Memory engrams: Recalling the past and imagining the future

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Summary Statement

Here we review the past, present and future of research examining how information is acquired, stored and used in the brain at the level of the memory engram.

Print Summary

Background

The idea that memory is stored as enduring changes in the brain goes back at least to Ancient Greece, but its scientific formulation had to wait until the turn of the 20th century when a German evolutionary zoologist Richard Semon introduced the term "engram" to describe the neural substrate responsible for storing and recalling memories. Essentially, Semon proposed that an event activates a subset of neurons that undergo persistent chemical and/or physical changes. Subsequent reactivation of the engram by some cues that were available at the time of the event induces memory retrieval. After Karl Lashley failed to find the engram supporting a maze memory, studies attempting to localize the engram were largely abandoned. Spurred by Donald Hebb's theory that augmented synaptic strength and neuronal connectivity are critical for memory formation, researchers identified brain regions in which enhanced synaptic strength was correlated with a specific memory. Nonetheless, the causal relationship between these enduring changes in synaptic connectivity with a behaviorally identifiable memory and the link to engrams at the level of the cell ensemble awaited further advances in experimental technologies.

Advances

Starting in 2009, significant advances in our understanding of engrams were made in two studies that applied complementary intervention methods to target engrams supporting specific memories in mice. One study demonstrated that killing the small portion of lateral amygdala neurons allocated to an engram disrupted subsequent memory retrieval (loss-of-function intervention). The other study showed that artificially reactivating a subset of hippocampal dentate gyrus neurons activated by a fearful experience induced memory retrieval in the absence

of external retrieval cues (gain-of-function intervention). Furthermore, “engram cells” showed enduring increases in synaptic strength and density, and preferential connectivity to downstream engram neurons forming engram networks. Subsequent studies identified engrams in other brain regions supporting several different types of memory. Together, these engram studies led to novel insights into how information is acquired, stored and used in the brain.

To highlight a few exciting advances, studies showed both increased intrinsic excitability and synaptic plasticity work hand-in-hand to form engrams, and these processes are also implicated in memory retrieval and consolidation. Relative neuronal excitability determines which neurons are allocated to an engram and this process also organizes multiple engrams in the brain. It is now possible to mimic and artificially manipulate memory encoding and retrieval processes to generate false memories, or even create a memory in mice without natural sensory experience (implantation of a memory). Moreover, “silent” engrams were discovered in mice made amnestic by drugs or in mouse models for studying early Alzheimer’s disease; memories that could not be retrieved by natural recall cues were retrieved by artificial reactivation of these silent engrams. Endogenous engram silencing may contribute to the change in memory observed with time or changing circumstance. Further, a conversion of an engram from a silent to an active state and *vice versa* was found to be critical for normal time-dependent development of memory and is implicated in fear memory extinction. Together, these findings are defining an engram cell as the basic unit of memory storage. An emerging concept is that a given memory is stored in functionally connected multiple engram cell ensembles dispersed in multiple brain regions, each ensemble providing an unique component of the overall memory.

Outlook

The ability to identify and manipulate memory engram cells and their circuits has introduced a new era of memory research. Many questions remain. In the short-term, it will be important to discover the precise experience-specific memory substrate in engram ensemble circuits, how engrams change over time, how engram structure impacts memory quality, strength and precision, and the role of silent engrams in these processes. In the long-term, the goal is to leverage these rodent engram findings to humans with memory or information-processing deficits. The development of low-to non-invasive technology may enable new therapies in people based on knowledge about engrams.

Memory is the ability to use the past in service of the present or future (*1, 2*). Memory is central to our everyday lives and defines who we are. Without it, we are condemned to an eternal present. That memory persists suggests an internal representation of a past event is stored in the brain that later can be reconstructed and used. Richard Semon, an evolutionary zoologist turned memory theorist, introduced the term "engram" in 1904 to describe such memory representations (*3, 4*). Semon defined an engram as "…the enduring though primarily latent modifications in the irritable substance produced by a stimulus…" (p. 12)(*5, 6*). He postulated a fundamental "Law of Engraphy" in which "all simultaneous excitations… form a connected simultaneous complex of excitations which, as such, act engraphically, that is to say leaves behind it a connected, and to that extent, unified engram-complex" (*7*)(1923, pp. 159-160). An engram is roughly equivalent to a "memory trace". As much of the experimental work discussed here derives directly from Semon's original definition and theory, we use the term "engram".

Semon's innovative ideas were largely overlooked or dismissed during his life. However, his theories eerily foreshadowed many prominent contemporary memory concepts (*8-11*). Semon defined an engram as a physical change in some aspect of brain state, but was suitably cautious when asked to speculate on the precise neural mechanisms underlying an engram, "To follow this into the molecular field seems to me…a hopeless undertaking at the present stage of our knowledge and for my part, I renounce the task (1923, p. 154)(*7*)".

A few years later, though, Karl Lashley, a geneticist turned psychologist, took up this challenge by systemically attempting to localize an engram in the mammalian brain (*12-14*). In a series of studies, Lashley trained rats to solve a maze. Hypothesizing that some critical component of the engram supporting this maze memory is localized in the cortex, Lashley removed tissue of varying sizes from varying cortical locations before assessing the rats' memory for the maze. Although the amount of tissue removed correlated with overall memory impairment, the location did not. After more than 30 years of searching, Lashley failed to find an engram, and declared the engram elusive.

Donald O. Hebb, a psychologist, memory theorist and student of Lashley, developed a cell assembly theory (similar to Semon's engram complex) that incorporated more of what was known about the brain at the time (*15*). A cell assembly is formed, Hebb hypothesized, between reciprocally interconnected cells that are simultaneously active during an event. Sufficient activity within the cell assembly induces growth and/or metabolic changes that strengthen the

connections between these cells [neurons that fire together, wire together, (*16*)]. These synaptic and metabolic changes (perhaps including changes in intrinsic neuronal excitability) have implications for cell assembly function. For instance, reactivation of only a fraction of assembly cells was hypothesized to produce reactivation of the entire assembly [a process termed pattern completion (*15*)]. In contrast, destruction of a small number of assembly cells would not necessarily produce catastrophic failure of the entire cell assembly. Interestingly, Semon also proposed similar properties for engrams (*5*).

Together, these (and other) historic scientists helped define and describe an engram. However, there was a paucity of studies examining the biological basis of engrams. A PubMed search of the word "engram" reveals that from 1960 until 2008 only 4.8 papers per year were published using the term. There was a marked uptick in the number of "engram" papers published since 2009 (average of 19 papers per year), with 43 papers published in 2018 alone. The dramatic increase in the use of the term "engram" in memory research seems to reflect recent excitement in this field. From what does this excitement arise? And, more importantly, is this excitement justified?

Here, we review the current state of engram research. Guided by Semon, we define an engram as an enduring representation of an event (or experience). "Engram cells" constitute critical cellular components of a given engram. These cells may (or may not) also be critical components of engrams supporting other memories. Engram cells are 1) activated by a learning experience, 2) physically or chemically changed by the learning experience, and 3) reactivated by subsequent presentation of the stimuli present at the learning experience (or some portion thereof), resulting in memory retrieval. It is important to note that an engram is not yet a memory, but provides the necessary physical conditions for a memory to emerge (*17*). A memory emerges when appropriate retrieval cues reactivate an engram in a process of memory retrieval which Semon named "ecphory".

We argue the recent excitement surrounding engram research stems directly from the development of novel tools that allow cell ensembles to be imaged and manipulated at the level of the individual cell. However, we appreciate that, even today, some scientists may agree with Semon that to examine the neurobiological basis of engrams is a "hopeless undertaking". Therefore, we begin by briefly reviewing the neurobiological evidence supporting the existence of engrams in the rodent brain and our collective ability not only to find, but also manipulate,

engrams to better understand memory. We adapt the criteria and experimental strategies discussed by Morris and colleagues (*18, 19*) in their benchmark evaluation of synaptic plasticity and memory. Specifically, we discuss evidence from three types of studies. First, observational studies should show that the same (or overlapping) cell populations are activated both by an experience and by retrieval of that experience and, furthermore, learning should produce cellular and/or synaptic modifications in these cells. Second, loss-of-function studies should show that impairing engram cell function after an experience impairs subsequent memory retrieval. Third, gain-of-function studies should show that artificially activating engram cells induces memory retrieval, in the absence of any natural sensory retrieval cues.

Pioneering studies, such as those by Richard Thompson, Joaquin Fuster and others (*20, 21*), greatly informed our current understanding of memory. However, because of space constraints, here we limit our discussion to rodent experiments examining memory of an experience that probe the engram at the cellular level.

EVAULATING EVIDENCE FOR THE EXISTENCE OF ENGRAMS

1. Observational studies

Typically, observational studies take advantage of immediate early genes (IEGs) such as *c-fos*, *arc*, or *zif268* (*22-24*) to visualize active cells. Cells active during a memory test are marked using IEG immunohistochemistry, while cells active during a training experience are "tagged" with temporally-inducible IEG promoters driving the expression of more enduring fluorescent (or other) reporter proteins (*25-28*). Above chance overlap between these two cell populations ("active during training" and "active during test") within a brain region (or throughout the brain) suggests an engram.

In the initial observational study, Mayford and colleagues (*26*) tagged neurons active during auditory fear conditioning. In this commonly used memory task, an initially innocuous tone (a conditioned stimulus, CS) is paired with an aversive footshock (an unconditioned stimulus, US). When subsequently re-exposed to the tone or conditioning context, rodents freeze (the active learned conditioned response, CR), showing that they remember the training experience (*29*). In this experiment, mice were replaced in the conditioning context three days after training and active neurons marked with zif268. Consistent with an engram supporting this

conditioned fear memory, the overlap of neurons active during training (tagged) and testing (zif268+) in the basal amygdala nucleus exceeded chance (~11% total cells) (*26*).

Similar results, across multiple brain regions, [including dorsal hippocampus (*25, 30-40*), amygdala (*26, 30, 34, 36, 40, 41*), and cortex (*27, 30, 40, 42*)] were reported for a variety of different memory tasks (including contextual fear conditioning, auditory fear conditioning and novel object exploration). Control studies revealed that tagged cells were only reactivated by the corresponding CS, and not by unrelated stimuli (*30*). Although most observational studies did not address directly the learning-induced changes hypothesized by Semon, overall, these results provide broad support for the concept of an engram. Causal studies, though, are necessary to show that these reactivated putative engram cells indeed function as the internal representation of an experience.

2. Loss-of-function studies

Loss-of-function studies attempt to "capture" critical engram cells and specifically disrupt their function before a memory test. Josselyn and colleagues (*43*) performed the first loss-of-function study at the cell ensemble level providing direct evidence supporting the existence of an engram. An allocation strategy was used to capture putative engram cells supporting an auditory fear conditioned memory in the amygdala lateral nucleus (LA). A small, random population of LA neurons was biased for inclusion (or allocation) into an engram using a neurotropic virus expressing CREB. CREB is a transcription factor that increases neuronal excitability (*44-49*) and dendritic spine density (*45, 50*), thereby biasing the inclusion of infected neurons into an engram. A virus expressing both CREB and an inducible ablation construct was used to specifically kill allocated neurons after training (*43*). Ablating CREB-overexpressing neurons after training robustly disrupted freezing to subsequent tone presentation, as if the engram was erased. [Insert Fig 1 loss-of-function about here.] Importantly, mice were capable of learning a subsequent fear conditioning task (showing overall LA function was not comprised) and ablating a similar number of non-CREB-overexpressing cells (non-engram cells) did not disrupt memory (showing specificity of memory disruption at the cellular level).

Additional studies using diverse methods to permanently or reversibly inactivate allocated or tagged neurons across several brain areas, in many memory tasks produced comparable results (*25, 33, 38, 48, 51, 52*). Together, these findings indicate neurons active

during an experience become engram cells and are indispensable (or necessary) for memory expression.

3. Gain-of-function studies

Gain-of-function studies attempt to induce memory retrieval in the absence of natural retrieval cues, by artificially reactivating engram cells. Tonegawa and colleagues (*53*) provided the first gain-of-function evidence for the existence of an engram. Hippocampal dentate gyrus (DG) neurons active during contextual fear conditioning (in which a context served as the CS and a footshock served as the US) were tagged (*26*) and expressed the excitatory opsin, ChR2 (channelrhodopsin 2; (*54*)). When tested in a non-training context, mice did not freeze. However, photostimulation of tagged engram cells was sufficient to induce freezing, the specific CR (*29*), even though mice had never been shocked in this non-training context. [Insert Fig 2 gain-of-function about here.] Importantly, light-induced freezing was not due to activation of pre-wired learning-independent neural circuits or a simple reflex response as similar photostimulation of tagged DG neurons failed to induce freezing if downstream CA1 neurons were silenced during training (thereby preventing learning) (*55*).

Optogenetic or chemogenetic (*56, 57*) artificial reactivation of tagged or allocated engram cells across several brain regions similarly induced memory expression for a variety of tasks without external retrieval cues (*27, 38, 58-64*). Therefore, artificial engram cell reactivation serves as a sufficient retrieval cue to "reawaken" a dormant/latent engram to induce memory expression, similar to Semon's original definition of ecphory ("the influences which awaken the mnemic trace or engram out of its latent state into one of manifested activity" (*5*) p.12). Together, with observational and loss-of-function findings, these data provide compelling evidence for the existence and manipulability of an engram in the rodent brain.

Mimicry Experiments

In natural memory retrieval, the sensory CS (e.g., the training context) is thought to reactivate engram cells to induce memory expression. The first gain-of-function study (*53*) was designed to mimic this process by directly reactivating engram cells via optogenetics. Optogenetic stimulation of engram cells has also been used to mimic a CS during memory formation. For instance, DG neurons active during novel context (Context A) exploration were

photostimulated when mice later received footshocks in a different context (Context B). Even though mice were not shocked in Context A, they froze. Mice also froze in context B (showing natural memory retrieval), but not in a third distinct context (Context C), indicating freezing was a context-specific, and not a generalized, response (*31*). Both natural and artificial memories could be retrieved by their respective CSs, indicating both memories retained their identities. Similar to a compound CS in which a tone and light both predict footshock, the strength of both memories were roughly 50% of a single "normally-induced" memory, suggesting cue competition between the natural and artificial CSs (cue competition, initially reported by (*65*)). Therefore, false memories may arise when a high valence event occurs or is recalled during the formation of another memory.

Mayford and colleagues (*66*) used a similar approach but tagged active neurons across the brain as mice explored a novel context (Context A). Chemogenetically reactivating these neurons while mice were fear conditioned in Context B produced a "hybrid or synthetic" context representation that was not retrievable by either context alone. However, mice froze in a test session that more closely matched the training conditions (placement in Context B while chemogenetically activating Context A engram cells), suggesting that this hybrid memory incorporated both natural and artificial cues. Differences in the spatial and temporal properties of artificial engram reactivation may account for the discrepant outcomes of these two artificial CS studies.

Neurons active during US presentation have also been tagged in these types of mimicry studies (*67*). Neuronal ensembles active during novel context exploration (CS) and footshock (US) were tagged separately in CA1 subfield of the hippocampus and the basolateral complex of the amygdala, respectively. Homecage synchronous optogenetic activation of these ensembles was sufficient to induce a false memory; mice froze in the tagged (but non-shocked) context, as if the CS and US had been paired.

Finally, one study asked if a memory could be made in the absence of natural stimuli (either CS or US). To satisfy a true memory implantation experiment, this experiment should satisfy several criteria (*68*). First, the "learning experience" should occur entirely within the brain via, for example, direct stimulation of putative CS and US neural pathways. Second, the presence of the implanted memory should be probed via presentation of a "real" external retrieval cue and not just the internal neural cue. Finally, behavioral manifestation of this

memory should reflect the predicted memory content, and be retrieved by the "trained" CS, and not to similar cues. In this study, optogenetic stimulation of a genetically-specific olfactory glomerulus was paired with optogenetic stimulation of either appetitive or aversive neural pathways (*68*). Following this entirely intracranial conditioning, mice showed either an attraction or aversion, respectively, to the real odor that activated this olfactory glomerulus. In short, a memory was made in the absence of experience. These results satisfy the mimicry criterion of experimental evidence outlined by Martin and colleagues (*18, 19*), and as such, provide yet another line of persuasive evidence for the existence of engrams.

WHAT HAS THE STUDY OF ENGRAMS TAUGHT US ABOUT MEMORY?

"Enduring changes" and the engram

The ability to label *in vivo* engram cells supporting a specific memory provided an opportunity to investigate the nature of the "enduring changes" proposed by Semon. Guided by Hebb's influential theory on the critical importance of synaptic plasticity in memory (e.g., (*18, 19*)), Tonegawa and colleagues were the first to show that learning augmented synaptic strength specifically in engram cells. First, one day after training, hippocampal DG granule engram cells tagged during contextual fear conditioning showed greater synaptic strength (higher AMPA/NMDA ratio) and increased spine density at entorhinal cortex junctions than non-engram DG cells (*55*). Second, compared to non-engram CA3 cells, downstream CA3 engram cells were more functionally connected with upstream DG engram cells (*55*). Moreover, the number and sizes of spines on CA1 engram cells tagged during contextual fear conditioning receiving input from CA3 engram cells was greater than non-engram CA1 cells (*69*) (consistent with the notion that engram cells that fire together, wire together (c.f. (*15*)) and this enhanced interregional connectivity between CA3 and CA1 engram cells correlated with memory strength and occluded LTP (suggesting an LTP-like mechanism previously occurred). Similarly, LA engram cells tagged during auditory fear conditioning showed enhanced synaptic connectivity with pre-synaptic neurons (*41, 70*). Finally, shrinking potentiated synapses in primary motor cortex (M1) engram cells supporting a motor memory disrupted subsequent performance of this, and not a similar, motor memory (*71*). Together, these studies are beginning to integrate previous research on synaptic plasticity with engrams and suggest preferential engram cell-to-engram cell connectivity is a necessary part of the enduring changes generated by learning.

Distributed engram ensembles

Although one specific brain region is often examined in memory engram studies, it is generally appreciated that an engram supporting a specific experience may be widely distributed throughout the brain. Engram cell ensembles in different brain regions may support distinct aspects of an experience. For instance, in contextual fear memory, hippocampal (DG, CA3, and CA1) engram cell ensembles may represent the context (*25, 33, 72-74*), while amygdala engram cell ensembles may represent affective/valence information (*53, 55, 59*), and cortical engram cell ensembles may represent distinct sensory information (*63, 75-77*).

Several studies examined brain-wide engram cell ensembles supporting contextual fear memories (*27, 78, 79*) (Roy, et al., 2019). For instance, Frankland and colleagues compared the brain-wide (84 brain regions) distribution of active cells following retrieval of recent (1d after training) versus remote (36d after training) contextual fear memory. Graph theory was used to construct functional connectome "memory maps" (*78*) and identify hub-like regions hypothesized to play privileged roles in memory retrieval. Subsequent chemogenetic inhibition confirmed that these identified hub regions were necessary for subsequent memory expression (*79*). Using a combination of TRAP2 (targeted recombination in active populations) technology and IEG immunohistochemistry to examine overlap between neurons active at contextual fear training and testing, Luo and colleagues (*27*) showed that retrieval of a remote (14d) contextual fear memory engaged more neurons in prelimbic cortex than retrieval of a recent (1d) memory, showing the engram changes over time (consistent with (*80*)). Finally, a preliminary study (Roy et al., 2019) mapped engram ensembles and candidate engram ensembles representing a contextual fear conditioning memory in 409 brain regions by combining the tissue clearing technique SHIELD (*81*) with a newly developed "engram index". Further, this study, using optogenetic and chemogenetic methods, showed that many of these engram ensembles are functionally connected and activated simultaneously by an experience, providing experimental evidence supporting Semon's "unified engram complex" hypothesis. Together, a concept has emerged that an experience is represented in specifically connected multiple engram ensembles distributed in multiple brain regions.

Engrams, place cells and sleep

Location specific firing of CA1 place cells is well established (*82*). Stable place cells may be important in engrams supporting spatial or contextual memories (*83-85*). Recently, McHugh and colleagues (*86*) contrasted the roles of CA1 place cells and engram cells in memory. While mice explored a novel context, engram cells were tagged and place cells identified using tetrode recordings. Most tagged engram cells were also place cells, but the majority of place cells were not tagged. Non-tagged place cells acted like traditional place cells (stable in the same context, but remapping in a novel context). In contrast, tagged-place cells fired in a context-specific manner, but with imprecise spatial information, and fell silent (did not remap) in a novel context. Therefore, engram cells provide general contextual information while non-tagged place cells provide precise spatial information.

Post-encoding reactivation or replay of hippocampal place cell firing, especially during slow-wave sleep (SWS) (*87, 88*), is thought to be important for memory consolidation (*89-92*). During SWS, hippocampal neurons fire in an oscillatory rhythm (termed sharp-wave ripples), tending to co-occur with rhythmic firing of cortical neurons (termed spindles) (*93*). Disrupting either sharp wave ripple-spindle coupling (*94, 95*) or sharp wave ripple-associated replay of hippocampal place cells impairs memory expression (*83, 84, 96, 97*). However, the precise role of these rhythmic oscillations with respect to engram cells is unclear. Sharp-wave ripples promote synaptic depression of CA1 hippocampal neurons (*98, 99*). A recent study suggests CA1 engram cells tagged during context exploration are more likely than non-engram neurons to participate in sharp-wave ripple events, perhaps allowing these engram cells to escape this SWS-induced synaptic depression (*99*). In this way, post-encoding reactivation of engram cells during oscillatory rhythms may help refine an engram by decreasing irrelevant "noise" of non-engram neuronal activity during memory consolidation.

LIFETIME OF AN ENGRAM

Next, we examine lifetime of an engram, starting with its birth.

Birth of an engram: Neuronal competition for allocation to an engram

Josselyn, Silva and colleagues first examined how engrams are formed. They discovered that in a given brain structure, eligible neurons compete against each other for allocation (or

recruitment) to an engram. Neurons with relatively increased intrinsic excitability win this allocation competition to become engram cells [see (*43, 48, 51, 60, 61, 100-104*)]. [insert Fig 3 Allocation about here]. Competitive excitability-based allocation occurs in other brain regions supporting different types of memories [e.g., dorsal CA1 region of hippocampus (*72-74*) (for a contextual fear memory), insular cortex (*105*) (conditioned taste aversion memory), and retrosplenial cortex (*106*) (spatial memory)].

In addition to aversive memories, LA neurons made more excitable during training were also preferentially allocated to an engram supporting a cocaine-cue rewarding memory (*51*). Similarly, increasing the excitability of a small, random portion of piriform cortex principal neurons resulted in their allocation to an engram supporting a rewarding or aversive olfactory memory, depending on the type of training (*107*). Excitability-based neuronal allocation is predicted by computational modeling (*108-110*), occurs endogenously (*41, 70*), and is consistent with previous research implicating intrinsic excitability in the formation of invertebrate memory traces (*111-114*). Together, these findings suggest at any given time, a small portion of eligible neurons in some brain regions are "primed" to become part of an engram (should an event occur), regardless of event valence.

Although stable place cells and engram cells in dorsal CA1 of the hippocampus differ (see discussion above), some mechanisms underlying their formation may be shared. In a given environment, only a small subset of CA1 neurons are place cells, as the majority of CA1 neurons are silent (*115*). Moreover, those neurons with relatively higher excitability immediately before placement in a novel environment are more likely to become place cells in that environment (*116-118*) and experimentally increasing the excitability of an initially silent cell biased this cell toward becoming a place cell (*119, 120*).

Observational and tagging experimental studies agree with computational theories (*121*) that an engram is sparsely encoded. That is, not all neurons within a given brain region become an engram cell supporting a particular memory. The size of an engram within a given brain region is stable and invariant to memory strength. For instance, the size of an LA engram (number of LA engram cells) is similar for an auditory fear conditioned, and a cocaine-cue, memory (*51, 101, 122*) and memory strength does not impact engram size (*70, 101*). Rather, a stronger memory engages a greater number of synapses between engram cells (*69*).

In addition to excitatory neurons, inhibitory neurons also play an important role engram

formation, particularly by constraining engram size. For instance, inhibiting parvalbumin-containing interneurons in the basolateral amygdala complex increased the size of an auditory fear memory engram in the LA through a process of disynaptic inhibition (*122*) whereas inhibiting somatostatin-containing interneurons increased the size of a DG contextual fear memory engram through a lateral-inhibition like process (*123*). Recent studies have also suggested that in the human cortex associative memories are stored in a balance of dormant excitatory-inhibitory engrams and these memories are expressed by disinhibition of inhibitory engrams (*124-126*). Further exploration of excitatory/inhibitory balance in engram formation, storage and retrieval would be much desired and necessary to achieve a complete understanding of how countervailing forces interact to support memory function.

Silent engrams

Engrams may be damaged, such that a memory becomes forever unavailable. However, engrams may also be temporarily silenced or become inaccessible, such that the engram is still "there", but cannot be retrieved by natural means. Silent engrams were first revealed in an experiment in which the protein synthesis inhibitor, anisomycin, was administered immediately after contextual fear conditioning. Consistent with the known retrograde amnesia produced by anisomycin (*124, 125*), mice administered anisomycin after training showed little freezing when replaced in the training context (*55*). However, optogenetic reactivation of DG neurons tagged during contextual fear training was sufficient for memory recovery. [Insert Fig 4 silent engram about here.] Silent DG engram cells (in anisomycin-treated mice) showed weaker physiological (increased synaptic strength) and structural (increased dendritic spine density) alterations than normal engrams cells (in control mice), indicating that disrupting these plasticity processes silenced the engram. However, optogenetic activation of DG engram cells circumvented this plasticity requirement. Consistent with this, genetic restoration of spine density [targeted overexpression of p-21 activated kinase (PAK 1)] also allowed a silent engram to be reactivated by natural retrieval cues (*126*).

That engrams may be silenced by disrupting synaptic efficacy/spine density and, silent engrams re-awakened by enhancing these processes was also shown for auditory fear conditioning (*127*). Rats were trained in a variant of an auditory fear conditioning task in which

the tone CS was replaced by optogenetic activation of axon terminals in the LA from neurons originating in medial geniculate nucleus and auditory cortex. Immediately after conditioning, LTD-type optogenetic stimulation impaired memory, suggesting this stimulation silenced the engram. LTP-type optogenetic stimulation allowed the memory to be retrieved (suggesting engram un-silencing). Subsequent LTD-type optogenetic stimulation again silenced, while LTP-type optogenetic stimulation once again allowed recovery of this memory.

These findings raise the question of whether engrams (and corresponding memories) in other amnesic conditions are truly "lost", or simply inaccessible. Silent engrams were reactivated by artificially stimulating engram cells in amnestic mice used to study the early stages of Alzheimer's disease (AD) (*128, 129*). APP/PS1 mice showed contextual fear memory deficits (*128*). However, optogenetic reactivation of ChR2-labeled DG engram cells induced robust freezing, comparable to control (non-AD) mice (*128*). Consistent with other examples of silent engram cells, DG engram cells in these mice used to study AD showed decreased spine density. However, LTP-type optogenetic stimulation at entorhinal cortex engram cell inputs onto DG engram cells not only restored DG engram cell spine density, but also the ability of natural retrieval cues to elicit memory expression (thereby un-silencing the engram)(*128*). Together, these findings in mice are consistent with reports that memory retrieval in people with early stage AD may be enhanced by particular retrieval cues (*130, 131*), suggesting that under certain conditions, a previously inaccessible memory may be retrieved in human AD, such that some engrams in early-disease brains may be silent, rather than completely lost.

Silent engrams in normal memory

Memory may change with time and circumstance. Are these changes mediated by endogenous engram silencing? This was explored in a social discrimination task in which mice show greater interaction with a novel, than a familiar, mouse. This social discrimination memory lasts roughly an hour after exposure to a familiar mouse (the training event), and is absent 24 hr after training (*132*). The dorsal CA2 to ventral CA1 (vCA1) hippocampal circuit plays a pivotal role in social discrimination (*133*), with a vCA1 engram representing the familiar mouse (*134*). Consistent with the time-course of social discrimination memory, the familiar mouse engram in vCA1 becomes silent an hour after training. However, artificially reactivating this engram 24h after training (when the social discrimination memory normally has dissipated) reinstates social

discrimination memory, as if the trained-but-forgotten familiar mouse is being remembered. Besides artificial engram reactivation, the accessibility of vCA1 engram (and social discrimination memory) is prolonged by interventions such as group housing. These findings provide the first hint that engram silencing may be one way in which the brain normally regulates mnemonic processes. Additional examples of silent engrams in normal memory processes are discussed below.

Silent engrams and extinction

Following conditioning, repeated presentation of the CS stimuli alone (in the absence of the US) produces a gradual decrease of the CR (*65*) - a phenomenon referred to as behavioral extinction. Therefore, after extinction training, the ability of the CS to induce memory expression is diminished, suggesting that the original engram may be silenced. Consistent with this notion, some fear extinction protocols induce synaptic depotentation (reversal of synaptic potentiation induced by fear conditioning) of LA neurons (*135*), and, shortly after extinction training chemogenetic activation of cells tagged brain-wide during context fear training may increase freezing levels (*136*).

With time after extinction training, CRs often recur (spontaneous recovery) (*137*, *138*), showing that the original fear memory is not erased (*139-142*). Moreover, contextual fear extinction may be supported by a novel fear extinction engram in the DG that is distinct from, and suppresses, the contextual fear DG engram with a time-course that corresponds to the emergence of spontaneous recovery (*38*). In this experiment, spontaneous recovery was observed remotely (29d), but not recently (6d), after extinction training. Moreover, the original fear engram was reactivated at the remote, but not recent, memory test. The opposite pattern of results was observed for active cells tagged after extinction training (the presumed fear extinction engram). Interestingly, artificial reactivation of the fear extinction engram prevented spontaneous recovery of the original fear memory, even at remote times. These results suggest the extinction engram suppressed or silenced the original fear engram, but, with time, the fear extinction engram was itself silenced. Activation of a remote DG contextual fear engram (labelled 25d after contextual fear conditioning) itself may also be important for subsequent fear

extinction (*37*). The extent to which DG neurons activated 25d after contextual fear conditioning overlap with DG neurons active during training, however, is unknown [see below (*36*)].

A recent preliminary study showed that fear extinction engram cells are formed in a genetically distinct and "reward-responsive" subpopulation of basal amygdala neurons. These fear extinction neurons suppress the fear neurons also present in basal amygdala and induce appetitive behavior when optogenetically stimulated (*143*). These findings in mouse are consistent with the results of a recent fly study (*144*) and highlight the similarities between fear extinction and reward processes across species.

Silent engrams and time

The representation of a memory in the brain may change with time. For instance, dorsal hippocampal lesions in rodents disrupt expression of contextual fear memories in the days, but not weeks, after training (*145-147*). At more remote times, cortical areas, including anterior cingulate cortex or medial prefrontal cortex (mPFC), become preferentially engaged (*80*). The time-dependent reorganization of memory reflects systems consolidation (*148, 149*). Systems consolidation was recently examined at the engram cell ensemble level, where findings indicate time-dependent silencing of active engrams and conversions of silent engrams to active engrams. (*36, 150*). During contextual fear conditioning, active mPFC neurons were labeled to express ChR2. When placed in the conditioning context, mice showed robust freezing when tested either 2d or 13d after training. However, the engram ensemble components supporting memory retrieval differed with time. Tagged mPFC neurons were reactivated 13d, but not 2d, after training, suggesting the mPFC engram was silent shortly after training, but active at longer delays. DG engram cells showed an opposite pattern; DG engram cells were reactivated shortly after training, but silenced more remotely. Similar to other instances of silent engrams discussed above, the mPFC engram cells shortly after training and the DG engram cells at longer delays after training, had reduced spine density, but optogenetic activation of these silent engrams was sufficient to induce memory retrieval. Interestingly, post-training tetanus toxin-induced inhibition of the input from DG engram cells to mPFC engram cells blocked the maturation of the silent mPFC engram cells to an accessible state, suggesting co-ordinated network function between different engram ensemble components is important in systems consolidation.

Memories may also become less precise and more generalized with time (*151-153*). According to memory transformation theory, changes in memory nature and quality correspond to changes in neural representations, with hippocampal-dependent context-specific memories transforming into gist-like memories stored in cortical structures over time (*154, 155*). The neural processes governing remote memory generalization at the engram level suggest the availability of the DG engram is critical for memory specificity (*156*). In this experiment, shortly after contextual fear conditioning (1 d) mice froze in the training context only, whereas at more remote time points (16 d after training) mice also froze in a non-shocked context. This finding is consistent with previous reports of contextual fear memory generalizing over time (*36, 152*). At the recent, but not remote, time DG engram cells showed greater connectivity to parvalbumin-expressing CA3 basket cells (thereby inhibiting CA3 pyramidal neurons) than non-engram DG cells, suggesting greater feedforward inhibition in DG-CA3 circuits helps maintain memory precision. Interestingly, optogenetic activation of DG engram cells 10 d after training did not induce memory retrieval (suggesting this engram had become unavailable), except if feed-forward inhibition was genetically enhanced. Moreover, mice with genetically-enhanced feedforward inhibition also showed precise memory, even when tested at more remote times. These data suggest that enhanced feedforward inhibition onto CA3 neurons maintains DG engram cell availability and delays the loss of context-specificity associated with remote memories. This finding also highlights another proposed natural state of an engram; an engram may be 1) unavailable (neither natural CSs nor artificial reactivation induces memory expression), 2) silenced (only artificial reactivation is sufficient to induce memory expression), 3) dormant/latent, as initially described by Semon (natural CSs may induce memory expression), or 4) active (inducing memory retrieval).

Engram silencing may represent a continuum, ranging from reactivated to latent to silent to unavailable or lost. Furthermore, different processes may mediate these distinct stages. For instance, similar to silencing a DG engram, post-training anisomycin administration silenced an LA engram supporting an auditory fear memory (*63*). However, if in addition to anisomycin, a peptide to induce autophagy (a mechanism of protein degradation) (*157*) was administered after training, then optogenetic reactivation of inputs to the LA was no longer sufficient to induce memory retrieval, suggesting autophagy damaged (erased), rather than simply silenced, the engram .

From engrams to knowledge

Thus far, we have discussed engrams supporting a single memory. Of course, animals (including humans) learn many things. Some of these experiences may be best remembered as distinct episodes, rich with details (*158-160*). However, in other circumstances it may be advantageous to link related experiences together, thereby creating a general concept or principle (*161-165*). How do different engrams representing different events interact? The mechanisms governing neuronal allocation to an engram supporting a single event also serve to either co-allocate neurons to overlapping engrams (thereby linking events) or dis-allocate neurons to non-overlapping engrams (thereby disambiguating events) (*100, 166-168*). [Insert Fig 5 memory linking and allocation about here.] In this way, relative neuronal excitability is not only critical for initial engram formation, but also in organizing different memories across the brain.

Neurons that are relatively more excitable than their neighbors at the time of an event are more likely to be allocated to the engram supporting that event memory (*100*). Increased excitability in engram cells is also maintained for several hours after an event. Therefore, if a related event occurs in this time-window, these same (or overlapping) engram cells are more excitable than their neighbors and thus co-allocated to the engram supporting the second event memory. Because these two event memories are co-allocated to overlapping engram cells, these two memories become linked (or integrated); thinking of one event automatically makes one think of the second. For instance, LA neurons allocated to one auditory fear memory were co-allocated to a second auditory fear memory if the second event occurred minute-hours (30 min-6h), but not 24h, after the first (*166*). Similarly, co-allocation of CA1 engram cells supporting memories of two distinct contexts was observed if exposure to the contexts was separated by a short time interval (*167*). Behaviorally extinguishing one memory produced extinction for the second memory, even though the second memory was not behaviorally extinguished, indicating that the two memories were functionally linked (*166*) [but see (*167*)]. Co-allocated memories may maintain their unique identity by engaging specific synapses within the shared engram cells (*63*). These data from rodents experiments agree with results from human memory experiments showing the representations of memories for events experienced close in time or with related content overlap, enabling generalization and flexible use of this shared information [e.g., (*163, 169-172*)].

Memory retrieval also transiently reactivates engram cells (*70, 166, 173*). This increase in excitability both enhances memory retrieval precision and efficiency (*173*) and opens a new "co-allocation window" (*166*), perhaps explaining how new information is integrated into pre-existing knowledge.

CONCLUSIONS AND PERSPECTIVES

Together, these data provide compelling evidence for the existence of engrams in rodent brains. We agree with one of the pioneers of cognitive psychology, Endel Tulving, who stated …"As a scientist I am compelled to the conclusion—not postulation, not assumption, but conclusion—that there must exist certain physical-chemical changes in the nervous tissue that correspond to the storage of information, or to the engram, changes that constitute the necessary conditions of remembering. (The alternative stance, that it may be possible for any behavior or any thought to occur independently of physical changes in the nervous system, as all your good readers know, is sheer mysticism)."(*174*) Furthermore, these findings indicate that information may not be represented in single cells (a theory that may have originated with Horace Barlow), instead suggesting the basic unit of computation in the brain is engrams or cell ensembles (similar to Hebb's theory) (*175, 176*). The combination of engram theory and novel tools that allow researchers to image and manipulate engrams at the level of cell ensembles facilitated many important insights into memory function. For instance, evidence indicates that both increased intrinsic excitability and synaptic plasticity work hand-in-hand to form engrams and that these processes may also be important in memory linking, memory retrieval and memory consolidation. Interestingly, disrupting plasticity in engram cells either by disease processes (as in mice that are used to study AD), amnestic drugs (such as protein synthesis inhibitors) or during some natural behaviors (housing condition in social discrimination memory, memory consolidation, and possibly fear extinction training) silences engrams such that they can no longer be accessed by normal sensory cues. However, these studies show that silent engrams and the events they represent are still "there" in the brain. Without studies focused on engrams, the existence of silent engrams, and the potential they hold for memory studies, would be unknown.

There may be a continuum of engram availability. Engrams may be entirely unavailable and not retrievable by even artificial means. Or, engrams may be silenced such that memories may be retrieved by artificially reactivation engram cells. The processes that silence or erase an engram, as well as strategies for un-silencing engrams are a subject ripe for further investigation. That it was possible to artificially reactivate silent engrams in mice designed to study the memory deficits of AD hint at the extraordinary translational potential of this line of research.

Although the term and concept of an engram has been controversial since the time of Semon, sparked by the introduction of new tools, there has been a recent surge in research interest in the engram. These studies have offered important insights into engrams and the memories they represent. However, several key questions remain. First, although the majority of observational studies reveal that the overlap between populations of neurons active during training and testing exceed chance levels, the overall correspondence between these two populations is relatively low (roughly 10-40%, depending on the study). That this overlap is not nearer to 100% suggests a number of possibilities. The methods to label active neurons may be imprecise (either "overtagging" or "undertagging" the "real" engram at training and/or testing). Alternatively, engrams may be dynamic, even over relatively short (days) periods of time, with cells "dropping in" or "dropping out" of the engram as it is refined or consolidated (*177*, *178*). It will be interesting to determine how the mechanisms of engram silencing contribute to and/or interact with this refinement process and the implications this may have on memory quality, precision or strength. Moreover, it will be important to determine how engrams change over more prolonged periods of time. For instance, do all engrams (engrams representing different types of memories such as episodic, semantic or even procedural or motor memories, with different valence) change over time, gradually engaging more cortical regions? Is there a role for top-down (mPFC to hippocampal) processing in the de-maturation of hippocampal engrams and a possible role of silent hippocampal engrams in remote memory recall?

Second, do engrams underlie specific memories in humans, and, if so, how can we leverage our knowledge of engrams in rodents to facilitate memory research in humans? There is good evidence for general engram-like memory representations in humans, but, to date, no compelling findings at the cellular ensemble level [see (*179*)]. To extend knowledge gained in engram studies in rodents to humans, it will be necessary to develop non- to low- invasive

methods to image and manipulate engrams at the single cell or specific ensemble level. Progress in this general area has been made by harnessing the power of reconsolidation (*180-183*) in which engram cells are specifically reactivated by memory retrieval. Pharmacological blockade of reconsolidation and non-invasive techniques that "update" memory during reconsolidation have shown some success in manipulating memories in humans (*184*).

Finally, it is important that the links between neuroscience and artificial intelligence (AI) are leveraged to inform both fields. Understanding how the (biological) brain encodes, stores and uses information, especially at the level of the engram, can help inspire the development of more intelligent machines. For instance, engrams and how engrams serve to link memories and organize information in the "wet brain" may motivate the development of novel algorithms and architectures of "*in silico* brains" to better allow these agents to form generalizations and schemas. In addition, AI, machine learning and deep neural networks may inspire or generate novel, testable theories at the level of the engram for neuroscientist to investigate.

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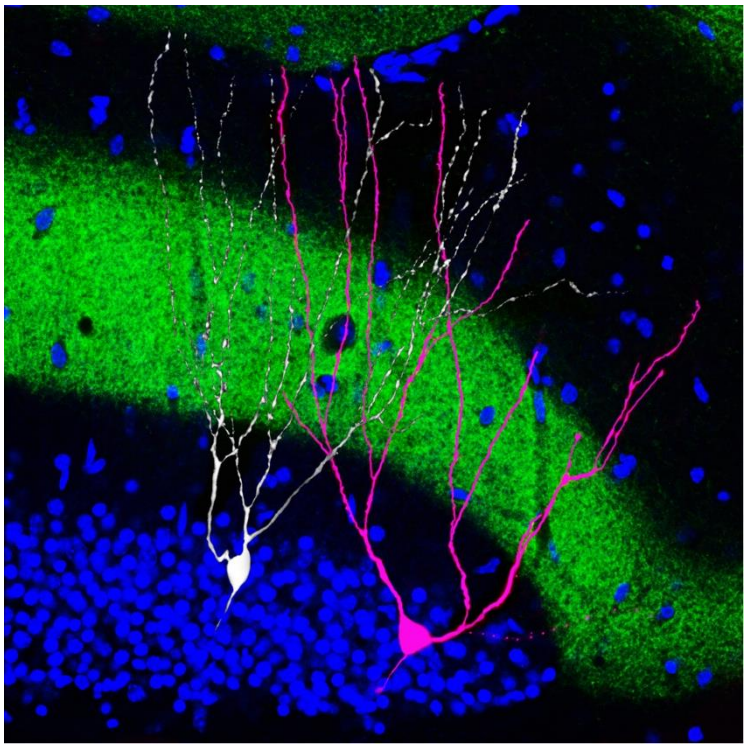
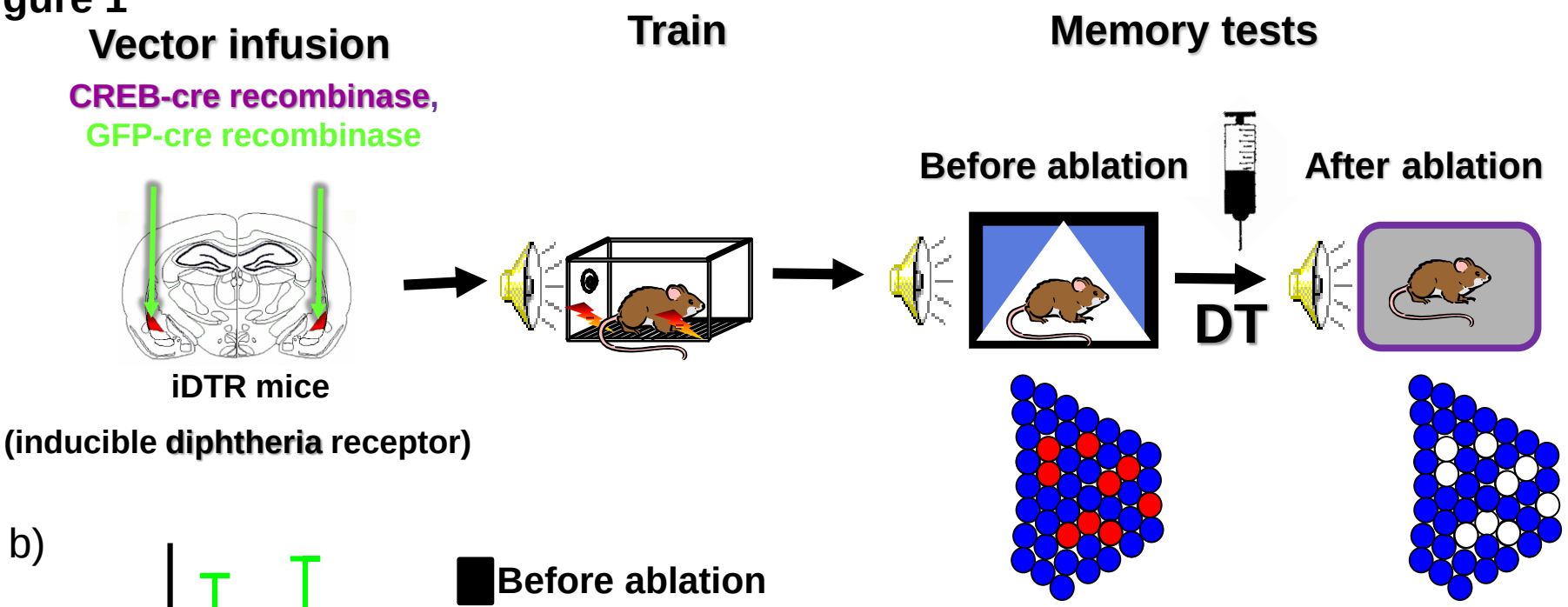


Figure 0. **Pair of biocytin-labelled engram (mCherry^{+}, pink) and non-engram (mCherry^{-}, white) dentate granule cells.** The pink color displayed by the engram cell is the result of the overlap between biocytin (white) and mCherry (red) signals. ChR2-EYFP^{+} Perforant Path axons are labelled in green. The nucei of the upper blade granule cells are labelled by NeuN (blue). A simultaneous patch clamping of these cells revealed enduring synaptic strengthening specifically in the engram-positive cells. From Ryan et al., Science (2014).

Figure 1


Vector infusion
CREB-cre recombinase,
GFP-cre recombinase
iDTR mice
(inducible diphtheria receptor)
Train
Memory tests
Before ablation
DT
After ablation

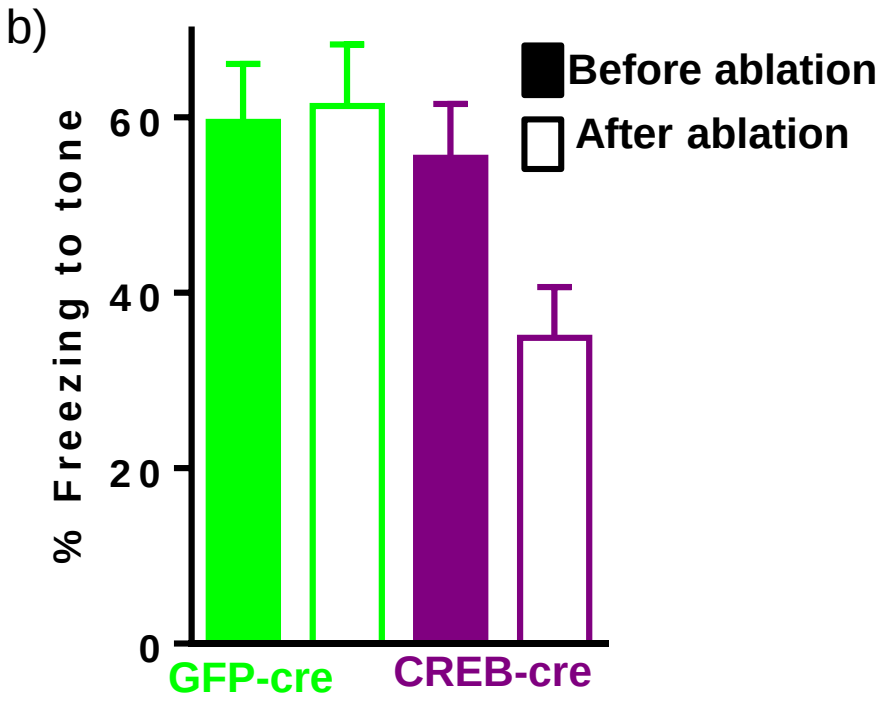

b)
% Freezing to tone
0
20
40
60
Before ablation
After ablation
GFP-cre
CREB-cre

Figure 1. Engram loss-of-function studies showing that selectively ablating neurons allocated to an engram disrupts subsequent memory retrieval. (a) Experimental design. Lateral amygdala (LA) principal (excitatory) neurons overexpressing CREB (infected with a virus expressing CREB) are preferentially allocated to an engram (red) supporting an auditory conditioned fear memory in which a tone is paired with a footshock. In control mice, neurons infected with a virus expressing GFP are not preferentially allocated to an engram (see Han et al., Science, 2007). Infected neurons (overexpressing CREB or GFP) were selectively ablated after training by using cre-recombinase-inducible diphtheria receptor transgenic mice and microinjecting a virus that either expressing CREB+cre recombinase or GFP+cre recombinase. In this way, a systemic injection of diphtheria toxin (DT) ablated only infected cells. (b) Before killing (closed bars) both groups showed robust memory (freezing to the tone CS). However, killing neurons overexpressing CREB disrupted memory whereas killing the same number of GFP-expressing neurons in control mice had no effect on memory. Adapted from Han et al., Science, 2009.

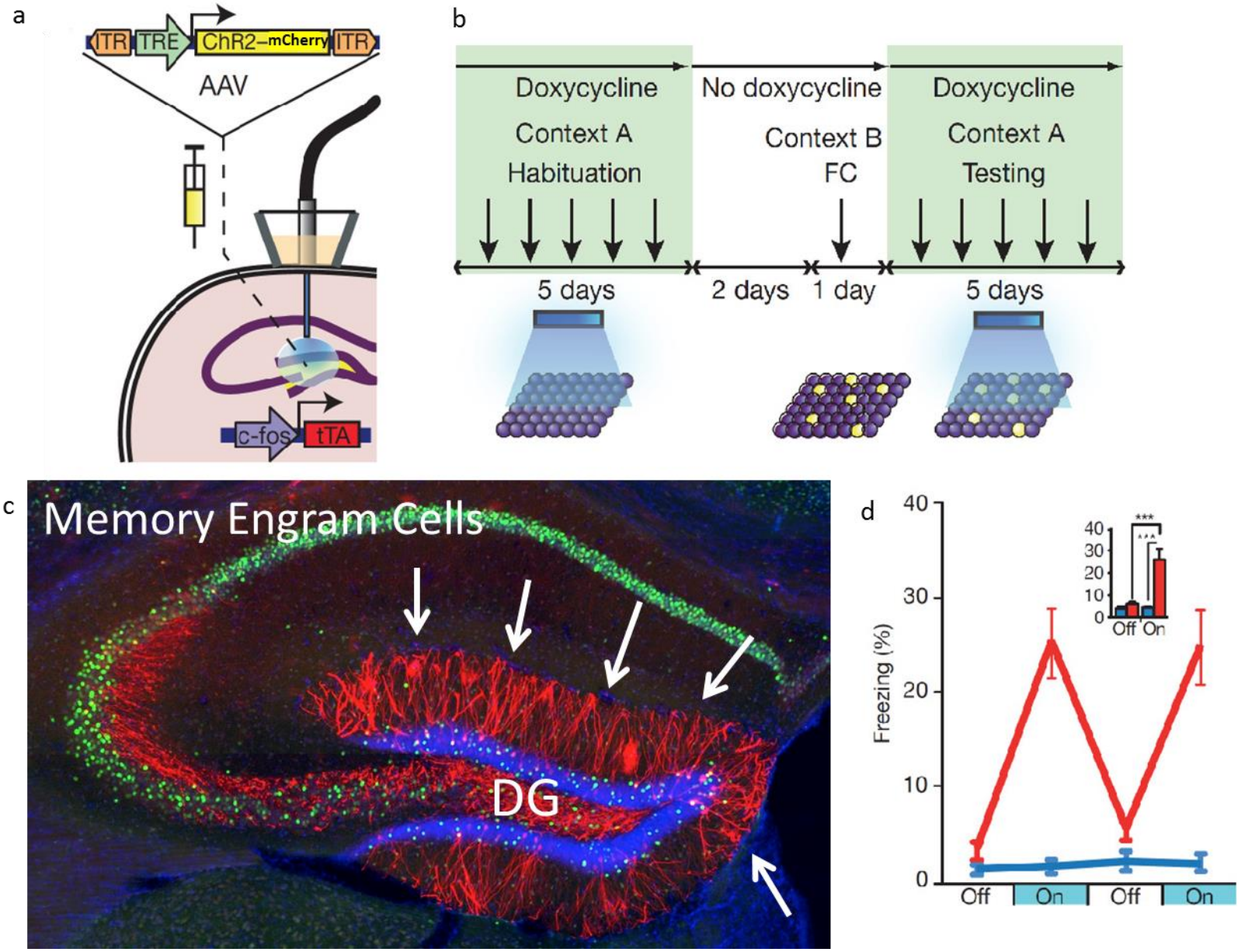

a
ITR
TRE
ChR2–mCherry
ITR
AAV
c-fos
tTA
b
Doxycycline
Context A
Habituation
No doxycycline
Context B
FC
Doxycycline
Context A
Testing
5 days
2 days
1 day
5 days
c
Memory Engram Cells
DG
d
Freezing (%)
40
30
20
10
0
Off
On
Off
On
***
40
30
20
10
0
Off
On

Figure 2. **Optogenetic activation of memory engram cells induced memory recall.** (a) Basic composition of the system. Virus expressing TRE-ChR2-mCherry and optic fibers are targeted bilaterally into the dentate gyrus (DG) of transgenic mouse line expressing c-fos-tTA. (b) Behavior schedules. Animals were habituated to context A with light stimulation while on Dox, trained in context B while off Dox, and texted again in context A with light stimulation while on Dox. (c) In the absence of Dox, DG neurons that are active during the formation of a memory are labeled with ChR2-mCherry. Note that not only the cell bodies but also dendrites and axonal fibers are stained (red). (d) Although light had no effect during pretraining habituation sessions (blue line), the animals showed light-dependent freezing behavior posttraining (red line), indicating the light-induced recall of a fear memory.

Figure 3

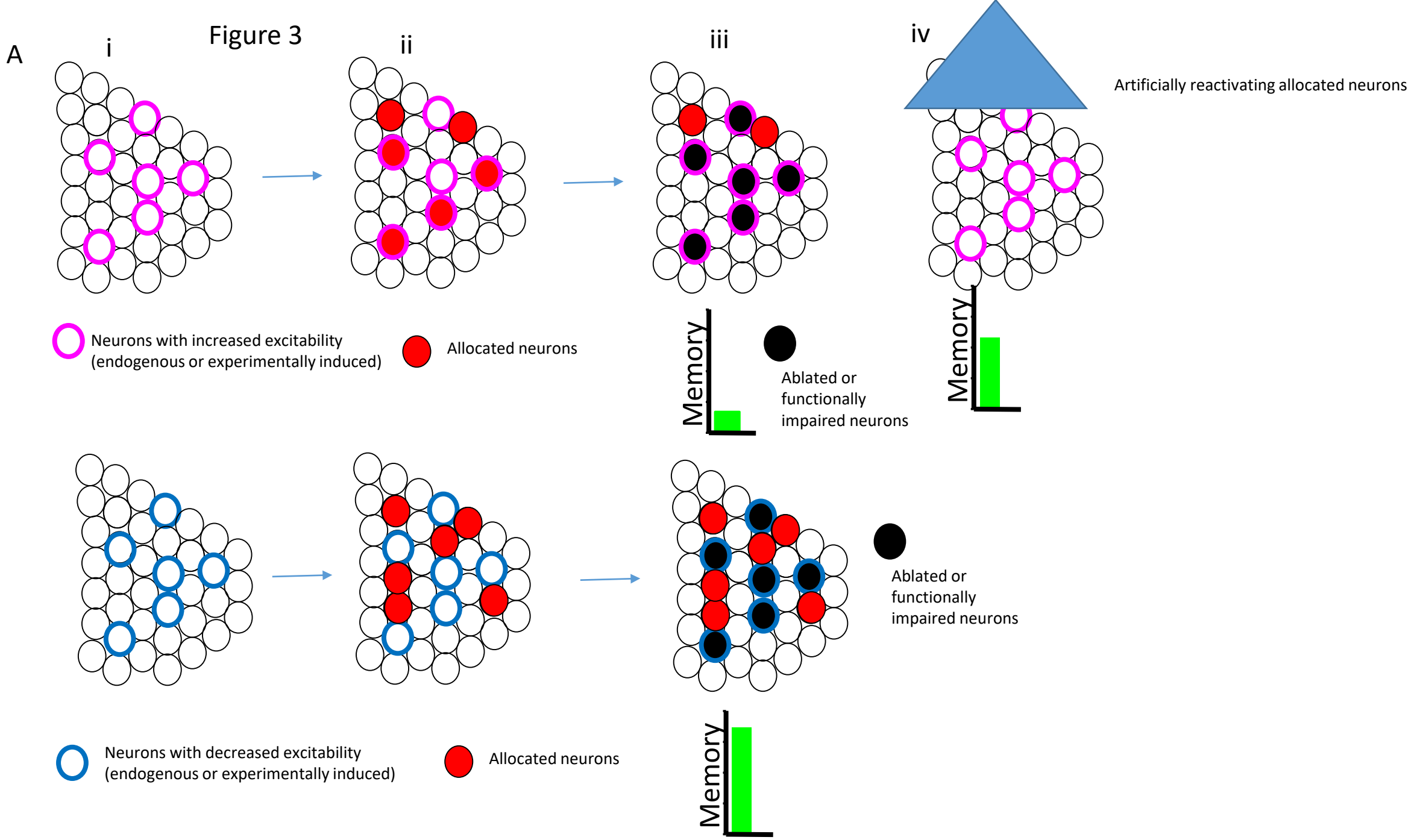

A
i
ii
iii
iv
Artificially reactivating allocated neurons
Neurons with increased excitability
(endogenous or experimentally induced)
Allocated neurons
Memory
Ablated or
functionally
impaired neurons
Memory
Ablated or
functionally
impaired neurons
Neurons with decreased excitability
(endogenous or experimentally induced)
Allocated neurons
Memory

Figure 3. Neuronal allocation to an engram.

Eligible neurons compete for allocation to an engram (solid red neurons) supporting a memory and neurons with increased relative excitability at the time of training “win” this competition for engram allocation.

A) i-ii) Allocation occurs endogenously (neurons outlined in pink), but excitability of a small portion of neurons can also be experimentally induced via CREB overexpression or by using excitatory optogenetic or chemogenetic actuators. Iii) Once allocated, these neurons become necessary/indispensable for subsequent memory expression. Ablating these neurons (neurons in black), and not a similar number of non-allocated neurons, results in amnesia for that particular event. iv) furthermore, artificially reactivating allocated neurons induces memory expression in the absence of a sensory retrieval cue.

B) i-ii) Eligible neurons with relatively decreased excitability (neurons outlined in blue) are excluded from the engram. iii) Ablation or disrupting the function of these neurons does not impact memory.

Figure 4: an adaption of this figure

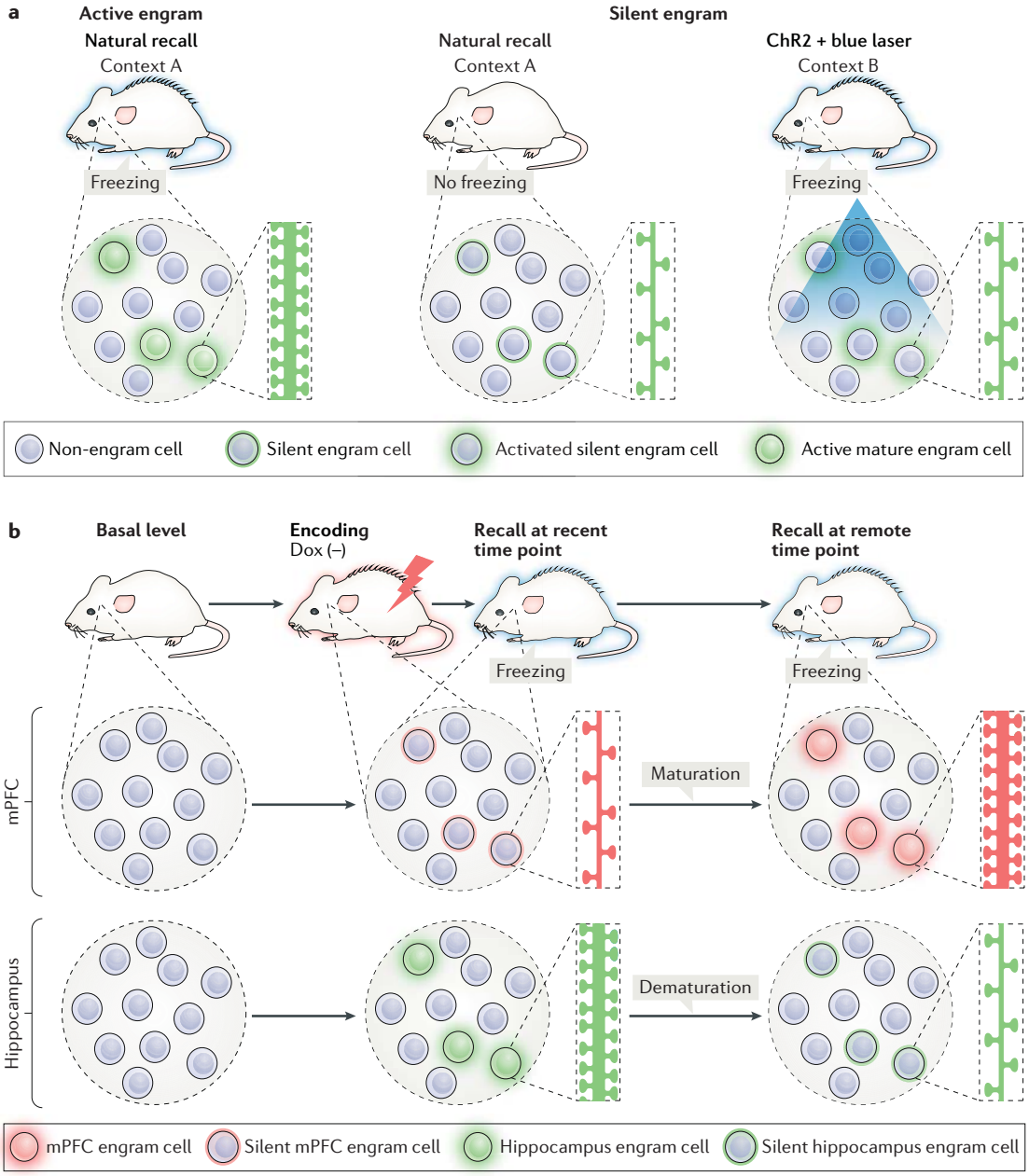


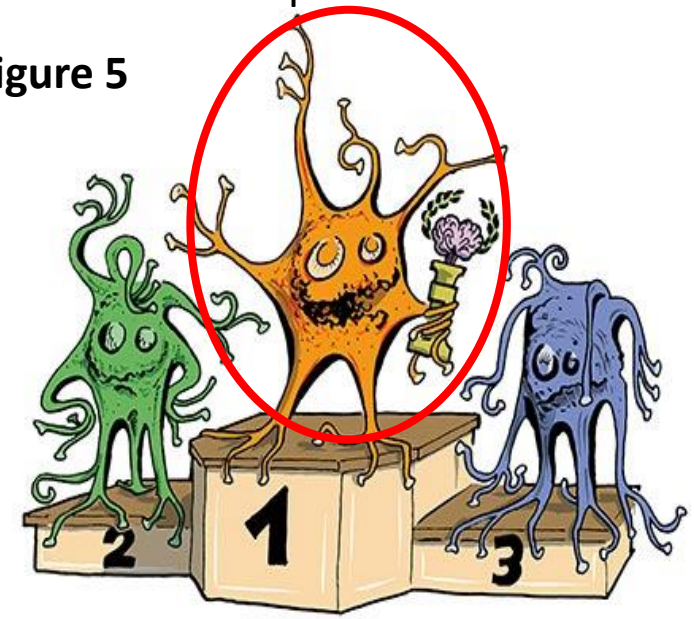
Fig. 3 | **Silent and active memory engrams. a** | Active engram cells typically show dense spines and are reactivated by natural cues. For example, in the left panel, the engram cells are reactivated by re-exposure to a context (context A) previously associated with an aversive stimulus, triggering activation of these cells and an appropriate behavioural response (freezing). Silent engram cells, such as those shown in the right two panels, contain more sparse spine density and are not reactivated by natural cues; however, if tagged with the light-activated channel rhodopsin 2 (ChR2), they can be artificially reactivated with blue laser light and can produce memory retrieval even in the absence of contextual cues[52,56,63,64]. **b** | In one study, medial prefrontal cortex (mPFC) engram cells were rapidly formed during contextual fear conditioning on day 1 and labelled with doxycycline (Dox) removed from the animal's diet. However, they were not reactivated with natural recall cues and displayed low spine density at recent time points. The immature mPFC engram cells functionally, structurally and physiologically matured during the subsequent few weeks and were active during retrieval at a remote time point, and displayed increased spine density. Conversely, hippocampal engram cells were rapidly formed during day 1 of training, at which point they were also functionally, structurally and physiologically mature. They gradually became silent with time, accompanied by a reduction in the density of dendritic spines[56].

unidirectional relationship between tagged, but inactive, and active cells.

We expect that there is a diverse set of underlying molecular and cellular features that define silent and active memory engram cells. One common difference between these cells that has been noted in all the aforementioned studies is a paucity of dendritic spines in the silent engram cells compared with their active counterparts[52,56,63]. There are also a number of genetic changes that take place during learning that are essential for

Figure 5

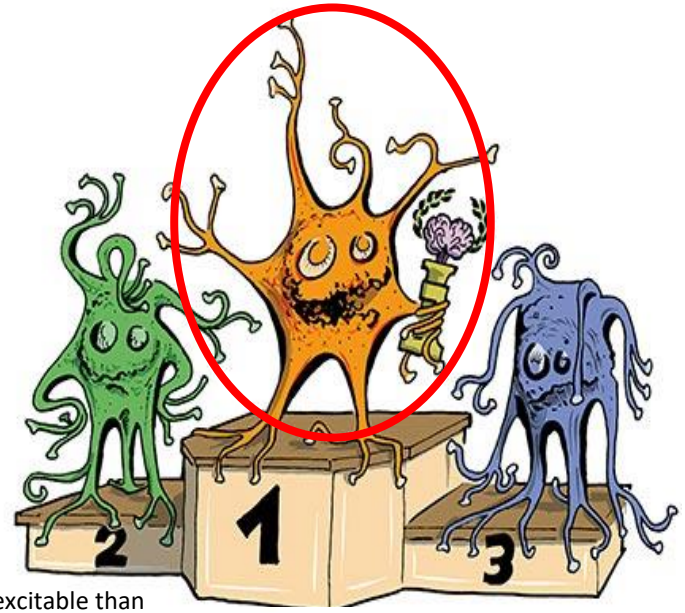
Experience 1



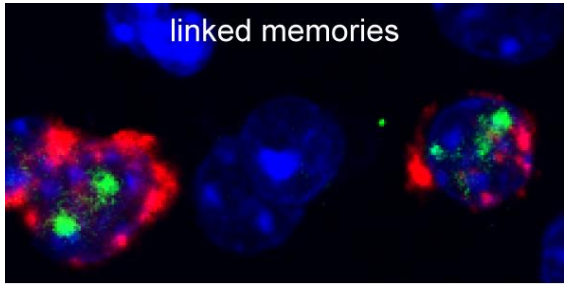
Neurons with increased excitability “win” the competition for allocation to an engram

6 h

Experience 2



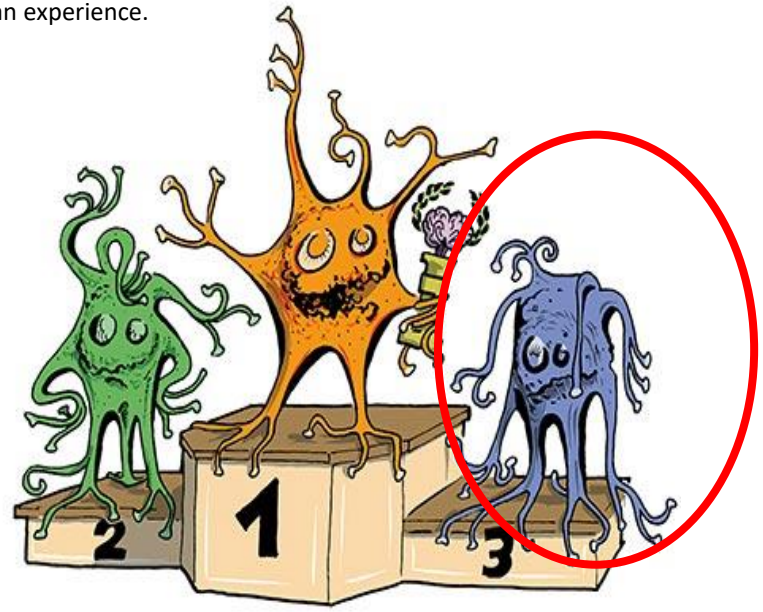
Allocated neurons remain more excitable than neighbors for some time after an experience.

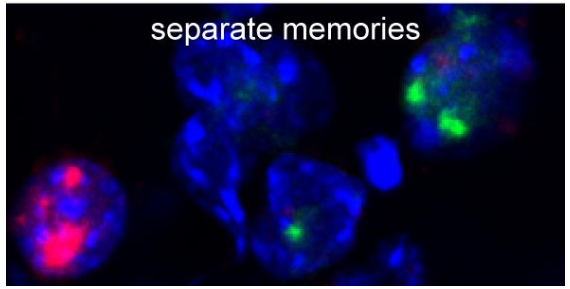


If a second related experience occurs within this time-window, a similar population of neurons are also allocated to the second memory.
By virtue of co-allocation, these two memories are linked.

24 h

After some time, allocated neurons become less excitable than their neighbors (“refractory”) and if a second experience occurs in this time window, a new population of more excitable neurons wins the competition for allocation to the engram supporting the memory.





By virtue of dis-allocation, these two memories are remembered separately.

Figure 5: Memory allocation and linking.

A) Related experiences occurring within a short-time period may be linked by virtue of co-allocation to overlapping engrams. Neurons (orange) with increased relative excitability at the time of Experience1 are allocated to the engram supporting the memory for that experience. These allocated neurons remain more excitable than their neighbors for some time after an experience (here, depicted as 6hr as for lateral amygdala pyramidal neurons and auditory fear conditioning). If a related experience occurs within this time window, these neurons allocated to Experience1 are still more excitable than their neighbors and tend to be allocated to the engram supporting the memory of Experience2. This co-allocation process links memories, such that remembering Experience1 reminds one of Experience2. This co-allocation is depicted in the histology figure at right. Neurons supporting Experience1 are visualized by arc mRNA (red) while Neurons supporting Experience2 are visualized by homer1a mRNA (green). Note overlap.

B) Neurons allocated to Experience1 gradually decrease their excitability and enter into a "refractory-like" phase (possibly with relatively decreased excitability). If Experience2 occurs during this time window, previous "loser" neurons are allocated to Experience2 as they are now more excitability than their neighbors. The engrams for the two experiences are dis-allocated (non-overlapping) and the two experiences are remembered separately. This dis-allocation is depicted in the histology figure at right. Neurons supporting Experience1 are visualized by arc mRNA (red) while Neurons supporting Experience2 are visualized by homer1a mRNA (green). Note lack of overlap. Adapted from Rashid et al., Science 2016