

REVIEW

The role of molecular diffusion within dendritic spines in synaptic function

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Spines are tiny nanoscale protrusions from dendrites of neurons. In the cortex and hippocampus, most of the excitatory postsynaptic sites reside in spines. The bulbous spine head is connected to the dendritic shaft by a thin membranous neck. Because the neck is narrow, spine heads are thought to function as biochemically independent signaling compartments. Thus, dynamic changes in the composition, distribution, mobility, conformations, and signaling properties of molecules contained within spines can account for much of the molecular basis of postsynaptic function and regulation. A major factor in controlling these changes is the diffusional properties of proteins within this small compartment. Advances in measurement techniques using fluorescence microscopy now make it possible to measure molecular diffusion within single dendritic spines directly. Here, we review the regulatory mechanisms of diffusion in spines by local intra-spine architecture and discuss their implications for neuronal signaling and synaptic plasticity.

Introduction

Neurons communicate with each other through synapses that organize to create functional circuits. Most excitatory synapses in the central nervous system are formed on dendritic spines, tiny protrusions that extend from dendrites (Bourne and Harris, 2008; Fig. 1 a). The spine typically has a head of 200–1,000-nm diameter, which is connected to the dendritic shaft via a neck of 100–200-nm width (Arellano et al., 2007; Fig. 1 b). The head contains postsynaptic density (PSD) proteins, the actin cytoskeleton, membrane structures, and organelles (Sheng and Hoogenraad, 2007; Fig. 1 c). The molecular composition of spine heads is different from that of the shaft. Because of its characteristic morphology, spines are thought to function as biochemically independent compartments by limiting molecular movement between the spine head and the rest of the dendrite (Adrian et al., 2014; Tønnesen and Nægerl, 2016). Clarifying this regulation is key to understanding how this unitary site of synaptic transmission is controlled. This is particularly crucial to our understanding about how changes in the postsynaptic site lead to synaptic plasticity.

The control of spine architecture is critical at excitatory synapses in the brain (Alvarez and Sabatini, 2007; Forrest et al., 2018). Excitatory synapses exhibit synaptic plasticity, which changes the strength of synaptic transmission through mechanisms at both pre- and postsynaptic sides (Citri and Malenka,

2008). This process is generally thought to be a basis for changes in neural circuits controlled by experiences—i.e., learning and memory (Humeau and Choquet, 2019; Magee and Grienberger, 2020). Here, the size and shape of spines are strongly correlated with the strength of synaptic transmission (Kasai et al., 2010). Spine volume is proportional to PSD area (Harris and Stevens, 1989) and the number of α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptors (AMPARs; Nusser et al., 1998; Matsuzaki et al., 2001). Recently, combinational analysis of electrophysiology and correlative light and EM (CLEM) revealed the linear relationship between PSD area and synaptic strength (Holler et al., 2021). Also, longer spine necks attenuate somatic potentials to a greater degree (Araya et al., 2006). Thus, structural and functional plasticity of spines is tightly regulated. Specifically, when synaptic transmission is strengthened (e.g., long-term potentiation [LTP]), spines grow (Matsuzaki et al., 2004). In turn, when synaptic transmission weakens (e.g., long-term depression), spines shrink (Zhou et al., 2004; Oh et al., 2013).

While many molecules involved in the plasticity of spine synapses have been identified (Sala and Segal, 2014), their mechanisms and regulations can only be discovered by monitoring the regulated changes in the composition and signaling properties of these factors within the confined space of the spine's cytoplasm. To this end, the development of local

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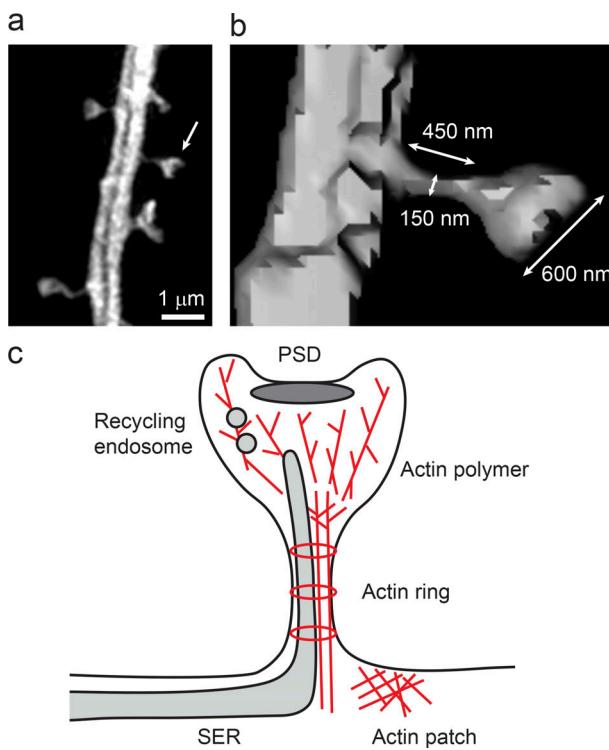


Figure 1. The shape and internal architecture of dendritic spines. (a) A super-resolution SIM image of a hippocampal neuron dendrite expressing GFP. (b) A surface image of a spine (arrow in a) reconstructed from a SIM image. (c) Schematic representation of a spine containing the PSD, actin cytoskeleton, recycling endosome, and SER.

photolysis of caged-glutamate played an important role (Matsuzaki et al., 2001). This method made it possible to induce structural plasticity locally at a single spine. Spine enlargement is induced by uncaging of caged-glutamate in the absence of Mg^{2+} or with postsynaptic depolarization in the presence of Mg^{2+} to activate N-methyl-D-aspartate-type glutamate receptors (NMDARs; Matsuzaki et al., 2004). Conversely, spine shrinkage is induced by low-frequency uncaging of caged-glutamate in the absence of Mg^{2+} or with postsynaptic depolarization (Oh et al., 2013). Shrinkage can also be induced by glutamate uncaging temporally coupled with back propagation action potential and uncaging of caged- γ -aminobutyric acid (GABA; Hayama et al., 2013). Glutamate uncaging-induced structural plasticity has also been seen to occur *in vivo* (Noguchi et al., 2019).

These methods have vastly improved our understanding of the molecular mechanisms of structural plasticity (Nishiyama and Yasuda, 2015). In particular, stimulus-dependent increases in spine size (structural LTP [sLTP]), which are thought to be associated with functional LTP, have been studied extensively as a model of LTP (Nakahata and Yasuda, 2018; Fig. 2). Strong synaptic input causes an influx of Ca^{2+} through NMDARs that activates Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and the downstream signaling cascades. This modification of signaling cascades can affect cytoskeletal organization and membrane trafficking, which are responsible for two subsequent cellular events. First, spine morphology is modulated

through cytoskeletal changes (Borovac et al., 2018). Second, synaptic transmission is enhanced by increased AMPAR insertion into the plasma membrane and movement to the PSD (Huganir and Nicoll, 2013). However, the molecular mechanisms that link these two phenomena are not fully understood (Herring and Nicoll, 2016). As sLTP progresses, the molecular composition within spines changes (Bosch et al., 2014; Meyer et al., 2014). Specifically, immediately after sLTP induction, actin-related molecules such as cofilin and actin-related protein 2/3 (Arp2/3) complex accumulate within a stimulated spine. On the other hand, scaffold proteins such as PSD-95 slowly accumulate over tens of minutes. SynGAP, which is localized at the PSD through interaction with PSD-95, escapes from spines immediately after stimulation and contributes to the expression of sLTP (Araki et al., 2015). These changes in molecular compositions immediately after stimulation may be due not only to molecule-specific binding but also to the physical regulation of diffusion (Obashi et al., 2019).

In addition to molecular localization, fluorescence lifetime imaging of FRET-based biosensors has made it possible to measure spatiotemporal changes in the activity of signaling molecules involved in sLTP (Yasuda, 2012). These studies have demonstrated a critical relationship between the time that a molecule spends within a spine and the rate of signal inactivation. This relationship determines whether an activated signaling molecule is confined within a single spine or escapes from the spine and interacts with effectors present in the adjacent dendritic shaft or nearby spines (Yasuda, 2017). The signal propagation into nearby spines is most likely related to heterosynaptic plasticity, where activated synapses influence neighbor synapses within the same dendritic segments (Oh et al., 2015; Colgan et al., 2018; Chater and Goda, 2021). Thus, diffusion is a central feature of the regulation of spine structural plasticity. However, because the size of spines is small relative to the spatial resolution of diffraction-limited fluorescence microscopy and measuring methods are limited, elucidation of the mechanism regulating diffusion within spines has been challenging.

To address this gap in understanding, researchers advanced fluorescence microscopy techniques, which enabled us to measure changes in the nanoscale localization, diffusion, and signal activities inside spines. These studies allow us to directly understand how spine structures physically limit molecular diffusion and reveal fundamental mechanisms that control the localization and biochemical signal transduction pathways in neurons. Here, we summarize recent findings that have revealed physical barriers within spines using super-resolution microscopy (Sigal et al., 2018; Table 1) and molecular dynamics measurements (Fig. 3 and Table 2), and we discuss how these barriers serve as a fundamental feature controlling neuronal signaling and synaptic plasticity.

Spine structures and diffusion

The complex physical structures of spines can impact the diffusion of molecules inside the spine cytoplasm and between spines and their parent dendritic shafts (Fig. 1c and Fig. 2). For example, consider the diffusional translocation of molecules

Lateral diffusion inside and outside of PSD

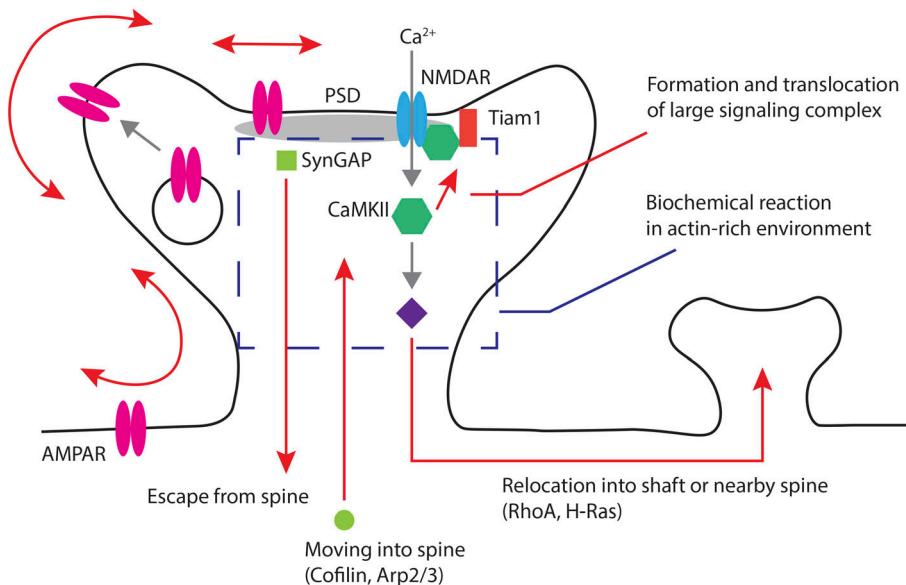


Figure 2. Molecular motion important in sLTP. Strong synaptic input causes an influx of Ca^{2+} through NMDARs that activates CaMKII and the downstream signaling cascades. This modification of signaling cascades can affect cytoskeletal organization and membrane trafficking, which regulate spine morphology. Spine morphology affects the molecular exchange between the spine head and the dendritic shaft and lateral diffusion of membrane proteins including AMPARs. Regulation of molecular movements through the spine neck affects the molecular composition within spines. This change affects signal propagation into nearby spines. For example, cofilin and Arp2/3 complex accumulate within spines. SynGAP and activated RhoA escape from spines. Reorganization of the actin cytoskeleton affects movement of large molecules and the formation of a large signaling complex containing CaMKII and Tiam1. Also, the structure of the PSD affects membrane protein diffusion and alters the synaptic trafficking of AMPARs.

between the PSD and dendritic shaft. For cytosolic proteins, because a spine is connected to the dendritic shaft through a narrow neck, proteins must pass through the neck by diffusion or slow active transport. A spine neck functions as a diffusion barrier because of its narrow width (Svoboda et al., 1996). Molecular complexes with actin filaments and related proteins, such as synaptopodin and ankyrin-G, that maintain this characteristic neck morphology may also affect diffusion. Furthermore, the cytoplasm within spines is likely to be not homogeneous but organized with multiple nanoscale domains with different biophysical properties (Frost et al., 2010a; MacGillavry and Hoogendoorn, 2015). Thus, these locally dense cytoskeletal and membranous structures can limit the molecular path of diffusion within a spine by specific binding interactions or nonspecific local

steric effects. These factors will change the residence time of proteins within spines.

Besides cytosolic proteins, membrane protein diffusion can be regulated by structures on and near the plasma membrane. For example, the cortical cytoskeleton affects the movement of membrane proteins (Kusumi et al., 2012). Furthermore, specialized membrane domains with a high density of membrane-associated structures, such as synaptic contact sites, accumulate many relatively immobile molecules and limit membrane protein diffusion (Trimble and Grinstein, 2015). Lastly, spines are not simply spherical. Boundaries between the spine shaft and neck—and also the spine head and neck—can contain high curvatures. Also, large spine heads contain a concave surface (Kashiwagi et al., 2019). Thus,

Table 1. List of super-resolution microscopy techniques

Technique	Principle	Resolution		Comments	Applications in spines and synapses
		Lateral (XY)	Axial (Z)		
CLSM		250 nm	500 nm		
TPLSM	Two-photon excitation	350 nm	700 nm	Deeper tissue penetration; adaptive optics further improve	Helmchen and Denk, 2005; Ji, 2017
STED	Stimulated emission (Vicidomini et al., 2018)	20–70 nm	500 nm	3-D STED increases axial resolution; chronic <i>in vivo</i> imaging is possible	Nägerl et al., 2008; Berning et al., 2012; Pfeiffer et al., 2018
SIM	Moiré effect with structured illumination (Wu and Shroff, 2018)	100 nm	250 nm	No need for special fluorophores; limited resolution improvement	Kashiwagi et al., 2019; Li et al., 2020
SMLM (PALM, STORM)	Photoactivation, photoconversion (Baddeley and Bewersdorf, 2018)	10–30 nm	30–60 nm	High spatial resolution; temporal resolution is relatively worse	Dani et al., 2010; Tang et al., 2016
ExM	Physical expansion of sample (Wassie et al., 2019)	4–20-fold improvement	4–20-fold improvement	Capable of combining with other imaging techniques, only for fixed samples	Gao et al., 2019; Sarkar et al., 2020 <i>Preprint</i>

CLSM, confocal laser scanning microscopy; SMLM, single-molecule localization microscopy; STORM, stochastic optical reconstruction microscopy; TPLSM, two-photon laser scanning microscopy.

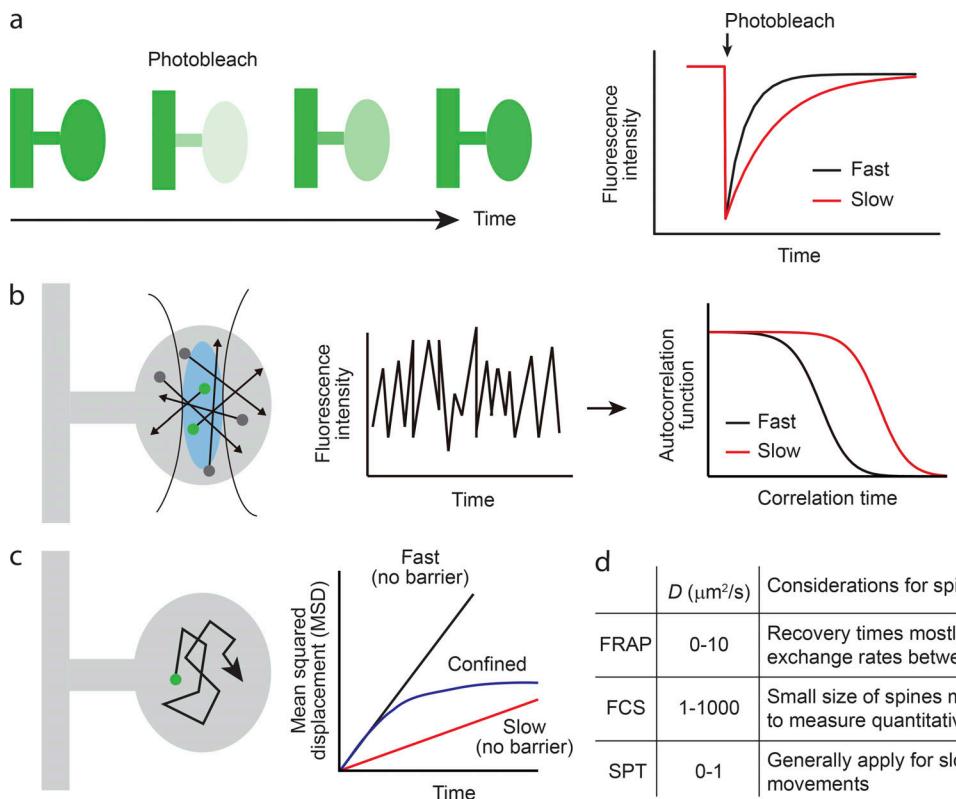


Figure 3. Imaging techniques to measure diffusion inside dendritic spines. (a) FRAP. Fluorescence intensity change is measured after photobleaching fluorescent molecules in a spine head. Fluorescence recovery rate is mostly determined by the exchange rate between spine and dendrite. (b) FCS. The fluctuation of fluorescence intensity from the detection volume fixed inside a spine head (blue region in left panel) is recorded as a function of time (center panel). Since the fluorescence intensity fluctuates as the molecules enter and leave the fixed detection volume, the characteristics of intensity fluctuation essentially contain information about local diffusion speed. To estimate the diffusion coefficient, the autocorrelation function of fluorescence intensity fluctuation is calculated (right panel). (c) SPT. In SPT, molecular trajectory is directly measured with video microscopy. To analyze the speed and pattern of molecular motion, mean squared displacement (MSD) is calculated. For diffusion without barrier, MSD increases linearly against time. On the other hand, for diffusion within the compartment, MSD converges to a certain value, which corresponds to compartment size. (d) Comparison of three measurement techniques.

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local concavities, undulations, and convexities may affect the possible path a molecule can take (Simon et al., 2014; Klaus et al., 2016). From all these factors, the shape and internal architecture of spines can have strong effects on diffusion for both cytosolic and membranous proteins.

Influences of spine morphology on diffusional coupling between spines and dendrites

Although the cytoplasm of spines is directly connected to the cytoplasm of dendritic shafts, a narrow neck is thought to limit diffusion of both cytosolic and membrane molecules between

Table 2. List of fluorescence molecular dynamic measurement techniques

Technique	Principle	Applications in spines and synapses
FRAP	Fluorescent molecules in a small region are photobleached, and subsequent movement of surrounding nonbleached fluorescent molecules into the photobleached area is monitored (Lippincott-Schwartz et al., 2018).	Svoboda et al., 1996; Bloodgood and Sabatini, 2005
FCS	Fluctuation of fluorescence intensity from the detection volume fixed at a specific position is recorded, and a temporal correlation is analyzed (Elson, 2011).	Chen et al., 2015; Obashi et al., 2019
RICS	Spatial correlation is analyzed from raster-scanned images (Digman and Gratton, 2011).	Obashi et al., 2019
SPT	The movement of a single particle is tracked using time-lapse imaging, and a trajectory is made and analyzed. To detect single particles, the density of fluorescence particles should be kept low (Shen et al., 2017).	Borgdorff and Choquet, 2002; Varela et al., 2016
SPT-PALM	Only a small number of photoactivatable fluorescent proteins in the field of view are activated and tracked until they are bleached (Manley et al., 2010).	Frost et al., 2010b; Nair et al., 2013

two compartments (Holcman and Schuss, 2011; Kusters et al., 2013; Ramirez et al., 2015). FRAP is a method that can be used to measure the diffusional speeds from an exchange rate between nonbleached and bleached molecules after bleaching fluorescent molecules in a small region (Lippincott-Schwartz et al., 2018; Fig. 3, a and d). Local photoactivation or photoconversion and subsequent measurements of fluorescence intensity is another technique comparable to FRAP (Bancaud et al., 2010). When fluorescence bleaching is performed in a spine head, the speed of fluorescence recovery mostly reflects the rate of molecular exchange between the head and the connected dendrite. Because the diffusion of small molecules in a head is faster than the rate of molecular exchange between spines and dendrites, the fast component of intra-spine diffusion is more difficult to detect in FRAP recovery curves (Svoboda et al., 1996). FRAP or photoactivation experiments of cytoplasmic and membrane-anchored fluorescent proteins showed that diffusional coupling between spines and dendrites varies between spines (Bloodgood and Sabatini, 2005; Ashby et al., 2006). Since the shape of spines is diverse, it has been proposed that this diversity underlies variability in spine-dendrite coupling. However, because the details of spine morphology cannot be analyzed with the spatial resolution of diffraction-limited fluorescence microscopy, a relationship between the shape of spines and diffusional coupling had not been directly demonstrated.

Recently, however, super-resolution microscopy has made it possible to analyze spine shape in living neurons with a spatial resolution of ~50 nm (Nägerl et al., 2008). Influences of spine morphology on diffusional coupling were verified experimentally for the first time by directly comparing the morphological features of spines and diffusional coupling. This comparison was achieved by stimulated emission depletion (STED) microscopy of spines combined with FRAP of YFP or Alexa dyes applied to the same spine (Takasaki and Sabatini, 2014; Tønnesen et al., 2014). These direct comparisons indicated that the diversity in diffusional couplings could be explained solely by the diversity of spine shapes for more than half of the measured spines. In other words, it was shown that for many spines, the exchange rate (τ) of small molecules within spines could be explained by a single-compartment model (Svoboda et al., 1996) described by the shape of the spine:

$$\tau = \frac{V \times L}{A \times D},$$

where V is the head volume, L is the length of the neck, A is the cross-sectional area of the neck, and D is the diffusion coefficient of molecules. Also, sLTP induction made a spine neck thicker and shorter (Tønnesen et al., 2014). This change in the spine neck complements the decrease in the coupling rate associated with the increase in the spine head volume. This coordinated morphological change appears to maintain molecular concentration in a spine.

Besides the work focusing on cytoplasmic proteins, the influence of spine shape on the diffusional coupling of membrane molecules has also been investigated (Adrian et al., 2017). The spine-dendrite diffusional coupling was tested by photoactivated localization microscopy (PALM) and photoconversion experiments using membrane-anchored mEos3.2 as a probe.

This study showed that even if spines have the same surface area and neck width, the diffusional coupling varies between different spine shapes. Therefore, a model spine was created based on the experimentally measured spine shape parameters, and a simulation was conducted on the model spine and compared with the experiment. As a result, although experimental results tended to provide slower diffusion kinetics than simulation values, experiments showed a good correlation with simulations based on the spine shape parameters alone.

Experiments have confirmed that spine morphology is a major factor determining the diffusional coupling for both cytoplasmic and membrane-bound molecules in dendrites. However, for some spines, the simulated and experimental results diverge. One possibility is that the effects of local intra-spine architectures on molecular diffusion vary for each spine. Another possibility is that there was insufficient spatial resolution for reconstructing the spine morphology. Although the above studies used rotationally symmetric shapes as model spines, actual spines are not rotationally symmetrical structures and generally have a more complicated morphology and surface features (Nägerl et al., 2008; Berning et al., 2012; Kashiwagi et al., 2019; Zaccard et al., 2020; Fig. 1, a and b). Thus, it is possible that estimations of spine shape were insufficient or that the fine structure of spines affects diffusion. In this regard, developing an analysis method for spine morphology from both the experimental and computational sides is key (Okabe, 2020a; Tamada et al., 2020). Recently, Kashiwagi et al. (2019) developed a 3-D structured illumination microscopy (SIM)-based nanoscale analysis of spine morphology. Direct comparison of SIM images and serial-section EM images revealed that the basic morphological features were highly correlated among these images. This indicates the high precision of SIM-based nanoscale spine analysis. To analyze spines computationally, SIM images were converted into a computational geometry, and morphological features were calculated. Then, these features were analyzed by principal component analysis. By mapping the temporal changes of spine morphology obtained by live-cell SIM imaging in the dimension-reduced feature space, the authors revealed that the spine population can be categorized based on different simplified morphological dynamics.

Also, expansion microscopy (ExM) is another new and important imaging technique for spine structural analysis (Wassie et al., 2019). However, it can only be applied to fixed samples. Since ExM samples are transparent, 3-D super-resolution imaging is available for thick samples with large volumes (Gao et al., 2019). With recent developments in sample preparation technology, ExM has the potential to investigate spine morphology and localization of multiple biomolecules and organelles within a single sample (Chozinski et al., 2016; Tillberg et al., 2016; Karagiannis et al., 2019 Preprint; Sun et al., 2021). Minimizing the distortion of isotropy during expansion will be important for nanoscale morphological analysis. In the future, combining dynamic fluorescence measurements and structural measurements gained from EM (CLEM) will be a powerful approach to evaluate the effects of spine ultrastructure on molecular diffusion in greater nanoscale detail (Maco et al., 2013; Taraska, 2015; Luckner et al., 2018).

Along with biochemical compartmentalization, dendritic spines have been proposed to be important for electrical compartmentalization (Yuste, 2013; Araya, 2014; Tønnesen and Nägerl, 2016). Spine morphology, particularly spine neck morphology, is thought to be critical for this effect (Cartailler et al., 2018). Several studies have sought to measure neck resistance based on morphological analysis using EM (Harris and Stevens, 1989; Tamada et al., 2020), super-resolution microscopy (Tønnesen et al., 2014), FRAP of small molecules (Svoboda et al., 1996; Tønnesen et al., 2014), glutamate uncaging (Araya et al., 2006; Takasaki and Sabatini, 2014), calcium imaging (Grunditz et al., 2008; Harnett et al., 2012), voltage imaging (Popovic et al., 2015; Acker et al., 2016; Kwon et al., 2017), and intracellular recordings directly from spine heads (Jayant et al., 2017). However, results were not completely consistent, and the degree of electrical compartmentalization is still unclear. Thus, the relationship between spine morphology and electrical signaling of the synapse is still an open question. Likewise, how morphological changes in the neck induced by LTP affect dendritic computation will be an important area of future study (Araya et al., 2014; Tazerart et al., 2020).

Actin cytoskeleton

The cytoskeleton in spines is primarily composed of actin (Hotulainen and Hoogenraad, 2010; Okabe, 2020b). Actin is present in high densities in both the head and neck regions (Korobova and Svitkina, 2010). Actin polymers are essential in controlling the localization of PSD molecules and in changing and maintaining spine morphology (Frost et al., 2010a; Bertling and Hotulainen, 2017). In addition to these functions, dense actin polymers in spines may regulate synaptic functions by controlling diffusion because the intracellular cytoskeleton and membrane structures influence diffusion (Novak et al., 2009). If this regulation occurs in spines, variations in the distribution of intra-spine structures can be a factor in the large deviations between the measured values of diffusional coupling and the value predicted from models. A ratio of the spine FRAP recovery time of Alexa dyes to that of YFP was comparable to that of hydrodynamic radii (Tønnesen et al., 2014). This suggests that the suppression of diffusion by actin polymers is weak for molecules with the size of GFP. However, suppressive effects on molecular diffusion by the cytoskeleton, such as actin polymers, is dependent on the size of molecules (Baum et al., 2014; Katrukha et al., 2017). Thus, diffusion of larger molecules may be influenced to a greater degree by actin polymers.

Because the shape of spines affects the recovery time of FRAP measurement, it is difficult to investigate the effects of intra-spine structure on molecular diffusion using FRAP alone. Therefore, there is a need for a method capable of measuring diffusion directly in confined spaces. Lu et al. (2014) measured the motion of mEOS2-fused CaMKII α in spines by single-particle tracking (SPT)-PALM. SPT can directly evaluate diffusional speed in spines because it analyzes the molecular movement trajectory of single molecules (Fig. 3, c and d). The SPT measurement showed that CaMKII α exhibited at least three different diffusion modes within spines: (1) a free diffusion component, (2) a component bound to immobile molecules, and (3) a

component moving at an intermediate velocity. Depolymerization of actin polymers by latrunculin A reduced the proportion of molecules with intermediate velocities in spines while concomitantly increasing the free diffusion component. Also, diffusional speeds of CaMKII α were slower and the ratio of the intermediate component was larger in spines than in dendrites. Because the transition between free and bound states would occur rarely during the measurement period due to the slow unbinding rate of CaMKII from actin polymers, transient binding alone does not explain the mechanism for the intermediate velocity. Although the details are unclear, CaMKII motion is restricted by actin polymers through a mechanism distinct from direct binding, including a molecular sieve effect or transient binding to actin-associated molecules.

Obashi et al. (2019) used fluorescence correlation spectroscopy (FCS) and raster image correlation spectroscopy (RICS) to measure the diffusion of biologically inert probes within spines. FCS is a method for estimating diffusion speed from the time taken for fluorescent molecules to pass through the detection volume excited by a high numerical aperture objective and is capable of measuring fast diffusion within a small cellular compartment (Elson, 2011; Fig. 3, b and d). RICS is another method for estimating diffusion speed from the spatial similarity of fluorescence intensity in a scanned image (Digman and Gratton, 2011). Since FCS and RICS are affected by the small size of spines due to the boundary effect, it is not possible to measure the diffusion coefficient accurately (Jiang et al., 2020). Still, by averaging, values proportional to the actual values can be obtained. Diffusion of GFP and GFP tandem pentamer (GFP5) were compared, and only diffusion of GFP5 within spines was enhanced by depolymerizing actin with latrunculin A treatment. Molecular dynamics simulation confirmed that the diffusion of molecules over the size of GFP5 was suppressed by actin polymers with a density (380 μ M) estimated from the values in the literature and experiments.

Together, these experiments support the idea that a mesh-work of dense actin polymers in spines acts as a physical barrier to the diffusion of larger (>100 kD) molecules (Fig. 4 a). Photo-activation experiments of intra-spine photoactivatable GFP (PA-GFP)-actin showed that there are at least three groups of actin polymers with different reorganization rates (Honkura et al., 2008). In addition, experiments with SPT-PALM of PA-GFP-actin showed that a rate of actin filament polymerization increased near PSDs (Frost et al., 2010b). PALM analysis also revealed that actin-related molecules within spines are arranged in a manner specific for each molecule (Chazeau et al., 2014). These results suggest that the diffusional control by actin polymers in spines may differ between each subcompartment.

It was also shown that reorganization of the actin cytoskeleton immediately after sLTP induction (Chazeau and Giannone, 2016; Mikhaylova et al., 2018) enhanced the diffusion of larger molecules within the spine head (Obashi et al., 2019). Further, FRAP experiments showed that diffusional coupling and synaptic translocation of large synaptic molecules, such as CaMKII and T cell lymphoma invasion and metastasis-inducing protein 1 (Tiam1), were facilitated at the initial phase of sLTP. Thus, the reorganization of actin polymers regulates molecular

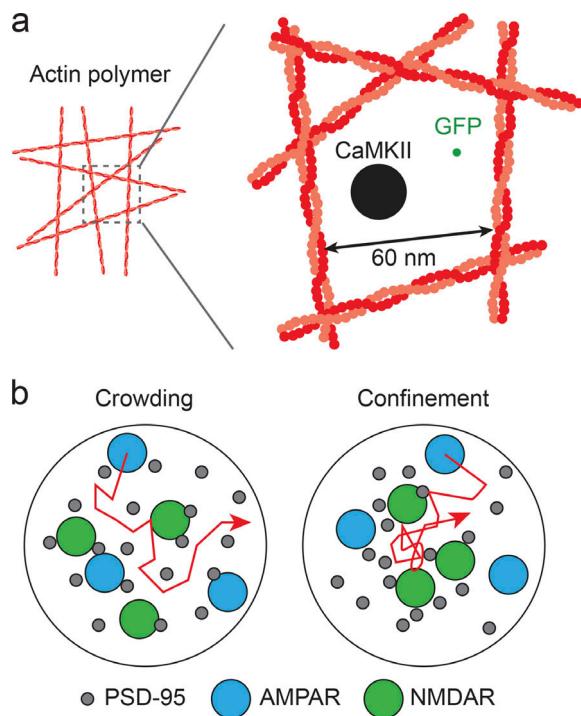


Figure 4. Diffusion within network of actin polymers and PSD. (a) Comparison of the size of actin polymer network and diffusion molecules. Average distance between actin polymers is estimated for actin polymers with 380 μ M (Obashi et al., 2019). GFP is represented as a diameter of 3 nm and CaMKII is represented as a diameter of 20 nm (Myers et al., 2017). (b) A schematic model of AMPAR diffusion within a crowded PSD. Different localization patterns of molecules cause different diffusion patterns. Such mechanisms will occur within the PSD. Density and size of molecules are based on the literature (Okabe, 2007; Li et al., 2016).

translocation between dendrites and the PSD in coordination with morphological changes of the spine neck (Tønnesen et al., 2014). The enhancement of molecular diffusion by actin may also be related to the formation of a large signaling complex containing CaMKII and Tiam1 and may be an important physical mechanism responsible for the initiation of sLTP (Saneyoshi et al., 2019).

Membranous organelles

Along with the cytoskeleton, dendrites also contain many membranous organelles and compartments, and some are present in spines (Bourne and Harris, 2008). Smooth ER (SER) and recycling endosomes are present in <50% of spines. Spine apparatus, which is composed of stacked SER, is present in 10–20% of spines. Localizations of these organelles change after LTP induction, and spines containing SER are larger than those without SER (Chirillo et al., 2019; Kulik et al., 2019; Perez-Alvarez et al., 2020). While mitochondria are abundant in dendritic shafts but rarely present in spines (Wu et al., 2017), synaptic activation relocates mitochondria into spines (Li et al., 2004). Therefore, variations in the diffusional coupling between spines and dendrites could be due to the heterogeneous localization of these organelles (Cugno et al., 2019). Holbro et al. (2009) compared the diffusional coupling of ER-containing

spines and ER-free spines by using an ER-targeted GFP probe. FRAP recovery times of RFP were not different among ER-containing and ER-free spines, indicating that the ER does not block cytoplasmic diffusion between spines and dendritic shafts. Understanding both spine morphology and the volume of ER within spines in the future will clarify the effects of excluded volume by the ER and other organelles in more detail.

Structures around spine necks

Molecules present in spine necks may physically control diffusion by forming a complex higher-order structure. Platinum replica EM showed the presence of Arp2/3 complex within necks and a longitudinal network of branched and linear actin filaments (Korobova and Svitkina, 2010). SPT-PALM of PA-GFP-actin also showed that actin polymers in necks are dynamically reorganized and that they are arranged in many orientations (Frost et al., 2010b). Thus, actin polymers in spine necks may affect molecular diffusion. Synaptopodin, for example, is an actin-binding protein located predominantly in spine necks. It is colocalized with the spine apparatus (Vlachos, 2012). Wang et al. (2016) used SPT to measure metabotropic glutamate receptor 5 (mGluR5) diffusion around necks. They compared the diffusion of mGluR5 around the necks of spines containing (or not containing) synaptopodin. The diffusion of mGluR5 decreases around spine necks near synaptopodin clusters. Further, latrunculin A treatment specifically enhanced the diffusion around spine necks near synaptopodin clusters. These results suggest that synaptopodin regulates the actin polymer network around spine necks. This actin complex can act as a diffusion barrier for membrane proteins.

Another protein that has been implicated in diffusional control of membrane proteins is ankyrin-G. Ankyrin-G forms nanodomain at perisynaptic membranes and in spine necks (Smith et al., 2014). AMPARs accumulated in spines with ankyrin-G clusters and showed slower spine-dendrite coupling. Ankyrin-G is the major cytoskeletal scaffold of the axon initial segment (AIS; Leterrier, 2018). Ankyrin-G and actin scaffolds densely accumulate at the AIS and inhibit diffusion of the membrane and cytoplasmic molecules (Winckler et al., 1999; Nakada et al., 2003; Song et al., 2009). Also, super-resolution microscopy recently revealed the presence of membrane-associated periodic skeleton composed of actin rings, spectrin, and accompanying proteins in the axon including the AIS (Xu et al., 2013; Zhong et al., 2014). At the AIS, the actin rings and associated structures act as a diffusion barrier to membrane proteins (Albrecht et al., 2016). Adding to the axon, membrane-associated periodic skeleton was also observed in the dendrites and spine necks (Bär et al., 2016; Sidenstein et al., 2016). Therefore, it is interesting to postulate that a molecular complex with actin filaments similar to the AIS is also present in spine necks and could regulate molecular diffusion in this small compartment.

Another cytoskeletal component, septin 7, localizes to the base of spines and acts as a diffusion barrier for membrane-bound molecules (Ewers et al., 2014). Recently, actin patches were found at the base of spines and were shown to be remodeled by synaptic activity. These structures modulate

microtubule entry into spines and the transport of lysosomes (Schätzle et al., 2018; van Bommel et al., 2019). It is interesting to ask whether actin patches at spine bases affect molecular diffusion. There are still many unknown features at the spine neck, and how these structures limit the diffusion of cytoplasmic and membrane molecules to control neuronal functions remains unclarified.

Molecular crowding in the PSD

The PSD is a membrane-associated structure containing densely packed postsynaptic molecules (Sheng and Hoogenraad, 2007). It was originally identified as an electron-dense structure in EM (Okabe, 2007). The number and location of receptors and adhesion molecules in PSDs are directly related to synaptic function (Chen et al., 2018). SPT studies indicate that AMPARs diffuse laterally into and out of PSDs and regulate synaptic function by controlling the number and location of AMPARs (Choquet and Hosy, 2020). Because there are many scaffold proteins in PSDs, membrane proteins including AMPARs accumulate in PSDs due to intermolecular binding. Furthermore, because the molecular density in PSDs is high, the accumulation of membrane proteins may be regulated by the suppression of mobility within the PSD and molecular exchange at the boundary of PSDs (Gerrow and Triller, 2010; Kokolaki et al., 2020).

To check this possibility, Li et al. (2016) combined FRAP, SPT, and Monte Carlo simulation to investigate the effect of molecular crowding of PSDs on the lateral diffusion of membrane molecules. When the intracellular domain size of membrane proteins was large, diffusion within the PSD and the exchange rate between the inside and outside of the PSD decreased. Super-resolution microscopy showed that the distribution of PSD-95, a major scaffolding protein of the PSD, within PSDs is not uniform (Fukata et al., 2013; MacGillavry et al., 2013; Nair et al., 2013; Broadhead et al., 2016; Gwosch et al., 2020). Interestingly, the simulation showed that the residence time of membrane proteins within PSDs was longer in the condition of experimentally measured PSD-95 distribution, while the residence time decreased with a random distribution of PSD-95 (Li et al., 2016).

Recently, the shape of PSDs inside spines induced by sLTP was analyzed by CLEM (Sun et al., 2019 Preprint). It was shown that rearrangements of PSD shape occurred immediately after induction of sLTP (<3 min), and the PSD took more complex morphology. This increased structural complexity persisted in the late phase (120 min). PSD size and the accumulation of PSD-95 increased slowly over several tens of minutes after sLTP induction (Meyer et al., 2014), whereas synaptic transmission efficiency increased immediately (Matsuzaki et al., 2004). This difference in time may be explained by a mechanism in which the acute ultrastructural changes of the PSD without net growth of the molecular assembly alter the mobility of AMPARs by changing the distribution of a physical barrier, leading to alternations in the number and localization of AMPARs (Fig. 4 b). In future studies, it will be necessary to clarify how coordination between intermolecular binding and physical diffusion barriers in PSDs supports both acute accumulation of AMPARs and their subsequent stabilization in stimulated spines. Further, 3-D SIM

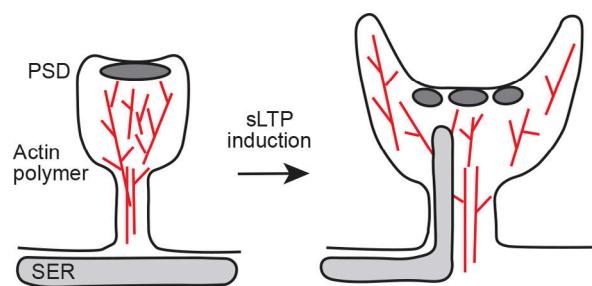


Figure 5. Changes in the shape and internal architecture of spines after induction of sLTP. At the initial phase of sLTP, a spine head expands. In addition, the spine neck becomes wider and shorter (Tønnesen et al., 2014), and a concave surface area of spine head is increased (Kashiwagi et al., 2019). The actin polymer network is reorganized (Obashi et al., 2019), and the SER visits within a spine transiently (Perez-Alvarez et al., 2020). Also, PSD shape becomes more complex (Sun et al., 2019 Preprint). These physical changes should occur in concert and will affect molecular composition and biochemical signaling through diffusional regulation. These physical changes will act as a precise temporal switch of sLTP induction.

imaging revealed that the concave surface of the spine head, which interacts with presynaptic membranes, is enlarged and stabilized by sLTP induction (Kashiwagi et al., 2019). In the future, it will be interesting to determine the relationships between concave membrane surfaces, PSD morphologies, and the dynamics of receptors and adhesion molecules at single spines.

In addition, although AMPAR has been thought to be present as a tetramer (Greger et al., 2007), recent observations of SPT have shown that the majority of diffusive AMPARs are monomers or dimers (Morise et al., 2019). Molecular diffusion in the monomer form increases an exchange rate between the inside and the outside of PSDs, making it possible to efficiently change the AMPAR composition within synapses. It remains to be seen whether other molecular complexes, such as NMDARs and cell adhesion molecules, also modulate their diffusion within the molecularly dense PSD by changing their oligomeric state.

Conclusion and outlook

Here, we have highlighted key recent findings on the relationship between molecular diffusion and physical barriers within spines. The regulation of molecular diffusion is important for sLTP expression (Fig. 2). Spine structural changes during sLTP will affect synaptic function in a coordinated manner (Fig. 5). For example, after sLTP induction, the actin network is reorganized and diffusion of large molecules is enhanced (Obashi et al., 2019). This facilitates the formation of large signaling complexes and the rearrangement of protein complexes within spines. At the same time, spine necks become wider and shorter, and spine heads enlarge (Tønnesen et al., 2014). Changes in actin and spine morphology enhance the molecular movement between the PSD and the shaft and are important for the relocation of proteins (Fig. 2). These structural changes occur in the early phase of sLTP. Thus, the cooperative regulation of diffusion might act as a precise temporal switch of sLTP induction. Also, this enhancement of molecular exchange affects the relocation of activated signaling molecules into the shaft or nearby synapses, which leads to heterosynaptic plasticity (Yasuda, 2017).

Potentiation of synaptic transmission requires synaptic trafficking of AMPARs (Choquet and Hosy, 2020). Although both spine morphology (Adrian et al., 2017) and PSD structure (Li et al., 2016) affect membrane protein diffusion, how structural changes associated with sLTP induction affect diffusion will be clarified in the future. Furthermore, the effects of transient SER visits (Perez-Alvarez et al., 2020) and structural changes around spine necks are an important area for future work. Although the relationship between structure and diffusion in sLTP is critical, the difficulty of measurements with a small single spine has made a comprehensive view difficult to obtain. Thus, future work will be necessary to clarify how structural changes affect diffusion and how this physical change to dendritic spines cooperatively modulates synaptic functions.

Although new imaging techniques have demonstrated the connection between diffusion and physical barriers, little is known about how changes in the movement of molecules alter synaptic functions (Reshetniak et al., 2020b). Because of the small volume of the spine, very small molecules with high diffusivity, such as Ca^{2+} , are expected to spread rapidly ($\sim 1 \text{ ms}$) by diffusion (Chen and Sabatini, 2012). For large molecules such as signaling complexes, it remains to be seen whether spatially uniform diffusion takes place or whether local heterogeneity in the spine cytoplasm results in a more complex pattern of diffusion. It is also necessary to clarify whether such changes affect local biochemical signaling events and molecular localizations. The number of molecules per spine could influence the magnitude of functional changes (Okabe, 2007; Ribrault et al., 2011). Furthermore, the changes in diffusion induced by alterations in spine structure will affect the stability of the structure. This will subsequently change the molecule's diffusivity. Thus, it will be interesting to investigate whether this type of mutual relationship exists within spines.

New imaging techniques will help to answer these questions. By applying fast 3-D SPT to intra-spine measurements, it will be possible to investigate the spatial heterogeneity of diffusion in single spines of living neurons in detail (Hou et al., 2020; Xiang et al., 2020). STED-FCS/fluorescence cross-correlation spectroscopy can also detect changes in intermolecular interactions (Lanzanò et al., 2017). In addition to the development of new measurement techniques, molecular dynamics simulations based on experimental data will become increasingly important in the future (Okabe, 2020a; Reshetniak et al., 2020a; Vasan et al., 2020). Spine morphology and intra-spine structures, which affect diffusion, are closely related. Thus, it is difficult to investigate the effect of one without changing the other experimentally. Molecular dynamics simulation is a useful tool to examine how molecular motion is adjusted by combining elements that are difficult to verify experimentally (Bell et al., 2019). Furthermore, the shape of spines and intra-spine components, such as the actin cytoskeleton, which are the structural basis of spines, differ from spine to spine. Here, a combination of quantitative measurements and simulations based on experimental data will help us to understand molecular events more quantitatively.

Although we reviewed work using fluorescence microscopy, details of spine morphology and intra-spine structures have also been revealed by EM at the nanoscale (Bourne and Harris, 2012;

Tao et al., 2018). However, it is difficult to observe specific molecular localizations with EM. On the other hand, super-resolution microscopy is suitable for obtaining a nanoscale picture of molecular positions within spines. Yet, it is still difficult to observe dense structures such as actin polymers (Kommaddi et al., 2018). Therefore, in the future, it will be essential to combine the advantages of each technique, observing internal structures at the nanoscale using EM and measuring molecular localization with super-resolution microscopy (CLEM; Taraska, 2019; Hoffman et al., 2020). Of course, dynamic intracellular structures such as lipid rafts and biomolecular condensates are also likely to affect molecular mobility (Sezgin et al., 2017; Chen et al., 2020). Thus, it will be key to overlay molecular mobilities from living cells over the static structural information of CLEM. We believe that combinations of multiple imaging modalities, along with modeling, will allow for a more in-depth understanding of synapses at the molecular level. These data will reveal how the elaborate architecture, density, and compartmentalization of subcellular components influence the highly tuned, dynamic, and changeable actions of synapses in the brain.

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