Intravital imaging of dendritic spine plasticity

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Dendritic spines are the postsynaptic part of most excitatory synapses in the mammalian brain. Recent works have suggested that the structural and functional plasticity of dendritic spines have been associated with information coding and memories. Advances in imaging and labeling techniques enable the study of dendritic spine dynamics *in vivo*. This perspective focuses on intravital imaging studies of dendritic spine plasticity in the neocortex. I will introduce imaging tools for studying spine dynamics and will further review current findings on spine structure and function under various physiological and pathological conditions.

Introduction

The study of the biological processes undergoing in the living brain has been one of the major challenges for neuroscientists. The strong and opaque mammalian skull has been preventing neuroscientists from studying the living brain. Until very recently, most of our understanding of the brain has come from studies on fixed brain tissue, neuronal culture, and brain slices. After the development of 2-photon laser scanning microscopy (TPLSM) in the 1990s,¹ intravital imaging of the central nervous system (CNS) has become possible. Imaging of the CNS with TPLSM enables researchers to follow the dynamic and time-dependent processes in the brain in a high spatial and temporal resolution. Some of the earliest intravital studies of the CNS were to investigate the plasticity of dendritic spines in transgenic mice expressing fluorescent proteins in the neocortex.^{2,3} These studies set up a revolutionary platform for the intravital imaging

of the CNS to study different neuronal cell types and processes in health and disease, for example microglia dynamics,⁴ astrocyte activity,⁵ cerebral angiogenesis,⁶ and amyloid plaques formation,⁷ etc.

Dendritic spines were first described by Santiago Ramón y Cajal more than a century ago.⁸ Since then neuroscientists have been fascinated by the function of these structures in the brain. Dendritic spines are small protrusions on dendrites, where the postsynaptic part of most excitatory synapses are located.⁹ Dendritic spines are highly dynamic, exhibiting different shapes and change over time.¹⁰ The structural and electrical properties of dendritic spines are crucial for local signal integration, signal transduction and molecular compartmentalization; therefore, they are vital for the function of the nervous system.¹¹ Studies in the past 2 decades have shown that substantial changes in spine number and morphology occur during development, learning, and different physiological and pathological conditions. With the development of microscopy and labeling technology, we now have much better understanding of dendritic spine structure and function.

In this perspective, I will focus on the intravital imaging of dendritic spine plasticity by using TPLSM in the neocortex. I will first discuss the tools and technical issues. Then I will further discuss the current findings on structural and functional plasticity of dendritic spines *in vivo*.

In vivo Labeling of Dendritic Spines

The advent of TPLSM has allowed live imaging of fluorescently labeled synapses in the living cortex. Genetic expression of fluorescent proteins or the use of synthetic

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dyes enables the imaging of both structural (presynaptic boutons and postsynaptic dendritic spines) and functional (calcium activity) dynamics of spines over time. Genetically encoded fluorescent proteins and sensors (e.g. calcium indicator: GCaMPs) can be expressed in transgenic animals, by *in utero* electroporation, or by viral systems. Different methods can label different populations of neurons to achieve desirable expression. Different approaches can also be combined in order to achieve multicolor labeling of different structures.

One of the commonly used transgenic mice is the *Thy1-YFP* H line, which expresses yellow fluorescent proteins in layer 5 pyramidal neurons in the cortex for spine imaging.¹² There are also different lines of transgenic mice that express various fluorescent proteins in different subsets of neurons. For example, the *Thy1-GFP* M line has sparse and Golgi-like pattern labeling of neurons in the cortex.¹² Transgenic animals can provide a relatively uniform and stable expression pattern compared to *in utero* electroporation or virus labeling and they are ready to use at most developmental stages. However, there are limited numbers of transgenic lines that are available for the imaging of synaptic structures. In addition, using transgenic animals provides less control over the temporal and spatial expression of fluorescent proteins. In some transgenic lines, the neuronal labeling is too dense to resolve refined structure in older age. *In utero* electroporation is another method used to express fluorescent proteins *in vivo*. Recombinant protein constructs are injected into the lateral ventricle of embryos through the uterine wall. Pulses of electrical field are applied through electrodes. Plasmids will be taken up by neurons and will express recombinant proteins. Specific layers of neuronal labeling at different cortical regions can be achieved by performing *in utero* electroporation at a specific developmental age (from E12-E18) and by controlling the electrode placement during electroporation.¹³ Labeling by *in utero* electroporation is usually sparse and expression is stable for imaging through adulthood (up to 4 months after electroporation).¹³ The virus infection system is another method

that is starting to become popular for expressing recombinant proteins *in vivo*. The adeno-associated virus (AAV) and lentivirus are 2 commonly used viral systems.¹⁴⁻¹⁶ The former system has been widely used for long-term expression due to its lower neuronal toxicity for longitudinal imaging experiments. With the use of specific promoters and Cre-recombinase viral vectors or mouse lines, different recombinant proteins can be expressed in specific neuronal types.¹⁷ Viral vectors also allow the expression of recombinant proteins in species other than rodent, e.g., zebra finches and monkeys.^{18,19} These methods can achieve a stable expression of fluorescent proteins, but the numbers and the spread of labeled neurons might vary from time to time. Other methods like synthetic fluorescent probes (Alexa or Lucifer yellow) and calcium sensors (e.g., Fluo-5 or OGB-1) loading are also useful for the imaging of dendritic spines with the combination of electrophysiology.²⁰⁻²²

Imaging Techniques: Transcranial Versus Cranial Window

TPLSM enables deep tissue imaging with low phototoxicity, therefore providing an excellent platform for intravital imaging.²³ Nonetheless, specific surgical procedures are still required to image the mammalian brain. For short-term studies, a small cranial opening in the skull is used for performing imaging and electrophysiological recording at the same time. For long-term studies, there are 2 commonly used techniques for imaging over days to months: the transcranial (thinned-skull) window and the glass cranial (open-skull) window techniques (Fig. 1).^{24,25} The transcranial window is prepared by thinning the skull surface to ~20 μm thickness to create an optically transparent transcranial window for imaging. Re-thinning of the skull is required for repeated imaging sessions over days.²⁴ The glass cranial window is achieved by the removal of a piece of skull followed by the implantation of a glass window.²⁵ Both methods require skillful surgical techniques for a successful preparation and each method has its own pros and cons (Table 1).²⁴⁻²⁸ One of the major concerns for choosing either method

is the immune response after surgical preparation. The implantation of a glass window can induce inflammation and activation of astrocytes and microglia in the brain.^{26,28} This immune response might perturb the brain's normal physiology and increase dendritic spine turnover.²⁶ Administration of anti-inflammatory drugs, dexamethasone, carprofen, sulfamethoxazole/trimethoprim, and prolonged resting time (10 - 14 days) after surgery before imaging are used to minimize the impact of the activated immune response.²⁵ For imaging of greater depth (> 100 μm), a chronic glass window provides better images compared to the transcranial window.^{24,27} Other imaging techniques, for example, microendoscope²⁹ and micropipism³⁰ can also be used for deep tissue imaging, e.g., imaging of the hippocampus or deeper cortical layers. However, these techniques also involve extensive disruption or removal of CNS tissue that is relatively invasive. In short, surgical preparation induced-inflammation and the length of recovery time after surgery should be taken into careful consideration when choosing different imaging techniques, and appropriate control experiments should be performed to avoid potential complication.

Structural Plasticity of Dendritic Spines

Dendritic spines are dynamic structures that are constantly changing in shape, appearing and disappearing, and are therefore a contrast to the mostly stable dendrites.^{3,31,32} The appearance and disappearance of spines on dendrites are usually measured by the rate of spine formation and elimination. A small portion of dendritic spines are constantly formed and eliminated, whereas the majority of others remain highly stable for months or even for the lifetime of the animal.^{33,34} The subtle changes in rate of spine formation and elimination occur in a homeostatic manner, leading to a relatively stable spine density. These changes of spines could have never been detected without *in vivo* longitudinal experiment.

It is not surprising that the turnover rate of dendritic spines is highest during

Figure 1. Schematic diagram of different preparations for *in vivo* imaging. **(A)** Open cranial window is made by removal a small area of skull and dura mater so that patch pipette can be inserted for cell labeling and electrophysiological recording. Pulsation of exposed brain is reduced by agarose. **(B)** Transcranial window is made by thinning down the skull to 20–40 μm thickness over a small area. **(C)** Glass cranial window is made by replacing the skull (~2–5 mm diameter) by cover glass. Dura mater remains intact. Agarose can be used to fill the space between the skull and neocortex, but it is optional. Cover glass and exterior is sealed by dental cement.

development. In neonates, dendritic spines and filopodia exhibit high motility. The dendritic spines are relatively small and thin, with a relatively large fraction of long filopodia at this stage.³⁵ Spine density continuously increase over a period of time, which is known as the spinogenesis stage.³⁶ After this initial stage of net spine gain, the rate of spine elimination outpaces formation, leading to a net loss of spines and a decrease in spine density in adolescence (before 2-months-old).^{34,37,38} This process is called spine pruning.³⁶ Newly formed spines are usually small and they are more susceptible for elimination compared to mature, large head mushroom spines.³⁹ A retrospective electronic microscopy (EM) study of previously imaged dendritic spines found that only a fraction of newly formed spines within a few days bear synapses, but once they are stabilized and have persisted over 4 days, they always bear synapses.⁴⁰ Small spines can be transformed to larger spines upon stabilization. Stabilization and maturation of spines continue during development and into adulthood. In adults, spine

formation and elimination reach equilibrium and the majority of spines are stable until the onset of aging.^{34,37,41} Yet, a small portion of adult spines (3–5%) remain transient and dynamic.³⁶ The remarkably stable dendritic spines provide a physical entity for memory storage, whereas the spines that remain plastic may contribute to the capacity for learning new things or short-lived memories.

External stimulus is critical for neurodevelopment. Sensory input plays an important role in the development and maturation of synaptic networks. In barrel cortex, sensory deprivation by unilateral all-whiskers trimming in adolescent mice reduced spine elimination, while leaving spine formation unaffected.³⁸ Interestingly, chessboard trimming, where each trimmed whisker is surrounded by intact whiskers, evoked robust changes in spine turnover.^{3,33} This paradigm induced adaptive functional changes in the neural circuit by stabilizing newly formed spines and destabilizing previously existing spines that persisted over 8 days before whisker trimming.³³ An enriched environment

also promoted spine turnover and the novel experience-induced newly formed spines persisted throughout life.³⁴ Similarly, monocular deprivation (MD) also induced a robust increase in spine turnover in the visual cortex. This enhanced spine turnover was transient. After restoring binocular vision, spine dynamics was returned back to baseline.⁴² However, the second MD did not further enhance spine turnover, but selectively enlarged new spines formed during the first MD, suggesting these spines were functional.⁴² All these findings suggest that sensory experiences evoke reorganization of synaptic circuits and reveal a structural based mechanism for functional plasticity and memory storage of prior experiences.

How memories are stored is one of the most fascinating and challenging questions in neuroscience. Learning-induced dendritic spine plasticity has been observed in different learning paradigms. It is thought that dendritic spines might serve as a structural substrate for both learning and memory.³⁹ Dendritic spine plasticity has been shown to correlate with

Table 1. Comparison of transcranial and glass cranial window

	Transcranial window ²⁴ (Thinned-skull window)	Glass cranial window ²⁵ (Open-skull window)
Pros	<ul style="list-style-type: none"> Chronic imaging can start immediately after surgery No detectable astrocyte or microglia activation if the skull thickness is > 20 μm Transcranial window can be re-thinned if there is skull regrowth 	<ul style="list-style-type: none"> Large window (~2–5 mm diameter) Theoretically unlimited imaging sessions (if there is no skull regrowth or dura thickening)
Cons	<ul style="list-style-type: none"> Small window (~0.2 mm diameter) Up to 5 imaging sessions for chronic imaging with intervals of days to a year (unlimited within 2 days) 	<ul style="list-style-type: none"> Activation of astrocyte and microglia, systemic anti-inflammatory medications and antibiotics administrations are required Need to wait ~1–2 weeks due to opaque period after surgery for chronic imaging Experiment is to be terminated if there is skull regrowth

memory acquisition and storage. In mice, learning novel motor tasks induced rapid formation of dendritic spines.^{34,43} The amount of spine formation and its survival strongly correlated with motor performance in the initial acquisition phase and later skill retention phase.^{34,43} Similar findings have also been observed in song birds. In juvenile zebra finches, song learning induced the rapid formation and enlargement of dendritic spines. Furthermore, the high level of spine turnover before song learning predicted the greater capacity for subsequent song learning and better song imitation.¹⁸ Interestingly, the same learning task can also induce different responses in different cortical regions. Auditory fear conditioning induced spine formation in auditory cortex.⁴⁴ However, in the frontal association cortex, auditory fear conditioning induced spine elimination and the amount of spine elimination correlated with fear memory retrieval (i.e. freezing response).⁴⁵ On the contrary, subsequent fear extinction induced spine formation, while reconditioning eliminated spines that were formed during fear extinction in a location- and cue-specific manner in the frontal association cortex.⁴⁵ This finding suggests input-specific neural circuitry is involved in the learning and unlearning of fear. While the same task has different effects in different cortical regions, different tasks can also induce location-specific spine dynamics on the same neuron. A recent study showed that mice learning different motor tasks (forward vs. backward running on rotarod) led to spine formation on specific dendritic branches from the same neuron.⁴⁶ In addition, this dendritic branch-specific spine formation was sleep-dependent, suggesting the important role of sleep for memory consolidation. Indeed, predominant spine elimination in the sleep state has been shown in the developmental brain.^{47,48} Glucocorticoid is a stress hormone that oscillates in synchrony with the circadian rhythm. It was found that training mice at glucocorticoid peaks promoted dendritic spine formation, whereas troughs were required for stabilizing newly formed spines that were important for long-term memory retention.⁴⁹ In short, these studies show that dendritic spine plasticity is important for memory

acquisition and storage. Mechanisms underlying learning and memory formation are neural circuit specific and they are regulated by different physiological processes.

Heightened dendritic spine turnover has been commonly observed in a variety of mouse models of neurological and psychiatric diseases. Increases in spine formation and elimination have been found in animal models for Fragile X syndrome,^{50,51} Rett syndrome⁵² and schizophrenia.⁵³ In neurodegenerative disease models, similar increases of spine turnover are observed and it will follow by subsequent net loss of spines, for example, in Alzheimer disease,^{54,55} prion disease⁵⁶ and Huntington disease.⁵⁷ In the stroke model, severe ischemia caused a rapid loss of spine and dendrite structure within 10 minutes. These damages were reversible if reperfusion occurred between 20–50 minutes.⁵⁸ Glia is another cell type that resides in the nervous system and plays an important role in neuroinflammatory response not only in pathological conditions but also in response to neuronal activity.⁵⁹ Therefore, it is important to understand the interactions between glia cells and synaptic remodeling. A few recent studies explored the role of glia cells (astrocyte and microglia) for spine plasticity. Astrocytic processes have been shown to have close contacts with spines in an EM study⁶⁰. A recent study showed that the motility of perisynaptic astrocytic processes that enveloped dendritic spines correlated with spine coverage and promoted spine stability in the mouse somatosensory cortex after whisker stimulation.⁶¹ Another study showed that a blockade of astrocytic glutamate uptake accelerated experience-dependent spine elimination during development.⁶² Microglia has also been found to closely interact with spines. It has been recently proposed that microglia is involved in synaptic pruning during development.^{63–65} Conversely, an experimental model of microglia depletion in the brain resulted in a reduction of motor learning-induced spine formation. This finding was recapitulated by selective depletion of brain-derived neurotrophic factor (BDNF) in microglia, suggesting the important role of microglia for spine formation in learning through the BDNF

signaling pathway.⁶⁶ These studies reveal the different roles of microglia during development and learning-induced synaptic structural remodeling. Taken together, the heightened spine turnover leading to spine instability shows that abnormalities of spine plasticity are a common hallmark for neurological diseases. It further concurs to the idea that structural plasticity is important for proper cognitive functions.

Functional Plasticity of Dendritic Spines with Calcium Imaging

The functional properties and the spatial distribution of individual synaptic input have a critical role in information processing in the mammalian brain.^{67, 68} Although most studies on dendritic spines have been focusing on structural plasticity due to various technical challenges, recent advances in calcium imaging techniques enable the observation of single spine activity *in vivo*.^{20,22,69} The first report of this approach combined whole-cell patch clamp recording and synthetic calcium sensor (OGB-1) to investigate the sound-evoked responses on individual spines in the auditory cortex.²⁰ Except for a few instances of local clustering of similar frequency tuned spines, most similarly tuned spines were found to be widely spread throughout both apical and basal dendrites in layer 2/3 pyramidal neurons.²⁰ Pure-tone stimulation at different frequencies revealed narrowly and widely tuned spines and spines tuned for specific frequency were intermingled on the same dendrite.²⁰ A similar approach from the same group also showed that some spines were activated uniquely by a single whisker, but were mostly activated by multiple whiskers in the vibrissal cortex.⁷⁰ These findings demonstrated that sensory inputs for the same feature are heterogeneously distributed on dendrites, without clustering on a specific segment.^{20,70} In contrast, a study on spontaneous activity in the somatosensory cortex showed an increased probability of co-activation of spines within 6 μm along the dendrites suggesting clustered synaptic plasticity.²¹ Reactivation of the same spines during spontaneous up states and auditory stimulation was observed in the auditory

cortex.⁷¹ The studies mentioned above used synthetic calcium indicators combining whole cell recording. These experiments are usually acute and short-term. The newly developed genetically encoded calcium indicators, GCaMP6, enable stable imaging of calcium activity on individual spines over a much longer period (27 days).⁶⁹ Using these new calcium sensors with higher sensitivity, faster kinetics and enhanced brightness compared to the older version of GCaMPs, it was found that orientation tuning of structurally persistent spines were largely stable over weeks in the visual cortex.⁶⁹ This calcium sensor provides a new avenue to study functional and structural dynamics of neural circuits over multiple spatial and temporal scales.

Conclusions

The use of intravital imaging techniques and the development of fluorescent probes have tremendously advanced our understanding of dendritic spine structure and function. Dendritic spines are constantly formed and pruned during development and adulthood. The structural and functional plasticity of spines are modulated by experience and learning. The formation and stabilization of newly formed spines together with the existing spines are orchestrated for neuronal network function and memory storage. Nonetheless, many questions remain unanswered. For example, what are the pre-synaptic inputs that modulate the structure and function of individual spines? What is the circuitry involved in learning and memory? What is the relation between structural and functional plasticity of individual spines? How do spines and dendrites encode, integrate and process information? What temporal and spatial signaling or electrical events govern spine plasticity? What molecular mechanisms underlie structural and functional plasticity *in vivo*? How do structural and functional plasticity of spines affect neuronal network function and subsequently affect animal behavior? Advancements in imaging techniques, together with electrophysiology, molecular genetics and tools for neuronal activity manipulation, such

as optogenetics, will help to reveal the mechanisms underlying neuronal network function in normal and pathological conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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