

## Review

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# Calmodulin Binding Proteins and Alzheimer's Disease

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**Abstract.** The small, calcium-sensor protein, calmodulin, is ubiquitously expressed and central to cell function in all cell types. Here the literature linking calmodulin to Alzheimer's disease is reviewed. Several experimentally-verified calmodulin-binding proteins are involved in the formation of amyloid- $\beta$  plaques including amyloid- $\beta$  protein precursor,  $\beta$ -secretase, presenilin-1, and ADAM10. Many others possess potential calmodulin-binding domains that remain to be verified. Three calmodulin binding proteins are associated with the formation of neurofibrillary tangles: two kinases (CaMKII, CDK5) and one protein phosphatase (PP2B or calcineurin). Many of the genes recently identified by genome wide association studies and other studies encode proteins that contain putative calmodulin-binding domains but only a couple (e.g., APOE, BIN1) have been experimentally confirmed as calmodulin binding proteins. At least two receptors involved in calcium metabolism and linked to Alzheimer's disease (mAChR; NMDAR) have also been identified as calmodulin-binding proteins. In addition to this, many proteins that are involved in other cellular events intimately associated with Alzheimer's disease including calcium channel function, cholesterol metabolism, neuroinflammation, endocytosis, cell cycle events, and apoptosis have been tentatively or experimentally verified as calmodulin binding proteins. The use of calmodulin as a potential biomarker and as a therapeutic target is discussed.

**Keywords:** Alzheimer's disease, calcium, calcium channels, calmodulin, calmodulin binding proteins, cholesterol metabolism, endocytosis, genome wide association studies, neuroinflammation

## INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia, with close to 50 million people worldwide currently suffering from dementia and the number expected to increase to 1 in 85 people by the year 2050 [1]. First described by the German psychiatrist Alois

Alzheimer, it manifests through progressive memory loss ultimately encompassing a loss of recognition of people, places, and things, eventually leading to death [2]. Currently, the cure for AD remains elusive, with treatments focusing on managing symptoms and attempts to slow neurodegeneration. While the initiating events leading to late onset AD (LOAD) remain to be discovered, the formation of amyloid plaques and neurofibrillary tangles remain as central culprits in the neurodegenerative aspects of the disease. More recently, numerous genes and several metabolic events

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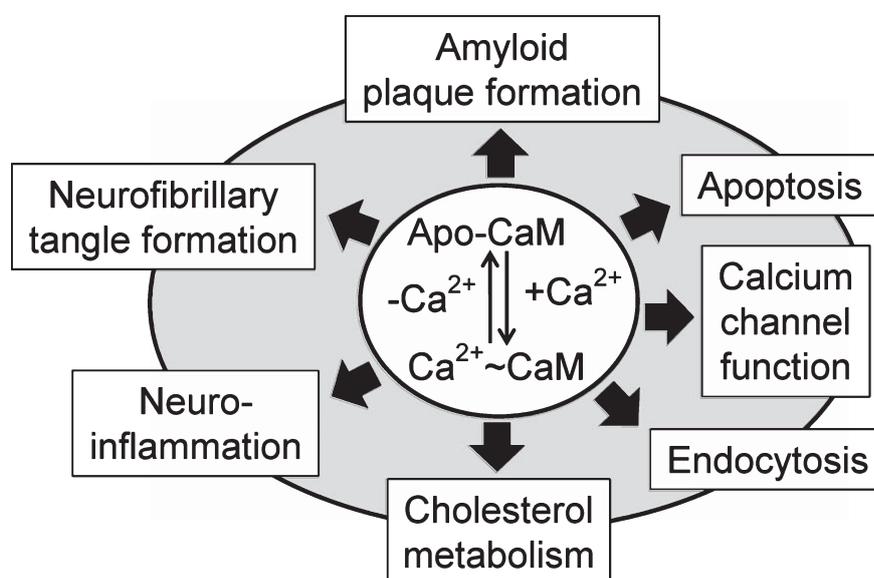


Fig. 1. Calcium-bound calmodulin (CaM) and its calcium-free form (Apo-CaM) are involved to many central events linked to Alzheimer's disease as discussed in this review.

have been identified as either central or contributing factors in AD.

A decade ago, O'Day and Myre [3] proposed the "Calmodulin Hypothesis" as an extension of the well-established "calcium hypothesis" of LOAD. In that study, a number of proteins linked to the production of amyloid- $\beta$  ( $A\beta$ ) were shown to possess putative calmodulin binding domains (CaMBDs) suggesting that they could be regulated by the small regulatory, calcium-binding protein calmodulin (CaM). Subsequently, two of the central proteins in this event, amyloid- $\beta$  precursor protein ( $A\beta$ PP) and beta-secretase (BACE1; beta-site  $A\beta$ PP cleaving enzyme 1) were each shown to bind to and be regulated by CaM [4, 5]. Non-amyloidogenic processing of  $A\beta$ PP in platelets was induced in a time- and dose-dependent manner by the calmodulin antagonist W7 [6]. Over the past 10 years, advancements have been made into understanding the pathways involved in both amyloid plaque and neurofibrillary tangle formation. Based on these new insights, here we first re-examine the role of calmodulin binding proteins (CaMBPs) in the formation of the two primary culprits linked to the symptoms and progression of AD: amyloid plaques and neurofibrillary tangles (Fig. 1). As summarized in Fig. 1, we then look at the function of calmodulin as a regulator of other events and recently identified proteins linked to the disease before looking at CaM as a potential therapeutic target.

## THE CALCIUM HYPOTHESIS AND CALMODULIN

The "calcium hypothesis" is one prominent model for AD [7]. It suggests that an imbalance of calcium levels in cells precedes the signaling pathway malfunctions and neuronal deterioration observed in neurodegenerative diseases. It has long been known that calcium ions ( $Ca^{2+}$ ) play a substantial role in normal physiology. In the brain,  $Ca^{2+}$  functions in neurotransmitter synthesis and release as well as in the control of membrane excitability [8]. The intracellular concentration of  $Ca^{2+}$  is kept within a tight range of  $10^{-7}$  to  $10^{-8}$  mol; variation from the norm, even if slight, is detrimental when it persists [8].

Calcium ions perform their function primarily through binding to membrane or cytoplasmic proteins. Arguably the primary calcium-binding protein and effector of calcium function is the small protein CaM. Highly conserved and ubiquitously expressed in all eukaryotes and in all cell types, it is a member of the EF-hand family of calcium sensors [9]. CaM possesses four E-F hands, two at the C-terminal and two at the N-terminal, allowing it to bind up to four  $Ca^{2+}$  ions [9, 10]. Each lobe (N, C) can bind up to two calcium ions (Fig. 2). The number and location of binding of this cation affects CaM's conformation and how it interacts with its target CaMBPs. Proteins do not bind to  $Ca^{2+}$ /CaM via traditional targeting sequences. In the absence of  $Ca^{2+}$ , the

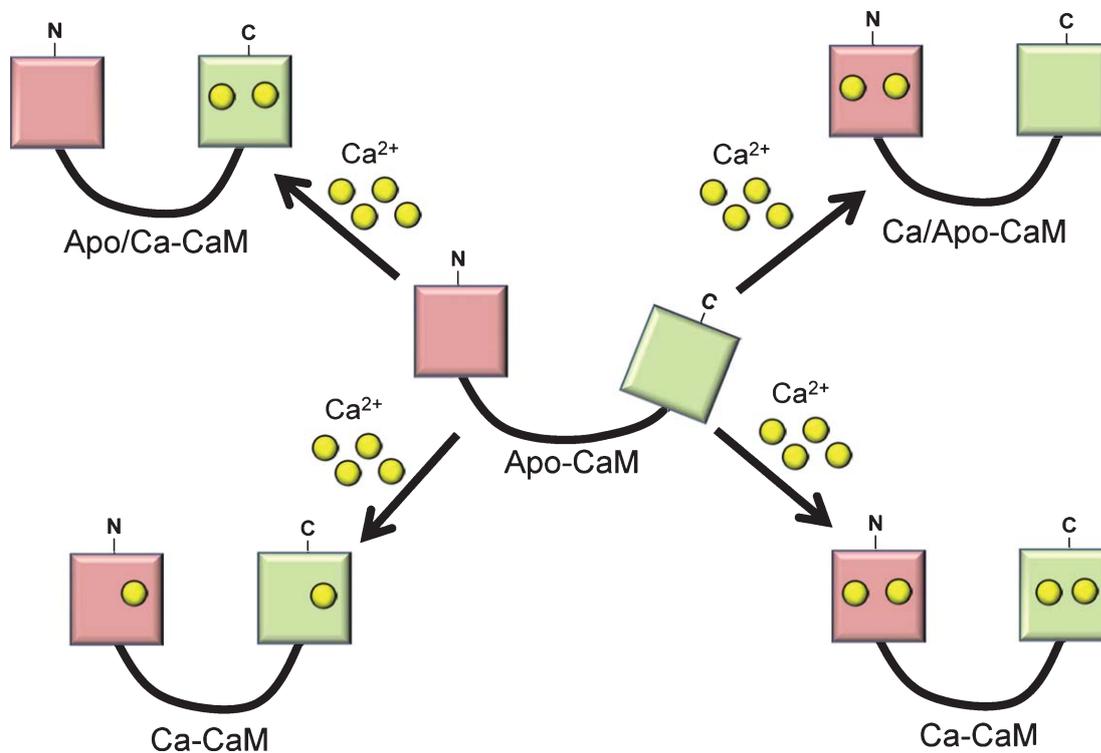


Fig. 2. Apo-Calmodulin (Apo-CaM) can bind up to two calcium ions in each of its N-term (pink) or C-term lobes (green). These binding options affect the conformation of CaM and its ability to bind to specific target CaM-binding proteins.

apo-CaM conformation allows the binding of the sub-group of  $\text{Ca}^{2+}$ -independent CaMBPs (Table 1).  $\text{Ca}^{2+}$ -independent binding occurs via well-defined IQ [(FILV)Qxxx(RK)Gxxx(RK)xx(FILVWY)] and IQ-like [(FILV)Qxxx(RK)xxxxxxxx] motifs. Upon  $\text{Ca}^{2+}$  binding, CaM undergoes significant conformational changes allowing it to bind to  $\text{Ca}^{2+}$ -dependent CaMBPs.  $\text{Ca}^{2+}$ -dependent binding involves a diversity of motifs and sub-classes that are defined primarily by the sequence and positioning of hydrophobic amino acids sometimes in conjunction with basic residues within the motif (e.g., 1–5–10 motif: xxx(FILVW)xxxx(FAILVW)xxxx(FILVW) where x=any a.a.) [11]. The “Calmodulin Target Database” categorizes the various types of CaM-binding motifs (Table 1; <http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html>). In addition to the major CaMBD categories (e.g., 1–8–14, 1–5–10, etc.), there are also a large number of others that do not fit into any of these specific categories (e.g., 1–12 and basic motifs) among other more recently discovered binding non-canonical motifs [12].

To add to this non-conventional binding interaction, Villarroel et al. [13] have presented evidence indicating that while CaM binds its calcium-dependent targets

via these CaMBDs in their normal orientation it can also bind to those sequences in the opposite direction. Furthermore, their data also suggests that upon binding to its target, CaM can undergo a conformational rotation further altering its structural relationship with that CaMBP. Considering the central importance of CaM to essential life processes, this complex protein interaction may be part of the reason CaM historically has eluded serving as a primary target in various diseases. Once the interactions between CaM and specific CaMBPs linked to AD are fully clarified, it may become possible to develop target-specific peptides to fight different aspects of the disease.

## CALMODULIN AND AMYLOID PLAQUE FORMATION

The Calmodulin Target Database identifies the presence and types of CaMBDs in protein sequences with over 90% accuracy [11]. Using this database, O'Day and Myre [3] first identified a diversity of potential CaMBPs linked to the formation of amyloid plaques. The “amyloid hypothesis” is arguably the predominant hypothesis for the symptoms and progression

Table 1  
Calmodulin binding domain (CaMBD) subclasses that have been identified and experimentally verified

<b>Calmodulin Binding Domains</b>	
<b>1-14 Subclasses</b>	
1-5-8-14	(FILVW) <sub>xxx</sub> (FAILVW) <sub>xx</sub> (FAILVW) <sub>xxxxx</sub> (FILVW)
Basic 1-8-14	(RK)(RK)(RK)(FILVW) <sub>xxxxxx</sub> (FAILVW) <sub>xxxxx</sub> (FILVW)
1-8-14	(FILVW) <sub>xxxxxx</sub> (FAILVW) <sub>xxxxx</sub> (FILVW)
1-14	(FILVW) <sub>xxxxxxxxxxxxx</sub> (FILVW)
<b>1-10 Subclasses</b>	
1-5-10	xxx(FILVW) <sub>xxxx</sub> (FAILVW) <sub>xxxx</sub> (FILVW)
Basic 1-5-10	(RK)(RK)(RK)(FAILVW) <sub>xxxx</sub> (FILV) <sub>xxxx</sub> (FILVW)
1-10	(FILVW) <sub>xxxxxxxx</sub> (FILVW)
<b>1-16 Subclasses</b>	
1-16	(FILVW) <sub>xxxxxxxxxxxxxxx</sub> (FILVW)
<b>Other Less Common Classes</b>	
1-12	(FILVW) <sub>xxxxxxxx</sub> (FILVW)
Basic (e.g., EGFR)	<u>RRRHIV</u> <u>RKRT</u> <u>LRRL</u> Q
Other (e.g., mGluR7A)	<u>KRKRS</u> <u>F</u> <u>KAVVTAA</u> <u>T</u> <u>M</u>
<b>IQ Subclasses</b>	
IQ	(FILV)Q <sub>xxx</sub> (RK)G <sub>xxx</sub> (RK) <sub>xx</sub> (FILVWY)
IQ-Like	(FILV)Q <sub>xxx</sub> (RK) <sub>xxxxxxx</sub>

Hydrophobic amino acids are highlighted; basic amino acids are underlined; Source: Calmodulin target database (<http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html>).

of AD [14]. It is based on the aggregation of A $\beta$  peptides plus a multitude of other components to form extracellular amyloid plaques in the brains of AD sufferers [15–17]. A $\beta$  peptides vary slightly in length and are formed through the regulated intramembrane proteolytic (RIP) cleavage of A $\beta$ PP at the carboxy-terminal fragment (CTF) by  $\beta$ -secretase and  $\gamma$ -secretase enzymes (Fig. 3) [18]. The main products of this pathway are A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> with A $\beta$ <sub>42</sub> being the more toxic fragment [19]. A $\beta$  peptides interfere with normal signaling leading to neuronal malfunction and, eventually, synapse loss [20]. On the other hand, there exists a non-amyloidogenic pathway of cleavage, where A $\beta$ PP is cleaved in the middle of the A $\beta$  sequence by  $\alpha$ -secretase thereby preventing the formation of the neurodegenerative A $\beta$  peptide [18].

CaM is significantly decreased in the brains of AD individuals [21]. In spite of this, the existing CaM can interact with several proteins in the amyloid pathway. Two independent groups have demonstrated experimentally that A $\beta$ PP binds to CaM [4, 6]. The main

$\beta$ -secretase enzyme in the brain that begins the amyloidogenic processing of A $\beta$ PP is BACE1 (Fig. 3). Its activity and expression are both increased in AD brains and its ablation completely prevents A $\beta$ PP cleavage through the amyloidogenic pathway in mouse models [22, 23]. BACE1 cleaves A $\beta$ PP at CTF-99 (and sometimes CTF-89), as opposed to cleavage at CTF-83 which is observed in the non-amyloidogenic pathway [18]. Most of the action of BACE1 on A $\beta$ PP occurs in endocytotic vesicles as opposed to when A $\beta$ PP is localized at the cell membrane [24]. BACE1 binds CaM with its activity being increased up to 2.5-fold by CaM in a dose dependent manner *in vitro* [5]. Sequence analysis of the enzyme indicates that binding most likely occurs via a 1-16 motif ((FILVW)<sub>xxxxxxxxxxxxxxx</sub>(FILVW)).

After BACE1,  $\gamma$ -secretase performs the final cleavage to produce A $\beta$ .  $\gamma$ -Secretase consists of four main subunits: nicastrin (Nic), anterior pharynx-defective 1 (APH-1), presenilin (PSEN), and presenilin enhancer 2 (PEN-2), which assemble in the order listed (Fig. 3)

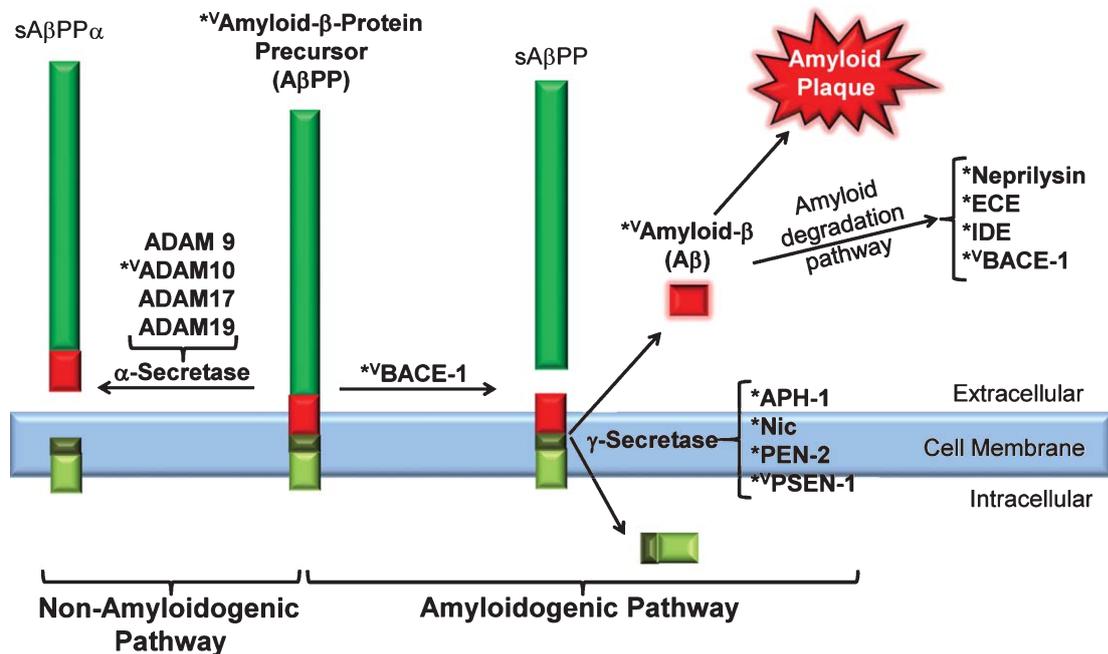


Fig. 3. Calmodulin binding proteins linked to the amyloidogenic and non-amyloidogenic pathways. amyloid- $\beta$  precursor protein (A $\beta$ PP); anterior pharynx defective 1 (APH-1);  $\beta$  site-amyloid converting enzyme 1 or  $\beta$ -secretase (BACE1); A Disintegrin And Metalloproteinase Family (ADAM 9,10,17, 19); endothelin-converting enzyme (ECE); insulin-degrading enzyme (IDE); nicastrin (Nic); presenilin enhancer protein 2 (PEN-2); and presenilin-1 (PSN-1); superscripts: \*putative CaMBD detected;  $\vee$ CaMBD experimentally verified.

[25]. PEN-2, the final subunit to be attached, leads to the activation of the enzyme complex [25]. Sequence analysis of the four  $\gamma$ -secretase subunits using the Calmodulin Database revealed that they all possess presumptive CaMBDs, with some having more than one binding motif [3]. Of these subunits, only PSEN has been experimentally verified to bind CaM [26]. Michno et al. [26] also showed that the loss of function of a single CaM copy inhibits  $\text{Ca}^{2+}$  dysregulation induced by PSEN in *Drosophila* neurons.

Several enzymes can degrade A $\beta$  including neprilysin, endothelin-converting enzymes (ECE), insulin degrading enzymes (IDE), and BACE1 (Fig. 3) [18, 27]. Aside from BACE1, none of the other A $\beta$  degrading enzymes have yet been experimentally confirmed to bind CaM, although all of them were found to possess putative CaMBDs [28]. In addition to binding to the enzymes which generate A $\beta$ , CaM was found to directly bind to A $\beta_{42}$  itself through a 1-8-14 motif ((FILVW)xxxxxx(FILVW)xxxxxx(FILVW)) [29]. When bound to CaM, A $\beta$  cannot inhibit the brain plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) and prevent  $\text{Ca}^{2+}$  entry into the cell. Thus the accumulated data presents a clear link between amyloid plaque formation,  $\text{Ca}^{2+}$ , and CaM revealing how the dysfunction in  $\text{Ca}^{2+}$  levels and A $\beta$  production contribute to each

other with CaM functioning at many levels during this interaction.

As mentioned above A $\beta$ PP binds to CaM *in vitro* in a  $\text{Ca}^{2+}$ -independent manner [6]. The non-amyloidogenic pathway involves  $\alpha$ -secretase cleavage of A $\beta$ PP at CTF-83 precluding the formation of A $\beta$  (Fig. 3). A $\beta$ PP processed in this non-amyloidogenic pathway appears to have a neuroprotective effect. The ADAM (A Disintegrin And Metalloproteinase) family of proteases, specifically ADAM9, 10, 17, and 19 are all thought to exhibit  $\alpha$ -secretase activity in neurons [18]. ADAM10, the predominant  $\alpha$ -secretase, binds CaM through an IQ-motif [30–32]. In contrast, ADAM17 does not bind CaM [30]. Importantly, W7 an antagonist of CaM stimulates cleavage of A $\beta$ PP via the non-amyloidogenic pathway [6, 33].

## CALMODULIN AND NEUROFIBRILLARY TANGLE FORMATION

Neurofibrillary tangles constitute the second major hallmark of AD. Consisting primarily of hyperphosphorylated tau, they have long been known to be a neuropathological feature of the disease; although their role in neurodegeneration has come into question [34]. Tau belongs to the family of microtubule-associated

Table 2

Putative calmodulin binding domain (CaMBD) classes in GWAS proteins linked to Alzheimer's disease. Appropriate hydrophobic (green), acidic (yellow), and IQ (cyan) amino acids are highlighted.

## CaMBD Classes in Proteins Linked to AD

### Cholesterol Metabolism

#### APOE

41GQRWELALGRFDYLRWVQ59 (Basic)

41GQRWELALGRFDYLRWVQ59

41GQRWELALGRFDYLRWVQ59

159EELRWRLASHLRKLRKLL167

159EELRWRLASHLRKLRKLL167

#### ABCA7 sp|Q8IZY2

1221LQALLKRFLLARRSRGLF1240

1221LQALLKRFLLARRSRGLF1240

1221LQALLKRFLLARRSRGLF1240

1221LQALLKRFLLARRSRGLF1240

1221LQALLKRFLLARRSRGLF1240

#### IQ-Like

1221LQALLKRFLLARRS1235

#### CLU tr|E7ETA7

198VAERLTKRYNELKSYQWKM217

### Endocytosis

#### BIN1 sp|O00499

25AGKIASNVQKKLTRAQEKVL40

#### IQ-Like

LQKLGKADETKDEQ

#### PICALM sp|Q13492

129RYLNEKAVSYRQVAFDF145

#### CD2AP sp|Q9Y5K6

211IRGIGFGDIFKEGSVKLRTR230

211IRGIGFGDIFKEGSVKLRTR230

211IRGIGFGDIFKEGSVKLRTR230

211IRGIGFGDIFKEGSVKLRTR230

#### EPHA1 sp|P21709

167NVERCSLGRLLTRRGYLA184

167NVERCSLGRLLTRRGYLA184

### Neuroinflammation

#### CD33 sp|P20138

279RFIVKTHRRKAARTAVGR296

#### CR1 sp|P17927

1090VVTYRCNLGSRGRKVFEL1107

1090VVTYRCNLGSRGRKVFEL1107

#### MS4A4E sp|Q96PG1

107NYLKNLSWRIMGSYLCF123

#### MS4A6A sp|Q9H2W1

101LSIATEKRLTKLLVH115

101LSIATEKRLTKLLVH115

ABCA7, ATP-binding cassette transporter member 7; BIN1, bridging integrator protein 1; CD2AP, CD2-associated protein; CD33, Myeloid cell surface antigen CD33; CLU, clusterin or apolipoprotein J (ApoJ); CR1, complement receptor type 1; EPHA1, ephrin receptor A1; MS4A4E, membrane-spanning 4-domains subfamily A, member 4; MS4A6A, membrane-spanning 4-domains subfamily A, member 6A; PICALM, phosphatidylinositol binding clathrin assembly protein.

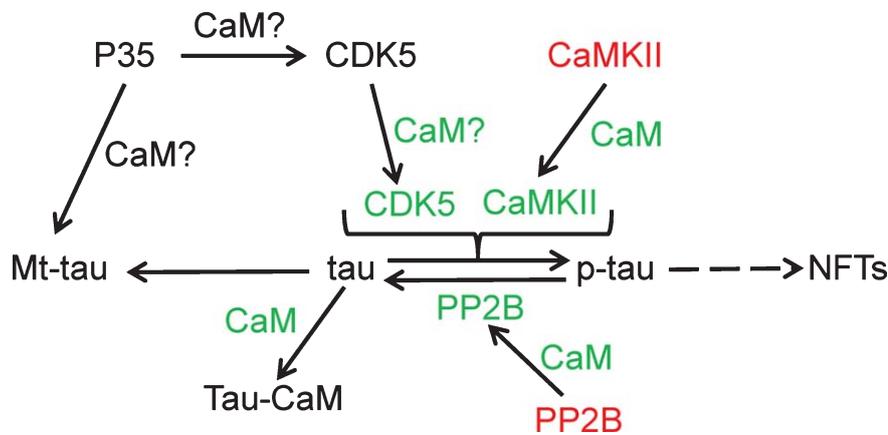


Fig. 4. Calmodulin binding proteins linked to tau phosphorylation. Calmodulin-dependent kinase II (CaMKII); cyclin-dependent kinase 5 (CDK5); calmodulin (CaM); neurofibrillary tangles (NFTs); CDK5 activator (P35); protein phosphatase 2B (PP2B); colors: green, stimulated/activated; red, inhibited.

proteins which function in microtubule assembly and stability [35]. Several early studies provided evidence for an *in vitro* association between tau and CaM that is  $\text{Ca}^{2+}$  dependent (Fig. 4) [36–38]. Tau's association with CaM prevents its binding to microtubules [36]. Furthermore, tau cannot be phosphorylated by protein kinase C *in vitro* when it is in a complex with CaM [37]. In spite of this extensive early association, no recent studies have been conducted on the direct interaction between CaM and tau.

Tau can undergo many post-translational modifications including phosphorylation, acetylation, glycation, and oxidation [39]. Phosphorylation is the best studied and important tau modification related to AD, with several kinases and some phosphatases working in concert to regulate phosphorylation at several specific serine, threonine, and tyrosine residues [39]. Of the kinases, two have been found to interact with CaM:  $\text{Ca}^{2+}$ /CaM-dependent protein kinase II (CaMKII) and cyclin-dependent kinase 5 (CDK5). In addition, protein phosphatase 2B (PP2B or calcineurin) is a well-established CaMBP that has been historically linked to tau dephosphorylation [40].

CaMKII phosphorylates tau *in vitro* at different sites including Ser262 which is in the microtubule binding domain of tau (Fig. 3) [41–44]. The enzyme was also found to phosphorylate tau in neurons at Ser416 [45]. CaM is responsible for activating CaMKII by binding to its regulatory segment through a 1–5–10 binding motif [10, 46]. However, CaMKII is capable of acquiring autonomy from CaM in the presence of high frequency  $\text{Ca}^{2+}$  spikes, despite it being able to phosphorylate tau more efficiently in the presence of CaM [43, 46].

It should also be noted that CaMKII has an established role in neuronal apoptosis which is observed throughout the course of the disease. Phosphorylation by CaMKII is a central pro-apoptotic event. Normally a murine double mutant 2 (MDM2) pathway mediates the degradation of CaMKII but this process is defective in AD lymphocytes [47]. The role of CaM in apoptosis has been reviewed by Berchtold and Villalobo [48].

CDK5 is another kinase with a well-established role in tau phosphorylation (Fig. 4). Various studies indicate that it phosphorylates a number of specific residues including Ser202, Thr205, Ser369, and others, both *in vitro* and *in vivo* [49–53]. Additionally, reducing CDK5 activity using RNA interference was found to reduce tau phosphorylation and the number of neurofibrillary tangles formed in transgenic mouse models, making it a possible target for AD treatment [54]. CDK5 has several activators, one of which is p35 [50]. Truncation of p35 into a shorter p25 fragment is observed in AD [50]. Unlike p35, p25 leads to constitutive CDK5 activation [50, 53]. The p10 fragment lost from p35 upon conversion to p25 is involved in the mutually exclusive binding of p35 to microtubules or CDK5 [55, 56]. The p10 segment also facilitates  $\text{Ca}^{2+}$ -dependent binding between p35 and CaM, which not observed with p25 (Fig. 4) [55]. It is possible that p35 contains a novel CaMBD since no canonical binding motifs were identified upon sequence analysis of the p35 peptide sequence [55]. Moreover, in the model organism *Dicystostelium discoideum*, CDK5 itself was found to bind CaM in a  $\text{Ca}^{2+}$ -independent manner [57]. The two CaMBDs found in *D. discoideum* CDK5 are highly conserved in human CDK5 indicating that the same interaction likely occurs in the human brain [57].

The role of the well documented CaMBP PP2B in tau dephosphorylation was established early but recently has been relegated to a less important role with the discovery of other phosphatases linked to the process. More to the point, studies on the function of PP2B in tau dephosphorylation have been contradictory. PP2B is a heterodimer made of A and B subunits. The A subunit is catalytic and known to be activated by CaM in a  $\text{Ca}^{2+}$ -dependent manner through a 1–8–14 binding motif while the B subunit is regulatory and is itself largely homologous to CaM and able to bind  $\text{Ca}^{2+}$  ions [58, 59]. Early studies *in vitro* showed PP2B's ability to dephosphorylate tau at positions including Ser262 and Ser369 (Fig. 4) [60–62]. However, when compared to other phosphatases such as protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1), it was found that PP2B is only responsible for 7% of the dephosphorylation activity which has been attributed to its low affinity toward tau [63]. Recent studies have reported an increased enzyme activity level of PP2B in AD brains [63]. While this seems contradictory to previous findings, this increase was ascribed to the increased levels of calpain I [64]. Calpain I is  $\text{Ca}^{2+}$  activated and its activity is increased in AD brains. It cleaves the A subunit of PP2B and renders it CaM-independent; the truncated form of PP2B has higher activity than the full length protein [64]. Therefore, the persistent high level of phosphorylation observed should indicate that PP2B does not play a major role in tau dephosphorylation. On the other hand, *in vivo* studies using PP2B-specific inhibitors resulted in hyperphosphorylated tau at positions including Ser262, Ser369, Thr181, and Thr231 [65–67]. To complicate matters further, CaM binding to PP2B, which normally activates the enzyme, was found to inhibit its association with tau [40].

#### **CAMKII AND PP2B ARE INVOLVED IN MEMORY STORAGE**

Furthermore, the two enzymes CaMKII and PP2B have been implicated as central players in deficient memory storage that occurs due to the  $\text{Ca}^{2+}$  deregulation that occurs in AD individuals. These enzymes often act antagonistically by phosphorylating (CaMKII) and dephosphorylating (PP2B) the same proteins. Long-term potentiation (LTP) has been well studied in the mammalian hippocampus as it is the region of the brain that is critical in the formation and retrieval of various forms of memory. The morphology of hippocampal neurons including dendritic spine structures and their density are essential for

memory storage. In the case of AD, progressive damage to the hippocampus results in an inability to form and store certain types of new memories concomitant with alterations in neuronal morphology, dendritic spine density, synapse formation, and deficits in LTP [68, 69]. Importantly, hyperactivation of the  $\text{Ca}^{2+}$ /CaM-dependent phosphatase calcineurin in the hippocampus has been reported for various models of AD and suggests that aberrant  $\text{Ca}^{2+}$  signaling contributes to deregulated interactions between CaM and calcineurin which impairs memory, increases tau phosphorylation and neuronal death [69, 70]. Short-term inhibition of calcineurin using the inhibitor FK506 was found to effectively reverse object recognition deficits in Tg2576 mice and improved dendritic spine loss, neuronal structure, and morphology in plaque bearing YFP-APP/PS1 transgenic mice [71]. Other work further demonstrated a direct connection between CaMKII activity in spines and spine loss in AD and aging brains [72]. Taken together, these data strongly suggest that the inhibition of calcineurin might protect hippocampal neurons and that the mechanism by which this occurs should be explored further. Recently, Berridge [73] has reviewed the positive role of CaMKII in memory formation and of PP2B in memory erasure. He has presented evidence arguing that the rapid memory erasure that occurs during wakefulness in AD individuals due to the action of PP2B prevents the ability of consolidating these memories during sleep which occurs in those not suffering from the disease. While much remains to be learned about this model, this again puts CaM and two of its primary targets—CaMKII and PP2B—at the center of issues facing those suffering from AD.

#### **CAM AND THE ACETYLCHOLINE RECEPTOR**

The oldest of all the models attempting to explain the causes and effects of AD, the “cholinergic hypothesis” is based on the decrease in acetylcholine neurocommunication [74, 75]. In AD, the levels of acetylcholine, acetylcholine receptors (AChR), and cholinergic neurons decrease [76]. Acetylcholine activates either inotropic nicotinic (nAChR) or metabotropic muscarinic (mAChR) receptors. The five subtypes (M1–M5) of mAChR are G-protein coupled receptors (GPCR) involved in  $\text{Ca}^{2+}$  signaling. M1 mAChR has been a primary therapeutic focus in the treatment of AD [77]. Like a number of verified GPCRs, M1 mAChR has a CaMBD on the cytoplasmic

i3 loop [78]. Peptides derived from this intracellular loop bind to CaM. To date these peptides have not been used therapeutically. It should be noted that CaM also has indirect but major effects on the function of the mAChR first through regulation via phosphorylation (CaMKII) and dephosphorylation (PP2B) and second through more complex processes such as the formation of multiprotein complexes including the binding and activation of TRPC6 channels that are also involved in Ca<sup>2+</sup> signaling [79].

### NMDAR, A CALMODULIN-BINDING RECEPTOR

The N-methyl-D-aspartate receptor (NMDAR) is a major Ca<sup>2+</sup> channel involved in synaptic plasticity and memory [80, 81]. The receptor binds glutamate allowing cations to enter the cell [80]. NMDAR levels are generally reduced in AD, likely as an adaptive mechanism in response to the increased receptor activity due to Ca<sup>2+</sup> dysregulation [82]. Likewise, Aβ can cause overactivation of the receptor leading to increased intracellular Ca<sup>2+</sup> levels, an effect which can be reduced through NMDAR antagonists [83]. In addition to affecting Ca<sup>2+</sup> homeostasis, NMDAR plays a role in the cell's antioxidant response, where it can augment the cell's defenses under conditions of oxidative stress [84]. On the other hand, overstimulation of the receptor can also lead to free radical generation which can be detrimental [85].

NMDAR is a heteromultimeric complex with the required presence of at least one monomer of each of NR1 and NR2. Different combinations of NR1 splice products and NR2 subunits vary among different cell types offering the receptor varied pharmacological and physiological properties [81]. CaM is capable of binding the NR1 subunit both *in vitro* and *in vivo* [86–88]. Binding occurs at two locations, both of which are in the cytoplasmic domain of NR1, reducing the mean time of the channel being open and the frequency at which it opens thus inhibiting Ca<sup>2+</sup> entry [81]. While its specific regulatory role is being elucidated, Apo-CaM can associate with the NR1 subunit at a different site than Ca<sup>2+</sup> dependent CaM binding. Upon Ca<sup>2+</sup> entry activated Ca<sup>2+</sup>/CaM induces a rapid structural shift in the NMDAR allowing for an efficient negative feedback cycle [89]. Binding of NR1 to CaM occurs through a novel 1–7 motif which is highly unusual and only observed in myristoylated alanine-rich C-kinase substrate (MARCKS) [90].

### OTHER ROLES FOR CALMODULIN IN ALZHEIMER'S DISEASE

Recent genome wide association studies (GWAS) have linked certain gene polymorphisms with LOAD [91–94]. Although the specific functions of each gene and its encoded protein remain to be elucidated, most are involved either in cholesterol metabolism, neuroinflammation or endocytosis [95]. Using the Calmodulin Target Database, the proteins discussed below were found to be putative CaMBPs but most remain to be experimentally verified (Table 2).

#### *Cholesterol metabolism*

The brain is rich in cholesterol, containing approximately 25% of the body's cholesterol content which is essential for myelin formation [96]. An association between cholesterol and AD was made when statins, which are used to treat elevated cholesterol levels, were found to reduce disease incidence [97]. Furthermore, levels of 24S-hydroxycholesterol, one of the first intermediates of cholesterol elimination, were higher in AD individuals than in healthy controls [98]. Since it cannot cross the blood-brain barrier, the brain's cholesterol is internally synthesized and must be shuttled around by lipoproteins containing ApoE [96, 99]. ApoE contains two putative CaMBDs each with 2-3 potential binding motifs (Table 2). While the role of CaM binding in ApoE function remains to be clarified, it is well established that the ApoEε4 variant is a significant risk factor for LOAD.

Clusterin (CLU) and ATP-binding cassette transporter A7 (ABCA7) are two other proteins involved in cholesterol metabolism with polymorphisms which are risk factors for LOAD [91–93]. Each possesses a single putative CaMBD as revealed through Calmodulin Target Database analysis (Table 2). The CLU protein is multifunctional affecting immunity and apoptosis in addition to playing a role in lipid metabolism [100]. With its expression increased in AD, CLU (also termed apolipoprotein J) is a component of the lipid particles that transport cholesterol [101]. ABCA7 belongs to the ABC transporter superfamily, which regulates cholesterol transport across the cell membrane [102]. However, it is likely that the highly homologous protein ABCA1, which is a proven CaMBP, plays a more prominent role in this process than ABCA7, according to mouse models. Binding through a 1–5–8–14 motif, CaM affects the stability of ABCA1 *in vivo*, preventing its degradation [103].

### *Neuroinflammation*

Inflammation of neurons and dysregulation of different aspects of the immune response are characteristic of AD brains (reviewed in [104]). GWAS identified several proteins that affect the immune response. All were found through the Calmodulin Target Database to contain presumptive CaMBDs, although none have so far been experimentally tested to confirm the interaction with CaM (Table 2). Cluster of differentiation 33 (CD33), which is upregulated in microglial cells of AD brains, impairs A $\beta$  clearance propagating plaque pathology [91, 105]. A second protein, the complement receptor 1 (Cr1) connects the complement system and CaM to AD although its exact role in propagation of pathology remains unknown [91, 106]. Moreover, polymorphisms in the membrane spanning 4-subfamily A (MS4A) proteins, MS4A4E and MS4A6A, were also found as risk factors for LOAD [91]. The function of the MS4A family remains poorly characterized, although, they may be components of Ca<sup>2+</sup> channels and thus, affect Ca<sup>2+</sup> homeostasis in neurons [107].

### *A $\beta$ PP endocytosis*

The A $\beta$ PP protein is synthesized in the rough endoplasmic reticulum and travels through the trans-Golgi network to the cell membrane where it can be brought back into the cell through receptor-mediated endocytosis [108]. The exact subcellular location where A $\beta$  is generated remains a topic of debate although endocytotic vesicles seem to be likely candidates since inhibition of endocytosis reduces the levels of A $\beta$  produced [109]. A number of putative CaMBPs related to vesicle assembly were also identified including the phosphatidylinositol-binding clathrin assembly protein (PICALM), the scaffolding protein CD2-associated protein (CD2AP), the sortilin-related receptor L (SORL1), ephrin type-A receptor 1 (EPHA1), and the bridging integrator 1 (BIN1) (Table 2) [28]. To date, only BIN1 has been experimentally confirmed as a CaMBP [110].

### *AD, calmodulin, and the cell cycle*

Although the amyloid cascade hypothesis dominates the AD research landscape, a number of alternative theories have been suggested in attempts to understand the pathogenesis of AD. In the developing brain neurons are integrated into complex synaptic networks after

they have completed their proliferation, migration, and differentiation. The cellular signals that tightly regulate neuronal connectivity and plasticity also serve to ensure neurons are maintained in a differentiated state while simultaneously preventing reactivation of signaling pathways that control proliferation and cell cycle progression. The protection from aberrant neuronal cell cycle re-entry occurs through strict regulatory mechanisms at specific cell cycle control checkpoints. However, neuronal plasticity and formation of neuritic extensions allows for progression of the cycle early in G1 phase which is followed by opposing signals for re-differentiation back into G0. The mechanisms controlling transient re-entry into the cell cycle is considered to be an essential event for synaptic remodeling (reviewed in [111]).

In AD, the G1/S phase check-point regulatory mechanisms break down to the point where individual neurons can proceed through S-phase and undergo full or partial DNA re-replication with consequent entry into the G2 phase of the cell cycle [112–114]. Normally, in cycling non-neuronal cells, the G2 phase prepares the cell for mitosis, but in the case of post-mitotic neurons, improper cycling halts during the G2 phase and triggers neuronal death by apoptosis. Evidence from a number of animal models of human neurodegenerative disorders strongly suggests that atypical cell cycle re-entry events precede neuronal apoptosis ultimately leading to cell death [114–117]. Upregulation or improper protein degradation alters the normal level of a number of cell cycle proteins, including proliferating cell nuclear antigen (PCNA), cyclin D1, CDK4, and cyclin B1, within the hippocampus and other AD-diseased brain regions. These markers of cell cycle re-entry are not found randomly dispersed throughout the AD diseased brain nor are they detected in age-matched control patient brains [118, 119]. This ectopic cell cycle re-entry (CCR) is believed to account for a significant fraction of cortical neurons that are lost in AD [120]. Even more intriguing is that cell cycle markers are not only one of the earliest cellular abnormalities detected in AD, but theoretically may contribute to AD pathology including tau phosphorylation, A $\beta$  formation, and neuronal calcium-ion dysfunction [121–123]. Seward et al. [124] further showed that CCR requires soluble A $\beta$ , tau, and concomitant activation of kinases including the Ca<sup>2+</sup>-calmodulin kinase II (CaMKII). This irregular cell cycle control within specific neuronal populations in the AD brain likely plays an early, yet crucial role for abnormalities associated with AD pathogenesis [117, 125, 126].

Studies in many cell types using a variety of approaches have implicated both  $\text{Ca}^{2+}$  and CaM as key regulators of distinct checkpoints in the cell cycle, including early G1, the G1 to S phase transition and G2/M transition. Calmodulin interactions with the CaMK family of CaMBPs act as important regulators of cell cycle progression (reviewed in [128]). Putative CaM-binding motifs have also been detected in a large number of cell cycle proteins including the cyclins (reviewed in [129]). Mitogenic stimulation leads to a variety of  $\text{Ca}^{2+}$ -CaM-mediated responses yet when the stimulation is permanent it causes changes in the sub-cellular distribution of CaM, which leads to changes in the total amount of CaM, alters cellular sensitivity to  $\text{Ca}^{2+}$  signals, and presents the potential for aberrant interactions between CaM and CaMBPs [130]. Although the deregulation of  $\text{Ca}^{2+}$ /calmodulin signaling in AD brains is not well understood, increased levels of CaM and decreased levels of phosphorylated CaMKII have been reported in the hippocampus of APP/PS1 mice [131]. These changes might be reflective of an aberrant involvement of CaM/CaMKII in the impairment of cell cycle control in AD. Indeed many different kinds of signaling pathways are changed in AD, and the relevance of the mitogenic upregulation that may induce cell cycle re-entry in the disease process is far from clear. Future studies will need to focus on identifying the mechanisms that regulate CaM and CaMKII expression in the hippocampus, as well as the downstream effector molecules involved, as this will potentially uncover new pathways for understanding the dysregulation of cell cycle control in AD brains.

### CALMODULIN AS A BIOMARKER

Since AD cannot be fully verified until autopsy, the search for minimally invasive biomarkers for the disease continues [132]. While cerebrospinal levels of A $\beta$ , tau, and phosphorylated tau are effective biomarkers, this approach is fairly invasive and not without problems. Generally blood tests for those same biomarkers have been less than rewarding. Esteras et al. [133] have shown that levels of CaM are significantly increased in lymphoblasts from AD individuals. In a subsequent study, the increased CaM levels in lymphoblasts and peripheral blood mononuclear cells from AD individuals were found to be significantly greater than from non-dementia persons or those suffering from other types of dementia including amyotrophic lateral sclerosis, dementia with Lewy bodies, and

frontotemporal dementia, among others [134]. These results suggest that CaM levels in peripheral blood cells have the potential to serve as a biomarker for various dementias including that resulting from AD.

### CONCLUSION

Considering the multiple regulatory functions that CaM carries out in all cells, it is not surprising that it has many links to the underlying events of AD. CaM is involved in the defining aspects of AD progression and pathogenesis including A $\beta$  generation, tau phosphorylation,  $\text{Ca}^{2+}$  homeostasis, cholesterol metabolism, neuroinflammation, A $\beta$ PP endocytosis, and apoptosis. While it seems to present itself as a primary target in combating the symptoms and progression of the disease, many issues appear to reduce its current appeal as a therapeutic target. It has both positive and negative regulatory roles, making blanket inhibition approaches untenable. Thus currently available pharmacologically proven CaM antagonists likely would not be an option for medical treatment. However, circumstances could change with the development of new CaM antagonists (e.g., [135]). Since most CaMBPs bind to CaM in unique ways and since some have multiple types of binding sequences, based on the technologies that exist for developing CaM antagonists it should be possible to develop target-specific pharmaceuticals.

The idea of targeting CaM and its CaMBPs is not without precedent. CaM is already a potential target for the treatment of Huntington's disease, another neurodegenerative disease. Huntington's disease is an autosomal dominant disorder due to a polyglutamine expansion in the huntingtin protein that is exacerbated by transglutaminase [136]. Huntingtin binds CaM and this binding can be disrupted using a CaM-peptide consisting of amino acids 76–121 of the CaM protein [137]. This peptide was then shown to reduce the level of transglutaminase-modified huntingtin and cytotoxicity in differentiated neuroblastoma (SH-SY5Y) cells. Subsequent treatment of a Huntington's mouse (R6/2) model with this CaM-peptide led to neuroprotection via the mechanisms established in the tissue culture cells [138]. Thus CaM-Huntingtin binding appears to present a potential therapeutic target in combating this disease. The identification of critical CaMBPs linked to AD may also open a similar door.

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