Spatiotemporal Regulation of Signaling In and Out of Dendritic Spines: CaMKII and Ras

Seok-Jin R. Lee¹ and Ryohei Yasuda*¹,²

¹Department of Neurobiology; ²Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA

Abstract: Recent advances in 2-photon fluorescence lifetime imaging microscopy (2pFLIM) in combination with 2-photon photochemistry have enabled the visualization of neuronal signaling during synaptic plasticity at the level of single dendritic spines in light scattering tissue. Using these techniques, the activity of Ca²⁺/Calmodulin-dependent kinase II (CaMKII) and Ras have been imaged in single spines during synaptic plasticity and associated spine enlargement. These provide two contrasting examples of spatiotemporal regulation of spine signaling: Ras signaling is diffusive and spread over ~10 µm along the dendrites, while CaMKII activation is restricted to the spine unde. Here we will discuss the mechanisms and roles of the different spatiotemporal regulation of signaling in neurons, and the impact of the spine structure upon these biochemical signaling processes.

INTRODUCTION

Dendritic spines are the sites of most excitatory synapses (>90%) on pyramidal neurons in the brain. Despite their relatively small size (~0.1 femtoliter), single spines can house several hundred signaling proteins and receptors [1, 2]. Even within a single pyramidal neuron, spines vary greatly in size and shape [3], from immature, filopodia-like ones to more mature, mushroom-shaped spines [4, 5]. Spine structure is dynamic: they change their morphology, appear and disappear quickly, sometimes on the minute time scale, in both activity-dependent and activity-independent manners [6-10]. The morphogenesis of spines is considered to be correlated with the functional plasticity of the spine [11, 12]. Spine shrinkage and enlargements are associated with decreases and increases in synaptic AMPA receptors (AMPARs), which is one of the main mechanisms for long-term potentiation (LTP) and depression (LTD), respectively [13-17]. Also, spine formation and pruning have been reported to contribute to LTP and LTD [18-20].

What are the roles of the spine structure? Spines are connected to their parent dendrites through narrow necks, which act as diffusion barriers [21, 22]. This structural feature is thought to biochemically isolate spine heads from their parent shafts to some degrees and to be important for spine specificity of synaptic plasticity [23, 24]. Also, the role of the spine structure as an electric compartment has been suggested to be important for dendritic computation [22, 25-28].

Consistent with the important roles of the structure and number of dendritic spines in biochemical and electrical signaling, aberrant morphology and density of dendritic spines have been observed in brain tissues from many disorders, including Down syndrome patients [29, 30], schizophrenia [31], and some forms of mental retardation [32]. Furthermore, early stages of neurodegenerative diseases such as Alzheimer’s diseases are associated with abnormal morphology, dysregulation and retraction of dendritic spines [33, 34] as well as attenuated synaptic plasticity caused by amyloid-beta oligomers [35, 36] and their interaction with the cellular prion protein (PrPc) [37].

The postsynaptic signaling mechanisms underlying synaptic plasticity, particularly LTP and LTD, have been extensively studied by a combination of pharmacology, electrophysiology and biochemistry [1, 38, 39]. The list of molecules involved in synaptic plasticity is continuously growing, and it is now clear that signaling is operated by complicated networks consisting of hundreds of proteins, which extensively interact with one another [1, 2, 40, 41]. Although these studies identified players in synaptic plasticity, many fundamental questions about signaling dynamics remain elusive. To what extent is biochemical signaling in individual dendritic spines isolated from their parent shafts? To what extent do dendritic spines compete or share resources with neighboring dendritic spines? How does the modification of a dendritic spine in its strength affect neighboring spines? These are some of the fundamental biological questions that have been actively pursued by many laboratories.

With recent advances in imaging techniques to monitor signaling in single synapses, our knowledge of signaling mechanisms underlying synaptic plasticity has broadened. Here, we will review some of the recent studies that provided new insights and answers to long-standing biological questions on dendritic spines with a focus on the underlying biochemical signaling regulated at the level of single synapses.

NEW TECHNIQUES TO STUDY SIGNALING IN SINGLE DENDRITIC SPINES

In order to image dendritic spines in thick tissues such as hippocampal slices, 2-photon excitation laser scanning microscopy (2pLSM) has become a standard tool [42]. 2-
Fluorescence Res 2 >10 to activation of paGFP i excitation of caged co under either applying a train of uncaging pulses to a single spine in lent to glutamate release from pr

ture A ble development of MNI

caled to confine the uncaging volume e
quir ephotol- tion provides great advantages in photochemistry such as photolysis of caged compound and photoactivation of fluorescence [42, 43].

Recent advances in 2-photon photochemistry techniques and 2-photon fluorescence lifetime imaging microscopy (2pFLIM) have greatly facilitated the study of intracellular signaling in single dendritic spines [42].

2-PHOTON PHOTOCHEMISTRY

Single-photon uncaging of caged compounds (caged Ca\(^{2+}\), caged IP\(_3\), etc) has been a useful biological tool [44-47]. However, 2-photon uncaging originally envisioned by Denk et al. to confine the uncaging volume to a more finite scale [43] required the development of caged compounds with enough 2-photon excitation cross section area.

The first demonstration of optical stimulation of a single dendritic spine in brain slices was achieved after the development of MNI-glutamate, a caged glutamate with appreciable 2-photon cross section [48]. In this study, AMPAR currents evoked by 2-photon glutamate uncaging were measured by whole cell patch clamp. The resolution of the technique was measured to be 0.5–1 µm and, the amplitude and kinetics of uncaging-evoked currents were similar to miniature AMPAR-excitatory postsynaptic current (EPSC). These results suggest that 2-photon glutamate uncaging is equivalent to glutamate release from presynaptic terminals. Later, the same group demonstrated that LTP and associated spine enlargement can be induced in single dendritic spines by either applying a train of uncaging pulses to a single spine in Mg\(^{2+}\)-free medium or pairing postsynaptic depolarization with uncaging pulses in the presence of Mg\(^{2+}\) [14].

Since the development of MNI-glutamate, only a handful of caged compounds have been successfully used for biology under 2-photon excitation. However, the list of available caged compounds is growing rapidly (Rubi-glutamate, Rubi-GABA, CDNI-glutamate) [49-51] and the development of new compounds will further the understanding of signaling in single spines.

Another useful technique developed recently is 2-photon excitation of photoactivatable GFP (paGFP) [22, 52]. Photoactivation of paGFP increases the fluorescence of paGFP >100 fold, providing an excellent signal to noise ratio [52]. By photo-activating paGFP tagged molecules in single spines with 2-photon excitation, the diffusion coupling of the molecule between spine and dendrite through the spine neck can be monitored [13, 14, 22, 53, 54].

2-PHOTON FRET/FLIM

Intracellular signaling dynamics have been studied using Fluorescence Resonance Energy Transfer (FRET) imaging in combination with signaling sensors made of fluorescent proteins [55, 56]. FRET is non-radiative energy transfer between two fluorophores due to dipole-dipole interaction, which occurs when the donor and acceptor fluorophores reside in proximity (nanometers) [57, 58]. The excitation energy of the donor is transferred to the acceptor, decreasing the donor fluorescence and increasing the acceptor fluorescence. Because the efficiency of FRET decreases sharply as the distance between the donor and the acceptor becomes larger than several nanometers, it can be used to measure protein-protein interactions or conformational changes of proteins [57, 58].

FRET can be quantified by imaging the ratio of fluorescence intensity of the donor and acceptor (ratiometric FRET) [57]. Advantages of ratiometric imaging include a simple optical setting and high signal-to-noise ratio. However, the ratiometric FRET is prone to an artifact caused by the local concentration ratio between the donor and the acceptor and wavelength dependent light-scattering.

Alternatively, one can use the fluorescence lifetime of the donor to quantify FRET [57]. Fluorescence lifetime is the time elapsed between fluorophore excitation and photon emission. The fluorescence decay curve following a short excitation pulse is mono-exponential typically with a nanosecond time constant. FRET accelerates the fluorescence decay in proportion to the FRET efficiency, and thus one can use fluorescence decay as a readout of the FRET efficiency [57]. Because this measurement involves only the donor fluorescence, the measurement is independent of donor/acceptor ratio and free from wavelength-dependent light scattering unlike ratiometric FRET. Furthermore, when multiple populations with different FRET efficiency co-exist, the fluorescence decay curve becomes multi-exponential, and each component can be de-convoluted to calculate the fraction of the donor binding to the acceptor [57]. The mean fluorescence lifetime of the entire populations can be measured by calculating the mean fluorescence lifetime, \( \tau = \left( \int F(t) dt \right) \left( \int F(t) dt \right)^{-1} \), where \( F(t) \) is the fluorescence lifetime decay after a short excitation pulse [57] (Fig. 1).

Since Miyawaki et al. reported the first genetically encoded Ca\(^{2+}\) sensors based on FRET and fluorescent proteins [59], many FRET-based sensors have been developed to measure signaling activity including the concentration of second messengers and the activity of protein kinases/phosphatases [55, 56]. Biosensors designed to change their FRET between activated and inactivated states of a protein of interest largely fall into two groups: bi-molecular sensors and mono-molecular sensors. Bi-molecular sensors report binding between two proteins associated with signaling activity [56]. Typically a target protein is tagged with the donor and another protein that specifically binds to the active state of the target protein is tagged with the acceptor. Activation of the target protein is associated with the binding of two proteins, increasing FRET [56]. FLIM, but not ratiometric FRET, should be used for this type of sensors, because the donor/acceptor ratio is not constant. In contrast, a mono-molecular sensor has both the donor and acceptor in the same polypeptide, fixing the donor/acceptor ratio allowing simple ratiometry to be used. For example, a typical kinase
activation reporter consists of a donor, a consensus substrate phosphorylation site for the kinase, a phospho-recognition motif and an acceptor all in the same polypeptide. When the kinase of interest is activated, the phosphorylation site becomes phosphorylated and binds to the phospho-recognition domain, changing the FRET efficiency [55].

Although FRET imaging techniques have provided insights into the spatiotemporal dynamics of intracellular signaling at the whole-cell level, it has been difficult to apply these techniques to the study of synaptic signaling. Limited fluorescence signal from the small structure of spines, and light scattering from tissue are the main issues. Recently, the combination of FRET signal imaging techniques with 2pFLSM have addressed these issues and enabled imaging FRET in single synapses with high sensitivity. Using 2-photon ratiometric FRET imaging, actin polymerization in single spines was monitored during spine growth and shrinkage by overexpressing both CFP-tagged and YFP-tagged actin monomers [60]. It has been also demonstrated that the combination of FLIM and 2pLSM (2pFLIM) provides extremely robust signal in small neuronal compartments in light scattering brain slices [61]. However, most FRET sensors are optimized for ratiometric imaging, and not for FLIM [58]. Recently, sensitive sensors for the activity of Ras [61] and CaMKII [13], molecules important for synaptic plasticity, optimized for 2pFLIM have been developed and successfully employed to measure the activity of these molecules in single dendritic spines.

The Ras sensor for 2pFLIM, FRas-F, is a bi-molecular sensor consists of H-Ras tagged with monomeric enhanced green fluorescent protein (mEGFP) and the Ras-binding domain of Raf (RBD) tagged with two monomeric red fluorescence proteins (mRFPs). When mEGFP-Ras is activated, mRFP-RBD binds to mEGFP-Ras, thereby increasing FRET [61]. Because RBD binding to Ras competes with Ras inactivation, the affinity between RBD and Ras was decreased by mutating RBD (R59A), allowing fast reversing kinetics of the sensor signal [61]. FRas-F in combination with 2pFLIM provided high sensitivity sufficient for measuring Ras activity in single spines in response to physiologically relevant stimuli [16, 61].

The CaMKII sensor optimized for 2pFLIM, Green-Camuia, is a mono-molecular sensor based on the original ratiometric CaMKII sensor Camuiia [62]. Camuiia is CaMKIIα with its ends tagged with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). When CaMKIIα is activated, it changes its conformation from a closed to an open form [63] thereby reducing FRET. Because the CFP-YFP pairs is not optimal for FLIM [58], the fluorophores were replaced by the pair of mEGFP and resonant energy transfer acceptor chromophore (REACH) [64, 65]. The sensor in combination with 2pFLIM allows ones to measure CaMKII activity in single spines during LTP with high temporal resolution (~ seconds) [13].

These new 2-photon based FRET techniques provided many new insights of the roles of spine morphology in the spatiotemporal regulation of biochemical signaling during synaptic plasticity and associated spine enlargement.

**THE SIGNALING DURING SPINE STRUCTURAL PLASTICITY AND LTP**

Repetitive NMDA receptor (NMDAR) activation causes the enlargements of spines associated with recruitment of AMPARs into the stimulated spines [14]. The opening of NMDARs in a spine causes ~micromolar Ca$^{2+}$ transients largely restricted to the spine [66-69] (Fig. 2A). The resulting Ras and CaMKII signaling dynamics in spines induced by NMDAR activation has been recently revealed using 2pFLIM and glutamate uncaging [13, 16].

**RAS**

A GTPase protein Ras constitutes an essential element in the signal transduction network that couples Ca$^{2+}$ elevations to diverse signaling cascades. Calcium-dependent Ras activation results in the phosphorylation of extracellular signal-related kinase (ERK) and phosphoinositide-3 (PI3K) and this Ras pathway extensively branches out to different forms of neuronal plasticity, including LTP [70, 71], regulation of dendritic excitability [72, 73], new spine formation [74, 75], spine structural plasticity [16] as well as dendritic protein synthesis [76] and gene transcription [77]. To measure the activity of Ras at the level of single synapses during synaptic plasticity, the Ras sensor FRas-F [61] was expressed in Hippocampal pyramidal neurons [16]. When a single spine underwent structural plasticity induced by a train of uncaging pulses to open NMDARs in Mg$^{2+}$-free medium, Ras activation reached its peak in 1 min after the stimulation. Subsequently, Ras activation spreads into dendrites over ~10 μm and invades neighboring spines [16] (Fig. 1).

Does the spreading of Ras occur even in unperturbed cells? In imaging experiments using a sensor, it is important to evaluate the effect of the overexpression of the sensor on signaling [58, 78]. For example, overexpression of FRas-F may saturate Ras binding partners or inactivation machinery,
causing larger Ras spreading. To address this concern, Harvey et al. measured the relationship between FRas-F expression level and the spreading width, and extrapolated to zero expression level. The width of Ras spreading only weakly correlated with the expression level of FRas-F, and the extrapolated value was similar to the measured value (~10 µm), confirming that spreading of Ras was not due to overexpression of FRas-F.

This study demonstrated that a biochemical signal triggered by NMDAR-mediated Ca\(^{2+}\) influx, which is mostly restricted to the stimulated dendritic spine, can diffuse out and spread over a short stretch of dendrite (~10 µm) sending signals to not only the stimulated spine but also nearby spines as well. In other words, at least for Ras signaling, spine morphology is not capable of constraining signaling within a dendritic spine. This study also shows that while Ras activation is required for the maintenance of structural plasticity of dendritic spines, it is not sufficient for the induction of structural plasticity. Neighboring spines with almost similar degree of Ras activation as the stimulated spine showed neither structural nor functional plasticity measured as uncaging-evoked AMPAR current. So what does activated Ras do in unstimulated, nearby spines while its activity takes ~10 min to fully return to the basal level of activity? What is the physiological function of the spreading of the activated signaling proteins during synaptic plasticity? Considering the role of Ras in synaptic plasticity, this observation calls for a new form of local plasticity that affects a group of spines on a short stretch of dendrite, which can be characterized as time- and location-dependent.

About that time, Harvey and Svoboda discovered a new form of plasticity that spans a similar length with the spread of Ras activity [15]. In this experiment, a spine is first stimulated with a usual train of uncaging pulses that induces synaptic plasticity (suprathreshold stimuli). Then within 5 minutes after the first stimulation, weak stimulation which does not produce plasticity by itself (subthreshold stimuli) was applied to a neighboring dendritic spine less than 10 µm away from the originally stimulated spine. Surprisingly, this subthreshold stimulation was now sufficient to induce long-lasting synaptic plasticity in time- (within 5 min after the initial suprathreshold stimulation) and location-dependent manner (within 10 µm from the originally stimulated spine). They found that this new form of plasticity is caused by diffusion of intracellular factors.

The spatiotemporal scale of the facilitation of plasticity seems to be very similar to that of Ras activation, and thus Ras would be the natural suspect which causes this phenomenon. In order to test if Ras is required for this form of plasticity, Harvey et al. applied an inhibitor of downstream signaling of Ras (U0126) between the first suprathreshold and second subthreshold stimuli [16]. They found the reduction of structural plasticity in response to subthreshold stimuli, but not to suprathreshold stimuli [16]. Furthermore, the subthreshold stimuli did not produce any additional Ras activation, suggesting that spreading of Ras is essential to produce the facilitation of plasticity [16].

CaMKII

Ras imaging clearly showed that signaling can spread from spines undergoing plasticity. However, spine enlargement and LTP are known to be spine-specific [14], and thus there must be a biochemical signal that is input-specific for synapse-specificity of synaptic plasticity. CaMKII has been a great candidate molecule for the synapse-specificity of synaptic plasticity given that it is sufficient for induction of LTP [79].

CaMKII consists of a dodecamer with each subunit acting as a kinase [80-82]. When [Ca\(^{2+}\)] increases, it binds to calmodulin and this Ca\(^{2+}\)-bound calmodulin binds to CaMKII [82]. CaMKII subsequently undergoes a conforma-
tional change from its closed conformation to open conformation, which exposes its kinase site and becomes active [63, 83]. Active CaMKII subunits autophosphorylate the T286 site of an adjacent subunit [84]. Once a subunit is autophosphorylated at T286, its activity becomes insensitive to the interaction to Ca\(^{2+}\)/calmodulin, thereby leaving them active after [Ca\(^{2+}\)] decays [85-88]. It has been proposed that this Ca\(^{2+}\)-independent, autonomous activity due to T286 phosphorylation may last long-term for hours or days to maintain LTP and ultimately learning [89, 90]. Consistent with the importance of T286 phosphorylation, mice with this autophosphorylation site mutated to alanine (T286A) are found to be deficient in LTP and spatial learning and memory [91] as well as experience-dependent cortical plasticity [92].

In a recent study by Lee et al., the activity of CaMKII was directly measured in single spines using Green-Camuia. When expressed in CA1 pyramidal neurons in organotypic cultures, it co-assembles with endogenous CaMKII subunits to form dodecamers [13, 83]. Stimulation of single spines via 2-photon glutamate uncaging to induce LTP caused FRET changes of Green-Camuia indicative of CaMKII activation only in the stimulated spines [13]. Contrary to the hypothesis of long-term autonomous CaMKII activity, CaMKII activity lasts only for ~1 min, while both structural and functional plasticity lasts more than 1 hour.

What is the role of T286 phosphorylation, if the wild type activity lasts only for ~1 min? Lee et al. found that the activity of wild type Green-Camuia decays double exponential time constants of ~6 s and 45 s whereas T286A mutant completely returns to the basal state in 2 seconds. Therefore, in the protocols used in Lee et al. to induce LTP by uncaging (30–45 uncaging pulses at 0.5 Hz), the stimulation frequency is not fast enough to accumulate activated T286A-CaMKII mutant. Instead, the T286A mutant inactivates before the next stimulation arrives. Hence, the autophosphorylation at T286 site is critical for delaying inactivation kinetics thereby allowing repetitive stimulation to accumulate activated CaMKII efficiently.

These new imaging data showing transient CaMKII activation does not support the theory in which long-term autonomous CaMKII activity is important for the maintenance of LTP. How do these results fit with previous biochemical studies? Fukunaga et al. reported persistent autonomous activity in response to LTP inducing stimuli [93]. Also, persistent T286 phosphorylation has been observed by several groups [94, 95]. However, Lengyel et al. reported that the persistent T286 phosphorylation is not associated with persistent autonomous activity which decays within ~2 min while LTP persists more than 60 min [95]. Therefore, although there may be some stimulus conditions that causes persistent autonomous activity [93], persistent CaMKII activity is probably not required for LTP maintenance [95]. With one exception [96], pharmacological inhibition of CaMKII after induction of LTP does not affect the maintenance of LTP, arguing against the role of CaMKII in the maintenance of LTP [97-99]. A more specific approach using a CaMKII mutant with a bigger ATP binding pocket and an ATP analog that specifically inhibits the mutant CaMKII [100] also suggests that CaMKII kinase activity is required only for the first 10 min of stimulation to induce LTP [101]. Furthermore, auto-inactivation of T286 phosphorylated CaMKII by T305/T306 phosphorylation with a half-life of 50 s has been proposed by a recent biochemical study supporting transient (~1 min) activation of CaMKII [102].

**PRINCIPLES OF SIGNAL COMPARTMENTALIZATION**

Ras and CaMKII provide two beautiful examples of how spine signaling is spatiotemporally regulated in a contrasting manner. CaMKII activation is transient (~1 min) and restricted within a dendritic spine while Ras activation persists longer (~5 min) and spreads over ~10 µm along the dendrite.
(Fig. 1). This clearly shows that some molecules are important for synapse specific signaling, while other molecules signal on a larger scale (Fig. 3). What is the basic principle underlying the diffusivity of biochemical signals in and out of dendritic spines?

The compartmentalization of signaling activity is in general determined by the balance between two factors: effective spine-dendrite diffusion coupling time constant \( \tau_{\text{coupling}} \) and inactivation time constant \( \tau_{\text{inactivation}} \) [67]. When \( \tau_{\text{coupling}} \gg \tau_{\text{inactivation}} \), the protein is inactivated before it diffuses out of the spine, and thus the activity is compartmentalized to the spine. In contrast, when \( \tau_{\text{coupling}} \ll \tau_{\text{inactivation}} \), the molecule is inactivated after it diffuses out of the spine, and thus the activity spreads into dendrites. For example, in the case of \( \text{Ca}^{2+} \), \( \tau_{\text{coupling}} \) is measured as 0.1 s and \( \tau_{\text{inactivation}} \) (extrusion in the case of \( \text{Ca}^{2+} \)) as 15 ms [67-69, 103]. Thus, in general, \( \text{Ca}^{2+} \) is extruded before it can diffuse out of the spine, making it compartmentalized within spines [67-69] (Fig. 2A). In spines with small \( \tau_{\text{coupling}} \), \( \text{Ca}^{2+} \) can spread into dendrites to some degree [68, 104].

One can measure \( \tau_{\text{coupling}} \) by photoactivating paGFP tagged molecule and measuring the decay of paGFP fluorescence (Fig. 2A). For cytosolic small molecules such as \( \text{Ca}^{2+} \) or fluorophores, \( \tau_{\text{coupling}} \sim 0.1 \text{ s} \) [67, 68], for cytosolic proteins such as paGFP, \( \tau_{\text{coupling}} \sim 0.5 \text{ s} \) [16, 22], and for membrane targeted proteins like H-Ras and MARCKS, \( \tau_{\text{coupling}} \sim 5 \text{ s} \) [16] (Fig. 2B). CaMKII interacts with many proteins in the postsynaptic density (PSD) and actin cytoskeleton, increasing \( \tau_{\text{coupling}} \) to double exponential time constants of \( \sim 1 \text{ min} \) and \( 20 \text{ min} \) [13, 105] (Fig. 2). In comparison to these values, \( \tau_{\text{inactivation}} \) was measured to be \( \sim 5 \text{ min} \) for Ras [16, 61] and double exponential time constants of \( \sim 6 \text{ s} \) and \( 45 \text{ s} \) for CaMKII [13]. Thus, the balance of the time constants is \( \tau_{\text{inactivation}} \gg \tau_{\text{coupling}} \) for Ras, and \( \tau_{\text{inactivation}} \ll \tau_{\text{coupling}} \) for CaMKII (Fig. 3). This explains why Ras signaling is diffusive and CaMKII activation is compartmentalized (Table 1).

The length constant of signaling activity – the mean distance an activated molecule travels before being inactivated – can be calculated using the following simple equation:

\[
L = \sqrt{D_{\text{eff}} \tau_{\text{inactivation}}}
\]

where \( D_{\text{eff}} \) is effective diffusion coefficient of the signaling molecule and \( \tau_{\text{inactivation}} \) is the time constant of inactivation [16]. In other words, the length constant is balanced by diffusion and inactivation of the protein. For cytosolic proteins, \( D_{\text{eff}} \) can be calculated from \( \tau_{\text{coupling}} \) as:

\[
D_{\text{eff}} = \frac{IV}{s \tau_{\text{coupling}}}
\]

where \( V \sim 0.1 \text{ µm}^3 \) is the volume of the spine, and \( l \sim 0.8 \text{ µm} \) is the spine neck length, \( s \sim 0.008 \text{ µm}^2 \) is the cross-section area of the spine neck. For membrane proteins, \( V \sim 1 \text{ µm}^3 \) is the surface area of the spine and \( s \sim 0.3 \text{ µm} \) is the circumference of the spine neck [21, 22]. \( D_{\text{eff}} \) and \( L \) calculated for \( \text{Ca}^{2+} \), Ras, and CaMKII are in Table 1.

The length constant \( L \) of \( \text{Ca}^{2+} \) and CaMKII are calculated to be \( \sim 1 \text{ µm} \) (Table 1), suggesting that they should be confined within the stimulated spines, consistent with previous experiments [13, 67-69, 105]. It should be noted that this also suggests that \( \text{Ca}^{2+} \) and CaMKII are confined within \( \sim 1 \text{ µm} \) length without the help of spine morphology. Indeed, compartmentalization of \( \text{Ca}^{2+} \) is observed in non-spiny neurons as well [106, 107]. In contrast with these molecules, the length constant of Ras is much longer (\( \sim 10 \text{ µm} \); Table 1). This length is consistent with the observed width of the activity spreading of Ras during synaptic plasticity [16].

**ROLE OF SPINE MORPHOLOGY IN COMPARTMENTALIZING BIOCHEMICAL SIGNALING**

Since the finding of spine structure by Santiago Ramon y Cajal in 1888, it has been speculated that spine morphology is important for isolating biochemical reactions within a synapse [23, 24]. However, the spatiotemporal dynamics of Ras and CaMKII suggest that spine morphology does not contribute much to compartmentalize biochemical signaling. If a

<table>
<thead>
<tr>
<th>( \text{Ca}^{2+} )</th>
<th>CaMKII</th>
<th>Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \tau_{\text{coupling}} )</td>
<td>0.1 s</td>
<td>60 s</td>
</tr>
<tr>
<td>( \tau_{\text{inactivation}} )</td>
<td>0.015 s</td>
<td>6 s</td>
</tr>
<tr>
<td>( D_{\text{eff}} )</td>
<td>( \frac{IV}{s \tau_{\text{coupling}}} )</td>
<td>100 ( \mu \text{m}^2/\text{s} )</td>
</tr>
<tr>
<td>( L )</td>
<td>( \sqrt{D_{\text{eff}} \tau_{\text{inactivation}}} )</td>
<td>1 ( \mu \text{m} )</td>
</tr>
<tr>
<td>Compartmentalization</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

\( \text{Fast component only} \)
protein does not interact with the PSD or cytoskeleton, the protein diffuses out of spines within a few seconds for membrane targeted protein and even faster for cytosolic proteins (Fig. 2). This diffusion coupling time is much faster than the typical biochemical time scale for the maintenance of synaptic plasticity (min to hour) (Fig. 2). Indeed, the Ras activation profile shows very little gradient between the stimulated spine and their parent dendrites (Fig. 1), demonstrating the ineffectiveness of spine morphology on membrane targeted proteins. Molecular size plays a small role in the diffusion constant, as the diffusion constant is proportional to the cubic root of the molecular mass (Fig. 2B).

To produce synapse specific activation of a protein for more than a few seconds, the molecule needs to interact with non-diffusible structures such as the PSD or cytoskeleton to limit their diffusion. Thus, the degree of the compartmentalization depends more on interactions of the molecule with the non-diffusible structures rather than spine morphology. Indeed, CaMKII compartmentalization is likely due to the ability of CaMKII to bind to the PSD or cytoskeleton. The “stickiness” of CaMKII can be observed by the difference between the effective diffusion constant in spines ($D_{eff} \sim 0.16 \mu m^2/s$, Table 1) and the diffusion constant of CaMKII in lysates (~25 µm²/s) [13, 108].

In contrast with slow signaling like Ras and CaMKII, spine structure is likely to be important for shaping Ca²⁺ dynamics by confining it in a small volume. Because the number of NMDARs is relatively independent of the spine volume and spine neck resistance is high, smaller spines experience higher Ca²⁺ elevation in response to the opening of NMDARs [68, 69, 109]. This may be important for differentiating plasticity in mature and immature spines [14, 68].

**NANODOMAIN SIGNALING**

Electron microscopy images of dendritic spines show specialized structures [3] such as the PSD and endocytic zone [110]. Receptors exchanging between synaptic and extra-synaptic sites for synaptic efficacy have been visualized by tracking diffusion of single synaptic receptors [111]. Hence, despite the relatively small size of a dendritic spine (~0.1 µm), there exists even more finite, localized, compartmentalized signaling within a dendritic spine. Furthermore, nanometer scale signaling complexes at the mouths of channels are considered to play important roles in producing channel-specific signal transduction [112-114].

LTP induction is thought to be channel specific, because LTP can be induced by the micromolar level of Ca²⁺ elevation due to the opening of NMDARs, but not by the similar level of Ca²⁺ elevation due to the opening of voltage sensitive calcium channels (VSCCs) [13, 115]. This specificity is important for input-specificity, because LTP in general requires pairing of postsynaptic depolarization and presynaptic activation to release Mg²⁺ block of NMDARs [39], and postsynaptic activation alone opens VSCCs to produce non-specific Ca²⁺ elevation in all spines [13, 115]. Thus, some of the signaling pathways underlying LTP should be preferentially activated by NMDARs.

At the single spine level, Ras and CaMKII activity has been measured with 2pFLIM [13, 16]. In these studies, channel specificity of these molecules was also measured. Diffusion of Ras is fast [116, 117], and its activity spreads long distances [16]. Thus, Ras signaling would not be able to produce local signaling at the nanometer scale. Consistent with this view, nanodomain Ca²⁺ does not play a role in Ras activation in response to action potentials measured with FRas-F in combination with 2pFLIM [61]. Further, during the uncaging-depolarization pairing protocol, Ras in all spines was activated [16]. Thus, Ras signaling is designed neither for synapse specificity nor channel specificity.

In contrast, CaMKII interacts with many channels including L-type VSCCs [118, 119], T-type VSCCs [120], P/Q-type VSCCs [121], dopamine receptors [122], NMDARs [123, 124], and thus should be able to detect Ca²⁺ through specific channels. To assess this possibility, Lee et al. measured CaMKII activity during the uncaging-depolarization pairing protocol [13]. During postsynaptic depolarization, although depolarization produces Ca²⁺ transients of similar amplitudes in spines and dendrites, CaMKII activation in spines was much smaller than in dendrites. Also, in spines, depolarization and uncaging produce similar elevations of Ca²⁺, but uncaging produces much higher CaMKII activation. Thus, CaMKII activation is likely to be channel specific. Consistent with this, depolarization-induced CaMKII activation was completely blocked by inhibiting L-type VSCC with Nimodipine in spines but not in dendrites, while global Ca²⁺ elevation was not affected by Nimodipine.

Lee et al. performed further experiments using Ca²⁺ chelators EGTA and BAPTA to test the hypothesis in which nanodomain Ca²⁺ at the inner mouth of VSCCs is responsible for CaMKII activation [13]. Although BAPTA and EGTA have similar dissociation constants to Ca²⁺, BAPTA is one hundred times faster at chelating Ca²⁺ ions than EGTA. Therefore, BAPTA can capture Ca²⁺ ions right away once they flow in through ion channels while Ca²⁺ ions can travel much longer distance before being chelated by EGTA [125]. Thus, BAPTA inhibits both global and nanodomain Ca²⁺ elevation, whereas EGTA inhibits global Ca²⁺ elevation without affecting nanodomain Ca²⁺ much. When cells were patch-clamped with an electrode containing 20 mM BAPTA, CaMKII activation in response to depolarization was completely inhibited. However, the same concentration of EGTA did not affect CaMKII activation significantly. Interestingly, unlike depolarization-induced CaMKII activation, uncaging evoked CaMKII activation shows similar sensitivity to EGTA and BAPTA, suggesting that NMDAR-mediated CaMKII activation requires global Ca²⁺. Because CaMKII is not activated by high Ca²⁺ through non-L-type VSCCs in response to depolarization, NMDAR-mediated CaMKII probably requires both nanodomain and global Ca²⁺.

The nanodomain signaling of CaMKII at L-type VSCCs and NMDARs is consistent with previous studies showing that NMDARs interact with CaMKII [118, 119, 123, 126]. However, the underlying mechanism for the difference between L-type VSCC-mediated CaMKII activation, which does not require global Ca²⁺, and NMDAR-mediated CaMKII activation, which requires both global and nanodomain Ca²⁺, remains unanswered. One possibility is that the difference in calmodulin distribution near channels causes this difference in the sensitivity in global Ca²⁺. Because the Ca²⁺ sensor for the activity of CaMKII is calmodulin, the distribution of calmodulin near Ca²⁺ channels should have a large
impact in shaping CaMKII activation dynamics. It has been reported that L-type VSCCs are highly enriched with calmodulin [127]. This calmodulin at L-type VSCCs might be locally activated in response to L-type VSCC activation, making this signaling insensitive to global Ca$^{2+}$ elevation. More experimental and theoretical studies on Ca$^{2+}$-calmodulin – CaMKII interaction in spines will be required to fully understand the mechanisms of CaMKII activation in nanodomains of calcium channels.

FUTURE

Imaging of the activity of a few proteins in single spines has already provided many insights into the principles and roles of the spatiotemporal regulation of signaling activity during synaptic plasticity. However, there are hundreds more proteins involved in synaptic plasticity [1, 2, 40] and thus we need to measure the activity of more proteins to disentangle the complicated signaling network in spines. This will answer many important questions, for example: are there signaling proteins whose activity lasts more than hours to maintain long-term synaptic plasticity? Are there signaling processes that are actively transported or regeneratively propagated from synapses to nucleus? Are there signaling processes that spread to negatively regulate synaptic plasticity, sharpening the synapse specificity of plasticity? We speculate that measuring the activity of almost any protein will provide us new surprises and insights.

Finally, although signaling in sub-spine structure and nanodomain signaling have been suggested to be important, current FRET imaging techniques cannot provide access to these compartments due to their limited spatial resolution. The combination of FRET imaging technique and recently developed super-resolution microscopy [128-130] may enable direct visualization of signaling dynamics on the nanometer scale and provide us with a more precise view about the sub-spine signaling.

ACKNOWLEDGEMENTS

We thank M. Patterson for critical reading of the manuscript. This study was funded by American Heart Association Predoctoral Fellowship (S.-J.R.L), Howard Hughes Medical Institute, Alzheimer’s Association of America, NIMH / NIH (R01MH080047) and NINDS / NIH (1R01NS068410).

REFERENCES

[31] Garey LJ, Ong WY, Patel TS, et al. Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. J Neurol Neurosurg Psychiatry 1998; 65(4): 446-53.
Spatiotemporal Regulation of Signaling


Spatiotemporal Regulation of Signaling

The Open Neuroscience Journal, 2009, Volume 3


Received: August 04, 2009

Revised: August 30, 2009

Accepted: September 19, 2009

© Lee and Yasuda; Licensee Bentham Open.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.