Studying Signal Transduction in Single Dendritic Spines

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Many forms of synaptic plasticity are triggered by biochemical signaling that occurs in small postsynaptic compartments called dendritic spines, each of which typically houses the postsynaptic terminal associated with a single glutamatergic synapse. Recent advances in optical techniques allow investigators to monitor biochemical signaling in single dendritic spines and thus reveal the signaling mechanisms that link synaptic activity and the induction of synaptic plasticity. This is mostly in the study of Ca^{2+} -dependent forms of synaptic plasticity for which many of the steps between Ca^{2+} influx and changes to the synapse are now known. This article introduces the new techniques used to investigate signaling in single dendritic spines and the neurobiological insights that they have produced.

Each neuron typically receives 1000–10,000 synaptic inputs and sends information to an axon, which branches to produce a similar number of synaptic outputs. Most excitatory postsynaptic terminals are associated with dendritic spines, small protrusions emanating from the dendritic surface (Nimchinsky et al. 2002; Alvarez and Sabatini 2007). Each spine has a volume of ~ 0.1 femtoliter, and connects to the parent dendrite through a narrow neck, which acts as a diffusion barrier and compartmentalizes biochemical reactions. Ca^{2+} influx into spines initiates a cascade of biochemical signals leading to various forms of synaptic plasticity including long-term potentiation (LTP).

Because LTP in hippocampal CA1 pyramidal neurons is a cellular mechanism that may underlie long-term memory formation, the sig-

nal transduction underlying LTP has been extensively studied by pharmacological and genetic methods (Bliss and Collingridge 1993; Derkach et al. 2007). It is now well established that LTP is induced by Ca^{2+} influx into dendritic spines through NMDA-type glutamate receptors (NMDARs), which induces the insertion of AMPA-type glutamate receptors (AMPA receptors) into the synapse, thereby increasing the sensitivity of the postsynaptic terminal to glutamate (Derkach et al. 2007; Kessels and Malinow 2009). An increase of release probability during LTP has also been reported (Enoki et al. 2009), and thus both pre- and postsynaptic mechanisms may contribute to LTP (Lisman and Raghavachari 2006).

Manipulations of signal transduction using specific pharmacological inhibitors or genetic

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perturbations have identified many signaling pathways that connect Ca^{2+} to LTP induction. For example, LTP requires the activation of many signaling proteins, including Ca^{2+} /calmodulin-dependent kinase II (CaMKII), extracellular signal-related kinase (ERK), Phosphoinositide 3 kinase (PI3K), protein kinase A and C, and GTPases such as Ras, Rab, and Rho (Kennedy et al. 2005). The list is continually growing, and the hundreds of implicated proteins form a complex signaling network whose contribution to LTP is still unclear (Bromberg et al. 2008).

Signaling dynamics in neurons have traditionally been measured using biochemical analyses (Bromberg et al. 2008). However, the spatiotemporal resolution of conventional biochemistry is limited, restricting analysis to the time scale of many minutes and requiring the homogenization of tissue containing millions of synapses and other cellular elements. Furthermore, resolving synaptically induced changes in signaling by biochemical analysis typically requires stimulating many synapses at the same time, which may produce unintended effects, for instance, excitotoxicity or homeostatic plasticity.

The size of dendritic spines is similar to the resolution of an optical microscope, permitting the optical analysis of biochemical signaling in each dendritic spine (Svoboda and Yasuda 2006). In particular, the advent of two-photon-based FRET techniques and the development of appropriate fluorescent reporters of specific bio-

chemical reactions (see below) have provided readouts for signal transduction with high spatiotemporal resolution in live brain tissue (Svoboda and Yasuda 2006; Yasuda 2006). This has provided detailed information about the dynamics of signal transduction in spines and dendrites, and insights into the molecular mechanisms of synaptic plasticity.

MONITORING SIGNAL TRANSDUCTION IN LIVE CELLS USING FRET

FRET Imaging of Intracellular Signaling

The dynamics of intracellular signaling have been studied using fluorescence resonance energy transfer (FRET) (Miyawaki 2003; Ni and Zhang 2010). FRET occurs when a donor and acceptor fluorophore are within nanometers of each other (Lakowicz 2006). The excitation energy of the donor is transferred to the acceptor owing to a dipole–dipole interaction, decreasing donor fluorescence and increasing acceptor fluorescence. The efficiency of FRET decreases sharply with the distance (r) between the acceptor and the donor:

$$Y_{\text{FRET}} = 1/[1 + (r/R_0)^6], \quad (1)$$

where R_0 is the distance that gives $Y_{\text{FRET}} = 0.5$ (Fig. 1A) (Förster 1993; Lakowicz 2006). R_0 is called the Förster distance, because it was calculated by Förster in 1947 and found to be a

Figure 1. FRET for readout of signal transduction. (A) FRET efficiency as a function of the pulse distance between donor and acceptor (when Förster distance = 5 nm). (B) Fluorescence decay after short pulse excitation under non-FRET, FRET, and mixture conditions.

function of several factors (Förster 1993; Lakowicz 2006):

$$R_0 = (8.79 \times 10^{17} J Q_D \epsilon_A \kappa^2 n^{-4})^{1/6}, \quad (2)$$

where J is the spectral overlap integral $J = \int f_D(\lambda) g_A(\lambda) \lambda^4 d\lambda$ (where f_D is the fluorescence spectrum of the donor normalized to the peak, g_A is the absorption spectrum of the acceptor normalized to the peak, and λ is the wavelength), ϵ_A is the molar absorption coefficient of the acceptor, Q_D is the quantum yield of donor, κ^2 is the orientation factor ($0 \leq \kappa \leq 4$), and n is the refractive index (~ 1.3 for solution). κ^2 depends on the relative orientation between the dipole moments of the donor emission and acceptor excitation. For random orientation, $\kappa^2 = 2/3$.

For good pairs of fluorescent donor and acceptor proteins such as enhanced cyan fluorescence protein (ECFP) and enhanced yellow fluorescent protein (EYFP) or enhanced green fluorescent protein (EGFP) and monomeric red fluorescent protein (mRFP), R_0 is ~ 5 nm. Because this distance is similar to the size of proteins, FRET can be used to detect intermolecular interactions between proteins tagged with fluorophores, or to detect intramolecular conformational changes of proteins tagged with both the donor and acceptor. Because Miyawaki et al. (1997) reported the first genetically encoded Ca^{2+} sensors based on FRET and fluorescent proteins, many FRET-based sensors designed to report the concentration of second messengers and the activity of protein kinases/phosphatases have been developed (Miyawaki 2003; Ni and Zhang 2010).

For imaging in biological systems, FRET is typically measured by taking the ratio of intensities of fluorescence of the donor and of the acceptor, which provides a fast and high-sensitivity measure of FRET (Lakowicz 2006). However, owing to the broadness of fluorophore absorption and excitation spectra, it is often impossible to excite the donor exclusively, or to separately measure donor and acceptor fluorescence (Wallrabe and Periasamy 2005; Lakowicz 2006). Because of this spectral bleed-through, the ratio of donor and acceptor fluorescence depends on the ratio of fluorophore

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concentrations as well as on the FRET efficiency. Correcting for this concentration dependence is not simple and can severely complicate the analysis of FRET-based measurements. To avoid this problem, most FRET sensors designed for ratio-metric imaging include both donor and acceptor in the same polypeptide, ensuring that the stoichiometry of the donor and acceptor is one to one (Miyawaki 2003; Ni and Zhang 2010).

FRET can also be measured by measuring the fluorescence lifetime of donor fluorophores using fluorescence lifetime imaging microscopy (FLIM) (Wallrabe and Periasamy 2005; Lakowicz 2006; Yasuda 2006). The fluorescence lifetime is the time elapsed from fluorophore excitation to photon emission, and is typically on the nanosecond scale. Because photon emission events occur stochastically, the fluorescence of a free donor after a short pulse excitation decays monoexponentially (Fig. 2) (Lakowicz 2006):

$$F(t) = F_0 \exp(-t/\tau_D). \quad (3)$$

When FRET occurs, the fluorescent decay of the donor is accelerated because its excited state is now reduced by both photon emission and by FRET. The fluorescence lifetime of the donor bound to the acceptor, τ_{DA} , is related to the FRET efficiency Y_{FRET} as (Lakowicz 2006)

$$Y_{\text{FRET}} = 1 - \tau_{DA}/\tau_D. \quad (4)$$

Because FLIM measures donor fluorescence only, it is not affected by changes in the concentrations of the donor and acceptor or by wavelength-dependent light scattering (Wallrabe and Periasamy 2005; Lakowicz 2006; Yasuda 2006). When the free donor and the donor bound to the acceptor coexist, the fluorescence decay becomes double exponential (Lakowicz 2006):

$$F(t) = F_0 [P_D \exp(-t/\tau_D) + P_{DA} \exp(-t/\tau_{DA})], \quad (5)$$

where P_D and P_{DA} are the proportions of donor that are free and bound to acceptor, respectively. Thus, one of the big advantages of fluorescence lifetime measurements is that the fraction of donor that is bound to acceptor is directly measured

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Figure 2. Imaging CaMKII activation during spine structural plasticity. (A) Schematic of the CaMKII sensor, Green-Camui α . (B) CaMKII activation during spine enlargement triggered by stimulation of a single dendritic spine. (Figure adapted from Lee et al. 2009; reprinted, with permission, from Nature Publishing Group © 2009.)

by fitting the fluorescence decay curve with double exponential curves (Lakowicz 2006).

Several methods to image fluorescence lifetime have been developed (Lakowicz 2006). For example, time-correlated single-photon counting (TCSPC) uses a low light level, at which at most one photon is detected after each laser pulse (no photon after most laser pulses), and measures the timing between the excitation laser pulse and the detection of the emitted photon. The resulting histogram of the measured delays indicates the fluorescence decay (Lakowicz 2006). By combining this with scanning microscopy, one can obtain the fluorescence decay for each pixel in the image. One drawback of TCSPC is that accumulation of photons sufficient for making the histogram takes a relatively long time. After each photon detection, there is some dead time (approximately microseconds) in the instrumentation, and when the photon-counting rate is higher than the inverse of the dead time, the image can be saturated. To avoid this, one needs to decrease the excitation intensity to decrease photon-counting rates;

thus, the system is optimal for dim samples in which one cannot waste any photons.

Combining Two-Photon Laser Scanning FRET Imaging

Two-photon laser scanning microscopy (2pLSM) enables imaging with high sensitivity and resolution in light scattering tissue (Denk et al. 1990; Svoboda and Yasuda 2006). Thus, combining FRET imaging techniques with 2pLSM allows monitoring of the biochemical state of small cellular compartments located deep within brain tissue (Svoboda and Yasuda 2006; Yasuda 2006). For this reason both 2pLSM ratiometric imaging and FLIM have been used to reveal the signaling cascades that act within dendritic spines to mediate LTP (see below).

Ratiometric FRET is accomplished in 2pLSM by splitting the fluorescence emitted by the donor and acceptor with dichroic mirrors and detecting the separated colors simultaneously by different photomultiplier tubes (PMTs). It has been shown that FRET between

CFP and YFP in single dendritic spines in brain slices can be imaged with two-color 2pLSM (Okamoto et al. 2004). Furthermore, because two-photon excitation of both fluorophores is achieved with a single laser and the image is built by laser scanning, the images of different color are automatically aligned.

More recently TCSPC was combined with 2pLSM (2pFLIM) to image FRET in single dendritic spines in brain slices with high sensitivity (Yasuda et al. 2006; Lee et al. 2009). Instrumentally the combination of 2PLSM and TCSPC is relatively straightforward, as both of them require a pulsed laser and scanning microscope (Yasuda 2006). The pulse rate of a typical mode-lock laser used for 2pLSM is ~ 80 MHz, making the interpulse interval (~ 12 ns) just long enough for measurements of fluorescence decay of typical fluorescent proteins (2–4 ns) after each pulse, while allowing measurements at close to the maximum repetition rate (Yasuda et al. 2006).

Designing High-Sensitivity Sensors for FRET and FLIM

The design criteria for FRET-based sensors are different for ratiometric imaging and FLIM (Yasuda et al. 2006). First, optimal donors for FLIM have monoexponential fluorescence decay, enabling easy calculation of binding fractions (Eq. 5). Although ECFP and its brighter variant Cerulean are the most common donors for ratiometric imaging, their fluorescence decay is multiexponential, making them unsuitable for FLIM. In contrast, EGFP and EYFP have monoexponential decays, making them ideal FLIM donors. As for acceptors, because ratiometric uses both donor and acceptor fluorescence, bright acceptors provide better signal-to-noise ratios. In contrast, FLIM measures donor fluorescence alone. Thus, the quantum yield of the acceptor can be very low, although the absorption coefficient needs to be high for high-yield FRET (Eq. 2). Thus, the recently developed resonance energy acceptor chromophore (REACH; a high-absorbing, dark EYFP variant), can be used as a FLIM acceptor (Ganesan et al. 2006; Murakoshi et al. 2008). Overall, whereas the

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ECFP-EYFP pairs are widely used for ratiometric imaging, the EGFP-mRFP, EYFP-mRFP and EGFP-REACH pairs are used for FLIM (Tramier et al. 2006; Yasuda 2006; Murakoshi et al. 2008).

Most sensors developed so far use ECFP-EYFP pairs and are optimized for ratiometric imaging (Miyawaki 2003; Ni and Zhang 2010). To convert them to FLIM sensors, one can simply replace the ECFP-EYFP pair with EGFP-mRFP or EGFP-sREACH (Murakoshi et al. 2008). For example, the dynamics of actin polymerization in individual spines were first imaged by a ratiometric sensor (Okamoto et al. 2004). The sensor consists of ECFP-actin and Venus (a brighter variant of EYFP) -actin (Okamoto et al. 2004). When ECFP-actin and Venus-actin copolymerize, FRET between CFP and Venus increases (Okamoto et al. 2004). Using this method, Okamoto et al. successfully used two-photon ratiometric imaging to visualize the polymerization of actin during spine structural changes induced by electric stimulation. Later the polymerization fraction was quantified with 2pFLIM in combination with a new actin polymerization sensor made of the mEGFP (monomeric EGFP: monomeric variant of EGFP) and sREACH (super REACH: high-folding REACH variant) pair (Murakoshi et al. 2008).

ACTIVATION PROFILE OF SIGNALING MOLECULES MEASURED BY FRET IMAGING

Two-Photon Photolysis to Stimulate Single Spines

To study signaling in single spines, it is necessary to trigger signaling events in a spatiotemporally controlled manner. The photolysis of caged compounds is one way to achieve this (Nerbonne et al. 1984; Walker et al. 1987; Li et al. 1998; Cai et al. 2004). In particular, because the development of MNI-glutamate, a caged glutamate with a high two-photon cross section, two-photon glutamate uncaging has been applied to study the physiology of single dendritic spines (Matsuzaki et al. 2001). Combining FRET imaging and two-photon glutamate uncaging allows one to study the spatiotemporal

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dynamics of signal transduction in response to single spine stimulation (Harvey et al. 2008b; Lee et al. 2009).

Compartmentalization of CaMKII Signaling

Ca^{2+} /Calmodulin-dependent kinase II (CaMKII) is the most abundant protein in the post-synaptic density (Cheng et al. 2006), and is required for LTP (Silva et al. 1992; Giese et al. 1998; Lisman et al. 2002), spine structural plasticity (Matsuzaki et al. 2004; Lee et al. 2009; Pi et al. 2010), and some forms of learning and memory (Giese et al. 1998; Glazewski et al. 2000; Frankland et al. 2001). CaMKII is a holoenzyme consisting of 12 subunits, and each subunit acts as a serine/threonine kinase (Rosenberg et al. 2006). When $[\text{Ca}^{2+}]$ increases, Ca^{2+} /Calmodulin binds to a CaMKII subunit (Braun and Schulman 1995; Lisman et al. 2002). This changes the structure of the CaMKII subunit from a closed form into an open form, exposing its kinase domain (Rosenberg et al. 2006; Chao et al. 2010). Activation of two adjacent subunits causes autophosphorylation of T286 (Lisman et al. 2002; Chao et al. 2010), which renders its activity independent of the interaction to Ca^{2+} /CaM, thereby keeping them activated after clearance of Ca^{2+} and dissociation of CaM (Saitoh and Schwartz 1985; Lou et al. 1986; Miller and Kennedy 1986; Yang and Schulman 1999). Because of this Ca^{2+} -independent, autonomous activity of phosphorylated CaMKII, some speculated that CaMKII is a biochemical memory that lasts more than hours to maintain LTP (Lisman and Zhabotinsky 2001; Lisman et al. 2002).

The importance of T286 phosphorylation in LTP and learning was confirmed by experiments using mice with the autophosphorylation site mutated to alanine (T286A) (Giese et al. 1998; Hardingham et al. 2003). The presence of the persistent activity, however, has been controversial: Persistent T286 phosphorylation was observed biochemically following LTP (Fukunaga et al. 1995; Barria et al. 1997), whereas other studies showed that the Ca^{2+} -independent activation of CaMKII decays much faster than T286 phosphorylation (Lengyel et al. 2004). Furthermore, pharmacological inhibition of CaMKII

after establishing LTP does not typically affect the maintenance of LTP for most inhibitors (Malinow et al. 1989; Otmakhov et al. 1997; Chen et al. 2001), although one antagonist derived from a natural CaMKII inhibitor reversed LTP maintenance (Sanhueza et al. 2007).

To test the hypothesis of synapse-specific, persistent activity of CaMKII, the ratiometric CaMKII sensor Camui α was developed (Takao et al. 2005). Camui α consists of a CaMKII α subunit in which its ends are tagged with ECFP and Venus. FRET between ECFP and Venus reports the conformational change associated with its activation (Takao et al. 2005). However, the sensitivity was not sufficient for imaging in single spines in slices. More recently, the sensor was further optimized for 2pFLIM. In the new sensor called Green-Camui α , the fluorophores were replaced to the pair of mEGFP and REACH (Ganesan et al. 2006; Murakoshi et al. 2008), which allowed measurement of CaMKII activity in single spines during LTP with high temporal resolution (approximately seconds) (Lee et al. 2009).

When a single spine was stimulated with two-photon glutamate uncaging to induce NMDAR-dependent LTP and associated spine enlargement, spine-specific CaMKII activation was observed (Lee et al. 2009). Contrary to the hypothesis that long-term autonomous CaMKII activity underlies the maintenance of LTP, CaMKII activity lasted only for ~ 1 min, whereas the plasticity lasted more than 1 h (Fig. 1). The study also clarified the impact of T286 phosphorylation as the activity of wild-type Camui α decays with the time constants of 6 sec and 45 sec, whereas the T286A mutant is inactivated within 1 sec. Thus, T286 phosphorylation can underlie a biochemical memory for less than 1 min, but not hours, allowing CaMKII to integrate brief (~ 0.1 sec) synaptic Ca^{2+} signals during repetitive stimulation (Sabatini et al. 2002; Noguchi et al. 2005).

Spreading of Ras Signaling

Ras is a small GTPase important for many forms of synaptic plasticity including LTP, synapse formation, spine structural plasticity, protein

synthesis in dendrites, and gene transcription (Wu et al. 2001; Zhu et al. 2002; Thomas and Hugarir 2004). Because Ras-dependent signaling occurs in many different compartments, including spines, dendrites, and the nucleus, the spatiotemporal dynamics of Ras signaling are considered important in determining downstream effects (Yasuda et al. 2006). Thus, to fully understand the mechanism of Ras signaling, it is essential to measure the spatiotemporal dynamics of Ras activation.

The first Ras activation sensor, Raichu, was designed for ratiometric imaging and consists of CFP-RBD-Ras (without CAAX) -YFP-CAAX in a single polypeptide. RBD is a Ras-binding domain that binds to GTP-bound but not GDP-bound Ras whereas CAAX is a membrane-targeted sequence derived from Ras itself (Mochizuki et al. 2001). Although this sensor worked in cell lines, it never was used for single synapse imaging. Also, the trafficking of this construct may be different from that of Ras. A next-generation Ras sensor, FRas, was designed specifically for 2pFLIM (Yasuda et al. 2006) and 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 (Yasuda et al. 2006). Because RBD binding to Ras competed with Ras inactivation, the affinity between RBD and Ras was decreased by mutating the RBD (R59A), allowing fast reversal kinetics of the sensor (Yasuda et al. 2006). FRas-F in combination with 2pFLIM provided sufficient sensitivity to measure Ras activity in single spines in response to physiologically relevant stimuli (Yasuda et al. 2006; Harvey et al. 2008b). It was further confirmed that the activation kinetics measured by FRas-F are weakly correlated with expression level of Ras (Harvey et al. 2008b).

By combining 2pFLIM-based Ras imaging with two-photon glutamate uncaging, one can monitor Ras signaling in dendrites while stimulating a single spine (Harvey et al. 2008b). When the NMDARs on a single spine are opened, Ras activity increased in the stimulated spine, and rapidly spread into adjacent den-

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drites and spines (Harvey et al. 2008b). Ras activity in surrounding spines is close to ~70% of that in the stimulated spine, but was not sufficient to induce plasticity. Because the expression of dominant-negative Ras or inhibition of the downstream kinase ERK causes inhibition of structural plasticity, these results showed that the Ras-ERK pathway is required, but not sufficient, for inducing structural plasticity. Pharmacological analysis indicated that the Ras-ERK pathway acts in parallel with the CaMKII pathway (Harvey et al. 2008b), which perhaps is required for the spine specificity of synaptic plasticity (Lee et al. 2009).

MODELING SPATIOTEMPORAL DYNAMICS

Degree of Compartmentalization in Spines

Once a protein is activated, the degree of compartmentalization is determined by competition between diffusion out of the spine and inactivation (Harvey et al. 2008b; Lee and Yasuda 2009). If the time constant of the spine-dendrite diffusion coupling (τ_{coupling}) is much greater than the time constant of inactivation ($\tau_{\text{inactivation}}$) (i.e., $\tau_{\text{coupling}} \gg \tau_{\text{inactivation}}$), the activity can be compartmentalized within the stimulated spine: In other words, the protein is inactivated before it spreads into the dendrite. However, this is not a sufficient condition for compartmentalized activity, because if an upstream inducer molecule spreads, the downstream effector molecule will show a diffuse pattern. Conversely, when $\tau_{\text{coupling}} \ll \tau_{\text{inactivation}}$, the activity of the molecule should spread into dendrites.

τ_{coupling} depends on several morphological factors of spines:

$$\tau_{\text{coupling}} = \frac{lV}{sD}, \quad (6)$$

where $V \sim 0.1 \mu\text{m}^3$ is the volume of the spine, $l \sim 0.8 \mu\text{m}$ is the spine neck length, $s \sim 0.008 \mu\text{m}^2$ is the cross-section area of the spine neck, and D is the effective diffusion constant of the molecule. For membrane proteins, $V \sim 1 \mu\text{m}^2$ is the surface area of the spine and $s \sim$

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0.3 μm is the circumference of the spine neck (Svoboda et al. 1996; Bloodgood and Sabatini 2005). Recently, several groups showed that τ_{coupling} is regulated in an activity-dependent manner (Bloodgood and Sabatini 2005; Grun-ditz et al. 2008). Thus, it is likely that synaptic activity regulates the degree of compartmentalization of biochemical signaling in dendritic spines.

The degree of compartmentalization has been measured for three molecules— Ca^{2+} , CaMKII, and Ras—and all of these molecules follow the rules laid out (Lee and Yasuda, 2009). For example, τ_{coupling} for Ca^{2+} is about 100 ms. By comparison, $\tau_{\text{inactivation}}$, or in the case of Ca^{2+} —the extrusion time constant—is ~ 20 ms, which is much faster than τ_{coupling} , producing compartmentalized Ca^{2+} elevation in spines in response to NMDAR activation (Sabatini et al. 2001). The spine-dendrite diffusion coupling for CaMKII is described with two time constants: $\tau_{\text{coupling}} \sim 1$ min and 20 min (Okamoto et al. 2004; Lee et al. 2009). The inactivation also has two time constants: $\tau_{\text{inactivation}} \sim 5$ sec and 45 sec (Lee et al. 2009). Thus, the law predicts that CaMKII is compartmentalized, as shown by the experimental results (Fig. 2) (Lee et al. 2009). On the other hand, Ras has slow

inactivation kinetics with $\tau_{\text{inactivation}} \sim 200$ sec (Yasuda et al. 2006; Harvey et al. 2008b), whereas the diffusion is much faster, $\tau_{\text{coupling}} \sim 5$ sec (Harvey et al. 2008b). Thus, Ras shows a diffusive activity pattern (Fig. 3).

Spatial Profile

Once a molecule spreads into dendrite, it produces a specific pattern of activity that is dependent on its diffusion and inactivation constants and that can be modeled with a one-dimensional reaction–diffusion equation (Noguchi et al. 2005; Harvey et al. 2008b),

$$\frac{\partial \Phi(x, t)}{\partial t} = D \frac{\partial^2 \Phi(x, t)}{\partial x^2} - \frac{1}{\tau_{\text{inactivation}}} \Phi(x, t), \quad (7)$$

where Φ is the spatiotemporal profile of activation, D is the effective diffusion coefficient, and $\tau_{\text{inactivation}}$ is the time constant of inactivation. We assume that the protein is activated at a spine located at $x = 0$, diffuses in the active state with diffusion constant D , and is inactivated at a rate given by $1/\tau_{\text{inactivation}}$. In this case, the

Figure 3. Imaging Ras activation during LTP. (A) Schematic of the Ras sensor. (B) Ras activation during LTP. (C) Spatial profile of Ras activation along the dendrite measured as a function of distance from the stimulated spine ($x = 0$).

steady-state solution is given by (Harvey et al. 2008b)

$$\Phi = \exp(-x/L),$$

where L is the length constant given by

$$L = \sqrt{D\tau_{\text{inactivation}}}. \quad (8)$$

Thus, the length constant of protein signaling is basically determined by two factors. For Ras, $D \sim 0.5 \mu\text{m}^2/\text{sec}$ (Lommerse et al. 2004; Murakoshi et al. 2004) and $\tau_{\text{inactivation}} \sim 200 \text{ sec}$ (Yasuda et al. 2006; Harvey et al. 2008b) yielding $L \sim 7 \mu\text{m}$, consistent with the measured length constant (Fig. 3) (Harvey et al. 2008b). Overall, these approaches can be used to predict the spatial profile of signal transduction from the diffusion coefficient and inactivation time constant as long as the upstream signaling molecules do not spread (Harvey et al. 2008b; Lee and Yasuda 2009).

SOURCE-SPECIFIC ACTIVATION OF SIGNALING MOLECULES VIA NANODOMAIN Ca^{2+}

Ca^{2+} nanodomains, the region near the mouth of open Ca^{2+} channels where $[\text{Ca}^{2+}]$ can be locally very high, have long been proposed to modulate signaling transduction (Neher 1998). The importance of nanodomain Ca^{2+} was shown by the different impacts of the Ca^{2+} chelators BAPTA and EGTA on the activation of Ca^{2+} -dependent processes (Neher 1998; Augustine et al. 2003). Although BAPTA and EGTA have similar dissociation constants for Ca^{2+} , BAPTA chelates Ca^{2+} ions 100 times faster than EGTA and can prevent Ca^{2+} ions from traveling more than a few angstroms by quickly capturing the ions after entry through the channel. In contrast, dependent on the chelator concentration, Ca^{2+} ions can travel many nanometers or even micrometers before being chelated by EGTA. Thus, BAPTA inhibits both global and nanodomain Ca^{2+} elevations, whereas EGTA selectively inhibits global Ca^{2+} elevations. It has been suggested that LTP is mediated via nanodomain

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signal transduction at specific subunits of NMDARs because, under some conditions, LTP induction has been found to be more sensitive to BAPTA and EGTA (Hoffman et al. 2002). Furthermore, pharmacological inhibition of NR2A and NR2B containing NMDARs can differentially impact LTP, suggesting that there may be subunit-specific signal transduction (Liu et al. 2004; Yashiro and Philpot 2008; Foster et al. 2010). Using CaMKII and Ras imaging techniques, we can now directly examine if the activation of these pathways is mediated via nanodomain signaling.

CaMKII

CaMKII interacts with many types of Ca^{2+} channels and transmitter receptors including L-type voltage-sensitive calcium channels (VSCCs) (Hudmon et al. 2005; Wheeler et al. 2008), T-type VSCCs (Welsby et al. 2003), P/Q-type VSCCs (Jiang et al. 2008), dopamine receptors (Liu et al. 2009), muscarinic receptors (Guo et al. 2010), and the NR2B subunit of NMDAR (Bayer et al. 2001; Merrill et al. 2005). This naturally leads to the hypothesis that opening of specific channels activates bound pools of CaMKII via nanodomain Ca^{2+} . Indeed, CaMKII imaging in spines and dendrites supports this hypothesis (Lee et al. 2009). When a neuron is depolarized, CaMKII is activated in both spines and dendrites, with higher activation in dendrites. The spine but not dendritic CaMKII activation is prevented by the L-type channel inhibitor nimodipine. However, the same inhibitor does not reduce the depolarization-evoked Ca^{2+} elevation, suggesting that L-type channels do not contribute to global Ca^{2+} under this stimulus. Thus, CaMKII in spines can sense small calcium flow through L-type channels, but is not sensitive to large Ca^{2+} increases mediated by other VSCCs, consistent with nanodomain-mediated activation.

To confirm Ca^{2+} -CaMKII nanodomain signaling, the sensitivity of CaMKII activation to BAPTA and EGTA was measured (Lee et al. 2009). When whole-cell recordings were obtained with an electrode containing 20 mM BAPTA, CaMKII activation in response to depolarization

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was inhibited. However, the same concentration of EGTA affected CaMKII activation to a smaller degree. Notably, unlike depolarization-induced CaMKII activation, glutamate uncaging-evoked CaMKII activation shows similar sensitivity to EGTA and BAPTA, suggesting that NMDAR-mediated CaMKII activation requires global Ca^{2+} . Because CaMKII is not activated by high Ca^{2+} influx through non-L-type channels in response to depolarization, NMDAR-mediated CaMKII probably requires both nanodomain and global Ca^{2+} .

The source-specific activation of CaMKII produces remarkable activation patterns during LTP induced by pairing postsynaptic depolarization and two-photon glutamate uncaging (Fig. 4) (Lee et al. 2009). The main purpose of the postsynaptic depolarization is to release Mg^{2+} block from NMDARs. However, depolarization also opens VSCCs, and produces large Ca^{2+} elevation in spines and dendrites whose amplitude can be larger than that owing to opening of NMDARs. In addition, whereas NMDAR Ca^{2+} lasts only for ~ 0.1 sec, depolarization-induced Ca^{2+} is more prolonged, and lasts seconds until VSCCs are inactivated and Ca^{2+} decays. However, as discussed above, CaMKII activation in response to depolarization produces high CaMKII activity in dendrites, but much lower in spines (Fig. 4). Subsequent uncaging activates CaMKII only in the stimulated spine (Fig. 4), indicating that only a fraction of CaMKII can be stimulated by large, depolarization-induced Ca^{2+} increases. This is consistent with the fact that depolarization itself

generally does not trigger LTP (Kullmann et al. 1992) even if, under some conditions, depolarization-induced CaMKII activation in spines can be sufficient to induce LTP in a synapse nonspecific manner (Kato et al. 2009).

Ras

Ras signaling is much more diffuse than CaMKII (Fig. 3), and thus source-specific signaling transduction may not occur. The relationship between global Ca^{2+} evoked by back-propagating action potentials and Ras activation in the dendrite has been measured—Ras activation is supralinearly related to Ca^{2+} elevation (Yasuda et al. 2006). However, when Ca^{2+} entry was reduced by pharmacological inhibition of specific classes of Ca^{2+} channels, the degree of Ras activation was independent of which channel was blocked and predicted by the amplitude of the remaining action potential-evoked Ca^{2+} increase (Fig. 5) (Yasuda et al. 2006). Hence, in contrast to CaMKII, Ras is activated by global Ca^{2+} and not by nanodomain Ca^{2+} .

THE FUNCTION OF SPREADING SIGNALS

The differential spread of activity for each signaling molecule suggests that movement of molecules across the spine neck may have important functions. One possibility is that it is required for spine-to-dendrite signaling to bring resources from the adjacent dendrite into stimulated spines. For example, during chemical LTP induction and in response to a

Figure 4. CaMKII activation during glutamate uncaging paired with depolarization. (Figure adapted from Lee et al. 2009; reprinted, with permission, from Nature Publishing Group © 2009.)

Figure 5. Supersensitive Ras activation as a function of Ca^{2+} concentration. (Image modified from Yasuda et al. 2006; reprinted, with permission, from Elsevier © 2006.)

signal that moves from spines to dendrites, endosomes containing transferrin receptors move from dendrites into spines (Park et al. 2004, 2006). In addition, AMPAR exocytosis during LTP occurs in dendrites near the stimulated spines as well as directly in the stimulated spines (Yudowski et al. 2007; Makino and Malinow 2009; Patterson et al. 2010). The exocytosis of AMPARs was imaged using superecliptic pHluorin, a pH-sensitive species of GFP. When the extracellular domain of the GluA1 subunit of AMPA receptor is in the endosome, fluorescence is quenched owing to low pH of that environment. This allows one to image surface AMPA receptors selectively (Yudowski et al. 2007; Makino and Malinow 2009; Patterson et al. 2010). Following photobleaching preexisting surface receptors, individual exocytosis events can be visualized as the appearance of new bright spots (Fig. 6). AMPAR exocytosis occurs over a $\sim 3 \mu m$ stretch of dendrite near the stimulated spine, producing a similar spatial profile to that of the Ras pathway (Fig. 6) (Makino and Malinow 2009; Patterson et al. 2010). Activity-dependent AMPAR exocytosis is indeed Ras-ERK dependent (Patterson et al. 2010), suggesting that the spreading of Ras produces a similar spatial pattern in the downstream effects.

Another interesting possibility is that of heterosynaptic cross talk. Recently, a new form of plasticity that spans a similar length as activated Ras was described (Harvey and Svoboda 2007). Harvey and Svoboda (2007) stimulated a spine

with a train of uncaging pulses that induces synaptic plasticity (suprathreshold stimuli). Within 1–5 min after the first stimulation, weak stimulation, which does not produce plasticity by itself (subthreshold stimuli), was applied to a neighboring dendritic spine less than $\sim 5 \mu m$ away from the originally stimulated spine. This subthreshold stimulus was now sufficient to induce long-lasting synaptic plasticity. The spatiotemporal scale of the facilitation of plasticity is very similar to that of Ras activation, leading to the hypothesis that the spread of activated Ras causes this phenomenon. To test if Ras is required for this form of plasticity, Harvey and Svoboda perfused an inhibitor of signaling downstream from Ras (U0126) between the first, suprathreshold and second, subthreshold stimuli. This manipulation prevented the structural plasticity triggered by the second, subthreshold stimulus, but not to the first, suprathreshold stimulus (Harvey et al. 2008b). Furthermore, the subthreshold stimuli did not produce any additional Ras activation, suggesting that spreading of Ras resulting from the first stimulus was essential to produce the facilitation of plasticity.

DISSECTING SIGNALING NETWORKS IN SINGLE DENDRITIC SPINES

Imaging signal transduction in single dendritic spines has provided many insights into the biochemical mechanisms underlying synaptic plasticity. The same techniques promise to elucidate

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Figure 6. Spatiotemporal profile of AMPA receptor exocytosis. (A) Exocytosis in a stimulated spine. (B) Exocytosis in the dendrite. (C) Spatial profile of AMPA receptor exocytosis (adapted from Patterson et al. 2010; reprinted, with permission, from the author and National Academy of Sciences © 2010).

the spatiotemporal profiles and functions of many other signaling pathways. In principle, we should be able to image all small GTPase proteins using sensors with a similar design to Ras, and recent work has examined Rho and CDC42 GTPases, which are also important for the morphogenesis of spines and the function of synapses (Murakoshi et al. 2011). Also, because most kinases change their conformation when activated, a design similar to that used for CaMKII may work for many other kinases. A general design for kinase sensors based on substrate and phospho-binding domains (Ni and Zhang 2010) also works for FLIM (Harvey et al. 2008a). Although it may not be possible to monitor the activation of all signaling proteins, combining pharmacological and genetic perturbation with FRET-FLIM imaging techniques will produce a detailed view of the signaling cascades that are active in situ (Harvey et al. 2008b; Lee et al. 2009). Furthermore, changes in protein interactions in

PSD—e.g., CaMKII binding to the NMDAR—are thought to be important for synaptic plasticity (Bayer et al. 2001, 2006) and will be amenable to analysis by FRET-FLIM imaging.

Another interesting and developing front is the optical activation and inactivation of intracellular signaling proteins. Genetically encoded photoactivatable GTPases have been realized by several groups (Machacek et al. 2009; Wu et al. 2009; Wang et al. 2010) and will allow testing of the sufficiency of GTPase activity for inducing specific cell responses. Further, by combining readout of signaling with FRET-FLIM and photoactivation/inactivation, the biochemical interactions between two signaling components can be directly measured with high spatiotemporal resolution (Wang et al. 2010). Thus, by developing more techniques to measure and manipulate signaling proteins optically, the mechanisms of signal transduction in dendritic spines will be much better understood.

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