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The Diversity of Calcium Sensor Proteins in the Regulation of Neuronal Function

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Calcium signaling in neurons as in other cell types mediates changes in gene expression, cell growth, development, survival, and cell death. However, neuronal Ca^{2+} signaling processes have become adapted to modulate the function of other important pathways including axon outgrowth and changes in synaptic strength. Ca^{2+} plays a key role as the trigger for fast neurotransmitter release. The ubiquitous Ca^{2+} sensor calmodulin is involved in various aspects of neuronal regulation. The mechanisms by which changes in intracellular Ca^{2+} concentration in neurons can bring about such diverse responses has, however, become a topic of widespread interest that has recently focused on the roles of specialized neuronal Ca^{2+} sensors. In this article, we summarize synaptotagmins in neurotransmitter release, the neuronal roles of calmodulin, and the functional significance of the NCS and the CaBP/calneuron protein families of neuronal Ca^{2+} sensors.

Calcium signaling in many cell types can mediate changes in gene expression, cell growth, development, survival, and cell death. However, neuronal calcium signaling processes have become adapted to modulate the function of important pathways in the brain, including neuronal survival, axon outgrowth, and changes in synaptic strength. Changes in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]i$) are essential for the transmission of information through the nervous system as the trigger for neurotransmitter release at synapses. In addition, alterations in $[Ca^{2+}]i$ can lead to a wide range of different physiological changes that can modify neuronal functions over time scales of milliseconds through tens of minutes to days or longer (Berridge 1998). Many of these processes have been shown to be dependent upon the particular route of Ca^{2+} entry into the cell. It has long been known that the physiological outcome from a change in $[Ca^{2+}]i$ depends on its location, amplitude, and duration. The importance of location becomes even more pronounced in neurons because of their complex and extended morphologies. $[Ca^{2+}]i$ also regulates neuronal development and neuronal survival (Spitzer 2006). In addition, modifications to Ca^{2+} signaling pathways have been suggested to underlie various neuropathological disorders (Braunewell 2005; Berridge 2010).

Highly localized Ca^{2+} elevations (Augustine et al. 2003) formed following Ca^{2+} entry though voltage-gated Ca^{2+} channels (VGCCs) lead to

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synaptic vesicle fusion with the presynaptic membrane and thereby allow neurotransmitter release within less than a millisecond. Differently localized and timed Ca^{2+} signals can, for example, result in changes to the properties of the VGCCs (Catterall and Few 2008) or lead to changes in gene expression (Bito et al. 1997). Postsynaptic Ca^{2+} signals arising from activation of NMDA receptors give rise to two important processes in synaptic plasticity, long term potentiation (LTP) and long term depression (LTD). LTP and LTD are examples of the way synaptic transmission can change synaptic efficacy and are thought to be important in modulating learning and memory. Importantly, the Ca^{2+} signals that bring about either LTP or LTD differ only in their timing and duration. LTP is triggered by Ca^{2+} signals on the micromolar scale for shorter durations, whereas LTD is triggered by changes in $[Ca^{2+}]i$ on the nanomolar scale for longer durations (Yang et al. 1999). Specific Ca^{2+} signals are likely to be decoded by different Ca^{2+} sensor proteins. These are proteins that undergo a conformational change on Ca^{2+} binding and then interact with and regulate various target proteins. Among those Ca^{2+} sensors that are important for neuronal function are the synaptotagmins that control neurotransmitter release (Chapman 2008), the ubiquitous EF-hand containing sensor calmodulin that has many neuronal roles, and the more recently discovered neuronal EF-hand containing proteins, including the neuronal calcium sensor (NCS) protein (Burgoyne 2007) and the calcium-binding protein (CaBP)/calneuron (Haeseleer et al. 2002) families. We will briefly review synaptotagmins and the neuronal functions of calmodulin but concentrate on the NCS and CaBP families of Ca^{2+} sensors.

SYNAPTOTAGMINS AND NEUROTRANSMITTER RELEASE

Synaptotagmins are transmembrane proteins mostly found associated with synaptic and secretory vesicles. There are multiple known isoforms of synaptotagmin (Craxton 2004) of which synaptotagmin I is the best studied. The role of synaptotagmins in neurotransmitter release has been the subject of intense investigations, which have been extensively reviewed (Chapman 2008; Rizo and Rosenmund 2008; Sudhof and Rothman 2009) and so only a brief outline is given here. Synaptotagmins bind Ca^{2+} with relatively low affinity ($Kd > 10\ \mu M$) through their two C2 domains (C2A and C2B) (Shao et al. 1998; Fernandez et al. 2001), which are functional in many but not all synaptotagmin isoforms. Ca^{2+} binding by C2 domains requires coordination of Ca^{2+} by both the protein and membrane lipids and this lipid interaction is a key aspect for its function. In synaptotagmin I, the C2A and C2B domains (Fig. 1) bind three and two Ca^{2+} ions, respectively (Shao et al. 1998; Fernandez et al. 2001). It is now well established that synaptotagmin I is a key sensor for evoked, synchronous neurotransmitter release in many classes of neurons (Fernandez-Chacon et al. 2001). Structure–function studies based on expression of specific mutants have been carried out in mice, worms, and flies. For example, disruption of Ca^{2+} binding to the C2B domain of synaptotagmin I has

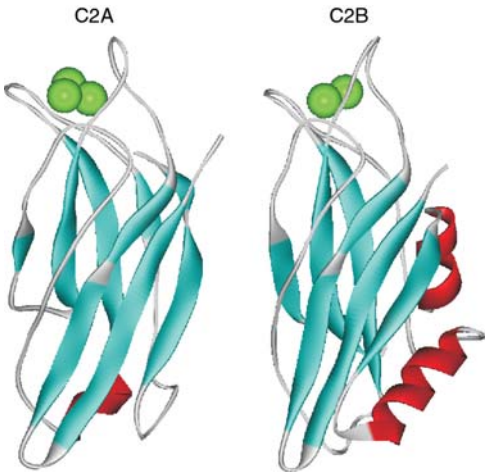

Figure 1. Structures of the C2A and C2B domains of synaptotagmin I. The structures show the isolated C2 domains in their Ca^{2+}-loaded state with the bound Ca^{2+} ions shown in green. The coordinates for the structures for the C2A and C2B domains come from the PDB files 1BYN and 1K5W, respectively.

been shown to have a more deleterious effect than disruption of Ca^{2+} binding to its C2A domain (Mackler et al. 2002; Robinson et al. 2002). The details of exactly how it triggers exocytosis and the function of other syntaptotagmin isoforms remain controversial. Membrane fusion requires the pairing and interaction of so-called SNARE proteins on vesicle and target membranes (Sollner et al. 1993). These can assemble into a SNARE complex that may form the minimal fusion machinery. For synaptic vesicle and neuroendocrine exocytosis, the SNARE proteins are SNAP-25, syntaxin 1, and synaptobrevin. In the case of regulated exocytosis, such as in neurotransmitter release, vesicle fusion is tightly regulated and requires a Ca^{2+} signal for activation. Ca^{2+} entry through VGCCs leading to Ca^{2+} elevation in local microdomains close to the mouth of the Ca^{2+} channel is able to trigger very rapid (within less than 1 ms) fusion of synaptic vesicles. Synaptotagmin can bind to both syntaxin and SNAP-25, and fast neurotransmitter release requires synaptotagmin (Geppert et al. 1994) probably prebound to assembled or partially assembled SNARE complexes (Schiavo et al. 1997; Rickman et al. 2006) so that Ca^{2+}-induced interaction with phospholipids can occur rapidly (Xue et al. 2008). It is still under debate how important synaptotagmin is in vesicle docking (de Wit et al. 2009) and how it acts at the plasma membrane in fusion itself (Tang et al. 2006; Hui et al. 2009). Synaptotagmin could act as a brake on fusion that is relieved on Ca^{2+} binding or have a positive role in membrane fusion (Chicka et al. 2008). A recent focus has been on the combined role of synaptotagmin and another SNARE interacting protein complexin in timing synaptic vesicle fusion (Sudhof and Rothman 2009), but much still remains to be learnt about the molecular basis of its function. While synaptotagmin is a key sensor for evoked neurotransmitter release, an alternative C2-domain containing protein, Doc2b, has been identified as a Ca^{2+} sensor for spontaneous neurotransmitter release (Groffen et al. 2010). Like synaptotagmin, Doc2b appears to function via interaction with SNARE complexes.

NEURONAL FUNCTIONS OF CALMODULIN

Calmodulin is a ubiquitously expressed Ca^{2+}-binding protein that can bind four Ca^{2+} ions through its four EF-hand domains (Chattopadhyaya et al. 1992). This protein has been highly conserved throughout evolution, is found in all eukaryotes, and is 100% identical across all vertebrates at the amino acid level. It is involved in the regulation of many essential physiological processes including cell motility, exocytosis, cytoskeletal assembly, and modulation of intracellular Ca^{2+} concentrations. The first two EF-hands of calmodulin form an amino-terminal globular domain that is joined by a flexible linker to a highly homologous carboxy-terminal region encompassing the third and fourth EF-hands. The carboxy-terminal pair of EF-hands has a much higher affinity for Ca^{2+} than the amino-terminal pair, which allows the two domains to behave independently at varying Ca^{2+} concentrations (Tadross et al. 2008). The highly flexible linker between the two domains can be bent dramatically upon binding to target proteins (Fig. 2) and is an essential property of calmodulin, which permits this protein to interact with a large and diverse array of interacting partners. The significant conformational changes on binding to its targets (Fallon et al. 2005) can increase its affinity for Ca^{2+}.

Calmodulin is present in brain at high concentrations (up to $\sim$100 μM). In addition to its more general functions, calmodulin also has a series of specific roles in transducing Ca^{2+} signals in neurons, including, for example, in the regulation of glutamate receptors (O'Connor 1999), ion channels (Saimi and Kung 2002), and proteins in signaling pathways such as neuronal nitric oxide synthase, and it can affect synaptic plasticity (Lisman et al. 2002; Xia and Storm 2005). One key direct function of calmodulin is in regulating the activity of VGCCs by binding to channel subunits (Catterall and Few 2008). Ca^{2+}-binding to VGCC-associated calmodulin can have a range of effects on channel function, including mediating Ca^{2+}-dependent facilitation or Ca^{2+}-inactivation (Lee et al. 2000; DeMaria et al. 2001; Catterall and Few 2008;

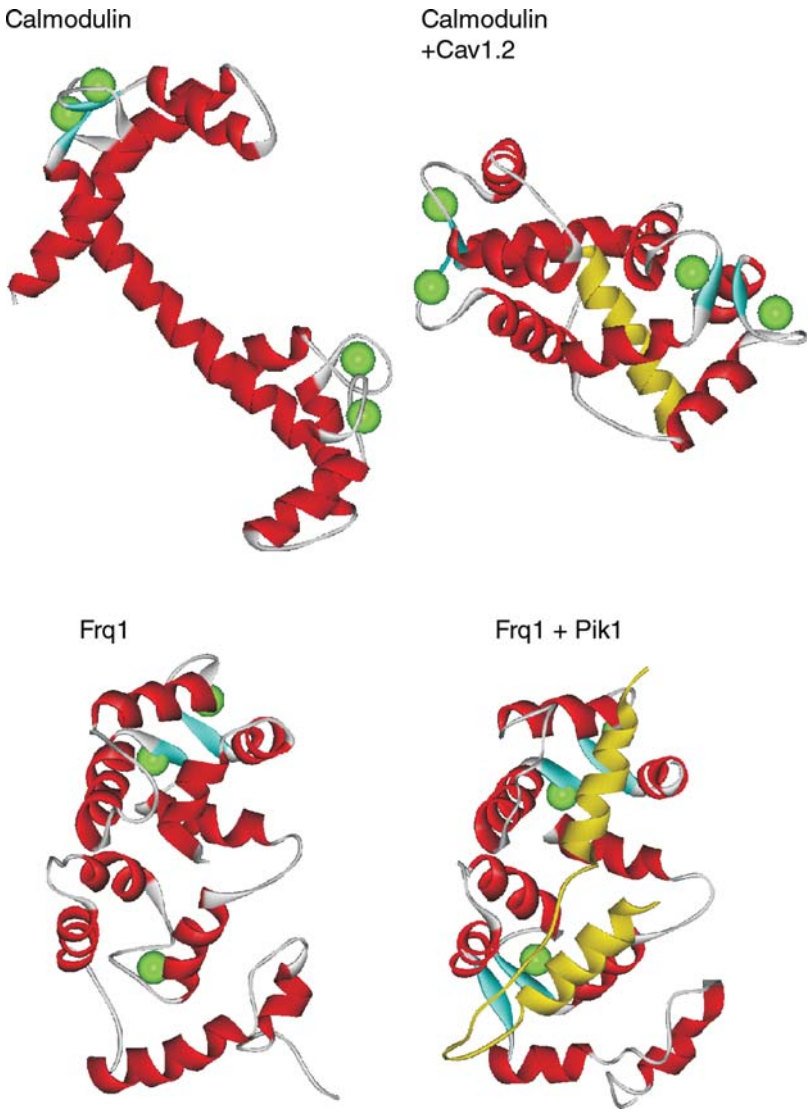

Figure 2. Comparison of the structures of Ca^{2+}-loaded calmodulin and yeast frequenin with and without bound target peptides. The structures at the top are of Ca^{2+}-bound calmodulin alone (PDB 1CLL) or in a complex with the IQ-like domain of the $Ca_v1.2$ Ca^{2+}-channel α-subunit (PDB 2F3Z). The structures at the bottom are of the Ca^{2+}-bound yeast frequenin (Frq1) alone (PDB 1FPW) or in a complex with the binding domain from Pik1 (PDB 2JU0). In each of the complexes, the target peptide is shown in yellow.

Liu et al. 2010). Calmodulin is also constitutively associated with and regulates opening of Ca^{2+}-activated potassium channels (Xia 1998; Schumacher et al. 2001) and other types of potassium channels (Wen and Levitan 2002). Two other major modes of action of calmodulin are exerted indirectly through its target proteins Ca^{2+}/calmodulin-dependent kinases (CaMKs) and calcineurin. CaMKs contribute to a number of regulatory pathways involving, for example, phosphorylation of AMPA receptors (Barria et al. 1997) and the nuclear transcription factor CREB (Deisseroth et al. 1998). Calmodulin also positively regulates presynaptic

release probability and this is mediated via activation of CaMKII (Pang et al. 2010). The Ca^{2+}-activated phosphatase calcineurin can dephosphorylate a wide range of neuronal proteins, leading to direct effects and effects through changes in gene transcription following activation of the transcription factor NFAT and its translocation into the nucleus. Calcineurin has also been implicated, for example, in synaptic plasticity (Malleret et al. 2001; Xia and Storm 2005). Although many aspects of neuronal function are known to be regulated by calmodulin, proteins related to calmodulin have been discovered in recent years, which are enriched or expressed exclusively in neurons. Duplication and diversification of the calmodulin gene may have given rise to these neuronal calcium sensing proteins so that they can carry out specific neuronal functions in higher organisms.

NCS PROTEIN FAMILY

Whereas calmodulin is ubiquitously expressed, the expression of other calcium sensing proteins can be restricted to particular tissues and cell types. A good example of this is the neuronal calcium sensor (NCS) family of proteins, which are primarily expressed in neurons or retinal photoreceptors. The NCS family of proteins is related to calmodulin but have distinct properties that allow them to carry out nonredundant roles that do not overlap with the functions of calmodulin. Members of the NCS protein family have been implicated in the regulation of neurotransmitter release, regulation of cell-surface receptors and ion channels, control of gene transcription, cell growth, and survival (Burgoyne 2007). The NCS proteins are encoded by a family of 14 genes in mammals with greater diversity stemming from alternative splicing of transcripts from a number of the genes. All NCS gene products harbor four EF-hand motifs and display limited similarity (<20%) to calmodulin (Burgoyne 2004).

NCS-1 is the most widely expressed of the NCS proteins and is thought to be the primordial NCS protein. The protein was first discovered (as frequenin) in *Drosophila melanogaster* (Pongs et al. 1993), where there are two very closely related genes (Sanchez-Gracia et al. 2010). Although initially thought to be neuronal specific (Nef et al. 1995), NCS-1 has also been identified in *Saccharomyces cerevisiae* (Hendricks et al. 1999). After this first appearance of NCS-1 in yeast, there has been a steady increase in the diversity of this family throughout evolution, which roughly correlates with increasing organism complexity. Five classes of NCS proteins have now been identified in higher organisms termed classes A-E (Burgoyne 2007). Class A contains NCS-1, which is present in yeast and all higher organisms. Class B consists of the visinin-like proteins (VSNLs), which appear first in *Caenorhabditis elegans*. Classes C and D evolved with the appearance of fish and comprise recoverin and the guanylyl-cyclase-activating proteins (GCAPs), respectively. Finally, class E contains the K^+ channel-interacting proteins (KChIPs), which are found in insects and evolutionary subsequent species (Burgoyne 2004). Mammals have a single NCS-1, five VSNL proteins (hippocalcin, neurocalcin δ, and VILIPs1-3), a single recoverin, three GCAPs, and four KChIPs. Expression of the recoverins and GCAPs is restricted to the retina, whereas the rest of the NCS family is found in varied neuronal populations (Burgoyne 2007). Although localization and expression studies have proven difficult because of cross-reactivity of antibodies, it has been established that certain neurons express several or all of the NCS proteins, but in general, the expression profile for each of the NCS proteins is unique (Paterlini et al. 2000; Rhodes et al. 2004). This suggests that despite the high sequence homology between the proteins, each is likely to perform distinct functions in specific cell types (Burgoyne and Weiss 2001).

Unlike calmodulin, not all EF-hands are functional in the NCS proteins and the most amino-terminal EF-hand is unable to bind Ca^{2+} in all family members. In the case of recoverin and KChIP1, only two of its four EF-hand motifs are functional in Ca^{2+} binding (Burgoyne et al. 2004; Burgoyne 2007). Unlike the dumbbell structure of calmodulin, the NCS proteins are compact and globular when Ca^{2+}-bound and they undergo limited

conformational change on binding to their target proteins (Ames et al. 2006; Pioletti et al. 2006; Strahl et al. 2007; Wang et al. 2007) (Fig. 2). NCS proteins also differ from calmodulin in that many have motifs that allow membrane association. KChIP1 and all the members of classes A–D are N-myristoylated, whereas certain KChIP2, KChIP3, and KChIP4 isoforms harbor palmitoylation motifs. In some cases, the membrane association conferred by these moieties is dynamically regulated by Ca^{2+} binding when a sequestered mysristoyl chain becomes solvent-exposed following a Ca^{2+}-driven shift in conformation as originally described for recoverin (Ames et al. 1997). VSNL proteins are also cytosolic at resting $[Ca^{2+}]_i$ but localize to the plasma membrane or Golgi complex upon Ca^{2+} elevation (O'Callaghan et al. 2002; Spilker et al. 2002; O'Callaghan et al. 2003b). Each of the NCS proteins displays distinct subcellular localizations, which are in part determined by additional interactions with specific phosphoinositides mediated by basic amino-terminal residues immediately proximal to the site of acylation (O'Callaghan et al. 2003a; O'Callaghan et al. 2005).

NCS proteins are multifunctional regulators of various proteins involved in processes ranging from trafficking and ion channel modulation to gene transcription (Burgoyne 2004), and the function of NCS-1 in particular has been intensively studied. NCS-1, the primordial NCS protein, is highly evolutionarily conserved, retaining 59% identity with its yeast ortholog, frequenin. It displays a high Ca^{2+}-binding affinity and is able to respond to small fluctuations in $[Ca^{2+}]_i$. NCS-1 is amino-terminally myristoylated and is constitutively associated with membranes including plasma and Golgi membranes (O'Callaghan et al. 2002), although in some cell lines, NCS-1 has been found to be partially cytosolic (de Barry et al. 2006) and it is able to rapidly exchange between membrane and cytosolic pools (Handley et al. 2010). In contrast to all other NCS family members, NCS-1 is not neuron specific and is expressed in neuroendocrine cells (McFerran et al. 1998) and at low levels in several nonneuronal cell types. NCS-1 has three functional EF-hand motifs, which have differing cation specificities. It has been suggested that under resting conditions when $[Ca^{2+}]_i$ is low ($\leq 0.1\ \mu M$), EF2 and EF3 are Mg^{++} bound, whereas EF4 is a Ca^{2+} specific binding site and remains vacant. In the Mg^{++} bound state, NCS-1 adopts a conformation, which reduces exposure of hydrophobic regions. This may be important in the prevention of nonspecific interactions in the absence of a specific Ca^{2+}-signal. In the presence of elevated $[Ca^{2+}]_i$, EF2 and EF3 become Ca^{2+}-occupied, simultaneously followed by Ca^{2+} binding to EF4 (Aravind et al. 2008). The Mg^{++} bound form of NCS-1 has a fivefold lower affinity for Ca^{2+} than the Mg^{++}-free/Ca^{2+}-free apo-form. This implies that Mg^{++} binding permits significant modulation of NCS-1 and is important in fine tuning its Ca^{2+}-sensing properties (Aravind et al. 2008; Mikhaylova et al. 2009).

Much current understanding concerning the function of NCS-1 derives from overexpression or knockout studies. Overexpression in *Drosophila* caused a frequency-dependent facilitation of neurotransmitter release (Pongs et al. 1993) and its importance for neurotransmissions has been confirmed by knockout of the two *Drosophila* frequenin genes (Dason et al. 2009). In *Xenopus*, overexpression caused enhanced spontaneous and evoked transmission at neuromuscular junctions (Olafsson et al. 1995) and over-expression was also found to increase Ca^{2+}-dependent exocytosis of dense core granules in PC12 cells (McFerran et al. 1998) and to enhance associative learning and memory in *Caenorhabditis elegans* (Gomez et al. 2001; Hilfiker 2003).

Knockout of NCS-1 (Frq1) in the yeast *Saccharomyces cerevisiae* is lethal because of its requirement for the activation of Pik1, one of the two yeast phosphatidylinositol-4 kinases (PI4Ks) (Hendricks et al. 1999). NCS-1 can also interact with the mammalian Golgi enzyme PI4KIIIβ and enhances its activity three- to 10-fold (Taverna et al. 2002; Haynes et al. 2005; de Barry et al. 2006). The interaction with Golgi-associated PI4KIIIβ suggests that it may regulate secretion through the modulation of phosphatidylinositol-dependent trafficking

steps (Hendricks et al. 1999; Zhao et al. 2001; Haynes et al. 2005). In support of this, NCS-1 has also been demonstrated to associate with another PI4KIIIβ regulator ARF1, a small GTPase critical to multiple trafficking steps in mammalian cells (Haynes et al. 2005; Haynes et al. 2007).

Knockout of NCS-1 in other organisms is not lethal but does generate specific phenotypes. In *Dictyostelium discoideum*, loss of NCS-1 function alters developmental rate (Coukell et al. 2004) and in *C. elegans* results in impaired learning and memory (Gomez et al. 2001). Knockdown of one of the two NCS-1 genes in zebrafish, ncs-1, prevents formation of the semicircular canals of the inner ear (Blasiole et al. 2005). The signaling pathway involving NCS-1, ARF1, and PI4KIIIβ (Haynes et al. 2005) modulates the secretion of components important for the development of the vestibular apparatus of the inner ear (Petko et al. 2009). Knockdown of NCS-1 or expression of a dominant–negative inhibitor based on an EF-hand mutation (Weiss et al. 2000) disrupted the induction of long-term depression in rat cortical neurons (Jo et al. 2008).

Many different binding partners have been identified for NCS-1 (Haynes et al. 2006; Haynes et al. 2007) (Fig. 3) and in some cases these interactions overlap with those of calmodulin (Schaad et al. 1996). This overlap using in vitro binding assays may not be physiologically meaningful because of substantial lower affinity of NCS-1 for known calmodulin targets (Schaad et al. 1996; Fitzgerald et al. 2008). NCS-1 has a higher affinity for calcium than calmodulin and therefore may preferentially interact with certain binding partners when the amplitude of a Ca^{2+}-signal falls below the threshold for activation of calmodulin. For example, both calmodulin and NCS-1 have been shown to interact with and desensitize dopamine D2 receptors but are likely to mediate their effects at different $[Ca^{2+}]_i$ (Kabbani et al. 2002; Woods et al. 2008). Functional analyses have confirmed that NCS-1 is a regulator of D2 receptors and that this function modulates learning in mice (Saab et al. 2009). Other NCS-1 target proteins (Fig. 3) appear to be specific for NCS-1 (Haynes et al. 2006). Various studies have implicated NCS-1 in the regulation of VGCCs (Weiss et al. 2000; Tsujimoto et al. 2002; Dason et al. 2009) but there is as yet no evidence for a direct interaction. Interestingly, in *Drosophila*, the effects of NCS-1 on both neurotransmission and nerve-terminal growth can be explained by a functional interaction with the VGCC *cacophony,* which is related to the mammalian P/Q-type VGCCs (Dason et al. 2009). In contrast, in this study, there was no evidence for an essential functional interaction with the fly PI4KIIIβ ortholog *four wheel drive*.

The example of NCS-1 illustrates how evolutionary pressures have fine-tuned Ca^{2+} sensors to carry out specialized neuronal functions. The individual properties of NCS-1 allow this protein to localize to discrete domains within the cell and interact with distinct target proteins under conditions of Ca^{2+} stimulation, which would not activate the archetypal Ca^{2+} sensor, calmodulin. Although NCS-1 has been found not to be neuronal specific and may carry out more generalized functions conserved through evolution in organisms from yeast onwards, further adaptive mutations have given rise to many more members of the NCS protein family, each tasked with dedicated functions.

Much less is known about the VSNL or class B proteins, although these appear to modulate various signal transduction pathways such as cyclic nucleotide and MAPK signaling (Braunewell and Klein-Szanto 2009). VILIP-1 has been found to regulate a class of purinergic receptors (Chaumont et al. 2008). They have been shown to have effects on gene expression and are also involved in traffic of proteins to the plasma membrane (Lin et al. 2002; Brackmann et al. 2005). Hippocalcin has been suggested to be involved as a Ca^{2+}sensor in long term depression in hippocampal neurons (Palmer et al. 2005) and shows a Ca^{2+}/myristoyl switch for translocation within such neurons (Markova et al. 2008). It has also been implicated in protection from neuronal apoptosis (Mercer et al. 2000; Korhonen et al. 2004).

Recoverin is expressed exclusively in the retina and is believed to have a role in light

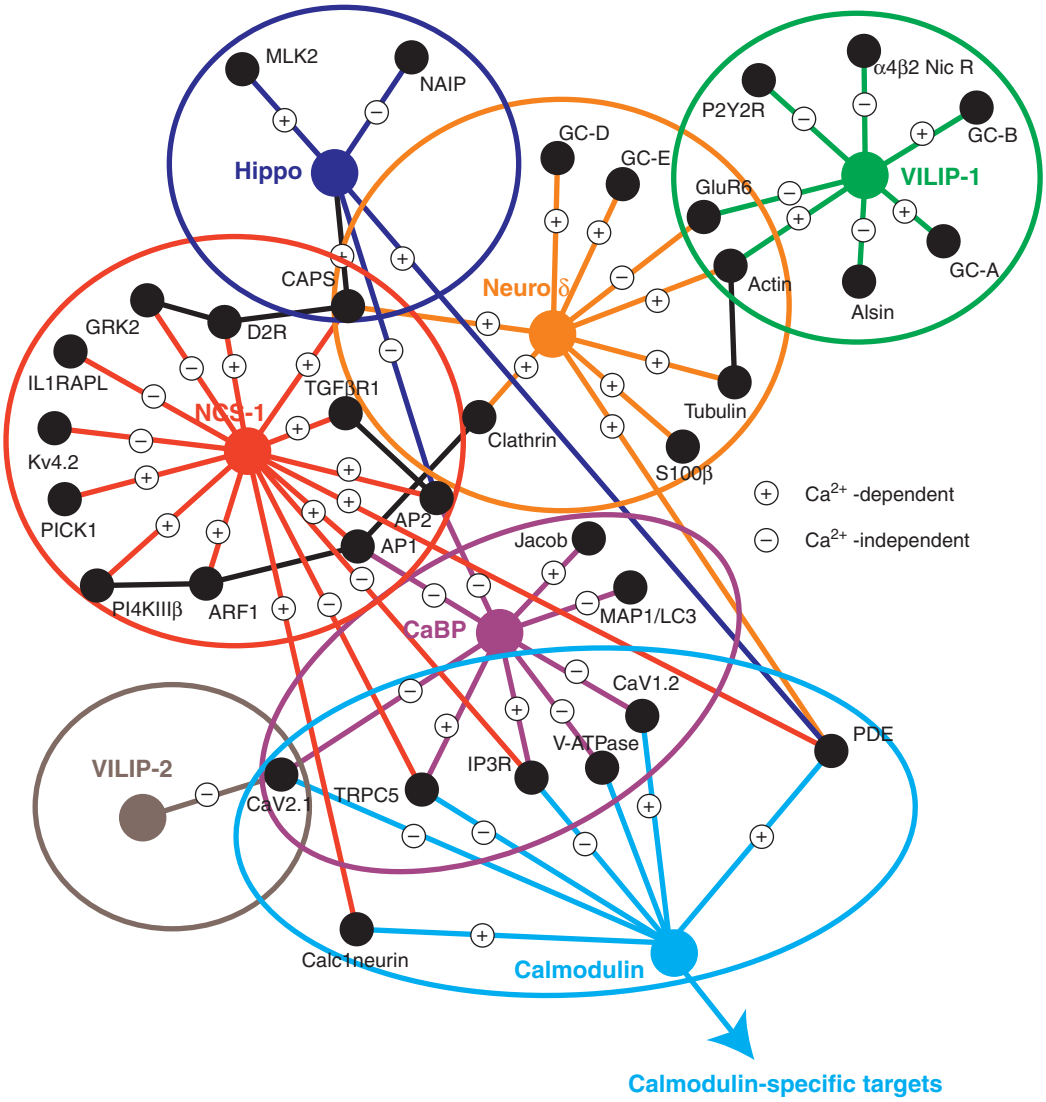

Figure 3. An interaction map showing protein–protein interactions made by some NCS proteins and CaBP1 compared to calmodulin. Known protein interactions for CABP1, hippocalcin, NCS-1, neurocalcin δ,VILIP1, and VILIP2. Links indicate where these target proteins have also been found to interact with calmodulin. It is also indicated whether these interactions require the Ca^{2+}-bound form of the protein or not.

adaptation and can enhance visual sensitivity (Polans et al. 1996; Sampath et al. 2005). Recoverin is found primarily in rod and cone cells of the retina (Yamagata et al. 1990; Dizhoor et al. 1991). Recoverin was predicted to prolong the lifetime of photolyzed rhodopsin by inhibiting its phosphorylation by rhodopsin kinase to extend the light response (Chen et al. 1995; Klenshin et al. 1995). The function of recoverin has been controversial and this hypothesis may be oversimplified. Discrepancies have been noted regarding the $[Ca^{2+}]_i$ required for rhodopsin kinase interaction, which may lie outside normal physiological limits but analysis of recoverin knockout mice have shown changes in photoresponses consistent with a

physiological role in inhibition of rhodopsin kinase (Makino et al. 2004).

The structure of recoverin has been extensively studied by X-ray crystallography and NMR studies to interrogate its structure in its Ca^{2+}-bound and -free forms (Flaherty et al. 1993; Ames et al. 1995; Tanaka et al. 1995; Ames et al. 1997; Ames et al. 2002; Weiergraber et al. 2003). Recoverin is composed of two distinct domains connected through a bent linker and forms a compact structure in the absence of Ca^{2+}. Unlike other NCS proteins, recoverin has only two functional EF-hand motifs. Upon binding of Ca^{2+}, the amino-terminal domain comprising EF-1 and EF-2 rotates through 45° relative to the carboxy-terminal domain driving extrusion of its buried myristoyl group to permit association with membranes and revealing a hydrophobic surface, which can mediate interaction with the target protein rhodopsin kinase (Ames et al. 2006). The residues involved in the interaction of the myristoyl group with the hydrophobic pocket are also conserved in the other members of the NCS family, however not all of the other family members display this Ca^{2+}/myristoyl switch (O'Callaghan et al. 2002; Stephen et al. 2007). NCS-1 and KChIP1 expose a similar hydrophobic surface upon Ca^{2+}-binding, which could be similarly important for target interactions (Bourne et al. 2001; Scannevin et al. 2004; Zhou et al. 2004b; Pioletti et al. 2006). In contrast, other NCS proteins are able to interact with certain binding proteins in the absence of Ca^{2+} and therefore Ca^{2+}-driven exposure of a hydrophobic surface cannot be the sole mechanism by which these proteins bind to effectors. Although extensive structural characterization of recoverin may go some way to inform an understanding of the general conserved structures of members of the NCS family, subtle differences in "active" surface residues of the individual proteins gives rise to their ability to interact specifically with a wide range of binding partners.

GCAPs are the only known activators of retinal guanylyl cyclases (GCs) (Palczewski et al. 2004) and are known to be physiological regulators of light adaptation (Mendez et al. 2001; Burns et al. 2002; Howes et al. 2002; Pennesi et al. 2003). They are unusual in that they activate GCs when in their Ca^{2+}-free form but become inhibitors of GCs at higher Ca^{2+} concentrations (Dizhoor and Hurley 1996). GCAP3 is expressed in cone cells, whereas GCAP1 and GCAP2 are expressed in rod cells, and despite GCAP1 and GCAP2 having the same function in the same cell type, the two proteins have different Ca^{2+} binding affinities for GC activation. This means that both proteins are required for GC activation over the full physiological Ca^{2+} concentration range, thus maximizing the dynamic range of GC activity (Koch 2006). The GCAPs are an example of how calcium sensors have become adapted to increase the dynamic Ca^{2+} sensitivity of important regulatory mechanisms in specialized cell types (Palczewski et al. 2004).

KChIPs have been found to associate with transient voltage-gated potassium channels of the Kv4 family (An et al. 2000) and can stimulate their traffic to the plasma membrane (Hasdemir et al. 2005). Four KChIP genes and a large number of expressed splice variants are present in mammals (Pruunsild and Timmusk 2005). Knockout of KChIP1 has revealed a potential role in the GABAergic inhibitory system (Xiong et al. 2009). The KChIPs are expressed predominantly in the brain but KChIP2 is also expressed in the heart, and knockout of KChIP2 causes a complete loss of calcium-dependent transient outward potassium currents and susceptibility to ventricular tachycardia (Kuo et al. 2001). KChIP3 is also known as DREAM or calsenilin, and has documented roles in transcriptional regulation (Carrion et al. 1999; Mellstrom and Naranjo 2001) and in the processing of presenilins and amyloid precursor protein, which are important in the pathogenesis of Alzheimer's disease (Buxbaum et al. 1998; Jo et al. 2004). Despite KChIP3 being implicating in three quite different functions, it is likely that these are all physiologically relevant. KChIP3 knockout mice show reduced responses in acute pain models because of changes in prodynorphin synthesis (Cheng et al. 2002), decreased β-amyloid production, and physiological defects consistent with changes to the Kv4 channels

(Lilliehook et al. 2003). Although many of the KChIPs and their isoforms may have overlapping functions, some differences between them are beginning to emerge (Holmqvist et al. 2002; Venn et al. 2008).

In support of key roles for the NCS family in higher organisms, a number of recent studies have implicated these proteins in the pathological progression of human neurological diseases in addition to the potential link with Alzheimer's disease via the interaction of KChIP3 with presenilins. VILIP1 may have a role in Alzheimer's disease because of an association with amyloid plaques in diseased brains (Schnurra et al. 2001). NCS-1 has been found to be up-regulated in patients with schizophrenia and bipolar disorders (Koh et al. 2003) and also interacts with interleukin-1 receptor accessory protein-like (IL1RAPL), a protein, which when mutated results in X-linked mental retardation (Bahi et al. 2003). The effects conferred by NCS proteins in all of these diseases would appear to be dependent on the up- or down-regulation of their expression. As yet few genetic links have been established between NCS proteins and the aforementioned diseases, suggesting epigenetic effects may be responsible. One idea is that epigenetic mediated alterations in NCS protein function may contribute to cognitive impairments observed in neurodegenerative states. Targeting of NCS protein function through this novel route may offer a future therapeutic approach for such diseases (Braunewell 2005).

The NCS protein family has evolved to carry out specialized neuronal functions that are separate to those of calmodulin. When attempting to decipher precisely why these proteins are particularly well adapted for carrying out functions in neurons, it is therefore relevant to compare their properties to those of calmodulin. Of note is their approximately 10-fold higher affinity for Ca^{2+} when compared to calmodulin. This higher affinity would allow the NCS proteins to be activated at much lower Ca^{2+} concentrations and, in combination with calmodulin, extends the dynamic range over which Ca^{2+} can regulate neuronal processes. In this way, responses to very slight or more dramatic changes in $[Ca^{2+}]_i$ would depend on which populations of calcium binding proteins are activated under particular conditions (Burgoyne and Weiss 2001). As mentioned previously, the individual expression patterns and subcellular localization of each of the NCS proteins is also likely to represent a key factor in their specific roles in neuronal cell signaling. The characteristic amino-terminal myristoylation or palmitoylation modifications, which allow these proteins to associate with membranes, may spatially partition them to relevant subcellular sites within the cell, leading to a faster and more efficient response to particular Ca^{2+} signals. Specific physiological outcomes will be determined by their distinct target proteins. The various members of the NCS family arose at points in evolution corresponding to increasing neuronal sophistication in higher animals. As such, these proteins represent an example of how the properties of calcium binding proteins have been fine-tuned to act in specific neuronal signaling pathways.

CaBP Family

The CaBPs are a relatively recently discovered family of EF-hand containing Ca^{2+}-binding proteins, which are only found in vertebrates (Haeseleer et al. 2000). They represent another example of a diverse family of Ca^{2+}-sensors capable of regulating discrete processes in the nervous systems of higher organisms. The CaBPs share sequence homology with calmodulin and also display a similar structural arrangement of EF-hand motifs. Each of the CaBPs has four EF hands, although, like the NCS proteins, they display different patterns of EF-hand inactivation (Fig. 4). In CaBPs 1–5, the second EF-hand motif is inactive with the exception of CaBP3, which also has an inactive EF-1 motif. CaBP3 is believed to represent a pseudogene as only the mRNA has been detected in cells and as yet no protein product has been found (Haeseleer et al. 2000). Two proteins were named CaBP7 and CaBP8 (Haeseleer et al. 2002), but bioinformatic analysis is more consistent with them being a conserved and distinct subfamily of CaBPs (McCue et al. 2010).

Figure 4. Schematic diagram showing the domain structure of calmodulin and members of the CaBP/calneuron protein family. Active EF-hand motifs are shown in red and inactive EF-hand motifs are shown in pink. Compared to calmodulin, the CaBPs have an extended linker region between their first EF-hand pair and their second EF-hand pair (shown in black). CaBP1 and CaBP2 have an N-myristoylation site (shown in blue). CaBP1 and CaBP2 have alternative splice sites at their N-terminus, which give rise to long and short isoforms (shown in orange). Calneurons 1 and 2 possess a 38 amino acid extension at their C-terminus (shown in purple).

We will therefore refer to them by their alternative names, calneuron 2 and calneuron 1, respectively (Wu et al. 2001; Mikhaylova et al. 2006). The calneurons, by contrast to the CaBPs, have a different pattern of EF-hand inactivation with active EF-hands 1 and 2 and inactive EF-hands 3 and 4 (Mikhaylova et al. 2006). The CaBPs also differ from calmodulin in that their central α helical linker domain connecting the carboxy- and amino-terminal EF-hand pairs is extended by four amino acid residues. This has been suggested to allow these proteins to interact with unique targets (Haeseleer et al. 2000).

A major difference compared with calmodulin is the ability of CaBP 1 and 2 and calneurons 1 and 2 to target to specific cellular membranes (McCue et al. 2009). CaBP 1 and 2 are amino-terminally myristoylated, which permits localization to the plasma membrane and Golgi apparatus (Haeseleer et al. 2000; Haynes et al. 2004). The precise amino-terminal sequence to which the myristoyl group is attached is also important in the targeting of these two proteins, as exemplified by the long and short splice isoforms generated from their genes, which show subtle differences in their localization. CaBP1-Long localizes predominantly to the Golgi and also displays some cytosolic localization, whereas CaBP1-Short localizes most prominently to the plasma membrane and to Golgi structures (Haeseleer et al. 2000; McCue et al. 2009). Alternative splicing of the CaBP1 gene generates a third protein product, caldendrin (Seidenbecher et al. 1998). This splice isoform is significantly larger than either CaBP1-Long or CaBP1-Short because of an amino-terminal extension, but caldendrin mRNA lacks the exon required for N-myristoylation and as a result the protein displays a markedly different subcellular localization to its shorter relatives.

N-terminal acylation is important in the localization of some CaBPs, but the calneurons appear to be targeted via a different mechanism. Like CaBP1 and CaBP2, calneurons 1 and 2 localize to internal membranes that colabel with Golgi-specific markers and also to vesicular structures (McCue et al. 2009; Mikhaylova et al. 2009). Calneurons 1 and 2 do not possess an amino-terminal myristoylation motif and differ from the rest of the CaBP family because of a 38-amino acid extension at their carboxyl terminus. Analysis of this sequence revealed a predicted C-terminal transmembrane domain with a cytosolic amino terminus. The carboxy-terminal domain resembles tail-anchor motifs

and directly localizes calneurons 1 and 2 to membranes particularly of the trans-Golgi network (McCue et al. 2009).

To date, only the structure of CaBP1-Short has been solved (Wingard et al. 2005; Li et al. 2009). This structural information may provide insight into the structures of the rest of the CaBPs. Analogy to calmodulin would suggest that the CaBPs should adopt a dumbbell like tertiary conformation consisting of an amino-terminal domain containing EF-1 and EF-2 and a carboxy-terminal domain containing EF-3 and EF-4, connected by a central linker. NMR analysis revealed that CaBP1 does indeed have two independent, noninteracting domains, joined by a flexible linker (Wingard et al. 2005). Investigation into the effects of Mg^{++} and Ca^{2+} binding has shown that as predicted the second EF hand of CaBP1 is incapable of binding divalent cations. EF-3 and EF-4 bind to both Mg^{++} and to Ca^{2+}, whereas EF-1 is thought to be constitutively Mg^{++} bound. Binding to either Mg^{++} or Ca^{2+} induces distinct conformations of this protein. Mg^{++} binding results in a global conformational change, whereas Ca^{2+} binding results in only a localized change in EF-3 and EF-4. These two conformational states may allow CaBP1 to interact with different target molecules driven by the ratio of Mg^{++} to Ca^{2+} (Wingard et al. 2005; Li et al. 2009). The Mg^{++} bound form of CaBP1 is similar to that of apo-calmodulin, but the Ca^{2+} bound form appears markedly different. This is perhaps unsurprising as neither of the amino-terminal EF-hands of CaBP1 bind to Ca^{2+} under saturating conditions and only EF-1 binds to Mg^{++}. This results in a constitutively closed conformation of the amino-terminal domain, whereas the carboxy-terminal domain can switch to an open conformation upon Ca^{2+} binding to EF-3 and EF-4. Comparison of the carboxy-terminal domain with that of calmodulin, however, still reveals differences in exposed hydrophobic residues thought to mediate target interactions (Wingard et al. 2005).

The structural differences between calmodulin and CaBP1 may go some way to explaining how they impose differing effects on the same target molecules. For instance, both CaBP1 and calmodulin bind to L-type Ca^{2+} channels with calmodulin causing Ca^{2+}-induced channel closure but CaBP1 promoting channel opening (Zhou et al. 2004a; Zhou et al. 2005). Both calmodulin and CaBP1 also regulate inositol 1,4,5-trisphosphate receptors (IP_3Rs) (Yang et al. 2002; Haynes et al. 2004; Kasri et al. 2004) with CaBP1 binding the type I IP_3R with 100-fold higher affinity than calmodulin. This high affinity binding may result from the exposure of a distinct hydrophobic patch revealed in the carboxyl terminus of CaBP1 upon Ca^{2+}-binding (Haynes et al. 2004; Li et al. 2009). This unique surface hydrophobicity profile is likely to be important for the specialization of CaBP1 function in the brain and retina, and the existence of splice isoforms is also likely to further fine-tune the actions of this Ca^{2+} sensor. The differing expression patterns, subcellular targeting mechanisms, and Ca^{2+} binding properties of the various members of the CaBP protein family would allow them to carry out highly specialized regulatory roles modulating important Ca^{2+}-channels in the central nervous system.

The majority of studies to date on CaBP1 have examined the functions of the longest splice isoform caldendrin and it is not yet clear whether the other splice isoforms of CaBP1 can carry out the same functions. CaBP1-Long and -Short have been found to have roles in the regulation of various Ca^{2+}-channels including P/Q-type ($Ca_V2.1$) channels (Lee et al. 2002), L-type ($Ca_V1.2$) channels (Zhou et al. 2005; Cui et al. 2007), TRPC5 channels (Kinoshita-Kawada et al. 2005), and IP_3Rs (Yang et al. 2002), which they apparently inhibit (Haynes et al. 2004; Kasri et al. 2004). The interaction of $Ca_V2.1$ with CaBP1 appears to rely acutely upon amino-terminal myristoylation. Wild type, myristoylated, CaBP1-Long enhances channel inactivation and shifts the activation range to more depolarizing voltages (Lee et al. 2002). An N-myristoylation mutant, however, was unable to mediate these effects and instead modulated channels in a similar fashion to calmodulin (Few et al. 2005). Differential modulation of L-type channels depending on the

splice isoform of CaBP1 has also been observed. CaBP1-Short has been shown to completely inhibit inactivation of $Ca_V1.2$ channels (Zhou et al. 2005), but caldendrin causes a more modest suppression and signals through a different set of molecular determinants (Tippens and Lee 2007). This suggests that the subcellular localization of CaBP1 splice variants is important for their function and there are likely to be individual roles for each protein. Interactions of caldendrin with other types of proteins have also been reported, such as its interaction with light chain 3 of MAP1A/B, a microtubule cytoskeletal protein (Seidenbecher et al. 2004), and an interaction with myo1c, a member of the myosin-1 family of motor proteins (Tang et al. 2007). Finally, a role for caldendrin in NMDA receptor (NMDAR) signaling has been reported via an interaction with a novel neuronal protein, Jacob. Upon extrasynaptic NMDAR activation, Jacob translocates to the nucleus to influence CREB activity, resulting in the stripping of synaptic contacts and an associated simplification of dendritic architecture. Synaptic NMDAR mediated synpatodendritic $[Ca^{2+}]_i$ elevation induces caldendrin binding to Jacob, inhibiting nuclear trafficking and maintaining dendritic organization. This interaction represents a novel mechanism of synapse to nucleus communication and highlights the important roles of CaBP family members in the mammalian central nervous system (Dieterich et al. 2008).

Little information is available concerning the function of CaBP2 apart from in vitro studies suggesting that it might stimulate CaMK activity (Cui et al. 2007). Initially, CaBP2 was detected exclusively in the retina, although it has also been identified in auditory inner hair cells (Cui et al. 2007). CaBP5 was also detected in inner hair cells as well as in the retina, but in contrast to CaBP2, was found to have a modest inhibitory effect on the inactivation of $Ca_V1.3$ channels in transfected cells (Cui et al. 2007). Little is known about the functions of CaBP5, but knockout mice displayed reduced sensitivity of retinal ganglion cells to light responses, implicating CaBP5 in phototransduction pathways. CaBP5 was also found to interact with and suppress calcium-dependent inactivation of $Ca_V1.2$ channels (Rieke et al. 2008).

CaBP4 is the most extensively characterized of the CaBP family. It is expressed in the retina, where it localizes to synaptic terminals and has also been detected in auditory inner hair cells. CaBP4 modulates voltage gated Ca^{2+}-channels and directly associates with the carboxyl terminus of the $Ca_V1.4$ $\alpha1$ pore-forming subunit, shifting the activation range of the channel to more hyperpolarized voltages in transfected cells (Haeseleer et al. 2004). CaBP4 has also been shown to eliminate Ca^{2+}-dependent inactivation of $Ca_V1.3$ channels, which is likely to be important in the modulation of these channels in inner hair cells, where Ca^{2+}-dependent inactivation is weak or absent, probably allowing the audition of sustained sounds (Yang et al. 2006). A stronger inhibitory effect has been noted for CaBP1, however, suggesting that CaBP4 may not be the key Ca^{2+} sensor involved in this process (Cui et al. 2007). The function of CaBP4 is modulated by protein kinase C ζ in the retina, with increased CaBP4 phosphorylation in light-adapted tissue. Phosphorylation prolongs Ca^{2+} currents through $Ca_V1.3$ channels and suggests that light-stimulated phosphorylation of CaBP4 might help to regulate presynaptic Ca^{2+} signals in photoreceptors (Lee et al. 2007). CaBP4 has also been implicated in neurotransmitter release at synaptic terminals because of its interaction with unc119, a synaptic photoreceptor protein important for neurotransmitter release and maintenance of the nervous system (Haeseleer 2008). Knockout of CaBP4 results in mice with abnormalities in retinal function, where rod bipolar responses are approximately 100 times lower than those observed in wild-type animals (Haeseleer et al. 2004).

The functions of calneurons 1 and 2 have only recently begun to be investigated in detail. Both have been found to inhibit the activity of PI4KIIIβ at low or resting $[Ca^{2+}]_i$. Overexpression of the proteins was also found to inhibit Golgi-to-plasma membrane trafficking, caused enlargement of the trans-Golgi network (TGN), and reduced the number of Piccolo-Bassoon positive transport vesicles. A molecular switch

for the production of phosphoinositides at the TGN is thought to be created by the opposing roles of NCS-1 and calneurons 1 or 2. At elevated Ca^{2+} levels, NCS-1 preferentially binds to PI4KIIIβ over the calneurons, activating the enzyme to drive enhanced TGN-to-plasma membrane trafficking (Mikhaylova et al. 2009). Patch clamping experiments have shown that over-expressed calneuron 1 can inhibit N-type Ca^{2+}-channel currents in 293T cells and this inhibition was not observed with a truncated calneuron 1 lacking its hydrophobic C-terminus, suggesting normal localisation is important in carrying out this function (Shih et al. 2009).

CaBPs have been directly or indirectly implicated in multiple neuronal diseases. Post-mortem brains of chronic schizophrenics have lower numbers of caldendrin-immunoreactive neurons, which express the protein at a much higher level. This loss of caldendrin in some neurons and up-regulation in others is likely to profoundly change synapto-dendritic signalling in schizophrenic patients (Bernstein et al. 2007). Changes in the distribution of caldendrin have also been observed in kainate-induced epileptic seizures in rats. Caldendrin translocates to the postsynaptic density only in rats that suffered epileptic seizures and may implicate the protein in the pathophysiology of the disease (Smalla et al. 2003). CaBP4 function has been convincingly linked to disease and mutations in this gene generate defects in retinal function. Knockout of CaBP4 was shown to cause a phenotype similar to that of incomplete congenital stationary night blindness patients (Haeseleer et al. 2004) and mutations in CaBP4 can cause autosomal recessive night blindness (Zeitz et al. 2006). Patients with mutations in the CaBP4 gene have been identified, which display congenital stationary night blindness. However, some patients with mutations display different phenotypes (Zeitz et al. 2006). In particular, a novel homozygous nonsense mutation has been reported in two siblings that resulted in severely reduced cone function but only negligible effects on rod function (Littink et al. 2009). It appears, therefore, that genetic mutations in CaBP4 underlie cone-rod synaptic disorders (Littink et al. 2009).

CONCLUDING REMARKS

It has become increasingly clear that a full understanding of how specific aspects of neuronal function are regulated in response to spatially and temporally distinct Ca^{2+} signals will require a detailed knowledge of the Ca^{2+} sensors involved. Some of these, like synaptotagmin, are specialized for particular neuronal functions, whereas others such as calmodulin may be involved in multiple cellular processes. In recent years, much has been learnt about the functions of the NCS family of Ca^{2+} sensors, although the functions of some of the family members are still unknown. Nor is it clear what the significance is of the multiple genes and splice variants of these proteins. The CaBPs have so far been much less studied and much remains to be learnt about the functions of each of these sensors. Further advances will require new insights into the molecular targets of each of the Ca^{2+} sensors, the molecular basis for their regulation of these targets, and more detailed dissection of the functional roles of each protein in identified neurons.

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