Activity-dependent remodeling of genome architecture in engram cells facilitates memory formation and recall

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The formation and preservation of long-term memories critically depend upon coordinated activity of neuronal circuits, intracellular signaling cascades and synaptic remodeling (Josselyn et al., 2015). These essential processes occur in specific cell populations known as the engram ensemble (Josselyn et al., 2015). The current model for engram formation suggests that an experience activates a sub-population of neurons that can be measured by the expression of immediate early genes (IEG), such as Arc (activity regulated cytoskeleton associated protein, Arg3.1) or cFos (Fos Proto-Oncogene, AP-1 transcription factor subunit). Activated engram cells are then physically or chemically modified during memory consolidation, where labile memory is transformed into a more stable and long-lasting state. Notably, the memory consolidation process occurs predominantly long after the initial stimulus had ceased and the engram cells are in a dormant or a quiescent state. Reactivation of the engram ensemble by subsequent presentation of the original stimuli results in memory retrieval. Therefore, temporal progressions of memory formation from learning to retrieval require several waves of delayed transcriptional and translational alterations (Josselyn et al., 2015). Nonetheless, the molecular mechanisms that enable this temporal and spatial synchronization remain elusive.

Epigenetic modulation and three-dimensional (3D)-genome architecture are emerging as key factors in mediating activity-dependent genes programs in numerus brain functions, including neurodevelopment, neurogenesis and neuronal properties (Malik et al., 2014; Rajarajan et al., 2016; Fernandez-Albert et al., 2019; Yamada et al., 2019). Here, we review recent studies that indicate an important role for chromatin accessibility and spatial nuclear architecture in facilitating critical aspects of learning and memory. This additional layer of evidence not only has significant implications for understanding neuronal plasticity, learning and memory, but also could enable the identification of novel disease risk factors in many cognitive and memory related disorders.

The term 'epigenetics' refers to external chemical modifications to the DNA or proteins associated with DNA that affect how cells will "read" and use the genes, without changing the DNA sequence itself (Rajarajan et al., 2016). In the cell nucleus, DNA exists in a complex with histone proteins, called chromatin. Epigenetic modifications alter chromatin structure and DNA accessibility. Generally, when chromatin is tightly packed or the DNA is blocked by methyl molecules (i.e., DNA methylation), nearby genes will not be expressed. However, open or relaxed chromatin state creates a permissible environment that enables the binding of the transcriptional machinery, and thereby controls and enhances gene expression (Rajarajan et al., 2016). Although high-resolution mapping of the epigenome, including whole genome DNA methylation and chromatin immunoprecipitation sequencing has enabled the linear genome to be understood, these data does not account for the spatial configuration of chromatin. Thus, a comprehensive exploration of the genome has to go far beyond measuring linear epigenetic properties. New approaches such as chromosome conformation capture (3C) techniques allow us to map 3D-chromatin contacts on a genome-wide scale and demonstrate multiple levels of nuclear organization (Rajarajan et al., 2016). These include the configuration of chromosome in territories and their interaction with one another and with the nuclear lamina, which has a profound effect on gene expression (Rajarajan et al., 2016). Furthermore, chromatin is segregated into two sub-nuclear compartments; 'A compartment', which is enriched for accessible chromatin and correspond to higher overall levels of transcription, and 'B compartment' that harbor the transcriptionally inactive and less accessible (heterochromatin) regions of the genome. Interestingly, early evidences suggest that neuronal activity induces re-organization in compartments and large scale 3D-chromatin structures (Rajarajan et al., 2016; Fernandez-Albert et al., 2019; Yamada et al., 2019), indicating that chromatin organization might be highly responsive to external stimuli.

The genome is further organized into self-interacting units called topologically associated domains (TADs) which are demarcated by clear boundaries. Furthermore, new observation from detailed 3D-contacts genomic maps indicated that smaller chromatin domains, so-called subTADs, are nested hierarchically within TADs. Nested subTADs are also demarcated by boundaries, however exhibit weaker insulation strength and might have different functional properties. How TADs, subTADs and compartment domains are uniquely defined by their structural and mechanistic properties remains an open question. Several lines of evidence support a model of bidirectional structure–function relationship which is determined by transcriptional programs, developmental phase and external stimuli (Rajarajan et al., 2016; Yamada et al., 2019; Beagan et al., 2020). Importantly, both TADs and subTADs tend to co-regulate gene expression by confining and stabilizing long range interactions between promoters and cis-regulatory elements, also known as enhancers (Rajarajan et al., 2016). The physical interaction between these regulatory DNA elements (promoter-enhancer) and transcription factors are known to facilitate and induce transcription. Additionally, unlike TADs which are highly stable genomic structures and are considered to be conserved across cell types and even between species (Rajarajan et al., 2016), promoter-enhancer interaction are vastly dynamic and hypothesized to be one of the major molecular mechanism acting at the interface between genome functionality and external stimuli. Thus, mapping activity–dependent chromatin organization is vital to understand the range of transcriptional responses that occur during brain and neuronal activity.

Nonetheless, this task could be extremely challenging for a few reasons; first, a functional hallmark of enhancers is that they act independent of distance and orientation to their target genes, and they can bypass thousands of base pairs of the linear genome by forming 3D-DNA loops (Rajarajan et al., 2016). In addition, individual enhancers are also found within introns. Second, one gene can be under the control of multiple enhancers (average of 4–5) and groups of putative enhancers in close genomic proximity, also

known as super-enhancers, can regulate multiple genes at the same time. Finally, the activity of enhancers can be restricted to a particular tissue or cell type, or to specific physiological, pathological or environmental conditions. In agreement with this notion, early *in vitro* studies on cortical neurons (Kim et al., 2010; Malik et al., 2014) showed relatively large activity-induced increase of two specific histone modification that usually demarked enhancers activity, acetylation of histone H3 Lys27 (H3K27ac) and monomethylation of histone H3 Lys4 (H3K4me1). These chromatin changes remained long after the neurons were depolarized and corresponded to elevated transcription. In another study, the authors used CRISPR-based dead Cas9 epigenome editing tools in mice to specifically block activity-induced histone acetylation at enhancers of Fos and Npas4 genes (Chen et al., 2019). Interestingly, this manipulation was shown to modulate the transcriptional bursting dynamics of these genes and demonstrate impaired hippocampal experience-dependent Fos gene expression, providing further support for the pivotal role of enhancer modulation in mediating proper neuronal activity and function. In agreement other *in vivo* studies reported genome-wide changes in chromatin accessibility shortly after neuronal activation (Su et al., 2017; Fernandez-Albert et al., 2019). These gained-accessibility loci were predominantly enriched at active enhancer regions and at binding sites for AP1-complex components, including c-Fos. Consistent with the *in vitro* studies, some chromatin changes remained stable for at least 24 hours (Su et al., 2017). A more recent study (Beagan et al., 2020) investigated alteration in 3D-chromatin loops during short- and long-term neural activity. The authors created high-resolution genome folding maps around rapidly transcribed genes, so called the IEGs (such as Arc/cFos) and secondary response genes (SRGs, such as Bdnf). The authors demonstrated that IEGs (Fos and Arc) connect to activity-dependent enhancers via simple, singular short-range loops that form shortly after induction, before maximum mRNA levels are reached. In contrast, the SRG (Bdnf) engages in both pre-existing and activity-inducible loops that form within 1–6 hours. This complex network of long-range loops, enables slower induction of transcription kinetics (Beagan et al., 2020). Indeed, the identification of chromatin folding, enhancer activity and other epigenetic modification in neurons was a first step toward understanding the function of these regulatory elements. However, further studies were required to address how physiological stimuli, such as those encountered during explicit learning tasks, impact chromatin modulation across different phases of memory.

Studying molecular changes over time has been one of the major challenges in the field, as the markers for neuronal activity, such as IEGs, return to baseline shortly after induction (Josselyn et al., 2015). This creates technical limitations to temporally track and separate activated neurons long after the stimulus ceased. Different approaches using activity-dependent expression of marker proteins were able to identify and manipulate specific cell populations that are activated during the process of long-term memory formation, thus providing a framework for exploring the engram ensemble (Josselyn et al., 2015). In our recent publication (Marco et al., 2020), we utilized a mouse model known as TRAP (targeted recombination in active populations) which uses the promoter of an IEG, Arc to drive permanent expression of a fluorescent reporter (eYFP) in a Tamoxifen-inducible manner (Josselyn et al., 2015). This allowed tagging of activated neurons during the learning phase and the longitudinal tracking of these neurons during memory consolidation. Re-exposing the mice to the conditioned stimulus followed by staining of the endogenous ARC proteins, allows the identification of the re-activated engram cells during memory recall. This approach allowed us to conduct molecular studies of memory consolidation and recall in neurons directly involved in memory generation. Following Pavlovian contextual fear conditioning paradigm (a commonly employed method to study aversive memories), hippocampi tissues were extracted from TRAP mice and four population of neurons were isolated: i) non-activated basal-state neurons, ii) activated neurons after learning, iii) late state tagged neurons (5 days after learning), denoting memory consolidation and iv) reactivated cells from memory recall. Our work provides the first comprehensive landscape of temporally distinct reorganization of chromatin accessibility and 3D-genome architecture during different phases of memory formation and the first ever transcriptional and epigenetic characterization of engram cell during memory recall.

Specifically, our findings show that at the chromatin level, memory formation is largely an enhancer-driven phenomenon. Comprehensive analysis of the chromatin state revealed that memory encoding leads to increased accessibility, predominantly on non-coding regulatory elements (intergenic and intronic). Moreover, these regions were significantly enriched with specific histone modification of enhancers, H3K4me1 and H3K27ac. Remarkably, many of these loci remained accessible and stable throughout all memory phases. These results are in line with previous publications, showing that stimulating neurons induces prolonged enhancer activity (Su et al., 2017; Fernandez-Albert et al., 2019) although these enhancers were not known to remain accessible over the extended timespan (5 days) of memory consolidation and recall. Notably, the accessibility on its own was not sufficient to induce a robust transcriptional response, suggesting that this is a priming event.

Further investigation of the late phase of memory consolidation revealed re-localization of large chromatin segments (sub-compartments) from B to A compartments (and *vice versa*), where many of the putative enhancers transformed from inactive to permissive environment. Within those compartments, we have identified large-scale reorganization of specific long- range interactions, where in each memory phase, the same promoters interact more frequently with a distinct subset of enhancers. Consistent with this data, Yamada et al. (2019) demonstrated that a motor learning paradigm that is represented by the granule neurons in the cerebellum promotes activity-induced alteration in promoter-enhancer interactions, sub-nuclear compartments organization and corresponding transcriptional programs. Moreover, conditional CRISPR based knockout of the Cohesin subunit, Rad21 in the granule neurons impaired the learning associated enhancer-promoter interactions and transcriptional re-programming.

Interestingly, long-range interaction dynamics during memory formation and recall indicated a 'loop competition' process where two or more enhancers, with opposing effects on transcription are competing to access the gene promoter. For example, during basal state, promoters interact with a 'silencer' protein and the corresponding transcriptional activity was weak. However, during memory consolidation, the same promoter moves to interact with an active enhancer and correspondingly leads to elevated gene expression. Importantly, *de novo* interactions of the gene promoter (regardless if it is with an 'activator' or 'silencer' enhancer), was largely afforded only after these enhancers

gained accessibility during memory learning, providing further evidence for a priming event and transcriptional lag. Together, these epigenetic and spatial chromatin changes contribute to long-lasting alterations in neuronal properties and function after the learning phase. Previous studies have also suggested that activity-induced expression of IEGs, such as c-Fos, might be coupled with localized transcription of non-coding RNAs on enhancers (Kim et al., 2010). In another study, the authors described a different mechanistic model (to the 'loop competition'), in which non-coding RNAs on enhancers act as decoys and release the target Arc promoter from negative regulators of transcription (Schaukowitch et al., 2014). Yet, all of these models are associated with IEGs, which require a mechanism to rapidly induce activation. The model of chromatin reorganization described in our study suggests a slow and sequential alteration of the enhancer landscape, which is more consistent with the later phases of memory consolidation and the timescale of synaptic and morphological changes observed after learning. Nonetheless, it appears that neurons use a wide range of machinery to coordinate gene expression programs in response to different forms of activity, and future studies should further explore these models under different environmental stimuli.

Finally, our studies illuminate for the first time the unique transcriptional landscape of reactivated engram cells. Importantly, our analysis revealed that with reactivation, engram neurons utilize a subset of *de novo* promoter-enhancer interactions, where primed enhancers were brought in contact with their respective promoters to up-regulate genes involved in mRNA transport and local protein translation in synaptic compartments. By utilizing high-resolution microscopy tools for imaging, coupled with immunohistochemistry and RNA in situ hybridization assays, we have found increased transport of Glutamate Ionotropic Receptor AMPA Type Subunit 1 mRNA to the dendritic shafts of reactivated engram neurons. This process was coupled with elevated protein levels in synaptic compartments, of members from the Eukaryotic Translation Initiation Factor family, which functions in the early steps of protein synthesis. Collectively, this data suggests that these synapses are functionally stronger and memory might be modified at this phase. What is the functional role of a priming event and a delayed transcriptional surge in the reactivated engram cell? Recent studies have reported that memory retrieval is involved in transient destabilization of the engram state, followed by a protein synthesis to re-stabilize the engram (Josselyn et al., 2015). We propose that the observed unique transcriptional signature of the reactivated engram (initiated during memory retrieval), is required for the active process of memory reconsolidation or memory extinction. In line with this hypothesis, ablation of engram cells was recently shown to impair fear extinction learning (Khalaf et al., 2018). This data provides further evidence that the engram may be updated continuously based on re-exposure and/or extinction and the molecular process we have highlighted may underlie how coordinated gene expression is required for this continuous refinement.

Collectively, our work provides the first evidence for a functional priming event in the initial stages of memory formation that is characterized by an increase in enhancer accessibility during encoding, without the expected transcriptional changes. Further on, these primed enhancers engaged in a *de novo* interactions with promoters, which corresponded to transcriptional changes during later phases of memory consolidation and recall. Moreover, this data provides new collection of targets, such as transcription factors, epigenetic modulators and unique set of genes that need to be investigated for their causal role in memory formation and recall.

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Date of submission: March 15, 2021
Date of decision: April 26, 2021
Date of acceptance: June 28, 2021
Date of web publication: September 17, 2021

https://doi.org/10.4103/1673-5374.324834
How to cite this article: *Marco A (2022) Activity-dependent remodeling of genome architecture in engram cells facilitates memory formation and recall. Neural Regen Res 17(5):991-993.*

Open peer reviewer: *Ana M M Oliveira, Heidelberg University, Germany.*
Additional file: *Open peer review report 1.*

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P-Reviewer: Oliveira AMM; C-Editors: Zhao M, Zhao LJ, Li JY; T-Editor: Jia Y