Specific Aims

Understanding how memories experienced across large time scale are associated together is crucial to understanding episodic memories. Recently it has been demonstrated in rodents that two neutral contexts experienced closer in time shared a larger proportion of neural ensemble. Furthermore, subsequent fear conditioning in the second context increased animals' freezing level in the first context, suggesting a transfer of fear memory retrospectively [1]. These results showed that memories that have a small temporal distance can be linked together. However, it remains unclear what factors may affect the temporal window of memory linking. Specifically, it is unknown whether the affective value of a memory influence the time window within which it may be linked to a previous memory. Moreover, it is not clear whether memory linking can happen prospectively, where the associated fear of previous context may be transferred to a later context. Furthermore, if there is prospective memory linking, it is interesting to see whether the temporal window of prospective memory linking is similar to those in retrospective linking, *i.e* whether memory linking is symmetric regarding the temporal order of memories. Thus, the main goal of this proposal is to study how affective value and temporal order affect the time window of memory linking.

In addition, the analysis of neural dynamic in memory linking experiments have been limited to comparing the number of overlapping active ensemble cells across different sessions. Although such analysis successfully provided strong support for behavior results, it reduces time dimension to a binary, all-or-none representation, thus precluding the possibility of understanding the temporal structure of ensemble as well as the evolving nature of population coding within session. By applying dimension reduction analysis such as principal component analysis (PCA), we can uncover the underlying structures of neural ensembles for individual sessions and compare their similarity across linking memories versus non-linking memories.

Aim 1: Test the hypothesis that negative valued memories have extended retrospective linking window comparing to neutral memories. It has been shown that two neutral contexts can be linked together when they are separated 5 hours apart, but not when they are 2 days apart. We have preliminary results suggesting that negative-valued context can be linked with a neutral context two days ago, and they have larger proportion of overlapping ensemble cells comparing to two neutral contexts. To test this hypothesis, we will use contextual fear conditioning along with *in vivo* calcium imaging in freely moving animals.

Aim 2: Test the hypothesis that prospective memory linking has a different temporal window comparing to retrospective memory linking. It is hypothesized that memory linking might be mediated through a sustained increase in neuronal excitability after the first memory. If such hypothesis is true, we should expect similar temporal window of memory linking regardless of which of the two memories is later associated with fear, as long as the temporal distance of the two memories stay the same. However, we have preliminary results showing that associating the first context with foot-shock did not induce increase of freezing level in the second context at 5 hours time interval, suggesting that prospective memory linking is not observed at time scales that induced retrospective memory linking.

Aim 3: Test the hypothesis that linked memories have higher similarity of ensemble structures. To test this hypothesis, we can apply PCA analysis to calcium traces recorded at different behavior sessions. The resulting principal components can be thought of as subset of cells that exhibit highly correlated firing. We can then calculate a correlation of the components across different sessions, and compare the correlation between linking contexts with those between non-linking contexts. We predict that the correlation of structured ensemble components are higher for linking contexts comparing to non-linking contexts.

A. Significance

Understanding how temporally distinct memories can be related together is essential to understanding episodic memory. It is generally believed that hippocampus make an important contribution to episodic memory in rodents. Traditionally, studies have focused on how familiar environments are coded in hippocampus. Most notably, we have extensive knowledge of how space is coded in distinct familiar settings, as well as how time is coded in learned task contingencies. On the other hand, it is found that different population of hippocampal neurons are engaged in memories at different times, forming a neuronal ensemble of the memory, and artificially stimulating a specific ensemble can create a "false" memory. It is thus hypothesized that the evolving nature of ensembles can serve as a timestamp for temporally discrete events, even when the experiences took place in identical familiar environments. At the same time, a subset of neurons that is presented in multiple ensembles could code for a stable representation of the environments. However, it was unclear whether such time-stamping has any behavior correlates, and moreover, whether memories happened at different times can be associated together.

Consistent with the time-stamping hypothesis, it is recently found in rodent hippocampus that the neuronal ensembles of two neutral contextual memories separated by 5 hours time interval has more overlapping cells than those separated by 2 days or 7 days. Interestingly, subsequent fear conditioning in the later context induce elevated freezing level in the former context when the two contexts are separated by 5 hours, indicating a transfer of fear memory from the second context to the first. Such results suggest a linking of two temporally distinct memories through overlapping neuronal ensembles. However, two important questions remain unclear for the memory linking phenomenon: **a) whether and how the subjective value of the experiences affect the temporal window of memory linking. b) whether and how the temporal order of the experiences affect the temporal window of memory linking.** From a psychological perspective, these two questions are highly relevant to our day-to-day experiences and thus important for the implication of memory linking, since: a) relating a highly favorable/traumatic memory to other (potentially causal) experiences is more beneficial than relating a neutral memory to others, so that the animal may learn to engage/avoid the same highly significant experience in the future. b) relating a memory to past experiences is more beneficial than relating a memory to future experiences, since only past experiences may have a causal role which is important to learn. Moreover, answering these two questions may hint on the potential mechanism of memory linking. It has been hypothesized that memory linking happens through excitability mechanism, where the ensemble neurons of first memory sustain an elevated excitability during the memory linking time window, and thus are more likely to be recruited during the encoding of the second memory, facilitating the linking of the two memory. If such hypothesis is true, we should expect an unbiased memory linking, where neither the affective value of the second memory nor the temporal order of the two memories would affect the time window of memory linking. Such result would further support the time-stamping hypothesis, and would suggest memory linking to be a result of constant spontaneous turn over of ensemble. Alternatively, if the affective value or the temporal order of the two memories has an effect on memory linking, it suggests a more top-down, potentially learning guided mechanism may be responsible for memory linking. Thus, the main goal of this proposal is to study the effect of affective value and temporal order on memory linking. The results would extend our knowledge on memory linking and more generally episodic memory.

In addition, the analysis of neural recording data in memory linking experiments have been limited to comparing overlapping ensemble cells that are active during recording sessions. Such analysis provided a simple estimation of similarities between ensembles and successfully supported behavior data. However, it reduces the time dimension of each recording session to a binary, "active-or-not" representation, precluding any analysis on the structure of the ensemble within session. This limitation is mainly due to the task-free and one-trial-learning nature of memory linking, where there is no task variables to align the recording data to, nor is there enough time for place cells to be formed and detected. Dimension reduction approaches, or more specifically principal component analysis (PCA) is a very useful tool in such circumstances, since it can transform higher dimension recording data to lower dimension, temporally structured components in an unsupervised manner. Thus another goal of the presented proposal is to apply PCA analysis to neural recording data during memory linking experiments. Such analysis could uncover the underlying structures of memory ensembles, and help us understand the nature of memory linking on the ensemble level.

C. Approach

Aim 1: Test the hypothesis that negative valued memories have extended retrospective linking window comparing to neutral memories.

To test the hypothesis, we will use contextual fear conditioning combined with calcium imaging in behaving mice. We choose contextual fear conditioning because it is a robust and well-established test for long-term memory, and moreover a strong memory can be formed within one learning session. We choose miniature calcium imaging due to its capability to record neuronal activities in behaving mice and to track same field-of-view across long period of time, which is essential for the purpose of memory linking studies. We will focus on recording in dorsal CA1 region since it is believed to make a major contribution to contextual memory.

Previously it has been shown that two neutral contextual memories separated by 5 hours interval have significantly larger overlaps in ensemble cells comparing to those separated by 2 days or 7 days. Furthermore, subsequent fear conditioning in second context induce significant elevated freezing level in the first context when the two contexts are separated by 5 hours, but not when they are separated by 2 days or 7 days. These results suggest that the time window of memory linking extends beyond 5 hours, but is shorter than 2 days.

We have preliminary results showing that when the second context is paired with fear during encoding, the time window of memory linking extends to 2 days. Specifically, when the animals received a delayed shock in the second context during encoding, there is higher overlap in ensemble cells between the first and second context during the retrieval test comparing to a “chance” level of overlap between one of the two context and another novel context, even when the two context are separated by 2 days. Whereas when the second context remains neutral during encoding, and subsequently associated with fear by an immediate shock, the ensemble overlap between the two contexts remained at a low level, consistent with previous findings.

The presented proposal will adopt a similar experimental design. Specifically, animals will be divided into two groups: “neutral” and “negative”, which represent the affective value of the shocking context during encoding. Both groups will explore context A, B and C for 10 minutes. The time point at which the animals experience A, B and C is spaced out in such a way that the temporal distances between each of them and the shocking context S is 7 days, 2 days and 5 hours respectively. During the exploration of the shocking context S, a 2 seconds long, 0.1 mA delayed shock will be delivered at fifth minute to the animals in “negative” group, but not “neutral” group. Both groups explore the shocking context S for 10 minutes. Then 2 days later, animals in the “neutral” group will be put back in context S, where a 2 seconds long, 0.1 mA immediate shock is delivered after 10 seconds of exploration. Finally, another 2 days later (that is, 4 days interval for “negative group”), each group of animals are further divided into 5 sub-groups, where their freezing levels are assessed in parallel for context A, B, C, S, as well as N, which is a novel context. Neuronal activities in dorsal CA1 are recorded with miniature endoscope throughout the whole experiment.

We expect to see significantly higher freezing levels in context B, C, and S when comparing to A or N in “negative” group, while only freezing levels in C and S are significantly higher when comparing to either A, N or C in “neutral” group. Furthermore, we expect in “negative” group that the overlap of neural ensembles between B and S, as well as between C and S, are significantly higher than the overlaps between A and S or N and S, which is supposedly at “chance” level. Whereas in “neutral” group, the overlaps between A and S, B and S, as well as N and S should all be low and at “chance” level, while only the overlaps between C and S are significantly higher than others. Taken together, these results would suggest that the paring of context S with shock during encoding extend the time window of memory linking to at least 2 days back, while the memory linking time window for a neutral context is only longer than 5 hours but shorter than 2 days.

The proposed experiments differ from preliminary studies in two important aspects: Firstly, the proposed experiments include various time-points within each group. This enable us to compare freezing level during retrieval testing and identify the temporal window of memory linking within group, after which the time window can be compared across group. The advantage for the within-group design is that we no longer have to compare freezing level across groups, especially when we are essentially adopting different fear conditioning paradigm for the two groups which may confound the interpretation of difference in freezing levels across group; Secondly, the proposed experiment divide each group into subgroups and test freezing levels in parallel, since the previous repeated testing paradigm might introduce confounding extinction effects, especially when we expect memory linking between some of the contexts.

Still, the interpretation of the behavioral data of the proposed experiment could suffer from another confounding factor — the time interval between the shock and the encoding of different contexts. An alternative interpretation of the expected behavior results would be that in "negative" group, the freezing level in context B is higher than those in A simply because the encoding of B happened closer to the shock, and thus associated stronger with the shock than A, regardless of memory linking, while such time-dependent associations decays non-linearly across time so that in "neutral" group the freezing levels in B and A are indistinguishable. However, such interpretation can be distinguished by the analysis of neuronal data, since if the shock is the driving factor of the observed behavior, there is no reason to expect the temporal location of the shock affect the ensemble overlaps between either A and S, B and S, or C and S. Thus the specific hypothesis of the effect of affective valence on memory linking can still be tested by the proposed experiments.

Aim 2: Test the hypothesis that prospective memory linking has a different temporal window comparing to retrospective memory linking.

Similar to Aim 1, we use contextual fear conditioning combined with calcium imaging to test the hypothesis.

We have preliminary results showing that when fear conditioning is carried out in the second context, the fear could transfer back to the first context when the two contexts are separated by either 5 hours or 1 day, but not when they are separated by 2 days or 7 days. However, if the fear conditioning is carried out in the first context, the freezing may transfer to the second context only when the two contexts are separated by 5 hours, but not when separated by 1 day, 2 days or 7 days. This result suggest a shorter prospective memory linking window.

The presented proposal use a similar setup. Specifically, animals are divided into two groups, "prospective" and "retrospective". The animals in "retrospective" group will explore context A, B and C before the shocking context S, while those in "prospective" group will explore A, B and C after the shocking context S. The time points at which the animals explore A, B and C are spaced out such that the temporal distance between the shocking context S and context A, B or C are 2 days, 1 day and 5 hours respectively. In both groups, animals explore context A, B, C, S for 10 minutes, where during exploration of context S, a 2 seconds, 0.1 mA shock will be delivered at the fifth minute. 2 days after the exploration are finished for the last context, each group will be further divided into 5 sub-groups, where their freezing levels are assessed in parallel in context A, B, C, S, as well as N, which is a novel context. Neuronal activities in dorsal CA1 are recorded with miniature endoscope throughout the whole experiment.

We expect to see elevated freezing level for context B, C and S comparing to A and N in "retrospective" group, whereas in "prospective" group freezing levels are only higher in C and S, but not A, B or N. Furthermore, we expect the overlap in ensembles between B and S as well as between C and S are higher in "retrospective" group, whereas in "prospective" group the overlap are only higher between C and S but not between B and S, when comparing to the "chance" level overlap between N and S. Taken together, these results would suggest that "prospective" memory linking has a shorter temporal window than "retrospective" temporal linking.

Aim 3: Test the hypothesis that linked memories have higher similarity of ensemble structures.

The raw videos from calcium imaging recording could be processed with an open-source analysis toolkit CaImAn implementing a constrained non-negative matrix factorization algorithm. After the process, a spatial matrix representing the spatial footprint of each putative neurons, as well as a temporal matrix representing the calcium traces of each putative neurons will be extracted from the raw data. A custom-written script is used to visually assess the accuracy of the extraction as well as manually refine the results. After this, neurons from different recording sessions are cross-registered based on the euclidean distances between the centroids of their spatial footprint, and a unique master index can be assigned to each neuron in the whole experiment.

For each recording session, given a matrix representing the calcium traces of N neurons along T time-steps (usually frames), a PCA analysis can be applied to extract R principal components, where each components contain a "neuron vector" $\vec{n}$ of length N, and a "temporal vector" $\vec{t}$ of length T. Thus the dimension of the data is reduced from $N \times T$ to $R \times (N + T)$. The PCA is carried out in a way so that: a) the "neuron vector" of each principle component represent a group of neurons that has a highly correlated firing pattern, and the "temporal vector" represent that averaged pattern treating the whole group as single neuron. b) a dot product can be computed with each "neuron vector" and "temporal vector", and the sum of R such dot products should closely reproduce the original $N \times T$ data. c) the R components should explain most of the variance in the original data, thus the value of R can be determined by thresholding the proportion of variance explained.

Once the principal components of each recording sessions are extracted, we can calculate a cross-correlation of the "neuron vector"s between any two session. We can then compare such correlation matrices between linked context and unlinked contexts. We expect to see higher correlations between linked contexts, suggesting that the temporally correlated structures within each ensemble are more likely to be preserved across linked contexts than across unlinked contexts.

The presented approach has two caveats that might require further refining: Firstly, a method to assess the quality of cross-registration is lacking. For this issue, an algorithm developed by Yaniv lab might be more suitable since it can also output the confidence of cross-registration. However, as long as the current approach does not produce systematic bias towards linked contexts, that is, as long as the field-of-view of recordings remain relatively stable, there is no reason to expect a significant artifact from presented methods. Secondly, the application of PCA analysis presume that the neuronal ensembles are structured such that subsets of cells fire together. It may fail to detect other temporal structures, such as sequence of firing. For this, other dimension reduction algorithms might address the issue.

References

[1] D. J. Cai, D. Aharoni, T. Shuman, J. Shobe, J. Biane, W. Song, B. Wei, M. Veshkini, M. La-Vu, J. Lou, S. E. Flores, I. Kim, Y. Sano, M. Zhou, K. Baumgaertel, A. Lavi, M. Kamata, M. Tuszynski, M. Mayford, P. Golshani, and A. J. Silva. A shared neural ensemble links distinct contextual memories encoded close in time. *Nature*, 534(7605):115–118, June 2016.