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Review

Mechanisms for localising calcineurin and CaMKII in dendritic spines

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ABSTRACT

Calcineurin and calmodulin-dependent protein kinase II (CaMKII) are both highly abundant in neurons, and both are activated by calmodulin at similar Ca^{2+} concentrations in the test tube. However, they fulfill opposite functions in dendritic spines, with CaMKII activity driving long-term synaptic potentiation following large influxes of Ca^{2+} through NMDA-type glutamate receptors (NMDARs), and calcineurin responding to smaller influxes of Ca^{2+} through the same receptors to induce long-term depression. In this review, we explore the notion that precise dynamic localisation of the two enzymes at different sites within dendritic spines is fundamental to this behaviour. We describe the structural basis of calcineurin and CaMKII localisation by their interaction with proteins including AKAP79, densin-180, α-actinin, and NMDARs. We then consider how interactions with these proteins likely position calcineurin and CaMKII at different distances from Ca^{2+} microdomains emanating from the mouths of NMDARs in order to drive the divergent responses. We also highlight shortcomings in our current understanding of synaptic localisation of these two important signalling enzymes.

1. Introduction

Ca^{2+} entry into dendritic spines is essential for triggering changes in both spine morphology and the strength of synaptic connections. Ca^{2+} signalling mechanisms have been most intensively researched in spines that lie postsynaptic to hippocampal CA3-CA1 synapses. These synapses are the leading model for understanding the molecular basis of synaptic plasticity, and their properties are thought to be characteristic of excitatory synapses in general. Ca^{2+} entry through NMDA-type glutamate receptors (NMDARs) in the postsynaptic membrane is the trigger for long-term changes in the strength of CA3-CA1 synapses [1], with the degree of Ca^{2+} influx determining the direction of plasticity [2]. Both low-frequency (*e.g.*, 1 Hz) homo-synaptic stimulation, and hetero-synaptic 'post' before 'pre' spike timing [3], trigger relatively small Ca^{2+} influxes into dendritic spines that induce long-term depression (LTD) [1]. Larger Ca^{2+} influxes that induce long-term potentiation (LTP) may be triggered by high-frequency homosynaptic stimulations (evenly-spaced or tetanic), and 'pre' before 'post' spike timing [3]. The molecular basis of Ca^{2+}-driven synaptic plasticity has been intensively researched as the process is thought to underlie learning and memory. Two highly abundant [4,5] Ca^{2+}/Calmodulin (CaM)-sensitive enzymes have emerged as the key inducers of LTP and LTD of excitatory glutamatergic synapses following Ca^{2+} influx: the phosphatase calcineurin and CaM-dependent protein kinase II (CaMKII). The two enzymes control the phosphorylation state of synaptic proteins including AMPA-type glutamate receptors (AMPARs), thereby determining the conductance and numbers of these receptors in the postsynaptic density (PSD) [6]. This is an effective mechanism for altering synaptic strength, as AMPARs are the major mediator of fast excitatory synaptic transmission. Calcineurin dephosphorylation of AMPARs at residue Ser845, following smaller influxes of Ca^{2+}, is an important component of LTD induction [7,8]. Larger influxes of Ca^{2+} activate Ca^{2+}/CaM-dependent protein kinase II (CaMKII) resulting in phosphorylation of proteins, including AMPARs at residue Ser831 to induce LTP [9–11]. Activated CaMKII is also thought to play a key structural role in LTP that supports enduring modifications in spine morphology [12]. Despite its status as the foremost mechanism for driving bidirectional synaptic plasticity [13], Ca^{2+} activation of calcineurin and CaMKII presents a paradox: how do enzymes that are activated with similar half-maximal Ca^{2+} and CaM concentrations in the test tube [14,15] respond to different modes of Ca^{2+} influx through NMDARs in dendritic spines?

Elevations in second messengers including Ca^{2+} are localised within neurons [16]. Two-photon fluorescence lifetime imaging microscopy shows that CaMKII activation can be insulated within single spines [17]. Experiments with fast and slow Ca^{2+} chelators indicate that Ca^{2+} signalling may be further localised at the sub-spinal level [18,19]. Ca^{2+} 'microdomains' are known to occur in the vicinity of L-type Ca^{2+} channels, and enable spatial decoding of Ca^{2+} signals by these channels [20]. Highly localised Ca^{2+} signalling is emerging as an important feature of cellular signalling in further contexts, including coupling of Ca^{2+} release from acidic organelles and the endoplasmic reticulum [21]. It is likely that highly localised elevation of Ca^{2+} in the vicinity of

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NMDARs is also important in dendritic spines. For example, localised activation of calcineurin in restricted Ca^{2+} microdomains could explain why only the phosphatase is activated during LTD. Such a model is consistent with the patterns of electrical sitmulation that induce LTP or LTD [1]. Investigation of localised cAMP signalling has revealed that cAMP cyclases, receptors, and phosphodiesterases are precisely co-ordinated within cAMP microdomains [22]. Protein-protein interactions involving anchoring proteins are fundamental to establishing this sub-cellular structure. In an analogous way, the internal structure of dendritic spines – in particular mechanisms for positioning CaMKII and calcineurin – will direct responses to localised Ca^{2+} signals. On this basis, the aim of this review is to consider how CaMKII and calcineurin are positioned in dendritic spines, and how this relates to their roles in responding to Ca^{2+} signals. We begin by discussing high-resolution structural information for calcineurin (Part 2) and CaMKII (Part 3), with a focus on describing sites on each enzyme that are known to mediate protein-protein interactions. After summarising the overall architecture of dendritic spines (Part 4), we consider how interaction sites on CaMKII and calcineurin are likely to determine the localisation of the enzymes in idealised 'naïve' spines (Part 5), and how this positioning may underlie their functions. Both calcineurin and CaMKII relocate in spines upon larger influxes of Ca^{2+} that drive LTP. In Part 6, we discuss the implications of this structural reorganisation.

2. Calcineurin: structural overview & key interaction sites

Calcineurin is a heterodimer consisting of a catalytic subunit (A) of ~60 kD that presents a helix following the catalytic domain that interacts with a myristylated B subunit of ~19 kD [23] (Fig. 1a). Calcineurin is highly expressed throughout the brain, accounting for 1% of total brain protein in hippocampus [4], and it was first identified as a major neuronal CaM-binding protein ('calci*neur*in') prior to its subsequent characterisation as the Ca^{2+}/CaM-dependent phosphatase known as protein phosphatase 2B (PP2B) [24]. The B subunit resembles CaM with four EF hands that can each coordinate Ca^{2+}. At resting cellular Ca^{2+} concentrations, high affinity sites 3 and 4 are fully Ca^{2+} occupied while sites 1 and 2 act as Ca^{2+} sensors. Ca^{2+} binding to sites 1 and 2 serves as a gateway for Ca^{2+}/CaM binding, which releases an autoinhibitory element to bring about a further ~10-fold increase in phosphatase activity [15]. *In vitro* enzymatic assays suggest that calcineurin is activated with a half-maximal Ca^{2+} concentration in the range of a few hundred nM to a few μM, depending on factors including CaM and Mg^{2+} concentration [25]. The half-maximal CaM concentration for activation *in vitro* is ~15–30 nM [26,27], much lower than the concentration of CaM present in dendritic spines with CaM accounting for ~1.5% of total protein in hippocampus [28]. Two interaction sites have been identified on calcineurin that enable interaction with both substrates and anchoring proteins that present short linear motifs encoded by the amino acid sequence 'PxIxIT' and/or 'LxVP'.

2.1. PxIxIT-type interactions

The PxIxIT motif was first identified in the calcineurin substrate NFAT [29], which enters the nucleus following dephosphorylation to regulate transcription. NFAT dephosphorylation by calcineurin can be antagonised using a high affinity peptide with the consensus sequence PVIVIT with comparable efficacy to the calcineurin inhibitor cyclosporine [30]. PxIxIT motifs bind at a site equivalent to the RVxF site on protein phosphatase 1 (PP1) [31], with the PxIxIT sequence adopting a β-strand conformation (blue, Fig. 1b) that packs against and extends a

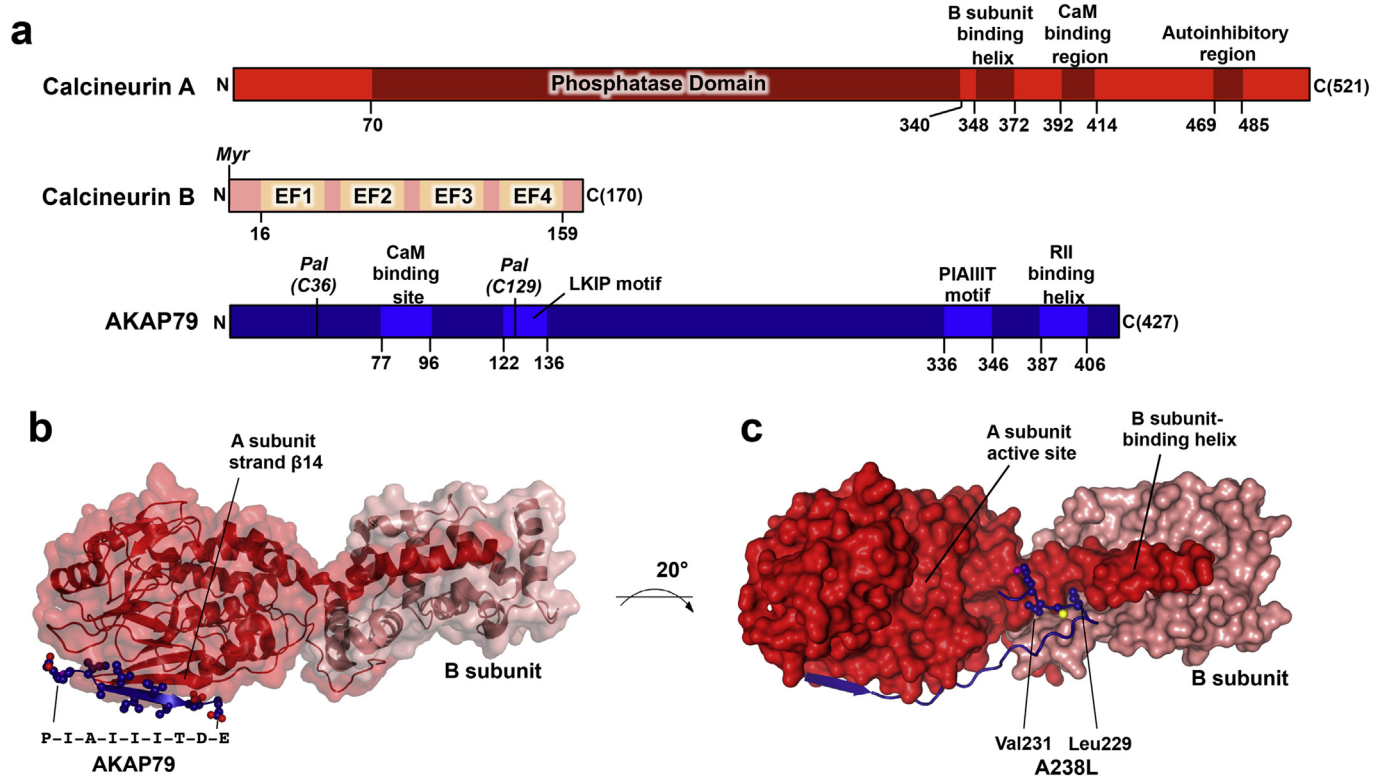

Fig. 1. Calcineurin interaction sites. (a) Topology diagrams for mouse calcineurin A and B subunits, and human AKAP79. Lipid attachment sites for myristyl ('Myr') and palmitoyl ('Pal') groups are indicated. (b) Cartoon representation of crystal structure (PDB ID 3LL8) of calcineurin (red) in complex with a peptide encompassing the constitutive PIAIIIT anchoring motif of AKAP79 (blue). (c) The location of the LxVP motif binding site on calcineurin is demonstrated by the structure of calcineurin in complex with the viral inhibitor A238L (blue), which contains the sequence 'LCVK' (PDB ID 4F0Z). A238L also contains a PxIxIT-type motif that binds in the conventional way to strand β-14. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

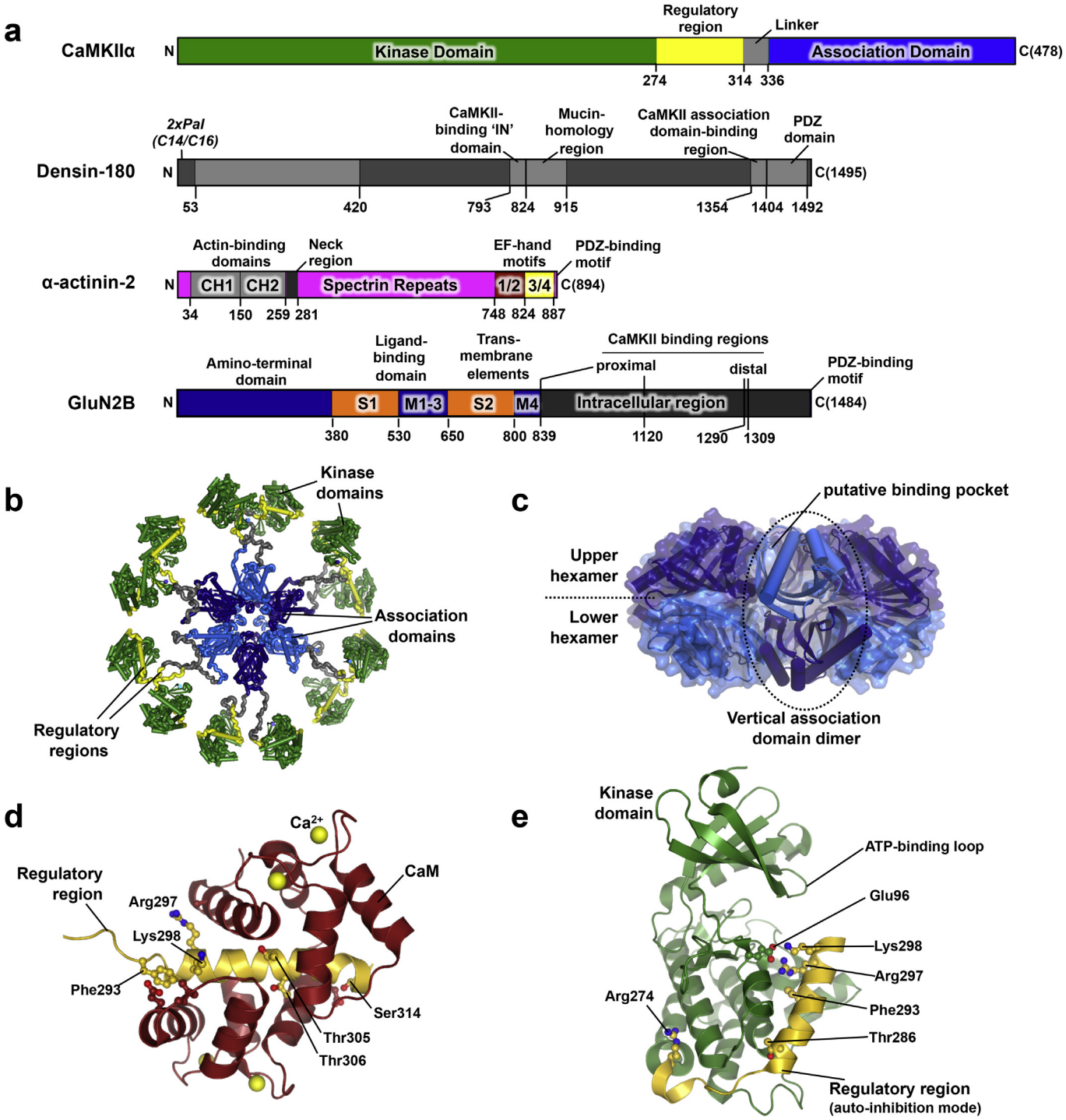

Fig. 2. CaMKII interaction sites. (a) Topology diagrams for mouse CaMKIIα and key interacting proteins within dendritic spines. Numberings are for mouse α-actinin-2, rat densin-180, and human GluN2B. Palmitoyl ('Pal') groups are indicated. (b) Structural overview of CaMKIIα with domains coloured according to the topology diagram in (a). The association domains of protomers at the centre of the dodecamer are alternately coloured light and dark blue (cryo-EM reconstruction, PDB ID 5UOW). (c) Side view of central association domain dodecamer (C-terminal 131 residues), taken from crystal structure of auto-inhibited dodecamer (PDB ID 3SOA). A vertical dimer – thought to act as a unit during subunit exchange – is highlighted. (d) Representation of the complex between Ca^{2+}/CaM (red) and the regulatory region of CaMKIIδ (yellow) from the crystal structure with PDB ID 2WEL. (e) Auto-inhibited human CaMKIIα kinase domain (PDB ID 2VZ6). Contacts between the kinase domain and regulatory elements are centred on the T-site that coordinates T286. Amino acids in panels (d) and (e) are numbered according to the equivalent positions in mouse CaMKIIα. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

β-sheet on the calcineurin catalytic domain (red, Fig. 1b) [32]. The PxIxIT binding site is located on a different face to the phosphatase active site (Fig. 1c). Interactions between calcineurin and PxIxIT motifs are relatively low affinity in comparison to, for example, interactions between protein kinase A and its anchoring proteins [33,34]. Roy and colleagues showed that calcineurin binds to a range of PxIxIT motifs with affinities ranging from 15 to 250 μM [35]. In dendritic spines, AKAP79 is the only protein besides NFAT known to interact with the PxIxIT binding site on calcineurin. AKAP79, which localises to the PSD [36], was first found to bind calcineurin using the yeast two-hybrid

assay [37]. AKAP79 contains the sequence 'PIAIIITD' in its C-terminus [38] about 30 amino acids upstream of an anchoring helix for type II PKA regulatory subunits. Although this sequence is not a perfect match for the 'PxIxIT' consensus, the crystal structure of calcineurin in complex with a peptide spanning AKAP79 amino acids 336–346 confirms that the PIAIIITD sequence from AKAP79 forms a β strand that interacts with strand β-14 in calcineurin, as predicted [39] (Fig. 1b). Hydrophobic side-chains project from either side of the β-strand formed by the AKAP79 PIAIIITD motif [39], unlike conventional PxIxIT motifs in which they only project on one side. This potentially enables calcineurin to bind to two copies of calcineurin at once. Consistent with this notion, crystallographic studies show that two copies of calcineurin can bind simultaneously to either side of β-strands formed by PVIVIT [32] and PIAIIITD [39] peptides. In addition, gas phase measurements [40] indicate that calcineurin can bind in a ratio of 2 calcineurin: 1 AKAP79. Conversely, interaction assays in solution [39,41], fluorescent imaging in cultured cells [39], and imaging by electron microscopy [41] support a binding ratio of 1 AKAP79: 1 calcineurin, with calcineurin binding to a single side of the PIAIIITD β-strand, as shown in Fig. 1b. Calcineurin interacts with the AKAP79 PIAIIITD motif in the low μM range [39,41], consistent with high occupation of the site in dendritic spines given the high concentration of calcineurin in the brain [4].

2.2. LxVP-type interactions

A second short linear motif that interacts with calcineurin, 'YLAVP', was subsequently identified in the NFAT family [42,43]. Motifs of this class align with the consensus ΦLxVP (where Φ is typically aromatic, and x cannot be glycine), and generally only interact with activated calcineurin [44]. A crystal structure of calcineurin in complex with the viral inhibitor protein A238L, which contains sequences conforming to both the PxIxIT and LxVP classes, shows how the LxVP-type motif of A238L binds at the interface of the calcineurin A and B subunits in proximity to the binding site for the immunosuppressant drug cyclosporine (Fig. 1c) [45]. The upstream PxIxIT-type motif of A238L interacts in the conventional mode with calcineurin strand β-14 (Fig. 1c). Calcineurin-interacting proteins may contain either or both of the two motif types, and there is no clear rule dictating spacing when both motifs are present [45]. AKAP79 is an example of a bipartite calcineurin-interacting protein [40], with the LxVP-type sequence 'LKIP' in its N-terminus mediating Ca^{2+}/CaM-dependent interactions with calcineurin [41]. Single particle analysis by electron microscopy shows that Ca^{2+}/CaM triggers a contraction in the AKAP79-calcineurin complex brought about by association of the AKAP79 LKIP sequence [41] with activated calcineurin. The functional effects of removing LKIP from AKAP79 are subtle in comparison to removing the PIAIIITD calcineurin anchoring site [41]. AKAP79 is itself regulated by CaM binding to a novel '1–4–7-8' type CaM interaction motif centered ~40 amino acids N-terminal to the LKIP sequence [26] (Fig. 1a). Pull down assays show that CaM association with this site, at slightly higher Ca^{2+} concentrations than required to activate calcineurin, is required for engagement of the LKIP motif with calcineurin [26]. Bioinformatic analyses suggest that there may be many uncharacterised LxVP-type calcineurin motifs [46]. Commonly applied protein-protein interaction screening approaches, such as the yeast two-hybrid assay or MS identification of proteins in immuno-precipitates from brain extracts, are typically performed in the absence of elevated Ca^{2+}. Therefore, there is scope for the identification of further proteins, in addition to NFAT and AKAP79, that employ LxVP-type interaction motifs in dendritic spines.

3. CaMKII: structural overview & key interaction sites

CaMKII is a predominantly dodecameric enzyme, as indicated by its Stokes radius [47], imaging by electron microscopy using protein purified from native [48,49] and transgenic [50,51] sources, and protein crystallography [52]. The enzyme assembles into two stacked rings of six subunits with 622 symmetry [53] held together by C-terminal association domains (blue, Fig. 2a) that pack together in the interior of the ring (Fig. 2b). A minor subpopulation of 14 subunit multimers is also thought to exist [51,54]. Of the four CaMKII isoforms, α and β are most highly expressed in the forebrain at a ratio of about three α per β subunit, with CaMKII partitioning into a mixture of α/β heteromers (ratio 2α:1β) and α-only but not β-only homomers [55]. All four CaMKII isoforms are highly spliced, particularly within the variable region that falls between the regulatory elements (yellow, Fig. 2) and association domains (blue, Fig. 2) [56]. CaMKIIα adopts a disc-shaped structure with a maximum diameter of ~25–30 nm (Fig. 2b) [50,51], whereas splice variants with shorter linker regions, such as β7, are more compact [14]. The half-maximal CaM (K_{CaM}) concentration for activation of CaMKII holoenzymes containing α and β subunits is in the range of 22–68 nM [57], with higher CaM sensitivity for higher proportions of β subunits [58]. Recent insights suggest that vertical CaMKII dimers (Fig. 2c) are the basic building block of holoenzymes [59], with activation triggering the inter-enzyme exchange of dimers over a time-scale of minutes [60] with a potential role for spiral-shaped (rather than disc-shaped) intermediates [54].

Unlike many protein kinases, CaMKII lacks a phosphorylation site in its activation loop. Instead, kinase activity is controlled by a key autophosphorylation site on residue T286 (residues are numbered by convention according to the mouse α isoform). Following CaMKII activation by Ca^{2+}/CaM [61], intersubunit autophosphorylation may occur at T286 [62,63] to generate an autonomously active form of the enzyme [64–66], although this form is less active than Ca^{2+}/CaM-bound CaMKII [67]. Autonomously active CaMKII is likely important for the maintenance of LTP. Consistent with this notion, T286A substitution in mice occludes LTP and leads to learning deficits [10]. Generation of autonomous activity is linked to the frequency of Ca^{2+} oscillations [68]. The concentration of Ca^{2+} that triggers autonomy is shifted higher in the presence of high concentrations of PP1 [69], which may contribute to ensuring that CaMKII is only activated by large influxes of Ca^{2+}. Withdrawal of Ca^{2+}/CaM following establishment of autonomy leads to phosphorylation at two further threonine residues (positions 305 and 306), within the Ca^{2+}/CaM-binding region whose phosphorylation prevents subsequent Ca^{2+}/CaM activation [70]. Mutation of these sites consequently affects synaptic plasticity and learning [71], for example, phospho-mimetic substitutions at positions 305 and 306 decrease CaMKII association with the PSD, occlude LTP and reduce learning [71]. In the following sections, we lay out mechanisms for localising CaMKII by association with the association domain (3.1), regulatory domain (3.2) or kinase domain (3.3).

3.1. Association domains interactions

The association domain of each CaMKII protomer within the holoenzyme folds into a wedge-like shape (Fig. 2b & c) with a prominent external N-terminal helix that packs against a six-stranded β-sheet [72]. In each dimer spanning the upper and lower rings of the holoenzyme, the N-termini of the external helices face towards one another [51,52]. The association domains of CaMKIIα assemble leaving a central hole of ~20 Å diameter [51,52], and with grooves lined by acidic residues presented on each vertical subunit interface. Interactions between the regulatory elements and these grooves underlie subunit exchange [54,60]. Densin-180 is the only documented interaction partner for the CaMKII association domain [73,74]. Densin-180 is a core protein of the PSD [75] that does not span the cell membrane [76,77] but interacts with the internal face of the membrane through palmitoyl groups attached near to its N-terminus [78]. The efficacy of binding to the CaMKII association domain depends on the splice variant of densin-180 [73], with splicing altering the sequence of a critical binding region of ~30 amino acids within the C-terminal region of densin-180 [73] (Fig. 2a). The exact residues on the CaMKII association domain involved in coordinating densin-180 are unknown. Although densin-180

can bind inactive CaMKII, approximately twice as much active CaMKII binds to a fusion of GST and the final ~250 amino acids of densin-180 (with CaMKII at 50 nM) [73]. According to a sedimentation assay, pT286-CaMKII binds to this GST-fusion with a K_d of ~200 nM [73]. Small-angle x-ray scattering shows that CaMKII activation increases the holoenzyme radius of gyration, consistent with release of kinase domains from centrally docked positions, adjacent to the hub domain [59]. Therefore, it is likely that the interaction involves the vertical outward-facing surfaces of the association domains, at least in part. Each protomeric association domain also contains a deep cavity formed by residues within its C-terminus (Fig. 2c), but no ligands have been identified to date for this putative binding pocket [72,79].

3.2. Regulatory domain interactions

The regulatory region of CaMKII (typically denoted as spanning residues 274–314 in mouse CaMKIIα) performs a dual role in controlling the activity and localisation of the kinase [14]. Ca^{2+}/CaM (red, Fig. 2d) wraps around a helix formed by amino acids 293–314 (yellow, Fig. 2d) to release autoinhibitory elements of the regulatory region [80] from the kinase domain to allow ATP binding, permit substrate phosphorylation [61,79], and expose T286 for trans-phosphorylation. Ca^{2+}/CaM binding also frees amino acids 274–291 to engage in inter-subunit interactions that enable cooperative activation [81]. Isothermal measurements reveal that CaM must overcome an energy barrier for release of the binding elements within CaMKII from the kinase domain [79]. Autophosphorylation at T286 traps CaM, decreasing the off rate by 1000-fold [82], whereas T305/306 phosphorylation occludes CaM binding [83,84]. The regulatory domain mediates two important protein-protein interactions besides CaM. First, CaMKIIβ binds directly to F-actin [85] using elements in exon V1 that is not spliced into the regulatory region of CaMKIIα [86], although mixed α/β holoenzymes also associate with F-actin [85]. F-actin binding does not activate CaMKIIβ [87]. The interface is disrupted by Ca^{2+}/CaM [88], which provides a mechanism to couple Ca^{2+} influx to CaMKII release from the actin cytoskeleton. This interaction enables CaMKIIβ to bundle F-actin *in vitro* [87] and, consistent with a key role for the β isoform in regulating the cytoskeleton, CaMKIIβ knock-down alters neurite extension and synapse formation [87,89]. Second, the regulatory domain of CaMKIIα interacts with the actin crosslinking protein α-actinin [74]. In mammals, there are four α-actinin isoforms with distinct expression patterns, including in the developing [90] and adult [91] brain. α-actinin-2 is localised to dendritic spines of glutamatergic neurons, where its expression increases postnatally [90,92]. Following its spectrin-like repeats, α-actinin-2 contains four EF hand motifs with homology to CaM (Fig. 2a). EF hands 3 and 4 of α-actinin-2 are thought to associate with the CaMKIIα regulatory region with no requirement for Ca^{2+} [93]. Consistent with this model, the interaction is sensitive to the phosphorylation state of T305 [94], and α-actinin competes with Ca^{2+}/CaM for binding to CaMKII [94]. Unlike CaM, α-actinin binding is not affected by CaMKII T306 phosphorylation [94]. α-actinin only weakly activates CaMKII [93], presumably because the absence of interactions with EF hands 1 and 2 does not release the autoinhibitory elements of CaMKII in the same way as CaM. This suggests that α-actinin's primary role is not to activate CaMKII and may instead be structural.

3.3. Kinase domain interactions

The autoinhibited kinase domains of all four human CaMKII isoforms adopt highly similar conformations [79]. For all isoforms, in the absence of T286 phosphorylation or Ca^{2+}/CaM binding, the regulatory elements (yellow, Fig. 2e) dock at two adjacent regions within the kinase domain (green, Fig. 2e). In human α and β CaMKII isoforms, contacts are primarily through the 'T' site (centred on the binding pocket for T286) rather than the 'S' (substrate binding) site [79] (Fig. 2e). E96 is oriented away from the ATP binding pocket in the autoinhibited kinase (Fig. 2e), which prevents binding of ATP [79]. In activated CaMKII, both S and T sites are available for interaction with substrates and interaction partners. GluN2B (formerly 'NR2B') subunits of NMDARs have emerged as the key interaction partner for activated CaMKII. NMDARs are tetrameric receptors composed of an obligatory NR1 dimer and differing type II subunits [95]. GluN2B [96–99] and, to a lesser extent, GluN2A [100] subunits bind to all activated CaMKII isoforms [101]. CaMKII binds with comparable affinity to two separate sites within the GluN2B C-terminal tail: a distal site between residues 1290–1309 [102] that encompasses a CaMKII phosphorylation site at S1303 [103]; and a less clearly defined proximal site within the region 839–1120 that does not undergo CaMKII phosphorylation [101,104,105]. S1303 phosphorylation drives the dissociation of autophosphorylated CaMKII from GluN2B peptides corresponding to the distal site [102]. Consistent with phosphorylation weakening the affinity of the interaction, the substitution S1303D in GluN2B impairs LTP [106]. Since T286-autophosphorylated CaMKII binds to GluN2A subunits with a reduced affinity compared to GluN2B [99], altering the ratio of GluN2A:GluN2B through brain development is thought to be a mechanism for altering synaptic propensity for LTP [106]. A two amino acid insertion in the distal binding site of GluN2A underlies the different affinities of GluN2A and B for CaMKII [107].

Disruption experiments with peptides that occupy either the S or S and T sites on CaMKII show that interaction with GluN2B involves occupation of the T-site [94,99,102,108]. In comparison, sequence surrounding the important regulatory residue S831 in GluA1 subunits does not associate with the T site, so long-term interactions between CaMKII and AMPARs do not occur. Higher peptide concentrations are required to disrupt the GluN2B-CaMKII interaction *in situ* than to inhibit CaMKII activity [109], suggesting that the interaction is very robust in the context of the PSD. Experiments with CaMKII variants bearing S or T site mutations suggest that residues prior to S1303 in NMDARs initially bind to the S-site before the autoinhibitory domain of CaMKII vacates the T-site enabling its occupation by GluN2B [108]. *C. elegans* CaMKII kinase domain crystal structures showing binding to adjacent regulatory segments (PDB ID 3KK8) [81], and association with the 18-mer peptide inhibitor CaMKIINtide (PDB ID 3KL8) [81], provide insights into possible binding modes for substrates such as NMDARs that occupy the T site. In addition to elements that interact with the association domain of CaMKII, densin-180 contains a sequence known as the 'IN' domain immediately N-terminal to its mucin-like domain (Fig. 2a, residues 793–824). This sequence also associates with CaMKII kinase domains, contingent on activation [97]. The K_d for interaction between CaMKII and this region of densin-180 is decreased by more than 100-fold by CaMKII activation to ~75–100 nM [74]. The inhibitor 'N-tide' potently inhibits this interaction consistent with occupation of the T-site by densin-180. Mutational analysis shows that the binding mode for the densin-180 'IN' domain is subtly different to GluN2B [97]. Interaction with the densin-180 IN domain selectively inhibits CaMKII phosphorylation of substrates that occupy the T-site. In summary, protein-protein interaction sites have been identified in each CaMKII sub-domain. This imbues CaMKII dodecamers with the potential to engage in highly complex patterns of interaction.

4. Overview of dendritic spine ultrastructure

Before considering how calcineurin and CaMKII are positioned within dendritic spines, it is important to highlight relevant features of dendritic spine ultrastructure [110]. The most commonly studied dendritic spines are postsynaptic to excitatory hippocampal CA3-CA1 synapses. These spines are supported by a complex cytoskeletal ultrastructure based upon actin filaments [111]. Analysis of synaptic ultrastructure has focused on prominent mushroom-shaped spines [112]. Electron microscope (EM) tomography shows that mushroom spines are supported by a network of thin F-actin filaments that are typically shorter than 100 nm [111]. F-actin branching is most dense at

the edges of the PSD, and lateral to the PSD [111,113]. The cytoplasmic face of the PSD connects to actin filaments [111]. The PSD itself is disc-shaped, with a core depth of ~30 nm, and a diameter of ~180–350 nm, depending on the number of AMPARs that are incorporated [114]. The number of NMDARs per PSD is not correlated with PSD width, whereas larger PSDs contain proportionately more AMPARs [114]. AMPAR endocytosis occurs in part within an 'endocytic zone' in the membrane immediately surrounding the PSD [6].

The core structure of the PSD comprises a lattice of vertical filaments, including membrane-associated guanylate kinase (MAGUK) proteins PSD-95 and SAP-97, linked to horizontal filaments such as SHANK [115,116] by scaffold proteins including GKAP [110]. The vertical filaments are typically spaced ~14 nm from one another [117]. In an average forebrain synapse, there are ~300 copies of PSD-95, 150 SHANK family proteins, and 150 GKAPs [110,118]. The composition of the average PSD changes markedly over development, which makes it challenging to establish general molecular mechanisms for synaptic plasticity [119]. For example, whereas the number of GluN2A subunits in the average PSD increases with age, the reverse is true of GluN2B subunits [119]. PSD proteins exhibit a laminar organisation according to immunogold-EM labeling, with PSD-95 sharing an axiodendritic plane with the NMDAR C-terminus in an electron-dense layer close to the postsynaptic membrane (Fig. 3a), and SHANK and GKAP positioned on the cytoplasmic face of the PSD, ~30 nm inside the membrane [120]. This deeper layer, which contains horizontal filaments including SHANK and homer and extends to about 50 nm inside the membrane, is known as the 'pallium' [120,121]. PDZ domain interactions play a key role in supporting the structure of the PSD. For example, the second PDZ domain of PSD-95 binds to the C-terminus of NMDARs [122]. Consistent with a key structural role for PSD-95, reducing expression of this MAGUK protein leads to breakdown of the molecular organisation of the PSD [117]. Immunogold labeling studies show that PSD-95 forms 17 nm filaments that run from N to C along the axiodendritic axis with the N-terminal PDZ domains engaging ion channels including NMDARs closer to the membrane, and the C-terminal SH3 and guanylate kinase (GK) domains engaging other proteins more distally [117] (Fig. 3a).

It is not straightforward to assign positions for CaMKII and calcineurin within the dendritic ultrastructure, since the localisation of both enzymes is altered by Ca^{2+} influx. To account for this complication, we will first consider dynamic postsynaptic positioning of CaMKII and calcineurin in two idealised cases: in non-stimulated ('naïve') synapses (section 5) and during large influxes of Ca^{2+} that trigger LTP (section 6).

5. Calcineurin and CaMKII positioning in dendritic spines engaged in naïve synapses

5.1. Positioning of calcineurin

The key postsynaptic anchoring site for calcineurin is the constitutive PIAIIITD anchoring motif presented within the C-terminus of AKAP79. AKAP79 is consistently identified in PSD extracts by immunoblotting [36], and quantitative mass spectrometry (MS) supports an approximate abundance of 20 copies of AKAP79 per PSD [118]. EM imaging of immunogold labelled AKAP79 in the dendrites of CA1 pyramidal cells reveals strong dendritic labeling of AKAP79 as expected, but suggests that AKAP79 is also present on membranes in the endocytic zone [123]. Consistent with this, subsequent studies have reported that a pool of AKAP79 resides on endosomes [124]. AKAP79 directly interacts with the C-terminal SH3 and guanylate kinase domains of the vertical filament proteins PSD-95 and SAP-97 through central binding elements [125]. This interaction provides a reference point to position AKAP79 within the PSD. The SH3 and GK domains of PSD-95 are located in a laminar plane ~15 nm from the postsynaptic membrane (Fig. 3a). The second PDZ domain of PSD-95 also directly interacts with the C-termini of NMDARs subunits [122]. This pattern of interactions is consistent with AKAP79 presenting an anchoring site for calcineurin close to NMDARs.

AKAP79 also binds directly to membrane phospholipids and a number of integral membrane proteins, consistent with it positioning calcineurin very close to the postsynaptic membrane. For example, the anchoring protein directly interacts with L-type Ca^{2+} channels [126]

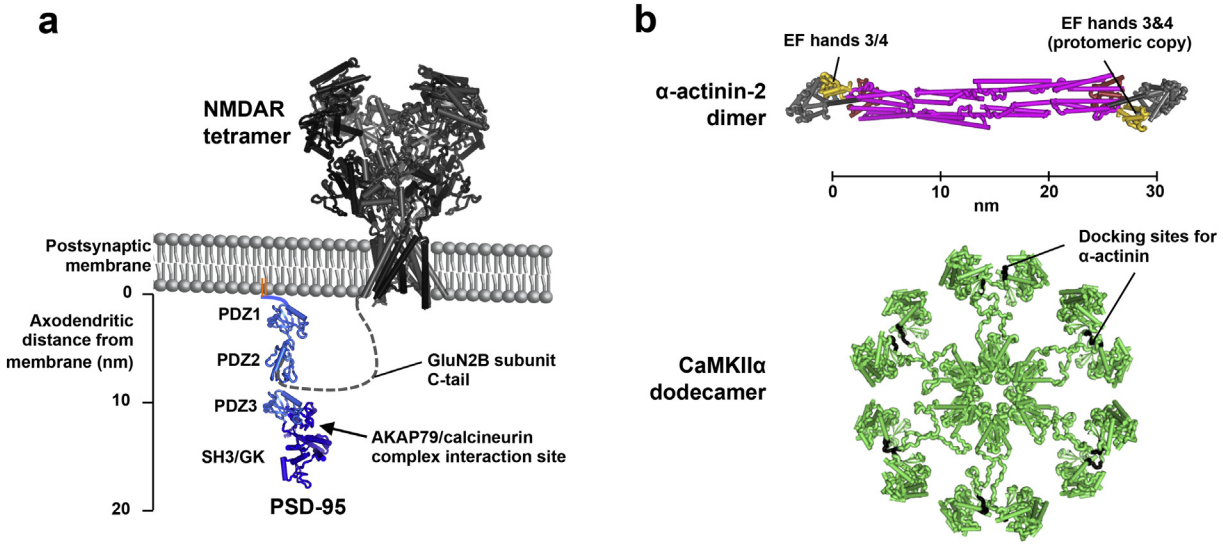

Fig. 3. Interactions involving inactive calcineurin and CaMKII in dendritic spines. (a) Model showing potential localisation sites for the AKAP79-calcineurin complex. The AKAP79-calcineurin complex likely bridges the internal surface of the postsynaptic membrane, and a binding site on the SH3/GK domains of MAGUK proteins including PSD-95. Given the association of NMDARs and PSD-95, this would position calcineurin close to the NMDAR mouth. The model was assembled using PDB files with the following IDs: 3GSL (PSD-95 PDZ domains 1 & 2), 1KJW (PSD-95 SH3-GK domains), and 5UOW (NMDAR tetramer). For simplicity, a single NMDAR tail is shown. Palmitoyl groups are shown in orange. Within the NMDAR tetramer, GluN1 and GluN2B subunits are coloured dark and light grey, respectively. (b) The crystal structure of dimeric α-actinin-2 (PDB ID 4D1E) is shown to scale alongside the most extended structural model of CaMKIIα to date, which was determined by cryo-EM (PDB ID 5U6Y). The domains of α-actinin-2 are coloured according to Fig. 2a. EF hands 3 & 4 (gold) within α-actinin-2 bind to a helix within the regulatory region of CaMKII (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and certain adenylyl cyclase isoforms [127]. AKAP79 contains three basic regions within its first 150 amino acids that associate with acidic phospholipids [128] and F-actin [129], and it is also palmitoylated at cysteines 36 and 129 [130,131]. Low-resolution models of the AKAP79-calcineurin complex from negative-stain EM show that AKAP79 assumes an extended conformation [41] that could enable simultaneous interaction with both the postsynaptic membrane and MAGUK C-terminal domains. Lipid and protein interactions involving AKAP79 point towards the existence of an anchoring site that would support calcineurin activation within Ca^{2+} microdomains centered on NMDARs following small influxes of Ca^{2+} that trigger LTD (Fig. 3a). This model is supported by investigations of the functional effects of removing the PIAIIITD anchoring site of the rat ortholog AKAP150: in genetically-modified mice, LTD can no longer be induced at CA3-CA1 synapses if either the PIAIIITD site is removed [132], or if AKAP150 is knocked out altogether [133,134]. In sum, studies of AKAP79/150 interactions, localisation and function indicate that this anchoring protein is critical for positioning calcineurin during the induction of LTD.

5.2. Positioning of CaMKII

Quantitation of the abundance of proteins in purified PSDs by gel electrophoresis shows that the average PSD contains ~80 CaMKII holoenzymes [135], corresponding to ~6% of total PSD mass, although a recent MS analysis reported a higher copy number [136]. In dendritic spines of CA1 stratum radiatum, CaMKII immunogold labeling density peaks about 40 nm inside the postsynaptic membrane, within the pallial layer [137]. Electron tomography of isolated PSDs supports the notion that most CaMKII associated with the PSD is positioned at a greater depth from the postsynaptic membrane than core proteins such as PSD-95 [138]. Within 25 nm of postsynaptic membranes in CA1 stratum radiatum, CaMKII labeling is concentrated to the lateral edges of the PSD [137]. This suggests that CaMKII can attach to PSDs in two ways: at sites within the pallial layer, and within the core but at the edges of the PSD. Since these studies were performed with PSDs extracted from brain – therefore deriving in some cases from synapses that have undergone LTP - it is not clear to what extent these two locations represent CaMKII anchoring locations in naïve synapses. Inactive CaMKII can bind α-actinin, actin, and – to some extent – densin-180 (Section 3). In this section, we consider how interaction with these proteins is likely to determine CaMKII localisation prior to influx of Ca^{2+}.

α-actinin forms an antiparallel dimer *via* central inflexible spectrin-like repeats to create a rigid rod with a double-headed actin binding capability [139] (Fig. 3b). The dimeric elongated conformation of α-actinin enables it to effectively crosslink actin filaments, and to tether F-actin to a range of intracellular targets. Its best-characterised role is at the sarcomeric *Z*-disk in striated muscle where it links F-actin and titin [140]. The ability of α-actinin to cross-link actin with diverse proteins is also utilised in other settings, such as in the assembly of focal adhesions in the leading protrusions of migrating cells [141], and in cytokinesis [142,143]. Knock-down or overexpression of α-actinin-2 in rat hippocampal neurons leads to striking defects in spine formation, consistent with an additional role for the protein in regulating dendritic actin dynamics [144]. α-actinin is sensitive to regulation by the plasma membrane phospholipid $PI(4,5)P_2$ [145] by an interaction with residues in the actin binding domain [146,147], which may underlie α-actinin's ability to regulate membrane dynamics. α-actinin-2 is highly localised to dendritic spines [90,144], and sub-synaptic immunogold labeling shows that it resides within the PSD but not at a single laminar depth. α-actinin-2 labeling is also observed on actin filaments in the spine neck [92]. α-actinin isoforms exhibit variable regional expression, with relatively low expression in CA1 dendrites [92].

The two copies of EF hands 3 & 4 in the α-actinin-2 dimer are separated by ~28 nm (yellow, Fig. 3b) [148], whereas the maximum separation of α-actinin-binding motifs in the CaMKII dodecamer is only ~22 nm (black, Fig. 3b) according to the most extended structural conformations proposed for the CaMKII holoenzyme [51]. Therefore, it appears unlikely that α-actinin operates by bridging two regulatory domains within a single CaMKII holoenzyme. For mixed α/β CaMKII holoenzymes, the ability of β subunits to bind directly to F-actin likely enables formation of ternary complexes between α-actinin, actin, and mixed CaMKII holoenzymes. In general, actin and α-actinin naturally organise into arrays with regular spacing [149], supported by proteins such as titin that bridge between them [140]. However, there is little evidence for the formation of actin lattices in dendritic spines [111]. α-actinin may also potentially stabilise CaMKII association with NMDARs [150], and densin-180 [74]. Overall, it is difficult to assign a precise localisation site for CaMKII anchored to α-actinin, as α-actinin engages in many additional interactions with no single sub-spinal locus.

Inactive CaMKII associates to some extent with densin-180 *in vitro via* the association domain, although the affinity of the interaction is increased by activation of the kinase [74]. Densin-180 is highly localised to spines, and it is enriched in PSD extracts [75]. Its ability to interact with elements of the horizontal filament protein SHANK, including the SH3 domain [151], indicates that at least part of densin-180 is located in the pallium of the PSD. Densin-180 contains a C-terminal PDZ domain that binds to a PDZ recognition motif [152] formed by the last ~6 amino acids of α-actinin [74]. Experiments with purified protein [96] show that ternary complex formation between CaMKII, densin-180 and α-actinin is possible, and densin-180 stabilises interaction between CaMKII and α-actinin [96]. This interaction mode may enable association of some CaMKII in the PSD pallium prior to LTP induction. Studies with densin-180 knock-out mice support the idea that the anchoring protein is involved in positioning CaMKII in dendritic spines [153]. Mice lacking densin-180 exhibit deficits in object-place recognition, and suffer from increased anxiety as measured by open field behavior [153]. Consistent with ternary densin-180/α-actinin/CaMKII complex formation *in vivo*, α-actinin levels in densin-180 knockout mice are reduced by one third in both total brain and fractionated PSDs. Intriguingly, resting levels of pT286-CaMKII - but not total CaMKII - are reduced in PSDs of mice lacking densin-180. Therefore, densin-180 may be more important for supporting tethering of activated CaMKII at the PSD [153].

6. Changes in sub-spinal location during large influxes of Ca^{2+}

Calcineurin anchoring by AKAP79 enables the phosphatase to sense the small, localised influxes of Ca^{2+} through NMDARs that induce LTD [133]. Our current understanding of the molecular architecture of the AKAP79 signalling complex suggests that AKAP79 initially positions the Ca^{2+}/CaM-sensitive elements of calcineurin within Ca^{2+} microdomains emanating from the mouths of NMDAR receptors (Fig. 3a). No equivalent anchoring site for CaMKII has been identified in naïve synapses. In spite of their similar inherent sensitivities to Ca^{2+}, this differential initial localization of calcineurin and CaMKII may partly explain why only calcineurin responds to small influxes of Ca^{2+} that induce LTD. However, both enzymes relocate following strong activation of NMDARs governed by changes in molecular interactions that are regulated by Ca^{2+}/CaM (Fig. 4a). In this section, we will consider how Ca^{2+}/CaM-induced changes in sub-spinal location may support long-lasting activation of CaMKII, and explain why calcineurin does not dephosphorylate AMPARs during induction of LTP.

6.1. Calcineurin dynamics

Binding of Ca^{2+}/CaM to a helix spanning position 79–86 in AKAP79 [26] leads to dissociation of the anchoring protein from *in vitro* membrane preparations containing the acidic phophoslipid $PI(4,5)P_2$ [128]. In a physiological context, strong NMDAR association triggers removal of rat AKAP150 from dendritic spines [129,132]. Dissociation from F-actin may also contribute to Ca^{2+}/CaM-triggered re-localisation of AKAP79 [129]. In this way, Ca^{2+} influxes can be expected to remove a

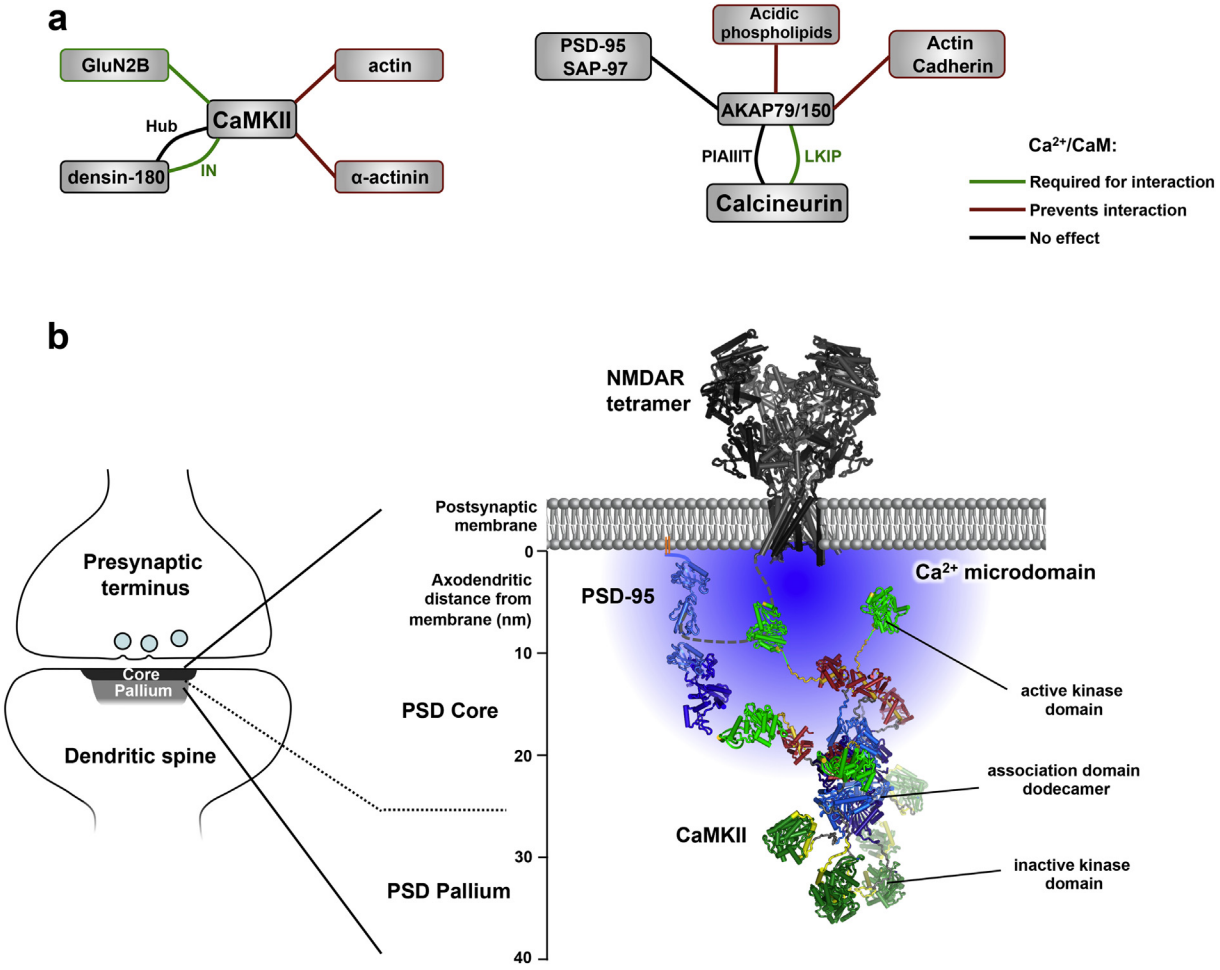

Fig. 4. Coordination of active CaMKII in the PSD. (a) Network diagram summarising molecular interactions involving calcineurin and CaMKII that are important for positioning the enzymes in dendritic spines. Links are coloured according to how they are affected by Ca^{2+}/CaM. The interaction between the CaMKII association and densin-180 is labelled 'Hub'; the interaction between CaMKII and the densin-180 IN domain is labelled 'IN'. (b) Structural model showing anchoring of active CaMKII within the PSD. The nearest neighbour distances of receptors and vertical filaments in the PSD is consistent with CaMKII associating 'side-on' with the PSD. This may enable the kinase to bridge localisation sites within the PSD core, including interactions sites within the tails of NMDAR subunits, and the PSD pallium. Interaction partners within the PSD pallium may include densin-180 and α-actinin (not shown). CaMKII is modelled using structures of the autoinhibited dodecamer (PDB ID 5UOW), and the complex of CaMKIIδ (11–335) and Ca^{2+}/CaM (PDB ID 2WEL). One implication of side-on CaMKII insertion is that CaMKII protomers within the same holoenzymes will have different access to localised Ca^{2+} microdomains (blue) emanating from the mouths of NMDARs. This may enable simultaneous interactions between active and inactive protomers within single CaMKII dodecamers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

major anchoring site for calcineurin in the vicinity of its substrates within the PSD. This may provide a mechanism for preventing dephosphorylation of Ser845 on GluA1 receptors during LTP. However, this raises a question of why Ca^{2+}/CaM-triggered release of AKAP79 from the PSD doesn't prevent AMPAR dephosphorylation by calcineurin during LTD. *In vitro* binding assays show that CaM associates with calcineurin at lower half-maximal Ca^{2+} concentrations than it does with AKAP79 [26]. Therefore, the window of Ca^{2+} concentrations within which LTD occurs may potentially trigger calcineurin dephosphorylation without dissociating the AKAP from PSDs. Finally, association of calcineurin with LxVP-type interactions motifs, dependent on Ca^{2+}/CaM, is an emerging calcineurin anchoring mechanism. However, besides AKAP79 [40,41], there are no reports of LxVP-type interactions within dendritic spines. This may be because interactions of this type are harder to detect than those that occur in the absence of elevated Ca^{2+}.

6.2. CaMKII dynamics

CaMKII distributes evenly in naïve dendrites [154,155]. Upon neuronal activation, the kinase assumes a punctate distribution and localises with PSD proteins unless NMDAR antagonists are present [108,154,155]. The speed of recruitment depends on the ratio of α to β subunits, with CaMKIIα/β heteromers accumulating at the PSD more slowly than CaMKIIα homomers [154] presumably due to direct interactions between β subunits and actin [85]. Approximately one fifth of CaMKII that localises at the PSD during LTP induction remains associated indefinitely, long after the triggering Ca^{2+} signal has subsided [108]. This indicates the presence of a 'trapping' mechanism [155]. Short-term retention of CaMKII at the PSD is dependent on phosphorylation of T286 as the non-phosphorylated T286A mutant dissociates over the same time course as the Ca^{2+} signal, whereas phosphomimetic T286D forms persistent puncta [155]. Mass spectrometry also shows that CaMKII phosphorylated at T286 is enriched in the PSD fraction, whereas CaMKII phosphorylated at T306 is predominantly cytosolic [156]. Together, these results paint a picture of diffuse CaMKII being recruited to the PSD upon NMDA-receptor stimulation, and remaining partially PSD-localised over long time periods. But what is the structural and molecular basis of this behaviour?

Activation of CaMKII enables the kinase to bind to the C-terminus of

NMDARs, and also increases the affinity of the holoenzyme for densin-180. Quantitative MS experiments reveal that GluN2B subunits are present in the PSD at approximately twice the abundance of densin-180, with the two together accounting for ~70 potential CaMKII anchoring sites per PSD [136]. Functional experiments are consistent with the more abundant GluN2B subunit C-terminal tail playing a key role in localising CaMKII at the PSD during LTP. In heterologous expression systems, diffuse CaMKII becomes punctate and co-localises with NMDARs upon NMDAR activation [99] in a Ca^{2+}-dependent manner [102,104,157]. In transgenic mice, induced expression of the C-terminal tail of GluN2B (839–1482) effectively chelates existing CaMKII and disrupts existing CaMKII/GluN2B interactions, leading to reduced T286 autophosphorylation, reduced AMPA receptor phosphorylation, impaired LTP, and learning defects [158]. Furthermore, knock-in point mutations that disrupt the distal CaMKII anchoring site within the GluN2B subunit produce similar effects [159]. Binding to the NMDAR is thought to both enhance local phosphorylation by the kinase, and sustain the activity of the kinase. Experiments in HEK cells using a chimeric GluN2B construct fused with a CaMKII-substrate confirm that anchoring to the NMDAR increases CaMKII activity in the immediate vicinity of the anchoring site [160]. Binding of CaMKII to GluN2B inhibits phosphorylation of T305 and T306 by reducing the dissociation rate of CaM [104]. Moreover, when bound to GluN2B, CaMKII retains about 30% of its autonomous activity, in a manner independent of both T286 phosphorylation and CaM binding [104].

CaMKII dissociation from actin and actinin during LTP induction is thought to be critical for coupling changes in synaptic strength driven by AMPAR incorporation to modifications in the actin substructure of the spine [12]. Chemically-induced LTP leads to a marked decrease in CaMKII mobility not only inside the PSD but within ~600 nm of the structure extending into the spine [161]. This is perhaps surprising as CaMKII mobility might be expected to *increase* inside the spine after release from the actin cytoskeleton outside of the PSD. One potential explanation, consistent with the known stabilization of the actin cytoskeleton during LTP [162], is that release of CaMKII triggers higher rates of actin polymerization and branching that enable actin to act as a more effective 'sieve' to slow CaMKII diffusion. Clearly, there is still much to learn about CaMKII interplay with actin, actinin, and other cytoskeletal regulators [162]. How might active CaMKII be coordinated within the PSD? Identification of ring-shaped objects corresponding to CaMKII association domains within isolated PSDs using electron tomography [163] show that CaMKII dodecamers can embed within the core matrix of the PSD, in addition to a more prevalent anchoring site in the pallium [137,163]. It is difficult to be certain of the location of densin-180. It has been shown to interact with SHANK [151], which resides within the pallium, but its palmitoyltated N-terminus is also thought to associate with the postsynaptic membrane. The location of the NMDAR C-terminus is less ambiguous. Interaction between the C-terminus of NMDARs and the second PDZ domain of PSD-95 is compatible with the known vertical geometry of PSD-95, and places the C-termini of NMDAR GluN2A/B subunits (grey, Fig. 4b) ~8 nm from the membrane [115], and presumably very close to the mouth of the channel. Both CaMKII and the overall PSD are disc shaped. In representations of CaMKII association with the PSD, the two discs are usually arranged in parallel. However, orthogonally-angled CaMKII dodecamers might be able to bridge both the core NMDAR association site, and sites within the pallium (Fig. 4b). Electron tomography has revealed that both vertical filaments [117] and ionotropic glutamate receptors [135] are tightly packed in the PSD, with vertical filaments separated on average by only 14 nm [117]. Orthogonal insertion of CaMKII would therefore enable kinase domains to access NMDAR tails without major reorganisation of the tightly packed vertical filament framework of the PSD.

The importance of cooperative interactions through different protomers engaging different proteins is highlighted by experiments with monomeric CaMKII. Deletion mutants of CaMKIIα that lack the association domain, despite retaining the ability to become autonomously active, do not bind to immobilised fragments of the distal GluN2B region [102] and fail to accumulate at the PSD in neurons [108]. Moreover, GluN2B and other CaMKII interaction partners do not compete for binding to CaMKII [96], and thus the holoenzyme may act as a 'bridge' between different anchors in the PSD. Recent studies have established that abundant PSD scaffold proteins, including SynGAP and AIDA-1, move from the core to the pallium during LTP [121]. Conceivably, this could create space for insertion of CaMKII within the PSD core, although this remains to be tested. Finally, it is important to consider the implications of anchoring part of CaMKII within the immediate vicinity of the NMDAR receptor. Access to Ca^{2+} microdomains centered on NMDAR subunits, which would not activate CaMKII prior to localization, likely contribute to the sustained long-term association of a fraction of the kinase following the induction of LTP. In addition, the CaMKII holoenzyme is so large that it is likely that different kinase domains within a single dodecamer will respond differently to Ca^{2+} entry through NMDARs. This could support coordination mechanisms in which active and inactive CaMKII protomers within the same dodecamer engage in different types of protein-protein interaction. For example, active kinase domains in proximity to the postsynaptic membrane may bind NMDARs while, within the same CaMKII dodecamer, regulatory elements of inactive protomers lying nearer the pallium interact with actin and α-actinin (Fig. 4b).

7. Concluding remarks

In this review, we have considered how the precise positioning of calcineurin and CaMKII in dendritic spines underlies their ability to drive long-lasting changes in synaptic strength and spine morphology. Some important differences in the targeting of the two enzymes are emerging. Current evidence suggests that in naïve synapses calcineurin, but not CaMKII, is anchored in close proximity to NMDARs (Fig. 5a). Their different initial proximity to NMDARs may support activation of calcineurin but not CaMKII within restricted Ca^{2+} microdomains emanating from NMDARs during the induction of LTD. LTP triggers an

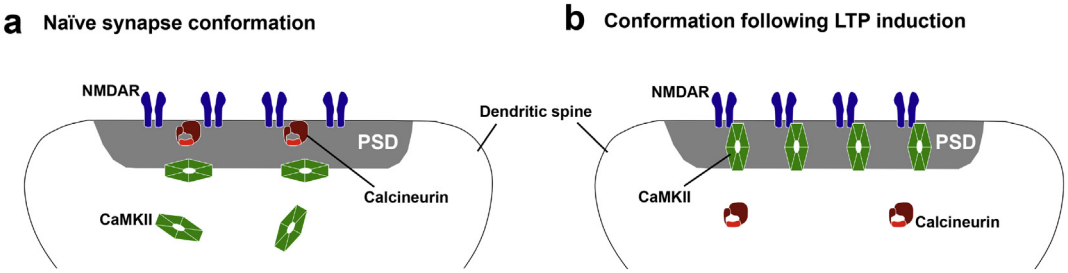

Fig. 5. Summary model of calcineurin and CaMKII coordination in naïve and potentiated spines. This simplified model shows calcineurin (red) and CaMKII (green) positioning in naïve (a) and recently potentiated (b) dendritic spines. The model highlights positioning relative to NMDARs (blue), which are the major Ca^{2+} entry point within the PSD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

approximate inversion of this anchoring pattern (Fig. 5b). Large influxes of Ca^{2+} during LTP induction trigger relocation of AKAP79 out of the PSD, which may shield substrates including AMPAR GluA1 subunits from calcineurin dephosphorylation. Furthermore, binding of CaMKII to the C-terminal tails of NMDARs likely contributes to enduring CaMKII activity in part by enabling the kinase to respond to local elevations of Ca^{2+} close to the receptor mouth. However, it is difficult to be confident in these proposed mechanisms without answering several outstanding structural questions regarding CaMKII and calcineurin in dendritic spines. Notably, there is no high-resolution structural data revealing how CaMKII associates with any of actin, α-actinin, densin-180, or its proximal interaction site in NMDARs. CaMKII associates with α-actinin in the absence of Ca^{2+}, but it is not clear whether this interaction supports CaMKII localization outside of the PSD, within the PSD pallium, or the core PSD. There is no information relating to densin-180 coordination within the PSD besides a reported interaction with the horizontal filament protein SHANK. Further outstanding topics include determining how actin and α-actinin assemble with mixed CaMKII α/β holoenzymes, and understanding how release of CaMKII from α-actinin is coupled to changes in the actin sub-structure of the spine during LTP. Finally, little effort has been made to search for LxVP-type calcineurin-interacting proteins that could associate with the phosphatase during LTP.

Technical advances in recent years should support efforts to resolve these outstanding questions. For example, super-resolution microscopy [116], and cross-linking coupled to mass spectrometry [164], may enable the positions of calcineurin and CaMKII complexes to be more accurately assigned within intact PSDs. Progress is also likely to be supported by determining structures of purified multi-protein sub-complexes involving CaMKII by single-particle cryo-electron microscopy, an approach that has also developed rapidly in the last decade [165]. In the long-term, insight may also be gained by incorporating a temporal component into structural models to understand how both the amplitude and timing of Ca^{2+} influxes affect CaMKII and calcineurin interactions in dendritic spines. Calcineurin [166], CaMKII [167,168], and their associated anchoring proteins [169] are emerging as important players in the development of neurological diseases. This should spur on efforts to understand how these remarkable enzymes regulate synaptic strength and the shape of dendritic spines at the structural and molecular level.

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