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Memory Engram Cells Have Come of Age

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The idea that memory is stored in the brain as physical alterations goes back at least as far as Plato, but further conceptualization of this idea had to wait until the 20th century when two guiding theories were presented: the "engram theory" of Richard Semon and Donald Hebb's "synaptic plasticity theory." While a large number of studies have been conducted since, each supporting some aspect of each of these theories, until recently integrative evidence for the existence of engram cells and circuits as defined by the theories was lacking. In the past few years, the combination of transgenics, optogenetics, and other technologies has allowed neuroscientists to begin identifying memory engram cells by detecting specific populations of cells activated during specific learning epochs and by engineering them not only to evoke recall of the original memory, but also to alter the content of the memory.

History and Definition of Memory Engrams

Does the brain store memories? This seemingly obvious theme in contemporary neuroscience was actually hotly debated by leading scholars of learning and memory as recently as a century ago. For some, it was obvious that memory is represented in the brain (that is, physically), but others argued that it is stored in the mind (that is, psychically) (Bergson, 1911; McDougall, 1911). In this paper, we will review the recent advances demonstrating that memory is indeed held in specific populations of neurons, referred to as memory engram cells, and their associated circuits. We will then sketch out a new perspective in the neuroscience of learning and memory, including potential applications for the development of therapeutic methods for brain disorders.

Semon's Engram Theory of Memory

In the first decade of the 20th century, Richard Semon, a German scientist who wrote two books on this subject (Semon, 1904, 1909), advocated the physical theory of human memory. Unfortunately, Semon's contributions were almost completely ignored by mainstream psychologists concerned with the human memory process until the late 1970s and early 1980s, when Daniel Schacter, James Eich, and Endel Tulving revived Semon's theory (Schacter, 1982; Schacter et al., 1978).

Semon coined the term "engram," which he defined as "…the enduring though primarily latent modification in the irritable substance produced by a stimulus (from an experience)…" (Semon, 1904). "Engram" is roughly equivalent to "memory trace," the term used by some contemporary neuroscientists. Semon's memory engram theory was built on two fundamental postulates termed the "Law of Engraphy" and the "Law of Ecphory" for memory storage and memory retrieval, respectively. The Law of Engraphy posits that "All simultaneous excitations (derived from experience)…form a connected simultaneous complex of excitations which, as such acts engraphically, that is to say leaves behind it a connected and to that extent, separate unified engram-complex," (Semon, 1923). The Law of Ecphory on the other hand posits that "The partial return of an energetic situation which has fixed itself engraphically acts in an ecphoric sense upon a simultaneous engram complex," (Semon, 1923). Thus, Semon's view of retrieval is reintegrative. Only part of the total situation (i.e., stimuli) at the time of storage needs be present at the time of recall in order for retrieval of the original event in its entirety to occur (Schacter et al., 1978). Semon's concept about memory retrieval is evidence for his amazing insightfulness, because it is nothing but the process of "pattern completion" theorized (Marr, 1970) and experimentally demonstrated many decades later (Leutgeb et al., 2004; Nakazawa et al., 2003).

The Contemporary Version of Semon's Engram Theory and the Definition of Engram Cells

Semon's conceptualizations of the memory process were novel at his time and were remarkably predictive of our contemporary state of memory research. However, he did not elaborate on the biological basis for the "simultaneous excitations" and "a connected unified engram complex." This is not surprising considering that the theory was put forward nearly a century before the rapid development of molecular, cellular, and genetic biology, and sophisticated imaging and electrophysiological technologies for the analysis of the nervous system. Incorporating our current knowledge about neurons, synaptic connections, and neuronal circuits into Semon's memory engram theory, we propose usage of the terms engram, engram cells, and other associated terminologies in these contemporary contexts as follows:

- "Engram" refers to the enduring physical and/or chemical changes that were elicited by learning and underlie the newly formed memory associations.
- "Engram cells" are a population of neurons that are activated by learning, have enduring cellular changes as a consequence of learning, and whose reactivation by a part of the original stimuli delivered during learning results in memory recall. Note that this goes beyond a correlational definition of the term.

- "Engram cell pathway" is a set of engram cells for a given memory connected by specific neuronal circuits. It's important to note here that these connections don't necessarily have to be direct.
- An "engram component" is the content of an engram stored in an individual engram cell population within the engram cell pathway.
- "Engram complex" refers to the whole engram for a given memory that is stored in a set of engram cell populations connected by an engram cell pathway.

The last three terms were introduced because the latest studies on engram cell populations have indicated that an engram of a given memory is not necessarily located in a single anatomical location, but is distributed in multiple locations connected in a pattern specific to the given memory, forming an "engram cell pathway." The term "engram component" denotes not necessarily the specific physiological content of the engram held by a given population of engram cells, but rather the type of represented mnemonic information.

Early Attempts to Localize Memory Engrams

Decades after the English translation of Semon's original book was published (Semon, 1921), the American psychologist Karl Lashley pioneered a systematic hunt for engram cells in the rodent brain by introducing lesions of varying sizes into different sites of the cerebral cortex and attempting to find associations of each of these lesions with the ability of the animals to solve a maze task. The results showed that the behavioral impairments were due to lesions introduced throughout the brain and that the severity of the impairments was proportional to the size of the lesions wherever the lesions were introduced. Based on these results, Lashley concluded that the putative memory engram cells are not localized in the cerebral cortex, leading him to formulate the Mass Action Principle (Lashley, 1950). As discussed below, Lashley's notion that engram cells for a specific memory are spread broadly and indiscriminately throughout the brain has not been supported by subsequent studies for at least several types of memory, including episodic memory. It is conjectured that Lashley's failure in identifying localized engram cells is because the maze tasks he used were too complex and required multiple regions of the cerebral cortex, and/or the primary sites of the storage of this type of memory may be in subcortical regions. Lashley's extreme view was wrong, but as will be discussed later in this article, a certain type of memory (e.g., contextual fear memory) could be distributed over limited, but multiple, brain regions (e.g., hippocampus and amygdala, etc.).

Years later, Canadian neurosurgeons Wilder Penfield and Theodore Rasmussen (Penfield and Rasmussen, 1950) serendipitously obtained the first tantalizing hint that episodic memories may be localized in specific brain regions. As a pre-surgery procedure, Penfield applied small jolts of electricity to the brain to reveal which regions were centers for causing seizures. Remarkably, when stimulating parts of the lateral temporal cortex, approximately 8% of his patients reported vivid recall of random episodic memories (Penfield and Rasmussen, 1950): one patient exclaimed, "Yes, Doctor, yes, Doctor! Now I hear people laughing - my friends in South Africa … Yes, they are my two cousins, Bessie and Ann Wheliaw." Another patient reported, "I had a dream. I had a book under my arm. I was talking to a man. The man was trying to reassure me not to worry about the book." This study had the first glance at what geneticists call "gain-of-function" or "sufficiency" evidence for the notion that the lateral temporal lobe (LTL) region harbors a biological locus for episodic memory. This work was complemented by a study conducted several years later by the American neurosurgeon William Scoville and Canadian neuropsychologist Brenda Milner (Scoville and Milner, 1957) that provided "loss-of-function" or "necessity" evidence. To treat the epileptic seizures of a young man (Henry Molaison [H.M.], who suffered seizures caused by a bicycle accident), Scoville resected a large portion of the medial temporal lobes from both hemispheres, including the hippocampus and the adjacent brain areas. As a consequence of this surgery, H.M. lost his ability to form new episodic memories (anterograde amnesia) as well as the ability to recall memories of episodes and events that occurred to him within a year prior to his surgery (graded retrograde amnesia). H.M.'s other types of memory, such as motor memory, were largely unaffected, indicating that episodic memories may be specifically processed in the MTL and, in particular, in the hippocampus.

These pioneering studies led to a notion that at least some types of memory, in this case episodic memory, may be stored in a localized brain region. In the meantime, memory has been classified into multiple types—declarative or explicit memory and non-declarative or implicit memory. Both explicit and implicit memories are further classified into subtypes, each of which is supported by one or more specific brain areas or systems (Squire, 2004). Numerous efforts have been made during the past 30 years to identify the sites where each of these types of memory is located by using lesion, physiological, or fMRI imaging methods combined with behavioral paradigms. Some of these efforts led to the identification of brain regions or brain systems that are crucial for their respective type of memory. Indeed, many of these studies advanced the field toward a better understanding of memory mechanisms (e.g., Olds et al., 1972; Fuster and Jervey, 1981; Miyashita, 1988) but could not identify a specific subpopulation(s) of neurons in these brain regions or systems that would satisfy all the criteria for engram cells as defined in our proposal of a contemporary definition of engram cells mentioned above (see Observational Studies). Meeting these criteria has required a combinatorial use of new technologies, like those harnessing immediate early genes (IEGs), transgenics, optogenetics, pharmacogenetics, in vitro and in vivo physiology of single cells, and behavioral paradigms. This has recently been accomplished, but thus far mainly for hippocampus- and/or amygdala-dependent classical conditioning memories. Thus, in this review, most (but not all) of our discussion will concern this type of memory. Readers are referred to other reviews for the discussion of earlier efforts to identify brain regions or systems that play important roles in various forms of memory (Horn, 2004; Horn et al., 2001; Martin and Morris, 2002; Christian and Thompson, 2003; Weinberger, 2004).

Identification of Engram Cells

The general criteria for the inclusion of a study in this review article is whether it implicated a specific subpopulation of

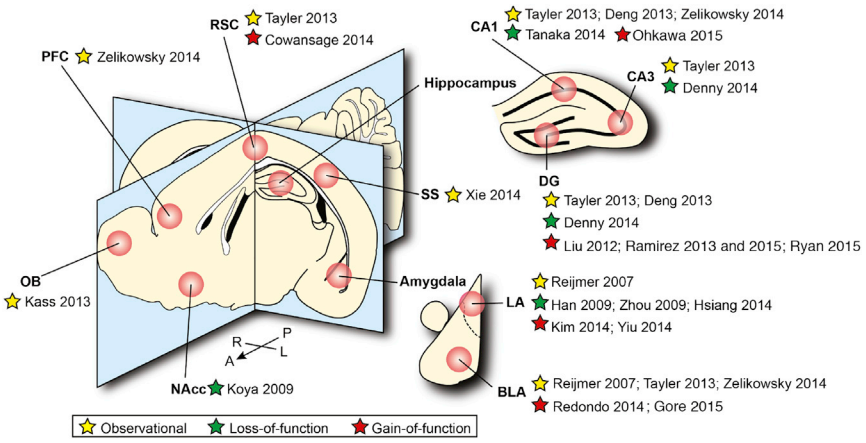

Figure 1. Anatomical Locations of Memory Engram Cell Populations Identified

Memory engram cell populations in different areas of the brain, as indicated by different types of supporting evidence (observational, loss of function, and gain of function) and representative studies published. Abbreviations: OB, olfactory bulb; PFC, prefrontal cortex; RSC, retrosplenial cortex; NAcc, nucleus accumbens; SC, sensory cortex; LA, lateral amygdala; BLA, basolateral amygdala.

neurons within a specific brain region in a particular memory as monitored by behavioral experiments. To demonstrate that specific populations of neurons qualify as cells harboring a component of the engram complex, multiple conditions must be met according to our proposed definition. One must demonstrate that these cells are activated by learning, that they undergo enduring physical or chemical changes, and that their reactivation results in recall of the originally formed memory. To design and conduct an experiment that will satisfy all the criteria of the definition at once seemed daunting. Thus, given the limited technologies available at the time of each study, the search for memory engrams and engram cells has advanced until recently with a limited goal in mind—namely to satisfy some, but not all, of the criteria.

Three Types of Supporting Evidence

The search for memory engrams conducted to date can be divided into the following types: observational, loss-of-function, and gain-of-function experiments (Gerber et al., 2004; Martin and Morris, 2002). Observational studies demonstrate correlations between certain activities of a studied cell population and the behavioral expression of a specific memory; loss-of-function studies show that a certain population of neurons is necessary for the behavioral expression of a specific memory; and gain-of-function studies indicate the activation of a certain population of neurons is sufficient for the behavioral expression of the memory (Figure 1, Table 1). Among the three types of evidence, evidence obtained by observational studies is usually non-causal and therefore weaker. Loss-of-function evidence is stronger because it reveals a specific cell population necessary for the expression of the memory, and gain-of-function evidence is the strongest because it demonstrates that activation of a specific cell population is sufficient to elicit the expression of memory. However, all of these types of evidence must be supplemented by a demonstration of learning-induced enduring changes in the putative engram cells.

Observational Studies

Many observational studies have implicated selected populations of neurons in specific memories across species, although none of these cells could entirely satisfy the proposed definition of engram cells. Among early studies, and across multiple modalities, notable and pioneering examples but which still belong to this category include Olds et al. (1972), Fuster and Jervey (1981), and Miyashita (1988). For instance, Olds et al. (1972) recorded electrical activity from multiple cortical and subcortical areas and found a variety of response latencies to auditory conditioned stimuli. The authors subsequently proposed that a subset of the brain areas analyzed (i.e., those in which response latencies matched or were shorter than responses in the inferior colliculus) indeed contained cells that comprised a ''learning center'' and were thus putative sites involved in processing a mnemonic record. A decade later, Fuster and Jervey (1981) recorded single-cell activity from the inferotemporal (IT) cortex of monkeys performing a visual delayed matching-to-sample task. Many cells responded differentially to the colors of the stimuli, and notably, many cells also responded differentially to color depending on whether or not attention mechanisms were engaged, thus demonstrating their behaviorally relevant role. Fittingly, the authors demonstrated correlations of these neuronal activities to the encoding, retention, and retrieval of visual information. Then, in 1988, Miyashita revealed a neuronal correlate of visual long-term memory by studying how the anterior ventral temporal cortex represented stimulus-stimulus associations. By training monkeys to perform a visual memory task and simultaneously recording from over 200 neurons, Miyashita found that single neurons could respond conjointly to temporally related, albeit geometrically dissimilar, stimuli (i.e., these neurons displayed stimulus selectivity during learning and could then become associated with unrelated stimuli), thus demonstrating a neuronal correlate of associative visual memory.

More recently, in flies, when a particular odor was paired with foot shock, defined neurons within the olfactory learning pathway, such as those in the antennal lobes and mushroom bodies, changed their responses selectively toward the odor used in the training, suggesting that cue-specific memory traces were formed within these cell populations as a result of learning (Liu and Davis, 2009; Yu et al., 2006). Similar neuronal activity changes induced by olfactory associative learning have also been reported in mice (Kass et al., 2013).

By examining the expression of IEGs such as c-Fos and Zif268 (Flavell and Greenberg, 2008), several groups of researchers found that selected cell populations active during the acquisition of a fear memory were preferentially reactivated during the recall of that memory in different areas of the mouse brain, such as the amygdala (Reijmers et al., 2007), the hippocampus (Deng et al., 2013; Tayler et al., 2013), multiple layer II cortical areas including the sensory cortex (Xie et al., 2014),

Table 1. Three Lines of Evidence for Memory Engram Cell Populations

Evidence	Technology	Brain Area(s)	Reference(s)
Gain of function	c-Fos-tTa/TetO-ChR2/ArchT	HPC, BLA	Liu et al., 2012; Ramirez et al., 2013; Redondo et al., 2014; Ramirez et al., 2015; Ryan et al., 2015; Ohkawa et al., 2015
	c-Fos-tTa/TetO-ChEF	RSC	Cowansage et al., 2014
	enhanced neural excitability via TRPV1 and capsaicin system	LA	Kim et al., 2014
	enhanced neural excitability via CREB overexpression, hM3DqDREADDs, ChR2	LA	Yiu et al., 2014
	c-Fos-ChR2	BLA	Gore et al., 2015
Loss of function	HSV-mediated CREB overexpression; inducable diptheria - toxin system	LA	Han et al., 2007, 2009
	CREB overexpression via allatostatinG protein-coupled receptor (AlstR)/ligand system	LA	Zhou et al., 2009
	enhanced neural excitability via CREB overexpression, hM3Dq DREADDs	LA	Hsiang et al., 2014
	cFos-lacZDaun02 inactivation system	NAcc	Koya et al., 2009
	cFos-tTa/TetO-CRE and AAV-FLEX-ArchT	HPC	Tanaka et al., 2014; Matsuo, 2015
	Arc-CreER^{T2}x R26R-STOP-floxed-EYFP	HPC	Denny et al., 2014
Observational (new studies)	catFISH	PFC, HPC, BLA	Zelikowsky et al., 2014
	in vivo optical imaging	OB	Kass et al., 2013
	c-Fos-tTA-TetO-H2B-GFP	neocortex, HPC, BLA	Tayler et al., 2013
	in vivo optical imaging	layer II cortex	Xie et al., 2014
	c-Fos-tTA/TetO-tauLacZ	LA, BLA	Reijmers et al., 2007
	c-Fos-tTA/TetO-tauLacZ	HPC	Deng et al., 2013
Observational (examples from older studies)	in vivo electrophysiology	various	Olds et al., 1972
	in vivo electrophysiology	inferotemporal cortex	Fuster and Jervey, 1981
	in vivo electrophysiology	inferotemporal cortex	Miyashita, 1988

Representative studies on memory engram cell populations categorized by types of supporting evidence (observational, loss of function, and gain of function), with methods used, brain areas involved, and publication listed.

and the prefrontal cortex (Zelikowsky et al., 2014). In humans, single-unit recordings identified cells in the hippocampus and surrounding areas that were reactivated only during the free memory recall of a particular individual, landmark (Quiroga et al., 2005), or episode (Gelbard-Sagiv et al., 2008). Collectively, these observational experiments showed that the activity of defined neurons is correlated with selected memories, suggesting engram cells may be contained in the populations of the neurons studied.

Loss-of-Function Studies

Unlike the aforementioned observational studies, loss-of-function studies manipulate the system by either eliminating or inhibiting memory-related neuronal populations to see if such manipulations cause the impairment of a memory. By randomly overexpressing the transcription activator cAMP response element-binding protein (CREB) in a small population of neurons in the lateral amygdala (LA), a group of researchers could make these cells more likely to be recruited to become a part of putative engram cells during a subsequent fear conditioning training (Han et al., 2007). Moreover, by selectively ablating these cells, but not a random population of neurons in the same region, using a diphtheria toxin-based method (Han et al., 2009) or inhibiting these cells with allatostatin (Zhou et al., 2009), two groups of researchers were able to interfere with the recall of the associated fear memory in mice. A similar technology has been used to identify the necessary role of retrosplenial cortex neurons in spatial navigation memory (Czajkowski et al., 2014). More recently, using an activity-dependent and inducible system based on the promoter of IEG *Arc* and a Cre recombinase activated by Tamoxifen, Denny et al. (2014) labeled neuronal populations that were activated in either the dentate gyrus (DG) or CA3 of the hippocampus during the acquisition of a contextual fear memory and subsequently inactivated these cells using optogenetics. This resulted in impairment of fear memory recall (Denny et al., 2014). Another study found similar memory impairments when labeled CA1 neurons were inhibited (Tanaka et al., 2014). Importantly, this study also showed that if a CA1 cell subpopulation that would otherwise be active during the encoding of an overlapping contextual representation were inhibited, the new representation would simply be stored in other CA1 cells instead. Thus, engrams can be stored in stochastically varying CA1 cell populations. In other words, inhibiting putative engram cells inhibits recall of that labeled memory but does not inhibit the learning of new memories of similar contextual content (but see Matsuo, 2015). Thus, the CA1 cells are not hard-wired to a given perceptual input.

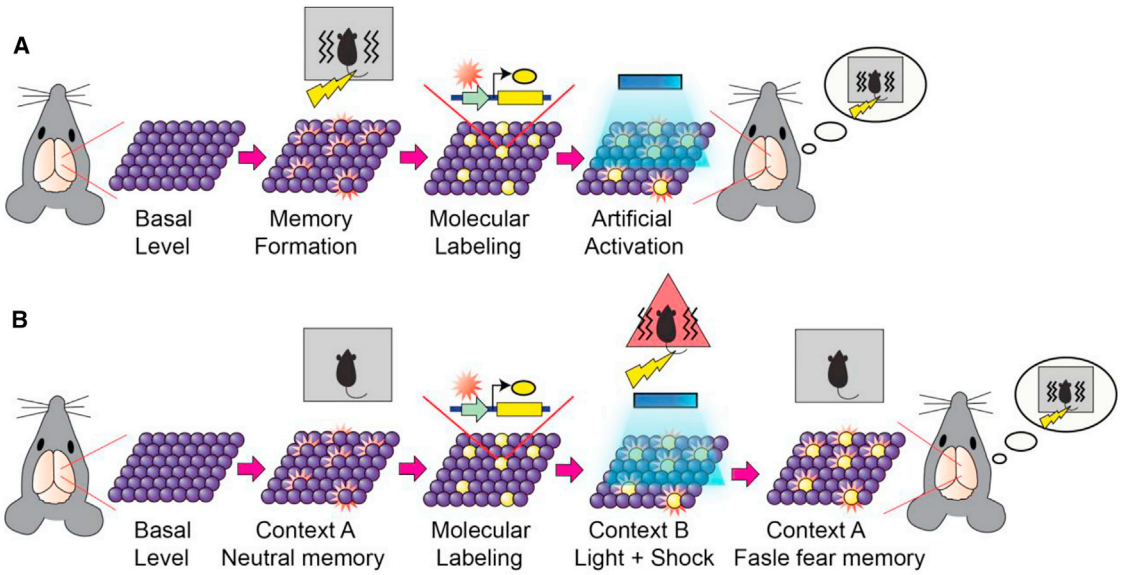

Figure 2. Optogenetic Manipulations of Memory Engram Cell Populations

(A) Light activation of memory engram cell population caused memory recall. Neurons active during the formation of a contextual fear memory were labeled by ChR2. When these neurons were artificially activated by light stimulation in a different context, the animals displayed freezing behavior, indicating the recall of the previous context associated with fear.

(B) Generation of a false contextual fear memory. Neurons active in a neutral context were labeled with ChR2 and later reactivated by light in a different context while the animals simultaneously received foot shock. When the animals were returned to the original neutral context, they displayed fear response, indicating the recall of a false memory associating the neutral context and the foot shock.

Still other studies have shown that contextual memory associated with a positive reinforcer such as cocaine could be blocked by either inactivating a minority of nucleus accumbens neurons that were previously active in the drug-associated environment in rats by the Daun02 method (Koya et al., 2009) or by suppressing a small population of LA neurons overexpressing CREB (and thus making these cells more active in the environment previously associated with drug administration) using a pharmacogenetics method in mice (Hsiang et al., 2014). Collectively, these experiments demonstrated that the disrupted cell populations impair a specific memory, supporting the notion that they contained an obligatory part of the engram complex.

Gain-of-Function Studies

The final and most technically challenging were gain-of-function studies, where researchers attempted to artificially reactivate a specific population of neurons activated by learning to mimic behavioral recall elicited by natural cues. Such studies are difficult due to the lack of proper tools that allow precise spatial and temporal control over the activity of defined neuronal populations. With the advent of optogenetics, such manipulations have, however, become possible. By combining the activity-dependent, doxycycline-regulated c-*fos*-tTA system and ChR2-mediated optogenetics, researchers were able to label with ChR2 a sparse population of DG neurons in mice that were activated by contextual fear conditioning memory. Subsequently, when these cells were reactivated by blue light in a context different from the original one used for the conditioning, these animals displayed freezing behavior as evidence of fear memory recall (Figure 2A) (Liu et al., 2012).

Gain-of-function studies also identified engram cell populations outside of the hippocampus. Two groups of researchers were able to overexpress CREB in a small population of LA cells, which made them preferentially recruited into the memory trace formed during fear conditioning (Kim et al., 2014; Yiu et al., 2014). They labeled these cells with exogenous receptors, TRPV1 or DREADD (designer receptors exclusively activated by designer drug) receptor hM3Dq. When they artificially activated these labeled neurons by administrating the receptor ligands capsaicin (for TRPV1) or CNO (for hM3Dq) in a novel environment, the animals showed fear memory recall. More recently, retrosplenial cortex (RSC) neurons activated during the formation of a fear memory were also shown to be able to elicit fear memory recall when re-activated in a novel context with optogenetics (Cowansage et al., 2014). All these experiments demonstrate that selected neuronal ensembles that are activated by learning are capable of eliciting memory recall once they are reactivated, thus providing the most direct evidence for the existence of memory engram cells. Given such evidence, the remaining task is to identify the nature of the enduring changes that are elicited in these cells by learning. We shall discuss this in the following section.

Learning-Dependent Enduring Changes in Engram Cells and Their Connections

Semon did not elaborate in his engram theory of memory about the nature of "…the enduring though primarily latent modification in the irritable substance produced by a stimulus…" (Semon, 1921). The guiding hypothesis regarding this issue has been that suggested by Canadian psychologist Donald

Hebb, who proposed that neurons encoding memory stimuli undergo enduring strengthening of some of their synapses through their co-activation with presynaptic cells: neurons that "fire together wire together" (Hebb, 1949). Starting with Bliss and Lomo's discovery of long-term potentiation (LTP)(Bliss and Lomo, 1973), in support of Hebb's idea, a large number of studies have been dedicated to the characterization of LTP and other facets of synaptic plasticity and their potential role in learning and memory. Activity-dependent increases of the size and density of dendritic spines (structural plasticity) have also been proposed as contributing to memory encoding (Bailey and Kandel, 1993; Holtmaat et al., 2006; Matsuo et al., 2008). There have also been studies suggesting that a cell-wide alteration, such as augmented intrinsic excitability, may contribute to memory (Daoudal and Debanne, 2003). However, until very recently none of these studies could link these activity-dependent alterations of synapses and neurons directly to specific engram cells, which are activated by learning and whose reactivation by specific recall cues elicited behavioral recall.

In this section, we shall first discuss those studies in which the synaptic or cellular changes observed were shown to be correlated with a mnemonic behavior. We will then refer to a very recent study in which an enduring change has been demonstrated in a population of DG granule cells that would satisfy the other criteria for engram cells—namely, activation by learning and memory recall by reactivation.

Learning-Dependent Alterations of Synaptic Strength

Even in those cases in which some links between synaptic or cellular changes and memory were made, until recently these links have been shown only by investigating globally one or more broad brain region(s), rather than a specific population of cells (i.e., putative engram cells) that were specifically activated by a given learning task and whose reactivation elicits behavioral recall. Since a form of LTP inducible in the CA1 region of the hippocampus by high-frequency stimulation (HFS) in vitro is NMDA receptor dependent (Malenka and Bear, 2004), efforts have been made to test whether this form of synaptic plasticity has an essential role in hippocampal-dependent memory. The results of early pharmacological blockade experiments conducted with an NMDA receptor (NMDAR) antagonist AP5 supported the notion that LTP is essential for spatial learning (Morris et al., 1986), and the validity of this notion was demonstrated with more specific targeted genetic ablation of the NMDAR in the CA1 region of the hippocampus (Tsien et al., 1996; but see Bannerman et al., 2012).

In a more recent study (Nonaka et al., 2014), the authors subjected transgenic mice in which the promoter of an IEG, Arc, drives the expression of dVenus, a destabilized version of the fluorescent protein Venus (Eguchi and Yamaguchi, 2009), to contextual fear conditioning. They found that fear conditioning induced presynaptic potentiation only in the cortical input to the dVenus positive cells in the basolateral amygdala (BLA). These data support the notion that synaptic plasticity in a subset of BLA neurons contribute to fear memory storage. However, they did not offer evidence indicating that reactivation of dVenus positive cells could evoke specific behavioral recall. (In addition, it should be mentioned that in this study the temporal window of Arc labeling was relatively lengthy, meaning that BLA neurons were potentially labeled indiscriminately for hours before the targeted behavioral experience.) In another recent study, researchers conditioned rats to associate a foot shock with optogenetic stimulation of auditory inputs into the amygdala. Optogenetic delivery of LTD-inducing stimuli (i.e., low-frequency stimulation) to the auditory input inactivated the memory of the shock, while subsequent optogenetic delivery of LTP-inducing stimuli (i.e., high-frequency stimulation) to the auditory input reactivated the memory of the shock (Nabavi et al., 2014). These data provided a causal link between these synaptic processes and memory. However, this study did not directly demonstrate that these synaptic processes (i.e., LTP and LTD) occurred in the same amygdala cell population that was activated by the initial conditioning (i.e., engram-containing cells).

In order to claim that an observed increase of synaptic strength indeed reflects a component of learning-dependent physical/chemical changes in engram cells, at least three criteria should be met. First, the increase should be observed only in a population of cells activated by the specific learning. Second, this increase should be dependent on plasticity associated with the learning episode. Third, reactivation of these cells results in behavioral recall. These criteria have been met in a recent study (Ryan et al., 2015), where hippocampal DG granule cells that were activated by contextual fear conditioning were labeled with *c-fos* promoter-driven fluorophore mCherry. In parallel, presynaptic entorhinal cortex cells were labeled with ChR2 driven by a constitutive CaMKII promoter so that perforant path axons that synapse onto DG engram and non-engram cells could be simultaneously stimulated with light while recording ex vivo by patch clamping. 1 day after training, the AMPA/NMDA excitatory postsynaptic current ratio was significantly higher in $mCherry^{+}$ engram cells compared to $mCherry^{-}$ non-engram cells in the same hippocampal slices (Ryan et al., 2015). Importantly, when mice were treated with the amnestic protein synthesis inhibitor anisomycin immediately after training, the engram cell-specific increase in synaptic strength was abolished and retrograde amnesia resulted. However, when anisomycin was administered 24 hr after training (outside the synaptic consolidation window), no effect on engram cell synaptic strength was observed and no amnesia occurred. Thus, the engram cell-specific increase in synaptic strength is a form of plasticity that is directly attributable to the target training experience. This set of findings was replicated using orthogonal measurements of spontaneous excitatory post-synaptic currents (Ryan et al., 2015). In the future, the generality of these findings should be demonstrated by expanding to engram cells in other areas of the hippocampal and other brain regions.

Learning-Dependent Structural Plasticity

Dynamics of the formation and elimination of individual dendritic spines in the neocortex of mice in response to sensory stimulation and motor learning (i.e., synaptic structural plasticity) have been investigated using two-photon laser scanning microscopy in vivo. Two recent studies showed the close association of synaptic structural plasticity in the neocortex with motor learning and novel sensory experience. In one study (Xu et al., 2009), training in a forelimb reaching task resulted in rapid (within an hour) formation of post-synaptic dendritic spines on the output pyramidal neurons in the motor cortex. The new spines induced

during learning were preferentially stabilized during subsequent training and endured long after training stopped. Furthermore, different motor skills were encoded by a different set of synapses. In another study (Yang et al., 2009), training on an accelerated rotarod (but not on a slowly rotating rotarod) over 2 days led to an increase in spine formation in the primary motor cortex. Furthermore, a novel sensory experience provided by switching animals from a standard to enriched housing environment resulted in an increase in spine density in 1–2 days in the barrel cortex. In addition, these newly formed spines survived experience-dependent elimination during subsequent periods. Thus, these studies suggest that durable motor memories are stored largely in stably connected synaptic networks.

While the aforementioned studies were conducted for relatively slow-forming motor skill memories, structural plasticity associated with fast-forming tone-fear conditioning memory (Matsuo et al., 2008) and its extinction has also been reported (Lai et al., 2012; for a review, see Maren and Quirk, 2004). In the former study, the authors observed newly recruited GluR1-counting AMPA receptors in the CA1 subregion of the hippocampus, specifically in mushroom-type spines 24 hr after learning. However, non-engram cells were not directly studied, making it difficult to discern whether or not the changes observed were specific to a defined set of cells active only during learning. In the latter study, by imaging postsynaptic dendritic spines of layer V pyramidal neurons in the mouse frontal association cortex, these authors found correlations between fear memory expression and spine elimination, as well as fear memory extinction and spine formation. Amazingly, spine elimination and formation induced by fear conditioning and extinction, respectively, occurred on the same dendritic branches in a cue- and location-specific manner within a distance of 2 μm. Furthermore, reconditioning following extinction eliminated spines formed during extinction, suggesting that within vastly complex neuronal networks, fear conditioning, extinction, and reconditioning lead to opposing changes at the level of individual synapses. Do these spine dynamics reflect what occurs in the cell populations that store the engrams for tone-shock association memory?

Recently, one study investigated structural plasticity in the DG engram cells holding an engram component for the contextual information of the CFC experience (Ryan et al., 2015). These DG cells were activated by the training experience and labeled with ChR2. Their optogenetic reactivation evoked the context-specific fear memory recall, satisfying a key criterion for engram-bearing cells. Ex vivo patch clamp recordings followed by confocal microscopic imaging revealed that $ChR2^{+}$ engram cells had significantly greater intrinsic cellular capacitance and dendritic spine density than $ChR2^{-}$ non-engram cells. Anisomycin administration immediately after training, but not 24 hr later, abolished the engram cell-specific increase in cellular capacitance and dendritic spine density. These data provided direct evidence for increased structural plasticity in memory engram cells that parallel the engram cell-specific synaptic plasticity discussed above. In the future, it will be necessary to improve the temporal control of engram labeling methodology in order to observe what changes may be happening during and immediately after training experiences.

Learning-Dependent Augmentation of Cellular Excitability

Another candidate for enduring physical or chemical changes that may be evoked by learning in memory engram cells is increased cell-wide excitability. Several studies showed LA cells in the amygdala could be genetically engineered to have higher levels of cell-wide excitability, even prior to specific learning, by overexpressing a transcription factor, CREB. After tone-fear conditionings, ablation of these high-CREB, high-excitability cells impaired fear memory expression, suggesting that the memory engram is preferentially allocated to these cells (Yiu et al., 2014; Zhou et al., 2009). It would be interesting for future studies to measure if excitability is further augmented in these LA cells in response to training as a putative mechanism underlying enduring storage of memory information.

Engram Cell Pathway and the Sufficiency/Necessity Issue

While there is sufficient evidence against Lashley's notion of the broad storage of a memory throughout the cerebral cortex, this does not mean that the memory engram is localized only in a single neuronal population. Semon's use of the phrase "engram complex" suggests that he was considering that the entire engram for a particular memory is composed of multiple components. Indeed, the data collected to date indicate that contextual memory engrams are present in multiple hippocampal subregions, each contributing to the overall memory of the context (Ji and Maren, 2008; Lee and Kesner, 2004; Leutgeb et al., 2004). For contextual fear or reward memory, distinct subpopulations of BLA cells are also recruited to provide engrams for negative or positive valence (Redondo et al., 2014; Gore et al., 2015). Thus, a notion of distinct "engram cell pathway" emerges for each distinct type of memory. How each engram component in the pathway contributes to the overall engram complex is a matter of great interest. Studies on this topic have begun only recently, but engram pathways for a few additional memories can already be conceived. For the memory of an episode that has not only a contextual component, but also a sequence of events, DG and CA3 engrams, which retain context information, may form an engram pathway with CA1 engrams, which may provide information about the temporal sequence of the events through their time cells (MacDonald et al., 2011). Yet another example of an engram pathway would be for tone fear conditioning memory. In this case, the auditory information may be stored in an engram in the auditory cortex (Weinberger, 2004), the context in which the tone occurred may be stored in the hippocampal engrams, and the association of tone, context, and foot shocks may be stored in amygdala engram cells (Ehrlich et al., 2009). These three populations of engram cells, each harboring a distinct engram component may constitute an engram cell pathway for the tone conditioning memory engram complex. It is also possible that potentiated synapses in engram cells may be just a contributing element of a memory engram complex, and a specific pattern of connectivity between different types of engram cell populations along the engram cell pathway may be the true content of a memory engram complex (Hebb, 1949). Indeed, a recent study suggested that for CFC, the preferential connectivity of DG engram cells with engram cells in

downstream CA3 and BLA is the crucial substrate for the consolidated memory (Ryan et al., 2015).

Redundancy and Compensations

As the memory engram pathway is not necessarily linear, parallel pathways can also contribute to an engram complex. One study has shown that blocking the CA1 activity by prolonged optogenetic inhibition during the recall of remote memory resulted in elevated activity in the anterior cingulate cortex (ACC). This compensation mechanism bypassed the requirement of CA1 and resulted in normal remote memory recall (Goshen et al., 2011).

A recent study also showed that blocking dorsal hippocampus activity by local microinfusion of glutamic receptor antagonists interrupted natural contextual fear memory recall when the animal was returned to the original fear-conditioned context, but light activation of memory engram cells in the RSC was sufficient to overcome this impairment and rescued the behavior phenotype (Cowansage et al., 2014). These results suggest the existence of multiple functional engram pathways for a given memory. The animals may preferentially use one default pathway for normal memory recall, but under certain conditions other latent pathways could be brought on line and compensate for the default one.

Adequate Spatial and Temporal Resolutions

The complexity of engram pathways also means that proper test conditions should be applied to demonstrate necessity and sufficiency for engram cell populations in the pathways. Both temporally and spatially appropriate perturbations are required to reveal the role of a given engram cell population. As to the former, earlier lesion and pharmacological blockade experiments had suggested that CA1 is not necessary for the recall of remote memory, yet acute inhibition of CA1 with optogenetics caused defects in remote memory recall (Goshen et al., 2011). Further investigation showed that with more temporally extended optogenetic inhibition, which mimicked the effects of lesion and drug treatment, additional structures such as ACC compensated for the inactivation of CA1 (Goshen et al., 2011). Therefore, the necessity of CA1 for remote memory recall can only be revealed with such acute treatments. It is reasonable to suspect that similar temporal dynamics may also apply to the engram cells in other brain areas (Do-Monte et al., 2015).

Spatially, non-selective inhibition of all dorsal DG neurons had no effect on memory recall, making them seem unnecessary for this process (Kheirbek et al., 2013). However, the memory was impaired if a selected small fraction of the DG neurons previously active during training were inhibited during recall, indicating that DG engram cells are indeed necessary for memory recall (Denny et al., 2014). Similarly, selective activation of an engram cell population in DG induced the recall of a previously formed fear memory (Liu et al., 2012), demonstrating the sufficiency of engram cells for memory recall in DG, while non-selective activation of dorsal DG neurons not only failed to induce artificial memory recall, but actually abolished natural memory recall in the original context (Kheirbek et al., 2013). The latter results may be due to neuronal competition and lateral inhibition among different subpopulations of cells within the subregion, as reported by other studies (Han et al., 2007; Tanaka et al., 2014), and illustrate the precise spatial resolution needed to properly characterize the contribution of engram cells.

Geneticists often perform epistasis analysis to map out the molecular pathways inside the cells, and similar principles can also be applied to engram cell pathways. In an analogous approach, a recent study activated engram cells in the hippocampus while simultaneously inactivating putative engram cells in downstream areas, such as the nucleus accumbens, which thereby blocked the hippocampus engram cells' behaviorally relevant effects. Similar approaches can be utilized by future experiments to examine the functional interactions among different engram cells across a variety of brain regions to gain further knowledge about the structure, function, and layout of such engram pathways (Ramirez et al., 2015). Even so, in trying to map these engram pathways, it is critical to keep in mind that flexible and dynamic systems are involved; as such, non-rigid models are required, with changing necessity and sufficiency for different components.

Engram Cell Manipulations

Memory engram technology permits not only the identification of engram cells, but also the engineering of these cells. This opens up the possibility of manipulating memory under a variety of conditions, though it's important to bear in mind that these studies have just begun and here we discuss three cases, all in mice. They are: creating a false memory, switching memory valence, and attenuating depression-related behavior by activating a positive memory. These studies not only might help to expand our knowledge about how memory is stored and retrieved, but could also reveal neural circuits underlying interactions of memory with other cognitive functions such as imagination and emotion.

Creating a False Memory

Memory is constructive in nature, and the act of recalling a memory renders it labile and highly susceptible to modification (Bartlett, 1932; Nader et al., 2000). In humans, memory distortions and illusions occur frequently, which often results from incorporation of misinformation into memory from external sources (Loftus, 2003; Roediger and McDermott, 1995; Schacter and Loftus, 2013). Cognitive studies in humans have reported robust activity in the hippocampus during the recall of both false and genuine memories (Cabeza et al., 2001). However, human studies performed using behavioral and functional magnetic resonance imagining techniques have not been able to delineate the brain regions and circuits that are responsible for the generation of false memories. In rodents, two lesion studies (McTighe et al., 2010; Romberg et al., 2012) investigated object recognition memory in rats with perirhinal cortex lesions and found that the subject tended to treat novel objects as familiar, thus leading to a type of false recognition. However, studies on false memories in animal models are rare, which may be a contributing factor in the slow progress in the elucidation of the potential neuronal mechanisms underlying human false memory.

Considering the fact that humans have a rich repertoire of mental representations generated internally by processes such as conscious or unconscious recall, dreaming, and imagination (Schacter et al., 2007), one possible cause of episodic false memory is that the memory of a past experience becomes associated with a current external event of high valence. Using

a method that permits optogenetic labeling and manipulation of memory engram cells (Liu et al., 2012), a recent study tested this possibility in mice (Ramirez et al., 2013). They labeled the contextual engram cells in the DG with ChR2 by exposing mice to a context A. On the second day, as the labeling window was shut down, and the mice received foot shocks in a distinct context B as their context A engram cells were artificially activated with pulses of blue light. When the animals were replaced in context A on the third day to test for context A-specific fear memory, the animals froze despite never having received foot shocks in context A (Figure 2B). The freezing was not due to generalization because the mice did not freeze above the background level in another distinct context C. These mice also froze when tested in context B, indicating they also formed a genuine context B-shock association memory. However, the level of freezing in context B was significantly lower than that of a group of mice that did not receive blue light delivery on day 2 while foot shocks were delivered, suggesting that formations of the false and genuine memory on day 2 were in competition. An important additional observation made in this study is that although the mice with the false fear memory for context B did not freeze in a distinct third context C, they did freeze significantly when blue light was delivered in context C, indicating the engram for the false memory is light-reactivatable as is the engram for the genuine memory, which Liu et al. had demonstrated (Liu et al., 2012). Moreover, using the same cFos-driven ChR2 labeling strategy, a recent study demonstrated that in addition to optogenetically driving a hippocampal contextual engram, BLA cells responding to a stimulus of high valence can be simultaneously activated to form an association with the hippocampal-driven contextual memory (Ohkawa et al., 2015). The synchronous activation of the hippocampus cells representing a conditioned stimulus (e.g., context) and the BLA cells representing an unconditioned stimulus (e.g., foot shocks) thus led to the creation of a new associative fear memory and shared similar molecular mechanisms as the formation of a genuine fear memory (e.g., protein synthesis dependence and NMDA dependence).

These studies showed that at least some form of false memory is generated by an association of internal brain activity representing recall of a past experience with the current external or internal experience of high valence (Ramirez et al., 2013; Ohkawa et al., 2015). Since the underlying neurophysiological mechanisms for such an association are similar to the one that occurs when a genuine memory is formed, it is not surprising that the subject behaves as if the (false) memory was formed by a perceived real experience.

While further studies are necessary to assess the relationship between the artificially induced false memories in these animal models and human false memories, the optogenetically induced false memory is consistent with the temporal context model (TCM) in humans, which posits that contextual memory reactivation can be linked to novel information that is presented at the time of reactivation (Gershman et al., 2013; St Jacques and Schacter, 2013). A crucial point here is that the formation of false memories in humans often occurs as a result of recombining mnemonic elements of discrete experiences into a new, reconstructed memory that is not a veridical representation of the past. These memories in humans are not formed de novo and require pre-existing memories as a scaffold onto which distinct experiences can be incorporated to update the memory itself (Garner et al., 2012; Gershman et al., 2013; Tse et al., 2007). The optogenetically induced false memory in mice shares this feature of human false memories.

Switching Memory Valence by Manipulating Engram Cells

While most studies on engram cells have focused on their properties in one anatomical region, diverse engram components within an engram cell pathway range across multiple brain regions. For instance, the hippocampus and the associated cortex are known to play a crucial role in episodic memories by associating the emotionally neutral components of the episode: information like what, where, and when (Anderson and Phelps, 2001; Zola-Morgan et al., 1991). On the other hand, the amygdala is known to be the main hub for the storage of the emotional valences associated with experiences. The amygdala receives inputs from all sensory modalities, including processed inputs from the hippocampus, perirhinal and entorhinal cortex, and prefrontal cortex (Sah et al., 2003; Senn et al., 2014; Trouche et al., 2013). Neurons in the amygdala respond to positive as well as negative values (Paton et al., 2006), and inactivating the amygdala prevents the association between neural stimuli and emotion in both an anterograde (Miserendino et al., 1990) and retrograde manner (Han et al., 2009).

Given the distinct properties of the hippocampus and the amygdala, do engram neurons in these two brain regions link up to form and drive a specific memory (i.e., contextual fear conditioning)? If so, does the contextual component of a hippocampal engram have the flexibility to associate with different engram components (i.e., fear or pleasure) in the amygdala? These issues were addressed recently by applying the memory engram technology to fear- (foot shocks) or reward- (male mice interacting with female mice) conditioned mice (Redondo et al., 2014; Gore et al., 2015). In the former study, the contextual component (context A) of the context-specific fear or reward conditioning engram complex in the hippocampal DG was labeled with ChR2 following the protocol established by Liu et al. (Liu et al., 2012), and the resulting fear or reward memory was confirmed with a novel optogenetic place avoidance or place preference test. These animals were then subjected, in a distinct context (context B), to a second conditioning with unconditioned stimuli of the opposite valence (from foot shocks to female exposure or vice versa) as their context A engram cells were reactivated by blue light pulses using the false memory inception protocol applied by Ramirez et al. (Ramirez et al., 2013). This led to a switch of the overall valence of the memory from negative to positive or positive to negative that DG cells were capable of driving, corresponding to the order of two successive conditionings. Furthermore, it was shown that the negative to positive switch was achieved not only by the prevalence of the reward memory, but also by the diminishing of the fear memory. The reversal of the dominant valence associated with the DG memory engram was also demonstrated at the cellular level by comparing the level of engram reactivation in the BLA after DG optogenetic engram stimulation in experimental and control mice. Only mice that underwent the memory reversal protocol showed a reduction in DG engram to BLA engram functional connectivity. Intriguingly, this

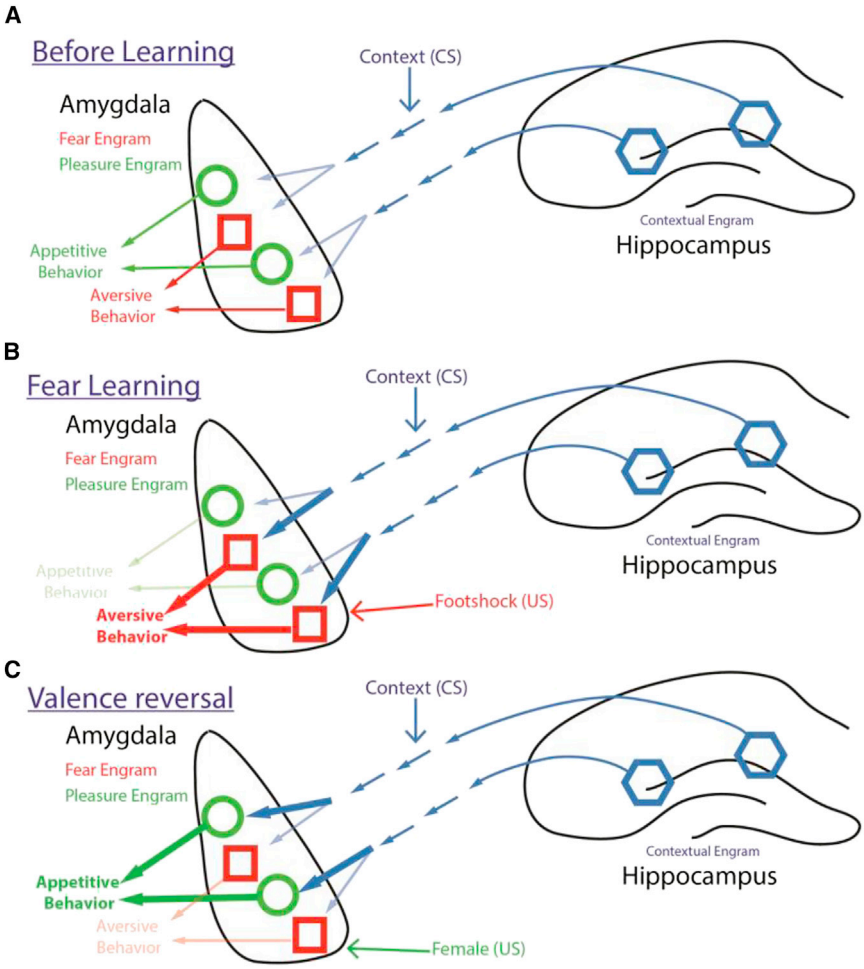

Figure 3. Summary Graph Depicting the Three Steps in a Valence Reversal Experiment

(A) Before learning, the contextual information from the hippocampus has the potential to drive output neurons in the amygdala specialized in producing either a fear or an appetitive response.

(B) During fear learning, the convergence of the contextual information (CS) with the foot shock (US) onto amygdala fear response neurons strengthens the functional connectivity between hippocampus (context) and amygdala (fear).

(C) If the same hippocampal inputs are active while the animal interacts with a female (US), there is a rewiring of the connectivity between the hippocampus and the amygdala. Hippocampal neurons encoding context (CS) are now capable of driving appetitive responses.

switch of valence was not observed when the BLA engram was labeled and its light activation was used as the protocol. These results indicate that in the DG, the outputs of the neurons sufficient to activate the memory engram of a given neutral context have functional plasticity such that the valence of a conditioned response evoked by their reactivation can be reversed by re-associating this contextual memory engram with a new unconditioned stimulus of an opposite valence (Figure 3).

Countering Depression-like Behavior by Activation of a Positive Memory Engram

The emerging picture of the interaction between the neural circuit governing memory valence and the circuit encoding neutral components of episodic memory is that the engrams for the latter, like the one in the DG, is free to associate with either positive or negative valence engrams in the BLA (Gore et al., 2015). The development of new technologies that permit engineering of these engrams might open up the possibility of adding a novel approach to the classical approaches for the treatment of psychopathologies (Wolpe, 1958). For example, depression is characterized by a pervasive and persistent blunted mood that is accompanied by motivational impairments and a loss of interest or pleasure in normally enjoyable activities. However, how positive episodes interact with psychiatric disease-related impairments at the neural circuit and systems levels remains largely unknown (Keller et al., 2000).

A recent study demonstrated in mice that optogenetic reactivation of engram cells formed in the DG by a naturally rewarding experience was sufficient to acutely suppress depression-related behavior (Ramirez et al., 2015). This study further showed that glutamatergic transmission from the amygdala's axonal terminals to the nucleus accumbens (NAcc) shell is necessary for the real-time antidepressant-like effects of the reactivated DG engram cells. Notably, the NAcc has recently been identified as a potential therapeutic node for deep brain stimulation (DBS) to alleviate anhedonia (Schlaepfer et al., 2008) in humans, and previous reports had also identified BLA axonal terminals onto NAcc as sufficient to support self-stimulation and reward-seeking behavior in a D1 receptor-mediated manner (Britt et al., 2012; Stuber et al., 2011). It is important to note here that directly reactivating cells associated with a positive memory is qualitatively different from exposing depressed subjects to naturally rewarding experiences, which would normally activate these corresponding brain areas in the healthy brain. In the psychiatric diseased-related state, acute administration of naturally rewarding external cues may not have access to, or activate, the positive engram cells' representations associated with a rewarding experience. Direct optogenetic stimulation of these cells may be able to override this obstacle.

It is intriguing to speculate that the acute behavioral changes observed during optogenetic stimulation (Nieh, et al., 2013; Ramirez et al., 2015) may reflect the degree to which directly stimulating neurons might bypass the plasticity that normally takes antidepressants weeks or months to achieve, thereby temporarily suppressing the depression-like state.

Still, the neural underpinnings inducing and correlating with long-lasting rescues have remained poorly understood. In mice, Ramirez et al. (2015) and Friedman et al. (2014) found

that chronic reactivation of DG engram cells previously active during a positive experience, and direct activation of the VTA dopaminergic reward system, respectively, had antidepressant-like behavioral consequences that outlasted acute optical stimulation. Friedman et al. (2014) also identified an optogenetically induced increase in K^+ channels and normalization of VTA firing rates as crucial contributors to the antidepressant-like effects. In Ramirez et al.'s study, while the causal link between chronically reactivated positive memory engrams and the corresponding rescue of behaviors remains elusive, many tantalizing hypotheses surface, including a normalization of VTA firing rates, epigenetic and differential modification of effector proteins (e.g., CREB, BDNF) in areas up- and downstream of the hippocampus, and a reversal of neural atrophy in areas such as CA3 and mPFC or hypertrophy in BLA. Together, these studies provide causal evidence in animal models that sparse populations of cells can be directly manipulated in a terminal-specific and activity-dependent manner to modulate a specific behavioral program associated with psychiatric disease-related states.

Conclusions and Perspectives

A set of cogent evidence for the long-sought memory engram and engram cells has now come of age. The evidence has been obtained by combining multiple technologies, each addressing a specific level of complexity: molecular and cellular neurobiology, physiological recording and multiphoton imaging, transgenic and virus vector-mediated gene insertions, and optogenetic and pharmacogenetic manipulations of neurons and their circuits as animals undergo mnemonic behaviors. The evidence falls into three types. A large number of earlier observational studies provided correlative evidence between physiological and structural properties of neurons in a given area of the brain, and one or more aspects of mnemonic behavior. The second line of evidence has been based on a loss-of-function strategy, with numerous studies demonstrating that animals or humans suffering from physical or chemical lesions of restricted brain areas, or animals with pharmacological manipulations, are impaired in a certain aspect of mnemonic behaviors. However, these early loss-of-function studies could not pinpoint the specific cellular subpopulations that are essential for a specific mnemonic behavior, while recent studies overcame these limitation by taking advantage of transgenic, optogenetic, or pharmacogenetic technologies (Denny et al., 2014; Han et al., 2009; Zhou et al., 2009).

The final and most technically challenging type of evidence has been gain-of-function evidence. To obtain such evidence, a specific population of neurons that is activated by learning first had to be identified, and then a method had to be developed by which a subsequent reactivation of the cells would elicit behavioral recall of the specific memory without relying on the natural recall cues. This was accomplished by combining the activity-dependent, doxycycline-regulated c-*fos*-tTA system and ChR2-mediated optogenetics to elicit a hippocampal-dependent contextual fear memory (Liu et al., 2012). This finding was extended to neurons in the retrosplenial cortex for the same memory task (Cowansage et al., 2014). Furthermore, the data obtained by applying pharmacogenetic methods to CREB-over-expressing LA cells, known to be required for tone fear conditioning, reinforced the gain-of-function evidence, although these studies did not demonstrate that the cells manipulated were initially activated by learning (Kim et al., 2014; Yiu et al., 2014).

While memory engram theory has clearly come of age, a number of important issues remain to be investigated. One is the nature of the "enduring changes" that occur in the engram cells and their connections. A first study by Ryan et al. (2015) along this line provides evidence for the long-held hypothesis that synaptic strengthening, as well as a change in structural plasticity, did occur specifically in engram-positive cells as opposed to engram-negative cells in the same hippocampal subregion (i.e., DG). The demonstration of these learning-induced changes strongly argues that they are indeed cells that carry an engram component, rather than cells necessary for performance. However, this study did not determine the in vivo firing patterns of the engram cells (e.g., are they place cells? What firing pattern would they show in vivo before and after recall cues are delivered, etc.?). Moreover, the integrative evidence for engram cells has been obtained to date in this one study, and only for contextual fear memory in DG (Ryan et al., 2015). Memory, however, appears in many different forms (e.g., emotional, procedural, working, semantic, perceptual), each supported by one or more distinct brain regions and systems. The basic technology used to identify engram and engram cells for classical conditioning memories may, in principle, be applicable to other types of memories. However, significant modifications of the technology may be needed to identify engram and engram cells for each type of memory. For instance, procedural or habit memories develop slowly with multiple rounds of training. Can one identify the putative habit engram cells and elucidate how they may change as training is repeated? Can one identify early habit memory engram cells and accelerate the process of learning by optogenetic activation of these cells? Or can one perform the converse experiment and inhibit the process of motor learning? An additional example includes the memory for a temporal sequence of events—a crucial component of episodic memory. Are these engram cell ensemble(s) that hold the sequence information identifiable by the current engram cell technology? Or is the technology effective only for the memories of individual events, and will other methods have to be invented to identify the mechanism that orchestrates the sequence of the expression of these individual event engrams? These studies are expected to reveal both common and memory type-specific features of engrams and engram cells.

The universality, causality, and detailed kinetics of the enduring changes in the engram cells, as well as their connections, during the encoding versus subsequent cellular consolidation phase will have to be determined. This line of thought takes us to other highly interesting questions that can be explored by the engram manipulation technology. For instance, what roles do protein and mRNA synthesis play in engram cells? It is generally thought that learning elicits new rounds of transcription and translation in the soma and dendrites of neurons that have encoded stimuli selected from experience. These molecular events are thought to stabilize the storage of the memory information encoded initially by a rapid macromolecule synthesis-independent process. However, this view has been challenged by

the finding that post-training protein synthesis is dispensable for the retention of 1-day-old memory (Ryan et al., 2015) and by the hypothesis that the strengthened synapses and increased spine density accomplished during the consolidation phase is to enable more efficient recall (Ryan et al., 2015; Tonegawa et al., 2015). Further studies seeking to resolve these issues will be greatly aided by also performing within subjects' analyses (i.e., light off versus light on epochs during a single session) while subsequently measuring physiological and structural changes in engram-positive and engram-negative cells.

Another exciting prospect for memory research triggered by engram manipulation technology is to elucidate the pathways comprising the engram complex for various types of memory and to identify the unique role of each contributing engram cell population. To date, this notion of an engram pathway has been investigated for contextual conditioning memories, for which context-related engram cells in the DG and fear- or reward-related engram cells in the BLA were identified (Redondo et al., 2014; Gore et al., 2015). But, it is likely that several other hippocampal and entorhinal cortical sites located between the DG and BLA for the signal transfer are likely to hold unique engrams as well. It would be extremely interesting to identify their nature and the dynamic interactions between engrams in multiple brain regions. The demonstration that the valence-regulated behavior of animals can be controlled by manipulating memory engram cells along the HPC→BLA→NAcc functional circuit is another example of an exciting advance made by the engram pathway notion (Ramirez et al., 2015).

Memory plays a wide-ranging role in a variety of cognition and behaviors, such as emotion, decision, attention, and awareness/consciousness. The effort to discern how the neural circuits underlying these cognitive functions intersect with memory engram circuits, in health and in disorder, will be greatly aided by memory engram identification and manipulation technologies. One day, probably in a not so distant future, we may even be able to combine the knowledge obtained by these studies with minimally invasive technologies such as wireless optogenetics (Kim et al., 2013) and magnetogenetics (Chen et al., 2015) to develop novel therapeutic methods for a variety of brain disorders.

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