

# Two-Photon Imaging of Dendritic Spine Development in the Mouse Cortex

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**ABSTRACT:** Dendritic spines are the postsynaptic sites of most excitatory synapses in the mammalian brain. With the advent of two-photon microscopy and transgenic mice expressing fluorescent proteins, dendritic spines can now be imaged in the living cerebral cortex over time scales ranging from seconds to years. Recent studies with this *in vivo* imaging approach have begun to provide important insights into the development and plasticity of dendritic spines in the intact

brain. Here, we review these studies and discuss technical requirements for image acquisition. We envision that intravital two-photon imaging at the level of individual synapses will greatly expand our current understandings of how neuronal networks are assembled and modified throughout life. © 2008 Wiley Periodicals, Inc. *Develop Neurobiol* 68: 771–778, 2008

**Keywords:** two-photon microscopy; imaging; dendritic spines; synapses; development; plasticity

## INTRODUCTION

Dendritic spines are small protrusions extending from dendritic shafts to receive the vast majority of excitatory inputs in the brains of diverse species (Shepherd, 1996; Yuste and Bonhoeffer, 2004). As the postsynaptic sites of neuronal connections, dendritic spines have been extensively investigated in the effort to understand how neuronal circuits are established during development and subsequently altered in life. Although many aspects of spine development and plasticity in living animals still remain unclear, it is well-established that dendritic spines are highly dynamic structures, particularly during postnatal development when enormous number of synaptic connections are rapidly made (Rakic et al., 1986; Harris

and Stevens, 1989; Huttenlocher, 1990; Dailey and Smith, 1996; Ziv and Smith, 1996; Dunaevsky et al., 1999; Engert and Bonhoeffer, 1999; Toni et al., 1999; Matsuzaki et al., 2004). As animals mature into adulthood, substantial changes in spine number and morphology could still occur during the learning process and under pathological conditions (Greenough et al., 1973; Terry et al., 1991; Kleim et al., 1997; Fiala et al., 2002; Knott et al., 2002; Duan et al., 2003; Tsai et al., 2004; Zhang et al., 2005).

Our current understanding about dendritic spine dynamics has mainly come from studies in fixed brain tissues, live neuronal cultures, and brain slices. While studies of fixed or *in vitro* preparations have provided a wealth of information on spine development, there is a great need to examine changes of dendritic spines directly in the living intact brain. Because of the complexity and variability of the brain, it is often difficult to extrapolate dynamics of dendritic spines from single-time-point observations of fixed preparations. The use of fixed preparations can at its best reveal the net change in the number or size of spines but does not provide information on the rates of spine elimination and formation. Although *in vitro* preparations

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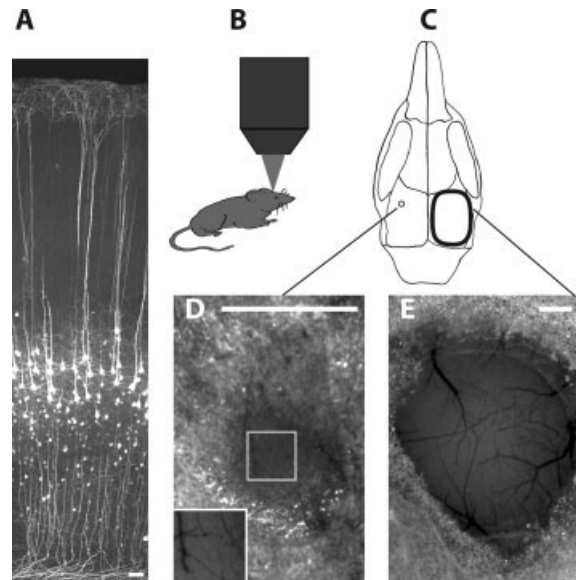
allow for direct observation of spine dynamics under defined conditions, they are compromised in that long-range connections between brain areas are inevitably severed and complex patterns of brain activity are absent. Thus, technical limitations of fixed or *in vitro* preparations have prevented investigators from addressing some basic questions on spine development and maintenance. For example, to what degree does the process of spine development *in vivo* resemble that *in vitro*? How long do spines live in a healthy brain? How does experience from the outside world modify the development and plasticity of spines? What is the relationship between spine plasticity and the development of psychiatric disorders? It is evident that the best way to answer these questions is to directly follow changes of dendritic spines in the living intact brain.

Recent technical advances have made it possible to image individual dendritic spines over extended periods of time in the mammalian brain. Specifically, the use of two-photon microscopy (TPM) allows live imaging of fluorescently labeled synapses in the living cortex several hundred micrometers deep from the pial surface (Denk et al., 1990; Theer et al., 2003). In addition, the expression of green fluorescent protein (GFP) and its spectral variants in specific cell types in the brain permits repeated imaging of individual synaptic structures in living animals (Feng et al., 2000; Lendvai et al., 2000; Grutzendler et al., 2002; Sin et al., 2002; Trachtenberg et al., 2002; Gan et al., 2003; Niell et al., 2004; Stettler et al., 2006). In recent years, investigators have applied TPM for imaging dendritic spines in GFP/YFP expressing transgenic mice, in attempt to address some of the long-standing questions related to spine development. Below, we review these studies and discuss technical requirements that are critical for spine imaging.

### TECHNICAL REQUIREMENTS FOR *IN VIVO* IMAGING OF DENDRITIC SPINES

Because dendritic spines are tiny protrusions on the surface of dendrites and exist in high abundance, imaging spines in the cortex requires the use of transgenic mice that have strong expression of genetically encoded fluorescent markers in a small subset of neurons (Feng et al., 2000). So far, two-photon imaging of spine dynamics has been mainly performed with transgenic mice that overexpress YFP or GFP predominantly in a subset of layer V pyramidal neurons driven by the Thy-1 promoter (Grutzendler et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2005a,b; Zhang et al., 2005; Zuo et al., 2005a,b;

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**Figure 1** Transgenic mice and surgical preparations for *in vivo* two-photon imaging. (A) A subset of layer V pyramidal cells express YFP in the barrel cortex of YFP H-line mice. These cells extend their apical dendrites to layer I where spines can be imaged with two-photon microscopy. (B) Illustration of an anesthetized animal under a two-photon microscope. (C) Surgical preparations for *in vivo* imaging can be accomplished either by thinning the skull of  $\sim 200 \mu\text{m}$  diameter region to about  $20 \mu\text{m}$  in thickness (small circle on the left hemisphere) or by removing a large piece of skull ( $\sim 4\text{--}5 \text{ mm}$  in diameter) and replacing with a glass coverslip (large circle on the right hemisphere). (D) A typical thinned-skull preparation in which the imageable region ( $\sim 200 \mu\text{m}$ ) is indicated by a square. The inset shows the blood vessels located under the thinned-skull window and within the square. The blood vasculature was imaged with a CCD camera (after adding artificial cerebrospinal fluid to the thinned-skull window) and was used as a map for repeated imaging of the same region. (E) A typical open-skull preparation. Scale bars: (A)  $50 \mu\text{m}$ ; (D, E)  $500 \mu\text{m}$ .

Majewska et al., 2006). Although many mouse lines expressing fluorescent proteins have been generated (Feng et al., 2000), some might not be suitable for chronic imaging of individual spines if the labeling is simply too faint to image or too dense to distinguish one spine from another.

In addition to neuronal labeling, to image fluorescently labeled dendritic spines in the cortex, the skull of the animals needs to be thinned to  $\sim 20 \mu\text{m}$  in thickness (thinned-skull preparations) [Fig. 1(C,D)]. Alternatively, a piece of skull can be removed and the exposed cortical region covered with a glass coverslip (open-skull preparations) [Fig. 1(C,E)]. To obtain stable image stacks of dendritic branches and spines, mice generally need to be anesthetized and their heads fixed with a custom-made metal plate or

bar glued on top of the skull. For chronic imaging of the same sets of dendritic spines, it is necessary to map the blood vasculature and dendritic branches as landmarks for relocation of the originally imaged area [Fig. 1(D,E)]. The detailed procedures of animal surgery and intravital imaging have been described elsewhere (Grutzendler and Gan, 2005; Holtmaat et al., 2005b; Xu et al., 2007). Because thinned- and open-skull preparations give rise to different degrees of baseline spine dynamics (Grutzendler et al., 2002; Trachtenberg et al., 2002), the choice of these two preparations for imaging spines will be discussed further in this review.

The final requirement that needs to be considered for imaging spines *in vivo* is the type of microscopy to be used. Strong expression of GFP or YFP in Thy-1 transgenic mice allows spines in the superficial cortical layer to be visualized under conventional epifluorescence or confocal microscopes (through thinned- or open-skull windows). However, TPM is the method of choice because it provides superior images of spines in the cortex several hundred micrometers deep from the pial surface (Denk et al., 1990; Theer et al., 2003). This depth of imaging is possible because TPM uses two far- or infra-red photons to excite a single fluorophore and thereby significantly reduces light absorption and scattering within biological specimens. Furthermore, the absence of excitation out of the plane of focus in TPM minimizes phototoxicity and allows efficient detection of emission light. For these reasons, TPM is essential for vital imaging of spines in the cortex; and as described below, its use has generated several new insights into the development and plasticity of dendritic spines in living animals.

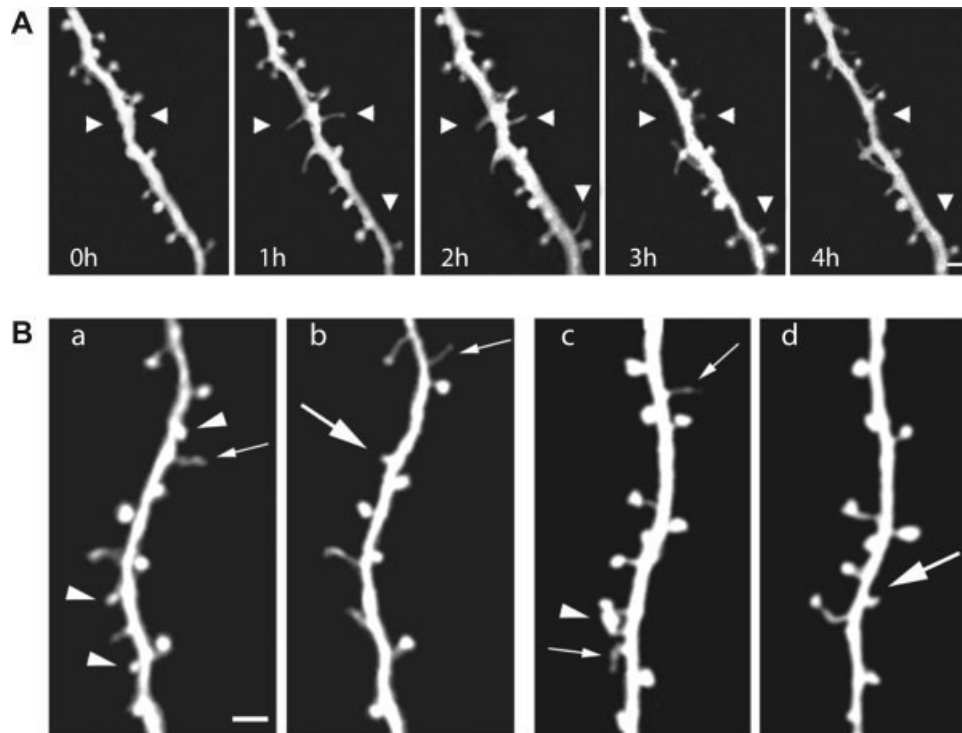
## DENDRITIC SPINE FORMATION

Dendritic spines appear early during development shortly after dendritic processes are extended from neurons. Time-lapse imaging of neuronal cultures and brain slices has shown that spines form either directly from dendritic shafts or are transformed from dendritic “filopodia,” long and thin protrusions without bulbous heads (Dailey and Smith, 1996; Ziv and Smith, 1996). In young developing brain, electron microscopic (EM) studies indicate that some filopodia do not have any synaptic contacts with axons, whereas others make several synaptic contacts (Fiala et al., 1998). In addition, the vast majority of dendritic filopodia undergo rapid extension and retraction within minutes to hours (Dailey and Smith, 1996; Ziv and Smith, 1996; Dunaevsky et al., 2001; Portera-

Cailliau et al., 2003; Tashiro et al., 2003). These observations suggest a model that dendritic filopodia play a pivotal role in spine formation, possibly by “capturing” the potential axonal contacts in nearby regions and eventually resulting in the formation of new spines.

Consistent with the role of filopodia in spinogenesis, *in vivo* imaging of YFP-expressing mice showed that dendritic filopodia are indeed highly dynamic and sometimes evolve into spines. In young adolescent mice at 1 month of age, ~12% of dendritic protrusions in different cortical regions are filopodia while the remaining are spines. Time-lapse imaging showed that ~50–60% of these filopodia were formed and eliminated over 4 h [Fig. 2(a)], whereas only ~1–2% of spines turned over within the same period (Zuo et al., 2005a). While the majority of filopodia underwent rapid turnover, ~15% of filopodia formed a bulbous-head and persisted over 4 h. Importantly, ~35% of these newly formed spine-like protrusions persisted over 24 h and ~13% lasted over 48 h (Zuo et al., 2005a). These protrusions are morphologically indistinguishable from pre-existing spines. Thus, *in vivo* observations indicate that similar to findings from *in vitro* studies, a small percentage of filopodia are transformed into more stable thin- or mushroom-like dendritic spines, providing further evidence for filopodia as spine precursors. The fact that most filopodia do not result in spines alludes to a highly selective nature of forming and maintaining contacts between filopodia and their appropriate axonal partners.

While the theory in which dynamic filopodia find their partners and mature into spines seems to describe an important rule of spine development, it remains unclear whether filopodia are precursors of most (if not all) spines or if the majority of spines are formed directly from the dendritic shaft. Another important question is related to the temporal sequence of “spinogenesis” and “synaptogenesis”: do neurons make synaptic contacts before dendrites produce spines or is it the other way around? The definitive answers to these questions require the use of combinatorial approaches for simultaneous monitoring pre- and postsynaptic cells at high temporal resolution as they are making contacts and subsequently identifying whether such contacts are synaptic in nature with electron microscopy. Recently, *in vitro* and *in vivo* studies with such approaches have suggested that dendritic protrusions that have emerged from dendritic shafts make physical contact with axonal terminals within tens of minutes and have apparent synaptic contact within a period of many hours (Knott et al., 2006; Nagerl et al., 2007). In addition, filopodia/



**Figure 2** Time-lapse imaging of dendritic filopodia and spines in barrel cortex. (A) Time-lapse imaging (1 h intervals) in 1-month-old mice reveals that filopodia (arrowheads) undergo rapid extension and retraction, whereas spines on the same dendritic branches remain stable. (B) Repeated imaging of a dendritic branch from an animal of 4–6 weeks old reveals spine elimination (arrowheads) and formation (large arrows) as well as filopodium turnover (small arrows) in control (a, b) and whisker-trimmed mice (c, d). Whisker trimming reduces spine elimination while has no significant effect on spine formation *in vivo*. Scale bars: 2  $\mu\text{m}$ . (Modified with permission from Zuo et al., *Neuron*, 2005, 46, 181–189, ©Elsevier).

spines show various degrees of motility even though they are in contact with presynaptic axonal terminals (Konur and Yuste, 2004; Deng and Dunaevsky, 2005). Together, these findings are consistent with the view that dendritic protrusions transiently sample the surrounding neuropil and initiate contacts with presynaptic terminals. Furthermore, because most of the newly formed protrusions are eliminated within hours to days after they are formed (Zuo et al., 2005a), it appears that the formation of functional spines with synapses involves a protracted selective process.

### SPINE PRUNING IN LATE POSTNATAL DEVELOPMENT

A hallmark of synapse development is that a substantial net loss of synapses occurs during late postnatal development. In the cerebral cortex of humans and other mammals, rapid synaptogenesis during early

postnatal life is followed by a substantial (~50%) loss of synapses/spines that extends through adolescence (Huttenlocher, 1979, 1990; Rakic et al., 1986; Markus and Petit, 1987; Lubke and Albus, 1989; Rakic et al., 1994; De Felipe et al., 1997). In adulthood, the number of spines remains relatively constant until aging-related loss of synapses occurs. These studies suggest that in late postnatal life, neuronal circuits undergo significant rewiring and synapse elimination is fundamental to the maturation of the nervous system. However, these studies from fixed postmortem tissues could not determine the dynamic behavior of spines and the nature of spine loss. Because spines are appearing and disappearing, the decrease in spine number could be due to more elimination of existing spines or instead due to slower addition of new spines.

To address this issue, TPM was used for *in vivo* imaging of spines of layer V pyramidal cells. It was found that in young adolescent mice (1 month of age), 13–20% of spines were eliminated and 5–8%

were formed over a 2-week interval in visual, barrel, primary motor, and frontal cortices, indicating a cortical-wide loss of spines during this developmental period (Zuo et al., 2005a). Furthermore, from 1 to 4 months of age, a ~25% net loss of spines occurs as the result of a higher rate of spine elimination than that of spine formation in both visual and barrel cortices. A recent study examining spine dynamics in the visual, somatosensory, and auditory cortices in ~1.5-month-old mice also found a net loss of spines over 1 or 3 weeks (Majewska et al., 2006). These observations are consistent with previous studies from fixed tissues showing that synaptic density in the mammalian cortex decreases substantially from infancy until puberty (Rakic et al., 1986; Markus and Petit, 1987; Huttenlocher, 1990; De Felipe et al., 1997). Importantly, these *in vivo* imaging studies indicate that the major reorganization of the cortex during late postnatal life is the elimination of existing connections between neurons. Because a substantial loss of synapses occurs across different regions of the developing brain in monkeys and humans, sculpting early established synaptic connections is likely a fundamental process in the developing brain and occurs in many cell types and cortical layers in diverse species.

## SPINE STABILITY IN THE ADULT CORTEX

Dendritic spines appear to be inherently plastic because substantial and rapid changes in spine number can occur in response to environmental challenges and under pathological conditions (Greenough et al., 1973; Terry et al., 1991; Knott et al., 2002; Duan et al., 2003; Kirov et al., 2004; Tsai et al., 2004; Zhang et al., 2005). However, it is unknown to what degree dendritic spines undergo remodeling in a normal and healthy adult brain. The answer to this question is important for understanding how long-term information is stored in neuronal circuits. For example, if the majority of adult spines remain throughout life, it would suggest that memory and basic cortical functions can be stably maintained by synaptic connections that are established during development. On the other hand, if adult spines are highly dynamic and turnover multiple times within the life-time of an animal, it would suggest that long-term information might instead be stored in a dynamic fashion in highly plastic synaptic networks.

The first two studies using *in vivo* imaging of GFP/YFP expressing dendritic spines generated fundamentally different views on the stability of spines in the adult mouse cortex (Ottersen and Helm, 2002). One study showed that in the adult mouse primary

visual cortex (>4 months of age), spines in apical dendrites from layer V pyramidal neurons are remarkably stable with ~4% turnover per month (Grutzendler et al., 2002). However, a different study in the barrel cortex suggested that spines are highly plastic in adulthood: ~20% of spines turnover within 1 day and ~40% turnover within a week (Trachtenberg et al., 2002). Subsequent studies continue to show substantial differences in spine dynamics. Zuo et al. showed that adult spines exhibited low rates of elimination and formation in barrel, primary motor, and frontal cortices, similar to that previously reported in the primary visual cortex (Zuo et al., 2005a). Furthermore, >70% of spines in adult barrel cortex could be maintained over an 18-month interval. On the other hand, Holtmaat et al. observed that ~20% of adult spines turnover within 4 days and ~30% turnover within 1 month in barrel and visual cortices (Holtmaat et al., 2005a).

The different results described above lead to contradictory views on the structural plasticity of spines in the mature brain and have different implications for information storage and maintenance in neuronal circuits. Various factors such as surgical techniques, animal ages, and transgenic mouse lines might all contribute to different spine dynamics observed in adult mouse cortex (Grutzendler and Gan, 2006). Using the same transgenic mouse line, a recent study examined the impact of cranial window type used for *in vivo* imaging on adult spine dynamics (Xu et al., 2007). It was found that spines were remarkably stable under thinned-skull windows (~200  $\mu\text{m}$  in diameter), but quite plastic under a large open-skull glass window (~4–5 mm in diameter). In addition, the use of open-skull preparations causes a substantial loss of spines within the first 2 weeks after surgery followed by a high spine turnover that lasts for at least an additional 3–4 weeks. Extensive glial activation was also found for at least 1 month after surgery under open-skull windows but not in the case of thinned-skull preparations. These observations suggest that the discrepancy in adult spine dynamics is at least partially related to cranial window types used for *in vivo* imaging (Grutzendler and Gan, 2006; Xu et al., 2007). Because open-skull preparations are valuable for certain experiments which cannot be done otherwise, it is important to develop new ways in the future to make open-skull windows with minimum perturbations to the brain and suitable for spine imaging (e.g., perhaps using a small open-skull window; Majewska et al., 2006; Nishiyama et al., 2007).

It is important to note that the above studies mainly imaged spines in apical dendrites of layer V pyramidal neurons in the cortex. It remains unclear

whether the observations of spine stability can be generalized to the entire dendritic tree of layer V pyramidal cells or different neuronal types. Studies in other brain areas such as the hippocampus and the olfactory bulb have demonstrated high levels of stability of adult dendritic branches and spines over hours to days (Mizrahi and Katz, 2003; Mizrahi et al., 2004). Recent imaging studies of inhibitory cortical neurons and adult-born neurons in the olfactory bulb have shown that a small degree of changes in dendritic branches and spines do occur in the mature brain (Lee et al., 2006; Mizrahi, 2007). Thus, it appears that under laboratory housing conditions, a large percentage of neuronal connections are likely stable in adulthood and may persist for the duration of a mouse's life. To what degree spines can remain stable over a lifespan under natural environment and in species such as monkeys and humans requires further investigation.

### EXPERIENCE-DEPENDENT REMODELING OF DENDRITIC SPINES

A fundamental aspect of the nervous system is that the patterns of neuronal connectivity and the animal's behavior are profoundly influenced by experience throughout life. Recently, two-photon imaging has been used to examine the effect of sensory experience on the rates of dendritic spine elimination and formation in mouse barrel cortex, a cortical region in which sensory input can be easily manipulated. By trimming all the whiskers on one side of the mouse facial pad daily, Zuo et al. showed that during late postnatal development, when extensive spine loss occurs, sensory deprivation over weeks preferentially reduces the rate of spine elimination rather than formation (Zuo et al., 2005b) [Fig. 2(b)]. Furthermore, as spines become stabilized in adulthood, sensory deprivation over a period of weeks has no significant effect on the rate of spine elimination. Because sensory deprivation reduces the net loss of spines during late postnatal development, an important insight from this study is that experience-dependent sculpting of neuronal connectivity seems vital to the maturation of the brain. Furthermore, sensory experience appears to have different roles in regulating the number of synapses depending on the animal's developmental stage.

The effect of sensory experience on spine dynamics has also been examined by trimming every other whisker in a chessboard pattern on one side of the facial pad. Unlike trimming all the whiskers which deprives all sensory inputs, chessboard deprivation removes only half of whiskers while leaving adjacent

whiskers intact. Trachtenberg et al. found that in 5–10 week-old mice, chessboard trimming over 4 days had no effect on overall spine density but increased spine turnover (Trachtenberg et al., 2002). Consistent with this finding, a recent study showed that chessboard trimming for weeks continue to alter spine dynamics without impact on spine density in adult barrel cortex (Holtmaat et al., 2006). In another study, Zuo et al. found that similar to all-whisker trimming, chessboard deprivation over 2 weeks preferentially reduces spine elimination without effect on the rate of spine formation in young adolescent mice (Zuo et al., 2005b). The effect of chessboard trimming however is less robust than that under all-whisker trimming. In addition, chessboard trimming over 2 weeks had no significant impact on the rates of spine elimination and formation in adult mice.

It is important to mention that different types of cranial windows were used for imaging experience-dependent spine dynamics in the studies described above. Because baseline spine dynamics differ in different studies, it remains unclear to what degree spine turnover is affected by sensory experience. Because substantial changes in the number of synapses can occur following various learning and injury paradigms even in the mature brain, the mature brain must retain some capacity to form new synapses and rewire its circuitry. Thus, although *in vivo* imaging studies have shown that adult spines can form and retract only to a limited degree under normal and sensory-deprived environments, it remains to be determined to what degree spine dynamics can be altered under enriched environment and learning conditions.

### CONCLUDING REMARKS

Recent intravital two-photon imaging studies have begun to provide important insights into dendritic spine development in the intact mammalian brain. Many basic questions regarding spine development and plasticity remain to be addressed with the *in vivo* two-photon imaging approach. Although much progress has been made in the past decades on the gene regulation of spine development, most of the studies so far have been focused on the role of individual genes in regulating spine density from a single-time-point observation. It is thus important to combine *in vivo* two-photon imaging and mouse genetics to determine the role of different genes in various phases of spine formation and maintenance. Furthermore, psychiatric disorders and neurodegenerative diseases are frequently associated with aberrant spine structure and plasticity in critical brain regions.

Abnormal formation and pruning of spines during development may compromise the establishment of a functional brain. Because spines are remarkably stable in adults, such developmental defects may have long-lasting effects later in life. Two-photon imaging, in combination with animal disease models, will likely provide important insight into the onset, progression, and treatment of psychiatric and neurological diseases.

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