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Review

Investigating the transition from recent to remote memory using advanced tools

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ABSTRACT

Remote memories, weeks to decades long, are usually the ones most important to the organism, as the longevity of a memory is tightly connected to its significance. Retrograde amnesia studies in human patients as well as lesions and immediate early gene expression investigation in animal models, suggested that the hippocampus has a time dependent role in memory consolidation. Namely, that as a memory matures it becomes independent of the hippocampus and instead depends on extra-hippocampal areas. However, accumulating evidence implies that this temporal segregation is not as rigid as originally proposed. In this review we will focus on the integration of new methods, such as chemogenetics, optogenetics and calcium imaging, which enable genetic specificity as well as high temporal and spatial resolution. Using these methods, recent studies have started to resolve the inconsistencies of past findings by observing and manipulating neural ensembles in different brain regions. We then discuss how these techniques can be applied to investigate the cellular underpinnings of memory across multiple time points, and employed to study the contribution of various cell types to remote memory.

1. Introduction

Memory consolidationis a complicated process in which an impermanent learnt association is turned into a semi-stable form (short term memory), which can be potentially maintained for long durations (long term memory)(McGaugh, 2000). Consolidation processes occur on different time scales following the acquisition stage: Recent memory involves relatively fast processes, which occur during the first hours following learning on the synaptic level (Frankland and Bontempi, 2005). Due to the well-defined time frame of this process, it has been vastly studied, and a lot is known about the different cascades involved in its formation (Abel and Lattal, 2001; Matynia et al., 2002). Remote memories, on the other hand, are consolidated over much longer time periods, in processes which involve multiple brain regions that change to enable the persistence of the memory – termed together "systems consolidation" (Frankland and Bontempi, 2005). The time dependent role of different brain regions and the mechanisms that underlie remote memories are debatable and not well understood.

In this review we will highlight the main discoveries from pioneering studies tackling remote memory, focusing on hippocampal-dependent tasks, which often reported contradictory findings. We will then discuss innovative techniques that enable the monitoring and manipulation of neuronal activity with better specificity and higher temporal and spatial resolution than previously available, which now uncover new concepts regarding remote memory. Finally, we will discuss different ways to incorporate these methods in future directions in remote memory research that have not been explored yet, and may transform the field.

2. The dynamic nature of remote memories

Prominent theories suggested that the consolidation of remote memories is a dynamic process in which memories first require hippocampal activation, and later become independent of the hippocampus and instead rely on extra-hippocampal regions (Wiltgen et al., 2004). This hypothesis is based on human studies in retrograde amnesia patients with hippocampal lesions that exhibit damaged recent memory but intact remote memory, suggesting that the hippocampus is necessary for newer memories, but not remote ones (Squire et al., 2015).

Further support for this hypothesis comes from multiple animal studies that used both lesions and gene expression studies to study the time dependent roles of these areas in memory. Several studies showed that lesioning hippocampal activity impairs recent but not remote memory (Gonzalez et al., 2013; Maviel et al., 2004; Ross and Eichenbaum, 2006; Wiltgen et al., 2010) while damage to frontal areas impairs remote but not recent memory (Fitzgerald et al., 2015;

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Frankland et al., 2004; Maviel et al., 2004; Teixeira et al., 2006). Moreover, the hippocampus was found to be more active during recent spatial memory retrieval compared to remote retrieval, as indicated by higher metabolic activity (Bontempi et al., 1999). Later studies testing immediate early gene (IEG) expression strengthened this theory by showing higher expression levels in the hippocampus during recent memory retrieval compared to remote memory, and the opposite pattern in frontal areas (Frankland et al., 2004; Maviel et al., 2004; Ross and Eichenbaum, 2006; Teixeira et al., 2006).

Nevertheless, several lines of evidence suggest that this temporal segregation is not truly that rigid, and in fact the hippocampus and the frontal areas may actually be involved in both recent and remote memories: First, studies found that the role of the hippocampus in memory consolidation exceeds the early memory stage: Elevated IEG levels in the hippocampus were found following remote retention of a spatial memory (Bonaccorsi et al., 2013; Goshen et al., 2011), as well as following contextual fear retrieval, even a year after conditioning (Lux et al., 2016). Importantly, different subdivisions of the hippocampus might show differential activation patterns during recent, as well as remote, memory retention (Barry et al., 2016; Gusev et al., 2005; Lux et al., 2016). Lesioning the hippocampus following reactivation of a previously learnt context-fear association can damage remote memory retention (Debiec et al., 2002; Shimizu et al., 2000)(but see (Milekic and Alberini, 2002)). Moreover, one study found that hippocampal damage following training impaired both recent and remote contextual and auditory-cued fear conditioning (FC). The degree of impairment was correlated with the extent of the lesion (Sutherland et al., 2008). A different study showed that animals that were fear conditioned with a lesioned hippocampus acquired intact freezing response during the training stage. However, they showed a deterioration of contextual memory as time went by, implicating the relevance of the hippocampus in the maintenance of remote memories (Zelikowsky et al., 2012). Similarly, damage to the hippocampus disrupted recent as well as remote spatial memory (Teixeira et al., 2006). Interestingly, naturally occurring changes in the hippocampal network induced by the incorporation of new cells into the dentate gyrus (DG) also disrupts recall (e.g. (Akers et al., 2014; Ishikawa et al., 2016)).

Second, a number of studies found an early involvement of frontal areas in recent memory consolidation: Lesions to frontal areas during early consolidation could impair remote memory retention (Blum et al., 2006; Einarsson and Nader, 2012; Gonzalez et al., 2013; Lesburgueres et al., 2011; Sierra et al., 2017; Stern et al., 2013; Takehara-Nishiuchi et al., 2006). Consistently, studies found elevated IEG in frontal areas during early consolidation (Gonzalez et al., 2013; Gusev and Gubin, 2010). Finally, one study reported that only simultaneous lesioning of both the hippocampus and the anterior cingulate cortex (ACC) impairs contextual fear memories (Einarsson et al., 2015). Studies investigating the roles of various epigenetic mechanisms in remote memory also suggest that the temporal separation between different brain regions is rather ambiguous (e.g. (Day and Sweatt, 2010; Graff et al., 2012; Lesburgueres et al., 2011; Miller et al., 2010; Zovkic et al., 2014)). Taken together, the gene expression and epigenetic studies point to a non-trivial temporal gene regulation dynamics of memory consolidation in various brain regions. As will be detailed in section 3, the integration of new perturbation and imaging methods has already shed some light on these contradictory findings.

2.1. Memory precision and generalization

As time goes by, most memories become less precise and more generalized − conveying the “gist” of a context, rather than its precise identity (Biedenkapp and Rudy, 2007; Ruediger et al., 2011; Wheeler et al., 2013; Wiltgen and Silva, 2007). The generalized version of the memory is hypothesized to be stored in extra-hippocampal areas, whereas the hippocampus is necessary for the retrieval of precise memories, even at remote times (Pedraza et al., 2016; Pedraza et al., 2017; Wiltgen et al., 2010; Winocur et al., 2007). Most of the studies tackling this issue are based on the observation that following FC training, animals show fear responses not only in the conditioned context, but also in a novel, neutral context during remote, but not recent recall, indicating that their memory is less precise following long retention intervals. Taking advantage of this phenomena, studies have shown that the hippocampus expresses less IEGs during distinct memory retrieval in comparison to a generalized one (Cullen et al., 2015; Wiltgen et al., 2010), possibly due to feedforward inhibition (Ruediger et al., 2011). Additionally, a marker of the mTOR signaling pathway, relevant for synaptic plasticity and memory consolidation, is elevated in the hippocampus for non-generalized contexts, whereas its levels in the ACC are elevated for generalized contexts (Gafford et al., 2013). Moreover, lesioning the dorsal hippocampus impairs precise recent memory retrieval while ventral hippocampus and ACC silencing decreases remote generalization of contextual fear (Cullen et al., 2015; Einarsson et al., 2015). Interestingly, a recent study has shown that time dependent generalization also occurs in a spatial task: whereas in recent times, mice learned specific locations, following longer retention periods they performed according to the overall statistics of the environment. This type of learning was impaired following pharmacogenetic inhibition of the medical prefrontal cortex (PFC) (Richards et al., 2014).

Nevertheless, employing different training procedures can generate precise long-lasting memories. For example, using multiple pre-exposures to a neutral context along with the fear-conditioning context, it was shown that mice show less remote contextual generalization than following the standard FC protocol in which the neutral context is only apparent in the test stage. Hippocampal lesions impaired this contextual discrimination during recent but not remote retrieval in comparison to controls (Wang et al., 2009), indicating that precise memories do not necessarily require an intact hippocampus. However, the authors note that the lesioned mice had a more fragile version of the memory, which is less robust than when it is supported by hippocampal regions.

Taken together, these studies show that the hippocampus is involved in the maintenance of precise remote memories, though certain memories may persist independently of this region. Future research incorporating various behavioral paradigms, as well as new techniques enabling higher temporal and spatial resolution, may help refine the exact role of the hippocampus in the preservation of precise memories as time passes by.

2.2. Extinction of remote contextual memory

Following the acquisition of a fear memory, repeated exposures to the conditional stimulus (CS) in the absence of the unconditional stimulus (US) result in a reduction of fear expression, a phenomenon named extinction (Quirk and Mueller, 2008). Most animal studies have focused on the extinction of a recently acquired fear response, where the CS is often a cue (reviewed in (Herry et al., 2010; Ji and Maren, 2007; Kim and Jung, 2006; Quirk et al., 2006)). Remote contextual fear extinction, however, is far less understood, though accumulating evidence indicates that both cortical and hippocampal regions are involved in this process (Awad et al., 2015; Corcoran et al., 2013; Graff et al., 2014; Ye et al., 2017). Specifically, one study found that the PKA signaling pathway in the retrosplenial cortex is involved in remote extinction (Corcoran et al., 2013). Others showed that the infra-limbic cortex is necessary for both recent and remote extinction (Awad et al., 2015; Ye et al., 2017).

Interestingly, while extinction of recently acquired contextual fear memories is persistent, this is not the case for remotely acquired memories. Rather, when extinction takes place following a long interval after contextual conditioning, the fear response spontaneously recovers after the extinction training (Costanzi et al., 2011; Graff et al., 2014; Quirk et al., 2010). The persistence of remote fear memory was shown

to be based on an epigenetic mechanism, specifically reduced histone acetylation in the hippocampus, which decreases plasticity compared to the recent time point (Graff et al., 2014; Lattal and Wood, 2013).

3. Contradictory evidence can be resolved by the integration of new research techniques

The studies described so far were mostly limited to correlational experiments or lesion studies, which could not be selectively targeted to specific cells. Moreover, the temporal resolution of these methods is low, and must be considered when interpreting studies that use them. In the following sections we will discuss studies employing new techniques to selectively and precisely manipulate cell populations of choice, and how they challenge and complement earlier studies. Moreover, we will discuss the potential of the new methods to resolve some of the contradictions in the field, and raise new questions for future studies.

3.1. Genetically targeted manipulations of neuronal populations lead to new insights

Two powerful approaches now enable researches to manipulate discrete cell populations based on specific genetic markers: (1) Optogenetics, employing light-sensitive opsins activated by a specific light wavelength, offers genetic specificity, bidirectional effects (excitation or inhibition) and a temporal resolution in the order of milliseconds. (2) Chemogenetics, employing Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). The most commonly used DREADDs are engineered G-protein coupled receptors that can recruit the endogenous Gq, Gi or Gs pathways by the application of an otherwise inert drug (reviewed in (Roth, 2016)). Chemogenetics offers genetic specificity, bidirectional effects (excitation or inhibition) and a temporal resolution in the order of minutes to hours. Both methods are vastly superior to the traditional physical or pharmacological lesions, and their distinct time resolutions make each one of them suitable to answer different needs (Table 1).

Many studies investigated the time dependent necessity of the hippocampus and frontal areas by lesioning different brain regions, yet their low temporal resolution could possibly give rise to compensatory pathways (e.g. (Lesburgueres et al., 2011)). To overcome the temporal delay, we −optogenetically inhibited CA1 activity only during the time of retrieval, and showed that this precise manipulation disrupts both recent and remote contextual fear memory. However, when prolonged optogenetic inhibition (mimicking the temporal timeframe of previous pharmacological studies) was employed, remote memory was intact, similarly to results obtained in lesion studies (Goshen et al., 2011). Optogenetic ACC inhibition had no effect on recent memory, and similar to genetic and pharmacological lesion disrupted remote memory, regardless of the temporal precision of the manipulation. The temporal precision of optogenetics was used since to show the different roles of the DG in acquisition, retrieval, and extinction of contextual memory (Bernier et al., 2017; Kheirbek et al., 2013). These studies demonstrate the importance of temporal precision in memory investigation, offered by modern optogenetic tools.

The lower temporal resolution of DREADDs makes them less suitable for precise manipulations (for example (Varela et al., 2016)), but nonetheless advantageous when prolonged manipulations are required. For example, one study used a Gi-DREADD to chronically silence different brain regions for 10 consecutive days following contextual FC acquisition (Vetere et al., 2017) (see Section 3.2), which cannot be done using optogenetics. Moreover, DREADDs can be used to manipulate cells during tasks which are long and have special environmental designs, such as a Moris water maze (Richards et al., 2014). DREADDs can also be used to induce higher neuronal excitability, without introducing synchronous activity in the network (Cai et al., 2016). As can be seen, the characteristics of each method make them appropriate for different research questions.

Table 1
Classical and modern manipulations used to study remote memory. The table lists the major differences between the available techniques, pointing to their benefits and drawbacks.

	Lesions	Pharmacology	Optogenetics	Chemogenetics
Surgery	Acute insertion of electrode or infusion needle	Chronic cannula insertion	Viral injection and insertion of chronic ferule	Viral injection
Procedure before and during behavior	None	Infusion of pharmacological solution through cannula (requires restraint or anesthesia)	Connection of optic fibers to ferule. Fiber usually attached during behavior	Designer drug delivered i.p. or orally (in drinking water or food)
Direction of manipulation	Inhibition only	Inhibition or excitation	Inhibition or excitation	Inhibition or excitation
Cell-type specificity	None	Limited	Yes	Yes
Activity-based specificity	None	None	Yes	Yes
Effect on axons of passage	Damaged in mechanical lesions and spared in excitotoxic lesions	Possible (depending on the pharmacological agent)	None (unless specifically targeted)	None (unless specifically targeted)
Spatial resolution	Very low, Widespread damage, usually beyond the target region.	Low, Widespread infusion, usually beyond the target region.	High, if opsin expression is restricted by viral vector-delivery. Medium when the region is determined by light delivery in transgenic animals.	High, if DREADD expression is restricted by viral vector-delivery. In transgenic animals: Low if designer drug is injected to the target region via a cannula. Very low if delivered i.p. or orally
Temporal resolution	None, Irreversible lesion	Low Minutes to days − depending on the drug.	High Millisecond precision	Low Several hours
Induction of synchronous activity (for excitation only)		Possible, Depending on the pharmacological agent	Yes for all excitatory opsin except step-function opsins.	No. If expression and dose are calibrated properly. Possible at high expression or high designer drug dose

3.2. A global brain-wide perspective on remote memory

As mentioned above, the relatively simple framework in which there is a clear division of labor between the hippocampal and frontal areas at different time points (Frankland and Bontempi, 2005) needs to be updated to a more comprehensive model to fit the accumulating contradictory evidence. The hippocampus recruits different brain circuits during recent and remote memory (Goshen et al., 2011), and due to the dynamic evolution of remote memories, and the multiple brain regions involved in concert in their consolidation and retrieval, it is highly important to determine the functional connectivity of different brain networks.

An ambitious study attempted to investigate this issue by analyzing brain-wide IEG expression to identify regions that were simultaneously activated during recent and remote contextual fear memory retention (Wheeler et al., 2013). During remote memory, a brain network consisting of numerous regions (e.g. cortical, thalamic, hypothalamic) was recruited. Moreover, frontal areas became more correlated with each other, as well as with other regions including the hippocampus, as time elapsed. The authors concluded that despite the finding that the hippocampus shows reduced IEG levels during remote compared to recent memory retrieval, its stable or even increased activity correlation with frontal areas implicates that it remains involved in contextual fear memories. By studying different brain regions in the context of a brain-wide network, the study revealed the importance of different regions as a measure of their connectivity and not solely of their relative activity levels as is often done in IEG expression studies. Based on the observed correlations, they suggest that the brain has a small world architecture, where most brain regions have a limited number of connections, and only a few are highly connected hubs. To test this model, a follow up study investigated the effect of silencing numerous brain regions using DREADDs following contextual fear training during remote retention (Vetere et al., 2017). It reports that silencing brain regions serving as main hubs with high functional connectivity (e.g. CA1, the intermediate part of the lateral septal nucleus, the reuniens thalamic nucleus and the laterodorsal thalamic nucleus) caused a significant reduction in contextual freezing, as predicted by their model. Inhibition of 17 other brain regions predicted to have low connectivity by the model had no significant effect on memory retention. The availability of whole brain clearing and imaging using tools like CLARITY can significantly enhance brain-wide imaging of fixed tissue and illuminate the global anatomy of such networks (Ye et al., 2016).

Brain-wide measurements of activity can also be performed in vivo. For example by measuring the LFP coherence between different areas (retrospleniel cortex, dorsal hippocampus, ACC and the anterior dorsal thalamus) during retrieval of recent and remote contextual fear memory (Corcoran et al., 2016). Differential coherence ratios depended on the memory stage, and predicted the retrieval success of the mice in remote memory. Oscillatory synchrony between the medial PFC and the hippocampus has been suggested as an indication of bi-directional information processing, and might be relevant for remote memory (Eichenbaum, 2017). Calcium imaging over multiple brain regions (see Section 3.4) can open new avenues of investigation of memory networks in live animals.

To better understand the global networks underlying memory, several methods can be used to examine the anatomical connectivity between relevant regions, and manipulate them. For example, cells in the medial entorhinal cortex (MEC) were infected with an inhibitory opsin, and light was delivered to the terminals at different memory stages in several downstream brain regions to recognize necessary and sufficient projections from this area (Kitamura et al., 2017). One interesting finding was that the MEC-PFC projection activity during training was necessary for intact contextual remote retrieval, exemplifying how specific connections, rather than entire regions, can influence memory processes. Some studies have used a similar approach using optogentic activation instead of inhibition (e.g. (Warden et al., 2012)), however when employing this method one needs to rule out the possibility that activating the terminals might affect the cell bodies as well (Fenno et al., 2011). A more specific approach is to first target monosynaptic connections using retrograde viruses. Afterwards, a virus carrying an opsin gene is injected to the area where the projecting cells reside, and light is administered to the terminal area. One study found such monosynaptic projection between the ACC and the CA1 and CA3 regions, and used optogentic activation to show that the hippocampal neurons activated during acquisition (i.e. engram, see next section) are later recruited by this top-down projection (Rajasethupathy et al., 2015).

The innovative studies described above have already started to illuminate the roles of various projections in memory, yet further research is required to better define the functional connectivity involved in remote memory. For example, *trans*-synaptic retrograde viruses can be used in combination with optogenetics to reveal multi-step circuits and dissociate the roles of specific projections in different memory types, as was lately done in a study about recent memory circuitry (Xu et al., 2016). Taken together, these studies emphasize the importance of investigating networks across multiple brain regions over time, a field that can now employ a combination of new techniques for both fixed and live imaging. Such experiments have the potential to refine the findings obtained in past studies which used lesions and pharmacology, methods that cannot be selectively targeted to specific projections and sometimes affect axons of passage.

3.3. Exploring and manipulating engrams using genetically targeted tagging of active neurons

A common view of memory is that the same population of neurons encoding it via synaptic alterations is later activated when the memory is recalled (reviewed in (Holtmaat and Caroni, 2016), though see (Arshavsky, 2006) for an alternative account). This neuronal population is called an “engram”. Engrams were found in multiple brain regions, during memory acquisition, consolidation and retrieval in various paradigms, from FC to drug related memory (Cruz et al., 2013; Silva et al., 2009; Tonegawa et al., 2015).

Pioneering studies investigated the formation of engrams during learning in order to determine which cells are more likely to become part of an engram. Interestingly, neurons that are highly excitable during acquisition are more likely to be allocated to a memory engram than neighboring neurons. Selective ablation of such neurons in the amygdala impaired conditioned cue memory, whereas selectively increasing neuronal excitability resulted in recent memory enhancement (Han et al., 2007; Han et al., 2009; Josselyn et al., 2015; Yiu et al., 2014; Zhou et al., 2009). Elevating excitability of DG neurons during contextual fear acquisition induced higher freezing levels during both recent and remote retrieval compared to controls (Sekeres et al., 2012).

Different studies investigated the fate of engrams as a memory ages, and used optogenetic tools to manipulate them at different time points and test the effect on memory retrieval. An important tool used for such studies is the Tet-Tag transgenic mouse line, allowing the expression of a gene of choice in neurons that were activated during a specific time window (Reijmers et al., 2007). Specifically, in such transgenic mice, an IEG promotor (i.e. cFos or Arc) can be coupled to the tetracycline transactivator (tTA). The mice chronically receive Doxycycline which prevents the tTA from binding to its target tetracycline-responsive element (TRE) site. In the absence of Doxycycline, the tTA can bind to the TRE, thus enabling tagging of neurons that were active during a specific time-frame. One study (Tanaka et al., 2014) used this system to tag reactivated cells by first expressing a fluorophore in the cells activated during contextual FC acquisition using the Tet-Tag system, and then performing a cFos staining following retrieval. They showed that the relative reactivation percentage of CA1 neurons was correlated with the freezing response. Another study used a similar method and found that the percent of reactivated (double stained) cells in the

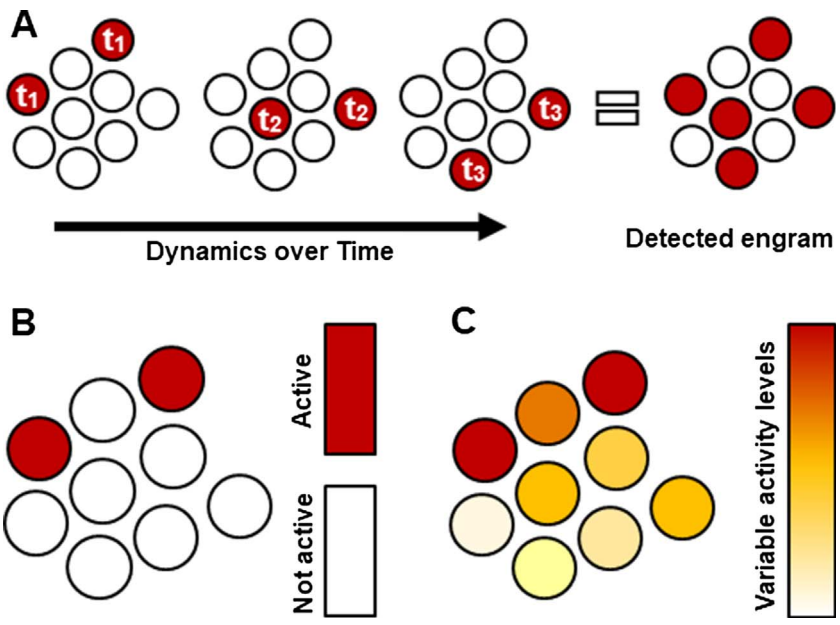

Fig. 1. Monitoring cell ensemble dynamics. Using IEG tagging methods, cells that are sufficiently activated (above the specific IEG transcription threshold) are marked. (A) The detected engram (right) consists of cells that were tagged at specific time points in a temporal window of at least an hour, and hence does not reveal the underlying temporal changes. Calcium imaging, on the other hand, allows temporally precise detection of ensemble dynamics. Furthermore, calcium imaging can be used to trach the same population over days, to detect long-term engram dynamics. (B) Differential activity levels are not apparent in the IEG driven engram. Each neuron either crosses the threshold for detectable detection, or not. (C) Using calcium imaging differential activity levels can be deduced from the calcium transients.

hippocampus and several cortical areas was above chance for both recent and remote recall, whereas reactivation in the amygdala was only high during recent retention (Tayler et al., 2013) (but see (Kitamura et al., 2017)). This may indicate that engrams are formed and maintained in both cortical and hippocampal regions as the memory ages, supporting the hypothesis that these regions do not have temporally discrete roles.

Other groups employed engram tagging to monitor spine dynamics in the neurons activated during memory acquisition. Without tagging, one could only find general patterns of spine dynamics during remote fear retrieval, without knowing if the observed spines belong to cells that were part of an engram. For example, such studies reported increased spine density in the ACC and decreased spine density in the hippocampus (Restivo et al., 2009; Vetere et al., 2011). By tagging hippocampal neurons active during memory acquisition it was shown that these specific cells have lower spine densities compared to non-active cells shortly after learning (Sanders et al., 2012). A later study showed that engram cells in the PFC have higher densities at long intervals compared to short ones after acquisition, while the opposite pattern was observed in the DG (Kitamura et al., 2017).

Several studies took advantage of the Tet-Tag system to not only image activated neurons, but manipulate them as well. For example, a population of DG neurons that were active during FC acquisition was targeted in Tet-Tag mice injected with a viral vector inducing the expression of an excitatory opsin. When these cells were optogenetically activated at a later time point in a different context, freezing was induced, suggesting that reactivation of hippocampal engram cells is sufficient to induce recent recall (Liu et al., 2012; Ryan et al., 2015) as well as remote recall (Kitamura et al., 2017). A later study used a similar approach to express the inhibitory opsin eArch in the CA1, and showed that optogenetically silencing CA1 engram cells abolished contextual freezing (Tanaka et al., 2014). Interestingly, similar contexts recruited similar neural activation patterns, or in other words gave rise to overlapping engrams. Thus, silencing a specific engram disrupted the retention of the generalized contextual memory only if it was encoded by an overlapping population of neurons as the conditioned one (Tanaka et al., 2014). A recent study showed that inhibiting PFC engram cells resulted in reduced freezing during remote, but not recent recall, whereas activating such cells induced a fear response following both intervals. Finally, inhibiting the terminals of PFC engram cells found in the amygdala resulted in remote memory deterioration (Kitamura et al., 2017). Tet-Tag mice can also be used to express DREADDs, though this approach so far yielded less potent results (Garner et al., 2012).

A different approach to tag activated cells within a short time window is the IEG promoter-Cre-ER^{T2} mouse lines, which can be used to selectively express a gene of choice in activated cells in the presence of tamoxifen by crossing it with a Cre dependent reporter mouse line (Guenthner et al., 2013). Using this approach, Denny and colleagues showed that recently acquired contextual memory retrieval did not generalize to a different context. The reactivation rate of CA3 and DG neurons in the retrieval stage was higher when animals were re-exposed to the conditioned context than to a novel one (Denny et al., 2014). However, remotely acquired contextual memory generalized to a novel context, and correspondingly did not show differential reactivation patterns for the conditioned and novel contexts. Optogenetic silencing of the CA3 and DG neurons recruited during acquisition impaired remote memory retrieval (Denny et al., 2014).

To conclude, studies using activity dependent tagging of neurons enable the investigation of specific neuronal populations, unlike studies which use manipulations of entire brain regions, leading to new insights regarding remote memory. For example, this method has shown that the reactivation of engrams found in hippocampal regions is sufficient for remote memory recall, hinting that this region is still relevant for memory long after acquisition. Nevertheless, while these methods have many advantages, they are still limited in several respects. First, the low temporal resolution (in the order of hours) does not allow the investigation of temporal sequences that may be relevant to the memory encoding, consolidation and retrieval processes. Second, cell tagging is dependent on the specific IEG threshold that reflects a specific action potential pattern (Fields et al., 2005), which certain active cells may not reach and therefore be missed (Fig. 1). Finally, expression is not genetically targeted to specific neurons, and can sometimes be found in various cells types (Chen et al., 2016; Ramirez et al., 2015). In the next section we will discuss imaging techniques, which provide higher temporal resolution and are independent of IEG thresholds, and the promising findings regarding remote memory obtained by employing them.

3.4. Investigating neuronal ensembles underlying memory formation and retrieval

Major advancement in calcium imaging techniques now enables researchers to conduct time-lapse experiments in which the same

population of neurons or smaller cellular compartments is observed for long intervals (Jercog et al., 2016). The temporal resolution of this method is higher than that of the IEG driven methods described previously. Specifically, the activity of cells can be identified on significantly shorter time scales (i.e. in the order of milliseconds to seconds) and with a different threshold (Fields et al., 2005). It should be noted, though, that calcium indicators can affect the natural calcium dynamics within cells and may lead to cytotoxicity after prolonged expression (Tian et al., 2009).

Taking advantage of these benefits, pioneering studies used a miniature microscope in combination with an endoscope lens in order to image neuronal ensembles in freely behaving animals over time: They showed that the CA1 ensembles which encode distinct contexts are more similar when they are acquired in temporal proximity than when they are separated by a long duration of time (Cai et al., 2016; Rubin et al., 2015). Interestingly, studies performed in the amygdala also showed that the neurons recruited to encode a memory are more similar for temporally adjacent events (Grewe et al., 2017; Rashid et al., 2016). Finally, one study showed that PFC engrams consist of relatively "silent" non-selective cells, which become more discriminative (i.e. show a larger activity difference between the conditioned context to a neutral context) as time progresses (Kitamura et al., 2017).

A different approach used to investigate the time-lapse activity of neurons during different memory stages is 2-photon imaging in combination with a virtual reality (Lovett-Barron et al., 2014; Rajasethupathy et al., 2015). This technique has a much higher resolution than the wide-field miniature microscopes, and importantly it has the ability to optically differentiate between cells along the z-axis. Thus, it enables imaging of different cell layers or compartments within single cells across long durations (Sheffield and Dombeck, 2015). Contextual fear can be learned in head fixed mice under a 2-photon microscope and studies using such an apparatus have already yielded interesting results regarding recent memory (Lovett-Barron et al., 2014; Rajasethupathy et al., 2015), but to our knowledge no work has been done for remote time scales. Simultaneous imaging of multiple brain regions (Sofroniew et al., 2016) can also be beneficial for the brain-wide search for the neural underpinnings of remote memory. However, the surgical procedure required for this method, i.e. implanting chronical windows over the cortex and/or cannulas above deep structures, poses some limitations on the regions which can be scanned in terms of distance and depths.

Finally, high resolution 2-photon imaging also enables tracking dendritic spines for long durations in vivo, both in the cortex and in the hippocampus. Using this technique, several studies found that the spines in different regions of the cortex are mostly stable in the adult animal, following a more plastic stage during early development (Grutzendler et al., 2002; Yang et al., 2009; Zuo et al., 2005). This stability was suggested as a mechanism enabling the storage of persisting memories, whereas transient spines could serve as a mechanism enabling new learning (Kasai et al., 2003). Studies examining the hippocampus using 2-photon imaging have found various spine turnover rates (percentage of eliminated or newly gained spines) in the adult mouse CA1 region (Attardo et al., 2015; Gu et al., 2014), a discrepancy that future studies may resolve.

In summary, calcium imaging techniques have already begun to expand our knowledge about remote memory acquisition, consolidation and retrieval. Taken together with the reactivation studies described previously, the findings obtained in these studies raise interesting questions regarding the importance of temporal dynamics within brain networks: Reactivation of an engram using tagging techniques is sufficient, in some cases, for recall, despite the fact that it does not convey the temporal code of the neurons. Moreover, whereas calcium imaging studies indicate that only a small portion of the memory acquisition engram is activated during remote recall, reactivating the original engram is sufficient for retrieval. Future work can use 2-photon imaging in combination with spatially and temporally precise optogentic tools to reactivate specific ensembles and determine the role of time dependent network activity in memory. In addition, monitoring specific neurons and manipulating them at single cell resolution, in contrast to the tagging experiments in which only entire networks can be manipulated, can help determine the sufficiency of single cells for memory recall. Studies can also benefit from these methods in the investigation of various cell types which cannot be specifically targeted using tagging techniques as the brain changes during systems consolidation.

4. In the future

4.1. From discrete to continuous sampling: studying remote memory at multiple time points

Experiments that test remote memory usually compare different biological markers like IEG driven fluorescence at discrete time points, often at acquisition and recall only. Time lapse monitoring of the cells between the initial acquisition and the remote retention is ideal for chronic experiments in which the biological marker can be repeatedly tracked in live mice. To this end, new methods enabling real time IEG monitoring in-vivo using transient IEG dependent fluorescence can be beneficial (reviewed in (Kawashima et al., 2014)). As mentioned before, calcium imaging of neuronal ensembles has already proven useful in the investigation of memory consolidation, and can be expanded to the examination of temporal activation patterns (Fig. 1), which might be relevant for memory consolidation and retrieval (Malvache et al., 2016). Importantly, extensive recording will likely provide large amounts of data. In order to analyze and interpret it, there is a need for automation processes as well as theoretical frameworks, which can provide testable hypothesizes.

4.2. Taking manipulations a step further

During recent years, the manipulation toolbox available to neuroscientists has expanded dramatically. The development of DREADDs and opsins, which can be genetically targeted to a set of specific cells distinguished by either cell type or activity has enabled researchers to selectively manipulate populations of interest in various regions of the brain in a reversible fashion with different temporal resolutions (Goshen, 2014; Roth, 2016). Such tools have enabled researchers to study more naturalistic brain circuits without the emergence of compensatory pathways and manipulate engrams following their tagging during memory acquisition (Tonegawa et al., 2015). Newly developed DREADDs and optogenetic tools now allow bidirectional manipulation for the same cell population. Specifically, DREADDs that are controlled by different ligands have been developed (Vardy et al., 2015), as well as viral cassettes that express both excitatory and inhibitory opsins activated by different wavelengths (Rashid et al., 2016) − both of which can be used in the remote memory field. Furthermore, red shifted opsins now allow temporally and spatially precise 2-photon optogenetic manipulation in combination with imaging (Emiliani et al., 2015). Together, these techniques can be used to investigate and manipulate specific cells within an engram as well as temporal sequences that occur during consolidation and retrieval.

4.3. Investigating the contribution of different cells types to remote memory

Most of the studies investigating remote memory focused on pyramidal or granular neurons. As of yet, only a few directly tested the contribution of other neuron classes or glia to these processes. Studies have shown that inhibitory and neuromodulatory circuits contribute to memory consolidation and precision (Lovett-Barron et al., 2014; Murchison et al., 2004; Ruediger et al., 2011; Stefanelli et al., 2016; Zhang et al., 2005). Future effort employing new methods may further illuminate their role in remote memory. Furthermore, the contribution of non-neuronal cells, such as astrocytes, has not received much

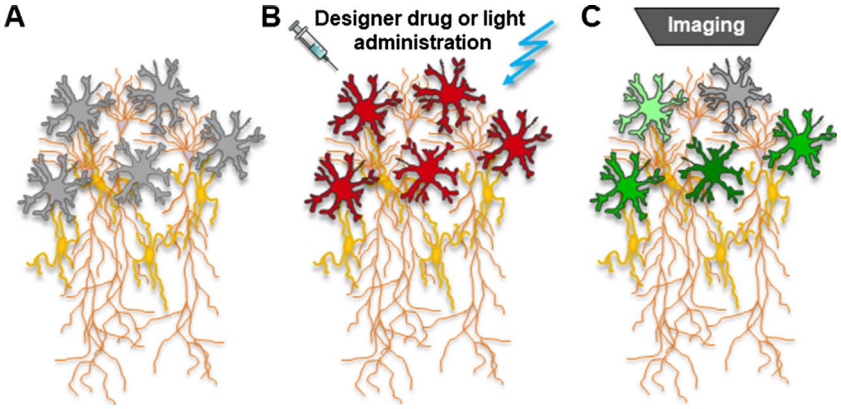

Fig. 2. Investigating the contribution of various cell types to remote memory. (A) Multiple cell types, including for example pyramidal excitatory neurons (orange), GABAergic inhibitory interneurons neurons (yellow) and astrocytes (grey), may be involved in systems consolidation. Genetic tools now allow specific targeting of each population separately. (B) Optogenetic or chemogenetic actuators (opsins or DREADDs; red) can be targeted to a specific population (for example astrocytes) using viral injections and/or transgenic mouse lines. This will allow the manipulation of a pre-defined cell type to determine its necessity and/or sufficiency to the different stages of memory. (C) Calcium indicators (green) can be targeted to a specific population (for example astrocytes), to image the activity of this population at the different stages of memory. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

attention, despite current findings which implicate they may be highly relevant for memory (Adamsky and Goshen, 2017). It should be noted that the IEG driven methods, like Tet-Tag or TRAP, are not selective for specific cell types, which means that the expression driven by them may represent engrams that include more than one cell type. In order to investigate cell sub-types separately, several things can be done: The first and relatively simple approach is to determine the different cell types of a given engram by immunohistochemistry or in situ hybridization. Second, to selectively tag and manipulate a genetically dissectible cell population, it is possible to use viral cassettes driven by unique promoters like CaMKII for excitatory neurons, ChAT for cholinergic neurons or GFAP for astrocytes. These promoters can also be used in a Cre dependent fashion in combination with activity dependent mouse lines, in order to specifically monitor and manipulate an active cell population of choice (Fig. 2).

5. Conclusions

Studying remote memory is challenging due to slow dynamics of this process, and the difficulty to pinpoint the relevant time frame for investigation. New innovative techniques enabling imaging and manipulation of cellular ensembles now enable neuroscientists to illuminate the brain-wide circuitry underlying memory as it is consolidated and retrieved. Taking advantage of these methods has already started to shed light upon the neuronal underpinnings of remote memory, though many questions remain open. Future studies may determine the involvement of various cell types in the different memory stages, and provide a less discrete temporal account of memory formation.

Conflict of interest

none

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