Ca^{2+} sensor proteins in dendritic spines: a race for Ca^{2+}

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Dendritic spines are believed to be micro-compartments of Ca^{2+} regulation. In a recent study, it was suggested that the ubiquitous and evolutionarily conserved Ca^{2+} sensor, calmodulin (CaM), is the first to intercept Ca^{2+} entering the spine and might be responsible for the fast decay of Ca^{2+} transients in spines. Neuronal calcium sensor (NCS) and neuronal calcium-binding protein (nCaBP) families consist of Ca^{2+} sensors with largely unknown synaptic functions despite an increasing number of interaction partners. Particularly how these sensors operate in spines in the presence of CaM has not been discussed in detail before. The limited Ca^{2+} resources and the existence of common targets create a highly competitive environment where Ca^{2+} sensors compete with each other for Ca^{2+} and target binding. In this review, we take a simple numerical approach to put forth possible scenarios and their impact on signaling via Ca^{2+} sensors of the NCS and nCaBP families. We also discuss the ways in which spine geometry and properties of ion channels, their kinetics and distribution, alter the spatio-temporal aspects of Ca^{2+} transients in dendritic spines, whose interplay with Ca^{2+} sensors in turn influences the race for Ca^{2+}.

Keywords: Ca^{2+}, neuronal calcium signaling, neuronal calcium sensor, calcium-binding protein, dendritic spine, binding affinity, calcium dynamics, protein-protein interaction
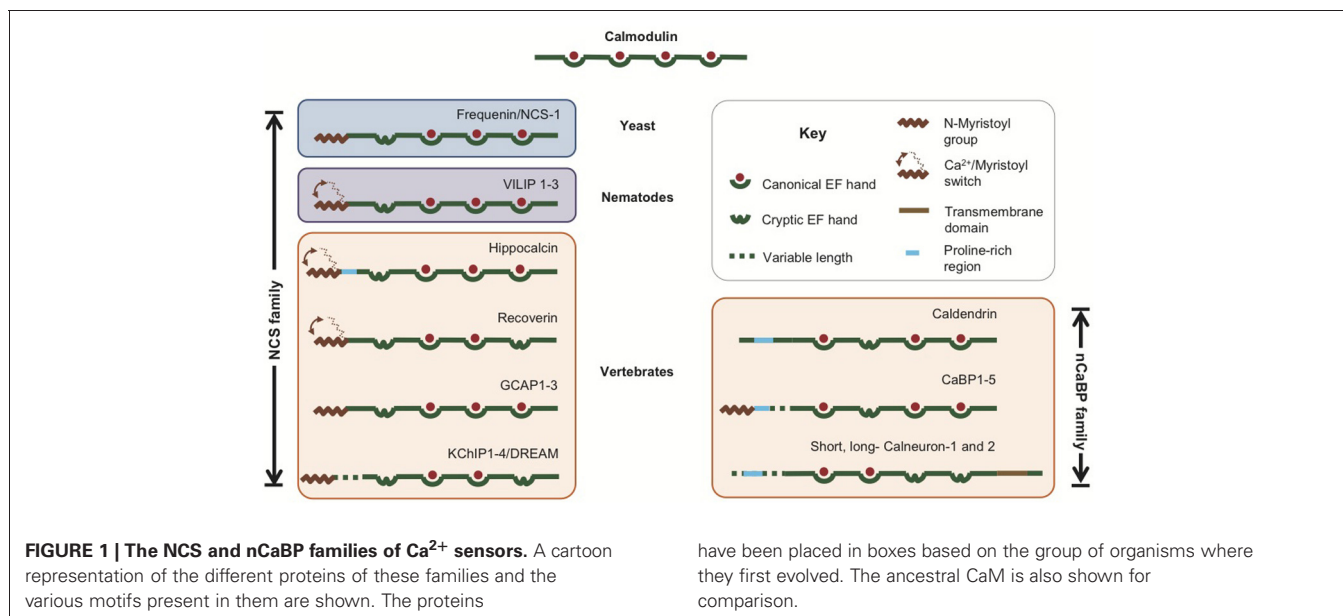
INTRODUCTION

In the human brain, spinous synapses on pyramidal neurons are the most abundant synapse type in the cerebrum and Ca^{2+} signaling in spines has been extensively studied. A largely overlooked area of neuronal Ca^{2+} signaling, though, is the functional role of EF-hand Ca^{2+}-binding proteins of the calmodulin (CaM) superfamily in dendritic spines. Traditionally, these proteins have been assigned to the neuronal calcium sensor (NCS) and neuronal calcium-binding protein (nCaBP) families, all of which are evolutionarily related to the ancestral CaM (**Figure 1**). Being particularly abundant in brain and retina, members of the NCS and nCaBP family have been implicated in a plethora of different cellular events (see Burgoyne, 2007; and Mikhaylova et al., 2011), although their exact synaptic function is largely unknown.

EVOLUTION OF NCS AND nCaBP FAMILIES OF PROTEINS

The NCS family of Ca^{2+} sensors (**Figure 1**) has been named after a group of proteins initially thought to be specifically expressed in neurons (De Castro et al., 1995). This group originated from the ancestral frequenin/NCS-1 and has diversified during evolution. Several reviews cover the topics of evolution and function of the NCS family of proteins (Burgoyne, 2007; Mikhaylova et al., 2011). Briefly, on the basis of sequence analysis, these proteins have been grouped into five classes, labeled in the order of their appearance during evolution (Burgoyne and Weiss, 2001; Burgoyne, 2007). Class A consists of NCS-1 or frequenin which appeared first in yeast. Visinin-like proteins or VILIPs evolved first in *Caenorhabditis elegans* and constitute the class B. With the evolution of the vertebrate eye, two new classes—C and D arose

which comprise recoverin and guanylate cyclase activating proteins (GCAPs). Class E includes the voltage-gated K$^+$ channel (K$_v$) interacting proteins or KChIPs and appeared first in insects. The mammalian genome encodes a single NCS-1, five VILIPS (hippocalcin, neurocalcin-δ, VILIPs1-3), a single recoverin, three GCAPs (GCAP1–3) and four KChIPs (KChIP 1–4) which exist in multiple isoforms. The various proteins of the NCS family show roughly <20% sequence identity with CaM. They possess four EF-hands out of which only two or three are capable of binding Ca^{2+}. All of the members except KChIP2 and KChIP3 show an N-terminal myristoylation consensus sequence (**Figure 1**). This post-translational modification is important for their membrane localization. The Ca^{2+} binding is generally cooperative in most of the members and they show a much higher affinity for Ca^{2+} compared to CaM, while many of them also bind Mg^{2+} (Mikhaylova et al., 2011).

The nCaBP family of proteins (Seidenbecher et al., 1998; Haeseleer et al., 2000; Wu et al., 2001; Laube et al., 2002; Mikhaylova et al., 2006, 2009, 2011; McCue et al., 2010a,b) comprising caldendrin/CaBPs 1–5 and calneurons-1 and -2 arose much later during evolution and are found only in vertebrates (**Figure 1**). With respect to their EF-hands, they show a greater similarity to CaM than the NCS-1 family. It is, therefore, believed that the nCaBP family has evolved directly from the ancestral CaM (Seidenbecher et al., 1998; Haeseleer et al., 2000; Wu et al., 2001; Mikhaylova et al., 2006, 2011; McCue et al., 2010a). Like the NCS family, the nCaBPs too possess cryptic EF-hands and a few members are also N-myristoylated (CaBPs 1 and 2). A common distinctive feature is the presence of four extra amino

FIGURE 1 | The NCS and nCaBP families of Ca²⁺ sensors. A cartoon representation of the different proteins of these families and the various motifs present in them are shown. The proteins have been placed in boxes based on the group of organisms where they first evolved. The ancestral CaM is also shown for comparison.

acids in the linker region between the two EF-hand pairs in Caldendrin/CaBPs (Haeseleer et al., 2000). Besides this common feature, the family members show diversity in the N-terminal region, which, in case of caldendrin, CaBP1 and CaBP2, is due to alternate splicing (Haeseleer et al., 2000; Laube et al., 2002; Mikhaylova et al., 2011). Calneurons (also called CaBP7 and 8) are a subfamily that has evolved independently from caldendrin/CaBPs with a different EF-hand organization, much higher Ca²⁺-binding affinities and a carboxy-terminal transmembrane domain (Wu et al., 2001; Mikhaylova et al., 2006, 2009; McCue et al., 2009, 2011; Hradsky et al., 2011). They have been assigned to the nCaBP family largely based on sequence similarity (Mikhaylova et al., 2006, 2011; McCue et al., 2010a).

THE RACE FOR Ca²⁺: NCS AND nCaBPs IN DENDRITIC SPINES

Dendritic spines are considered as microcompartments of Ca²⁺ signaling (Yuste and Denk, 1995; Yuste et al., 2000; Sabatini et al., 2001) with faster Ca²⁺ decay kinetics than their parent dendrites (Cornelisse et al., 2007). "Fast" Ca²⁺ buffers such as calbindin D28K are thought to be important for this increased rate of decay of Ca²⁺-transients in spines immediately after the closing of Ca²⁺ channels (Keller et al., 2008). It has also been assumed that these fast buffers are the first to intercept Ca²⁺ entering the spine. In a landmark study by Faas et al. (2011), it was found utilizing 1-(2-Nitro-4,5-dimethoxyphenyl)-N,N,N′,N′-tetrakis[(oxycarbonyl)methyl]-1,2 ethanediamine (DM-nitrophen)-Ca²⁺ uncaging experiments, that CaM binds Ca²⁺ at a faster rate than previously thought and the Ca²⁺-association to the N-terminal lobe turned out to be even faster than those of calbindin. Notably, calbindin D28K is absent in CA3 pyramidal cells and is expressed only at very low levels in a sub-population of CA1 pyramidal cells (Sloviter, 1989; Czarnecki et al., 2005; Jinno and Kosaka, 2010). Other important Ca²⁺ buffers, like parvalbumin and calretinin are also not expressed in

CA1 and CA3 pyramidal neurons of the hippocampus (Sloviter, 1989; Baimbridge et al., 1992; Résibois and Rogers, 1992; Czarnecki et al., 2005). Thus, CaM, with its fast Ca²⁺-binding ability, high abundance, and ubiquitous expression, is most likely the principal buffer in these neurons (also discussed in Kubota et al., 2008). Its Ca²⁺-dependent targets are numerous and regulate diverse cellular events, making it a very important Ca²⁺ sensor as well. An important question thus arises—how do other NCS and nCaBP proteins function in the presence of CaM? With respect to the abundance and fast association rate of CaM and the steep and short Ca²⁺ transients in spines, would other sensors have a chance at all to compete for Ca²⁺ binding? In many cases, CaM and NCS/nCaBP proteins associate with the same target with different functional outcomes (**Figure 2**). However, it is still essentially unclear how they can compete with CaM and with each other for target interactions particularly in dendritic spines. In this opinion type review, we focus on these questions and provide some numerical reasoning which might be useful for future experiments. Keeping non-specialist readers in mind, we approach these questions in a step-wise manner and although some of the initial assumptions present an over-simplified view of this very complex and dynamic system, we hope that this approach will help to better appreciate the complexity of neuronal Ca²⁺ signaling and the race for Ca²⁺.

ABUNDANCE AND AFFINITIES OF Ca²⁺ SENSORS

Table 1A provides an estimate of the abundance and Ca²⁺-binding affinity of five important EF-hand Ca²⁺ sensors found in dendritic spines of hippocampal pyramidal neurons. The precise protein concentrations of these sensors in neuronal subcompartments such as the synapse are unknown. A detailed discussion on the concentration of CaM in spines is available in Faas et al., 2011. For most brain regions including cerebral cortex, hippocampus, caudate nucleus, striatum, and amygdala, an average CaM
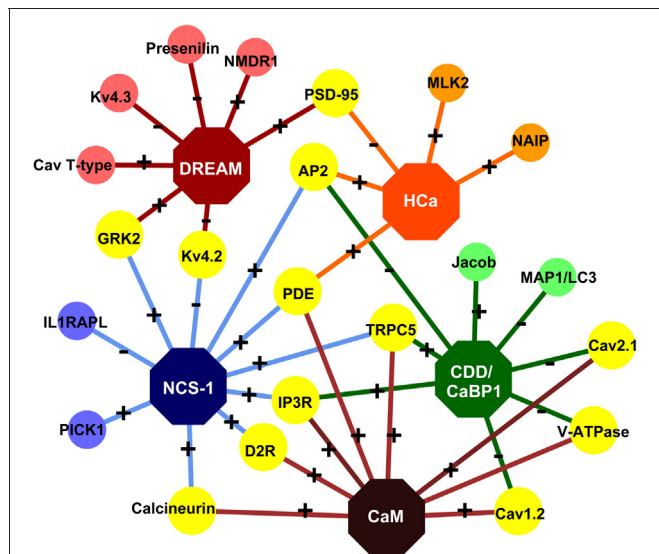
FIGURE 2 | The NCS/nCaBP interactome. Some of the known targets of the NCS and nCaBP family members in the spine are shown. Specific interaction partners are color-coded according to the color of the sensor. Common interaction partners are shown in yellow. "+" and "−" indicate Ca²⁺-dependence and -independence of an interaction, respectively. AP2: Clathrin adaptor protein 2 [Haynes et al. (2006)]; Calcineurin [Schaad et al. (1996); Xia and Storm (2005)]; Cav1.2: Voltage-gated (L-type) Ca²⁺ channel [Zhou et al. (2004), (2005); Tippens and Lee (2007); Dick et al. (2008)]; Cav2.1: Voltage-gated (P/Q-type) Ca²⁺ channel [Lee et al. (2000), (2006); Few et al. (2005)]; CavT-type: Voltage-gated (T-type) Ca²⁺ channel [Anderson et al. (2010)]; CDD: Caldendrin; D2R: Dopamine D2 receptor [Bofill-Cardona et al. (2000); Kabbani et al. (2002); Woll et al. (2011)]; GRK2: G protein-coupled receptor kinase 2 [Kabbani et al. (2002); Ruiz-Gomez et al. (2007)]; HCa: Hippocalcin; IL1RAPL: Interleukin like-1 receptor accessory protein-like protein [Bahi et al. (2003)]; IP3R: Inositol 1,4,5-Trisphosphate Receptor [Hirota et al. (1999); Yang et al. (2002); Schlecker et al. (2006)]; Jacob [Dieterich et al. (2008)]; Kv4.2: Voltage-gated potassium channel 4.2 [An et al. (2000); Nakamura et al. (2001)]; Kv4.3: Voltage-gated potassium channel 4.3 [An et al. (2000)]; MAP1/LC3: microtubule-associated protein 1A/1B light chain 3 [Seidenbecher et al. (2004)]; MLK2: Mixed-Lineage Kinase 2 [Nagata et al. (1998)]; NAIP: Neuronal apoptosis inhibitory protein [Mercer et al. (2000); Lindholm et al. (2002)]; NMDR1: NMDA Receptor NR1 subunit [Zhang et al. (2010)]; PDE: cyclic nucleotide phosphodiesterase [Schaad et al. (1996); Haynes et al. (2006)]; PICK1: Protein Interacting with C-Kinase 1 [Jo et al. (2008)]; Presenilin [Buxbaum et al. (1998)]; PSD-95: postsynaptic density-95 protein [Jo et al. (2010); Wu et al. (2010)]; TRPC5: Transient receptor potential channel 5 [Kinoshita-Kawada et al. (2005); Ordaz et al. (2005); Hui et al. (2006)]; V-ATPase: Vacuolar Type H+-Adenosine 5-Triphosphatase [Haynes et al. (2006)]. The figure was created with the help of Cytoscape 2.8 [Cline et al. (2007)].

concentration of about 100 μM has been reported (Vargas and Guidotti, 1980; Kakiuchi et al., 1982; Klee and Vanaman, 1982; Kitajima et al., 1983; Sano and Kitajima, 1983; Teolato et al., 1983; Biber et al., 1984). The values reported for the hippocampus range from 74 to 156 μM (Kakiuchi et al., 1982; Klee and Vanaman, 1982; Biber et al., 1984). With the exception of hippocalcin, which is very abundant in hippocampus (**Table 1**), other sensors will most likely be expressed at much lower levels with an average cellular concentration that is estimated to range between 1 and 10 μM (Furuta et al., 1999; Burgoyne, 2007; Mikhaylova et al., 2011).

Following excessive synaptic activity, the induction of back-propagating dendritic action potentials (bAPs) may result in Ca²⁺ levels up to 50 μM within a dendritic spine (Faas et al., 2011). As a starting point, we therefore, considered a hypothetical situation in which Ca²⁺ sensors equilibrate with the 50 μM Ca²⁺ that enters the spine. This assumption is most likely not valid for all sensors as Ca²⁺ transients occurring during a single action potential are very brief and favor fast buffers (Markram et al., 1998). The actual time required for equilibration depends on the association and dissociation rates of Ca²⁺ binding (Markram et al., 1998) and this has to be correlated with the rate of Ca²⁺ influx. We discuss this aspect in detail in a later section. Thus, an equilibrium will possibly even not be reached during slower transients and high frequency dendritic spiking. In addition, since the structural unit that binds Ca²⁺ is a single EF-hand motif, we have initially treated the spine as a bag full of many EF-hands with different affinities for Ca²⁺, corresponding to the global affinity of the parent protein. This is also not a realistic assumption as EF-hand motifs pair up to form EF-hand domains and these domains, even within a single protein, show distinct affinities, binding and dissociation rates as well as cooperativity in Ca²⁺-binding (Grabarek, 2006; Gifford et al., 2007). Based on these simplistic assumptions, we calculated the parameters, E_b, which is the concentration of Ca²⁺-bound EF hands of a particular protein, and P_{sat}, the maximum concentration of that protein that can possibly get saturated with Ca²⁺. These calculations were done for each protein separately and independent of the others. We have also calculated the buffer capacities (κ_B; Neher and Augustine, 1992) of these proteins at resting [Ca²⁺] of 100 nM in order to give a general idea about the steady state distribution of their Ca²⁺-bound forms during smaller Ca²⁺ transients. The Ca²⁺ buffer capacity of a spine, which is a function of the cumulative buffer capacities of its Ca²⁺-binding proteins, determines the peak amplitude and the decay rate of its Ca²⁺ transients. The kinetic profile of a Ca²⁺ transient in the spine with a higher buffer capacity has a smaller peak amplitude and a lower decay rate than the spine with lower buffer capacity. Based on the calculations in **Table 1A**, we ranked the sensors in different categories, shown in **Table 1B**. Since this review focuses mainly on the rising phase of Ca²⁺ transients, we have skipped the category of buffer capacity in **Table 1B**.

Dendritic spine heads of hippocampal pyramidal neurons have an average diameter of 0.5 μm and a volume of 0.062 fL (Harris and Stevens, 1989), and contain Ca²⁺ buffers at 210 μM concentration (Cornelisse et al., 2007). If [Ca²⁺] increases up to 50 μM in the spine, this means that ∼2000 Ca²⁺ ions enter the spine at which point, >6000 Ca²⁺ binding protein molecules, roughly accounting for >20,000 EF hands, out of which ∼4000 belong to CaM alone, must compete with each other to bind Ca²⁺. Under the category of total abundance (P_t) in **Table 1B**, CaM appears to be a clear winner in the race for Ca²⁺ by a very large margin, followed by hippocalcin. However, owing to its much greater affinity to Ca²⁺, the concentration of Ca²⁺-saturated hippocalcin exceeds that of Ca²⁺-saturated CaM (given as parameter P_{sat}). Provided Ca²⁺ saturation is essential for the activation of a sensor, in the competition for binding a low abundant Ca²⁺-dependent target having the same affinity for all the sensors, hippocalcin would out-compete CaM. Other sensors,

Table 1A | Concentration, affinity, and other parameters of selected neuronal Ca^{2+} sensors and CaM.

Protein	P_t (μM)	N	K_{dCa} (μM)	E_t (μM)	E_b (μM)	P_{sat} (μM)	κ_B at 100 nM Ca^{2+}	References
Hippocalcin	35.6	3	0.324	106.8	49.72	16.57	192.48	Furuta et al., 1999; O'Callaghan et al., 2003.
NCS-1	10[#]	3	0.440[§]	30	29.37	9.79	45.27	Aravind et al., 2008
DREAM	10[#]	2[§]	~1	20	19.37	9.68	16.53	Osawa et al., 2005
Caldendrin	10[#]	2[§,α]	7[α,δ]	20	16.54	8.27	2.78	Wingard et al., 2005
CaM	100	4	5.85[§]	400	49.18	12.29	66.10	Faas et al., 2011

[#]*Estimated approximate cellular levels;* [α] *Value published for the isoform, S-CaBP1; P_t, Total protein concentration; N,* [§] *number of functional EF hands. Those that bind Mg^{2+} constitutively have been excluded (e.g., DREAM, Caldendrin); K_{dCa},* [δ] *Global dissociation constant, which is the geometric mean of dissociation constants of individual sites of Ca^{2+} binding. In case of NCS-1 and Caldendrin, the dissociation constant of Mg^{2+}-bound protein/isoform is shown. In case of CaM, the geometric mean of the global dissociation constants (geometric mean of the T- and R- forms of an individual EF- hand domain) of the N- and C- terminal EF- hand domains [Faas et al. (2011)] is shown. E_t, Concentration of functional EF-hands = P_t*N; E_b, Concentration of Ca^{2+}-bound EF-hands obtained by solving the equation, $E_b(K_d + Ca_t + E_t - E_b) - (E_t \times Ca_t) = 0$, where Ca_t = total Ca^{2+} concentration = 50 μM. P_{sat}, maximum concentration of Ca^{2+}-saturated Protein = E_b/N; κ_B Buffer capacity = $K_a[E_t]/(1 + [Ca^{2+}]K_a)^2$, where $K_a = 1/K_{dCa}$ [Neher and Augustine (1992)].*

Table 1B | Ranking of various Ca^{2+} sensors under different categories (Absolute values calculated in Table 1A are shown in brackets).

Rank	P_t (μM)	E_t (μM)	K_{dCa} (μM) in decreasing order	E_b (μM)	P_{sat} (μM)
1	CaM (100)	CaM (400)	Hippocalcin (0.324)	Hippocalcin (49.72)	Hippocalcin (16.57)
2	Hippocalcin (35.6)	Hippocalcin (106.8)	NCS-1 (0.44)	CaM (49.18)	CaM (12.29)
3	NCS-1, Caldendrin and DREAM (10)	NCS-1 (30)	DREAM (1)	NCS-1 (29.375)	NCS-1 (9.79)
4		Caldendrin and DREAM (20)	CaM (5.85)	DREAM (19.37)	DREAM (9.68)
5			Caldendrin (7)	Caldendrin (16.54)	Caldendrin (8.27)

which include NCS-1, DREAM, and caldendrin with a low P_{sat} value, would have a much lesser chance to interact with this target under these conditions.

As stated earlier, the validity of the equilibrium assumption made above depends on the Ca^{2+} binding kinetics of the Ca^{2+} sensors (or their EF-hands) and the Ca^{2+} influx rate. Differences in the binding kinetics of Ca^{2+} sensors could result in a non-equilibrium concentration distribution of Ca^{2+}-bound proteins leading to P_{sat} values different from those in **Table 1A**. Obtaining this non-equilibrium concentration distribution necessitates a thorough understanding of the kinetics of all these sensors. Unfortunately, the kinetic data for most of these sensors is not available in the literature.

ENRICHMENT AND SEQUESTRATION OF Ca^{2+} SENSORS

While CaM is soluble and probably uniformly distributed in the cytosol (but see below), most NCS and nCaBP proteins are enriched in specific sub-cellular compartments, such as plasma membrane, golgi, endoplasmic reticulum (ER), and post-synaptic density (PSD; an electron-dense region with post-synaptic membrane thickening and enriched with cytoskeletal elements, scaffolding proteins and neurotransmitter receptors). The mechanism for membrane attachment is largely based on an N-terminal myristoyl group that provides a lipid anchor that interacts with certain phospholipids unique to the membranes of the organelle (O'Callaghan et al., 2005; Mikhaylova et al., 2011). While in case of hippocalcin, this myristoyl

group is buried in the apo protein and gets exposed in a Ca^{2+}-dependent manner—the so called Ca^{2+}-myristoyl switch-, the myristoyl group in NCS-1 is probably constitutively exposed and membrane-bound. Caldendrin gets enriched at the PSD by a yet unknown mechanism (Seidenbecher et al., 1998; Laube et al., 2002).

The protein concentration (P_t) stated in **Table 1A** estimates the global concentration of the proteins in neurons. Could accumulation at a specific organelle be effective enough to increase the concentration of these proteins to levels greater than CaM? In general, a diffusible Ca^{2+} sensor with at least two canonical EF-hands and at a global concentration of 10 μM (which is thought to be roughly the expression level of major EF-hand Ca^{2+} sensors other than CaM in brain), would have to get enriched at synapses by at least a factor of 20 in order to match the abundance of CaM. Is this degree of accumulation achievable? The PSD has a thickness of ~40 nm (Takashima et al., 2011) and an area of 0.08 μm^2 (Arellano et al., 2007). The average volume of the PSD and the postsynaptic membrane is therefore ~0.003 μm^3, which is ~20 times lesser than the spine head volume. Caldendrin, which gets enriched at the PSD in an activity-dependent manner (Smalla et al., 2003) probably meets this criterion in the PSD but not in the entire spine. Hippocalcin, which is already more abundant in the hippocampus than other EF-hand sensors, can get concentrated by dozens of times upon translocation to the membrane and as a consequence might even surpass the abundance of CaM at synaptic membranes (Dovgan et al., 2010).

In parallel to the above enrichment, sequestration of CaM might be another way to increase the relative abundance of NCS and nCaBPs as compared to CaM. RC3/neurogranin is a protein belonging to the IQ-motif family of CaM binding proteins with an estimated abundance of $\sim$60 μM (Huang et al., 2004) in dendritic spines of CA1 pyramidal neurons. It preferentially binds to apo-CaM with a high affinity and, therefore, sequesters it at low [Ca^{2+}] (Gerendasy et al., 1994) and upon binding it reduces the affinity of CaM for Ca^{2+} (Gaertner et al., 2004). The affinity of RC3 and CaM binding reduces significantly at higher [Ca^{2+}] and gets completely abolished upon phosphorylation of RC3 by protein kinase C (Gerendasy et al., 1994). Due to these properties, the interaction between RC3 and CaM at low resting [Ca^{2+}] has important implications on the availability of free CaM for Ca^{2+} dependent as well as independent targets (Gerendasy et al., 1994). Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is one of the most abundant proteins in the PSD that might similarly sequester CaM. The concentration of its subunits ranges from 100 to 200 μM (Lisman and Zhabotinsky, 2001). It has been shown that autophosphorylation of CaMKII causes a 100-fold reduction in dissociation rate of CaM due to which CaM stays bound to phosphorylated CaMKII long after [Ca^{2+}] returns to basal levels (Meyer et al., 1992). Since NCS and nCaBPS have not been reported to associate with CaMKII, it will be interesting to experimentally test if chelation of CaM by CaMKII could create open slots for target interactions of other Ca^{2+} sensors.

Ca^{2+}-INDEPENDENT PRE-ASSOCIATION OF NCS AND nCaBPs WITH TARGET MOLECULES

Another way to circumvent the problem of limited Ca^{2+} resources for target interactions is a Ca^{2+}-independent pre-association with a binding partner. Caldendrin is the best example for this mode of operation. Regarding its EF-hand containing C-terminal domains it is the closest relative of CaM and shares this region with its shorter splice isoforms. Interestingly, caldendrin modulates the activity of Ca$_v$1.2 (L-type) Ca^{2+} channels *via* different molecular determinants than the shorter splice isoform, caldendrin-S1 (also called S-CaBP1), which is, however, barely expressed in brain (Laube et al., 2002; Zhou et al., 2005; Tippens and Lee, 2007), indicating that the structures of the isoforms may be very different. An important feature of caldendrin and S-CaBP1 is that they bind many of their targets, e.g., Ca$_v$1.2, Ca$_v$2.1 Ca^{2+}-channels, LC3, V-ATPase, and Inositol 1,4,5-trisphosphate receptors (InsP(3)Rs) in a Ca^{2+}-independent manner (**Figure 2**; Kasri et al., 2004; Seidenbecher et al., 2004; Zhou et al., 2004, 2005; Few et al., 2005; Haynes et al., 2006; Lee et al., 2006; Tippens and Lee, 2007) whereas Ca^{2+}-binding increases the affinity of the association and triggers the actual signaling event. Although CaM is also a subunit of complexes with many enzymes and ion channels, such a pre-association would be very advantageous to convey a signal faster than other Ca^{2+} sensors, given that there is no other pre-association with another sensor at the target site and thereby could provide a molecular mechanism by which signals can be transduced to a specific target interaction irrespective of Ca^{2+}-concentrations and CaM levels.

The idea of a signalosome-like protein preassembly that provides a clear advantage in terms of accessibility of a target site within Ca^{2+}-nanodomains is not experimentally supported yet. A Ca^{2+}-dependent increase in the binding affinity for a target within such a pre-associated signalosome could overrule all advantages of CaM in the race of Ca^{2+}-binding in spines. This is clearly conceivable since Ca^{2+}-affinities of EF-hand domains can increase in the target-bound form (Dukhanina et al., 1997; Peersen et al., 1997). In this respect, it is also worth mentioning that levels of macromolecular crowding impact the conformation of EF-hand domains and potentially their Ca^{2+}-affinity (Wang et al., 2011). The impact of macromolecular complexes in spines could be substantial, given the high protein content and the compact structure of the PSD.

VARIABILITY AND INHOMOGENEITY OF DENDRITIC SPINES AND THEIR INFLUENCE ON THE RACE
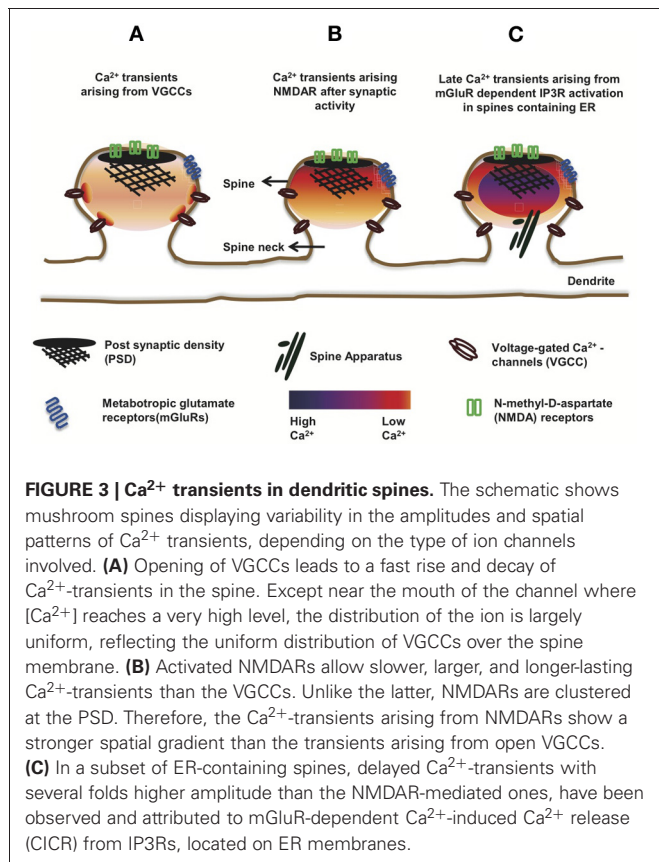
Dendritic spines display spatio-temporal gradients in cytosolic Ca^{2+} concentration and Ca^{2+} amplitudes. This variability reflects the diversity of various factors including the kind, number, and distribution of Ca^{2+}-channels and pumps, the mechanisms that regulate their activity, and the diffusability of Ca^{2+} and Ca^{2+}-bound buffers. These factors can influence the race for Ca^{2+} and will be described in more detail below.

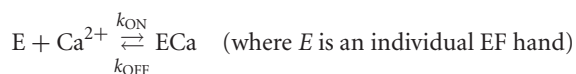### THE INTERPLAY BETWEEN Ca^{2+} ION CHANNELS AND Ca^{2+} SENSORS

The principal sources of Ca^{2+} in spines are voltage-gated Ca^{2+} channels (VGCCs), InsP(3)Rs, ryanodine receptors (RyRs), and Ca^{2+} permeable glutamate receptors, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and N-methyl-D-aspartate receptors (NMDARs). VGCCs, NMDARs, and AMPARs are located on the plasma membrane, whereas, InsP(3)Rs and RyRs line the membranes of smooth ER invading the spine.

The opening of VGCCs leads to a fast rise in Ca^{2+} concentration with a rise time constant of 3.24 ms (Cornelisse et al., 2007) which decays slowly with a time constant of $\sim$15 ms (Sabatini et al., 2002). On the other hand, NMDARs open slowly (rise time of Ca^{2+} >100 ms) and allow for longer-lasting and much larger Ca^{2+} influxes. Kubota and Waxham (2010) elegantly described the critical impact of Ca^{2+} injection rates of VGCCs and NMDARs on Ca^{2+} dynamics. VGCCs have a high injection rate ($\sim$1.4 ions/μs) but stay open for a very short time. Therefore, the opening of a VGCC leads to an accumulation of Ca^{2+} very close to the mouth of the channel before it diffuses away (**Figure 3A**). On the other hand, NMDARs have a much lower injection rate ($\sim$0.07 ions/μs) and remain open for a longer time. Ca^{2+} that enters the spine following the opening of these receptors can diffuse to a considerable distance ($\sim$140 nm from the channel) before the next ion enters (Kubota and Waxham, 2010). What properties should a Ca^{2+} sensor have, in order to respond to these diverse signals? The answer lies in the binding kinetics of the individual EF-hands.

In the previous paragraphs, we considered only the global Ca^{2+} affinity of a Ca^{2+}-binding protein which indicates only an average of affinities of its individual EF-hands. The dissociation

FIGURE 3 | Ca²⁺ transients in dendritic spines. The schematic shows mushroom spines displaying variability in the amplitudes and spatial patterns of Ca²⁺ transients, depending on the type of ion channels involved. **(A)** Opening of VGCCs leads to a fast rise and decay of Ca²⁺-transients in the spine. Except near the mouth of the channel where [Ca²⁺] reaches a very high level, the distribution of the ion is largely uniform, reflecting the uniform distribution of VGCCs over the spine membrane. **(B)** Activated NMDARs allow slower, larger, and longer-lasting Ca²⁺-transients than the VGCCs. Unlike the latter, NMDARs are clustered at the PSD. Therefore, the Ca²⁺-transients arising from NMDARs show a stronger spatial gradient than the transients arising from open VGCCs. **(C)** In a subset of ER-containing spines, delayed Ca²⁺-transients with several folds higher amplitude than the NMDAR-mediated ones, have been observed and attributed to mGluR-dependent Ca²⁺-induced Ca²⁺ release (CICR) from IP3Rs, located on ER membranes.

constant (inverse of affinity) is an equilibrium constant, given by the ratio between the OFF and ON rate constants of a reversible reaction.

$$\text{E} + \text{Ca}^{2+} \underset{k_{\text{OFF}}}{\overset{k_{\text{ON}}}{\rightleftarrows}} \text{ECa} \quad \text{(where } E \text{ is an individual EF hand)}$$

Due to subtle variations in structure, individual EF-hands have different ON and OFF rates of Ca²⁺-binding and, therefore, are fine-tuned to different Ca²⁺-binding affinities (Grabarek, 2006; Gifford et al., 2007). To be able to respond to a Ca²⁺ signal, the binding kinetics of EF-hands must match the rate of Ca²⁺ entry. CaM has four EF hands organized into two domains. These EF-hands display cooperativity and allosterism in Ca²⁺-binding. An apo-EF hand is in the tensed or T-state, which binds the first ion to change to the relaxed or R-state. Although it was previously estimated that the N-terminal domain has a faster ON-rate of Ca²⁺ binding than the C-terminal domain (Kubota et al., 2007), an important finding by Faas et al. (2011) was that the ON-rate of Ca²⁺ binding of the N-terminal lobe of CaM [$k_{\text{on}(T)} = 7.7 \times 10^8 \text{M}^{-1}\text{s}^{-1}$] was faster than important Ca²⁺ buffers like calbindin [$k_{\text{on}(T)} = 7.5 \times 10^7 \text{M}^{-1}\text{s}^{-1}$] and calretinin [$1.8 \times 10^6 M^{-1}s^{-1}$; Faas et al., 2007], making it the prime "fast" buffer. In spite of the fast binding rate, this domain has a much lower affinity ($K_d = 12.7 \mu\text{M}$) than the C-terminal domain ($K_d = 2.7 \mu\text{M}$) owing to its faster OFF-rate. When a VGCC opens, the instantaneous rise in Ca²⁺ concentration and the short

duration for which the channel stays open would enable only those sensors with a fast binding capacity without much regard to their affinity since the Ca²⁺ levels attained are reasonably high. These features match perfectly with the fast binding N-terminal EF-hands of CaM. Along these lines, the N-terminal EF-hand domain could also be important for the role of CaM in long-term potentiation (LTP), which is associated with a steep rise in [Ca²⁺] to high amplitudes (Byrne et al., 2009). However, the function of CaM or its individual domains as Ca²⁺ sensors also depends on the kinetics of downstream target interaction. Only those targets are physiologically relevant whose association rate with CaM or one of its EF-hand domains is greater than the dissociation rate of Ca²⁺ from that domain. This is especially important in the case of the N-terminal domain of CaM, since it has a faster dissociation rate [$k_{\text{off}(R)} = 2.2 \times 10^4 \text{s}^{-1}$] than the C-terminal domain [$k_{\text{off}(R)} = 6.5 \text{s}^{-1}$] (Faas et al., 2011). Unfortunately, although a number of Ca²⁺-dependent targets of CaM have been reported in the literature, the interaction kinetics of only a few of them is known.

The C-terminal lobe is unlikely to participate during the fast Ca²⁺ transients described above, due to its slow association rate. The possibility of a selective participation of the C-terminal domain during longer-lasting Ca²⁺ transients of long-term depression has been pointed out earlier (Byrne et al., 2009). The fast-binding of the N-terminal EF hands of CaM would be of no particular advantage in case of NMDAR-mediated Ca²⁺ influx. Their fast OFF-rates make CaM a low affinity sensor, rendering it less sensitive toward changes in Ca²⁺ concentration during NMDAR-mediated Ca²⁺ transients. The slow kinetics of NMDARs would not only engage the slower Ca²⁺ binding EF-hands/proteins, but might preferentially activate those Ca²⁺-binding proteins which are more sensitive to gradual changes in [Ca²⁺], such as the high affinity proteins of the NCS family, e.g., NCS-1 ($K_{\text{dCa}} = 440 \text{nM}$; Aravind et al., 2008) and hippocalcin ($K_{\text{dCa}} = 324 \text{nM}$; O'Callaghan et al., 2003), assuming that their Ca²⁺ association rate is not lower than the diffusion rate of Ca²⁺. Though their binding rates have not been determined, an advantage remains with these sensitive proteins even if they are slower in binding Ca²⁺ than CaM. It is hence not surprising that these two proteins play a crucial role in mGluR-dependent and NMDAR-dependent synaptic plasticity (Palmer et al., 2005; Jo et al., 2008, 2010). Importantly, it has also been shown that the Ca²⁺ influx through NMDARs, and not through VGCCs, is mainly responsible for the translocation of hippocalcin (Dovgan et al., 2010).

The VGCCs and NMDARs also differ in their distribution over the spine membrane. Based on a model by Keller et al. (2008), the VGCCs are uniformly distributed over the membrane. Therefore, their opening results in a uniform distribution of Ca²⁺ over the entire spine volume (**Figure 3A**). Although [Ca²⁺] can reach more than $10 \mu\text{M}$ at the mouth of an open VGCC (Simon and Llinás, 1985), these microdomains of high Ca²⁺ exist only within a few nanometer around the channel and dissipate within microseconds of channel closing (Sabatini et al., 2002). Intriguingly, the N- and C-terminal domains of CaM complexed to a VGCC (Ca$_v$1–2) might show distinct selectivity toward these local (nano domain of the complex) and global changes

in [Ca^{2+}] arising from the "host" VGCC (Tadross et al., 2008). The C-terminal domain might transduce local (nano domain) Ca^{2+} signals and the N-terminal domain global signals and is reportedly also capable of switching its selectivity between local (Ca$_v$1) and global(Ca$_v$2) changes in [Ca^{2+}] (Dick et al., 2008; Tadross et al., 2008).

In contrast to VGCCs, NMDARs are clustered at the PSD. This leads to a longer lasting Ca^{2+} gradient extended across the spine during an excitatory post-synaptic potential (EPSP) (Keller et al., 2008; **Figure 3B**). This scenario might favor caldendrin, which is also enriched in the PSD. Although caldendrin and CaM bind Ca^{2+} with similar affinity, it is conceivable that the greater physical proximity of caldendrin might render it a strong competitor for CaM.

Interaction partners can influence the Ca^{2+} binding kinetics and affinity of Ca^{2+}-binding proteins. RC3/Neurogranin interacts with apo-CaM (discussed in an earlier section) and increases the k_{off} of the C-terminal lobe of CaM (Gaertner et al., 2004), thereby reducing its affinity. On the other hand, another CaM target- CaMKII decreases the k_{off} of both N- and C-terminal lobes of CaM (Gaertner et al., 2004). The regulation of Ca^{2+}-binding kinetics of CaM by its targets could explain how it is able to decode a variety of Ca^{2+} signals. Alteration of Ca^{2+}- binding kinetics and affinity of CaM can not only influence Ca^{2+}/CaM-dependent signaling pathways, but also regulate the amounts and duration for which free [Ca^{2+}] is available for other Ca^{2+} sensors; an idea that is supported by mathematical models (Kubota et al., 2007, 2008).

Depolarization of spines is the key event that regulates the Ca^{2+} entry via VGCCs and NMDARs, which, as discussed above, is very influential in the race for Ca^{2+}. During synaptic activity, AMPARs function as the major source of spine depolarization, necessary to activate VGCCs as well as to remove the Mg^{2+} blockage of NMDARs (Bloodgood et al., 2009; Holbro et al., 2010). The arrival of bAPs to a spine is another source of spine depolarization. Co-incident pre- and post-synaptic activity which involves glutamate release at the synapse preceding the arrival of bAPs in a millisecond time window, is responsible for the non-linear amplification of Ca^{2+} transients that underlie LTP (Helias et al., 2008; Holbro et al., 2010; Hao and Oertner, 2011). NMDARs, with their dependence on both glutamate binding and voltage-dependent unblocking have been considered to play the role of a co-incidence detector for the induction of LTP. However, it has also been suggested that NMDARs alone are not sufficient for co-incidence detection, but that it is the spine that acts as a co-incident detector of pre- and post-synaptic activity and the degree of depolarization of the whole spine is the key element for the induction of LTP (Hao and Oertner, 2011). The depolarizing currents from AMPARs sensitize the NMDARs and VGCCs, so that their I-V curves reach the steepest zone where even a slight further depolarization drastically increases Ca^{2+} influx into the spine (Holbro et al., 2010). A negative feedback loop provided by small conductance Ca^{2+}-activated K$^+$ (SK) channels controls the depolarization of spines. The Cav2.3 class of VGCCs activate SK channels, which shunt the synaptic current and dampen Ca^{2+} fluxes through NMDARs by promoting Mg^{2+} blockage of these receptors (Ngo-Anh et al., 2005; Bloodgood and

Sabatini, 2007; Bloodgood et al., 2009). Spine head depolarization and the non-linear amplification of Ca^{2+} transients upon co-incident pre- and post-synaptic activity have been observed to be sharpest in spines that were well isolated from the dendrite (Holbro et al., 2010). This suggests the importance of spine neck dimensions in synaptic plasticity. The spine geometry also correlates well with the number of ion channels present on its membrane. Both the AMPAR and NMDAR-mediated currents increase with increase in the size of spine head. Intriguingly, the postsynaptic Ca^{2+} increase is lower in larger spines (Nimchinsky et al., 2004; Hayashi and Majewska, 2005; Noguchi et al., 2005). The importance of spine geometry in biochemical and electrical compartmentalization of spine heads is discussed in detail later.

THE ROLE OF SMOOTH ENDOPLASMIC RETICULUM (SER)

The smooth endoplasmic reticulum (SER), which is also called spine apparatus in spines, is a major internal store of Ca^{2+} and houses Ca^{2+} pumps such as sarco/ER Ca^{2+}-ATPase (SERCA) and Ca^{2+}- sensitive Ca^{2+} channels, namely, InsP(3)Rs and RyRs. While the SER is, undoubtedly, a major player in the regulation of cytosolic Ca^{2+} in a cell in general, its precise role in shaping the Ca^{2+} dynamics in dendritic spines in particular, is a matter of debate.

Emptage et al. (1999) found that although SER does not play any role in bAP-stimulated Ca^{2+} transients, they have a significant contribution in NMDAR-dependent Ca^{2+} dynamics. Upon depletion of the internal Ca^{2+} store by SERCA blockers and by application of RyR antagonists, a significant reduction in NMDAR-dependent Ca^{2+} transients was observed. This led the authors to conclude that during a single synaptic event, the Ca^{2+} current through activated NMDARs is too small in itself to be detected. What is actually detected is a much larger Ca^{2+} influx through RyRs that were triggered by the small Ca^{2+} currents coming from the NMDARs (Emptage et al., 1999). This phenomenon in which the SER releases the stored Ca^{2+} through its Ca^{2+}-sensitive channels, is known as Ca^{2+}-induced Ca^{2+} release or CICR (for review, see Rose and Konnerth, 2001).

Contradictory to this observation, other groups later found no significant role of CICR in either bAP- or NMDAR-dependent Ca^{2+} transients (Sabatini et al., 2002; Holbro et al., 2009). Both spines, that contained or lacked ER, showed no difference in NMDAR-dependent Ca^{2+} signals (Holbro et al., 2009); however, the authors observed delayed Ca^{2+} transients in a subset of ER-positive spines with more than five-fold larger amplitude arising in the order of 100 ms later than the NMDAR-dependent transient (**Figure 3C**). These large Ca^{2+} waves were abolished in the presence of group I metabotropic glutamate receptor (mGluR) or InsP(3)R blockers as well as upon depletion of Ca^{2+} stores, but persisted even in the presence of NMDAR blockers. Group I mGluRs are located in a perisynaptic zone surrounding the AMPARs and NMDARs and their role in mGluR-dependent long-term depression (mGluR-LTD) has been well studied (Lüscher and Huber, 2010). Holbro et al. (2009) observed that the induction of mGluR-dependent LTD was limited to ER-positive spines. Therefore, they suggested that the ER plays a role in mGluR-dependent LTD which involves CICR by activated IP3Rs.

Notably, only about 20% of the CA1 dendritic spines contain SER (Spacek and Harris, 1997; Toresson and Grant, 2005; Holbro et al., 2009). One can, therefore, speculate that in ER containing spines, upon the arrival of large Ca^{2+} waves, the concentration of Ca^{2+} might rise to a level where all Ca^{2+} sensors can be completely saturated, probably obsoleting the race for Ca^{2+}, albeit further intensifying the race for targets.

SPINE GEOMETRY AND THE RACE FOR Ca^{2+}

A spine is considered to be a micro-compartment of depolarization and Ca^{2+}-dynamics distinct from the dendrite owing to the diffusional resistance of the spine neck. The idea that the spine neck acts as an electrical resistor is supported by the finding that (1) VGCCs present on the spines get activated by synaptic but not dendritic depolarization (Bloodgood et al., 2009); (2) voltage pulses arriving at a spine head from the soma get attenuated linearly with increasing length of the spine neck (Araya et al., 2006); (3) potential changes arising in a spine due to synaptic activity are largely restricted to its volume and their invasion into the parent dendrite are limited by the spine neck (Araya et al., 2006; Bloodgood et al., 2009). Therefore, an electrical resistance to movement of ions provided by the spine neck controls the activation of depolarization-dependent Ca^{2+} channels and thereby controls the amplitude of Ca^{2+} transients within the spine boundaries.

Besides electrical resistance, the spine neck also provides a diffusional barrier for ions and other molecules (Hayashi and Majewska, 2005; Biess et al., 2007; Grunditz et al., 2008; Schmidt and Eilers, 2009; Sabatini et al., 2002). The residing actin meshwork within the neck could, therefore, act as a molecular sieve for the entry and exit of NCS and nCaBPs from spines in a manner similar to CaMKII (Byrne et al., 2011). The spine neck diameter has been found to be correlated with spine head volume and it has been observed that Ca^{2+} dynamics varies with spine volume, in part due to the faster diffusion allowed by wider spine necks, and in part because of the relationship between the number of AMPA and NMDA receptors and the spine head size (discussed above; Hayashi and Majewska, 2005). Neuronal activity has been shown to regulate the diffusional properties of the spine neck (Bloodgood and Sabatini, 2005), the plasticity of which, can further control the spatio-temporal aspects of Ca^{2+} dynamics in spines (Segal, 2001; Grunditz et al., 2008). Future studies with super-resolution microscopy and live-imaging will resolve these issues and potentially also answer the question how dynamic the spine geometry actually is.

CONCLUSIONS AND FUTURE DIRECTIONS

In the next coming years, it will be imperative to learn more about the biophysical features including their precise ion binding properties, their actual concentration in spines and the sequence of filling of EF-hand motifs of NCS and nCaBPs, to judge the physiological relevance of their synaptic protein interactions. How the presence of one Ca^{2+} sensor would influence the Ca^{2+}-binding to another sensor or target is another interesting question to be addressed. Super resolution microscopy to identify nanodomains, fast spectroscopic methods to study *in vitro* protein kinetics and advanced modeling might also help to address at least some of the unresolved issues. Finally, a systematic analysis of the synaptic interactome of NCS and nCaBPs will help to appreciate their synaptic role. Compelling evidence for this synaptic role is, with the exception of hippocalcin, caldendrin, and NCS-1, still lacking and the conditions under which these proteins will eventually "meet" Ca^{2+} in the synapse still remain to be established.

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