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Molecular Mechanisms of the Memory Trace

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Abstract

Over the past half-century, we have gained significant insights into the molecular biology of long-term memory storage at the level of the synapse. In recent years, our understanding of the cellular architecture supporting long-term memory traces has also substantially improved. However, the molecular biology of consolidation at the level of neuronal systems has been relatively neglected. In this opinion article, we first examine our current understanding of the cellular mechanisms of synaptic consolidation. We then outline areas requiring further investigation on how cellular changes contribute to systems consolidation. Finally, we highlight recent findings on the cellular architecture of memory traces in rodents and how the application of new technologies will expand our understanding of systems consolidation at the neural circuit level. In the coming years, this research focus will be critical for understanding the evolution of long-term memories and for enabling the development of novel therapeutics which embrace the dynamic nature of memories.

Keywords

memory trace; consolidation; neural systems; engram

Memory: From Synapses to Systems

Memory is the canvas upon which we paint the portrait of our lives, and research in the last half-century has provided tremendous insight into this canvas. We now know that the consolidation of long-term memories requires synaptic plasticity; that this plasticity depends on key molecular signaling cascades; and that these cascades serve to strengthen particular synaptic connections to consolidate memories in discrete brain networks (for review see [1]). There has been particularly strong progress in identifying the electrophysiological [2],

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genetic [3], proteomic [4], and epigenetic [5, 6] underpinnings of long-term memory (LTM; see Glossary) consolidation [1, 7]. Recently, with the advent of techniques for labeling active neuronal ensembles, we have also acquired the ability to resolve the cellular architecture of the memory trace/engram in mammals at the level of neural circuits [8].

Three key events in the mid-20th century catalyzed the search for molecular mechanisms of LTM traces. First, the formalization of a theory by Donald Hebb in the late 1940s that LTMs require concomitant activation and strengthening of pre- and post-synaptic neurons [9]. Second, the identification by Milner and colleagues in the 1950s that the hippocampus is critical for forming explicit (i.e., episodic) LTMs [10, 11]. Third, the discovery by Bliss and Lomo in the early 1970s that long-term potentiation (LTP; i.e., enhanced efficacy of presynaptic transmission and post-synaptic excitability) may serve as a substrate for LTM [12]. Together, these three findings fortified a view that LTP, long-term depression (LTD) and spike-timing dependent plasticity [13] may provide a mechanism for acquiring and consolidating LTMs. While the *in vivo* roles of LTP and LTD in learning and memory have been debated [14-16], there is a general consensus that the consolidation of LTMs requires *de novo* mRNA transcription and protein synthesis [17], as evidenced by their requirement in the late-phase of LTP [18] for episodic (e.g., hippocampal) memories in rodents [19]. Indeed, we have learned a great deal about the cellular and molecular mechanisms of LTM consolidation at the level of the synapse. However, there are still a number of important questions about the cellular and molecular mechanisms of systems consolidation (for etymological considerations on synaptic and systems consolidation see [20] and Glossary).

In this opinion article, we first provide an overview of what is known about the molecular biology of synaptic consolidation. We then focus on candidate cellular mechanisms of systems consolidation that require further investigation. Finally, we highlight recent studies on the organization of LTM traces in the mammalian brain and consider how certain technological breakthroughs will help to elucidate the molecular biology of systems consolidation.

Molecular Mechanisms of Long-Term Memory Storage

Reductionist approaches in the mid-20th century employed a number of model systems to identify the molecular mechanisms of synaptic consolidation (for review see [1, 21, 22]). Research using the marine snail *Aplysia californica* was particularly helpful in providing insight into the differences between short- and long-term memories. These studies revealed that short-term memories require increased pre-synaptic glutamate release as well as changes in post-synaptic glutamatergic receptor activity [23, 24] mediated by the covalent modification of existing proteins at preexisting synapses [21]. In contrast, long-term memories require *de novo* gene transcription [25], new protein translation [26], and synaptic growth at pre- and post-synaptic terminals [27, 28]. Of critical importance were findings that revealed that in a number of instances both mitogen activated protein kinase (MAPK) and protein kinase A (PKA) act in combination on cAMP response element binding protein (CREB) in the nucleus to consolidate a LTM [22]. In short-term memories, PKA functions in the cytoplasm to alter synaptic transmission. By contrast, in long-term memory the catalytic subunit of PKA translocates to the nucleus to phosphorylate CREB-1, which then

modulates the transcription of genes containing cAMP response elements [29]. This transcriptional mechanism recruits a host of additional genes including the immediate-early gene CCAAT box/enhancer-binding protein (C/EBP) which, via dimerization with an activating protein [30], drives the transcription of genes necessary for synaptic growth (e.g., elongation-factor 1α). Importantly, MAPK indirectly regulates CREB-1 via the removal of CREB-2 – a protein which represses CREB-1 activity in the basal state [31, 32]. Moreover, MAPK also guides the internalization and redistribution of neural cell adhesion molecules to sites of new synaptic growth [33]. These studies illuminated how a signal originating from an activated synapse triggers a specific intracellular signaling cascade to alter nuclear function and synaptic connectivity in order to consolidate a LTM.

The subsequent extension of these biological mechanisms of memory from *Aplysia* to mice was critical for elucidating (1) the cross-species preservation of these signaling cascades (i.e., from long-term facilitation in *Aplysia* to the late-phase of LTP in rodents) and (2) the precise role of these molecules in the storage of episodic or hippocampus-dependent memories. Research using *Aplysia* and mice helped bridge the postulate of Hebb with the findings of Milner, Bliss, and Lomo to provide a biological framework for the synaptic consolidation of LTMs. Studies have since elaborated on how these proteins and pathways interact and how they are intricately regulated [34-36]. This research has also identified the function of several immediate-early genes and other proteins as important regulators of different phases of LTP, as well as certain stages of LTM. Examples of such proteins include cellular feline osteosarcoma (c-Fos), Zif-268 [37], activity-regulated cytoskeleton protein (Arc), calcium-calmodulin dependent protein kinase II (CaMKII; [38]) and protein kinase C (PKC) isoforms (for review see [35, 39]). Notably, both c-Fos and Arc are associated with LTP, and increases in their expression are observed within minutes following an experience (for review see [39, 40]). For these reasons, much of the work examining the cellular organization of memory traces or engrams in rodents has leveraged these properties to selectively tag neuronal ensembles recruited to a memory trace (e.g., [41]).

Persistence of Long-Term Memories

An enduring focus in the study of the molecular biology of memory storage has been in the search for mechanisms which allow a LTM to persist. Many of the transcriptional events and post-translational modifications of proteins are short-lived (i.e., on the order of a few hours to days). This raises the challenging question: How is a LTM maintained in synaptic connections that are so dynamic [42] in a manner that allows a memory to persist throughout the life-span of an organism? In the early 1980s, it was hypothesized that the persistence of memories requires an “intramolecular autocatalytic” reaction [43-45], that is, a molecular mechanism that once activated persists in a self-sustaining manner. Due to the ability to autophosphorylate at a specific threonine residue, calcium calmodulin dependent protein kinase II (CAMKII) became an attractive candidate mechanism for the maintenance of LTMs [46]. Another protein-kinase, protein-kinase-M-zeta (PKMζ), an atypical isoform of PKC, has also been proposed as a necessary component for the maintenance of LTP and the persistence of LTMs given that only the catalytically active form of the gene is transcribed following stimulation [47, 48]. Despite controversy [49], PKMζ is a particularly interesting candidate in the persistence of LTMs in that its mRNA is transported to dendrites and locally

translated upon induction of LTP. Moreover, PKMζ regulates the endocytosis of GLUA2-containing AMPA receptors in addition to possibly regulating the structure of dendritic spines [50].

Similar to PKMζ, mRNA of the immediate-early gene Arc is also transported to activated synapses [51] where it too regulates AMPA receptor endocytosis [52]. Emerging studies on the properties of Arc have found that its function may be more complex than previously thought in that the protein contains a structural likeness to group-specific antigen proteins expressed by viruses [53, 54]. Moreover, Arc protein can bind its own mRNA and transport it across the synapse through extracellular vesicle [53-55]. These studies are of considerable importance in that they suggest a non-canonical mechanism for the synaptic transmission of information that would constitute a novel mechanism for the molecular control and possible persistence of LTMs [53].

At the turn of the century, work from our laboratory identified functional prions as another molecular candidate for the persistence of long-term memories [56]. Like their pathogenic counterparts, functional prions contain Q/N rich “prion-like” domains that promote aggregation. However, functional prions are distinguished from pathogenic prions in that their aggregation is tightly regulated and serves a physiological function. These functional prions exist in a soluble conformation until activated, at which point they oligomerize, become self-sustaining, and contribute to the consolidation of LTMs [56]. One particular functional prion, the RNA-binding protein cytoplasmic polyadenylation binding protein-3 (CPEB-3), was identified by this laboratory as important for the maintenance of LTP and hippocampal-dependent memories in mice [57]. These studies, along with work on the function of Arc, raise several intriguing questions: Can a self-sustaining molecule transport RNA between the pre- and post-synaptic compartments of the activated synapses of a memory trace and modify the synaptic architecture of a LTM? Can RNA-binding proteins and epigenetic modifications to the RNA that they carry serve as a synaptic substrate for storing information in select neurons during systems consolidation, or under conditions of massive dynamic change (see Figure 1A; [58])?. Indeed, different types of RNA (e.g., mRNA, miRNA, snRNA, etc.) perform fundamentally different roles in LTMs [59, 60] and the synaptic transfer of exosomally-packaged RNA between neurons [61] may be an important mechanism that warrants further investigation.

Research examining the function of RNA-binding proteins and RNA trafficking between synapses of a memory trace at multiple time points following acquisition of LTMs will lend new insights into the self-sustaining molecular machinery which contribute to systems consolidation. To answer these questions, future research must blend activity-dependent tagging strategies (detailed below) with novel tools that offer temporally-precise molecular control over these molecules such as optically-controlled protein degradation [62], optically-controlled dominant-negative protein inhibition [63], and real-time molecular imaging as has recently been accomplished with Arc [64]. By combining these *in vivo* approaches with techniques in single-cell sequencing (e.g., patch-seq., act-seq, etc. [65, 66]) to isolate neurons of a memory trace at various time points, we will gain a deeper insight into how the molecular landscape changes during systems consolidation. These tools also offer the ability

to determine the necessity and sufficiency of molecules such as ARC, CPEB-3, and others yet to be identified in systems consolidation [62, 63].

Beyond the traditional transcriptional and proteomic machinery of LTMs, recent studies have also identified the roles of a variety of post-translational epigenetic modifications in LTMs [6, 67, 68]. For example, methylation and acetylation of DNA as well as RNA [69] have a powerful influence over LTMs [70, 71]. The temporally-dependent emergence of epigenetic modifications to the DNA of key learning and memory proteins such as calcineurin following LTM acquisition has been suggested to contribute to systems consolidation and LTM maintenance [72]. The importance of site-specific epigenetic modifications may also extend to the transgenerational inheritance and persistence of specific LTMs [73]. However, much remains to be learned about the role of epigenetic modifications with regard to systems consolidation. Newer tools which provide high temporal control over RNA and epigenetic modifications such as transcription activator-like effectors (TALEs), chemo-optical modulation of epigenetically regulated transcription (COMET; [74]), and optically activated CRISPR/Cas9 [75] represent sophisticated new approaches for assessing the temporal necessity and sufficiency of epigenetic marks and synaptic RNA regulation. Moreover, integrating these tools with strategies to access select memory traces will enhance our understanding of which mechanisms modulate memory persistence and systems consolidation.

Memory Traces in mammalian systems

For nearly a century, the question of how long-term memories are stored within the mammalian brain has been an area of intense interest and debate [76-80]. Recent technological advances in tagging and optically controlling active neuronal ensembles have invigorated research into the cellular representation of discrete LTMs in mice [81]. This work has relied on viral delivery and antibiotic-dependent expression strategies [8, 82] to leverage the properties of immediate-early genes such as c-Fos and Arc to tag neuronal ensembles recruited to a particular memory.

The creation of transgenic mice that utilize a tetracycline-transactivator (tet) ON or OFF system or more recently a Cre-ERT2 fusion system [82] to achieve selective targeting of neurons activated during the acquisition of a LTM has been essential. In the tet-OFF system, a transgenic animal expresses the tTA gene (a fusion of the TetR repressor protein with the c-terminal domain of a herpes simplex virus protein) under the control of an immediate early gene promoter [8]. The activity of tTA can be inhibited by the antibiotic doxycycline. Removal of doxycycline from the diet enables tTA to bind to a Tet operator located within a tetracycline-response element (TRE) promoter to transcribe a target gene. By driving a viral transgene with a TRE promoter, regional and temporal expression of specific genes (e.g., opsins, designer receptors exclusively activated by designer drugs (DREADDs), fluorophores, toxins, etc.) can be achieved in a specific subset of recently activated neurons.

Using activity-dependent tagging approaches, research during the past decade has applied these and other novel technologies to dissect how neurons that are activated during the formation of a LTM can regulate distinct phases of hippocampal-dependent memories. For

example, recent work has found that optogenetic reactivation of c-Fos$^+$ neurons in the dentate gyrus (DG), a subregion of the hippocampal formation important for the acquisition and retrieval of contextual fear memories [83], tagged during the acquisition of a long-term fear memory is sufficient to elicit retrieval of a recently acquired LTM in a novel environment [84, 85]. Perhaps even more striking is the finding that when the hippocampal *Cornu Ammonis* field 1 (CA1) region or DG c-Fos$^+$ neurons labeled in a neutral context are optogenetically stimulated during the acquisition of a long-term fear memory, these neurons are recruited to the fear memory trace, and fear behavior can be elicited in the once-neutral context [85, 86]. Conversely, optogenetic silencing of DG and CA3 ARC$^+$ neurons or CA1 c-Fos$^+$ neurons tagged during acquisition can inhibit the recall of a LTM [82, 87]. Consistent with the idea that fear memories require brain-wide networks [88], newer studies have demonstrated the functional contribution of neuronal ensembles in cortical areas such as the retrosplenial cortex [89]; and limbic areas such as the amygdala during the acquisition and recent retrieval of LTMs [90]. One particularly interesting discovery is that, if neurons labeled in a neutral context are re-activated during the retrieval of a fear memory, they can interfere with recall of the recently acquired LTM [85, 86]. These observations have been extended to show how activating neuronal ensembles associated with different types of memory (e.g., positive memories) can disrupt aversive LTMs and potentially serve as a therapeutic intervention [91]. These studies reveal the delicate spatial and temporal constraints under which neuronal ensembles represent discrete LTMs in the mammalian brain.

Much of this work has focused on recently acquired LTMs (i.e., a few days), but studies are now beginning to focus on remote LTMs (i.e., a few weeks old or older) to examine how memories evolve during systems consolidation (for review see [92]). This is important because with the passage of time, systems consolidation relies progressively more on cortical areas and less on the hippocampus in a process that may be mediated by hippocampal sharp-wave ripples (for review see [93, 94]; but also see recent evidence that cortical areas are recruited early-on for systems consolidation [89, 95-97]). Recent work has found that by tagging the subset of DG neurons that are still active during the remote recall of a fear memory, and subsequently activating those neurons during fear extinction, the extinction of an aversive memory at remote time-points is facilitated [98]. Moreover, optogenetic silencing of neurons initially recruited to the memory trace in the prefrontal cortex at remote, but not recent, time-points is sufficient to disrupt LTMs [97] – a finding which reinforces an early-role for the prefrontal cortex as well as the slow maturation of region- and gene-specific epigenetic marks in systems consolidation [72, 95, 99-101]. Together, these studies highlight how specific neuronal populations in different brain regions can have varying influence over a particular LTM with the passage of time. They also raise the important question: What are the molecular mechanisms which control how cortical cells mature to store information important for retrieving particular memories?

Studies are now beginning to examine the molecular and structural mechanisms which guide the allocation of particular neurons to a memory trace – a focus which will be critical to understanding how information is preserved during systems consolidation. This research has concentrated on answering questions such as: What are the molecular mechanisms that control the initial allocation of a neuronal ensemble to a particular memory trace? Or what

differentiates stronger LTMs from weaker ones within discrete circuits? While it is generally thought that neurons with higher excitability are initially recruited to form a LTM trace [102], the molecular mechanisms which drive this allocation have been elusive. Key studies have found that in addition to its role in regulating transcription, elevated levels of CREB in neurons increases neuronal excitability and increases the likelihood that specific neurons (e.g., in the lateral amygdala) are recruited to a memory trace (see Figure 1A; [103-105]). Moreover, research has uncovered fundamental rules about neuronal allocation within a LTM trace. For example, similar, but non-identical, aversive memories (i.e., fear conditioning using different tones or contexts) that are acquired closely in time recruit an overlapping ensemble of neurons in the amygdala and CA1 [90, 106]. Furthermore, these ensembles can represent each memory in a synapse-specific manner (see Figure 1B; [105]). However, when the acquisition of similar LTMs has a greater temporal separation, the likelihood of this overlap diminishes. While neuronal ensembles of a particular memory are linked across brain regions, when similar memories differ in strength, stronger memories are initially differentiated by greater synaptic connectivity between CA3→CA1 neurons recruited to a memory trace relative to weaker memories [107]. It is still unclear how connectivity at the circuit-level for a particular memory evolves during systems consolidation given that synapses themselves are dynamic [42]. However, these studies are an important step forward in identifying the biological mechanisms of systems consolidation in mammals. They provide a foundation to interrogate, for the first time, the molecular mechanisms that control how distinct LTMs evolve and persist within shared neuronal ensembles (see Figure 1B).

The research on memory traces discussed above highlights a number of key organizing principles. First, levels of CREB may determine the excitability of neurons and guide which neurons are recruited to a LTM trace [103]. Second, a LTM trace is represented by a discrete subset of neurons, while activation of other neurons recruited to a different memory trace can interfere with recall. Third, and consistent with past work [27, 28], the initial strength of a LTM trace is represented by the number of pre- and post-synaptic connections between different brain regions (as shown using elegant virus-mediated tracing techniques [107]). Fourth, similar memories may recruit a shared neuronal ensemble, but this is constrained by the temporal separation of acquiring each memory in addition to a synapse-specific representation of each memory [105]. Fifth, with the passage of time, LTMs rely more heavily on cortical structures in a process that involves the delayed maturation of cortical neurons that were, in fact, initially recruited to the memory trace [95, 97, 99, 100]. While we have primarily focused on aversive learning and memory, these studies provide a framework for research to now examine the molecular biology of systems consolidation and for understanding how LTMs evolve over time within discrete neural circuits.

Concluding Remarks

In the coming decade, research can capitalize on new technologies to elucidate the molecular mechanisms (e.g., synaptic RNA, RNA-binding proteins, and epigenetic regulation of RNA) which govern systems consolidation and the persistence of LTMs (see Figure 1 and Outstanding Questions). By combining activity-dependent tagging strategies with neural-circuit targeting strategies and novel techniques for molecular interrogation, this research

focus is poised to obtain important insights into how memories are stored and preserved across time. New techniques such as iDISCO [108], CLARITY [109], *in vivo* optical control of proteins [62, 63], optical control of epigenetic marks [74], next-generation sequencing [65, 66], optical control of gene editing [75], *in vivo* transcriptional imaging [64], and viral mediated-tracing tools [110, 111] will make a significant contribution to furthering our knowledge. Importantly, a focus on the molecular biology of systems consolidation will provide a critical framework for how memories are preserved in the face of biological and circuit dynamicity. The past half century has provided considerable insight into the molecular mechanisms of synaptic consolidation. Progress in the coming decades will provide the necessary scaffold for understanding the molecular biology of systems consolidation and how memories persist across our lifespan. This progress will be an essential component in the development of better-targeted treatments for disorders of memory such as Alzheimer's disease, age-related memory loss, post-traumatic stress disorder, and many others.

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Glossary

Acquisition/encoding
the initial processing of information within a defined neuronal ensemble for a specific experience or event on times-scales lasting seconds or minutes.

Extracellular vesicle
membranous compartment that is capable of packaging and transferring proteins, RNA, etc. from pre-synaptic to post-synaptic cells

Long-term Memory (LTM)
the storage of information for a specific experience or event on a prolonged time scale such as days, weeks, months or years following acquisition.

Molecular mechanisms of long-term memory
the transcriptomic, proteomic and epigenetic mechanisms that are involved in long-term memory consolidation.

Memory trace/Engram
the cellular representation of a long-term memory by neuronal ensembles across different brain regions.

Persistence
the preservation of a stored long-term across neuronal ensembles in the timeframe of days, weeks, months, or years following the initial storage.

Storage/consolidation

the conversion of short-term memory into long-term memory within a defined neuronal ensemble in the timeframe of hours.

Synaptic consolidation
the molecular mechanisms associated with the long-term potentiation of a synaptic connection and linked to a long-term memory.

Systems consolidation
the maturation of a long-term memory to more heavily recruit cortical/neocortical brain regions.

Recall/Retrieval
the reactivation of a neuronal ensemble which has stored a specific long-term memory.

Recent LTM
a long-term memory acquired previously in the timeframe of days or less

Remote LTM
a long-term memory acquired previously in the timeframe of weeks or longer

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Highlights

- The molecular biology of Synaptic consolidation is relatively well-defined. By contrast, the molecular mechanisms of systems consolidation remain poorly understood.
- Recent technological developments have helped advance our understanding of the cellular representation of memories in the brain.
- One of the goals of future research is to clarify how non-canonical forms of synaptic transmission (e.g., exosomally transported RNA, associated RNA-binding proteins, and epigenetic modifications to the transported RNA) may contribute to systems consolidation.
- Integrating tools for examining these non-canonical cellular and molecular mechanisms with tools for tagging select circuits of a memory trace will be highly informative for elucidating the molecular mechanisms of systems consolidation.

Outstanding Questions

- Is the storage of long-term memory influenced by exosomally transported RNA and associated RNA-binding proteins? Can this transfer modify synaptic connections or store information in the face of dynamicity during systems consolidation?
- How do epigenetic modifications to DNA and exosomally transported RNA evolve within a defined long-term memory trace? Can these epigenetic modifications contribute to long-term memory during systems consolidation?
- What are the molecular mechanisms that preserve a long-term memory as neocortical areas undergo progressively greater recruitment over time?
- Some memories are expected to initially recruit partly overlapping neuronal ensembles. What are the molecular mechanisms that regulate the systems-level consolidation of such memories? How are these overlapping representations preserved during systems consolidation?
- How do highly-similar memories evolve within neuronal ensembles during systems consolidation?
- Can overlapping ensembles represent similar memories that differ in strength? And if so, what are the molecular mechanisms which allow for these long-term memories to co-exist and persist during systems consolidation?

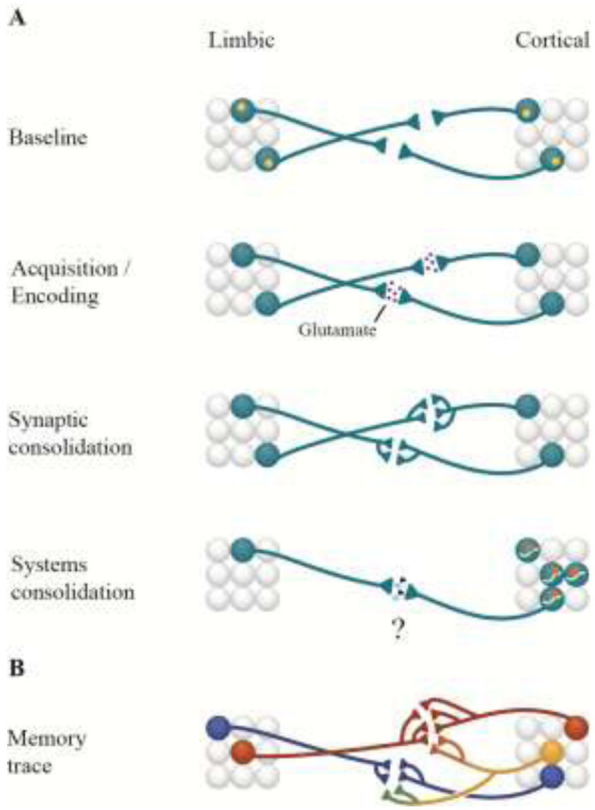

Figure 1.

Molecular Mechanisms of the Memory Trace. (A) At baseline, increased levels of CREB (yellow circles) are thought to determine which neurons are preferentially incorporated into a memory trace (blue circles). Acquisition/encoding of a long-term memory involves increased glutamatergic transmission between neurons of a memory trace. Synaptic consolidation and the transition from short-term to long-term memory involves the transcription of new genes, new proteins, and synaptic growth. Systems consolidation involves a greater reliance on cortical areas with the passage of time, in addition to epigenetic changes (e.g., methylation; orange circles) in genes involved in learning and memory. An important future direction (question mark) is in understanding if/how RNA-binding proteins (light blue diamonds) and modifications to the RNA they carry as well as RNA packaged into exosomes (black triangles) contribute to systems consolidation. (B) Recent progress has dissected some of the principles of how individual memories are represented in the brain. Stronger memories (orange circles) involve greater initial synaptic connectivity between brain regions relative to weaker memories (blue circles). Moreover, similar memories acquired close in time recruit an overlapping ensemble of neurons (yellow circle). However, these neurons can represent individual memories in a synapse-specific fashion (light orange vs. green processes). An important direction for future research would be to identify the molecular mechanisms which control how similar or stronger memories within overlapping ensembles are preserved during systems consolidation.