nature portfolio

Peer Review File

Parallel processing of past and future memories through reactivation and synaptic plasticity mechanisms during sleep

Corresponding Author: Professor Kaoru Inokuchi

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript is from a group that has been investigating the role of learning-activated ensembles, sometimes referred to as engram cells, using immediate early gene expression for tagging purposes, for many years now. In this latest manuscript, the authors set out to understand the role of offline periods (i.e., awake, sleep) either before or after an experience, in terms of engram cell dynamics and their relationship to neighboring non-engram cells. They link the well-known role of sleep and memory reactivation to engram cells and provide evidence for this process occurring together with the preparation of related/other engram cells for the next learning event. These observations are based on in vivo calcium imaging with single cell resolution. The group also uses neural network modeling to investigate the role of synaptic plasticity in engram cells during these offline processes.

Overall, the techniques used are appropriate, the conclusions are based on their data, and the conceptual advance is interesting/sufficient for the field. There are several minor questions that would be addressed to strengthen this work (in the order that they appear):

- Suppl. Fig. 2G: For the correlation matrix repetition analysis, how did the authors choose 60 sec? Please plot comparable data from the entire session A as well as only for the last 60 sec of session A. Do engram cells exhibit a higher correlation, independent of the time window selected?
- The authors indicate that the thresholds of <0.6 (weak) and >=0.68 (active) for the normalized dot product was used to identify ensembles that are active in other sessions. Though they mention that the results stayed the same for higher thresholds, these data have not been provided in the manuscript. It will improve our confidence to be able to review/refer to such data using a higher threshold for active ensembles in the study.
- Suppl. Fig. 4A and 4D: Why are the MS scores always zero except for session A in non-engram cells, which is different from main Figure 1D's non-engram scores.
- Fig. 2C: A big difference between NREM and REM is seen for online emerging and stand by ensembles in engram populations (i.e., more in the REM data). Why is this and what could be a role for these REM ensembles? This point is worth discussing in the main text.
- Fig. 5A: The authors should add a sleep epoch after session B to compare/contrast results of their model with Fig. 3F.
- Fig. 6: The authors tried to examine the role of offline plasticity in their model by removing both synaptic depression caused by SWRs and homeostatic synaptic plasticity in postlearning sleep. Given that they have a validated model, it would be conceptually more interesting to remove synaptic depression and homeostatic plasticity one at a time to reveal potential unique contributions to postlearning sleep ensemble dynamics.
- Discussion: I was surprised to see that the authors did not discuss the relevance of their neural network model of engram cells in relation to recent studies (e.g., a couple of papers from the Claudia Clopath lab). This should be included to highlight the unique features and potential similarities of the different engram models (including this study's), which is very important for the field moving forward.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This manuscript by Ghandour and colleagues sought to ask how ensembles of neurons are selected to be recruited to represent subsequent experiences encoded across time. To do this, the authors combined in vivo calcium imaging with activity-dependent ensemble tagging to investigate this question. They placed mice in a novel environment (A) with a pre- and post-recording in their homecage around the experience. Sleep was recorded during the pre- and post-recordings. The following day, mice were re-exposed to context A as a form of memory retrieval, and then exposed to a novel context B for the formation of a new memory. The authors recorded calcium dynamics in hippocampal CA1 neurons throughout all stages of the experiment, and also recorded sleep (via EEG & EMG) during the pre- and post-recordings on Day 1. The authors also used a technology to label cFos-expressing ensembles among their recorded neurons using a Dox off system that requires delivery of 365nm light for engram cells to turn from green to red and be identified in the recorded field-of-view. The pre-recording revealed that ensembles of cells were active prior to an experience which would then go on to be active during learning, reactivated offline, and reactivated during retrieval to stably represent that memory long-term. These results are consistent with past reports of both preplay and replay in the hippocampus. Next, the authors questioned how these cells representing one experience can give way to a new ensemble of cells that are pre-configured to be active during a subsequent novel experience (in context B). They found that during the post-recording after context A exposure, new cells became active which would go on to represent the novel context B the following day ("Engram-to-be" cells). The authors claim that this could be a mechanism by which cells are selected for subsequent novel experiences. The authors then investigated how networks of neurons could use plasticity rules to drive this change in ensemble dynamics, so they developed a neural network model of the hippocampus. They replicated their empirical result in the model that ensembles of hippocampal cells emerged during the post-learning period after context A exposure, and they then manipulated synaptic plasticity during sleep in the model to ask whether synaptic plasticity during post-learning sleep is necessary for the emergence of this new preconfigured ensemble. The authors conclude that synaptic plasticity is required for the emergence of preconfigured ensembles that go on to represent novel experiences.

The manuscript is interesting and timely, considering it is of great interest in the field how memories encoded across time are integrated and separated. In particular, replay and preplay are two phenomena that have been observed in the hippocampus, and it remains unclear how cells active around an experience go on to represent the memory for that experience versus other memories across time. The authors present a mechanism by which cells could be selected for subsequent experiences, which could have strong implications for the study of how multiple memories are formed and stored in the brain. This makes this manuscript novel and a worthwhile contribution to the field. However, there are significant conceptual concerns that detract from my enthusiasm for the manuscript. I highlight the major and minor points below:

Major Points

- There is a lack of consistency of the readout of each analysis and the rationale for using each analysis. For example, the authors switch between an ensemble overlap measure, correlations, sum of correlations, population vector distance, etc. without justifying why one measure is better than another, especially since they are all measures of representational similarity. I would appreciate if the authors could provide the results of the analyses with one method of measuring ensemble similarity. If the results are difficult to interpret altogether, I would appreciate if the authors could speculate about the discrepancies between the results.

- While it is interesting that ensembles of cells emerge during post-experience sleep periods, it is unclear whether this is a direct result of experience or whether the passage of time could explain this result. One possible control experiment the authors may consider is repeating this experimental procedure with homecage recordings on day 1 in the absence of context A exposure, followed by exposure to contexts on the following day. This way, the "engram-to-be cells" can be defined purely based on homecage activity (for example, during an awake state). Similarly, I wonder whether the engram-to-be cells might be influenced by the presence of context A recall just prior to exposure to B. While I do not think this warrants another experimental group, some speculation in the discussion would be useful.

- On page 31, the authors describe that sleep recordings were done for 1-4 minutes, which is quite a short period of time, and past reports have demonstrated that ensemble reactivation varies as a function of time after an experience (e.g., Giri et al., 2019). First, it would be useful if the authors could provide cell counts across their sessions. In typical calcium imaging recordings in hippocampus, one-minute recordings are incapable of detecting calcium transients from substantially large ensembles of cells. Additionally, since the timing of the sleep can be variable relative to experience and the activity dynamics could be variable within sleep states, it is unclear if a one-minute recording will allow for a representative sample. Would it be possible to perform continuous calcium imaging recordings for tens of minutes?

- This manuscript makes a claim about the formation of memories through ensemble reactivation around an experience; however, there is no direct measure of memory behaviorally, making it difficult to know whether the mice are retaining a stable memory of the context, and perceiving context A as different from context B. I do not suggest that the authors re-run the entire experiment using a behavioral paradigm that has a behavioral readout; however, the current manuscript makes a claim about memory where the evidence for a stably formed memory is not strong. Therefore, I believe that the authors should soften their claims about whether these homecage ensemble dynamics are directly relevant to the formation of memories and their neural representations. If the authors decide to make a claim about memory, stronger evidence of the retainment of a memory would be needed.

- The model that was developed to manipulate synaptic downscaling is an interesting expansion upon the original

experiment, but there is no empirical evidence that synaptic downscaling is a mechanism that drives the emergence of an engram-to-be ensemble in a post-learning period following an experience. The authors should emphasize that these results are purely in silico and do not necessarily reflect the biological in vivo condition. Future results will be necessary to disentangle the contributions of synaptic plasticity in driving the recruitment of new cells for encoding future events.

Minor Points

- In lines 49-50, the authors claim that how “these engram cells behave in periods prior to learning and how they affect future learning, is yet to be studied.” However, significant work has been done on this topic. For example, Dragoi & Tonegawa, 2011 demonstrated that ensembles are active prior to learning of an experience (termed preplay); Farooq & Dragoi, 2019 demonstrated that these preconfigured ensembles emerge in development; and Mocle et al., 2024 demonstrated how excitability plays a role in the development of preconfigured ensembles. I would appreciate if the authors could describe in further detail what has come before to set up why this study is an important contribution above and beyond what has already been done in past years.

- On line 705, the authors state that calcium activities during sleep were recorded as previously described, but there is no citation for where this information is previously described.

- In Figure 4B, why did the authors decide to change the definitions of the engram cells to not comprise cells active during context A retrieval? In Figure 2A, the ensembles are defined by their activity during learning and reactivation during retrieval. This is also consistent with past reports of ensemble reactivation (e.g., Reijmers et al., 2007; Cai et al., 2016). Do the results in Figure 4C-F change if the cells must be reactivated during retrieval of context A to be part of the context A engram?

- In Figure 4A, on the right side, the representative schematic of ensemble co-activities does not seem to add up, unless I am reading it wrong. For example, in the first column, there are no “Common Engram” cells active; however, in the summed up values below, it says that there is 1 cell active from this ensemble. In the second column, one “Common Engram” cell seems to be active, but the summed up values say that there are 0. If I am misreading this schematic, I would appreciate if the authors could write a clearer explanation of the analysis.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
Ghandour, Haga, Ohkawa et al. investigated the role of sleep in the recruitment of engram cells. They provided compelling evidence that learning-induced patterns of activity are highly stereotypical across memory stages (i.e., pre- and post-learning sleep as well as memory retrieval), pointing to neural activity prior to and following learning being predictive of engram recruitment and memory consolidation, respectively. They also found that the activity of cells not allocated to a first memory engram is reorganized during post-learning sleep such that a subset of these cells is recruited to a second memory engram. In addition, they proposed a computational model that suggested that synaptic plasticity during post-learning sleep can capture key features of their experimental data. Together, their results shed light on the interplay between sleep and memory allocation, complementing previous studies that have focused on the role of intrinsic excitability in memory recruitment (Zhou et al., Nat Neuro, 2009; Yiu, Mercaldo et al., Neuron, 2014).

Major Points
1. The key findings of the paper rely on identifying ensembles using an NMF-based approach outlined in the manuscript. Could you use an alternative method to identify ensembles in each session (e.g., an alternative unsupervised clustering technique, or a supervised approach contrasting cell activity in A’ and B on Day 2) and perform the same analyses to verify that the key results of the paper also hold in this case (e.g., ensembles activated during prelearning sleep are allocated to a subsequent engram)? This would considerably increase the robustness of the results.
2. There has been considerable evidence that memory engrams are dynamic, with cells dropping into and out of engrams following initial learning (Tomé, Zhang et al., Nat Neuro, 2024; DeNardo et al., Nat Neuro, 2019). Fig. 2B-C seems to suggest that your data also support a dynamic memory engram (e.g., stand-by and isolated patterns could indicate cells dropping out of the engram).
2.1. Could you quantify the fraction of cells dropping out of the engram (i.e., activated in learning A but not activated during retrieval A’) and the fraction of cells dropping into the engram (i.e., not activated in learning A but activated during retrieval A’)?
2.2. Do cells that drop into and out of the engram show distinctive activity patterns during pre- and postlearning sleep? For instance, are cells that drop into the engram not activated during pre- but activated during postlearning sleep? Are cells that drop out of the engram not activated during postlearning sleep?

Minor Points
3. Suppl. Fig. 1B: The y-axis needs a label.
4. Line 100-101: Specify the measure of dispersion (i.e., standard deviation, standard error of the mean?) in 7% (+- 1.5) and 93% (+- 1.5).
5. Suppl. Fig. 2G: Caption is unclear.

6. Reference 42: Missing year.
7. Line 111-113: Can a given cell have a high participation in multiple ensembles identified by NMF? This seems to be the case based on the schematic in Suppl. Fig. 3B, but this needs to be stated in the main text. If this is not the case, this should also be stated in the main text and the schematic in Suppl. Fig. 3B needs to be corrected.
8. Line 117-119: Why was 0.6 considered a “weak threshold”? This needs further elaboration.
9. Suppl. Fig. 3C: The plot and caption are unclear. The legend of the plot is hard to understand. MSs should be spelled out in the caption. Overall, the plot and caption need to be modified so the reader can understand them.
10. Line 119-120: The authors claim that “Comparable results were obtained regardless of higher thresholds” but this needs to be backed up by a proper analysis with different thresholds.
11. Suppl. Fig. 3D-G: The y-axis label in the bottom plot of each panel reads “Overalp (All)” but the caption says “relative intensities across all the sessions”. Are these the same?
12. Line 137-138: The following is unclear: “engram ensembles decreased significantly from the retrieval session (A’) to a different context (B)”. Do you mean the MS of the engram decreased?
13. Suppl. Fig. 4B: Please specify in the caption the definition of discrimination index used in this analysis.
14. Suppl. Fig. 5E: Shouldn’t the denominator for calculating false negatives and true negatives be 5+6+7+8?
15. Suppl. Fig. 6D: Missing measure of dispersion for UP in the pie chart.
16. Suppl. Fig. 6E-F: Missing y-axis label.
17. Suppl. Fig. 6C-F: Mismatching panel letters and the respective captions.
18. Suppl. Fig. 8B: Missing x-axis label.
19. Suppl. Fig. 8D: y-axis label should be neuron index.
20. Fig. 4C: Are the individual data points for “Specific engram & engram-to-be” really so close to each other across all sessions?
21. Suppl. Fig. 10: Caption should be for B-C. Please also indicate in the caption the difference between the top, middle, and bottom plots in panels B-C (e.g., different samples?).
22. Fig. 4: Captions of panels E and F are swapped.
23. Line 271-272: Include reference to the learning rule.
24. Line 895: The index on the left-hand side of the equation should be i and the upper index of the second summation on the right-hand side should be Ninh.
25. Line 896: Do inhibitory neurons in your model of CA1 receive feedforward (CA3) or recurrent (CA1) excitatory inputs? The notation on the right-hand side of the equation is not consistent with either case and needs to be adjusted.
26. Line 902-903: Do you mean “[then] simulated time evolution of the dynamics for 20 ms”?
27. Line 919/925/927-928: Do the synaptic learning rule, the synaptic depression rule, and the synaptic scaling rules apply only to E-to-E weights from CA3 to CA1 excitatory neurons or also to E-to-I weights from CA3 to CA1 inhibitory neurons? Based on lines 910-911, it seems that they only apply to CA1 excitatory neurons. However, this should be made clear.
28. Fig. 5D: Would the results hold if you compared engram vs. other nonengram and engram-to-be vs. other nonengram separately?
29. Line 303: Fix reference to Fig. 1E.
30. Fig. 5F: Could you perform the same analysis for the other sessions (i.e., prelearning sleep, context A, and context B) and verify that the same conclusions hold similar to the experimental data in Fig. 4C?
31. Fig. 6C: Results of statistical tests are not shown in the plot, colors of legend do not match colors of the data points, and comparisons between each pair of groups (i.e., similar to Fig. 5) are missing (currently only comparing common engram and engram-to-be to every other group).
32. Suppl. Fig. 9: Panels B-E and G-J are not referenced in the main text, only in the methods (line 786).
33. Line 29-31: Please clarify that the proposed model suggests a potential role of synaptic depression and scaling in the reorganization of the activity of nonengram cells.

(Remarks on code availability)
The instructions to run their code are clear.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
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(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors did a good job of addressing my comments thoroughly by updating the main manuscript text and providing several additional experimental/modeling results in the manuscript. This, in my opinion, has improved the manuscript. I have no further issues.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I thank the authors for a thorough response to my suggestions for the manuscript, which have strengthened the claims that the authors originally concluded. I recommend the manuscript for publication provided the authors address the following minor concerns.

Minor Points
1. I previously brought up a concern that the emergence of preconfigured ensembles during post-learning sleep may just be a result of the passage of time. The authors addressed this concern by performing an awake homecage recording on Day 0, Day 1 pre-learning, and Day 1 post-learning. The authors found that there was no difference in the activity of engram-to-be cells during these three awake time points (Supplemental Figure 8C). I agree that this lends support for the idea that awake cell activity is not modulated by time across days. However, I would like the authors to include a similar comparison for the cell activity during sleep at each of these time points (i.e., S0 vs S1 vs S2). If the activity of engram-to-be cells on Day 0 and Day 1 pre-learning is no different during sleep, while the activity of engram-to-be cells on Day 1 post-learning is different during sleep, I believe these results would effectively demonstrate that the sleep-specific changes in activity are not just a result of the passage of time.

2. I recognize that the authors are unable to record calcium dynamics for longer than ~12 minutes out of concern of photobleaching. However, I find the seizure-like activity provided in supplemental video “Phototoxicity demo2.tif” to be concerning and would like the authors to confirm that this animal was excluded from the datasets analyzed in the manuscript. While the authors speculate that this activity may have arisen from phototoxicity, it is possible that this animal exhibited aberrant epileptiform activity, not driven by phototoxicity necessarily, but the longer duration of recording time allowed an opportunity for this underlying pathology to be observed. Since the main claims in this manuscript depend on the “co-occurrence” of activity between neuronal ensembles, it is important to demonstrate that these results are not driven by aberrant epileptiform burst events during which large fractions of the recorded neurons are synchronously active (which would likely not represent the healthy neurotypical brain). To alleviate this concern, I would like the authors to compute the percent of cells that co-fire together across time (e.g., every calcium imaging frame), with the rationale that there should not be any timepoint where >50% cells are co-firing at one time (suggesting aberrant activity). If there are such frames that exist, it would be more difficult to interpret the results, considering the ensemble co-occurrence may be driven by seizure-like activity.

3. Figure 4C has a typo in the y-axis label where “egram” should be “engram.”

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I appreciate the time and effort you have dedicated to address my comments. I am particularly glad that you have conducted a series of new analyses to substantiate and expand the claims in the manuscript. I have recommended your manuscript for publication and I look forward to seeing it online.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
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(Remarks on code availability)

We thank you for handling our manuscript and the reviewers for their helpful and constructive comments. We have addressed all of the reviewers' comments, and the point-by-point responses to the comments are described below. In the revised manuscript, all corrections are shown with track change. We hope that our revised manuscript is now suitable for publication in *Nature Communications*.

Reviewer #1 (Remarks to the Author):

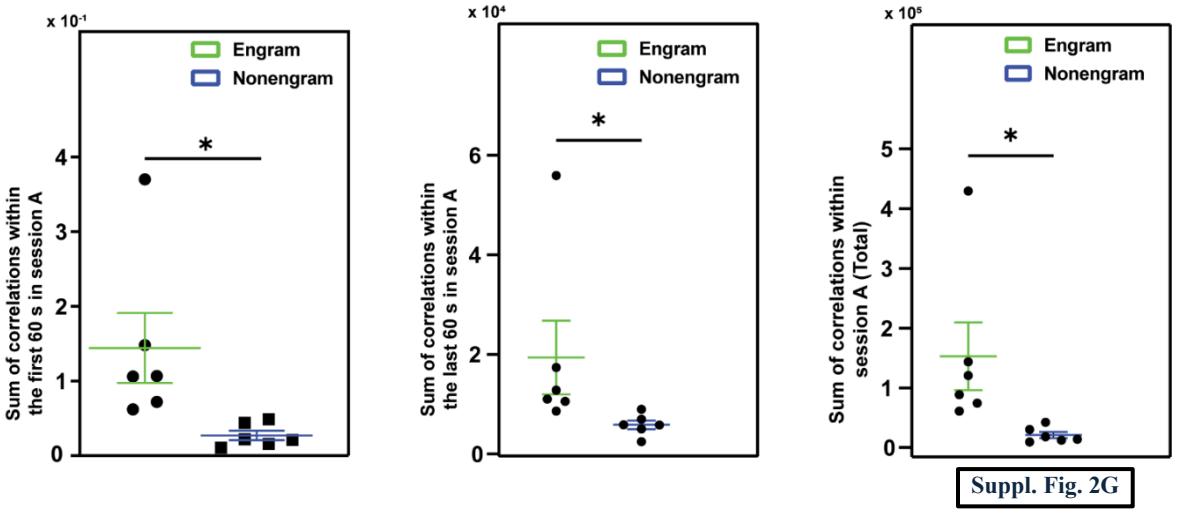
This manuscript is from a group that has been investigating the role of learning-activated ensembles, sometimes referred to as engram cells, using immediate early gene expression for tagging purposes, for many years now. In this latest manuscript, the authors set out to understand the role of offline periods (i.e., awake, sleep) either before or after an experience, in terms of engram cell dynamics and their relationship to neighboring non-engram cells. They link the well-known role of sleep and memory reactivation to engram cells and provide evidence for this process occurring together with the preparation of related/other engram cells for the next learning event. These observations are based on in vivo calcium imaging with single cell resolution. The group also uses neural network modeling to investigate the role of synaptic plasticity in engram cells during these offline processes.

Overall, the techniques used are appropriate, the conclusions are based on their data, and the conceptual advance is interesting/sufficient for the field. There are several minor questions that would be addressed to strengthen this work (in the order that they appear)

We appreciate the reviewer's careful reading, positive feedback, and constructive comments, which have truly helped us to increase the quality of the manuscript. We have taken all of the comments into consideration while revising the paper, as detailed below.

- Suppl. Fig. 2G: For the correlation matrix repetition analysis, how did the authors choose 60 sec? Please plot comparable data from the entire session A as well as only for the last 60 sec of session A. Do engram cells exhibit a higher correlation, independent of the time window selected?

We thank the reviewer for pointing out this interesting point, the reviewer asked whether engram cells exhibit a higher correlation, independent of the time window selected. To directly answer this question, we calculated the sum of correlations in both the first and last 60 seconds as well as in the total time (see panels below).
Engram cells showed significantly higher correlations in all cases, this suggests that engram cells exhibit a higher correlation, independent of the time window selected.
Based on the reviewer's comment, we have incorporated the total sum of correlations for the total time into the revised manuscript. This addition aims to make the results clearer and more accessible to the reader, ensuring there is no confusion regarding the findings (Fig.Supp.2G).



Suppl. Fig. 2G

The authors indicate that the thresholds of <0.6 (weak) and >=0.68 (active) for the normalized dot product was used to identify ensembles that are active in other sessions. Though they mention that the results stayed the same for higher thresholds, these data have not been provided in the manuscript. It will improve our confidence to be able to review/refer to such data using a higher threshold for active ensembles in the study.
We apologize for the lack of clarity in our previous explanation. First, we would like to clarify that the actual threshold for the dot product (θ) is 0.6, not 0.68. The number "8" in our previous mention was a reference to the threshold we used in our earlier study (Ghandour et al., Nat Commun. 2019), which we re-applied in the current study. To ensure the appropriateness of this threshold for our current data, we conducted an analysis across a range of thresholds. In Supp. Fig. 3C, the x-axis represents the dot product (θ) threshold range (0.1–0.9), and the y-axis displays the p-value for the difference between engram and non-engram patterns at each threshold. Thresholds below 0.6 seem quite low and couldn't detect differential reactivations across sessions. We have added more explanation in the revised manuscript and to the figure legends to make it clearer for the readers.

Page: 6 “We calculated the similarities between session A and other sessions using different thresholds (ranging from 0.1 to 0.9) (Supp. Fig. 3C). Thresholds below 0.6 displayed comparable activity across all sessions, which appeared less stringent, while thresholds above 0.6 maintained similar trends (Supp. Fig. 3C).”

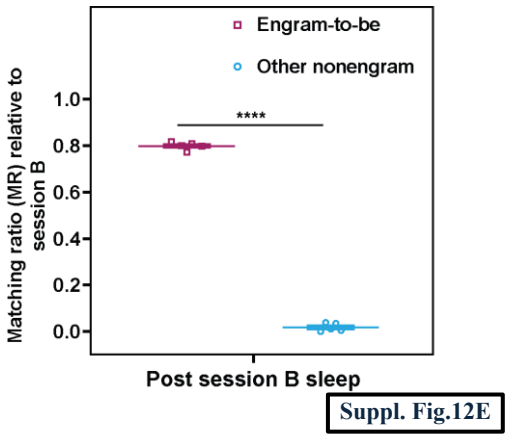
- Suppl. Fig. 4A and 4D: Why are the MS scores always zero except for session A in non-engram cells, which is different from main Figure 1D’s non-engram scores.
We appreciate the reviewer for highlighting this point. The key distinction between the main Fig. 1D and Supp. Fig. 4A and D lies in the calculation method. In supp. Fig. 4A and D the analysis was restricted to the same number of non-engram and engram cells. In which, decreasing the number of non-engram cells decreased the non-engram’s spurious correlations and thereby the matching score values. In brief, all the overlap between ensembles obtained from the non-engram cells with ensembles in other session was lower than the threshold (0.6). Next, in the matching score analysis, we only count the number of correlated ensembles across sessions with overlap above the threshold (0.6), the score became zeros in supp. Fig. 4A and D dataset. This was done to ensure that the observed differences in Fig. 1 were not influenced by sample size discrepancies, thereby confirming that the findings were not artifacts of uneven cell counts. In other words, we compared the activity of engram cells with the same number of non-engram cells. The matching score of the nonengram dropped to zero when comparing the same number of cells.

- Fig. 2C: A big difference between NREM and REM is seen for online emerging and stand by ensembles in engram populations (i.e., more in the REM data). Why is this and what could be a role for these REM ensembles? This point is worth discussing in the main text.
We appreciate the reviewer for raising this intriguing point. We believe that REM sleep could play a more significant role in the emergence of new ensembles and potentially in forming new pattern connections that are absent during awake states. However, we did not observe a significant difference between the two sleep stages in our current data. This remains an important question for future investigations, and we have included this point in the discussion section of the revised manuscript for further clarification.
Page 20: “Another noticeable point is that REM sleep shows a tendency of high ensemble percentage in online emerging and stand-by patterns, this could be related to the nature of REM sleep in the emergence of new ensembles (Cai et al., PNAS 2009; Kareem 2024; Fayed et al., 2024).”.

- Fig. 5A: The authors should add a sleep epoch after session B to compare/contrast results of their model with Fig. 3F.

Based on the reviewer's request We simulated a sleep session after the session B, and analyzed the matching ratio between activity patterns in the session B and the following sleep. We confirmed that replay (pattern matching) occurred in engram-to-be cells but not in other non-engram cells. This result corresponds to the experimental result shown in Fig. 3F. In the revised manuscript. We added the new data in Suppl. Fig. 12.

Page 16: "Furthermore, we also simulated a sleep session after context B and evaluated the matching between activity patterns in the context B and the following sleep. We confirmed that the matching of engram-to-be cells was higher than that of non-engram cells (Suppl. Fig. 12E), which corresponds to experimental results in Fig. 3F."



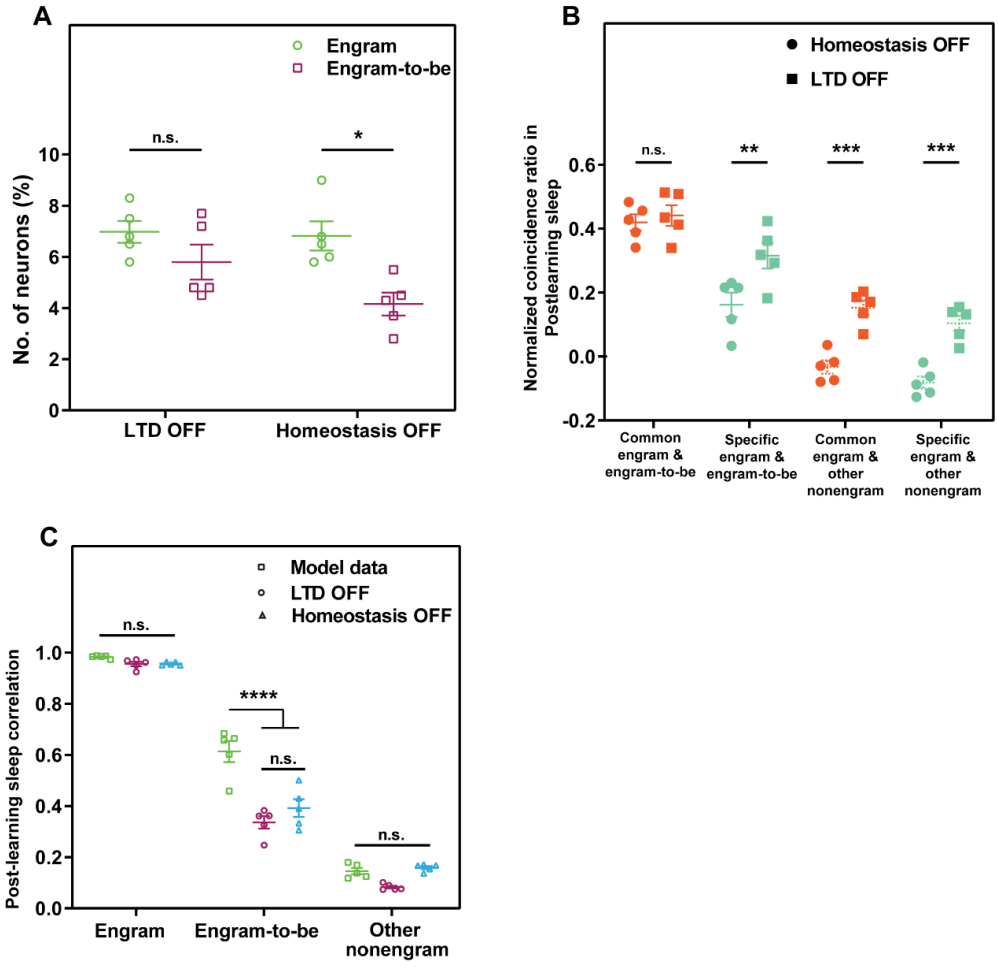
Suppl. Fig.12E

- Fig. 6: The authors tried to examine the role of offline plasticity in their model by removing both synaptic depression caused by SWRs and homeostatic synaptic plasticity in postlearning sleep. Given that they have a validated model, it would be conceptually more interesting to remove synaptic depression and homeostatic plasticity one at a time to reveal potential unique contributions to postlearning sleep ensemble dynamics.

We thank the reviewer for highlighting this interesting point. Based on the reviewer's request, we performed the manipulations one at a time (synaptic depression OFF or homeostatic synaptic plasticity OFF).

When synaptic depression alone was deactivated, the coincidence ratio increased nonspecifically between all cell types (Suppl. Fig.13B), suggesting that assembly formation was high but non-selective to any experience.

On the other hand, deactivating homeostatic plasticity alone results in a lower number of engram-to-be cells (Suppl. Fig.13A). Turning off homeostatic plasticity in sleep directly impairs the formation of engram-to-be assemblies while turning off synaptic depression leads to an overall increase in activity of all cell types without being selective to any experience. Both models, with either synaptic depression or homeostatic plasticity off, displayed low engram-to-be correlations during post-sleep sessions (Suppl. Fig.13C). Thus, both synaptic depression and homeostatic plasticity are required for the emergence of new specific ensembles for the new learning.



Suppl. Fig.13

We have added this data as Suppl. Fig. 13 and added this explanation in the revised manuscript.

Page: 17"Moreover, we tested the effects of shutting off synaptic depression and homeostatic plasticity individually. Disabling homeostatic plasticity during sleep directly impaired the formation of engram-to-be assemblies (Suppl. Fig.13A). When synaptic depression was

turned off, all cells were typically more active in all sessions, nonspecifically increasing the coincidence ratio (Suppl. Fig.13B). Both models, with either synaptic depression or homeostatic plasticity off, displayed low engram-to-be correlations during post-sleep sessions (Suppl. Fig.13C). This suggests that both mechanisms are essential for the emergence of specific ensembles and highlights the importance of sleep-related plasticity for the formation of engram-to-be cells."

Discussion: I was surprised to see that the authors did not discuss the relevance of their neural network model of engram cells in relation to recent studies (e.g., a couple of papers from the Claudia Clopath lab). This should be included to highlight the unique features and potential similarities of the different engram models (including this study's), which is very important for the field moving forward.

We appreciate the reference to the related modeling works. In the revised manuscript, we cited several articles from Clopath lab (Delamare et al., 2023; Tome et al., 2024; Delamare et al., 2024) and discussed the relationship with our model. Briefly, the offline learning mechanisms (synaptic downregulation and scaling during offline periods) in our model are different from those in the previous models (the modulation of neuronal excitability and plasticity of inhibitory circuits) although functions may be partly similar (e.g. potentiation of inhibitory synapses and downregulation of excitatory synapses). These different mechanisms may work independently or cooperatively, or different mechanisms may correspond to the difference of brain regions. Importantly, the emergence of engram-to-be cells has not been reported in the previous models because those models do not have offline mechanisms to actively recruit non-engram cells for next memory formation. This difference highlights the novelty of our model that actively creates new cell assemblies during sleep.

Page: 21-22

"Recently, neural network models have been proposed for the formation and the drift of memory engrams in hippocampus46,67,68. Those models employed a recurrent network model, the modulation of neuronal excitability and plasticity of inhibitory circuits, by which the model successfully explained how common and specific engrams emerge and evolve during learning and offline periods. However, the emergence of engram-to-be cells has not been reported in the previous models because those models do not have offline mechanisms to actively recruit non-engram cells for next memory formation. Furthermore, the offline learning mechanisms (synaptic downregulation and scaling during offline periods) in our model are different from those in the previous models although functions may be partly similar (e.g. potentiation of inhibitory synapses and downregulation of excitatory synapses). These multiple mechanisms may work independently or

cooperatively. Otherwise, different mechanisms may correspond to the difference of brain regions such as dentate gyrus46 and CA1 (our model)."

Reviewer #2 (Remarks to the Author):

This manuscript by Ghandour and colleagues sought to ask how ensembles of neurons are selected to be recruited to represent subsequent experiences encoded across time. To do this, the authors combined in vivo calcium imaging with activity-dependent ensemble tagging to investigate this question. They placed mice in a novel environment (A) with a pre- and post-recording in their homecage around the experience. Sleep was recorded during the pre- and post-recordings. The following day, mice were re-exposed to context A as a form of memory retrieval, and then exposed to a novel context B for the formation of a new memory. The authors recorded calcium dynamics in hippocampal CA1 neurons throughout all stages of the experiment, and also recorded sleep (via EEG & EMG) during the pre- and post-recordings on Day 1. The authors also used a technology to label cFos-expressing ensembles among their recorded neurons using a Dox off system that requires delivery of 365nm light for engram cells to turn from green to red and be identified in the recorded field-of-view. The pre-recording revealed that ensembles of cells were active prior to an experience which would then go on to be active during learning, reactivated offline, and reactivated during retrieval to stably represent that memory long-term. These results are consistent with past reports of both preplay and replay in the hippocampus. Next, the authors questioned how these cells representing one experience can give way to a new ensemble of cells that are pre-configured to be active during a subsequent novel experience (in context B). They found that during the post-recording after context A exposure, new cells became active which would go on to represent the novel context B the following day ("Engram-to-be" cells). The authors claim that this could be a mechanism by which cells are selected for subsequent novel experiences. The authors then investigated how networks of neurons could use plasticity rules to drive this change in ensemble dynamics, so they developed a neural network model of the hippocampus. They replicated their empirical result in the model that ensembles of hippocampal cells emerged during the post-learning period after context A exposure, and they then manipulated synaptic plasticity during sleep in the model to ask whether synaptic plasticity during post-learning sleep is necessary for the emergence of this new preconfigured ensemble. The authors conclude that synaptic plasticity is required for the emergence of preconfigured ensembles that go on to represent novel experiences.

The manuscript is interesting and timely, considering it is of great interest in the field how memories encoded across time are integrated and separated. In particular, replay and preplay are two phenomena that have been observed in the hippocampus, and it remains unclear how cells active around an experience go on to represent the memory for that

experience versus other memories across time. The authors present a mechanism by which cells could be selected for subsequent experiences, which could have strong implications for the study of how multiple memories are formed and stored in the brain. This makes this manuscript novel and a worthwhile contribution to the field. However, there are significant conceptual concerns that detract from my enthusiasm for the manuscript. I highlight the major and minor points below:

We thank the reviewer for the thorough review, which has helped us improve the quality of the manuscript. We have taken all the comments into consideration while revising the paper, as detailed below.

Major Points

- There is a lack of consistency of the readout of each analysis and the rationale for using each analysis. For example, the authors switch between an ensemble overlap measure, correlations, sum of correlations, population vector distance, etc. without justifying why one measure is better than another, especially since they are all measures of representational similarity. I would appreciate if the authors could provide the results of the analyses with one method of measuring ensemble similarity. If the results are difficult to interpret altogether, I would appreciate if the authors could speculate about the discrepancies between the results.

We appreciate the reviewer for highlighting this important point.

- Providing the results of the analyses with one method (NMF) for engram-to-be:

We have additionally performed the NMF analysis on the engram-to-be cells during post-learning sleep (Suppl Fig.8E). In line with our hypothesis, engram-to-be cells showed significantly higher matching scores across sleep sessions. This result supports the conclusions drawn from the population vector distance (PVD) analysis (Fig. 3F).

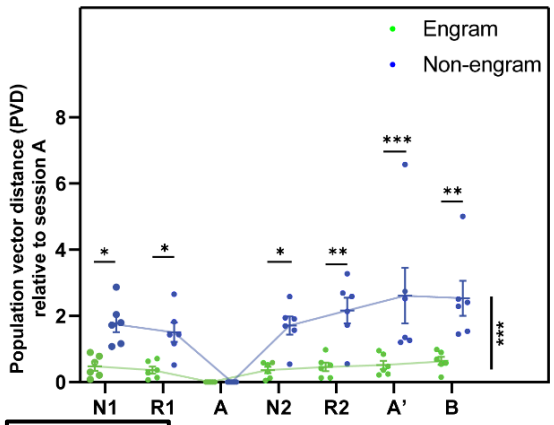
We have added the following statement on the non-negative matrix factorization (NMF) for engram-to-be (page 13).

The NMF analysis of engram-to-be cells during post-learning sleep revealed that, as expected, these cells exhibited higher matching scores across sleep sessions in reference to session B (Suppl Fig. 8E), suggesting that the engram-to-be ensembles were reactivated during subsequent sleep. These findings highlight the role of post-learning sleep in reinforcing and stabilizing engram-to-be cell activity, further supporting their involvement in memory processes.

- The rationale behind using different types of analyses:

The core findings of our engram and non-engram ensemble analysis are rooted in the NMF-based approach. Using NMF, we identified candidate ensembles of engram-to-be cells. To validate these engram-to-be cells, we additionally employed alternative analysis methods

(such as sum of correlations and population vector distance) to ensure the reliability of the results obtained by NMF.
In the revised manuscript, we have additionally performed PVD on engram cells (Suppl. Fig.4E). Engram cells show smaller distances than non-engram cells across sessions



Suppl. Fig. 4E

relative to session A, indicating that their activity remained stable both before and after learning. This further reinforces the conclusions of our NMF analysis (Fig. 1D). Thus, both analytical methods of measuring ensemble similarity led to the same conclusion.

Moreover, both engram and engram-to-be cells show a higher sum of correlations for their representative sessions A (Suppl. Fig. 2G) and B (Fig.3D), respectively.

- We have added an explanation in the revised manuscript about the use of each analysis and its rationale, and the alternative methods used for validation are added to the supplementary figures to keep the consistency of the read-out.

Population Vector Distance for engram cells (page 7).
"To further support the results, we have employed the population vector distance (PVD) to track the activity of the engram and non-engram ensembles across sessions (Suppl. Fig.4E). Engram cells show smaller distances across sessions in reference to session A, showing that the activity of engram cells remained stable and similar before and after learning. In contrast, the activity of non-engram cells was more variable overall, with a decreased similarity in response to the learning session (A). This result further supports the conclusions driven from the NMF analysis."
Sum of Correlations engram-to-be (page 10).
"Next, we calculated the correlation repetition analysis for the engram-to-be cells compared with the other nonengram cells, to check if they have the same feature of engram cells during learning."

- While it is interesting that ensembles of cells emerge during post-experience sleep periods, it is unclear whether this is a direct result of experience or whether the passage of time could explain this result. One possible control experiment the authors may consider is repeating this experimental procedure with homecage recordings on day 1 in the absence of context A exposure, followed by exposure to contexts on the following day. This way, the "engram-to-be cells" can be defined purely based on homecage activity (for example, during an awake state). Similarly, I wonder whether the engram-to-be cells might be influenced by the presence of context A recall just prior to exposure to B. While I do not think this warrants another experimental group, some speculation in the discussion would be useful.

We appreciate the reviewer for highlighting the key factor behind the emergence of ensembles. The reviewer suggested considering whether the emergence of engram-to-be cells depends on the context A exposure or not and whether engram-to-be cells are influenced by context A recall.

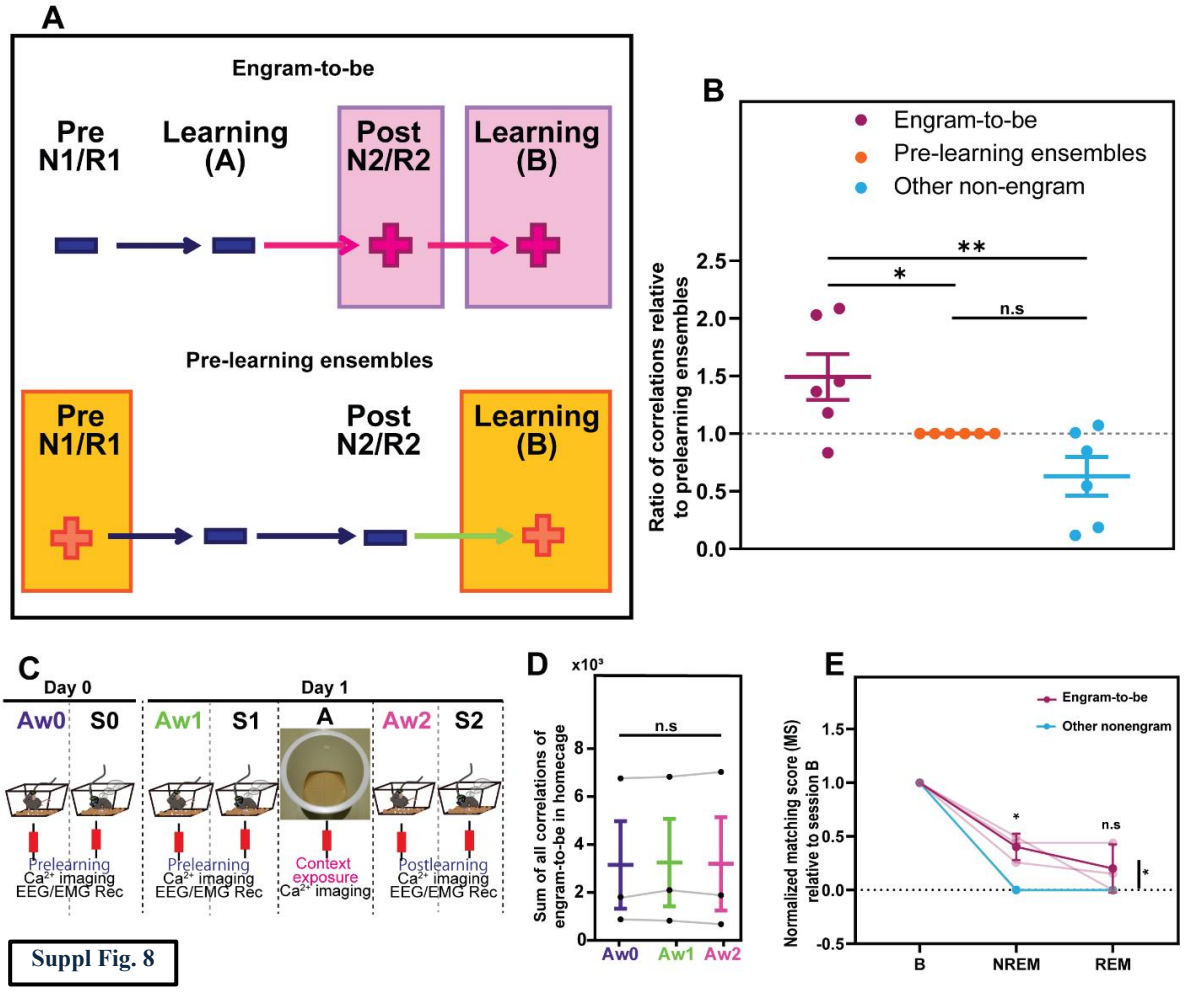
1. Time- or experience-dependency of the emergence of the engram-to-be cells:

To assess the necessity of the postlearning sleep (N2 and R2) ensembles activation, we have picked up cells constituting ensemble patterns that are active during pre-learning sleep and session B, but not in N2 and R2 sleep, which were termed as pre-learning ensembles (Suppl. Fig. 8A). Then, we compared the activity of engram-to-be and pre-learning cells during session B. Correlated activity of engram-to-be cells were significantly higher than the pre-learning cells (Suppl. Fig. 8B). Since the preN1/R1 and postN2/R2 sleeps were recordings from the same day with learning A in between, this result indicates that the difference in engram-to-be and pre-learning ensembles in learning B is due to the experience (learning A). This further denotes the importance of the post experience sleep activation for the engram-to-be preparation and next learning. In accordance with our argument, post-experience sleep, rather than sleep in general, is correlated with new ensembles emergence.

In addition, we have analyzed an awake home cage activity of engram-to-be cells one day before (Day0; Aw0), immediately before (Day1; Aw1), and after (Day1; Aw2) the learning. Those three time points track engram-to-be awake home cage activity as a function of time with and without learning experience (A). In accordance with our argument, engram-to-be cells show comparable activity across the three time points in awake (Supp. Fig.8C, D), which were significantly lower than that in learning B. This suggests that awake activity of engram-to-be cells remained unaffected by the learning experience. Thus, passage of time alone or an exposure to an experience without sleep cannot explain emergence of engram-to-be ensembles and post-experience sleep might play a key factor in their emergence.

Finally, in the model in Fig 5, the simulation, which assumes the experience-dependent formation of engram circuits in CA3-CA1, results in the generation of engram-to-be cells. This further suggests strongly the experience-dependent emergence of engram-to-be.

We have added these new analyses as Suppl. Fig. 8A-D and explanation to the revised manuscript. (page 11-12).



Suppl Fig. 8

2. Regarding the second point, whether the engram-to-be cells might be influenced by the presence of context A recall just prior to exposure to B.

We have calculated the co-occurrence in retrieval session A' and found that the co-occurrence between common engram and engram-to-be cells was not significantly different from the co-occurrence between specific engram and engram-to-be cells (Fig.4C). This suggests that retrieval of previous experience does not affect the emergence of engram-to-be cells.

We have added this point in page 13-14.

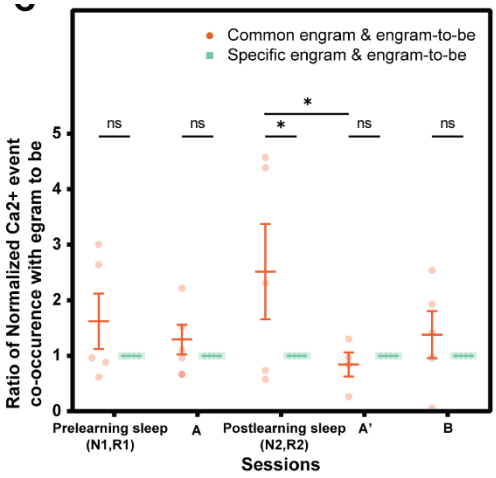


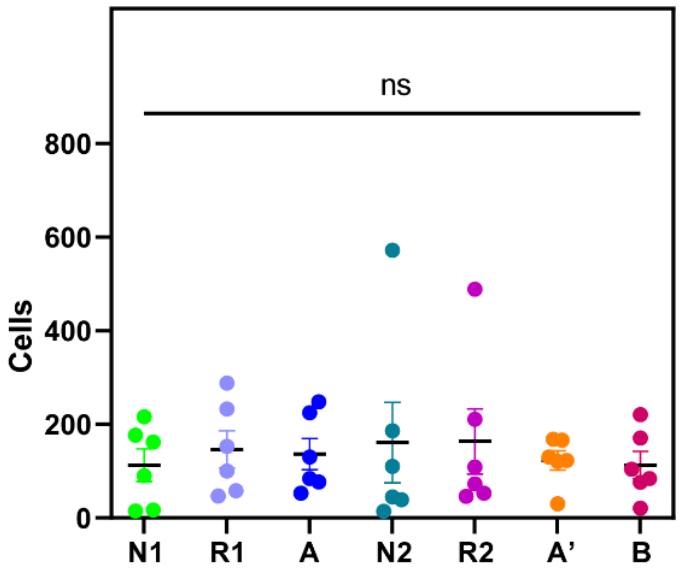
Fig. 4C

- On page 31, the authors describe that sleep recordings were done for 1-4 minutes, which is quite a short period of time, and past reports have demonstrated that ensemble reactivation varies as a function of time after an experience (e.g., Giri et al., 2019). First, it would be useful if the authors could provide cell counts across their sessions. In typical calcium imaging recordings in hippocampus, one-minute recordings are incapable of detecting calcium transients from substantially large ensembles of cells. Additionally, since the timing of the sleep can be variable relative to experience and the activity dynamics could be variable within sleep states, it is unclear if a one-minute recording will allow for a representative sample. Would it be possible to perform continuous calcium imaging recordings for tens of minutes?

We thank the reviewer for raising this important point. Sleep recordings were primarily conducted during the first occurrences of NREM and REM within the first hour of sleep, as our previous reports from both CA1 (Ghandour et al., Nat Commun 2019) and ACC (Abdou et al., Nat Commun 2024). This was due to technical limitations related to total recording time including pre-learning sleep, learning, post-learning sleep, and new learning etc., as we aimed to avoid photobleaching. However, to ensure an adequate number of cells are detected during sleep sessions, we performed cell activity counts for all sessions. Both sleep and awake sessions showed comparable numbers of detected cells across mice (as shown below). You can find sample videos of sleep recordings during both NREM and REM here: Sample Videos (please click). We have added this data as Suppl. Fig. 2H in the revised manuscript.

Additionally, we would like to clarify that each sleep stage recording lasted at least 1-2 minutes, and cumulatively, we recorded more than 12 minutes over several days. We have also attempted to extend the recording to 10 minutes for each sleep session, but this led to significant photobleaching. Furthermore, towards the end of these longer recording sessions, we observed seizure-like neural activities in the field of view, which may have been caused by phototoxicity. You can view a sample of these extended recordings here: Extended Videos (please click).

We have added the following statement in page 5.
We have confirmed that sleep recorded sessions show a comparable detected number of cells to awake sessions (Suppl. Fig. 2H).



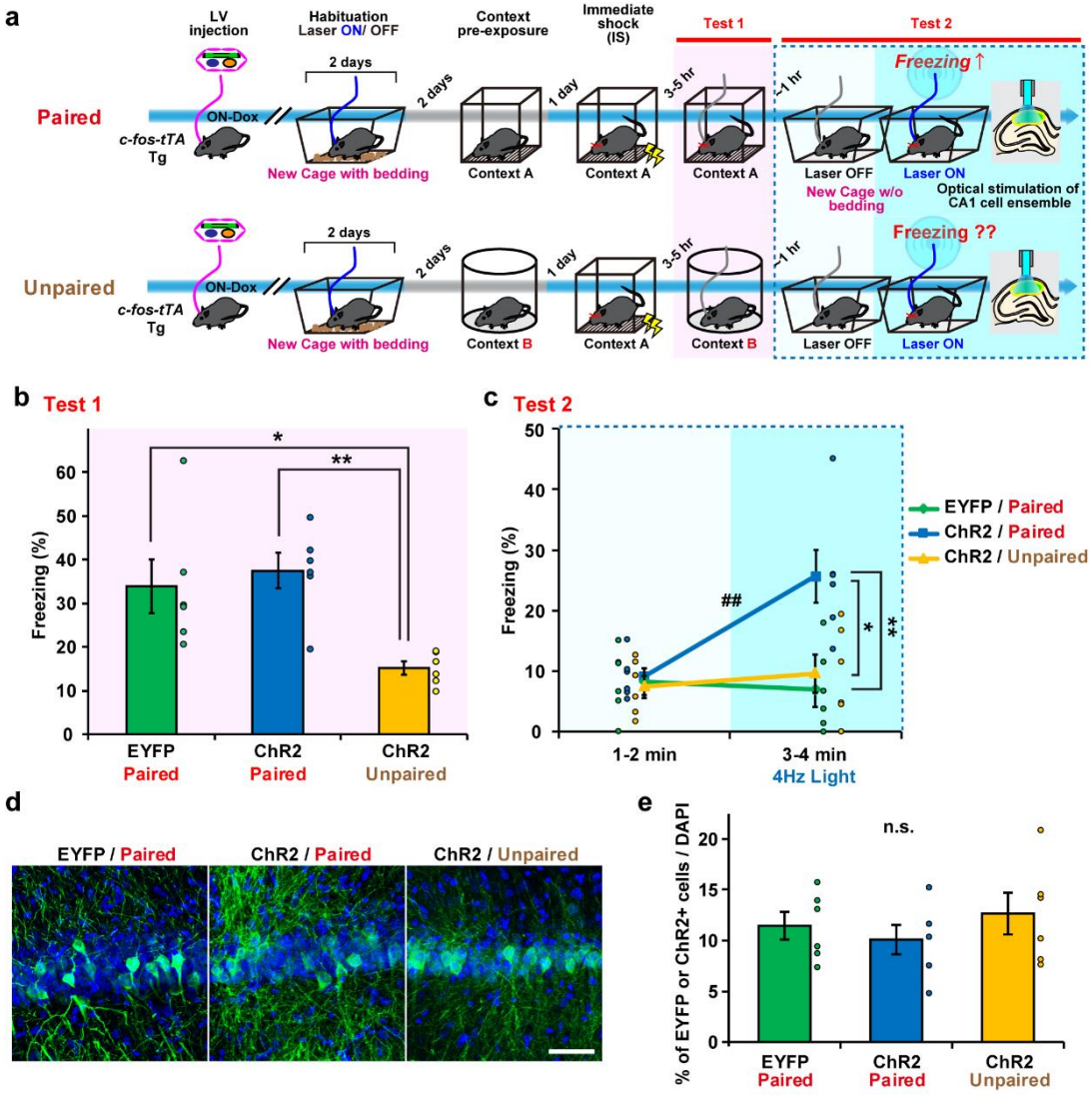
Suppl. Fig. 2H

- This manuscript makes a claim about the formation of memories through ensemble reactivation around an experience; however, there is no direct measure of memory behaviorally, making it difficult to know whether the mice are retaining a stable memory of the context, and perceiving context A as different from context B. I do not suggest that the authors re-run the entire experiment using a behavioral paradigm that has a behavioral readout; however, the current manuscript makes a claim about memory where the evidence for a stably formed memory is not strong. Therefore, I believe that the authors should soften their claims about whether these homecage ensemble dynamics are directly relevant to the formation of memories and their neural representations. If the authors decide to make a claim about memory, stronger evidence of the retainment of a memory would be needed.
We had already made sure in our previous study that these cells indeed represent the contextual memory. Activating these cells can retain the original contextual memory (see panel below) (Figure 1 in Ghandour et al., Nature Communications, 2019; figure below). "Context preexposure facilitation effect (CPFE) paradigm was used to investigate contextual memory per se. Mice explored a novel context under Dox removal (OFF Dox), during which the context engram was labelled. One day later, they received a foot shock in the same context (paired) or a different context (unpaired) during ON Dox, which prevented any additional labelling of cells associated with the shock information. In this paradigm, mice can associate context A (the pre-exposure context) with a temporally

separated immediate shock (IS) delivered in the same context. Therefore, re-exposure of the mice to the original context induces freezing behavior in the paired, but not the unpaired, group. This result clearly demonstrates that pre-exposed context information is associated with fear information only in the paired group. Later on, optogenetic activation at 4 Hz of the CA1- labelled neurons in a neutral context retrieved the fear memory in the ChR2 paired group, but not in the ChR2 unpaired or the EYFP group"

We have added an explanation to the revised manuscript.

Page 5: "We previously showed that cells labeled by this system represent contextual engram cells (Ghandour et al., Nature Communications, 2019).



- The model that was developed to manipulate synaptic downscaling is an interesting expansion upon the original experiment, but there is no empirical evidence that synaptic downscaling is a mechanism that drives the emergence of an engram-to-be ensemble in a post-learning period following an experience. The authors should emphasize that these results are purely in silico and do not necessarily reflect the biological in vivo condition. Future results will be necessary to disentangle the contributions of synaptic plasticity in driving the recruitment of new cells for encoding future events.

We agree with the reviewer, and we have indicated in the revised manuscript that these results were done only in silico and further biological evidence is required to support these results.

Page 21: "As all the model manipulations were performed solely in silico, additional biological evidences are needed to validate these findings."

Minor Points

- In lines 49-50, the authors claim that how "these engram cells behave in periods prior to learning and how they affect future learning, is yet to be studied." However, significant work has been done on this topic. For example, Dragoi & Tonegawa, 2011 demonstrated that ensembles are active prior to learning of an experience (termed preplay); Farooq & Dragoi, 2019 demonstrated that these preconfigured ensembles emerge in development; and Mocle et al., 2024 demonstrated how excitability plays a role in the development of preconfigured ensembles. I would appreciate if the authors could describe in further detail what has come before to set up why this study is an important contribution above and beyond what has already been done in past years.

We have modified the introductory part in the revised manuscript to include more previous work and emphasize the contribution of our study to the existing literature.

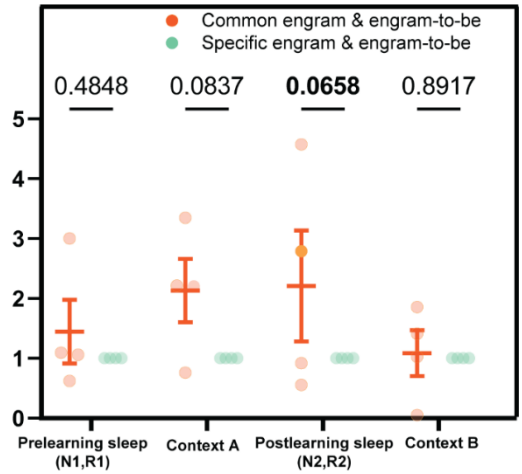
Page 3

"Moreover, hippocampal intrinsic dynamics form a repertoire of preexisting assemblies of high temporal sequential events ahead of time (known as preplay) (Dragoi et al, 2011, McKenzie et al., 2021, Valero et al., 2022), forming a network of preconfiguration (Dragoi. Et al 2011, Valero et al., 2022). Coding principles in neuronal networks might reflect selection of remarkably excitable neurons of properly connected cells that share the same birthdate and emerge together during development (Farooq et al., 2019). However, how engram cell dynamics orchestrate during offline pre- and postlearning stages and affect future learning and upcoming future engram cells is not well understood."

- On line 705, the authors state that calcium activities during sleep were recorded as

previously described, but there is no citation for where this information is previously described.
We thank the reviewer for pointing this out, we have added the reference to this sentence.

- In Figure 4B, why did the authors decide to change the definitions of the engram cells to not comprise cells active during context A retrieval? In Figure 2A, the ensembles are defined by their activity during learning and reactivation during retrieval. This is also consistent with past reports of ensemble reactivation (e.g., Reijmers et al., 2007; Cai et al., 2016). Do the results in Figure 4C-F change if the cells must be reactivated during retrieval of context A to be part of the context A engram?
We would like to emphasize that, throughout this paper, engram cells are defined as cells that are c-fos positive in learning session A (including Figures 2A and 4B). The basis for this, as explained in response to the major point, is that previous research shows that optogenetic activation of cells positive for learning context A enables mice to recall the memory of context A (Ghandour et al, 2019). In Fig 4B, engram cells are also defined according to this principle. The only exception in this paper is Suppl Fig. 6D, E, where engram cells are defined as cells positive for both A and A'. This stricter definition was used to demonstrate the validity of defining engram-to-be cells.
However, for caution, we also followed reviewer's advice and recalculated the co-occurrences between the common engram & engram-to-be cells and the specific engram & engram-to-be cells, taking into account the ensembles active during the retrieval session (A'). Common engram ensembles are defined as those active in sessions N1/R1, A, N2/R2, A', and B, while specific engram ensembles are active in sessions N1/R1, A, N2/R2, and A' only. By adding this additional criterion, one of the mice showed zero ensembles for the specific engram and was excluded from the analysis, even though, engram ensembles appeared in A and A' in all mice. The remaining four mice showed similar activity levels in before and after including the retrieval session (please see below panel). Individual data for N2/R2 session: Mouse ID#1 before (2.30) and after (2.78), ID#2 before (4.57) and after (4.57), ID#3 before (0.57) and after (0.55), ID#4 before (0.73) and after (0.91). Although statistical significance was lost in post-learning sleep, the trend remains. This change in significance is due to decreasing sample size because of the additional criterion.



- In Figure 4A, on the right side, the representative schematic of ensemble co-activities does not seem to add up, unless I am reading it wrong. For example, in the first column, there are no "Common Engram" cells active; however, in the summed up values below, it says that there is 1 cell active from this ensemble. In the second column, one "Common Engram" cell seems to be active, but the summed up values say that there are 0. If I am misreading this schematic, I would appreciate if the authors could write a clearer explanation of the analysis.

We thank the reviewer for pointing this mistake, we have corrected the schematic diagram in the revised manuscript to match the active cells and summed up values.

Reviewer #3 (Remarks to the Author):

Ghandour, Haga, Ohkawa et al. investigated the role of sleep in the recruitment of engram cells. They provided compelling evidence that learning-induced patterns of activity are highly stereotypical across memory stages (i.e., pre- and post-learning sleep as well as memory retrieval), pointing to neural activity prior to and following learning being predictive of engram recruitment and memory consolidation, respectively. They also found that the activity of cells not allocated to a first memory engram is reorganized during post-learning sleep such that a subset of these cells is recruited to a second memory engram. In addition, they proposed a computational model that suggested that synaptic plasticity during post-learning sleep can capture key features of their experimental data. Together, their results shed light on the interplay between sleep and memory allocation, complementing previous studies that have focused on the role of intrinsic excitability in memory recruitment (Zhou et al., Nat Neuro, 2009; Yiu, Mercaldo et al., Neuron, 2014).

We thank the reviewer for the thorough review, which has helped us improve the quality of the manuscript. We have taken all the comments into consideration while revising the paper, as detailed below.

Major Points

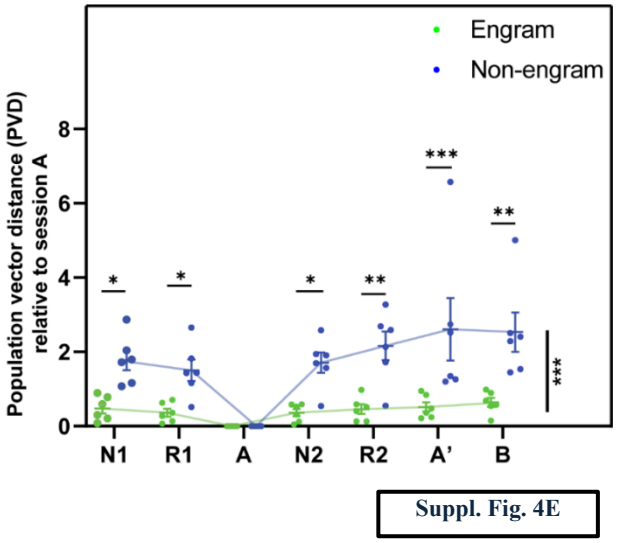
1. The key findings of the paper rely on identifying ensembles using an NMF-based approach outlined in the manuscript. Could you use an alternative method to identify ensembles in each session (e.g., an alternative unsupervised clustering technique, or a supervised approach contrasting cell activity in A' and B on Day 2) and perform the same analyses to verify that the key results of the paper also hold in this case (e.g., ensembles activated during prelearning sleep are allocated to a subsequent engram)? This would considerably increase the robustness of the results.

Based on the reviewer's request to increase robustness of the results by using another analytical method, we have calculated the eigenvectors and their corresponding eigenvalues across two distinct sessions in engram and nonengram cells, to identify dominant patterns or modes of activity in each session. Then, we employed the restricted Mahalanobis distance, in which if the distances between sessions are small, then members of clusters are close/similar to each other. The population vector distance (PVD) tracks the activity of the engram and non-engram ensembles across sessions. Restricted PVD ensures a fair comparison between populations of different sizes (engram and non-engram). Engram cells show smaller distances across sessions in reference to session A, showing that the activity of engram cells remained stable and similar before and after learning. In contrast, the activity of non-engram cells was more variable overall, with a decreased similarity to session (A).

Moreover, we have attempted to apply clustering methods, including k-means and PCA-ICA, to identify potential ensembles within the engram cells. However, these methods were ineffective in detecting multiple distinct ensembles. This limitation primarily stems from the low number of engram cells, which significantly reduces the statistical power of clustering algorithms, as these methods often struggle to handle small, underrepresented groups effectively.

NMF is a decomposition method rather than a clustering method. Clustering methods (e.g. k-means) assign the instantaneous co-firing into a pattern. However, NMF allows the occasional co-activation of patterns in the same time bins (some overlapping is allowed). It

makes NMF a unique method for detecting ensembles without over-categorization. Therefore, the usual clustering method cannot be a direct substitute for NMF.
Instead, PVD is added as it offers a robust way to focus on population-level dynamics and quantify meaningful similarities across sessions without being biased by population size differences.
We have added the data to Suppl. Fig.4 panel E and added the following statement in the revised manuscript.
Page 7: "To further support the results, we have employed the population vector distance (PVD) to track the activity of the engram and non-engram ensembles across sessions. Engram cells show smaller distances across sessions in reference to session A, showing that the activity of engram cells remained stable and similar before and after learning. In contrast, the activity of non-engram cells was more variable overall, with a decreased similarity in response to the learning session (A). This result further supports the conclusions driven from the NMF analysis."



Suppl. Fig. 4E

2. There has been considerable evidence that memory engrams are dynamic, with cells dropping into and out of engrams following initial learning (Tomé, Zhang et al., Nat Neuro, 2024; DeNardo et al., Nat Neuro, 2019). Fig. 2B-C seems to suggest that your data also support a dynamic memory engram (e.g., stand-by and isolated patterns could indicate cells dropping out of the engram).

2.1. Could you quantify the fraction of cells dropping out of the engram (i.e., activated in learning A but not activated during retrieval A') and the fraction of cells dropping into the engram (i.e., not activated in learning A but activated during retrieval A')?
2.2. Do cells that drop into and out of the engram show distinctive activity patterns during

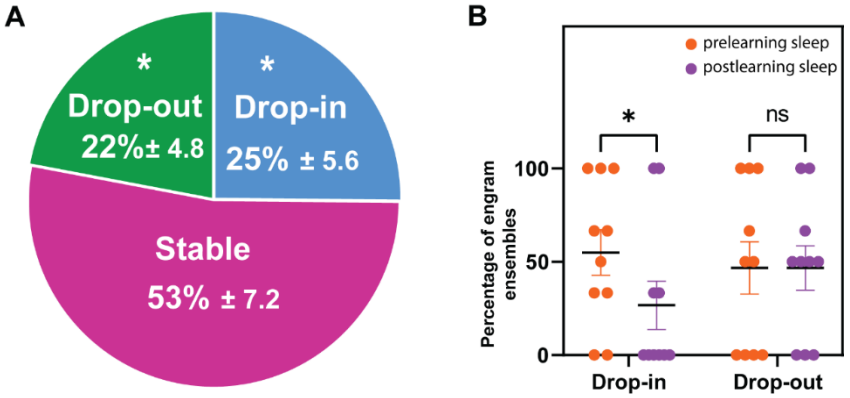
pre- and postlearning sleep? For instance, are cells that drop into the engram not activated during pre- but activated during postlearning sleep? Are cells that drop out of the engram not activated during postlearning sleep?

2.1. We appreciate the reviewer for raising this intriguing point. As requested, we calculated the number of drop-in and drop-out engram ensembles based on their activity in sessions A and A'. Interestingly, the number of drop-in and drop-out ensembles were quite similar, comprising 25% and 22% of the total, respectively. Stable ensembles, which were active in both sessions A and A', accounted for approximately 53% of the total engram population. This suggests that while sub-populations within the engram population exhibit dynamic activity, the overall engram population remains relatively stable.

2.2. We then tracked the activity of these identified drop-in and drop-out ensembles across sleep stages (pre- and post-learning sleep). Contrary to our expectations, the drop-in ensembles showed higher activity during pre-learning sleep sessions, rather than post-learning sleep. Meanwhile, the drop-out ensembles displayed no significant differences in activity between the two sleep sessions.

We have added these 2 panels as supplementary figure 5 and this explanation in the revised manuscript.

Page 8-9: "Recent studies have supported the concept of dynamic memory engram (Tome et al., 2024, Denardo et al., 2019), as suggested by previous research. Building on this idea, we explored the dynamism of the engram by calculating the fraction of drop-in and drop-out engram ensembles. Drop-in ensembles refer to those not activated during the learning session (A) but become active during the retrieval session (A'), whereas drop-out ensembles are active during learning but not during retrieval. Stable ensembles, in contrast, are those activated in both learning and retrieval sessions. Our findings revealed that drop-in and drop-out ensembles each constituted approximately 25% of the total engram population, while stable ensembles accounted for roughly 50% (Supp. Fig. 5A). This suggests that while individual ensembles may vary, majority of the engram population remains consistent, with a similar proportion of ensembles dropping in and out. Additionally, we



Suppl. Fig. 5

tracked the activity of these ensembles during both prelearning and postlearning sleep. Drop-in ensembles displayed higher activity during prelearning sleep compared to postlearning sleep, whereas drop-out ensembles exhibited similar activity levels across both sleep sessions (Supp. Fig. 5B)."

Minor Points

3. Suppl. Fig. 1B: The y-axis needs a label.

We have fixed the plot.

4. Line 100-101: Specify the measure of dispersion (i.e., standard deviation, standard error of the mean?) in 7% (+- 1.5) and 93% (+- 1.5).

We have added the measure of dispersion (standard error)

5. Suppl. Fig. 2G: Caption is unclear.

We have modified the caption to be clearer.

6. Reference 42: Missing year.

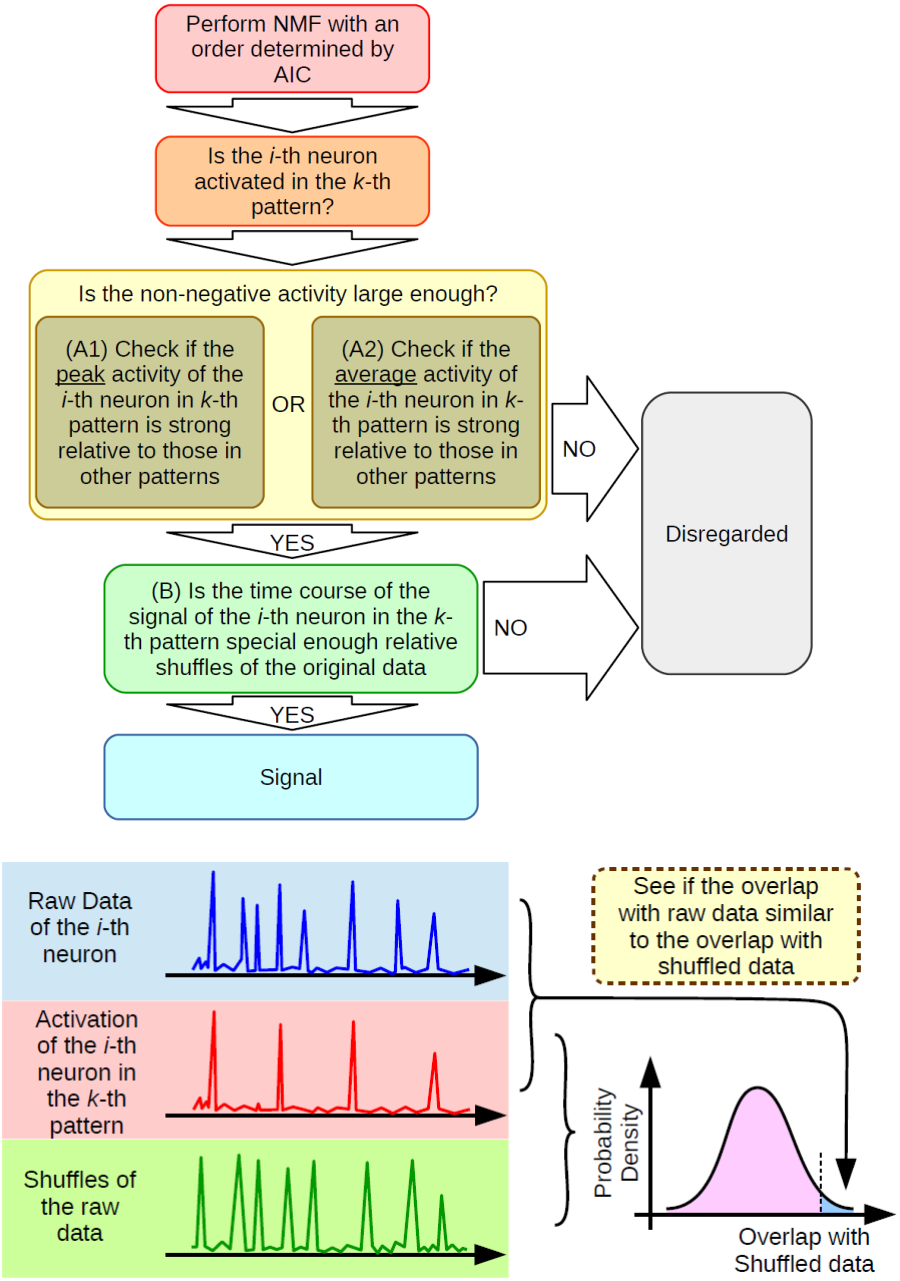
We have fixed the reference.

7. Line 111-113: Can a given cell have a high participation in multiple ensembles identified by NMF? This seems to be the case based on the schematic in Suppl. Fig. 3B, but this needs to be stated in the main text. If this is not the case, this should also be stated in the main text and the schematic in Suppl. Fig. 3B needs to be corrected.

A given cell can be a part of more than one ensemble depending on its activity and the timing of that activity. In simple terms, neurons are identified as part of a given ensemble if their activity in that ensemble is stronger compared to their activity in the other ensembles. If not, they are excluded. Additionally, if the timing of the neuron's activity in that ensemble is significantly different from shuffled data, it will be included as part of the ensemble. If a neuron does not meet these criteria, it is discarded. (please see the panels below for further illustration).

We have added this explanation in the revised manuscript and the methods section, cell ensemble analysis, NMF.

Page 6: Main text: "A given cell can be identified in several ensembles based on their activity and time course of its activity compared to shuffled data."



Page 45: Methods section: "Neuron (i) identified in an ensemble (k) if the i-th neuron in k-th' ensemble peak activity or average activity is stronger relative to those in other patterns, if not it would be discarded. Then, if the time course of the i-th neuron in the k-th ensemble significant enough compared to shuffles of the original data, will be considered as a participating neuron in the ensemble. If the i-th neuron did not pass any of these filters, it would be discarded."

8. Line 117-119: Why was 0.6 considered a “weak threshold”? This needs further elaboration.
We apologize for the unclear illustration. We have compared activities of different thresholds from 0 till 0.9 (Supp. Figure 3C), below 0.6 seems quite low and couldn’t detect differential reactivations across sessions. We have added an explanation in the revised manuscript.
Page 6-7: “We calculated the similarities between session A and other sessions using different thresholds (ranging from 0.1 to 0.9) (Supp. Fig. 3C). Thresholds below 0.6 displayed comparable activity across all sessions, which appeared less stringent, while thresholds above 0.6 maintained similar trends (Supp. Fig. 3C).”

9. Suppl. Fig. 3C: The plot and caption are unclear. The legend of the plot is hard to understand. MSs should be spelled out in the caption. Overall, the plot and caption need to be modified so the reader can understand them.
We have modified the legend and the plot and spelled the matching scores (MSs) to be easily understood by the readers.

10. Line 119-120: The authors claim that “Comparable results were obtained regardless of higher thresholds” but this needs to be backed up by a proper analysis with different thresholds.
We apologize again for the unclear illustration. Given the importance of this comment we added also more explanation to the revised manuscript. Same as comment 8 and 9.
Page 6: “We calculated the similarities between session A and other sessions using different thresholds (ranging from 0.1 to 0.9) (Supp. Fig. 3C). Thresholds below 0.6 displayed comparable activity across all sessions, which appeared less stringent, while thresholds above 0.6 maintained similar trends (Supp. Fig. 3C).”

11. Suppl. Fig. 3D-G: The y-axis label in the bottom plot of each panel reads “Overalp (All)” but the caption says “relative intensities across all the sessions”. Are these the same?
We have modified the plot to be the same as the caption.” Relative intensities across all sessions”.

12. Line 137-138: The following is unclear: “engram ensembles decreased significantly from the retrieval session (A’) to a different context (B)”. Do you mean the MS of the engram decreased?
We meant the discrimination ratio calculated from the MS. To make it clearer to the readers we have revised as follows.
Page 7: This is consistent with our previous finding that the high MS of engram cells was maintained in replay (N2 and R2) and retrieval (A’) sessions, and that MS discrimination

ratio of the engram ensembles decreased significantly from the retrieval session (A') to a different context (B; Suppl. Fig. 4B).

13. Suppl. Fig. 4B: Please specify in the caption the definition of discrimination index used in this analysis.
We have added the equation used to calculate the discrimination ratio in the caption of Suppl. Fig.4B.

14. Suppl. Fig. 5E: Shouldn't the denominator for calculating false negatives and true negatives be 5+6+7+8?
We thank the reviewer for noticing this mistake. For Supp. Fig. 5E (now Supp. Fig. 6E) we meant to calculate the total percentage of ensembles that are true negatives and false negative, that's why the denominator was different between the two groups. We have corrected the caption to be 2+4+6+8 for the true negative to be divided by all the true negative, while the false negative should be divided by all the true positives 1+3+5+7 ensembles.

15. Suppl. Fig. 6D: Missing measure of dispersion for UP in the pie chart.
We thank the reviewer for noticing this mistake. We have corrected the plot.

16. Suppl. Fig. 6E-F: Missing y-axis label.
We thank the reviewer for noticing this mistake. We have corrected the plot.

17. Suppl. Fig. 6C-F: Mismatching panel letters and the respective captions.
We thank the reviewer for noticing this mistake. We have corrected the plot.

18. Suppl. Fig. 8B: Missing x-axis label.
We thank the reviewer for noticing this mistake. We have corrected the plot.

19. Suppl. Fig. 8D: y-axis label should be neuron index.
We thank the reviewer for noticing this mistake. We have corrected the plot.

20. Fig. 4C: Are the individual data points for "Specific engram & engram-to-be" really so close to each other across all sessions?
The individual data points for the specific engram and engram-to-be are all normalized to one. This ratio comparison normalizes each common engram and engram-to-be to the corresponding specific engram and engram-to-be in that particular session. This adjustment helps account for differences between awake and sleep sessions.

21. Suppl. Fig. 10: Caption should be for B-C. Please also indicate in the caption the difference between the top, middle, and bottom plots in panels B-C (e.g., different samples?).
We thank the reviewer for noticing this mistake. We have corrected the plot.

22. Fig. 4: Captions of panels E and F are swapped.
We thank the reviewer for noticing this mistake. We have corrected the plot.

23. Line 271-272: Include reference to the learning rule.
We have included the following description.
Page 14: (see Methods "Simulation model and procedure" for details of the learning rule).

24. Line 895: The index on the left-hand side of the equation should be i and the upper index of the second summation on the right-hand side should be Ninh.
We thank the reviewer for pointing out the invalid expression. We have corrected the equation.

25. Line 896: Do inhibitory neurons in your model of CA1 receive feedforward (CA3) or recurrent (CA1) excitatory inputs? The notation on the right-hand side of the equation is not consistent with either case and needs to be adjusted.
We thank the reviewer for pointing out the misleading expression. Our model has recurrent inhibition in CA1 but we wrote invalid notation (N_CA3) by mistake. We have corrected the equation, and explicitly described that inhibition was recurrent feedback in CA1.

26. Line 902-903: Do you mean "[then] simulated time evolution of the dynamics for 20 ms"?
We thank the reviewer for noticing this mistake. We have modified the logical flow.

27. Line 919/925/927-928: Do the synaptic learning rule, the synaptic depression rule, and the synaptic scaling rules apply only to E-to-E weights from CA3 to CA1 excitatory neurons or also to E-to-I weights from CA3 to CA1 inhibitory neurons? Based on lines 910-911, it seems that they only apply to CA1 excitatory neurons. However, this should be made clear.
We thank the reviewer for pointing out the unclear point. We applied only E-to-E weights from CA3 to CA1. We explicitly explained this point in the Methods section (Simulation model and procedure) as follows.

Page 49: We fixed I-to-E and E-to-I weights throughout the simulation. We applied synaptic plasticity to only E-to-E synaptic weights w_{ij}^{EE}.

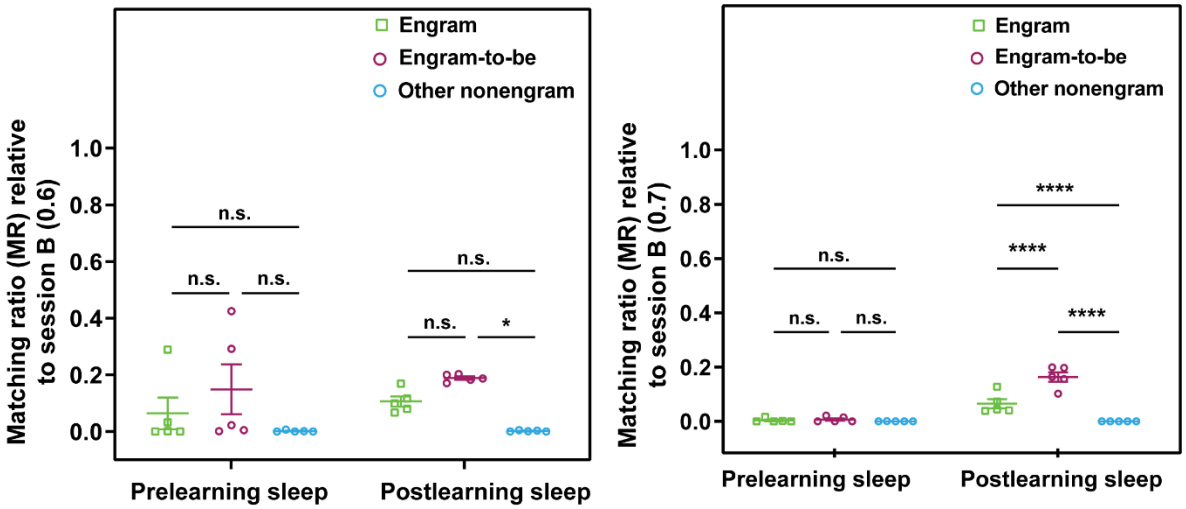
Furthermore, to avoid confusion, we corrected w_{ij}^{E} to w_{ij}^{EE} in synaptic plasticity rules in these equations.

28. Fig. 5D: Would the results hold if you compared engram vs. other nonengram and engram-to-be vs. other nonengram separately?

We attempted to separate engram cells from engram-to-be cells in Fig. 5D. However, when they were separated, the matching ratios (MR) in the pre-sleep sessions became unstable. In most trials, the MR during pre-sleep was near zero, with a few outlier trials showing higher MR (left figure). This instability might be due to the small number of engram cells involved.

These outliers disappeared when we increased the threshold of cosine similarity in MR (0.6 to 0.7) (right figure). This suggests that cosine similarity tends to be larger in the simulation of the model, especially when the size of an analyzed neural population is low. Although there was such a quantitative difference, results were qualitatively same between the experiment and the model even if engram and engram-to-be cells were separated.

In the revised manuscript, we added these results in Supplementary Figure 12 as panels C and D. In the text, we have added in page 15-16 “We obtained the qualitatively same result



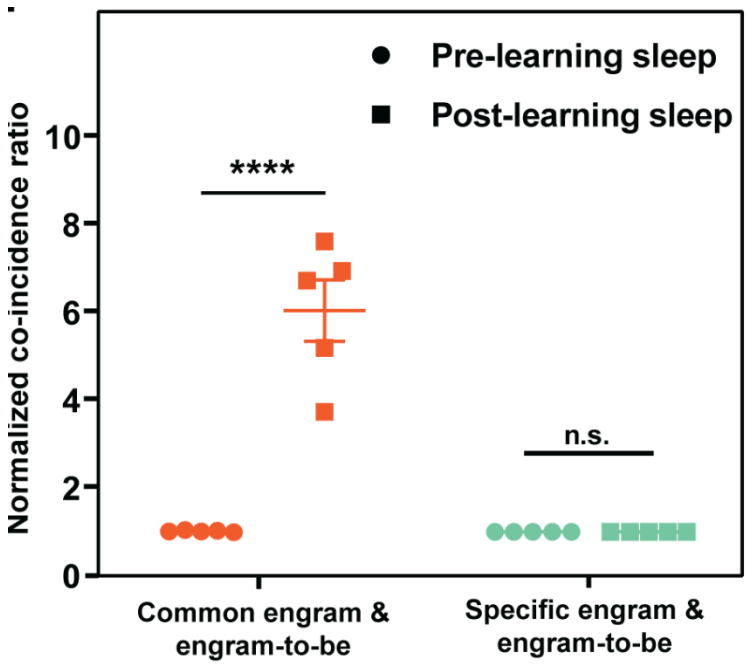
Suppl. Fig. 12 C&D

when we separately analyzed engram and engram-to-be cells, although the matching ratio became unstable due to limited population sizes (Suppl. Fig. 12C), repeating the same analysis using a higher threshold (0.7) showed same tendency (Suppl. Fig. 12D), further confirming that the new ensembles activated in context B emerged after the first learning

(context A). Furthermore, we also simulated a sleep session after context B and evaluated the matching between activity patterns in the context B and the following sleep. We confirmed that the matching of engram-to-be cells was higher than that of non-engram cells (Suppl. Fig. 12E), which corresponds to experimental results in Fig. 3F."

29. Line 303: Fix reference to Fig. 1E.
We thank the reviewer for noticing this mistake. We have fixed the reference.

30. Fig. 5F: Could you perform the same analysis for the other sessions (i.e., prelearning sleep, context A, and context B) and verify that the same conclusions hold similar to the experimental data in Fig. 4C?
We additionally analyzed only prelearning sleep but not context A and B because we calculated activity patterns in context A and B only once in our model (statistical analyses were not available in those periods). In the additional analyses, we found that our previous definition of the coincidence ratio results in unstable results because of its unnecessary complexity. Therefore, we simplified the definition of the coincidence ratio (see Methods in the revised manuscript) which did not largely alter the results shown in the previous manuscript. With simplified coincidence ratio, we confirmed that our model qualitatively reproduces the experimental result (the figure below).
In the revised manuscript, we added this result in Supplementary Figure 12. We have also



Suppl. Fig. 12F

added in text page 16, "We also confirmed that this difference between common engram

and specific engram emerged in postlearning sleep but not in prelearning sleep (Suppl. Fig. 12F), which qualitatively reproduces the experimental results in Fig. 4C."

31. Fig. 6C: Results of statistical tests are not shown in the plot, colors of legend do not match colors of the data points, and comparisons between each pair of groups (i.e., similar to Fig. 5) are missing (currently only comparing common engram and engram-to-be to every other group).
We thank the reviewer for noticing this mistake. We have corrected the plot and added statistical results on the plot and the comparisons between each pair of groups.

32. Suppl. Fig. 9: Panels B-E and G-J are not referenced in the main text, only in the methods (line 786).
We thank the reviewer for noticing this mistake. We have mentioned Suppl. Fig. 9 (now Suppl. Fig. 10) in the revised manuscript (page 13).

33. Line 29-31: Please clarify that the proposed model suggests a potential role of synaptic depression and scaling in the reorganization of the activity of nonengram cells.
We have revised the abstract by incorporating this suggestion. Furthermore, we have added the following in page 22.
"The proposed model suggests that synaptic depression and scaling play a key role in reorganizing the activity patterns of non-engram cells, allowing for flexibility and efficient network adaptation, essential for encoding new memories."

Reviewer #3 (Remarks on code availability):
The instructions to run their code are clear.
We appreciate the positive feedback from the reviewer.

Reviewer #4 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
We thank the reviewer for the thorough review, which has helped us improve the quality of the manuscript.

Reviewer #5 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and

to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for the thorough review, which has helped us improve the quality of the manuscript.

We thank you for handling our manuscript and the reviewers for their helpful and constructive comments. We have addressed all of the reviewers' comments, and the point-by-point responses to the comments are described below. In the revised manuscript, all corrections are indicated in blue. We hope that our revised manuscript is now suitable for publication in *Nature Communications*.

Reviewer #1 (Remarks to the Author):
The authors did a good job of addressing my comments thoroughly by updating the main manuscript text and providing several additional experimental/modeling results in the manuscript. This, in my opinion, has improved the manuscript. I have no further issues.
We thank the reviewer for his/her careful reading and efforts to increase the impact of the study.

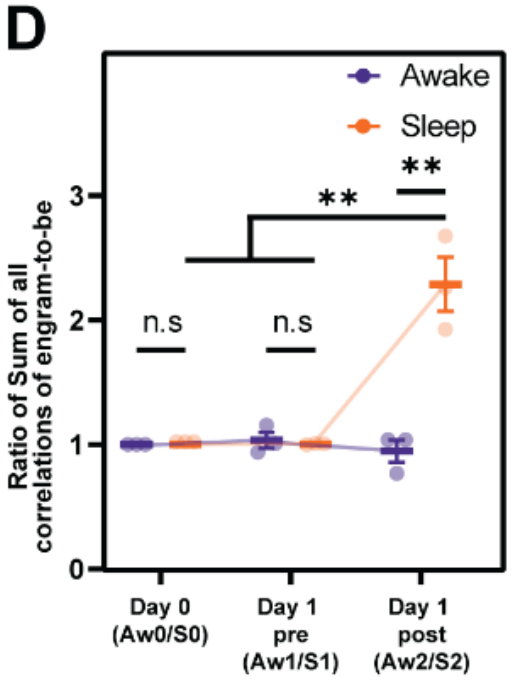
Reviewer #2 (Remarks to the Author):
I thank the authors for a thorough response to my suggestions for the manuscript, which have strengthened the claims that the authors originally concluded. I recommend the manuscript for publication provided the authors address the following minor concerns.
We thank the reviewer for his/her careful reading and efforts to increase the impact of the study.

Minor Points
1. I previously brought up a concern that the emergence of preconfigured ensembles during post-learning sleep may just be a result of the passage of time. The authors addressed this concern by performing an awake homecage recording on Day 0, Day 1 pre-learning, and Day 1 post-learning. The authors found that there was no difference in the activity of engram-to-be cells during these three awake time points (Supplemental Figure 8C). I agree that this lends support for the idea that awake cell activity is not modulated by time across days. However, I would like the authors to include a similar comparison for the cell activity during sleep at each of these time points (i.e., S0 vs S1 vs S2). If the activity of engram-to-be cells on Day 0 and Day 1 pre-learning is no different during sleep, while the activity of engram-to-be cells on Day 1 post-learning is different during sleep, I believe these results would effectively demonstrate that the sleep-specific changes in activity are not just a result of the passage of time.
We thank the reviewer for pointing out this important analysis. We have calculated the sum of correlations in S0, S1 and S2 in the same manner as Aw0, Aw1 and Aw2 as requested by the reviewer. As expected, only S2 shows higher correlations compared to both Aw2 and

other sleep (S0 and S1) sessions. We have added this data in the revised manuscript to Supp.Fig.8D

Page 11

“In addition, we have analyzed an awake and sleep home cage activity of engram-to-be cells one day before (Day0; Aw0 and S0), immediately before (Day1; Aw1 and S1), and after (Day1; Aw2 and S2) the learning. Those three time points track engram-to-be awake and sleep home cage activity as a function of time with and without learning experience (A). In accordance with our argument, engram-to-be cells show comparable activity across the three time points in awake sessions (Supp. Fig.8C, D), while only postlearning sleep (S2) exhibits higher correlations compared to both awake and other sleep sessions (S0 and S1). This suggests that the learning experience specifically influences the activity of engram-to-be cells during postlearning sleep, but not during wakefulness.”



Supp. Fig.8D

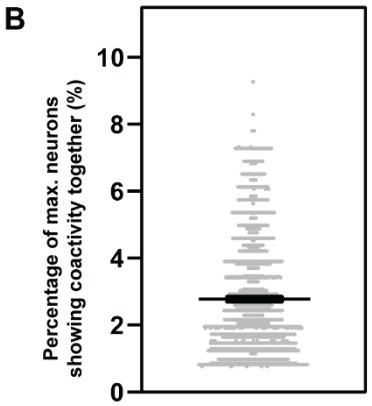
2. I recognize that the authors are unable to record calcium dynamics for longer than ~12 minutes out of concern of photobleaching. However, I find the seizure-like activity provided in supplemental video “Phototoxicity demo2.tif” to be concerning and would like the authors to confirm that this animal was excluded from the datasets analyzed in the manuscript. While the authors speculate that this activity may have arisen from phototoxicity, it is possible that this animal exhibited aberrant epileptiform activity, not driven by phototoxicity necessarily, but the longer duration of recording time allowed an opportunity for this underlying pathology to be observed. Since the main claims in this

manuscript depend on the “co-occurrence” of activity between neuronal ensembles, it is important to demonstrate that these results are not driven by aberrant epileptiform burst events during which large fractions of the recorded neurons are synchronously active (which would likely not represent the healthy neurotypical brain). To alleviate this concern, I would like the authors to compute the percent of cells that co-fire together across time (e.g., every calcium imaging frame), with the rationale that there should not be any timepoint where >50% cells are co-firing at one time (suggesting aberrant activity). If there are such frames that exist, it would be more difficult to interpret the results, considering the ensemble co-occurrence may be driven by seizure-like activity.

We appreciate the reviewer for highlighting this critical point. First, we confirm that any mice exhibiting such activity were excluded from the dataset and analysis. These mice were in the early stages of establishing our imaging protocol. Additionally, as per the reviewer’s request, we quantified the maximum number of neurons displaying coactivity within a single frame of all mice. Our analysis shows that no single frame exceeds 10% of the total population coactivity across all mice and recorded frames, with a mean coactivity of approximately 3% of the total population. This confirms that all recorded frames reflect normal activity, excluding any unhealthy phenomena. We have added this data to the revised manuscript as Supp. Fig. 11B.

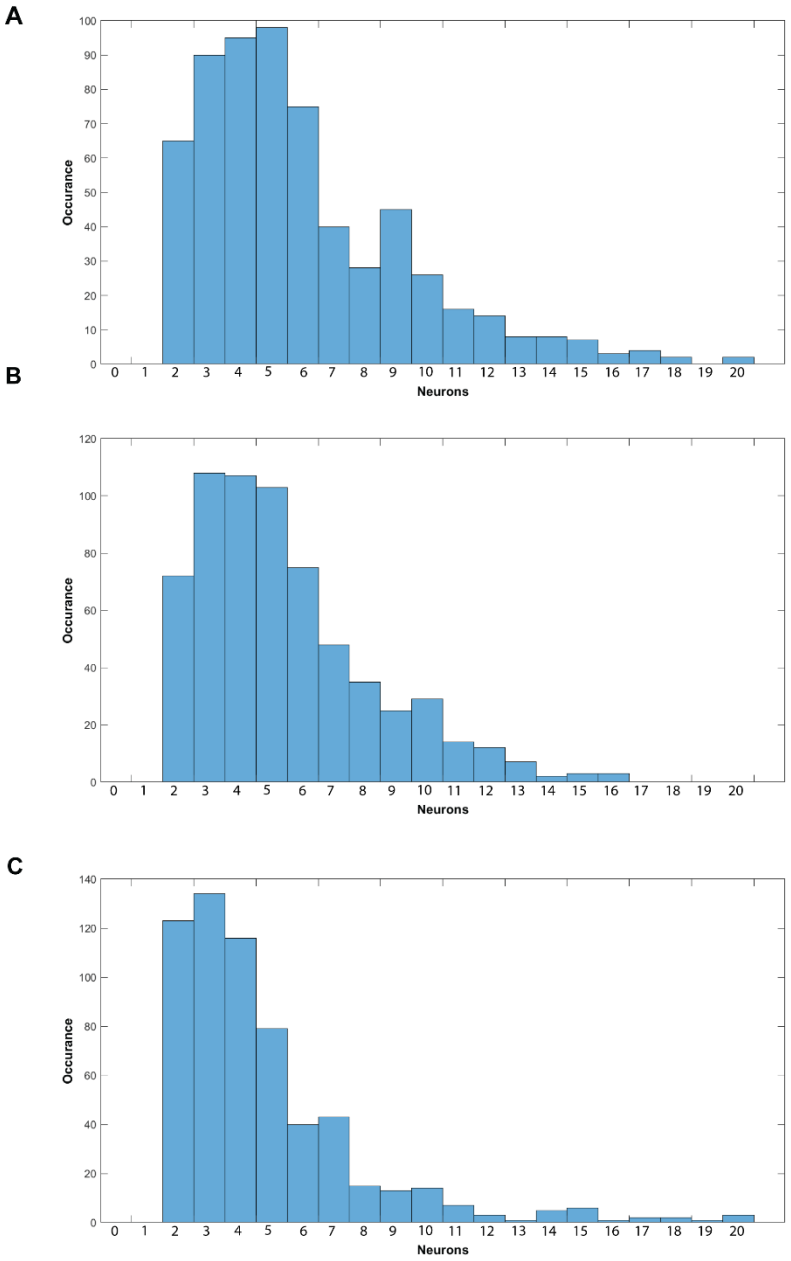
Page 14

“The maximum number of coactive neurons in a single frame was approximately 3% of the total population, with no frame exceeding 10% of the total population (Supp. Fig. 11B).”



Below, we provide representative raw data from three mice, displaying histograms of the number of neurons and their respective occurrences. The majority of occurrences fall between 3 and 6 neurons, with no more than 20 neurons (~9%) active in a single frame. This reassures the reviewer that the data does not contain any seizure-like or epileptic

activity. These data were not included in the revised manuscript but are provided here for confirmation.



3. Figure 4C has a typo in the y-axis label where “egram” should be “engram.”
We thank the reviewer for noticing this typo. We have fixed the typo in the revised manuscript.

Reviewer #3 (Remarks to the Author):
I appreciate the time and effort you have dedicated to address my comments. I am particularly glad that you have conducted a series of new analyses to substantiate and expand the claims in the manuscript. I have recommended your manuscript for publication and I look forward to seeing it online.
We thank the reviewer for his/her careful reading and efforts to increase the impact of the study.

Reviewer #4 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
We thank the reviewer for his/her careful reading and efforts to increase the impact of the study.

Reviewer #5 (Remarks to the Author):
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We thank the reviewer for his/her careful reading and efforts to increase the impact of the study.