Finding the memory engram

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Since the time of Plato and Aristotle (circa 350 BCE), memory is believed to be stored as enduring changes in the brain. However, the first scientific articulation of this concept only emerged in the 20th century when Richard Semon introduced the term “engram” to describe the neural substrate for storing and recalling memories. Since then, we have made great progress in studying engrams, to the point that we are able to visualize and manipulate them. Here, I will talk about new technologies that allowed us to tag and manipulate memory at the level of individual neurons.

1. Engram’s heroes

It was the German zoologist Richard Semon (1859–1918) that first proposed a conceptualization of a defined, tangible memory and coined the term engram, becoming a founding hero in the field. In his two monographs on memory (Semon, 1921; Semon 1923), Semon proposed that there are engrams (or memory traces) that are the physical substrate of memory. He defined engrams as enduring changes in the brain that result from a particular experience and whose underlying physical substrate can remain dormant until the appropriate external and/or internal cues result in its direct reactivation, thereby leading to retrieval. However, Semon's engram theory was very controversial for its time, as it proposed that memory units are passed from one generation to another.

Nonetheless, Semon is considered a pioneer with regards to memory research, since he not only introduced useful memory terminology, but also proposed a conceptual framework of how information is represented in the brain over time. This framework was foundational for developing criteria that researchers still use to evaluate memory (Josselyn et al., 2017). Even Semon's controversial idea regarding the inheritance of memory has become more broadly accepted and nowadays is an active area of study (Gräff and Mansuy, 2008; Dias and Ressler, 2014). For instance, it is widely accepted that mechanisms such as epigenetic modifications (i.e., modifications of DNA that do not alter its sequence) are an important component of engrams and can be transmitted to future generations.

Years later, the American scientist Karl Lashley (1890–1958) attempted to localize engrams in the brain by systematically lesioning various functionally connected brain areas in rodents performing visual discrimination and maze learning tasks (Lashley, 1950). He found that

the amount of tissue lesioned degraded memory, but more remarkably, where the tissue was lesioned made no difference. Karl Lashley's work suggested that the engram could not exist in any specific part of the rodent's brain, but that memory was widely distributed throughout the cortex. His pioneering lesion experiments provided support for the notion that memory is orchestrated by various functionally and/or structurally connected circuits that coordinate their activity to enable the retention of information. In other words, memory is made possible by joint activity within and across neural circuits and is not tied to single structure.

A remarkable body of work emerged after Lashley's initial attempts to isolate an engram. In particular, new technologies such as transgenic mouse models allowed us to find fixed location of memories and prove that engrams exist in many regions of the brain. However, rather than providing an exhaustive historical overview of engram research, I will focus on discussing a novel, state-of-the-art genetic strategy that can be used to tag the individual neurons forming an engram.

2. Genetic strategy to label engrams

Over the course of the last 10 years, we have made much progress on identifying and manipulating engrams. As aforementioned, engrams are defined as populations of neurons (neural ensembles) that are: 1) activated by learning; 2) have enduring cellular changes as a consequence of learning; and 3) whose reactivation by a part of the original stimuli delivered during learning results in memory retrieval or memory expression. Recent transgenic mouse models developed by our laboratory and others have enabled the identifications of engrams in several brain regions such as the hippocampus (HPC), prefrontal cortex (PFC) and amygdala (Denny et al., 2014; Liu et al., 2012; Ramirez et al., 2013, Ramirez et al.; 2015; Reijmers et al., 2007).

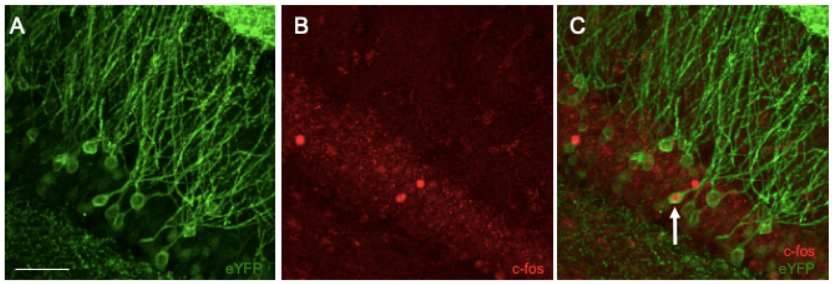

Figure 1. Characterization of the ArcCreER^{T2} mice. (A) EYFP^{+} DG cells corresponding to memory encoding. **(B)** c-Fos^{+} DG cells corresponding to memory retrieval. **(C)** Representative co-labeled EYFP^{+}/c-Fos^{+} cell corresponding to engram. Scale bars represent 100 mm.

The ingenuity of these mouse models lies in their ability to "tag" neurons by monitoring activity of immediate early genes (IEGs) as a proxy for neural activity. IEGs are genes that are activated by experience and lead to changes in the neuron that allow to encode and store memory. Arc and c-Fos are two examples of IEGs.

In particular, Dr. Denny created an Arc activity-dependent tagging system, the ArcCreER^{T2} mice, that enabled to label memory engrams representing individual memories (**Figure 1**). In

these mice, upon injection of the drugs 4-hydroxytamoxifen (4-OHT) or tamoxifen (TAM), a fluorescent protein, the enhanced yellow fluorescent protein (EYFP) is expressed in all neurons activated during the encoding of a memory, and therefore expressing Arc. Then during memory retrieval, either Arc or c-Fos will be expressed in all neurons that are active at that time. The overlap between the cells activated during encoding with the cells activated during memory retrieval, represents an engram or memory trace.

The ArcCreER^{T2} mice line is unique in that it allows for a *permanent tag of activated neurons* (indeed, **the encoding cells are indelibly labeled with the EYFP**) and thus, a comparison of the cells activated during memory encoding with those most recently activated during memory expression. This system is also flexible - we can tag these cells with a fluorescent reporter or express optogenetic or pharmacogenetic tools to manipulate them at a later time point.

Here, I will use these mice to address questions such as: Can we identify and manipulate engrams? Do engrams change across the lifespan? Can we tag and manipulate engrams to change the corresponding memory?

3. Behavioral methods to study engrams

While learning and memory may be studied in humans, in order to fully elucidate the underlying biological mechanisms these processes, model organisms such as rodents are invaluable tools. Studies on learning and memory in rodents rely on behavioral tasks. Two such tasks are the fear conditioning (CFC) and pattern separation (PS) tasks.

CFC consists of a training phase and a testing (re-exposure) phase. In the CFC training, mice are exposed to a conditioning chamber (context A) where they receive a mild electric foot shock. During CFC testing, days later, memory for the context-shock association is assessed by returning mice to the context A for 5 minutes and measuring time spent freezing (e.g., time spent in total immobility). If mice remember that they received the shock in A then they will show a freezing response when re-exposed to A (Mastrodonato et al., 2018).

In the PS test, mice are exposed to two contexts daily after the initial CFC training. One context is a similar, novel context that does not hold any negative valence (context B) and the other context is the training context where the mice were previously shocked (context A). Each day, memory for the context-shock association is assessed by returning mice to the chambers for 3 minutes and measuring time spent freezing. Mice usually can learn the dissociation between the two contexts in about 7 days (Sahay et al., 2011). However, during aging or in fear-related disorders, such as anxiety, the ability to discriminate between context A and B becomes impaired; this phenomenon is known as fear generalization. When fear is generalized in the PS paradigm,

context B may be said to be perceived as fearful even if it is safe. In the next paragraphs, I will describe the use of CFC and PS in some of my experiments.

4. Identification and manipulation of engrams

In a first set of experiments, we tagged the engram cells activated during CFC and then we manipulated them to prove their necessity for memory expression. As expected, mice administered a CFC test showed a freezing response in the context where they received the shock (context A). Most importantly, when we processed the brains of these mice, we found an increased reactivation of engrams cells in two regions of the HPC, the Dentate Gyrus (DG) and Cornu Ammonis 3 (CA3).

To prove the necessity of these engram cells for the CFC memory expression, we used a technique, optogenetics, in which a laser light can activate or inactivate the neurons that were previously activated by any experience and are now "tagged" with light-sensitive channels. We optogenetically inactivated the neurons associated with the CFC while mice were re-exposed to the context A. In doing so, we blocked the freezing response (the CFC memory was not expressed anymore). With this study we proved for the first time that memory engrams representing a CFC experience exist in the hippocampus regions DG and CA3 and they are necessary for the retrieval of the CFC memory.

5. Engrams changes with aging

Aging is accompanied by a progressive decline in cognitive function. Greater than 40% of the population over 65 years of age suffer from age related cognitive decline (ARCD), which threatens quality of life for older adults and presents challenges to the healthcare system (Small et al., 2002). Memory function, especially hippocampus-dependent memory function, is particularly sensitive to the effects of aging (Nilsson, 2003; Rosenzweig and Barnes, 2003). For example, the ability to distinguish between two similar contexts (i.e., pattern separation (PS)), is compromised, leading to increased generalization and inappropriate pattern completion (Krishna et al., 2012; Stark et al., 2010; Ally et al., 2013). One of the main challenges in our field has been determining mechanism, onset, and progression of memory decline, which we must first understand before designing targeted treatments. Assessing the memory performance in mice at multiple time points (e.g., mid- to late-life) is imperative both to identify the onset of memory deficits and to track the time course of cognitive decline.

Both rodent and postmortem studies have suggested that aging affects the DG and CA3 hippocampal subregions, reducing their neural plasticity (i.e., their ability to change in response to stimuli) inducing memory loss (Barnes 1994). Moreover, immediate early gene (IEG) expression (e.g., Arc/Arg3.1 or c-Fos) decreases with age, especially in the DG (Small et al., 2004; Desjardins et al., 1997; Weber et al., 2015), raising the possibility of decreased overall functionality or integrity of DG cells is causal in memory loss. Therefore, the DG and downstream CA3 subregion are ideal targets in determining the onset, as well as the neuronal and behavioral characteristics, of ARCD.

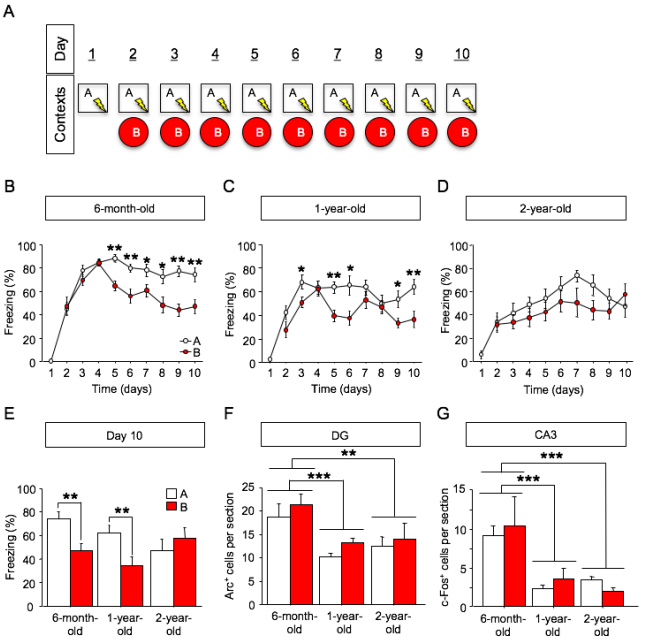

Figure 2. Aged mice are impaired in pattern separation and have decreased Arc DG and c-Fos CA3 expression. (**A**) PS behavioral design. (**B**) Six-month-old mice begin to PS starting on Day 5. (**C**) One-year-old mice have a variable performance, but continually PS on days 9 and 10. (**D**) Two-year-old mice never learned to PS. (**E**) Freezing levels on Day 10 of the PS protocol. Whereas the 6-month-old and the 1-year-old mice can discriminate, the 2-year-old mice can discriminate between the two contexts. (**F**) One-year-old and 2-year-old mice have significantly reduced Arc expression in the DG when compared with 6-month-old mice. (**G**) One-year-old and 2-year-old mice have significantly reduced c-Fos expression in CA3 when compared with 6-month-old mice.

Using the ArcCreER^{T2} mice, we have recently characterized the behavioral features and IEG dynamics that accompany normal aging. We found the most significant memory impairment in aged mice was in PS – aged mice could not discriminate between an aversive, context (A) (where they received a mild foots shock) and a similar, neutral context (B) (where they did not receive any shock) (**Figure 2A-2E**). Moreover, these age-related impairments in PS were accompanied by decreased IEG expression in the DG and in CA3 (**Figure 2F-2G**). We hypothesized that aging produced weakened DG memory engrams; specifically, with aging, there is reduced neuronal activity in the DG and this results in non-specific, generalized memories. We also believed that aged mice could not discriminate because the aversive context A memory was contaminating the neutral context B memory.

In order to test our hypothesis, we used a technique (i.e., optogenetics) to stimulate and target DG engrams. We labeled an engram corresponding to the neutral B context and we stimulated this engram in the neutral B context in order to promote memory retrieval. The aged PS deficit was rescued via optogenetic activation as evidenced by decreasing fear generalization in aged mice. However, optogenetic stimulation of a non-specific, neutral context engram did not ameliorate the PS deficit, suggesting that the behavioral results were engram specific. Furthermore, chronic stimulation of a neutral context engram improved PS following the stimulation period and also increased IEG expression in the DG in aged mice. These findings

support a model in which ARCD may result from contaminated, non-specific engrams and that targeting the DG may be a successful method for reversing ARCD.

6. Engrams changes during adolescence

The previous study in old mice convinced me that age heavily affects engrams and their corresponding memories. And I therefore wondered: how are memories formed and stored at a younger age? Or in other words: what happens to the engram cells during adolescence?

It is known that adolescence is a sensitive period for fear memory disorders, such as anxiety disorders. More than half of anxiety diagnoses are first made in childhood or adolescence, and an earlier age of anxiety onset is associated with a more chronic, non-remitting course. As aforementioned, PS impairment is a defining feature of anxiety disorders, in which danger cues are generalized to perceptually similar safety cues, leading to fear overgeneralization.

Despite the onset of anxiety disorders in late childhood/early adolescence, a crucial gap in studies of anxiety is the lack of data concerning fear generalization development from early adolescence to adulthood and the engram mechanisms underlying PS during development. Gaining this knowledge would help understanding when and where to intervene in anxiety disorders and to promote preventive therapeutic interventions against pathological anxiety.

Studies from our and other labs have implicated the HPC in controlling PS behavior. For instance, the large number of cells and sparsity of firing within the DG are believed to support contextual fear discrimination. Consistent with this view, perturbations of DG function are associated with impaired context discrimination and increased context fear generalization in aging mice. Hippocampal CA3 region has also been implicated in fear generalization. However, the role of the HPC in fear generalization have not been fully investigated during adolescence.

During my stay at Italian Academy, I compared PS between adult and adolescent mice. I found that adult mice discriminate between an aversive context A and a neutral context B and express more fear in context A than in context B. This behavior correlates with an increased activation of DG encoding ensembles in context A but not in context B. Moreover, in adult mice, we have found that optogenetic inhibition of context A results in decreased freezing in context A, but not in context B, suggesting context specificity of these engrams.

In contrast, adolescent mice are not able to discriminate between the aversive context A and neutral context B, expressing fear similarly in contexts A and B and equal DG engram activation in both contexts (e.g., there is no context specificity and mice do not have more co-labeled cells in A than in B). For my future experiments, I hypothesize that in adolescent mice, optogenetic inhibition of the DG ensembles will result in decreased freezing in both contexts A

and B, suggesting the lack of context specificity and perhaps, the driving factor in increased fear generalization. Similarly, if the aversive context A engram is influencing activity of the neutral context B engram (e.g., proactive interference), then by tagging context B before adolescent mice encode context A, I hypothesize that excitation will result in decreased freezing in context B, but not in context A and thus, decreased fear generalization.

Conclusion

In this paper I have highlighted the ArcCreER^{T2} mice as a state-of-the-art model that allows for permanent whole brain tagging of memories with single cell resolution; I have also discussed a number of factors such as time, context and age that affect behavioral performances. By correlating behavioral memory performances with the activity of the engrams during encoding or during retrieval, we can find brain regions that are either altered by these behaviors or that we can target to improve memory. Overall, by understanding how memories are formed, stored, and retrieved, we can find better treatments for pathologies characterized by memory loss, such as depression, anxiety or Alzheimer's disease.

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