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Postsynaptic signaling during plasticity of dendritic spines

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Abstract

Dendritic spines, small bulbous postsynaptic compartments emanating from neuronal dendrites, have been thought to serve as basic units of memory storage. Despite their small size (~0.1 femtoliter), thousands of species of proteins exist in the spine, including receptors, channels, scaffolding proteins and signaling enzymes. Biochemical signaling mediated by these molecules leads to morphological and functional plasticity of dendritic spines, and ultimately learning and memory in the brain. Here, we review new insights into the mechanisms underlying spine plasticity brought about by recent advances in imaging techniques to monitor molecular events in single dendritic spines. The activity of each protein displays a specific spatiotemporal pattern, coordinating downstream events at different microdomains to change the function and morphology of dendritic spines.

Introduction

In the central nervous system, most excitatory postsynaptic terminals reside in dendritic spines. A mature spine forms a mushroom-shaped structure: a small spherical head (~0.5 μm in diameter) connected to the dendrite through a thin neck (~0.1 μm in diameter) [1]. The neck limits the diffusion of cytoplasmic and membrane molecules in and out of the spine head [2–5]. Elevation of Ca^{2+} concentration in spines (~ micromolars [4, 5]) initiates biochemical signal transduction that leads to the expression of various forms of synaptic plasticity, including long-term potentiation (LTP) and depression (LTD) [6]. At Schaffer Collateral synapses in the hippocampus, synaptic plasticity is associated with morphological plasticity of dendritic spines: spines display long-term enlargement [7–9] and shrinkage [10] during LTP and LTD, respectively. Signaling involved in LTP and the associated spine enlargement in these synapses has been especially well studied as a prominent memory model. It has been revealed that LTP is caused by a combination of many postsynaptic processes coordinated in time and space, including reorganization of actin cytoskeleton, exocytosis from endosomes and insertion of AMPA receptors (AMPA receptors) into synapses [11, 12]. In turn, these events lead to an increase in the sensitivity of postsynaptic sites to glutamate [11–13] or increasing the release probability from the presynaptic terminal [14–16]. Depending on the stimulation paradigm, LTP and associated spine enlargement can be maintained for more than several hours [17, 18]. This form of LTP requires synthesis of new

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proteins [17–20]. Signaling mechanisms regulating these events have also been extensively studied, and tens of signaling proteins have been identified to be important for LTP [21].

Recent progress in imaging techniques has enabled molecular events at the level of the single-synapse to be visualized, and such studies have provided new insights into the molecular mechanisms underlying LTP and associated spine enlargement. In this review, we summarize recent findings that have revealed the spatiotemporal dynamics of molecular processes that occur in dendritic spines during the initial ~30 min of morphological and functional plasticity.

Molecular reorganization in spines during LTP

During LTP induction in Schaffer Collateral synapses in response to repetitive uncaging of caged-glutamate [7], high frequency electrical stimulation[7] or theta-burst electrical stimulation [19], spine morphology has been shown to dramatically change, growing over 2–5 fold in size within ~1 min (Figure 1). This is followed by a decay in volume over the next few minutes, followed by stabilization (for more than 1 hr) at a volume 1.5–2 times as large as the original volume [7, 19]. Recent imaging studies have revealed some of the mechanisms of this amazingly dynamic process, and it has become clear that the induction of LTP and spine enlargement require many cellular events that regulate the actin cytoskeleton, membrane, and postsynaptic density (PSD) within ~1 min, perhaps reorganizing the whole spine structure.

Reorganization of the actin cytoskeleton

The actin cytoskeleton plays an essential role in sustaining and modulating the morphology of spines [12, 22]. In spines, actin filaments undergo rapid treadmilling by adding actin monomer at one end (barbed end) and depolymerizing at the other end (pointed end) [23]. The dynamics of actin treadmilling in dendrites and spines has been studied by measuring fluorescence recovery after photobleaching (FRAP) of green fluorescent protein (GFP)-tagged actin monomer [23] or fluorescence decay after photoactivation of photoactivatable GFP (paGFP)-tagged actin [24]. These studies revealed that the treadmilling process resulted in an exchange between the actin monomers in the spines with those in dendritic shaft within 1 min (dynamic pool). In addition, there is a stable pool that is not exchanged for many minutes [23, 24]. The actin treadmilling produces a net flow of actin monomer from the tip toward the neck of the spine [24]. Single particle tracking of individual actin monomers revealed that the orientation of actin filaments is not well-regulated in spines: each actin monomer moves in all directions, but net ensemble flow is from the tip to the neck [25, 26]. Consistent with these data, direct imaging of actin filaments in spines using platinum replica electron microscopy (EM) revealed that actin filaments are not oriented regularly, but rather appear like that of tangled yarn [27].

During spine enlargement, rapid actin polymerization perhaps provides mechanical force required for pushing out the membrane of the stimulated spine [7, 8]. An imaging study using fluorescence resonance energy transfer (FRET) between yellow fluorescent protein (YFP)-actin and cyan fluorescent protein (CFP)-actin also supported this idea: the filamentous (F-) / monomeric (G-) actin equilibrium rapidly shifts to F-actin within ~5 min of LTP induction, and the change is maintained for more than 30 min [8]. Also, an analysis of paGFP-actin displayed that the stable pool at the spine neck grows within ~2 min and is stabilized over an hour [24].

Reorganization of PSD proteins

Recent technical advances have dramatically improved our understanding of the architecture of the postsynaptic density (PSD) and its regulation during LTP. The Proteomic analyses

have identified hundreds of proteins in the PSD (reviewed in [28]). Further, EM tomography reconstruction has enabled the visualization of non-labeled molecules in the PSD directly, and revealed that PSD95, the major PSD scaffold, forms vertically oriented filaments against the membrane, linked by unknown horizontal filaments [29, 30]. By using super-resolution optical imaging, the precise location of proteins in the PSD, as well as presynaptic terminals, has been measured [31]. Live imaging using GFP-tagged PSD95 has revealed that the shape of the PSD is not static, but is constantly changing on a timescale of minutes [32]. This morphological change is actin-dependent [32], suggesting that actin reorganization during LTP may have an impact on the conformation changes that occur at the PSD.

The dynamics of PSD proteins in single spines during LTP has been imaged using paGFP-tagged PSD95 and Shank [33]. Tagged proteins were photoactivated in a single spine, and the movement of these molecules was monitored following 2-photon glutamate uncaging at the same spine. Under basal conditions, these molecules stayed in the spine for more than ~30 min. Upon uncaging, however, both proteins rapidly diffused out of the spine and were exchanged by non-photoactivated proteins. The phosphorylation of PSD95 at Serine 73 by Ca^{2+} /Calmodulin-dependent kinase II (CaMKII) was found to be responsible for its dissociation from the PSD [33].

Surprisingly, the number of labeled proteins (ie. PSD95 and Shank) within the PSD were not changed during spine enlargement, even after ~30 min of stimulation [33]. This is in sharp contrast to CaMKII [34–36] and AMPARs [37–39], of which enrichment occurs at the same time and to the same degree with the volume change. Because the size of the PSD and spine volume are well correlated under basal conditions [40], PSD size may increase at a much later time-point. Consistent with this view, it has been reported that PSD95 enrichment in newly formed spines occurs many hours after spine formation in slices [41, 42] and *in vivo* [43]. In contrast to the decoupling in the timing of spine volume and PSD size increases during LTP and spinogenesis, PSD shrinkage and loss occurs at the same time (within ~1 min) with spine shrinkage and loss of spines [44].

Endosome trafficking and AMPAR insertion

Spine enlargement during LTP requires the addition of membrane area to the spine. This may be done by diffusion of excess membrane from the dendrite [45] or exocytosis of endosomes [9]. Ultrastructural studies have shown that some spines contain a relatively large fraction of internal membrane in endosomes [9]. Further, inhibition of postsynaptic exocytosis inhibits spine enlargement and LTP [9, 19, 46–48]. These results suggest that the additional membrane required for spine enlargement may come from exocytosis. The exocytosis of endosomes is also important for providing AMPARs to the surface of the spine, which is an important process of LTP [11, 13].

Recently, a technique to image individual exocytosis using a superecliptic pHluorin (SEP)-tagged GluA1 AMPAR subunit, or transferrin receptor, has been developed. SEP fluorescence is quenched in acidic conditions within endosomes and is de-quenched by exocytosis [9, 37, 49]. Thus, by pre-bleaching the existing surface SEP-tagged receptors, one can image exocytosis of single endosomes [38, 39, 48, 50–52]. It has been shown that chemically-induced LTP increases the rate of exocytosis of GluA1-containing endosomes in spines and dendrites [38, 48, 50]. Further, during LTP induced in a single dendritic spine with 2-photon glutamate uncaging, the rate of exocytosis of GluA1-containing endosomes was observed to increase in the stimulated spine as well as in the adjacent dendrite within 5 μm of the stimulated spine [38, 39]. Due to the lateral diffusion of AMPARs into the stimulated spine as well as direct exocytosis, the total number of AMPARs in the stimulated spines increased within ~1 min following LTP induction [38, 39]. The recruitment of AMPARs into the PSD requires CaMKII-dependent phosphorylation of stargazin, an

auxiliary subunit of AMPARs, and subsequent trapping of phospho-stargazin within the PSD [53–55].

The molecular machinery involved in the endosome trafficking during LTP has also been extensively studied. Two subtypes of soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins have been identified as being important for plasticity: syntaxin 13 [46], which directs traffic from early endosomes to the recycling endosome, and syntaxin 4 [48], which is involved in exocytosis at the plasma membrane. Rab small GTPases [9, 46, 56] and the motor proteins Myosin Va [57] and Vb [58] have also been shown to be important for regulating endosome trafficking during LTP.

Spatiotemporal activation of signaling molecules during plasticity of single dendritic spines

The recent development of 2-photon fluorescence lifetime imaging microscopy (2pFLIM) and new FRET sensors (Box 1) has enabled the visualization of signaling activity triggered by Ca^{2+} elevation in single dendritic spines. This has revealed the detailed signaling processes linking Ca^{2+} and molecular reorganization during LTP. We discuss such findings for Ca^{2+} and a number of downstream signaling molecules in the following sections.

Ca^{2+}

Development of 2-photon glutamate uncaging combined with 2-photon Ca^{2+} imaging has greatly facilitated the study of Ca^{2+} signals in dendritic spines [5, 59, 60]. Glutamate uncaging at a dendritic spine with a 2-photon laser can activate glutamate receptors on the spine with kinetics and amplitude similar to those evoked by presynaptic glutamate release [61]. When the Mg^{2+} block of NMDA receptors (NMDARs) is released by removing extracellular Mg^{2+} or depolarizing the neuron, glutamate uncaging can produce Ca^{2+} elevation to the micromolar level in the stimulated spine (Figure 1) [5, 36]. The Ca^{2+} elevation lasts only ~ 0.1 s, and is largely restricted to the stimulated spine [5, 36]. By repeating glutamate uncaging (0.5–2 Hz, 30–60 pulses), LTP and associated spine enlargement can be induced in the stimulated spine but not in adjacent spines (Figure 1a) [7]. During this process, Ca^{2+} elevations show a train of Ca^{2+} transients [36] (Figure 1b).

CaMKII

CaMKII is one of the most abundant proteins in the PSD [62], and is required for hippocampal LTP and some forms of learning and memory [63]. A holoenzyme of CaMKII consists of 12 subunits (mainly α and β in spines [62]), arranged in two hexameric rings [64]. When $[\text{Ca}^{2+}]$ increases, Ca^{2+} binds to calmodulin, and Ca^{2+} /calmodulin binds to a CaMKII subunit [63, 64]. This causes a conformational change of CaMKII to expose its kinase site, activating the subunit. When two adjacent subunits are activated, they undergo transautophosphorylation at Thr-286 [63, 64], which enable the subunits to maintain its activity even after calmodulin dissociation [63]. It has been hypothesized that this “autonomous” CaMKII activity persists for more than hours [65, 66], and may act as a biochemical memory to maintain LTP [67].

CaMKII activity during LTP was imaged using 2pFLIM in combination with a FRET based CaMKII sensor (Box 1) [36]. When a single spine is stimulated with 2-photon glutamate uncaging to induce LTP, CaMKII is rapidly activated within ~ 10 s in the stimulated spine, displaying a pattern that is restricted to the spine [36]. Contrary to the theory of persistent CaMKII activity, the activity decayed rapidly after cessation of uncaging with time constants of 6 s and 45 s (Figure 1d). Interestingly, when the autophosphorylation site was mutated (T286A), the activity completely returned to the basal state within a second and

thus failed to accumulate its activity during repetitive glutamate uncaging (Figure 1b) [36]. These results suggest that T286 phosphorylation is required for sustaining the activity of CaMKII for the time scale of seconds, not hours, and thus for the integration of the short (~ 0.1 s) Ca^{2+} elevation (Figure 1b). However, this experiment does not discard the possibility that a small fraction of CaMKII (for example, a pool of molecules bound to NMDARs [68, 69]) have persistent kinase activity.

During the short CaMKII activation, CaMKII may phosphorylate PSD95 and stargazin, leading to PSD disassembly and AMPAR confinement within the PSD, respectively [33, 53] (but see [70]) (Figure 2). In addition, CaMKII activation may directly regulate actin organization. Because CaMKII β can bind to actin filaments, dodecameric holoenzyme can bundle actin filaments to stabilize spine structure [71]. When CaMKII β is activated, it dissociates from actin filaments, thereby losing the ability to bundle actin filaments [71]. Thus, CaMKII activation during the induction of LTP may destabilize actin, allowing later actin extension [71]. CaMKII β knockout mice displayed deficits in LTP and learning while kinase-dead knock-in (R303A) mice displayed normal LTP and learning behavior [72], further supporting the idea that CaMKII β mainly plays a structural role rather than an enzymatic role.

Ras

One of the CaMKII downstream targets is Rat sarcoma (Ras) [73]. Ras was initially identified as an oncogene protein, and the function of Ras signaling in cell growth, division and survival has been extensively studied [74]. In neurons, Ras is known to be important for regulating various forms of neuronal plasticity and adaptation, including synapse formation, spine morphological plasticity, plasticity of neuronal excitability, dendritic protein synthesis, and gene transcription [73, 75]. Ras is active when bound to GTP, and inactive when bound to GDP [74]. This GTP-GDP cycle is regulated via the interaction with GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) [74]. GAPs promote the hydrolysis of GTP to GDP to inactivate Ras, whereas GEFs promote the exchange of GDP for GTP to activate Ras [74].

The activity of Harvey Ras (HRas), a major subtype of Ras, during LTP has been imaged with 2pFLIM [76, 77] (Box1). When LTP was induced in a single spine, Ras activity increased at the stimulated spine in ~ 1 min, and spread along its parent dendritic shaft over ~ 10 μm [77]. Ras activity in surrounding spines was $\sim 70\%$ as high as that of the stimulated spines, but still was not sufficient for inducing plasticity. The activation of HRas was partially inhibited by the CaMKII inhibitor KN-62, confirming that HRas is downstream of CaMKII [73, 77]. The link between CaMKII and Ras is not clear, but it has been suggested that CaMKII regulates activity of some GEFs and GAPs [78]. Overexpression of dominant negative HRas or inhibition of downstream extracellular signal-regulated kinase (ERK) inhibits LTP and spine enlargement [73, 77]. Thus, these studies suggest that the Ras-ERK pathway is required, but not sufficient, for inducing morphological plasticity (Figure 2).

The spatiotemporal pattern of HRas activity resembles that of activity-dependent AMPAR exocytosis [38, 39, 77]. Further, the activity-dependent increase of AMPAR exocytosis was found to be dependent on the Ras-ERK pathway [39]. Thus, the spreading of Ras signaling seems to be important for producing a similar pattern of exocytosis events (Figure 2). In addition, the spreading of Ras was found to be important for the priming of LTP: when LTP is induced in a spine, LTP can be induced in neighboring spines with weak stimuli that usually do not induce LTP [77] (Figure 2).

Rho and Cdc42

The Rho family GTPases, including Ras homolog (Rho), Cell division cycle 42 (Cdc42) and Ras-related C3 botulinum toxin substrate (Rac), are close relatives of Ras, and are key players in regulating the actin cytoskeleton [74, 79, 80]. Like Ras, the activity of Rho proteins is regulated by a GTP-GDP cycle caused by GEFs and GAPs [74]. Rho is also regulated by Rho GDP-dissociation inhibitor (GDI), which binds to inactive Rho GTPases and controls the interaction of Rho with membranes [74]. The function of these proteins for actin-mediated cell morphological changes [81], migration [82] and polarization [80] has been well characterized in non-neuronal cells. In neurons, Rho GTPases are known to be important for regulating spine morphology: in general it is thought that Rho activation causes spine loss and shrinkage by inhibiting actin polymerization, while Cdc42 and Rac activation increases the number of spines by promoting actin polymerization [83].

To image Rho activity in single dendritic spines undergoing structural plasticity, FRET-FLIM sensors for RhoA, a major subtype of Rho in neurons, and Cdc42 have been developed using a design similar to the Ras sensor (Box 1) [84]. Upon single-spine stimulation with 2-photon glutamate uncaging, the activity of RhoA and Cdc42 increased rapidly in the stimulated spines within ~1 min, and decayed over 3–5 min (Figure 1) [84]. This transient activity was followed by a sustained activation lasting more than ~30 min (Figure 2). Although RhoA and Cdc42 were similarly mobile, the spatial patterns of RhoA and Cdc42 signaling were different: RhoA activity diffused out of stimulated spines, and spread along their parent dendritic shafts over ~5 μ m, while Cdc42 activity was restricted to the stimulated spines (Figure 2) [84, 85]. Like Ras, both Cdc42 and RhoA activation were partially inhibited by inhibiting CaMKII signaling, suggesting that these molecules are also downstream of CaMKII [84].

Activation of Cdc42 and RhoA during LTP is perhaps important for regulating actin organization [83]. Inhibition of Rho or its downstream Rho kinase (ROCK) preferentially inhibited the transient phase of structural plasticity, while inhibition of Cdc42 or downstream p21-activated kinase (PAK) inhibits the maintenance of spine enlargement [84] (Figure 2). The activation and requirement of Rho signaling in the initial phase of the spine enlargement may be surprising, as previous studies have suggested that Rho activation caused spine loss and shrinkage (reviewed in [83]). However, considering the extreme stability of the basal actin structure, Rho activation may be important for disassembling the actin network, allowing the later growth and restabilization of the network in a larger form.

The sustained activation of Cdc42 and RhoA (Figure 1c) suggests that actin polymerization is continuously regulated during the sustained phase of LTP (Figure 2). Consistent with this hypothesis, partial inhibition of actin polymerization with low concentrations of Latrunculin A or cytochalasin B/D inhibits the maintenance of morphological plasticity [7] and LTP [86, 87]. Also, FRET imaging of the F-/G-actin ratio shows a long-term shift in the equilibration toward F-actin [8].

Spatiotemporal signal integration during morphological plasticity

Aligning various signals on multiple time scales (Figure 1b–d), we can now visualize how the short Ca^{2+} signaling, which lasts only ~0.1 s, can be relayed to long-lasting changes in spines. First, the initial Ca^{2+} signal is integrated by CaMKII activation over seconds to ~1 min. Subsequently, small GTPase proteins including Cdc42 and RhoA are activated by CaMKII, which expands the time scale to tens of minutes. Following the peak of small GTPase activity, spine enlargement peaks at ~2 min. RhoA and Cdc42 display sustained activity similarly to the spine volume change (Figure 1b–c).

CaMKII, RhoA, Cdc42, and HRas during these processes also display various length constants in their activity profile (Figure 1, 2) [85]. While Ca^{2+} elevation and CaMKII and Cdc42 activities are compartmentalized in the stimulated spine, HRas and RhoA activities spread out of the stimulated spine over $\sim 5 \mu\text{m}$, invading the adjacent dendrites and spines. In particular, Cdc42 activity is spine-specific and lasts more than 30 min, suggesting that Ca^{2+} -CaMKII-Cdc42 signaling constitutes spine-specific signaling spanning from 0.1 s to more than 30 min (**green in Figure 2**). Other signaling molecules, such as activated RhoA and HRas, spread into the dendrite from the stimulated spine. These spreading signals are important for heterosynaptic metaplasticity, such as priming of LTP in adjacent spines [18, 77, 88] and spine formation in the adjacent dendrite [89].

The mechanisms to produce localized activity of signaling proteins in dendrites have been reviewed elsewhere in detail [85, 90]. Basically, the degree of compartmentalization can be determined by the distance a molecule can diffuse before it is inactivated. In particular, small GTPase proteins HRas, RhoA and Cdc42 have similar structure and mobility, yet they have very different spatial patterns (Figure 2), providing the basis to mathematically model the spatial spreading of molecules. Using a simple model in which a molecule is activated in a spine, and inactivated by GAPs homogeneously distributed in the dendritic shaft, the spatial profile of small GTPase proteins was reproduced *in silico* (Box 2). This local excitation-global inhibition mechanism was also proposed as the mechanism for producing the spatial gradient of intracellular signaling in other systems such as chemotaxis of the amoeba *Dictyostelium*, as well as chemotaxis of neutrophils in the mammalian immune system (reviewed in [91]).

Conclusions

Recent studies that have utilized a variety of new imaging techniques have provided us with a more detailed understanding of the molecular processes of synaptic plasticity. In particular, imaging signal transduction with 2pFLIM has enabled the visualization of how signaling cascades temporally integrate signals in single dendritic spines from ~ 0.1 s to several tens of minutes (Figure 2), as well as how spatially distinct events are orchestrated (Figure 1 and 2). Since actual signaling networks contain hundreds of components, the monitoring of more nodes of the networks and the simultaneous observations of the activity of a few kind of molecules, will achieve a more complete view of signal transduction in spines (Box 3). In addition to imaging methods, the manipulation of a signaling node with photo-activatable proteins [92–95] will also provide more information of the function of signaling networks in single dendritic spines. A better understanding of single spine dynamics may ultimately help to understand how memories are encoded at the cellular level.

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Box 1. Visualizing molecular interactions with 2-photon fluorescence lifetime imaging microscopy (2pFLIM)

Fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM)

FRET is the distance/orientation-sensitive phenomenon that occurs between two fluorophores due to dipole-dipole interaction. Since FRET efficiency decays steeply as the distance between fluorophores (donor and acceptor) increases over nanometers, it can be used as a molecular-ruler to detect the conformational changes or interaction between proteins tagged with fluorophores [96–98]. Fluorescence lifetime, the time elapsed between fluorophore excitation and photon emission, is a sensitive and quantitative measure of FRET [96]. The fluorescence lifetime can be measured as the time constant of fluorescence decay (nanoseconds) after excitation with a short laser pulse (< 0.1 ns). Usually, free donor shows mono-exponential decay, and this decay rate is accelerated by FRET. When multiple populations, for example non-FRET and FRET populations, co-exist, the fluorescence lifetime decays in a multi-exponential manner. Thus, one can deconvolve the fraction of donor interacting with acceptor. Compared to other readouts based on the wavelength shift (for example, ratiometric FRET imaging), the obtained value is more robust against the local concentration change in the donor-to-acceptor ratio or wavelength-dependent light scattering [96, 99] (Figure 1a).

Overview of FRET sensor optimized for FLIM

The FRET sensor for FLIM needs to be optimized in a manner different from other imaging techniques. First, a combination of the bright donor and dim acceptor provides better signal-to-noise ratio, as FLIM uses only donor fluorescence. Second, donor with mono-exponential fluorescence decay is preferable for calculation of the fraction of donor bound to acceptor. Although the enhanced cyan fluorescent protein (ECFP)-enhanced yellow fluorescent protein (EYFP) pair is the most popular pair for the ratiometric FRET imaging, they are not an optimum pair for FLIM [98, 100]. This is because CFP-YFP is a dim donor-bright acceptor pair and CFP fluorescence decays in a multi-exponential manner. The enhanced green fluorescent protein (EGFP)-monomeric red fluorescent protein (mRFP), EGFP-monomeric Cherry (mCherry) or EGFP-super resonance energy-accepting chromoprotein (sREACH; non-radiative YFP) pairs provide much better and robust signal for FLIM, as GFP is much brighter than CFP, and shows mono-exponential decay [76, 101, 102].

FRET_{SENSOR FOR CaMKII}: Since many kinases, such as CaMKII, change their conformation when they are activated, a FRET probe sensing the conformational change of a kinase can serve as an indicator of the activity of the kinase. An example is a CaMKII FRET sensor named Camui [36, 103], which senses the conformational change of CaMKII by FRET between fluorophores attached to the both ends of the molecule (Figure 1b).

FRET_{SENSORS FOR SMALL GTPASE PROTEINS}: Activity of a small GTPase protein including Ras, Rho and Cdc42 can be monitored by measuring the interaction between the small GTPase protein fused to a donor fluorophore and small GTPase protein binding domain (Ras binding domain or RBD; chosen from its effectors) fused to acceptor [77, 84]. As RBD binds selectively to the active protein, activation of the small GTPase protein leads to increase in FRET (Figure 1c).

Box 2: Modeling the spatial profile of small GTPase proteins

The spatial profile of small GTPase activity along the dendrite, $C_D(x)$ (x is the contour distance from the neck of the stimulated spine along the dendritic shaft), relative to activity in the head of the stimulated spine, C_{head} , can be mathematically formulated using a diffusion-reaction model [85]. Assuming that small GTPase is activated in the stimulated spine, diffuses out of the spine, and is inactivated by GAP homogeneously distributed along the dendrite, the distribution of the activity on the dendritic shaft (C_D) as a function of time (t) and the distance along the dendrite (x) is described as:

$$\frac{C_D(x)}{C_{head}} = \alpha_{neck} \exp(-|x|/\lambda), \quad (3)$$

where α_{neck} is the gradient of activity at the spine neck and λ is the length constant of the decay along the dendrite. α_{neck} and λ are given by:

$$\alpha_{neck} = \frac{1}{4\pi r_D S_{head}^{-1} D^{1/2} \tau_D^{-1/2} \tau_{neck} + 1} \quad (4)$$

$$\lambda = \sqrt{D \tau_D}$$

where r_D is the radius of the dendrite ($\sim 0.4 \mu\text{m}$) [5], S_{head} is the surface area of the spine head ($\sim 4 \mu\text{m}^2$ during spine enlargement), D is the diffusion coefficient ($\sim 0.5 \mu\text{m}^2/\text{s}$) [84]. The equation fits well to the measured spatial profile, when one free parameter τ_D is obtained by fitting [85] (Figure I).

Box 3: Outstanding questions

- Is morphological plasticity of spines required for LTP and learning and memory?
- How is the molecular composition of the spine different after induction of LTP? Does the size of the PSD grow during LTP?
- How can the dynamic actin cytoskeleton maintain the stable spine structure? Is the cytoskeleton destabilized before extension during LTP?
- Among over one hundred GEFs and GAPs, which molecules are responsible for activation of small GTPase proteins during LTP?
- Can imaging techniques be scaled-up to allow for the visualization of hundreds of signaling proteins in spines?

Figure 1. Visualization of signaling molecules and spine volume changes in stimulated spines
 (a) Visualization of Ca^{2+} , Ca^{2+} /calmodulin-dependent kinase II (CaMKII), Ras homolog A (RhoA), and Cell division cycle 42 (Cdc42) activation during morphological plasticity in single spines using 2-photon fluorescence lifetime imaging microscopy combined with 2-photon glutamate uncaging. Warmer color indicates higher levels of activation. The white arrowheads indicate stimulated spines. Scale bars (white) are 1 μm . Images adapted, with permission from [39] (Ca^{2+} and CaMKII panels), [36] (Cdc42 and RhoA), and [84] (spine volume changes).

(b–d) Time-courses of signaling activity and spine volume changes in stimulated spines. Please note that (c) and (d) represent the same data, illustrated at time-intervals immediately after (d) and much later after (c) stimulation. The time courses of Ca^{2+} and CaMKII were adapted from [36], and RhoA, Cdc42, and spine volume changes are from [84]. The timecourses of CaMKII in (c) and (d) were originally taken under the condition of 45 pulses at 0.5 Hz [36], but the data points corresponding to the 30th–45th pulses were removed so that the plot approximately represents the one in response to 30 pulses stimulation. The time course of mutant CaMKII (T286A) was normalized to the peak of the wildtype CaMKII [36]. Autophosphorylation at T286 results in CaMKII activation independent of Ca^{2+} /calmodulin, and thus, the activity decays slower than Ca^{2+} [36]. Unlike wildtype, the T286A mutant fails to integrate Ca^{2+} signals [36]. These findings indicate that CaMKII activation peaks rapidly after Ca^{2+} stimulation, followed by RhoA and Cdc42 activation. Subsequent changes in spine volume then occur.

Figure 2. Signal transduction underlying spine morphological plasticity and long-term potentiation (LTP)

Spatiotemporal regulation of signaling cascades triggered by NMDAR activation in single dendritic spines in response to glutamate uncaging. NMDAR activation increases spine Ca^{2+} concentration, leading to activation of Ca^{2+} /Calmodulin-dependent kinase (CaMKII) [36]. It further activates downstream Rat sarcoma (Ras) [77], Cell division cycle 42 (Cdc42) and Ras homolog A (RhoA) [84]. CaMKII phosphorylates postsynaptic density 95 (PSD95) and causes dissociation of the postsynaptic density (PSD). Rho kinase (ROCK) and p21-activated kinase (PAK) are activated downstream of Rho and Cdc42, respectively. Exocytosis of AMPARs show similar patterns as Ras activation [38, 39] and requires Ras activation [39]. Trapping of diffused AMPARs into the PSD requires stargazin phosphorylation by CaMKII [53]. Fluorescence lifetime images are adapted from [77, 84] with permission. Color-coded intensity map of AMPAR exocytosis is adapted from [38] with permission. Warmer color indicates higher levels of activation/ receptor exocytosis. The white arrowheads indicate stimulated spines.

Box 1 Figure I. Fluorescence resonance energy transfer (FRET) sensor for fluorescence lifetime imaging (FLIM)

(a) Theoretical fluorescence lifetime curves of fluorescent protein (i.e. GFP as donor). The free donor at the excited state typically decays mono-exponentially (black line). When FRET occurs by the binding of acceptor to donor, the donor lifetime in the excited state is shortened (red line). For mixed population, the decay curve follows a multi-exponential curve (blue line). Thus, the population of donor bound to acceptor can be calculated from the curve.

(b) Schematic illustration of a CaMKII sensor. CaMKII takes compact form when it is inactive, but the binding of Calmodulin (CaM) induces the opening of CaMKII, increasing the distance between donor (monomeric EGFP or mEGFP) and acceptor (sREACH) and decreasing FRET [36].

(c) Schematic illustration of a RhoA sensor. The activation of mEGFP-RhoA induces the binding of Rho binding domain of Rhotekin (RBD) flanked by two mCherry molecules, and increases FRET [84].

Box 2 Figure I. Spatial spreading of small GTPase activity upon single-spine stimulation

Upon single-spine stimulation with 2-photon glutamate uncaging (indicated by the orange circle at the tip of spine), Cell division cycle 42 (Cdc42) is activated and localized in the spine, whereas the activity of Ras homolog A (RhoA) and Harvey Rat sarcoma (HRas) diffuse into the dendrite (*top*). The spatial profile of Cdc42, RhoA, and HRas activities measured with 2-photon fluorescence lifetime imaging microscopy were plotted as a function of the contour distance along the dendrite from the stimulated spines (*bottom*; at the stimulated spine, distance = 0). The curves were obtained by fitting the data with Eq. 3. For each protein, the activity in the stimulated spine was normalized to 1. Figure is adapted, with permission, from [85].