Shedding light on learning and memory: optical interrogation of the synaptic circuitry

Ju Lu, **Yi Zuo**

Department of Molecular, Cell and Developmental Biology, University of California Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA

Abstract

In the quest for the physical substrate of learning and memory, a consensus gradually emerges that memory traces are stored in specific neuronal populations and the synaptic circuits that connect them. In this review, we discuss recent progresses in understanding the reorganization of synaptic circuits and neuronal assemblies associated with learning and memory, with an emphasis on optical techniques for *in vivo* interrogations. We also highlight some open questions on the missing link between synaptic modifications and neuronal coding, and how stable memory persists despite synaptic and neuronal fluctuations.

Introduction

Learning and memory are vital brain functions, but their physical substrate long remains a mystery. In his Croonian Lecture delivered to the Royal Society of London in 1894 [1], Ramón y Cajal presciently remarked that with ' cerebral gymnastics' or 'mental exercise,' 'associations already established among certain groups of cells would be notably reinforced … in addition, completely new intercellular connections could be established thanks to the new formation of [axonal] collaterals and dendrites.' Essentially, his view amounts to the idea that the plasticity of neural connections, both structural and functional, is fundamental to information processing and storage in the brain. This 'connectionist' tenet foreshadows Donald Hebb's famous postulate [2], commonly summarized as ' fire together, wire together.' As the discovery of long-term potentiation (LTP) [3] provides a plausible cellular substrate of sustained synaptic changes, this school of thought has received tremendous attention, culminating in the 'synaptic plasticity and memory' hypothesis [4] that 'activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the encoding and trace storage of [the] memory.' Over the past several decades, a vast amount of knowledge [5,6] has accumulated on the molecular mechanisms and electrophysiology of various forms of synaptic plasticity, mostly with *ex vivo* preparations. As advancement in optical microscopy and molecular tools continue to promote *in vivo* interrogation of the synaptic circuit in learning and memory

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Nothing declared.

with single synapse-level resolution, our review will focus on findings arising from such endeavors.

Plasticity at individual synapses

Most early studies on synaptic plasticity used electrical stimulation of axonal inputs *en bloc*. This is a significant limitation, as synaptic inputs are hardly synchronous *in vivo*. The development of photo-uncaging of neurotransmitters addressed this problem [7]. In this technique, a photochemical protecting group is covalently linked to the bioactive molecule to render it inert; light irradiation severs the covalent bond and releases the caged substrate. The invention of two-photon (2P) microscopy [8], which confines excitation to a small volume around the focal point, further suggests that one may leverage 2P absorption for photo-uncaging with high spatial resolution. Matsuzaki *et al.* [9] showed that repeated photo-release of glutamate on single dendritic spines (postsynaptic sites of most excitatory neurons) induces a rapid, selective, and persistent enlargement of small spines, which is accompanied by electrophysiological changes consistent with LTP. This work establishes that LTP has a structural correlate and can indeed be induced in individual synapses. On the other hand, low-frequency glutamate uncaging results in the shrinkage of dendritic spines, a structural correlate of long-term depression (LTD) [10,11], and combining glutamate uncaging with optogenetic stimulation can induce spike timing-dependent plasticity at single spines [12]. To characterize the structure–function relationship of individual synapses in the living brain, Kasai *et al.* used *in vivo* patch clamp recording and 2P imaging to confirm a correlation between the maximum amplitude of electrical current induced by glutamate uncaging and the spine head volume [13]. Recently, the same group applied this technique to examine synaptic plasticity induced by 2P uncaging *in vivo*, and observed similar enlargement and shrinkage of spines in the neocortex as in hippocampal slices [14]. Furthermore, neurotransmitter uncaging can also induce spine formation [15] or elimination [16]. These studies demonstrate that the neuronal network can be reconfigured at the level of individual synapses.

Imaging learning-associated synaptic dynamics

As Bliss and Lømo prudently pointed out [3], the fact that the synapse can undergo LTP with repetitive stimuli *ex vivo* does not guarantee that this capacity is exploited in the intact animal under physiological conditions. To investigate synaptic plasticity *in vivo* during and after learning, researchers have followed structural changes of synapses with 2P microscopy.

Postsynaptic structural plasticity

To date, most works on the structural plasticity of synapses analyze the emergence and disappearance of spines, which are associated with synapse formation and elimination, respectively [17]. Such structural dynamics represents a topological reorganization of the neuronal network. Motor learning (e.g. single-pellet reaching, cued lever-press, running on an accelerating rotarod) promotes spine formation on pyramidal neurons in the mouse primary motor cortex (M1) [18–20] (Figure 1a–c). In addition, different sets of synapses may encode distinct motor memories (Figure 2a), as practicing novel, but not previously

learned, motor tasks further promotes synaptogenesis [19]. Auditory fear conditioning increases synapse formation between lateral amygdala axons and L5 pyramidal neurons in the auditory cortex [21••]; fear extinction preferentially removes the new spines induced by fear conditioning [22]. Notably, learning-induced spine formation is spatially structured over the dendritic arbors (Figure 1c). Clustered spine formation has been observed both in M1 during motor learning [23] and in the retrosplenial cortex with contextual fear learning or training in the Morris water maze [24••]. Sibling dendritic branches of L5 pyramidal neurons also exhibit different degrees of spine formation enhancement after motor learning [25]. Cued lever-press promotes spine dynamics on the distal branches of the apical, but not the perisomatic, dendrites of L2/3 pyramidal neurons in M1 [20]. Such spatial structures of spine dynamics may reflect the combined effects of postsynaptic molecular signaling cross-talks [26] and presynaptic axons' inhomogeneous distribution and differential activity patterns.

Besides spine turnover, spine head size, a correlate of synaptic strength, also changes with learning. In the forebrain nucleus HVC of juvenile zebra finches, hearing a tutor song triggers the enlargement of stable spines [27]. In mouse M1, when spines form in a cluster, succedent new spine(s) are added as the first new spine is enlarged [23]. Recently, Roth *et al.* [28•] observed an increased *a*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor level at a subset of spines in the mouse motor and visual cortices as the mouse learns a new motor skill. These results are consistent with mechanistic models of LTP and agree with the Hebbian theory of the strengthening of task-relevant neuronal circuits (Figure 1b,c).

Presynaptic structural plasticity

Learning also affects the structural dynamics on the presynaptic side. In the mouse M1, rotarod training differentially impact corticocortical and thalamocortical axonal boutons [29]. In the cerebellum, acrobatic motor learning (sequentially traversing elevated obstacles) reduces the formation of axonal varicosities on parallel fibers [30]. One plausible interpretation is that some inputs are strengthened by forming multi-synapse boutons (MSBs) [31], while the overall synaptic strength remains constant as the total number of presynaptic varicosities is reduced. This idea is consistent with electron microscopy reconstruction of Purkinje cell synapses after acrobatic learning, which reveals not only the selective induction of MSBs but also the comparatively small size of synapses near MSBs [32]. Such concerted synaptic strengthening and weakening are reminiscent of the findings in the hippocampus [33]. Interestingly, learning may also add new axonal boutons onto an existing dendritic spine, forming a multi-synapse spine [21••]. In principle, adding a new presynaptic or postsynaptic element to an existing synapse may strengthen it without changing the connectivity between the parent neurons, or create new fan-in or fan-out connections, or serve as a transitory state in swapping synaptic partners. The computational implication of such configurations calls for further studies.

Synaptic manipulation alters learning and memory

A demonstration of correlation does not establish a causal role of synaptic changes in learning and memory: one needs to employ manipulations. Nabavi *et al.* [34] made a significant progress in showing such a causal link. They generated associative memory in the rat by pairing a foot shock with optogenetic stimulation of auditory inputs to the amygdala, a region essential in fear conditioning. Optogenetically induced LTD inactivated the fear memory, which could be reinstated by optogenetic LTP. Similarly, optogenetic LTD in amygdalar neurons receiving thalamic afferents persistently attenuates learned fear [35]. Abdou *et al.* [36•] further showed that optogenetic LTP or LTD of synapses specific to one memory selectively affects the recall of that memory only. However, the plasticity induction protocols in these studies lack synapse-level precision and do not recapitulate the physiological conditions in natural learning.

To achieve spine-specific control, Hayashi-Takagi *et al.* [37] expressed AS-PaRac1 (a light-activatable version of the synaptic signaling protein Rac1), which can induce spine shrinkage with blue light, in the mouse motor cortex. They trained the mouse to run on a rotarod, and then selectively weakened the potentiated synapses to cause the mouse to forget the motor skill, showing the necessity of synaptic changes in memory formation (Figure 1d). It is conceivable that synapse-specific expression of optogenetic actuators [38], combined with novel microscopy that can access many targets in 3D with millisecond precision in parallel [39], will pave the way for the proof of sufficiency, that is, to synthesize a memory by artificially inducing synaptic changes without behavioral training.

From synaptic circuit to cell assembly

The selective strengthening of synaptic connections gives rise to the 'cell assembly,' subset of cells that can '[act] briefly as a closed system.' [2]. Such cell assemblies provide a natural candidate for the physical substrate of memory traces or 'engrams' (Figure 2b), which have been demonstrated in a number of elegant works using activity-dependent cell labeling with optogenetics to activate, erase, and synthesize memory [40]. Recently the advent of genetically encoded calcium indicators has enabled researchers to characterize the establishment, maintenance, and evolution of neuronal assemblies by monitoring the activities of the same neurons longitudinally. Such studies have revealed interesting dynamism of neuronal assemblies. Generally, during motor learning, the movement-related activities of neuronal populations in the mouse motor cortex [41,42] and dorsolateral striatum [43] progressively converge and stabilize, corresponding to performance improvement. The functional reorganization of neuronal ensembles during learning is also cortical layer-specific [44].

On the other hand, a recent study [45] shows that when the mouse performs a hippocampus-dependent spatial memory task in a virtual environment, granule cells in the dentate gyrus exhibit a stable code, whereas neuronal ensembles in CA1 and CA2/3 represent the environment in a precise but dynamic way: although the number of place cells is similar for each context across days, individual cells shift their firing locations in the same context over days. This is consistent with the previous finding of a fluctuating membership of CA1

neurons in the place-coding ensemble when the free-moving mouse repeatedly explores an environment [46]. A spatial code with high day-to-day dynamism also emerges in the retrosplenial cortex [47]. Notably, such dynamism is not restricted to spatial memory. An earlier work on zebra finches [48] shows that the ensemble dynamics of projection neurons in the premotor nucleus HVC are globally stable, but individual neurons drift in and out of the ensemble. The dynamic representation of movement at single cell level is also observed in L2/3 neurons of M1 [44,49]. In mice repeatedly engaged in a virtual navigation task, neurons in the posterior parietal cortex exhibit a systemic representational drift. The drift makes the performance of fixed-weight linear decoders of kinematic information (position, velocity, and head direction) degrade significantly over time, which may be compensated by a biologically plausible synaptic plasticity rule [50••,51••]. Overall, these works suggest that the stability of a neuronal assembly, which accounts for the reproducibility of behavioral outputs, may be an emergent property at the ensemble level, while individual neurons only participate in the ensemble transiently. To use an analogy, when a vortex forms upon unplugging the bathtub, the structure of the vortex persists for some time, but the constituent water molecules are constantly in flux.

Outlooks and open questions

Many questions remain regarding how the cellular engram arises from synaptic interactions of its constituent neurons, and how neuronal network activities in turn modify the synaptic circuit. Here we highlight a few of them. To address these challenges, we need the synergy between conceptual innovations and technical advances.

Who are the presynaptic partners in structural dynamics of synapses?

Most *in vivo* studies on synaptic plasticity so far have focused on the postsynaptic spines only, due to the sparse neuronal labeling necessitated by the limited resolution of 2P microscopy. Yet the activity patterns experienced by synapses on the same dendritic branch may be dramatically disparate, dictated by their presynaptic partners [52,53]. Thus, identifying the parent neurons of the presynaptic axons becomes ineluctable for further dissection of the cellular engram. Molecular tools based on protein engineering [54] and viral vectors with low cytotoxicity [55] will enable trans-synaptic labeling of neuronal connections *in vivo*.

What are the functional roles of newly formed synapses in memory recall?

Only a fraction of newly formed synapses persist beyond the learning period [19]. What determines their fate? Are the persistent synapses active during memory recall in a consistent way? Novel volumetric imaging techniques [56,57] that can image synaptic activities will help address these questions.

How does the neuron integrate cell type-specific and pathway-specific synaptic inputs, and how does learning affect that?

In addition to the excitatory synapses discussed above, the placement and dynamics of inhibitory synapses exert a significant impact on the signal detection and integration of the neuron [58]. Even with the knowledge of the type and location of all synapses, the plethora

of nonlinearities in dendritic integration and localized dendritic electrical events [59] highlight the need to treat a neuron, with its elaborate dendritic arbors and myriad of synaptic inputs, as a network rather than a point [60]. Further complicating this picture are the global changes in neuronal excitability, which may modulate the allocation of neurons to memory traces [61]. By combining anatomical and functional imaging data, one may build morphologically and biophysically realistic computational models with predictive power [62•] to address such questions.

How does the synaptic circuit maintain a stable representation of information despite spontaneous synaptic turnover and remodeling?

Numerous works have shown that synaptic connections in the living brain are volatile [63,64]. Properties such as spine and axonal bouton volume, which are correlated with synaptic strength, may fluctuate considerably under baseline conditions. Moreover, synapses may be formed or eliminated under baseline conditions without any explicit learning and memory process. These phenomena raise an acute question: how can a synaptic network maintain a stable memory with unstable synapses? A recent work [65•] proposes an interesting scheme to store memory as limit-cycle attractors. Such ideas await experimental tests and further theoretical elaborations.

How can a fluctuating neuronal assembly encode reproducible behavior? What are the underlying synaptic mechanisms?

If population dynamics are confined to a low-dimensional manifold, it may yield a stable representation of information in spite of single neuron variability [66,67]. However, certain synaptic plasticity may still be required to compensate for the neuronal drifting [51••]. It would be desirable to monitor synaptic and somatic activities of a neuronal population simultaneously to uncover such mechanisms.

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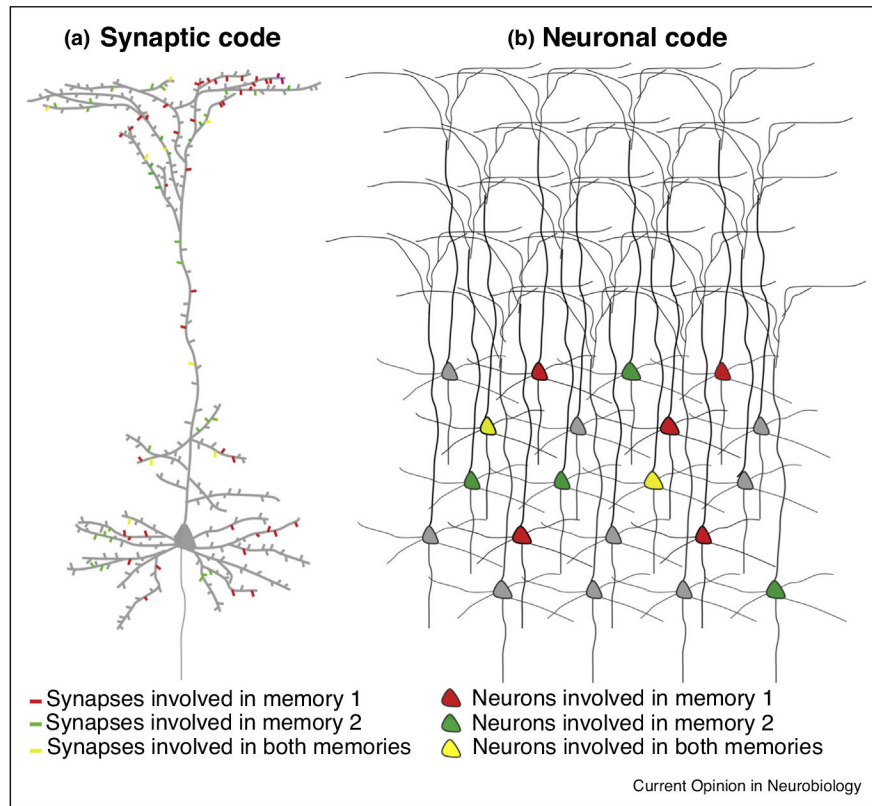
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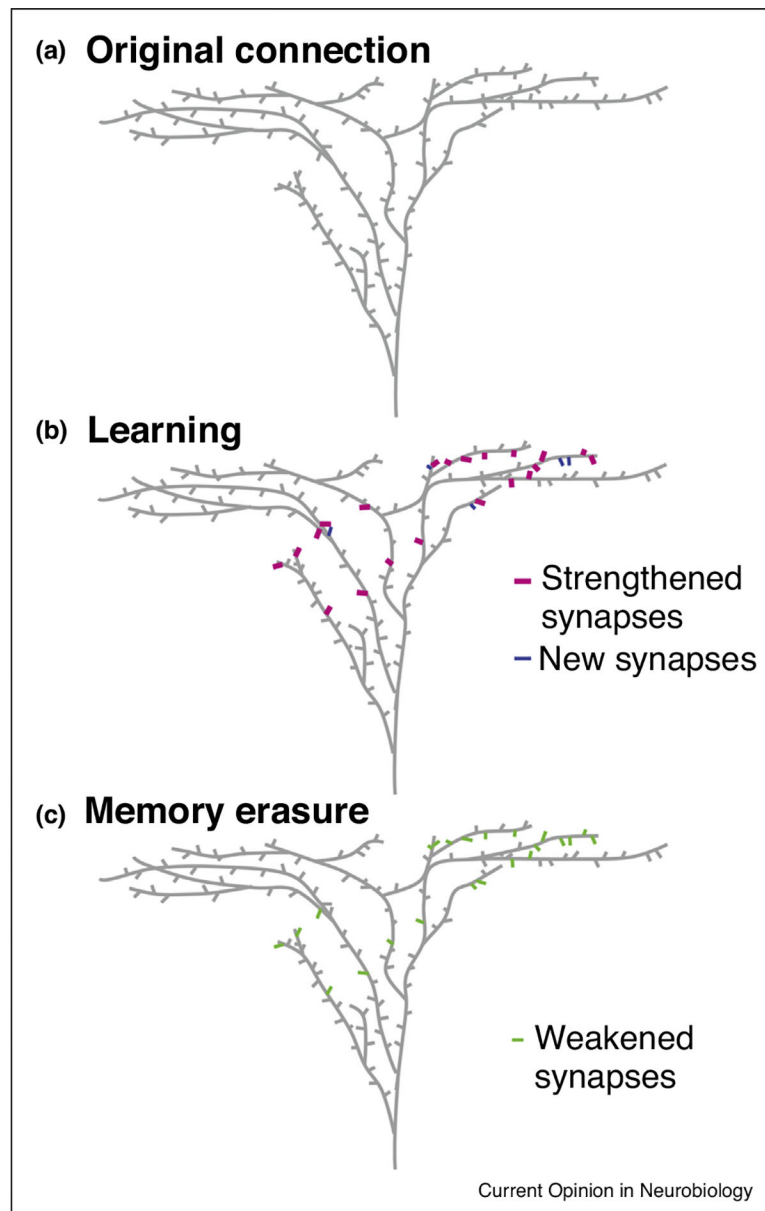
Figure 1.
Learning strengthens specific neuronal connections via synaptic modifications.
(a) A schematic drawing of spines on the apical dendritic arbor of a pyramidal neuron. **(b)** During learning some existing synapses (magenta) are strengthened and new synapses (blue) are formed, leading to stronger connections between the input axons (not shown) and the pyramidal neuron. **(c)** Selective weakening of the strengthened and newly formed synapses (green) erases the acquired memory.

(a) Synaptic code   **(b) Neuronal code**

- Synapses involved in memory 1   ▲ Neurons involved in memory 1
- Synapses involved in memory 2   ▲ Neurons involved in memory 2
- Synapses involved in both memories ▲ Neurons involved in both memories

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Figure 2.
Memory coding at synaptic and neuronal levels.

Two different memories may recruit two partially overlapping sets of synapses on the same neuron **(a)** and/or two partially overlapping sets of neurons **(b)**.