Review of: "Neural Activity in Afferent Projections to the Infralimbic Cortex is Associated with Individual Differences in the Recall of Fear Extinction"

Kubra Gulmez Karaca, Marloes Henckens[1]

1 Radboud University Medical Center

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This study investigated the neuronal activation patterns during extinction recall in rats, focusing on the IL-projecting neurons, given the pre-established functions of the IL cortex in fear extinction recall. For that, the authors used retrograde viral GFP tracing into the IL and determined Fos activity in IL afferents, as well as overall Fos expression in projection areas, after fear recall, extinction recall, and a no-behavior control group. The authors dissociated good vs. bad extinction rats, and correlated this activity with the inter-individual differences in extinction retention performance.

Our specific comments for the study are listed below:

1. *Not all conclusions of the study are supported by the data.*The authors claim throughout the manuscript that (in their own words) “Outside of the IL-projecting cells, increased neural activity was observed in good extinction rats in select regions of the claustrum and ventral hippocampus. Our results suggest that successful extinction recall is orchestrated by specific PVT projections to the IL and non-IL targeting cells in the claustrum and ventral hippocampus”. However, their data does not support these strong arguments for the claustrum and ventral hippocampus. Neither in the hippocampus, nor in claustrum the authors identified a significant difference in neuronal activation pattern between the good or bad extinction performers (only significant difference that was identified was between the home-cage and good performers group). So, the authors should be very careful in making such strong arguments and conclusions based on their data, and should rephrase these conclusions accordingly.
2. *Comparison of GFP expression across different experimental groups is lacking.*The authors infused a retrograde tracer into the IL and compared the number of activated (i.e., Fos-expressing) GFP-expressing cells in different brain regions across different experimental groups (extinction groups, home-cage, fear recall). However, before making such comparison, it is critical to provide experimental evidence that GFP expression is similar in different experimental groups. Because GFP expression can directly be influenced by surgical conditions (suboptimal viral delivery, slight changes in stereotaxic coordinates etc.) and thus unintentionally bias the results. Therefore, the authors should compare GFP expression between different experimental groups and show similarity. Furthermore, the dataset includes many data points with 0s in every brain region analyzed. Especially in Figure 4, it is impossible to distinguish individual data points as majority of them are clustered at 0. If possible, the authors should use more clear graphs. At least they should provide raw numbers of the analysis.
3. *Definition of extinction recall score is unclear.* The authors applied an extinction score to distinguish the rat’s

performance into good or bad extinction. Scientific justification of how the groups are divided seems arbitrary (top third versus bottom two/third). Why not using only the top third for bad extinction and bottom third for good extinction? That would avoid (close-to) overlapping values between groups. In any case, the authors should clearly explain in the manuscript how this division is justified. Furthermore, they should also provide the delta in % freezing upon tone administration (baseline-to-tone) during extinction/fear recall tests to be able to compare the group performances with the existing literature.

4. *More detailed information on freezing scores during fear recall testing would be preferred.*The authors administered 4 presentations of the tone during fear memory recall test and showed the average percentage in Figure 2D. However, the presentation of consecutive tones *per se* could lead to an extinction during the testing session itself. To clarify i) if such reduction in freezing occurred between the different presentations of the tone during the test, and if such, ii) whether this reduction differed between experimental groups, the authors should report the freezing rate to each tone presentation (so, dividing the block into 4 separate data points) during testing. They should also comment on why the fear recall group is exposed to the context B only 48 h later without being exposed to the context B 24 h later (as in the extinction group).

Additional minor comments:

1. *Representative immunohistochemistry images in Figure 3G should be changed.*Why is there Fos expression in GFP channel? Is it caused by fluorescent signal bleeding between different channels? It also seems like the images are acquired from different locations on the sample. Each channel should be displayed from the same location (point of imaging).
2. *Inconsistency of the y-axis scale in Figure 4C-D.*Although Figure 4C-D show the same parameter on their y-axes, the scale dramatically changes between the two figures. The authors should correct this. If this is indeed the case, then they should provide an explanation for this discrepancy.
3. *Throughout the manuscript, there is no clear explanation of why these regions have been selected as regions of interest.* The authors should justify why they selected analyzing these regions.
4. *When comparing the GFP count in different brain regions (Figure 3) a paired test should be used, since the comparison is performed on the same subject.* Did the authors use a paired test for this comparison? If not, why not? This information should be included in the statistics section.

Suggestions for future directions:

1. The authors identified significant changes in the activity of IL afferents in the PVT (Figure 4K-L). This result seems promising to follow up. It would be relevant to further dissociate these cells (excitatory, inhibitory (also subpopulations)) to get a better understanding. These experiments could even be performed on the same slices.