from the BLA or the PRh was collected from all three groups at three timepoints after stage 2: 30, 90 and 270 minutes. Twelve genes of interest were chosen. Three of these have been implicated in first order fear conditioning: Egr1, Arc and BDNF (REF). The remaining genes, in addition to being implicated in first order fear, are involved in pathways that have previously been shown to mediate discrimination of first- and second-order fear memories: DNMT3a, PKCb1, PKIa, Prkaca, MAPK1, Map2k7, CamkIV, CamkIIa, CamkIIb (REF).

At 30 minutes, we found that second-order fear promotes the significant changes in the expression of CamkIV, DNMT3a and MAPK1 I the BLA. In contrast, in PRh only one gene PKCb1 was significantly different from First Order group.



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Figure 2. Fold change of genes 30 minutes after acquisition. (a) BLA (b) PRh

Ninety minutes after conditioning, we found that 4 genes exhibited significant difference between two groups. In addition to CamkIV, PKIa, DNMT3a and EGR1 were shown to have s significant decrease in expression compared with First Order group. In PRh the only different gene was mapk1.

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Figure 3. Fold change of genes 90 minutes after acquisition. (a) BLA (b) PRh

By 270 minutes after fear learning, we found that most of the genes returned to the baseline and did not demonstrate statistically significant differences both in BLA and PRh. Th only exception was mapk1 in BLA.

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Figure 4. Fold change of genes 270 minutes after acquisition. (a) BLA (b) PRh

Figure 1. demonstrates the changes in gene expression in the BLA 30, 90, 270 minutes after second-order conditioning. Most of the genes have not shown significant differenses in 2nd Order group compared with 1st Order group at the time points of interest. Only CamkIV?, Dnmt3a appeared to show group-dependent changes at 30 minutes. At 90 minutes there were 3 genes discriminated 1st Order and 2nd Order group, while at 270 minutes there was only 1 gene.

To assess the specificity of the changes in gene expression to the BLA, we performed PCR for the same genes in a neighboring region of the brain, the perirhinal cortex (PRh). There were not any significant changes in gene expression registered for this part of the brain (Figure 2).

Within the BLA, multiple genes were significantly different at both 30 and 90 minutes, but not 270 minutes. Next, we employed a principal component analysis (PCA) to establish the relative contribution of the genes to overall similarities and differences between the samples. Further, we wanted to determine whether we could use the gene expression data to form two distinct clusters, thereby confirming our *a priori* groups of first- and second-order fear conditioning. For the PCA we used an *n* x *m* matrix, where rows (observations) correspond to individual BLA samples and columns (variables) that corresponded to the 12 genes of interest. To determine the number of principal components, eigenvalues and proportion of variance captured were analyzed in a Scree plot (Figure X).

Describe 30 minutes data first

1. Scree plot
2. Two 2D plots (1v2, 1v3)
3. 3D plot
4. Contribution to variation plot

Describe the 90 minutes data

1. Scree plot
2. Two 2D plots (1v2, 1v3)
3. 3D plot
4. Contribution to variation plot

This indicated that three principal components accounted for 82.9% of the variation, allowing for a close approximation of the dataset.

The relationships between the variables and the level of their correlation with PC1 and PC2 can be observed on a variable correlation plot (Pic). Here, it can be seen that variability in PC1 is accounted for the CaMKIV, CaMKIIb and EGR1 that are highly correlated between each other, while PKC.b1 underlies the variability in PC2.

*Experiment 2: EGR1 Knockdown*

1. Behavior 1: Infusion 1h before second order
2. Behavior 2: Infusion 6h after second order

Given that we find it disrupts second and first order within the consolidation window, and that outside of the second order consolidation window, it does not, we then shought to determine if there was an influence of EGR1 knockdown on reconsolidation of second order.

1. Behavior 3: Reconsolidation after second order (need to include model)

First, we sought to determine the genes that regulate second-order conditioning fear in BLA of rats.

As it can be seen from the picture (table? Panno? ) expression of only four genes out of 14? has been shown to have statistically significant differences in 2nd Order group. Expression of CamkIV, Egr1 was lower at 90 minutes, while mapk1 was higher at 270.

Among those four genes three were studied Lay et al.(2018) found that CaMKII/IV, ERK/MAPK pathways and DNA methylation.

Contributions are presented in

Second, to investigate the patterns of gene expression that discriminate 2nd Order group principal component analysis has been deployed.

PCA plot (pic) clustered the samples based on their similarity in a sense of gene expression. At both 30 and 90 minutes samples from 2nd order and 1st Order groups formed distinctive clusters, which supports the idea about differences in gene expression occurring while regulating 2nd order fear.

To estimate the role of genes in discriminating 2nd order group from 1st order we compared the loadings which reflect the degree of correlation between original variables (genes) and new principle components

At 30 minutes principal component 1 and principal component 2 explained 45.7% and 25.2% of data variance respectively. PC1 has positive associations with Arc (loading: 0.407), CamkIIa, CamKIIb (loadings are 0.383 and 0.385 respectively), EGR1 (loading: 0.359) and map2k7 (loading: 0.355). PC2 has negative associations with PKCb1 (-0.480), mapk1(-0.461), Dnmat3a (-0.444) and CamkIV(-0.420).

At 90 minutes pattern of changes between two groups of interest has changed. Egr1, Dnmt3a and CamkIIa

At 270 the changes between groups fucked off