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Dynamic and heterogeneous neural ensembles contribute to a memory engram

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In the century since the notion of the 'engram' was first introduced to describe the physical manifestation of memory, new technologies for identifying cellular activity have enabled us to deepen our understanding of the possible physical substrate of memory. A number of studies have shown that memories are stored in a sparse population of neurons known as a neural ensemble or engram cells. While earlier investigations highlighted that the stability of neural ensembles underlies a memory representation, recent studies have found that neural ensembles are more dynamic and fluid than previously understood. Additionally, a number of studies have begun to dissect the cellular and molecular diversity of functionally distinct subpopulations of cells contained within an engram. We propose that ensemble fluidity and compositional heterogeneity support memory flexibility and functional diversity.

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In the early twentieth century, Richard Semon introduced the term 'engram' to describe the physical manifestation of memory, defined as 'the enduring though primarily latent modification in the irritable substance produced by a stimulus' [1]. The biological basis for the engram was elusive, however, as early investigators were unable to find a specific engram within the cortex [2,3]. In recent years, new technologies for identifying and controlling cellular activity have enabled us to deepen our understanding of the possible physical 'trace' of memory. Several studies have shown that memories are stored in a sparse population of neurons, defined as engram cells or a neural ensemble [4–10]. While earlier studies highlighted that the stability of a neural ensemble underlies a stable memory representation, recent studies have suggested that neural ensembles are more fluid than previously thought [11–15]. Additionally, several groups have begun to dissect the cellular and molecular diversity of functionally distinct subpopulations of cells contained within an engram [16•,17•]. We propose that memory ensembles comprise two additional properties that have only recently been explored: ensemble fluidity that supports memory flexibility and the compositional heterogeneity of subensembles that contributes differentially to memory functions.

Prior studies leveraged immediate-early gene tagging strategies as a way to identify which cells were activated during a learning or memory recall session [18]. By taking a 'snapshot' of which cells were activated during both learning and memory recall, investigators sought to find the engram—the physiological trace or storage site of the memory. In a study in the hippocampus using fluorescent *in-situ* hybridization measuring the expression of the immediate-early gene Arc, investigators found that many of the cells initially activated during encoding of an environment were reactivated when the animals re-entered the environment 20 min later [19]. Similarly, another study found that amygdala cells initially activated during tone fear conditioning (tone paired with a shock) were likely to be reactivated when the animals heard the tone a week later, recalling the fear memory [20,21•]. Furthermore, the behavioral memory of the tone paired with the shock (assessed by the degree of freezing) was positively correlated with the amount of reactivation of the neural ensemble that was activated during initial learning. These studies suggest that memories are stored in neuronal ensembles and reactivation of those ensembles contributes to memory retrieval and subsequently to behavior.

If a memory is stored in a sparse neural ensemble, then silencing these cells should impair the brain's ability to retrieve the memory. Conversely, artificially activating these cells should induce the brain to retrieve the memory. To test the first hypothesis, an allocation strategy was used to bias a tone-fear memory to be stored in a subset of CREB+ cells in the amygdala. Later during recall when the tone was played, investigators silenced the CREB+ neurons (by ablating or temporarily silencing those

neurons), which inhibited the ability of the animal to recall the tone-fear memory [21•,22•]. To test if artificially reactivating the neural ensemble was sufficient to recall a memory, an immediate-early gene tagging approach was used to tag hippocampal neurons that were activated during context conditioning (shock paired with a novel context) [23•]. Animals were later placed in a different and safe context, and when the neural ensemble tagged from context conditioning was artificially activated with an optogenetic strategy, the animals froze, suggesting that they were recalling at least some aspect of the fear memory. The memories stored in the ensembles were context specific, such that artificially activating a tagged ensemble of a neutral context (as a control) did not exhibit an expression of fear. Similar findings have been reported using a chemogenetic strategy [24]. Importantly, these findings have been reported for multiple types of memory [25–29] and across brain regions [9,30,31].

In addition to these studies, an extensive literature in hippocampal physiology also supports the theory that memories are stably encoded in neural ensembles. Using *in vivo* electrophysiology, early studies found that hippocampal neurons ('place cells') fire according to the animal's position in space [32–34]. Many studies have shown that place cells reliably fire when animals return to the same spatial location and this stability can be preserved across weeks, making these cells suitable for long-term storage of a cognitive map and possibly contributing important spatial information to specific engrams [34–37]. Critically, disruption of ensemble-specific reactivation of place cells (replay) impairs spatial memory [38]. Conversely, pairing intracranial stimulation with place cell reactivation during sleep can reinforce specific spatial memories and induce later awake spatially goal-directed behavior in mice [39]. Together, these studies strongly suggest that the memory engram exists in a stable neural ensemble and that this ensemble is necessary and sufficient for memory recall.

Memory engrams are flexibly updating with new information

While a number of *in vivo* electrophysiology studies have found hippocampal place cells to reliably fire again when animals return to the same spatial location, most of these studies compare place cell stability within one day because reliably recording from the same cells across multiple days has been challenging for electrophysiology. One study successfully recorded the same place cells across 2 days and found that place cells had more similar firing patterns within 6 hours than within 30 hours when the animal was exploring the same environment [40,41•]. Similar findings were reported using an *in vivo* imaging method which can record from the same cells across weeks [42•,43]. The probability that place cells would fire again when the animal was exposed to the same environment and was performing the same behavior decreased across weeks. Nevertheless, many of these place cells retained stable spatial information when they fired again days or weeks later. In other words, many place cells continued to fire with high fidelity in the same spatial location even though the probability of firing decreased across time. This ability to decode stable information in hippocampus despite changes in firing rates over time is not limited to spatial information but also extends to strategic information in rats trained on a rule-switching task [44•]. This phenomenon is known as representational 'drift,' where tuning is mostly stable while individual cell activity rates change across minutes to days. Representational drift has been reported across the brain [12].

Interestingly, the drift observed from *in vivo* data in place cell studies is consistent with immediate early gene results. While it is difficult to make direct comparisons across studies as the methods differ, it is worth noting that experiments using *in vivo* recording techniques found that when an animal returns to the same environment within minutes to hours the ensemble activity correlation of place cells is ~90% but drops to ~60% or lower across days [40,42•,43]. This decrease in reactivation of the same cells across time is generally consistent with observations from immediate early gene studies. When an animal returns to the same environment within 20 min, the ensemble reactivation in hippocampal CA1 is ~90% [19] but decreases to 30–50% days later [25,27]. While the exact reactivation rates differ across studies and methods, there seems to be consistency in that the relative reactivation rates of neural ensembles decrease across time.

This dynamic property of ensemble activity may provide a mechanism that allows certain features of a neuronal population to meaningfully track time while maintaining accurate tuning properties. One study supporting this theory showed time-dependent changes in spatial representations in the hippocampus [43]. Spatial tuning curves varied from day to day yet were sufficient to decode an animal's position along a linear track. Surprisingly, these activity patterns were also sufficient to decode the session in which the recording occurred. This finding led the authors to conclude that the variance in population activity could contain information about relative temporal distance between two similar encoding events [40,43].

Another possibility is that ensemble drift is not related to tracking time, but that instead, the function of dynamic ensembles might be to impose a strategy where neurons 'take turns' encoding new information to prevent too many memories from being allocated to the same population [Mau *et al.*, in press]. Long time intervals between encoding episodes, for example, can result in mostly non-overlapping neuronal ensembles [45•,46•]. While the temporal distance between the two episodes could

theoretically be distinguished with this pattern of memory allocation, it is equally likely that temporal distance is not interpreted by a downstream reader these neurons project to as implied by Rubin *et al.* [43]. Instead, time-dependent variance in ensembles could merely reflect the shift in priorities for which neurons receive incoming new memories, and the function of neuronal drift would be to distribute memories to neurons that were not recently recruited. This process could be beneficial for memory systems if plasticity is saturated in the population of neurons that make up an engram, which could occlude memory formation and memory-updating in these overworked populations [47]. To overcome this challenge, drift may support the turnover of ensembles to facilitate the availability of 'new cells' to encode new information to integrate with the prior memory or to encode a new, distinct memory altogether.

Endogenous changes and fluctuations in cellular activity may contribute to drift and serve as a mechanism for updating memories, linking those encoded close in time, while separating memories encoded at more distant time points [15,48], [Mau *et al.*, in press]. One study focused on the hippocampus showed that 5 hours after context learning, the ensemble that encoded the memory had increased cellular excitability and this transient increase in intrinsic excitability allocated a second distinct context memory to be encoded by many of the same neurons as the first context memory [45•]. However, days later when excitability returned to basal levels, memory for a new context was no longer preferentially allocated to the neurons of the prior ensemble but was encoded instead in an independent ensemble of neurons. Sharing a neural ensemble between two memories functionally linked the two such that recall of one memory triggered recall of the temporally linked memory (encoded 5 hours apart, but not 24 hours apart, Figure 1). Similar findings have been observed in the lateral amygdala, where two different cued fear conditioning sessions administered 6 hours apart were more likely to be encoded by an overlapping population of neurons than those encoded a day apart [46•]. These memories were also shown to be behaviorally linked: extinguishing one fear memory also extinguished the other. While the studies described here focused on how distinct memories can be linked during encoding, other studies have demonstrated that transient increases in intrinsic excitability in ensemble cells can also occur during memory retrieval [49], priming the retrieved memory to be updated with new information [50].

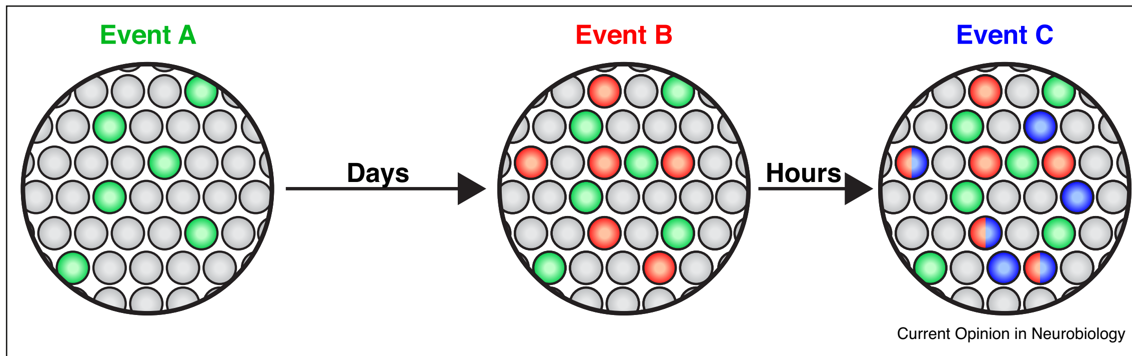
Similar findings have been reported in human studies investigating how memory representations are linked across time. Functional imaging in humans revealed that neural representations for object pairs heavily overlapped if spaced 30 min apart, but not 24 hours apart [51]. Within the same temporal block of imaging, hippocampal activity patterns are more similar than when separated by an intervening event, suggesting that episodes are segregated based on time [52]. Furthermore, fear from aversive memories can transfer to neutral memories if encoded close in time [53,54]. Taken together, these findings across species, techniques, and behaviors suggest that endogenous changes and fluctuations in cellular excitability across time and experience can support the linking and updating of memories (Figure 1).

Memory engrams are heterogeneous in their composition

Earlier work on memory focused on engrams consisting mostly of excitatory neurons. Recent evidence, however, points to greater functional heterogeneity within ensembles than previously thought. Heterogeneous population activities have been observed during various types of learning. Diversity in neural firing dynamics in the hippocampus has been proposed to reflect the familiarity or novelty of learned information. One study found that familiarity was encoded by fast-firing, less-modifiable neurons where novel features of an experience were represented by a different set of slowly firing and highly plastic cells [55]. Another study investigated how hippocampal ensembles differentially represent context and space [56]. Similar to prior studies, Tanaka *et al.* found that a subset of CA1 cells expressed c-Fos after an animal experienced a novel context (A) and many of these cells were reactivated when the animal returned to the same context (A) but not when the animal explored another novel context (B), demonstrating context specificity of a neural ensemble. Interestingly, the cells that expressed c-Fos and reactivated during memory recall when revisiting context A did not fire in the same spatial location as during the initial memory encoding of this context. Surprisingly, between visits, the place fields of these cells had 'remapped' to a different spatial location within the same context. Non-c-Fos cells also, surprisingly, showed more stable spatial coding, as they were more likely to fire in the same spatial location during the recall session in context A. These data suggest that distinct hippocampal ensembles may store spatial and contextual information. Recent studies have also shown that subensembles contain different components of a memory, which are then orchestrated to constitute a memory [57–59]. In the hippocampus, hierarchical structures can organize relational information of multiple features of the environment [60].

While identifying subensembles is gaining increasing attention in the study of memory engrams, it is also important to highlight that distinct ensembles can encode the same information. For instance, multiple hippocampal ensembles can encode the same spatial context [61]. Elucidating both the divergent roles of subensembles, as well as the convergent roles of separate ensembles, will be critically important to understand the full complexity of

Figure 1

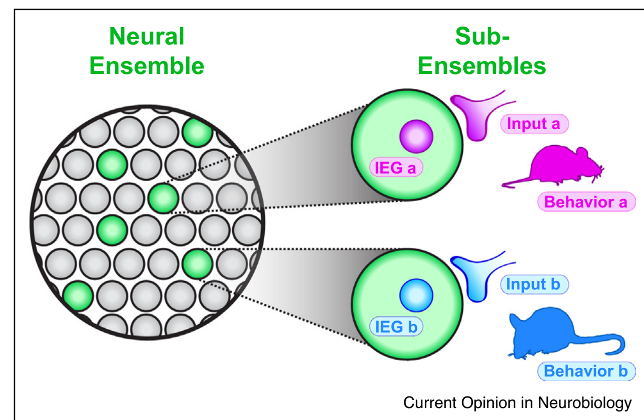


Neural ensembles are temporally dynamic.
A population of neurons whose firing patterns are tied to the encoding of and/or retrieval of a specific event in time is thought to comprise a memory trace, or engram. A sparse population of neurons that represent Event A are shaded in green and are distinct from the neurons that represent a separate episode occurring days later, Event B, shaded in red. Separate memory episodes that occur close in time to one another are more likely to share overlapping populations of neurons, as depicted in the cells shaded in blue corresponding to Event C occurring hours after Event B. This phenomenon is known as temporal memory-linking.

engrams. Very few studies have dissected how functionally distinct neuronal ensembles can be distinguished within an engram at the molecular and cellular levels. Genetically encoded activity reporters based on immediate early genes, such as c-Fos and Arc, commonly considered proxies for neuronal activity, have been used to identify neuronal ensembles in engrams. Until very recently, most studies have focused on ensembles defined by a single activity-dependent pathway [18]. However, activity-dependent pathways are known to be highly diverse: they respond differently to external stimuli and mediate distinct cellular and synaptic processes [62,63]. For instance, it is known that Arc proteins have virus-like capsid capabilities that can influence synaptic communication between neurons and underestimating this feature of Arc expressing neurons may miss critical network architecture features of how ensemble microcircuitry forms and is maintained [63,64]. Additionally, a recent study dissected two molecularly and functionally distinct ensembles within the dentate gyrus of the hippocampus underlying a contextual fear memory engram. Neurons that expressed c-Fos at learning, which mediates long-term potentiation of excitatory synapses, supported memory generalization, while neurons that expressed Npas4 at learning, which preferentially recruits inhibitory synapses onto excitatory neurons, regulated memory discrimination [17•] (Figure 2). The two molecularly distinct ensembles were both reactivated during recall, playing subtle but important roles in balancing memory generalization versus discrimination. These types of approaches to identifying how molecularly distinct cellular profiles can be linked to different physiological and behavioral functions have been appreciated in other areas of research. Bringing such molecular insights to the engram field, particularly as newer technologies combining imaging and transcriptomics tools emerge, will advance systems neuroscience at a more interdisciplinary level [65].

Most studies on memory engrams have focused on the storage of memories in a neural ensemble consisting of excitatory neurons hypothesized to store stimulus associations through persistent changes in excitatory synapse

Figure 2



Neural ensembles consist of heterogenous subpopulations.
Neurons belonging to an ensemble that represents a specific memory may be composed of distinct subpopulations of cells defined by molecular composition, circuit-specificity, and/or functional output. Different immediate-early genes (IEG, e.g. c-Fos, Npas4, Arc) that are commonly used to tag neurons tied to a specific event in an activity-dependent manner can label separate subpopulations of neurons that, for example, receive distinct inputs and are important for fundamentally different behaviors, shaded in magenta and blue, respectively.

strength and density [4,58,66]. In contrast, GABAergic interneurons are generally thought to inhibit excitatory neurons, and have been suggested to constrain ensemble size and to modulate memory strength and the specificity of learning [46•,67–70]. While several studies show that interneurons play an important supporting role in memory storage, recent evidence demonstrates that interneurons can also have a direct role in storing memories through their own functional plasticity. One study has shown that a subset of prefrontal somatostatin (SST) interneurons was activated during initial learning of tone-shock pairing and this ensemble of SST cells also exhibited enhanced plasticity (as shown by enhanced synaptic transmission) [16•]. Inactivating this specific SST ensemble reduced memory recall, while activating the SST ensemble elicited memory recall. This phenomenon was specific to SST neurons and was not seen in other interneurons (e.g. parvalbumin interneurons). Emerging evidence suggests that a memory engram contains different cell types and signaling pathways to engage different synaptic and circuit mechanisms to modulate memory-guided behaviors [71].

Conclusion and future directions

With the advent of more advanced experimental tools, the cellular and molecular properties of engrams can today be characterized in unprecedented ways [72–74]. We are discovering that engrams are more dynamic and fluid in their population codes than previously understood. Rather than mere noise in the system, these dynamic properties may support complex computational capacities that might be useful for updating and integrating new information with existing memories across time. Furthermore, it is becoming increasingly clear that the cellular makeup of an engram's constituent neurons is more heterogenous than it appears, contributing to more complex microcircuitry that needs further characterization. Developing newer analysis methods of neural activity using unsupervised approaches can help us identify clusters of neural activity by how they are internally related in the brain rather than by experimenter-imposed behavioral labels [75]. Such knowledge, when combined with other approaches, may reveal unexpected functional roles of population activity as well as the how different ensembles or subensembles of cells may contribute to a function. In addition to analysis tools, developing more sensitive approaches with increased spatial and temporal resolution when recording, tagging and controlling neural activity, including newer advancements in optogenetics, chemogenetics, multi-channel calcium imaging, activity-dependent engineering strategies, and holography technologies will allow for a more refined understanding of the importance of spatiotemporal dynamics in memory processing [76–81]. Development of more sensitive behavioral readouts of memory representations will also help to further engram research. Much of the engram literature to date has concentrated on fear conditioning related behaviors, which, while useful for their simplicity, are limited by simple behavioral readouts. Increasing the complexity of the behavioral tasks, as well as the ability to extract more subtle behaviors from existing paradigms to probe the functional diversity of memory representations will be needed to advance the engram field [82]. Improving our knowledge of the complexity of the temporal and cellular properties of memory engrams will enable to us to better understand the multifaceted nature of memory representations and how they contribute to behavioral outcomes.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Brian M Sweis: Conceptualization, Writing - original draft, Writing - review & editing. **William Mau:** Conceptualization, Writing - review & editing. **Sima Rabinowitz:** Conceptualization, Writing - review & editing. **Denise J Cai:** Conceptualization, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

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