Review

Spine Dynamics: Are They All the Same?

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Since Cajal's first drawings of Golgi stained neurons, generations of researchers have been fascinated by the small protrusions, termed spines, studding many neuronal dendrites. Most excitatory synapses in the mammalian CNS are located on dendritic spines, making spines convenient proxies for excitatory synaptic presence. When in vivo imaging revealed that dendritic spines are dynamic structures, their addition and elimination were interpreted as excitatory synapse gain and loss, respectively. Spine imaging has since become a popular assay for excitatory circuit remodeling. In this review, we re-evaluate the validity of using spine dynamics as a straightforward reflection of circuit rewiring. Recent studies tracking both spines and synaptic markers in vivo reveal that 20% of spines lack PSD-95 and are short lived. Although they account for most spine dynamics, their remodeling is unlikely to impact long-term network structure. We discuss distinct roles that spine dynamics can play in circuit remodeling depending on synaptic content.

Spine Visualization: Evolution from Classical Anatomy to Modern Imaging Methods

For over a hundred years now, since Santiago Ramón y Cajal first published his seminal work using Golgi staining to reveal the fine architecture of neurons in fixed slices, the fine protrusions studding the dendrites of many neurons depicted in his detailed camera lucida drawings have fascinated generations of neuroscientists (Ramón y Cajal, 1893). In his Neuron Doctrine, Cajal postulated that the nervous system was composed of discrete neurons rather than a contiguous network of cells as was the popular theory espoused by luminaries such as Golgi himself (DeFelipe, 2015). Cajal also postulated that the dendritic protrusions, which came to be known as spines, were points of contact between two neurons and that, in reaching out from the dendritic shaft, they could facilitate diverse connections with axons from many different sources. Later, Sherrington and others introduced the concept of the synapse as the site of chemical neurotransmission between neurons (reviewed in Shepherd and Erulkar, 1997), although techniques available at the time were not able to resolve the synaptic cleft between a spine and an axonal bouton. It was not until the advent of electron microscopy (EM), that researchers were able to directly visualize the synapses on dendritic spines (Gray, 1959a, 1959b) and show that spines were the major sites of excitatory synaptic transmission (Uchizono, 1965).

For the next several decades, methods for visualizing neuronal structure were static, providing snapshots of the neuron at the time of fixation for EM or other light microscopy. Without the ability to track the same neuron over time, inferences about spine dynamics could only be made at the population level, by comparing groups of samples fixed at different ages or after different manipulations. In the mid 1990s, use of fluorescent dyes as cell fills allowed for the first repeated monitoring of spines, revealing their dynamic nature in cultured neurons (Fischer et al., 1998; Ziv and Smith, 1996) and brain slices (Dailey

and Smith, 1996). At the same time, the introduction of fluorescent proteins for neuronal visualization opened the door for their genetic labeling and long-term tracking (Chalfie et al., 1994; Chen et al., 2000a; Moriyoshi et al., 1996). However, the limitations of conventional light microscopy would have relegated such studies to in vitro culture or slice preparations if not for the fortuitous development around the same period of the ultrafast pulsed lasers that ushered in the age of two-photon microscopy (Denk et al., 1990, 1994). The combination of fluorescent cell fills with two-photon microscopy allowed for imaging of structures deep within scattering tissues such as the brain (reviewed in Denk and Svoboda, 1997; So et al., 2000). Using this technique, dendritic spines could be seen in situ in brain slices (Dunaevsky et al., 1999; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999) and later in vivo (Grutzendler et al., 2002; Holtmaat et al., 2005; Lendvai et al., 2000; Trachtenberg et al., 2002) changing their shape and size, as well as forming or disappearing across an animal's lifespan. Spine dynamics were shown to be responsive to both the animal's experience and environment (reviewed in Holtmaat and Svoboda, 2009), and changes in their size and shape have become accepted markers for changes in synaptic strength or synaptic presence (reviewed in Bhatt et al., 2009).

The view of spines as readily identifiable morphological surrogates for excitatory synapses is largely based on EM studies of the mature brain, showing that very few excitatory synapses onto excitatory neurons are located on the dendritic shaft, and the vast majority of spines contain a single excitatory synapse (Harris et al., 1992; LeVay, 1973). Despite this almost one-to-one correspondence between spines and excitatory synaptic presence assessed by EM, when visualizing an individual spine in vivo it is impossible to deduce whether it in fact contains a synapse by morphological criteria alone. This is particularly true when the spine is newly formed (Knott et al., 2006). Recently, methods for direct synaptic labeling are allowing a re-evaluation

of the relationship between spine and synapse dynamics. Here, we review our evolving understanding of spine dynamics and caution that not all spine changes necessarily reflect long-lived alterations in circuit connectivity. While most spines in the adult contain a post synaptic density visible by EM (Arellano et al., 2007b), a significant fraction lack the key excitatory synaptic scaffold protein PSD-95 required for synaptic stabilization (Cane et al., 2014; Villa et al., 2016). These spines lacking PSD-95 account for the majority of spine dynamics and likely represent an effort to test new partners by forming transient connections, few of whom eventually survive (Cane et al., 2014; Holtmaat et al., 2005; Villa et al., 2016; Zuo et al., 2005a). We discuss imaging strategies that can discriminate between transient and long-lasting spine remodeling and best practices for their faithful interpretation.

Spines as Synaptic Markers

In EM micrographs, spines characteristically contain what is classified as a type 1 asymmetric synapse, defined as a synapse containing a protein rich postsynaptic density (PSD) apposed by a presynaptic axon containing round presynaptic vesicles (Gray, 1959a, 1959b; Hersch and White, 1981; LeVay, 1973; Parnavelas et al., 1977). Type 1 synapses were linked with excitatory inputs after electrophysiology experiments in the cerebellum showed the excitatory nature of granule cell input onto Purkinje cell dendrites (Uchizono, 1965). Later, immuno-EM showed that axons positive for the excitatory neurotransmitter glutamate only form connections with type 1 synapses and not type 2 symmetric synapses, which lack a visible PSD and are typically inhibitory (DeFelipe et al., 1988). The PSD that defines type 1 synapses has been identified as a complex network of proteins forming a highly regulated scaffold that anchors the glutamate receptors at excitatory synapses (reviewed in Sheng and Hoogenraad, 2007).

During early neuronal development, the majority of type 1 synapses on pyramidal neurons are located on the shaft, but, as spines begin to form, excitatory synapses on spines gradually replace the dendritic shaft population (reviewed in Yuste and Bonhoeffer, 2004). In the adult, very few excitatory synapses onto excitatory neurons are located on the dendritic shaft, and the vast majority of spines contain a single type 1 excitatory synapse (Harris et al., 1992; LeVay, 1973). Excitatory synapses that have been observed on the shaft are generally formed on the dendrites of inhibitory interneurons that are typically aspiny (Ahmed et al., 1997; Bock et al., 2011; Bopp et al., 2014; Buhl et al., 1997). The minority of inhibitory interneurons that contain spines are less spiny than pyramidal neurons (Kawaguchi et al., 2006; Kuhlman and Huang, 2008). Whether on excitatory neurons (Harris et al., 1992; LeVay, 1973) or on spiny inhibitory neurons, most spines contain a single type 1 excitatory synapse (Kawaguchi et al., 2006; Kuhlman and Huang, 2008). Spine size linearly correlates with PSD size and the number of presynaptic vesicles (Harris et al., 1992), as well as with synaptic strength (Asrican et al., 2007; Béïque et al., 2006; Matsuzaki et al., 2001; Noguchi et al., 2005, 2011; Zito et al., 2009). The close correspondence between spines and excitatory synaptic presence and strength is the basis for considering spines as good morphological surrogates for excitatory synapses. Spine density
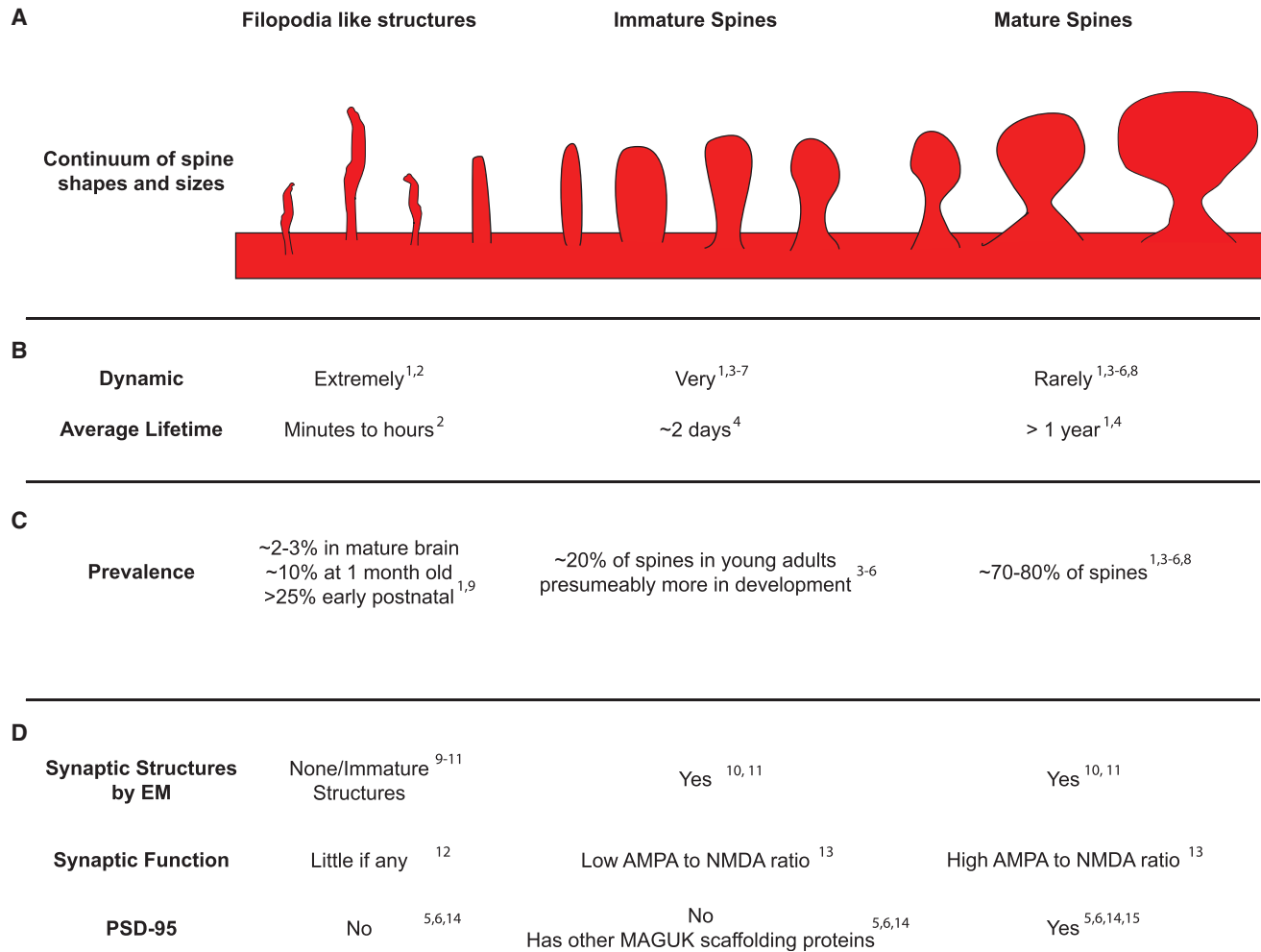
and size visualized through the use of sparse labeling methods such as the Golgi stain, injection of fluorescent dies, or transfection with genetically encoded fluorophore cell fills have been used to infer the locations of excitatory synapses in cultures, slices, and in vivo and as metrics to assess normal excitatory circuit health and development (reviewed in Rochefort and Konnerth, 2012).

Spine Morphological Categories

Dendritic protrusions are often grouped into 4 classes based on their morphologies: mushroom, thin, and stubby spines and filopodia (Peters and Kaiserman-Abramof, 1970). Mushroom shaped spines, defined by their characteristically large bulbous head and narrow neck, contain the largest excitatory synapses. Thin spines are smaller, lack the large bulbous head and thin neck, and contain smaller excitatory synapses. In the adult cortex and hippocampus, ~25% of spines are mushroom shaped while >65% are thin shaped (Harris et al., 1992; Peters and Kaiserman-Abramof, 1970). Large mushroom spines may be close to the upper limits of synapse size and strength and therefore have little range for synaptic strengthening. New, thin spines, carrying small or immature synapses, would have a greater potential for strengthening and may therefore be indicative of the capacity for plasticity in the local circuit. This has led some to describe mushroom spines as memory spines and thin spines as learning spines (reviewed in Bourne and Harris, 2007). The prevalence of thin spines declines during aging and cognitive deterioration (Dumitriu et al., 2010). However, manipulations that restore plasticity in older animals also increase the prevalence of thin spines (Hao et al., 2006). The third category of spines associated with excitatory synapse presence are the stubby spines. These spines are shorter and squatter than thin spines, lack a distinctive head and neck configuration, and are viewed as immature structures based on their prevalence during early postnatal development and relative scarcity in the mature brain (Harris et al., 1992).

The fourth category of dendritic protrusions are filopodia, the smallest structures protruding from dendrites, often described as thin, hairlike structures. The immature filopodia or stubby shapes account for approximately 10% of spines in the adult (Fiala et al., 2002; Harris, 1999; Harris et al., 1992; Peters and Kaiserman-Abramof, 1970). Many filopodia lack a clear type 1 synapse in EM micrographs (Arellano et al., 2007b), mostly due to the lack of a PSD, though they often present with synaptic characteristics such as a small cleft or a few synaptic vesicles (Fiala et al., 1998). Given their lack of clear type 1 synapses, one might think that filopodia should simply be excluded from spine counts that are used to represent synaptic density. However, this is not a trivial task due to the difficulty of distinguishing between spine morphological categories. Particularly difficult is drawing a clear distinction between filopodia and thin spines without the aid of EM.

The resolution of light microscopy for the discrimination of small morphological differences is limited, especially along the z axis (Holtmaat et al., 2009; Sorra and Harris, 2000). The ability to resolve a structure is dependent on the wavelength of light used to visualize that structure and the numerical aperture of the objective lens (Abbe diffraction limit), with axial resolution

A

Filopodia like structures   Immature Spines   Mature Spines

Continuum of spine shapes and sizes

B

	Filopodia like structures	Immature Spines	Mature Spines
Dynamic	Extremely[1,2]	Very[1,3-7]	Rarely[1,3-6,8]
Average Lifetime	Minutes to hours[2]	~2 days[4]	> 1 year[1,4]

C

	Filopodia like structures	Immature Spines	Mature Spines
Prevalence	~2-3% in mature brain ~10% at 1 month old >25% early postnatal[1,9]	~20% of spines in young adults presumeably more in development[3-6]	~70-80% of spines[1,3-6,8]

D

	Filopodia like structures	Immature Spines	Mature Spines
Synaptic Structures by EM	None/Immature Structures[9-11]	Yes[10, 11]	Yes[10, 11]
Synaptic Function	Little if any[12]	Low AMPA to NMDA ratio[13]	High AMPA to NMDA ratio[13]
PSD-95	No[5,6,14]	No Has other MAGUK scaffolding proteins[5,6,14]	Yes[5,6,14,15]

Figure 1. Spines Exist on a Continuum of Morphologies and Functions from Nonfunctional Filopodia-like Structures to Large Mature Spines
(A) Diagram showing continuum of spine shapes. These spines can be grouped into three separate categories, filopodia-like structures, immature spines, and mature spines. Distinguishing between these categories based on morphology is extremely difficult due to the limited resolution of light microscopy.
(B) The history of a spine can distinguish between these types.
(C) In adults, the majority of spines contain a mature synaptic contact, while 20% are either immature or filopodia like.
(D) The three categories of spines are difficult to distinguish based on any one category alone. However, by comparing across several criteria the differences become clearer. Reference key: (1) Zuo et al., 2005a; (2) Dunaevsky et al., 1999; (3) Trachtenberg et al., 2002; (4) Holtmaat et al., 2005; (5) Villa et al., 2016; (6) Cane et al., 2014; (7) Majewska and Sur, 2003; (8) Grutzendler et al., 2002; (9) Fiala et al., 1998; (10) Knott et al., 2006; (11) Arellano et al., 2007a; (12) Lohmann et al., 2005; (13) Zito et al., 2009; (14) Lambert et al., 2017; (15) Ehrlich et al., 2007.

being worse than lateral resolution. In general terms, the shortest resolvable distance between two objects is roughly one-half of the wavelength of light used to illuminate the sample (NA~1). Since the shortest wavelength of visible light is ~400 nm, this limits the lateral resolution of light microscopy to ~200 nm, which is quite large in cellular terms. Since multiphoton microscopy uses longer excitation wavelengths, typically greater than 800 nm, the lateral resolution limit is roughly in the ~400-nm range. The largest spines are on the order of 1 μm in diameter, and their sizes can range down to well below the diffraction limit in the case of thin spines and filopodia (for a more detailed discussion of the potential pitfalls for spine analysis, see Holtmaat et al., 2009). This technical limitation is confounded by the biological reality that spine morphology falls across a continuum of shapes and sizes rather than into distinct classes (Figure 1A)

(Arellano et al., 2007a; Portera-Cailliau et al., 2003; Yuste and Bonhoeffer, 2004). Some groups have developed quantitative measures for spine categorization, but classification inherently depends on subjective scoring criteria (e.g., neck width, length, etc.). These difficulties have led many to count any resolvable dendritic protrusion as a spine. While circumventing the issue of classification, this approach equates all protrusions with spines and glosses over the fact that not all protrusions necessarily contain an excitatory synapse. Further, as discussed below, not every synapse necessarily represents a mature and stable synaptic connection.

Spines Are Dynamic
Based on the fact that spine densities are higher during development than in adulthood, Cajal postulated that spines were

dynamic, in the sense that they could be added and subsequenty pruned, and went further to suggest that such changes could underlie learning and memory (Ramón y Cajal, 1893). The process of developmental spine pruning has since been seen in multiple experimental species and systems and has been shown to be responsive to an animal's environment and experience (reviewed in Markham and Greenough, 2004). The large-scale changes to dendritic arbor structure and spine numbers that occur during development are easily detected by comparing animals in different age groups using various cell fills in fixed tissue. Unfortunately, comparing fixed tissue samples cannot resolve subtle spine changes occurring in the same animal under different experimental conditions and cannot monitor spine turnover on a single cell over time.

The introduction of two-photon microscopy for neuronal imaging (Denk et al., 1990; Denk and Svoboda, 1997; Helmchen et al., 1999; Maletic-Savatic et al., 1999; Shi et al., 1999; Svoboda et al., 1996, 1997, 1999), combined with the development of methods to label individual cells with genetically encoded fluorescent cell fills (Chalfie et al., 1994; Chen et al., 2000a; Moriyoshi et al., 1996), has allowed for the first time in vivo longitudinal visualization of dendritic structure within intact brain circuits. From its first implementation, this modern anatomical method revealed with stunning clarity that there is considerable capacity for spine remodeling (Grutzendler et al., 2002; Trachtenberg et al., 2002), even across the stable excitatory dendritic scaffold of adult neurons in vivo (Lee et al., 2006; Mizrahi and Katz, 2003; Trachtenberg et al., 2002). Post hoc EM after imaging showed, anecdotally, that newly formed spines contain excitatory synapses (Trachtenberg et al., 2002), supporting the interpretation that spine addition and elimination represent excitatory synapse gain and loss, and therefore local remodeling of excitatory circuits.

Dendritic spine dynamics have been demonstrated across many different cortical regions (Attardo et al., 2015; Grutzendler et al., 2002; Hofer et al., 2009; Holtmaat et al., 2005, 2006; Majewska et al., 2006; Xu et al., 2009; Zuo et al., 2005a). They can be influenced by a wide range of sensory manipulations including whisker trimming (Holtmaat et al., 2006), monocular deprivation (Hofer et al., 2009), and retinal scotoma (Keck et al., 2008, 2011), consistent with the idea that they represent synaptic changes driven by the local circuit. Further, spine dynamics have been implicated in learning paradigms such as motor skill learning (Xu et al., 2009), song acquisition in songbirds (Roberts et al., 2010), and memory consolidation (Attardo et al., 2015; Yang et al., 2014). Each manipulation differentially affects the rate of spine dynamics in a circuit specific manner, i.e., only in the modality appropriate regions and cellular subtypes. For example, monocular deprivation in adult mice reduces the rate of spine dynamics in binocular visual cortex, specifically on the apical tufts of L5 neurons coursing through L2/3 but has no effect on the rate of spine dynamics on L2/3 neurons in the same region (Chen et al., 2012; Hofer et al., 2006; Villa et al., 2016). There are multiple comprehensive reviews covering the topic of how experience influences spine dynamics (Berry and Nedivi, 2016; Fu and Zuo, 2011; Holtmaat and Svoboda, 2009).

The lifetimes of newly formed spines fall into two distinct kinetic groups. The first of these are the persistent spines, which either form and remain for months to years or disappear and never return (Figure 2A). The second group consists of spines termed transient because they are added and then removed with a mean lifetime of ~2 days (Holtmaat et al., 2005) (Figure 2B). Thus, many groups classify dynamic spines present for less than 4 days as transient (Holtmaat et al., 2005; Keck et al., 2008; Trachtenberg et al., 2002; Villa et al., 2016). Transient spines tend to be smaller, but spines of all sizes and morphological subtypes from mushroom spines to filopodia can fall into either dynamic class (Holtmaat et al., 2005). The fraction of persistent versus transient spines varies with developmental age. In the mouse visual cortex and somatosensory cortex, only 35% of all spines persist at 3 weeks of age, increasing to 54% by 4–10 weeks and stabilizing at ~70% in the adult (Grutzendler et al., 2002; Holtmaat et al., 2005).

Spine dynamics in the adult are normally balanced between additions and eliminations (Holtmaat et al., 2005; Zuo et al., 2005a). This balance can be maintained even with decreased or increased spine turnover or can temporarily shift to favor additions or eliminations. In mice, sensory deprivation induced by trimming all the whiskers on one side of the face (Zuo et al., 2005b), or by monocular deprivation (Hofer et al., 2009), leads to a reduced rate of spine elimination and an increase in the persistence of newly formed spines in the contralateral cortex. Conversely, sensory stimulation by environmental enrichment or checkerboard whisker trimming increases spine turnover rates (Holtmaat et al., 2005; Trachtenberg et al., 2002; Yang et al., 2009). In the mouse motor cortex, learning induces rapid spine formation within hours, followed by subsequent pruning to pre-learning densities (Xu et al., 2009). The pruned spines are more likely to be ones preexisting prior to learning, while the newly formed spines are more likely to be stabilized. The maintenance of new spines correlates with the maintenance of the learned skill and the extent of new spine formation with the quality of the skill acquisition (Xu et al., 2009; Yang et al., 2009). The learning induced spines are very stable and able to persist for months after training (Yang et al., 2009). Thus, they are interpreted as representing new connections mediating the long-term encoding of the training memory in the cortex. This interpretation was elegantly reinforced in experiments where an optical probe was used to selectively shrink spines recently potentiated following a motor learning task, resulting in failure to learn the new task (Hayashi-Takagi et al., 2015). A similar interpretation has been proposed for spine dynamics in the hippocampus, where spines are extremely dynamic with an average lifetime of 1–2 weeks. This results in a complete turnover of the entire synaptic population within 4–6 weeks, which corresponds well with the temporary nature of memory storage in the hippocampus (Attardo et al., 2015).

These studies suggest that it is the stabilization step of spine dynamics, particularly in regard to spines newly formed in response to the manipulation or task, which best correlates with the consolidation and storage of newly learned information and behavior on a long-term basis. The overall increase in spine turnover, while not correlated with functional outcome per se, potentially creates fresh opportunities for the selective stabilization of newly salient contacts. Thus, increased spine turnover is
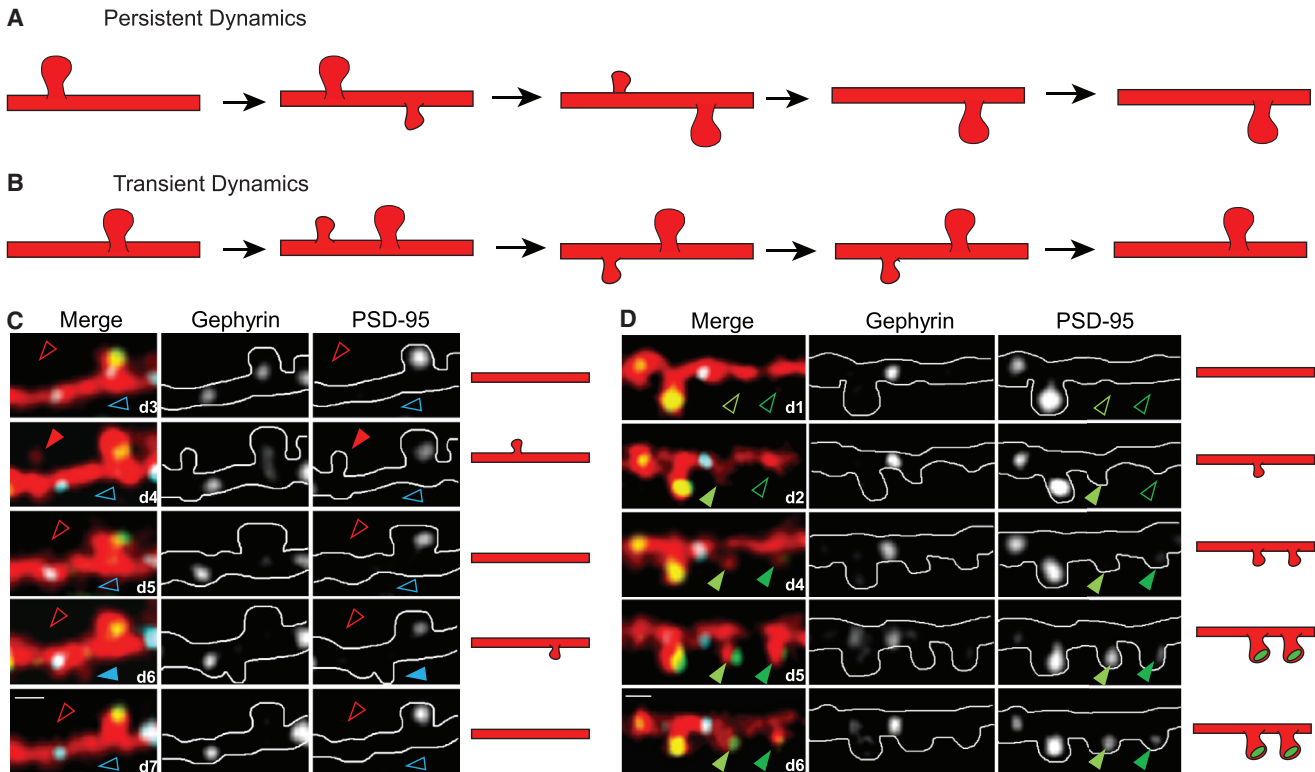
Figure 2. Dynamic Spines Fall into Either the Transient or Persistent Dynamics Categories
(A) Schematic illustration of two persistent dynamic events where a new spine forms and persists long term.
(B) Schematic illustration of two transient dynamic events that appear and disappear again with a few days.
(C and D) Examples of spine dynamics. Left, middle, and right panels show three-channel merge, Teal-gephyrin alone, and PSD-95-mCherry alone, respectively. Arrows denote dynamic spines: filled when spine is present and empty when spine is absent. (C) Shows the brief appearance and removal of spines without PSD-95 at separate nearby locations. (D) Shows formation of two new spines that gain a PSD-95 and persist. Adapted from Villa 2016).

permissive for circuit remodeling, but ultimately the stabilization step will determine whether a new spine is integrated into the local circuit and contributes to a long-term shift in that circuit's function.

Spine Maturation and Stabilization
Dynamic spines encompass the entire spectrum of spine morphologies and sizes. The smallest filopodia-like protrusions are highly unstable and can form or be removed within hours, while the largest mushroom type spines are more likely to remain stable for months to years (Figure 1B) (Grutzendler et al., 2002; Holtmaat et al., 2005; Trachtenberg et al., 2002; Zuo et al., 2005a). The relative representation of filopodia versus mushroom spine types, the extreme ends of the morphological spectrum, varies developmentally (Figure 1C) and goes hand in hand with the overall rate of spine dynamics. Filopodia are prevalent during early postnatal development, when synapse formation and selection is at its peak, and, with maturation, are replaced by larger spines (Fiala et al., 1998). In mice at 2 weeks of age, over half of all dendritic protrusions are filopodia like. This peak number drops to 10% at 1 month and to ~3% in the adult (Zuo et al., 2005a). The age-dependent drop in the number of filopodia concomitant with the increase in large spines coincides with the age-dependent decrease in the rate of spine dynamics

overall (Holtmaat et al., 2005; Majewska et al., 2006; Zuo et al., 2005a).

The maturation of small, highly dynamic spines or filopodia into larger, stable spines occurs not just at the population level, but also at the level of the individual spine. In both culture and in vivo, filopodia can form, stabilize, and grow into larger functional spines, suggesting that they represent an early stage of spine formation (Dailey and Smith, 1996; Ziv and Smith, 1996; Zuo et al., 2005a). Similar to the developmental progression from small, dynamic filopodia to larger, stable spines, new spines formed in hippocampal slice cultures after activity blockade often first appear as filopodia (Kirov and Harris, 1999; Petrak et al., 2005). The activity-dependent synaptic strengthening paradigm of long-term potentiation (LTP) also induces the proliferation of filopodia-like spines in cultured hippocampal slices, some of which are stabilized and maintained (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999).

Like spine emergence, spine stabilization is also activity dependent. Stimuli that in culture induce synaptic strengthening by LTP also increase spine volume and stabilize newly formed spines (Hill and Zito, 2013; Kopec et al., 2006; Lang et al., 2004; Matsuzaki et al., 2004; Nägerl et al., 2004). Conversely, synaptic weakening through induction of long-term depression (LTD) reduces spine volumes and can lead to the destabilization

and elimination of preexisting spines (Nägerl et al., 2004; Zhou et al., 2004). Thus, transitions in spine size and stability, from an immature, unstable filopodia or dendritic protrusion to a mature stable spine, goes hand in hand with the maturation and strengthening of the synaptic contact on that spine.

PSD-95 Recruitment Is Key to Synaptic Maturation and Spine Stabilization

It is perhaps unsurprising that the expression of PSD-95, a protein that is an integral part of the post-synaptic density and provides the scaffold for clustering and stabilizing glutamate receptors at excitatory synapses (reviewed in Kim and Sheng, 2004), has been intricately linked with synapse maturation and stabilization. The PSD-95 post-synaptic scaffold binds both NMDA and AMPA-type glutamate receptors, the NMDA receptor via a PDZ binding domain (Kornau et al., 1995), and the AMPA receptor via small transmembrane AMPA receptor regulatory proteins (TARPs) such as stargazin (Chen et al., 2000b; Nicoll et al., 2006; Schnell et al., 2002). During early postnatal development, when spine instability and synaptic immaturity are at their peak, PSD-95 expression is low, only later increasing to maximum levels in the adult (Al-Hallaq et al., 2001; Hsueh and Sheng, 1999). At these early times, other postsynaptic scaffolding proteins in the PSD-95 family predominate at excitatory synapses. For example, SAP102 expression is high during early cortical and hippocampal development and falls off into adulthood (Müller et al., 1996; Sans et al., 2000).

As seen with the developmental progression in spine morphologies, the developmental maturation of excitatory synapse scaffolding proteins is manifested not just at the population level, but also at the individual spine level. Tracking newly formed spines in hippocampal organotypic slice cultures shows that endogenous PSD-95 can take up to 24 hr to accumulate in a new spine that is later stabilized, but other proteins in the same family such as PSD-93, SAP102, and SAP97 appear much earlier and facilitate AMPA receptor recruitment to the nascent synapse (Lambert et al., 2017). These proteins contribute to the short-term stability of the spine (Lambert et al., 2017), but it is the ultimate activity-dependent recruitment of PSD-95 that is required for long-term stability (De Roo et al., 2008; Lambert et al., 2017). Electrophysiological experiments in acute slices also show that during synaptogenesis SAP102 acts as a scaffold for NMDA and the first AMPA receptors at the immature synapse, but it does not seem to have the same stabilizing effect that PSD-95 has at mature synapses (Elias et al., 2008).

In vitro studies expressing a fluorescently tagged version of PSD-95 suggest that most new spines initially lack PSD-95 (Chen and Featherstone, 2005; De Roo et al., 2008; Lambert et al., 2017; Waites et al., 2005). In disassociated neuronal cultures, PSD-95-GFP accumulation in spines occurs within a few minutes to hours after initial contact between the nascent spine and a presynaptic partner (Bresler et al., 2001; De Roo et al., 2008; Friedman et al., 2000; Okabe et al., 2001). In organotypic slice cultures, this process is much longer, and can take up to 24 hr (De Roo et al., 2008; Lambert et al., 2017). In these studies, the recruitment of PSD-95-GFP has been shown to be dependent on synaptic activity (De Roo et al., 2008; Taft and Turrigiano,

2013) and required for the activity-dependent stabilization of excitatory synapses and spines (Ehrlich et al., 2007), so much so, that its presence provides an excellent prediction for which spines will be stabilized (De Roo et al., 2008; Ehrlich et al., 2007; Taft and Turrigiano, 2013). Interestingly, the increase in PSD-95 levels that accompanies spine enlargement after LTP induction has a similar time delay to that of PSD-95 accumulation after contact between a nascent spine and its presynaptic partner (Bosch et al., 2014; Meyer et al., 2014).

In line with the correlation between PSD-95 presence and the stability of spines and synapses, knockdown of PSD-95 in hippocampal slice cultures leads to increased spine turnover rates, and a failure to add and stabilize spines after LTP induction (Ehrlich et al., 2007). In contrast, high levels of PSD-95 overexpression lead to an increase in spine density as well as average spine size suggesting a role for PSD-95 in spine stabilization and maturation (El-Husseini et al., 2000). PSD-95 overexpression in slice cultures results in a significant increase in AMPA-receptor-mediated transmission (Ehrlich and Malinow, 2004; El-Husseini et al., 2000; Schnell et al., 2002) and can mimic as well as occlude LTP (Ehrlich and Malinow, 2004; Stein et al., 2003). Knockdown of PSD-95 in slices results in reduced AMPA to NMDA ratios as well as preventing the normal developmental increase in synapses with functional AMPA receptors (Ehrlich et al., 2007). AMPA to NMDA ratios of excitatory postsynaptic currents are also reduced in PSD-95 knockout mice indicating a role for PSD-95 in the maturation of the excitatory synapse population (Béïque et al., 2006).

Yet, despite the lack of spine stability and the immature synaptic function after PSD-95 knockdown, spine morphology and asymmetric synapse structure (viewed by EM) in PSD-95 knockout mice are indistinguishable from their wild-type littermates (Béïque et al., 2006; Migaud et al., 1998). This suggests that PSD-95 does not necessarily represent excitatory synaptic presence per se, but rather, the presence of a mature, stable synaptic contact.

Not All Spines Are Created Equal

Recent expansion of two-photon spectral capabilities, allowing in vivo imaging of two or three different fluorescent proteins concurrently have enabled the tracking of synaptic components, such as PSD-95 for excitatory synapses and/or gephyrin for inhibitory synapses, independent of spine visualization (Cane et al., 2014; Chen et al., 2012; Gray et al., 2006; Isshiki et al., 2014; Villa et al., 2016). Concurrent monitoring of spine dynamics and the PSD-95 excitatory postsynaptic scaffold made it possible to address in vivo the relationships between the presence of PSD-95 and spine dynamics (Figures 2C and 2D).

A striking finding from these studies is that on any given day, ∼20% of spines do not contain fluorescently tagged PSD-95 (Cane et al., 2014; Isshiki et al., 2014; Villa et al., 2016). This seems contradictory to the large body of EM data demonstrating that only 2%–4% of spines in the adult brain lack an identifiable post synaptic density (Arellano et al., 2007b; Hersch and White, 1981; White and Rock, 1980). However, as we learned from PSD-95 knockout mice, the lack of PSD-95 does not necessarily preclude the existence of a synapse but rather indicates the lack of a mature synapse that contains stable AMPA receptors

(Béïque et al., 2006). Other studies have also shown functional spines in the absence of PSD-95. In organotypic hippocampal slice cultures, newly formed spines do not acquire PSD-95 for up to 24 hr after formation (De Roo et al., 2008; Lambert et al., 2017), yet glutamate uncaging experiments have shown that nascent spines can have synaptic responses similar to preexisting spines (Kwon and Sabatini, 2011; Zito et al., 2009). Thus, the 20% of spines that lack PSD-95 labeling likely contain a synaptic structure but are potentially lacking the stable AMPA receptor population conferred by the presence of PSD-95 (Figure 1D).

Examining spine dynamics in relation to fluorescently tagged PSD-95 presence in vivo shows that spines bearing PSD-95 puncta are more stable (Cane et al., 2014; Villa et al., 2016), consistent with previous PSD-95 gain- and loss-of-function studies (Ehrlich et al., 2007; El-Husseini et al., 2000; Woods et al., 2011). On the rare occasion that such spines are removed or added, they often first lose PSD-95 puncta before the spine retracts, or, alternatively, they gain a PSD-95 punctum and are stabilized (Cane et al., 2014; Villa et al., 2016) (Figure 2D). Conversely, spines that never bear a PSD-95 puncta, although they are only 20% of the spine population, account for ~80% of all dynamic spines (Cane et al., 2014; Villa et al., 2016). To a large extent, these spines are in the transient category (Villa et al., 2016) (Figure 2C) and likely bear an immature synaptic structure that is functional, but unstable. This illustrates that the majority of spine dynamics are transient in nature and do not involve the addition or elimination of PSD-95, the hallmark of a mature and stable glutamatergic synapse.

Why might the cell expend so much effort extending and retracting connections that ultimately fail to stabilize? Many newly formed spines will form connections onto preexisting multisynaptic boutons that already synapse with other spines, potentially competing with these spines for the same presynaptic bouton (Knott et al., 2006; Nägerl et al., 2007). In this scenario, transient spines may represent failed attempts to compete for a presynaptic contact, where during the competitive process they form immature synaptic contacts that fail to stabilize and form a long-term connection. Their lack of PSD-95, required for AMPA receptors and spine stabilization, is consistent with their transient existence.

In vivo, the process of PSD-95 recruitment is slower than in vitro and can take up to several days (Cane et al., 2014; Villa et al., 2016). In vivo PSD-95 recruitment is also less predictive for spine stabilization, but it does appear to be a pre-requisite in that process (Cane et al., 2014). The link between PSD-95 recruitment and spine stabilization introduces an alarming pitfall. Does expression of fluorescently tagged forms of PSD-95 affect synapse stability in vivo? Overexpression of PSD-95 in cell culture has been shown to increase the number of spines, perhaps by their artificial stabilization (El-Husseini et al., 2000). However, control experiments for the in vivo studies have not found evidence for an increase in spine densities when comparing imaged neurons that were labeled only with a cell fill versus ones labeled with fluorescently tagged PSD-95 in addition to a cell fill (Cane et al., 2014; Gray et al., 2006; Villa et al., 2016). This suggests that with use of a low expression vector, the amount of fluorescent PSD-95 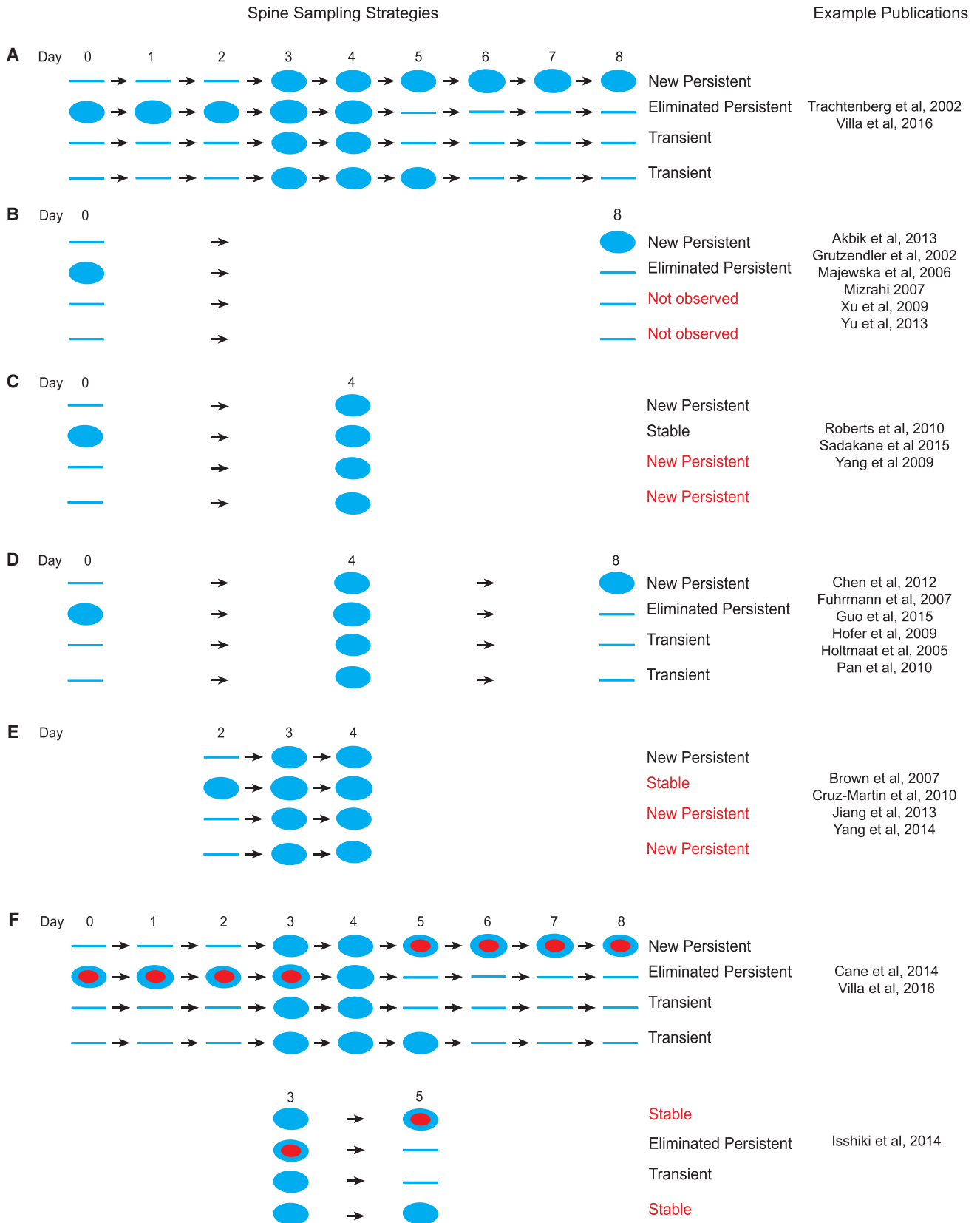fusion protein generated is unlikely to perturb the system. Such controls should be the norm for all experiments implementing new expression methods for synaptic labels.

Persistent versus Transient Circuit Changes

The ability to visualize tagged PSD-95, in combination with longitudinal in vivo imaging, revealed that in terms of synaptic content, the spine population can roughly be partitioned into two categories (see Figure 1 for a detailed comparison). The largest category of spines, which in adult mice comprises approximately 70%–80% of the population, are PSD-95-puncta-containing spines with mature excitatory synapses (Cane et al., 2014; Villa et al., 2016). These spines are mostly stable, but if they lose the PSD-95 punctum and disappear or are formed de novo and gain a PSD-95 punctum, their dynamics will result in a persistent synaptic gain or loss (Figure 2D). The addition or removal of spines that persist are the events most likely to reflect permanent changes in local circuit connectivity.

The second category of spines, comprising approximately 20% of the population, are those that lack PSD-95 puncta. These spines are highly dynamic and will only rarely be stabilized into a long-lasting change in connectivity (Figure 2C). When interpreting spine dynamics, it is important to keep in mind that the dynamics of persistent and transient spines have drastically different impacts on the local circuit. The ability to assign an individual dynamic spine to the persistent or transient category equates with determining whether its addition or elimination likely represent a long-term rewiring event in the local circuit or merely an attempt to form a new connection. A spine whose elimination or addition persist is a true indicator of local circuit remodeling, reflecting the complete loss or addition of a selected information channel. A transient spine mostly reflects opportunistic sampling of the local environment that fails to stabilize as a long-term change.

Identifying a persistent as opposed to transient spine is thus critical for determining whether its dynamics represent circuit remodeling. This is a better diagnostic than using morphological criteria, for example, that loss/gain of a mushroom spine represents a synaptic change, while that of a thin or filopodia-like spine does not. As discussed above, morphological criteria can only approximate the chance that a given spine is forming a mature and stable synaptic contact. Coupled with the inherent limitations of light microscopy for spine morphological classifications, it is a poor tool for predicting the long-term behavior of individual spines. The use of post hoc EM to determine whether a newly formed spine contains a mature excitatory synapse is typically considered definitive in terms of synapse validation. This too is problematic due to the anecdotal nature of small EM sample sizes. Moreover, the contrast between the 20% of dynamic, PSD-95 punctum lacking spines seen by in vivo imaging and the 97% of spines showing synaptic structure by EM suggests that EM cannot distinguish between immature synapses (PSD-95 negative with unstable AMPA receptors) on transient spines, and mature (PSD-95 positive with stable AMPA receptors) synapses on stable spines. Thus, aside from the experimental difficulty of validating every synaptic change by EM, it does not provide a distinction between spines harboring a transient versus mature synaptic structure.

Spine Sampling Strategies

Example Publications

A Day 0 1 2 3 4 5 6 7 8

New Persistent

Eliminated Persistent — Trachtenberg et al, 2002
Villa et al, 2016

Transient

Transient

B Day 0   8

New Persistent — Akbik et al, 2013
Grutzendler et al, 2002
Eliminated Persistent — Majewska et al, 2006
Mizrahi 2007
Not observed — Xu et al, 2009
Yu et al, 2013
Not observed

C Day 0   4

New Persistent

Stable — Roberts et al, 2010
Sadakane et al 2015
New Persistent — Yang et al 2009

New Persistent

D Day 0   4   8

New Persistent — Chen et al, 2012
Fuhrmann et al, 2007
Eliminated Persistent — Guo et al, 2015
Hofer et al, 2009
Transient — Holtmaat et al, 2005
Pan et al, 2010
Transient

E Day   2 3 4

New Persistent

Stable — Brown et al, 2007
Cruz-Martin et al, 2010
New Persistent — Jiang et al, 2013
Yang et al, 2014
New Persistent

F Day 0 1 2 3 4 5 6 7 8

New Persistent

Eliminated Persistent — Cane et al, 2014
Villa et al, 2016
Transient

Transient

3   5

Stable

Eliminated Persistent — Isshiki et al, 2014

Transient

Stable

(legend on next page)

We propose two useful criteria for the persistent versus transient distinction. The first would be tracking the spine over sufficient time and at sufficient intervals to confirm its persistence. The second would be the presence or absence of a PSD-95 punctum. The question is whether having an extended dynamic history without PSD-95 labeling, or PSD-95 labeling with only a few imaging sessions, are sufficient to determine whether or not a dynamic spine represents the elimination/formation of a new contact or merely a transient one that failed to stabilize. Would the same rules apply when characterizing baseline dynamics, comparing different genetic lines, or examining the effect of a specific manipulation? Let's examine several case studies.

Different sampling strategies have been used by various groups to assess the degree of spine dynamics and rewiring that occurs under different conditions in the cortex. These are roughly segregated into studies where the same neuron is repeatedly imaged many times and those with only two to three imaging sessions. Often, the strategy is related to the surgical preparation used by that lab. The former approach utilizes a cranial window, while the latter utilizes a thinned skull preparation. The pros and cons of these two methods have been extensively discussed elsewhere (Dorand et al., 2014). For this discussion, the salient difference between the two preparations is that cranial windows allow for multiple imaging sessions of the same region over long intervals, while skull thinning allows for the same site to be revisited only once, or at best twice, after the initial imaging session due to clouding of the skull after multiple thinning preparations. This limited number of sessions constrains the number of data points that can be collected, requiring judicious selection of the imaging intervals and careful interpretation of the results.

Whatever the sampling strategy, it is important to remember that imaging sessions merely sample a continuously dynamic system. Elimination events occurring on the first day of imaging, or new spines appearing on the last, will have limited history compared to those that can be tracked over several sessions. The more sampling that can be done, the more accurately the dynamics of the system can be characterized, as long as the imaging frequency is on the same timescale as the dynamic events, with the average lifetime of newly formed spines being about 2 days (Holtmaat et al., 2005).

On the exhaustive end of the sampling strategies is daily imaging for over a week (Figure 3A). This approach readily captures spine eliminations and additions bracketed by several sessions and allows for their definitive characterization into the persistent and transient categories. At the other end of the spectrum are strategies using only two imaging sessions. In this approach, one lacks the history needed to determine if newly formed or eliminated spines represent transient or persistent changes in circuitry (Figure 3B). However, if these two sessions are spaced far enough apart, most transient changes would not be captured as they can occur and reverse within a few days. This biases toward persistent changes that accumulate over time and form a larger fraction of the observed dynamics the longer the interval between the two time points.

What if the two sessions are closer together, say 4 days, which is closer to the average lifetime of transient spines (Holtmaat et al., 2005; Villa et al., 2016)? Many dynamic events would appear to persist (Figure 3C), although in most cases they are in reality short lived. Adding a third confirmatory session at a later time can resolve this problem (Figure 3D), but only if the time interval is longer than the lifetime of most transient events (usually at least 4 days) (Figure 3E). One solution in the case where imaging is limited to a total of two to three sessions is to sample different cohorts of animals at different intervals to build a picture of the formation and elimination process across the spine population. This strategy can identify a general propensity across the spine population but cannot resolve the behavior of specific spatially and temporally defined dynamic spines (data not shown; see Xu et al., 2009 for an excellent example of this method).

The addition of a synaptic label can sometimes help with categorization of spines into the persistent versus transient category, although in itself it is not a definitive criterion. Spines often briefly acquire a fluorescent PSD-95 punctum at some point after their appearance. The acquisition of the PSD-95 punctum is required for spine stabilization and persistence but does not necessarily predict whether or not the spine will ultimately stabilize and persist (Cane et al., 2014). Spines that last for at least 4 days and express PSD-95 are very likely to remain long term. The PSD-95 label is more useful when evaluating the significance of a spine elimination. Since the majority of spines with PSD-95 puncta on any imaging session are in the persistent

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Figure 3. Different Strategies Commonly Used for Sampling Dendritic Spine Dynamics

Different sampling strategies of a continuously dynamic system can give rise to different perceived outcomes in terms of transient versus persistent events. Outcomes marked in red font illustrate potentially erroneous conclusions.

(A). Daily imaging easily distinguishes between transient and persistent dynamic spines (blue circles indicate spine presence). This imaging interval is less than the approximately 1- to 4-day lifetime of transient spines (Holtmaat et al., 2005), so that the majority of transient and persistent events will be detected in this approach.

(B) Imaging twice with a longer interval, ranging from a week to over a month, will allow persistent dynamics to accumulate giving a good indication of long-term changes in connectivity. However, shorter, transient events that represent a plasticity-related sampling strategy will be missed.

(C) Imaging two times at relatively short intervals leads to the converse problem, where the inability to distinguish between transient and persistent spines leads to an overestimation of persistent dynamic spines.

(D) The addition of a third imaging session, longer than 4 days, allows the outcome of a dynamic event to be tracked in terms of spine survival and the correct scoring of transient versus persistent events.

(E) Imaging sessions that are performed too close together, hours or days apart, cannot be used to infer whether a spine will persist or is only transiently present since these intervals are within the mean lifetime of transient spines.

(F) PSD-95 (red) can be used in lieu of an extended dynamic history to differentiate between eliminated spines that do not form mature synaptic contacts and those that do. The following citations are included in this figure: Trachtenberg et al., 2002; Villa et al., 2016; Akbik et al., 2013; Grutzendler et al., 2002; Majewska et al., 2006; Mizrahi, 2007; Xu et al., 2009; Yu et al., 2013; Roberts et al., 2010; Sadakane et al., 2015; Yang et al., 2009; Chen et al., 2012; Fuhrmann et al., 2007; Guo et al., 2015; Hofer et al., 2009; Holtmaat et al., 2005; Pan et al., 2010; Brown et al., 2007; Cruz-Martín et al., 2010; Jiang et al., 2013; Yang et al., 2014; Cane et al., 2014; Isshiki et al., 2014.

category, the division of eliminated spines into those with and without PSD-95 can largely differentiate between the persistent and transient population (Figure 3F). Since the PSD-95 label is indicative rather than definitive, the best confirmatory data for whether spines are persistent or transient are additional imaging sessions to track spine survival. With the addition of PSD-95, the number of tracking sessions can be reduced.

Summary

For over a hundred years now, researchers have been using spines as morphological markers for excitatory synaptic presence, and their appearance and disappearance as indicators of excitatory circuit remodeling. While EM evidence suggests a one-to-one correspondence between the presence of a spine and an excitatory synapse, not all excitatory synapses are equivalent in terms of maturity and stability, and not all spine dynamics have equivalent meaning for the local circuit.

The ability to visualize PSD-95 in combination with longitudinal in vivo imaging reveals that spines roughly fall into two categories based on their synaptic content and dynamic behavior. The majority of spines, approximately 70%–80% in adult mouse neocortex, carry mature excitatory synapses scaffolded by PSD-95. Most PSD-95 positive spines are stable, but, on the rare occasion that they lose PSD-95 and disappear, or are formed de novo and gain a PSD-95, their dynamics will result in a persistent synaptic gain or loss. Although infrequent, this type of spine dynamics is the one most likely to reflect permanent changes in local circuit connectivity.

The second category of spines comprises all PSD-95 negative spines, which in the adult brain account for 20% of all spines. A few percentage of these, mainly in the filopodial category, may lack synaptic structure and function. The remainder contain a visible type 1 synaptic structure as assessed by EM and may have immature synaptic currents due to AMPA receptors with high turnover rates, but their synapses are unstable. The spines carrying such PSD-95 negative synapses are highly dynamic, and mostly transient, only rarely gaining PSD-95 or persisting. Their dynamics likely represent a sampling strategy for testing potential partners. A surprising aspect to this new finding is the large fraction of transient spines in the adult brain whose dynamics don't have long-lasting impact on circuit structure. On the flip side, this means that many spines in the adult are open to exploring alternative connections, implying a significant capacity for rewiring.

Aside from providing a useful tool for more reliable distinction of permanent versus more transient circuit remodeling, the ability to label and independently track different synaptic components as well as spine dynamics within an intact circuit provides new opportunities for the temporal and molecular dissection of mechanisms that underlie spine and excitatory synapse formation, stabilization, or elimination in vivo. As discussed above, PSD-95 appears to be required for the stabilization of spines and the maturation of excitatory synapses, but in vivo its presence is not a 100% predictive of future spine stability. There are many molecular players that have been implicated in synapse formation and maturation (Krueger-Burg et al., 2017; Krueger et al., 2012; Sala and Segal, 2014; Schreiner et al., 2017), and their roles in this process have yet to be determined.

Spine imaging has always been carried out in the anesthetized mouse due to the importance of stability when imaging small structures at diffraction limited resolution. In the case of events that happen on the scale of days, this is not unreasonable, since the time under anesthesia is usually short (~1 hr) as compared to the awake time between imaging sessions, with the morphological changes mostly occurring when the animal is awake. However, if imaging sessions become more frequent, for example, when examining faster molecular events, the burden of anesthesia may become intolerable to the mouse and there is concern that the dynamics may not be representative of the awake state. In the future, development of faster structural imaging approaches could facilitate molecular studies of synapse formation and elimination by reducing imaging time so that anesthesia is very short, or can be performed in the awake state.

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