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Signaling in dendritic spines and spine microdomains

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The specialized morphology of dendritic spines creates an isolated compartment that allows for localized biochemical signaling. Recent studies have revealed complexity in the function of the spine head as a signaling domain and indicate that (1) the spine is functionally subdivided into multiple independent microdomains and (2) not all biochemical signals are equally compartmentalized within the spine. Here we review these findings as well as the developments in fluorescence microscopy that are making possible direct monitoring of signaling within spines and, in the future, within sub-spine microdomains.

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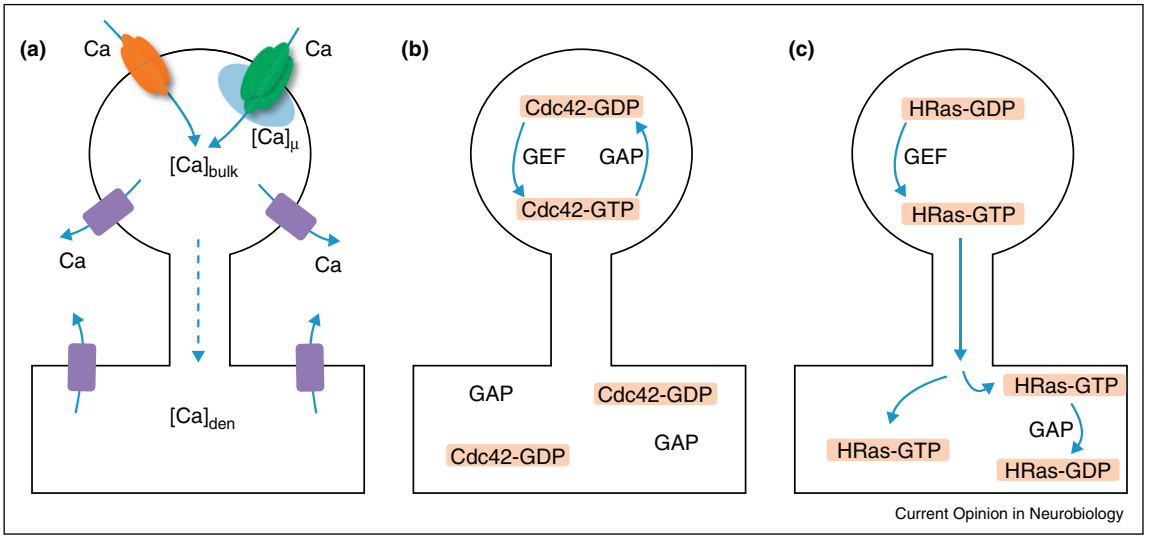
Introduction

In projection neurons of the mammalian brain, the post-synaptic sides of glutamatergic synapses are typically housed within specialized cellular compartments called dendritic spines [1]. In many parts of the brain, including the CA1 subfield of the hippocampus, each sub-femtoliter compartment is associated with one, and only one glutamatergic synapse, and spines are therefore considered morphological correlates of synapses. Each mature spine contains a post-synaptic density (PSD), which houses ionotropic glutamate receptors, other ion channels, scaffolding proteins, and enzymes that transduce and regulate synaptic signals. Spines exist in several morphological classes that are thought to correlate with different developmental stages of the associated synapse (reviewed [1,2]). Morphologically, each spine consists of a bulbous head that is separated from the parent dendrite by a thin neck, which can biochemically isolate the spine head. The biochemical isolation arises from the spine neck, which acts as a barrier to movement of ions, second messengers, and proteins, as well as from the action of enzymes and proteins that limit the half-life of signaling molecules in the spine. Such compartmentalization is thought to endow the associated synapse with spatially confined signaling modes.

The properties of individual spines and the signaling that occurs within them have been extensively studied in the CA1 region of the hippocampus in the context of the induction of long-term potentiation (LTP), a calcium dependent form of synaptic plasticity in which correlated pre and post-synaptic activity leads to strengthening of an individual synapse and enlargement of the associated spine [3,4]. For these reasons, the discussion below focuses on signaling cascades relevant to LTP induction in CA1 pyramidal neurons. Furthermore, many of the results discussed may only apply to mushroom spines, which are thought to be the most developmentally mature class of spines and, because of their relatively large size and high AMPA-type glutamate receptor content, have received the majority of experimental attention [5].

A biochemical cascade that is active in the spine head can be considered to occur in a spatially isolated manner if the duration of the signaling reaction or the lifetime of the signaling molecules (τ_{signal}) is short compared to the time constant of diffusion equilibration across the spine neck (τ_{equil}) – that is, $\tau_{signal} \ll \tau_{equil}$. In this case, the reaction or signal in the spine head will come to an end before significant mixing can occur between the spine head and the dendrite. This is certainly the case for synaptic calcium (Ca) transients, since under physiological conditions, Ca is extruded from the spine to the extracellular environment with a time constant of $\tau_{signal} \sim 15$ ms, which is much shorter than the typical mixing time constants $\tau_{equil} \sim 200$ ms [6]. (Note that this short Ca lifetime results at least in part from a low Ca buffering capacity in spines of CA1 pyramidal neurons, a feature that is not shared with CA2 pyramidal neurons [7].) Based on the diffusion constant of Ca and the small size of the spine head (<1 μm diameter, <1 fL volume), Ca diffuses and equilibrates within the spine head in only ~1 ms. Extrapolating from the studies of Ca, it is often assumed that, for many signaling molecules, the spine head operates as a uniform but diffusionally isolated signaling compartment in which bulk, or volume averaged, concentrations of second messengers and enzymes drive downstream reactions (Figure 1). This model appears to apply to some signaling cascades underlying LTP, which is triggered by the build-up of bulk Ca in the spine (see below) and can be induced in one spine independent of its closely spaced neighbors [8,9].

Figure 1



Modes of signaling in dendritic spines. **(a)** During synaptic activity Ca enters the head of a dendritic spine through multiple classes of ion channels (represented by the green and orange structures) and is rapidly extruded by the action of transporters and pumps (purple). Within microseconds of channel opening, the Ca concentration reaches tens of micromolar in the microdomain around the mouth of an open ion channel (blue shade, $[Ca]_{\mu}$). The localization of Ca-binding and Ca-sensitive proteins in this zone allows for Ca-dependent processes to be triggered by the opening of one class of ion channel and not by another. By contrast, Ca can also diffuse and equilibrate across the spine in milliseconds such that volume-averaged or bulk ($[Ca]_{bulk}$) Ca concentration results from the summed contributions of Ca entering through multiple sources. Ca-dependent proteins not physically associated with Ca channels will experience this lower $[Ca]_{bulk}$ and may be activated by Ca entering through multiple sources. Due to the high efficiency of Ca extrusion and the high resistance to Ca movement across the spine neck (dashed arrow) of mushroom spines in CA1 pyramidal neurons, only minimal Ca accumulates in the neighboring dendrite during synaptic stimulation ($[Ca]_{den}$). **(b)** The small GTPase Cdc42 is activated in the spine during LTP induction, presumably due to the local action of its cognate guanine nucleotide exchange factor (GEF). Despite the mobility of Cdc42-GTP, the action of GTPase-activating proteins (GAPs) in the spine head and dendrite triggers hydrolysis of the GTP before it can substantially accumulate in the dendrite shaft. For this reason, most the dendritic Cdc42 during synaptic stimulation remains bound to GDP and inactive whereas the GTP-bound Cdc42 is limited to the stimulated spine. **(c)** As for Cdc42, the small GTPase HRas is activated in a spine during LTP induction. However, probably due to the relatively lower amounts of cognate GAP in the spine and dendrite, HRas-GTP is able to enter the dendrite and diffuse before being inactivated. For this reason, following LTP induction at one synapse, active HRas is able to enter neighboring spines and reduce the threshold for subsequent LTP induction at the associated synapses.

Nevertheless, Ca-dependent signaling in the spine is more complex that the simple model presented above, and Ca-signaling microdomains, as discussed below, are now known to exist within the spine and the dendrite. Furthermore, the degree of diffusional isolation of the spine is highly molecule dependent such that, for example, within the family of small GTPases, some are functionally restricted to a spine whereas others can signal over relatively large time and space scales [10,11,12••,13••]. Such differential compartmentalization can confer differential temporal and spatial specificity during modification of synapses.

Signaling microdomains within spines

The existence of signaling microdomains is inferred from two classes of pharmacological experiments. First, and specific for Ca, is the differential actions of slow and fast Ca buffers of similar affinities such as EGTA and BAPTA. Both have affinities for Ca in the 200 nM range but EGTA, being a relatively unstructured molecule, has slow kinetics whereas BAPTA, which was designed to be a stiff and fast Ca buffer with rapid kinetics [14]. Within a microdomain, the slow buffer EGTA is unable to bind to Ca in the few microseconds necessary for Ca to diffuse from the source to the physically associated Ca sensor, thereby not affecting microdomain-Ca dependent processes. For example, in the presynaptic terminal, moderate concentrations of BAPTA, but not EGTA, can prevent Ca-dependent neurotransmitter release, and this was one of the original pieces of evidence to infer that vesicle fusion is driven by high and short-lived Ca microdomains located at the mouth of open Ca channels (reviewed [15,16]). Computer simulations suggest that the Ca concentration within nanometers of Ca channels rises and falls within microseconds of channel opening and closing, respectively, and can reach more than 10–100 μM [17,18]. The second type of experiments inferring signaling microdomains involve certain biochemical cascades that can be activated by one source of second messenger but not by a second source, even when both are located in the same microliter volume. Again using an example from the presynaptic terminal, Ca entry through

different classes of voltage-gated Ca channels (VGCCs) in the bouton shows differential efficiency in triggering neurotransmitter release (e.g. [19]). This indicates that all Ca is not the same and is used to infer physical relationships between particular VGCCs and the release machinery. Both lines of evidence were used to conclude that neurotransmitter release is not driven by bulk, volume-average Ca in the bouton, but rather by the short-lived, high-amplitude build-up of Ca that occurs around the mouth of open VGCCs. The physical association of VGCCs and the release machinery has been confirmed by biochemical analysis (e.g. [20]).

On the post-synaptic side, by contrast, similar analyses showed that EGTA blocks the sustained phase of LTP, suggesting that it is triggered by a build-up in bulk Ca and not by microdomain Ca (e.g. [21–23]). In addition, LTP also requires high Ca after the end of the induction protocol for ~1 s, orders of magnitude longer than the lifetime of a microdomain, again supporting the conclusion that bulk Ca build-up is important for LTP [24]. However, although intracellular Ca elevation is found to be sufficient to induce LTP, LTP induction requires opening of NMDA-type glutamate receptors (NMDARs) and not of VGCCs, both of which are found in spines [25]. At first glance, these two types of experimental results are hard to reconcile. The resolution of this apparent conflict may be that spine head Ca transients due to NMDAR opening are much longer lasting and, particularly in the face of depolarization and diminished Mg^{2+} block, much larger in amplitudes than those due to VGCC opening [6,26]. Thus, the privileged ability of NMDAR-dependent Ca influx to induce LTP may reflect the properties of the Ca transient and not the physical association of NMDARs and Ca sensors.

Although microdomain Ca may not be involved in LTP induction, functional Ca signaling microdomains do exist within dendritic spines. In rat hippocampal CA1 pyramidal neurons, Ca influx through nimodipine-sensitive, presumably L-type, VGCCs triggers a kinase cascade that reduces the probability of opening of R-type VGCCs [27]. Other sources of Ca, whose opening raise bulk spine head Ca to similar or higher levels and with similar kinetics, are unable to engage the same kinase cascade, indicating a privileged function of Ca entering through L-type channels. Similarly, Ca influx through L-type VGCCs, while not sufficient to induce LTP and comprising a very small and nearly undetectable fraction of synaptic Ca influx [6,27–29], selectively activates CAMKII in dendritic spines and is necessary for induction of LTP in some protocols [13••,30]. Therefore, these results indicate the existence of Ca signaling microdomains at the mouth of L-type VGCCs. Curiously, opening of non L-type VGCCs is sufficient to induce CAMKII in the dendrite during extended depolarizations [13••].

Separate studies indicate that Ca entry through R-type VGCCs in dendritic spines of mouse hippocampal CA1 pyramidal neurons has a privileged ability to open small conductance Ca-activated potassium (SK) channels [28,31••]. Blocking R-type VGCCs with the spider toxin SNX-482 has the curious effect of increasing the amplitude of synaptic Ca transients while preventing the Ca-dependent opening of SK channels. The activation of SK channels in spines dampens synaptic potentials and Ca influx; conversely, their blockade enhances these signals, thus favoring LTP induction and hippocampal dependent learning [32–34]. Interestingly, inhibition of SK opening represents the mechanism by which activation of post-synaptic muscarinic acetylcholine receptors modulates synaptic transmission, Ca influx, and LTP [31••,35••].

These studies of L-type and R-type VGCC signaling in spines demonstrate privileged coupling between a particular Ca source and the activation of a Ca-dependent process, indicative of functional signaling microdomains. However, a caveat of these studies is that, since it was necessary to monitor spine Ca signaling, they were performed with Ca indicators in the cell. Since Ca indicators must bind Ca, they are Ca buffers and, when used at typical sub-millimolar concentrations, may preferentially interfere with bulk Ca signaling and force microdomain Ca signaling to be the dominant mode [14,36,37].

Molecule specific compartmentalization

During the induction of LTP, the localized accumulation of Ca in a single stimulated spine leads to the spatially restricted and relatively short-lived (~10 s) activation of CAMKII [13••]. Beautiful recent studies exploiting fluorescent reporters of the activation of biochemical cascades demonstrate that LTP induction turns on at least three small GTPases – Cdc42, HRas, and RhoA – in the active spine [10,12••]. As recently reviewed [38], GTP-bound (i.e. active) Cdc42 is largely confined to the active spine whereas GTP-bound forms of HRas and RhoA are able to exit the spine, diffuse along the dendrite over relatively large distances (~10 μm), and enter neighboring, unstimulated spines [10,12••]. The entry of active HRas into neighboring spines lowers the threshold for subsequent LTP induction over the next ~10 min [10].

The differential ability of an activated GTPase to move out of the potentiated spine and reach neighboring spines depends on (1) the intrinsic mobility of the protein, (2) the distribution and kinetics of the cognate guanine nucleotide exchange factor (GEF), which activates the GTPase by loading it with GTP, and (3) GTPase-activating protein (GAP), which deactivate the GTPase by initiating hydrolysis of the bound GTP. Thus, the spatiotemporal domain of synaptically evoked GTPase signaling can be highly individualized for each GTPase. In fact, the three GTPases discussed above have similar mobility across the spine neck, indicating that the

differences in the spatial spread of their activated GTP-bound forms reflect secondary factors. For example, continued activation of Cdc42 by GEFs in the spine head coupled with rapid inactivation in the spine and in the dendrite shaft by GAPs would produce a maintained gradient across the spine neck [12••] (Figure 1). Thus the gradient of active Cdc42, as for Ca, reflects the location of sources and sinks and the short half-life of the activated (or for Ca, bound) molecule as compared with its diffusion coefficient.

Conversely, signaling cascades can be initiated in the dendrite shaft but subsequently enter and act in spines. This includes CAMKII and PKA, which have large inactive reservoirs in the parent dendrite that can be quickly engaged and mobilized (e.g. [39••,40]). The physiological importance of mobile dendritic CAMKII is unclear as CAMKII is present in resting spines and is activated by synaptic Ca influx; nevertheless, dendritic CAMKII moves into the spine during LTP induction or strong NMDAR activation [13••,40–45]. Recent work has highlighted that in many cell types PKA action on synaptic NMDARs enhances Ca influx through the open channel, which can have important consequences for the subsequent induction of plasticity [46,47••,48••]. In this manner, spatially diffuse signals, such as activation of PKA following stimulation of $G_{\alpha s}$-associated G-protein coupled receptors, can send a signal from the dendrite into the spine to modulate the function of post-synaptic terminals [39••]. Additional complexities of PKA signaling are posed by the PSD localization of A-kinase anchoring proteins (AKAPs), which provide scaffolds that place PKA at the mouth of ion channels, often in conjunction with phosphatases, phosphodiesterases, or other kinases [49,50]. This tight physical association between kinase and substrate once again suggests highly localized signaling. Indeed AKAPs regulate synapse structure, function, and plasticity in a variety of systems (e.g. [51–54]).

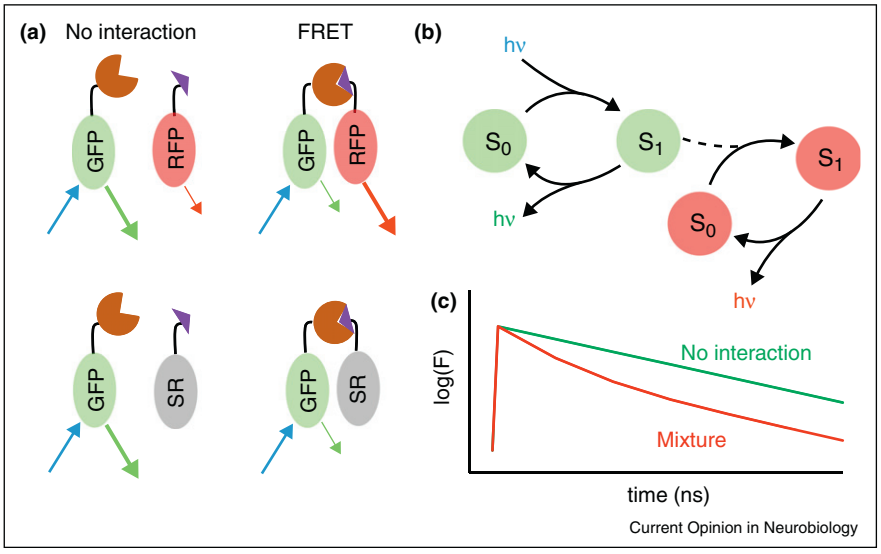
New microscopy approaches for the analysis of signaling in spines

The remarkable insight that has been gained into the signaling cascades that are active in individual spines during synaptic activity and plasticity is due to equally remarkable advances in fluorescence microscopy. In particular, fluorescence-lifetime imaging microscopy (FLIM), a method to measure Förster resonance energy transfer (FRET) (Figure 2), provides an inherently quantitative measure of protein–protein interactions or of intramolecular conformational state (reviewed [55]). This approach has been used with genetically encoded fluorescent sensors that change fluorescent state upon activation of the pathway of interest. In ratiometric FRET, the proximity of donor and acceptor fluorophores fused to two potentially interacting proteins is reported by the ability of donor excitation to trigger fluorescence emission by the acceptor [56] (Figure 2a). This technique is difficult to combine with 2-photon microscopy due to overlapping excitation spectra of many fluorophores under nonlinear excitation. In FLIM, the analysis is limited to the lifetime of the excited state of only the donor fluorophore, which is shortened when a potential path to transfer energy to a second fluorophore is present [55–57] (Figure 2b and c). The utility and robustness of this approach is greatly increased by the development of 'dark' acceptors that are capable of absorbing the energy from the excited acceptor but do not emit fluorescence [58,59]. When coupled with 2-photon laser scanning microscopy, FLIM allows measurement of signaling state in dendrites and spines with ~1 s and ~1 μm spatiotemporal resolution within complex brain tissue [10,11,12••,13••,60]. Furthermore, when combined with the use of 2-photon laser photolysis of caged glutamate to stimulate a single visualized dendritic spine, the biochemical signaling that underlies post-synaptic plasticity can be studied in exquisite detail (e.g. [12••]).

These approaches were used to reveal the spatiotemporal extent of signaling discussed above. For example, the ratio of GTP-bound and GDP-bound forms of small GTPases is determined by measuring the fluorescence lifetime of the GTPase fused to a donor fluorophore and a protein that binds the GTP-bound GTPase fused to an acceptor fluorophore [11,55]. Conversely, CAMKII activation is monitored by an intramolecular interaction within a doubly tagged CAMKII [13••,45,61]. Fluorescent reporters used for FLIM are often based upon closely related sensors developed for traditional ratiometric FRET (e.g. [62] for Ras and [45] for CAMKII). The use of FLIM with synthetic Ca indicators to measure intracellular Ca dynamics was reported nearly 20 years ago [63], but has yet to achieve widespread use. Nevertheless, development of FLIM reporters of protein–protein interactions, enzymatic activity, metabolic state, and ion concentration continues (e.g. [64–68]) and will probably accelerate.

A further imaging advance with great promise for the analysis of synaptic signaling, which has just started to provide real-time and functional information, is super-resolution fluorescence microscopy (reviewed in [69]). Since the sizes of synaptic elements – spine head, spine neck, synaptic cleft, PSD, presynaptic active zone, synaptic vesicles – are at best similar to and often far smaller than the diffraction limited resolution of confocal (150–200 nm) and 2-photon (~450 nm) microscopes, they cannot typically be accurately resolved. Stochastic optical reconstruction microscopy (STORM) exploits antibodies labeled with synthetic photoswitchable fluorophores to localize proteins with 3D precision of tens of nanometers [70••,71] and has been used to visualize the structure of the synapse in stunning detail [72••]. The conceptually similar approach of photoactivatable localization

Figure 2



Förster resonance energy transfer and fluorescence lifetime imaging microscopy. **(a)** *top*, Two potentially interacting proteins are tagged with green and red fluorescence proteins (GFP and RFP, respectively). When the proteins are not interacting (*left*), excitation of GFP with blue light results in robust green fluorescence and minimal red fluorescence. By contrast, when the proteins are physically associated and the fluorophores are properly aligned, energy is transferred from GFP to RFP such that blue light excitation results in less green fluorescence and more red fluorescence. In this example, GFP is the 'donor' and RFP is the 'acceptor' but it is also possible to use cyan and yellow fluorescence proteins, respectively, as well as other genetically encoded fluorophore pairs. *bottom*, As above but the RFP has been replaced with a mutated genetically encoded fluorophore sREACH (SR) that is capable of absorbing energy but has little or no fluorescence. The elimination of the fluorescence from the acceptor precludes its use for traditional FRET but simplifies FLIM. **(b)** GFP (green) transitions from the ground state (S0) to the excited state (S1) on absorption of a blue photon ($h\nu$). In the absence of FRET, decay from the excited state to the ground state is accompanied by emission of a green photon. The fluorescence is spectrally shifted relative to the excitation light because of energy loss due to molecular interactions and conformational changes in the excited state. When FRET occurs due to well aligned interaction between the two fluorophores, energy is transferred directly between the fluorophores without emission of a photon from the donor (dashed line). Absorption of energy by the RFP causes a transition from the ground (S0) to excited state (S1). Subsequent energy loss and decay back to the ground state results in emission of a red photon. When FRET occurs, the presence of an additional path for GFP out of the excited state reduces its lifetime in S1 compared to when there is no FRET. **(c)** Based on the simple two state model of GFP fluorescence portrayed in panel B, a short pulse of excitation light of GFP in the unbound ('no interaction') state abruptly increases fluorescence emission, which decays mono-exponentially. When plotted on a semi-log plot as a function of time, fluorescence emission decays linearly ($F(t) = F(0)\exp(-t/\tau_D)$), where F is the number of fluorophores, t is time and τ_D is the decay time constant of donor only fluorescence (i.e. without FRET)). Lifetimes of many genetically encoded and synthetic fluorophores are on the order of a few nanoseconds. When a mixture of bound and unbound GFP is present, the fluorescence emission decay is bi-exponential and has multiple linear components on a semi-log plot ('mixture') ($F(t) = F(0)[P_D \exp(-t/\tau_D) + P_{DA} \exp(-t/\tau_{DA})]$, where τ_{DA} is the decay time constant of donor fluorescence decay in the donor-to-acceptor FRET process, P_D and P_{DA} are the fraction of donor fluorophores that are free undergoing FRET, respectively ($P_D + P_{DA} = 1$).

microscopy (PALM) uses genetically encoded photoswitchable fluorophores [73,74]. Furthermore, combinations of photoswitchable and single molecule tracking approaches reveal single protein trajectories in living cells with nanometer resolution [75–77]. A third approach, stimulated emission depletion microscopy (STED), physically limits the distribution of excited fluorophores to a volume below diffraction limit [78] and has been used for live neuron imaging [79,80••,81], including analysis of the dynamics of intracellular actin and synaptic vesicles in real-time [82,83••]. Furthermore, STED has been combined with 2-photon excitation and is suitable for closed-loop adaptive optics to potentially allow nanoscale imaging deep in the brain tissue [80••].

These super-resolution fluorescence approaches are already revolutionizing the static analysis of synapse morphology, structure, and molecular organization and provide complementary approaches to electron microscopic tomography. The integration of these approaches with FLIM, Ca-sensitive fluorophores or state-sensitive fluorophores, promises to allow direct visualization of the real-time function of signaling in microdomains. These techniques will probably continue to reveal the extraordinary intricacy of organization and signaling within dendritic spines that underlies the rich phenomenological repertoire of synapse regulation and plasticity.

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