# What does engram encode?: Heterogeneous memory engrams for different aspects of experience

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# What does engram encode?: Heterogeneous memory engrams for different aspects of experience

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**Abstract**

Long-lasting synaptic changes within the neuronal network mediate memory. Neurons bearing such physical traces of memory (memory engram cells) are often equated with neurons expressing immediate early genes (IEGs) during a specific experience. However, past studies observed the expression of different IEGs in non-overlapping neurons or synaptic plasticity in neurons that do not express a particular IEG. Importantly, recent studies revealed that distinct subsets of neurons expressing different IEGs or even IEG negative-(yet active) neurons support different aspects of memory or computation, suggesting a more complex nature of memory engram cells than previously thought. In this short review, we introduce studies revealing such heterogeneous composition of the memory engram and discuss how the memory system benefits from it.

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**Abbreviations**

BLA, Basolateral Amygdala; CPFE, Context Preexposure Facilitation Effect; CA1, Cornus Ammonius 1; CA3, Cornus Ammonius 3; DG, Dentate Gyrus; IEGs, Immediate Early Genes; LTP, Long-Term Potentiation; LTD, Long-Term Depression.

## Introduction

The question of where and how memories are encoded and retrieved has been challenging scientists and philosophers for centuries. In 1904, Richard Semon brought up the concept of memory engrams [1]. He defined an engram as a physical change in the brain introduced by a particular experience. After encoding, specific cues can reactivate this engram leading to memory retrieval (ecophry). Despite highly systematic attempts to find the engram, the lack of certain technologies made it very difficult for Lashley and others to find evidence of long-lasting changes in the brain that represent particular experiences (memory trace) during the 20th century [2,3]. However, the progress in methods of labeling and manipulating specific cell groups helped find strong support for the existence of neuronal populations that contain the engram, so-called memory engram cells [4]. Josselyn and Tonegawa [5] describe three major engram evidence strategies: First, finding the activity of the same neurons during encoding and retrieval; second, manipulating this respective network of cells and investigating if this can cause retrieval (gain of function) or suppress (loss of function) respective behavioral output; third, implanting a memory by mimicking the encoding and retrieval process.

Activity-dependent neuronal tagging during a certain experience makes immediate early genes (IEGs) valuable for engram studies. Guzowski et al. (1999) enabled a significant step forward in this approach. They investigated the reactivation of a cell group during retrieval at two sequential time points by utilizing the distribution time of *Arc* mRNA from the nucleus to the cytoplasm [6]. Barth et al. (2004) sophisticated the IEG tagging technique. They developed a transgenic mouse line expressing a fosGFP fused protein under the *c-Fos* promoter [7]. This transgenic mouse line allowed immediate identification of c-Fos expressing cells and their subsequent electrophysiological investigation in vitro. However, the fosGFP protein revealed a similar degradation time as the endogenous c-Fos. Later development of transgenic mouse lines enabled more stable labeling of neurons expressing IEGs, allowing investigations over longer periods [8,9]. Both studies reported reactivation (IEG expression) of neurons during encoding and retrieval in many brain areas, including the hippocampus. Those early studies of IEG reactivation represented the first evidence of the existence of a memory engram.

Neves et al. (2008) conceptualized the importance of manipulating the physiology of neuronal networks active during encoding [10]. Rizzi et al. (2009) first reported their successful memory recall by optogenetically reactivating c-Fos tagged neurons. Liu et al. (2012) and Garner et al. (2012) were the first to publish similar findings [11—14]. They showed that this manipulation leads to memory retrieval. One critical factor in some of the above studies is that their optogenetic activation cannot imitate the natural sequential activation of engram cells. As mimicking the natural firing pattern of cells is challenging, another approach was taken by inhibiting neurons in the hippocampus that expressed IEGs (c-Fos or Arc) during fear conditioning and demonstrated a decrease in context-specific freezing behavior during inactivation [15,16]. These manipulation studies suggest that the neuronal activity of artificially activated IEG tagged neurons in the hippocampus plays an essential role in memory retrieval.

The third engram evidence strategy was first followed by Steinmetz et al. (1989) by performing classical conditioning of muscles reflexes [17]. The entire learning process took place intracranially by pairing the stimulation of two cerebellar regions. The restriction to intracranial stimulation only represents one out of two criteria proposed by Martin and Morris for implanting artificial memories [18]. Their second criterion expects the retrieval of the artificial implanted memory by an external event, which was accomplished by the study of Vetere et al. (2019). They took advantage of the well-known olfactory system and implanted a memory artificially via optogenetic stimulation of particular olfactory glomeruli and the aversion and reward mediating ventral tegmental area. This allowed the retrieval of this implanted memory via exposure to respective external odor stimuli [19]. On a similar note, in a study by Ramirez et al. (2013), channelrhodopsin-2 was introduced in c-Fos expressing cells during the exploration of a neutral environment [20]. Respective cells were later optogenetically activated during fear conditioning in a different environment. Yet, mice revealed subsequently increased freezing behavior in the previous neutral environment. The successful artificial memory implantation displays another support for the existence of memory engram.

While we find strong support for the existence of engrams in these studies, diverse and complex natures of memory engram cells and the content/aspect these cells are encoding remain to be elucidated. More specifically, IEG expression is mostly used as an activity marker, and respective cells are equated as engram cells. However, the functional heterogeneity of different plastic changes will remain essential to consider and might reveal a more complex nature of our memory system. Encoding any experience can lead to the storage of a broad range of different types of information such as different sensory modalities, internally generated signals, or higher-order information integrating them. Moreover, different types of computation support those diverse inputs. Nevertheless, many engram studies testing the causal relationship using neuronal manipulation did not have a strong focus on these aspects of memory. In this review, we highlight recent studies revealing the heterogeneous nature of memory engram cells. By introducing hippocampus-dependent memory as an example, we discuss how these different memory traces might comprehensively support our memories of episodic experiences.

## The complex induction and functionality of IEGs

IEGs have been used as neuronal activity markers in neuroscience fields. IEGs expression is generally low in quiescent cells but is transiently induced at the transcriptional level by extracellular stimulation [21]. As early as the 1980s, researchers have found that c-Fos is rapidly induced after electrical stimulation or administration of growth factors [22—26]. Significantly, high-frequency synaptic stimulation, which resembles the stimulation required to induce long-term potentiation (LTP), increased IEGs expression [27]. In line with previous results, Jiang et al. (2021) recently discovered that Arc-positive neurons showed increased correlated activity in hippocampal cell culture [28]. Besides directly manipulating cellular activity, IEG expression can also be induced by various extracellular signals, including growth factors, immunological and neurological signals (e.g., BDNF, IL-6), and sensory and behavioral stimulations (e.g. the studies by Gallo et al., Pfaus et al., Lanahan et al., Morgan et al., O''Donnell et al., Wheeler et al.. [29—33]). Because of these features, IEGs have provided a cellular method to label and examine the functionality of activated neurons. One of the biggest strengths of using IEGs to examine activity, compared to electrophysiology methods, is that IEG studies can provide large—scale activity analysis of multiple neurons and even multiple brain regions with simple immunohistochemical methods. For example, Wheeler et al. (2013) analyzed the c-Fos expressions of 84 brain regions during fear recall, revealing a critical thalamic-hippocampal-cortical network involved in long-term fear memory [34]. Other examples are provided by Kubik et al. (2007), who reviewed IEG evidence of hippocampal function and the subregional-specific contribution to spatial and contextual memory [35].

Alterations of gene expression underlie plastic changes in the networks in the brain and hence support memory [36]. As the first genes to undergo regulation of expression following cellular stimulation, IEGs have been proposed to function as "plasticity" markers. For example, *Arc* has been repeatedly shown to participate in molecular mechanisms of synaptic plasticity [37—40]. Consistent with this idea, *Arc* knock-out mice do not

express lasting LTP or long-term depression (LTD) in the hippocampus and are impaired for long-term memory in behavioral tasks [41] (but also see the study by Kyrke-Smith et al. [42]). Besides *Arc*, other IEGs, including *Zif-268* and *c-Fos*, have also been suggested to play a role in synaptic plasticity [29]. Although more commonly used as an activity marker rather than a plasticity marker, IEG expression is not always linked to increased cellular activities. For example, a seizure study demonstrated that high-frequency burst firings do not result in c-Fos expression in multiple brain areas [43]. By inhibiting medial septum input to the hippocampus, Miyashita et al. (2009) found that the behavioral induction of *Arc* transcription in CA1 and CA3 regions was abolished [44]. Because medial septal inactivation does not eliminate location-specific firing in these regions [45], their results suggested that increased cell firing is insufficient to induce *Arc* transcription. Given that increased spike rate does not always lead to IEG induction and that IEG-tagged neurons are causally linked to memory, it would be a more promising approach to examine physiological activities of the engram cells in order to determine what is encoded as memory [46]. These studies indicate that IEGs do not simply represent more or less activity of neurons.

Different IEGs should not be treated as unitary entities. One supporting evidence is that the signaling cascades leading to transcription of different IEGs differ [47]. The induction cascade of IEGs typically includes the following steps: (1) extracellular stimuli activate membrane receptors and in turn initiate a series of intracellular pathways, (2) kinases mediate the activation of transcription factors that initiate the expression of IEGs, (3) protein products of IEGs mediate the expression of downstream genes or participate in other forms of signaling [48,49]. Interestingly, different IEGs have their unique characteristics. For example, unlike c-Fos, Npas4 expression was selectively induced by only membrane depolarization, while c-Fos could also be induced by growth factors or neurotrophins, suggesting the constitutional difference in their induction pathways [50]. Additionally, the induction kinetics of IEGs varies. Experiments using artificial synaptic stimulation demonstrate that different IEGs have different stimulus thresholds for transcriptional induction [51]. For example, the c-Fos induction threshold is relatively high compared to others, which provides a good signal-to-noise ratio and allows anatomical mapping of c-Fos positive neurons during a complex behavioral paradigm [52]. In contrast, the expression of Zif-268 is more responsive to synaptic activities at physiological levels [53]. To further illustrate the point that IEGs should be viewed as different entities, here we compare Arc and Homer 1a. Although they share similar mechanisms for transcriptional activation (MAPK/ERK cascade) and can be expressed in the same neuronal population in the hippocampus, *Homer 1a* is slower in mRNA transcription and is subject to additional activity-dependent regulation [54]. Even a more striking difference is their functional outcome. They play a common role in molecular trafficking but interestingly produce the opposite outcomes in synaptic plasticity. Both Arc and Homer 1a mediate endocytosis and internalize AMPA receptors to reduce their surface expression [41,55]. However, while Homer 1a involves homeostatic scaling of recently potentiated spines [56], Arc preferentially targets inactive spines to increase contrasts of synaptic weights [40]. In summary, it is oversimplified to view IEGs solely as an all-or-none biomarker due to all the differences (in induction, kinetics, and functionality) leading to the final expression, and thus, the distinct roles of IEGs in memory should also be examined carefully.

## Memory engram cells are heterogeneous

To better understand the complexity in IEGs, it is important to determine a specific role of neuronal ensembles expressing each IEG. Past studies parsed out a particular IEG responding to a specific aspect of memory. The hippocampal IEGs would be a good example showing such unique contributions. For example, Jenkins et al. (2004) revealed c-Fos expression in the hippocampus increases when animals find a novelty in the spatial arrangement of familiar cues [48]. Rats experienced either a maze where the familiar visual cues were repositioned or a maze with familiar cues located at the same position as habituation sessions. Rats that experienced the relocated cues significantly increased c-Fos expression in the hippocampus and its related areas. Importantly, the hippocampal c-Fos did not respond to familiar cues themselves, suggesting that c-Fos expression most prominently responds to the novelty in the arrangement but not the novelty of elements. The c-Fos response in the hippocampus agrees with behavioral studies showing that hippocampal lesion leads to impairment in memory of object—location association but not of objects themselves [49,50]. Together, these findings indicate that the hippocampal c-Fos represents "relational" or "structural" features of the elements of experience.

In line with the notion, a series of studies using the contextual fear conditioning paradigm, including context pre-exposure facilitation effect (CPFE), revealed that the hippocampus contains conjunctive representations. Conjunctive representation refers to multiple stimulus elements combined into a single entity. Immediate shock experiments following pre-exposure to stimuli showed the hippocampus encodes co-occurrence of separated features making up the context. Importantly, a variant of the CPFE paradigm, the so-called "bucket experiment', showed that animals associated the shock with the hippocampal contextual representation acquired during the pre-exposure session rather than with the context experienced during shock presentation [51]. Importantly, under this behavioral

paradigm, the context pre-exposure increases c-Fos expression in the dorsal hippocampus [57]. The increased c-Fos positives are shown to be memory engram cells encoding the contextual information, which can be later associated with a shock representation stored in the basolateral amygdala (BLA) [58]. In their experiment, c-Fos positives in CA1 and BLA are labeled during pre-exposure and immediate shock, respectively. Simultaneous optogenetic stimulation of these two cell populations in their home cage results in freezing behavior during the later testing session in the preexposed context. These experiments suggest that c-Fos positive cells during context pre-exposure are the conjunctive representation and satisfy a requirement as memory engram cells.

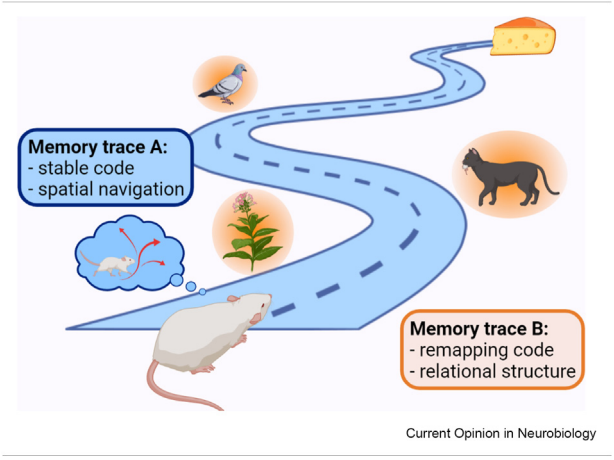
While c-Fos positive cells in the hippocampus encode contextual information as a conjunctive representation, it is not necessarily the case that other IEGs play identical roles. Experiments by Barbosa et al. (2013) showed that responses to novel relational structures differed between IEGs [59]. In the hippocampus, c-Fos expression responds to a novel location of the object, while Zif-268 did not show such specific responses to the contextual feature. Further, it has also been reported that there is no overlap between *Homer 1a* positive cells formed during pre-exposure and *Arc* positive cells during immediate shock. This observation indicates that these two IEGs make inconsistent contributions to the formation and expression of contextual memory [60]. Another recent example showed that different IEG-expressing engram cells mediate different computations. Sun et al. (2020) demonstrated a functional heterogeneity between c-Fos-expressing and Npas4-expressing engram cells in the dentate gyrus (DG) during contextual fear conditioning [61]. They found that c-Fos-expressing engram cells promote memory generalization, while Npas4-expressing engram cells promote memory discrimination. Moreover, the two classes of engram cells receive afferent projections that were differently modified by contextual fear conditioning, suggesting the engram cells engage distinct functional circuitry. Overall, these findings suggest that neuronal ensembles characterized by the expression of different IEGs do not have interchangeable roles for memory, even within the same subregion of the hippocampus.

## Reconciliation of hippocampal codes: A perspective from heterogeneous memory engrams

How does the heterogeneity in engram cells reconcile a coherent functioning of a specific brain structure? It is important to note that, in the hippocampus, the field has not reached a fully comprehensive view of its role in memory. Vast literature demonstrates its involvement in spatial navigation [62–66]. This cognitive process requires the more specific computation of locale information based on sensory cues and self-movement signals to navigate the animal from the current position to another location [67–70]. Extended views posit the hippocampal computation being navigation within 'memory space' or 'mental map' that allows sequences of events to be integrated into more generalized conceptual dimensions [71,72]. In these views, a critical question is whether the hippocampus computes the navigation within the abstract space or only provides a substrate to define the relational structure of events or cues [73]. The former requires a stable map for accurate computation (but also see the study by Kinsky et al. [74]). The latter can assimilate multiple maps for the same physical space depending on the nature of the experience. Previous studies do not reject these two hypotheses. As a support for the first view, the location-specific firing of place cells becomes more stable when the animal participates in a spatial task than the spatial map of randomly foraging animals [75]. Also, in line with the navigational computation by hippocampal place cells, Ormond and O'Keefe (2021) recently reported strong modulation of place fields by vectors pointing to a goal location [76]. In contrast, various internal factors influence the hippocampal activity and produce "remapping" of place fields [77–79]. As a striking example, contextual fear conditioning caused a robust remapping of place fields even though the animal was in the same physical space [80]. These studies raise two opposing views on the hippocampal role of memory and its contribution to spatial navigation.

The apparent incompatibility of the two hypotheses can be resolved by discarding an assumption that the hippocampus has the sole role in episodic memory. Here, we propose that the hippocampus has more than two different roles for episodic memory, each supported by distinct types of memory engram cells. As opposed to the over-simplified architecture of the hippocampal trisynaptic circuit, anatomical studies elucidated more complicated and diverse connections with extrahippocampal structures, possibly allowing flexible routing of different kinds of information (e.g. the study by Goode et al. [81]). Each hippocampal subfield is also more heterogeneous in multiple dimensions than previously thought, making various contributions to its physiology and function [82–87]. For example, a recent study found that DG represented sensory cues and spatial information in orthogonal populations of granule cells [88]. Importantly, non-homogeneous hippocampal wiring alone does not fully account for the heterogeneous memory engram cells. As we reviewed earlier, different IEGs have non-overlapping induction pathways and play diverse roles in plasticity. And different classes of engram cells characterized by different IEGs

**Figure 1**

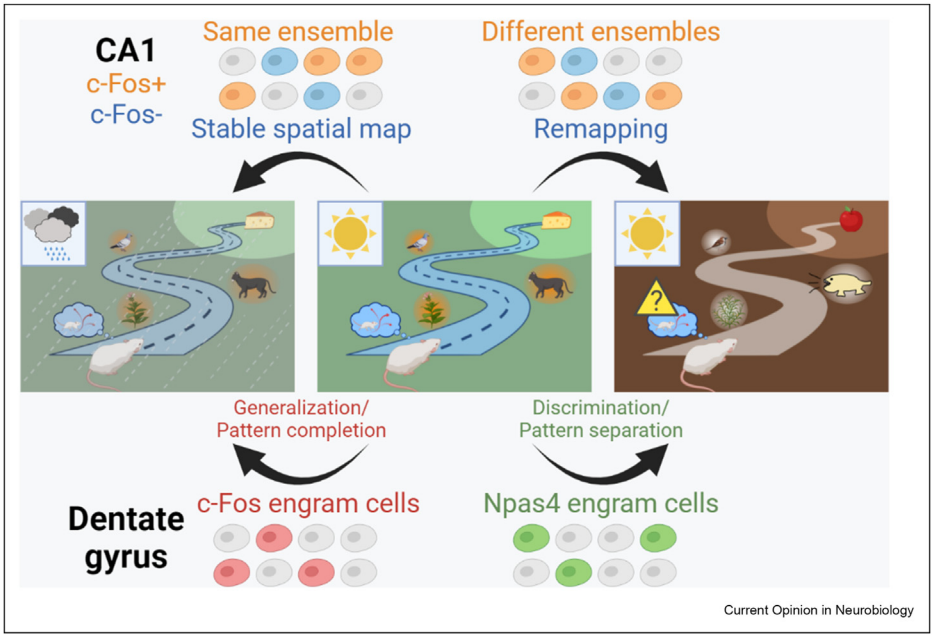



**Heterogeneous memory traces in the hippocampal CA1.** One type of memory trace supports spatial navigation through stable spatial maps. Another type of memory trace provides the relational structure of contextual elements in the experience.

do not always have identical roles for memory. This evidence favors a view that each memory is collectively and heterogeneously supported by different kinds of long-lasting changes [89,90].

The proposed scheme explains that spatial computation for navigation and storage of relational structure are achieved by two distinct classes of hippocampal memory engram cells. Upon exposure to a novel environment, only a fraction of place cells express c-Fos in CA1 of the hippocampus [46] (a similar observation with Arc is reported in the study by Lee et al.[91]). The IEG studies discussed earlier suggest these engram cells encode the relational structure of the contextual cues. Importantly, when revisiting the same environment later, c-Fos positive cells are more likely to change their firing locations, indicating their unreliability of computation for spatial navigation. On the other hand, the remaining population of place cells (c-Fos negatives) stably maintains their place fields. Notably, the formation of location-specific firing is mediated by experience-dependent hippocampal plasticity, suggesting that the c-Fos negative neurons undergo synaptic changes during the experience and thus are also a part of memory engram cells (e.g. the studies by Wilson et al., Geiller et al. [92,93]). A recent study supports this view and found preferential thalamocortical synaptic strengthening in c-Fos negative cells in the superficial layers of the barrel cortex after the olfactory association task [94]. These results suggest that, in the hippocampus, 'c-Fos positive engram cells' store the relational structure of the experience, and '"c-Fos negative engram cells" achieve computation of

**Figure 2**




**Different responses and roles of distinct classes of memory engrams.** In the CA1, c-Fos positive and negative pyramidal cells show different responses in their firing rates and place fields. In DG, c-Fos positive engram cells contribute to pattern completion and support context generalization, while distinct Npas4 positive engram cells play a role in pattern separation and context discrimination.

locale information for spatial navigation (Figure 1), again indicating heterogeneous neuronal ensembles to support hippocampal contribution to memory.

## Conclusion

Memory engram studies, combining IEG tagging and manipulation of neuronal activity, demonstrated a causal link between the activity in a subset of neurons and a specific piece of memory. However, further investigations of IEGs and their complexity are required to unveil the mechanisms underlying memory representation, encoding, and retrieval. Different factors activate different IEGs, producing various plastic changes in the network and making distinct contributions to the mnemonic processes. In that sense, memory engram is not a unitary entity.

The use of IEGs is still a powerful approach to disentangle their specific roles for memory. For example, c-Fos expression is significantly elevated in the hippocampus when the animal found a novel arrangement in the familiar cues, supporting the role of the c-Fos ensemble for encoding relational structure in the experience [95]. Importantly, the c-Fos positive cells are a neuronal subpopulation distinct from stable place cells in the CA1 and Npas4 positive granule cells in DG, suggesting each memory engram supports different aspects of the episodic experience for later retrieval (Figure 2) [46,61].

Cooperativity across heterogeneous memory traces might explain how representational drift in the hippocampal spatial maps achieves congruency of memory over time. Contrary to the expectation that representations of memory need to be stable, long-term imaging of $Ca^{2+}$ activity in the hippocampal neurons revealed continuous drifting in their representation of space [96–99]. Although firing locations of place cells are not stable, recent studies imply hippocampal neural codes independent of spike locations. Examples of these codes include context-specific firing rates of IEG positive cells [46,91] and synchronous activity that cannot be explained solely by overlaps of place fields [100]. If one of the representations is as stable as memory itself, functional coupling of these different representations provides reliable computation for spatial navigation while robustly retaining the hippocampal memory [101]. Further studies are required to test this prediction and understand how our brain stores memory under continuous changes of the network.

## Conflict of interest statement

Nothing declared.

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* of special interest
** of outstanding interest

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