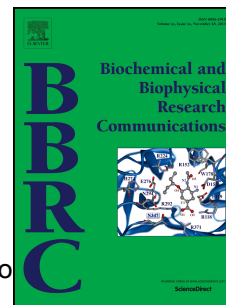


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High affinity binding of amyloid  $\beta$ -peptide to calmodulin: Structural and functional implications

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• **Title:** High Affinity Binding of Amyloid  $\beta$ -peptide to Calmodulin: Structural and Functional Implications

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**Abstract**

Amyloid  $\beta$ -peptides ( $A\beta$ ) are a major hallmark of Alzheimer's disease (AD) and their neurotoxicity develop with cytosolic calcium dysregulation. On the other hand, calmodulin (CaM), a protein which plays a major multifunctional role in neuronal calcium signaling, has been shown to be involved in the regulation of non-amyloidogenic processing of amyloid  $\beta$  precursor protein (APP). Using fluorescent 6-bromoacetyl-2-dimethylaminonaphthalene derivatives of CaM, Badan-CaM, and human amyloid  $\beta$ (1-42) HiLyte™-Fluor555, we show in this work that  $A\beta$  binds with high affinity to CaM through the neurotoxic  $A\beta$ 25-35 domain. In addition, the affinity of  $A\beta$  for calcium-saturated CaM conformation is approximately 20-fold higher than for CaM conformation in the absence of calcium (apo-CaM). Moreover, the value of  $K_d$  of  $0.98 \pm 0.11$  nM obtained for  $A\beta$ 1-42 dissociation from CaM saturated by calcium point out that CaM is one of the cellular targets with highest affinity for neurotoxic  $A\beta$  peptides. A major functional consequence of  $A\beta$ -CaM interaction is that it slows down  $A\beta$  fibrillation. The novel and high affinity interaction between calmodulin and  $A\beta$  shown in this work opens a yet-unexplored gateway to further understand the neurotoxic effect of  $A\beta$  in different neural cells and also to address the potential of calmodulin and calmodulin-derived peptides as therapeutic agents in AD.

**Keywords:** Amyloid  $\beta$ , Calmodulin, Badan-Calmodulin, Human amyloid  $\beta$  (1-42) HiLyte™-Fluor555, Calcium, Alzheimer's disease.

## 1. Introduction

A progressive loss of functional synapses has been noticed in hippocampal and cortical brain regions of patients with symptoms ranging from mild cognitive impairment (MCI) to early-mild Alzheimer's disease (AD) [1; 2]. The 42-residue-long amyloid  $\beta$ -peptide ( $A\beta_{1-42}$ ) is remarkably involved in AD. This peptide is prone to aggregation and also impairs synaptic function at both pre- and postsynaptic sites, although excitatory post-synapses are likely its early targets [3]. In addition, intracellular calcium homeostasis has been shown to be disrupted in both sporadic and familial forms of AD, and this can exacerbate  $A\beta$  formation and promote tau hyperphosphorylation [4; 5]. Dysregulation of intracellular  $Ca^{2+}$  buffering by  $A\beta$  can trigger a pathogenic feed-forward cycle leading to an altered synaptic morphology, to neuronal apoptosis, and eventually to cognitive impairment [6].

Calmodulin (CaM) plays a major role in neuronal calcium signaling, as a primary calcium binding protein relevant in cytosolic calcium buffering and also as a regulatory protein of other key effector proteins in calcium signaling pathways, reviewed in [7; 8; 9]. Noteworthy, nearly 30-years ago it was noticed that CaM is significantly decreased in the brain of AD individuals [10]. O'Day and Myre (2004) [11] raised the "calmodulin hypothesis" for late onset AD when they noticed that several proteins linked to the production of  $A\beta$  possess putative calmodulin binding domains (CaMBDs). However, the software-aided prediction of proteins that bind to CaM is hindered by three major issues: (1) the large conformational change of CaM upon  $Ca^{2+}$  binding [7; 8; 9], (2) proteins do not bind to  $Ca^{2+}$ /CaM via defined consensus targeting sequences, but through a diversity of motifs (e.g. 1-5-10 or 1-8-14 or 1-12 motifs or non-canonical motifs) involving hydrophobic amino acids and basic residues [12], and (3)  $Ca^{2+}$ -independent binding of proteins to CaM occurs via IQ- or IQ-like motifs showing certain degree of variability as well [8; 9]. Thus, direct experimental assessment of CaM interaction with each protein target is needed.

Actually, it has been shown that CaM can bind and regulate the functioning of both, amyloid  $\beta$  precursor protein (APP) and  $\beta$ -secretase (BACE1; beta-site APP cleaving enzyme 1) (reviewed in [9]), as

well as one hallmark of AD such as tau [13] and also  $\text{Ca}^{2+}$ /CaM-dependent protein kinase II (CaMKII) and cyclin-dependent kinase 5 (CDK5) involved in tau hyperphosphorylation [14]. Moreover, in a previous work [15] control dot-blot overlay assays suggested the occurrence of direct A $\beta$ 1-42 interaction with  $\text{Ca}^{2+}$ /CaM, which could, at least partially, account for calmodulin antagonism of the inhibition of brain plasma membrane  $\text{Ca}^{2+}$ -ATPase by A $\beta$ 1-42. However, dot-blot overlay assays neither allow to properly quantify the strength of the interaction between A $\beta$ 1-42 and CaM, nor to critically assess its functional relevance for each protein partner.

In this work, we have experimentally addressed the study of the binding of A $\beta$  to CaM, both in the presence and in the absence of  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$ /CaM and apo-CaM respectively), using fluorescence derivatives of CaM and A $\beta$ . The results have pointed out that (1)  $\text{Ca}^{2+}$ /CaM binding to A $\beta$  has a nanomolar dissociation constant, (2) the affinity of A $\beta$  for CaM is strongly altered by calcium binding-induced changes of CaM conformation, (3) this interaction involves the neurotoxic A $\beta$  domain 25-35, and (4) it also significantly slows down A $\beta$  fibrillation.

## 2. Materials and methods

### 2.1. Materials

All reagents and buffer components were of analytical grade purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. T34C and T110C variants of human expressed calmodulin were generated and labelled with Badan (6-Bromoacetyl-2-dimethylaminonaphthalene) in the same conditions as previously described for the T34C/T110C double mutant [16]. The labelled proteins were HPLC purified and freeze-dried. Unlabeled calmodulin from bovine testes was obtained from Sigma-Aldrich. Human amyloid  $\beta$  (1-42) HiLyte™-Fluor555 was obtained from AnaSpec (Freemont, CA). Unlabeled amyloid  $\beta$  (1-42) and amyloid  $\beta$  (25-35) were synthesized by StabVida (Caparica, Portugal). Lyophilized

peptides were dissolved in 1% NH<sub>4</sub>OH, and then diluted with PBS buffer to desired concentrations. Reconstituted peptides were aliquoted and stored at -20°C.

## 2.2. Fluorescence quenching studies

Fluorescence measurements were performed using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) at 25°C in 1 cm quartz cells with both excitation and emission slits of 10 nm.

Fluorescence titrations of 5 nM Badan-CaM(T34C) or 10 nM Badan-CaM(T110C) with unlabeled or labeled A $\beta$ 1-42 and A $\beta$ 25-35 were carried out by addition of varying amounts of stock solutions of amyloid peptides (20  $\mu$ M and 2  $\mu$ M) in 50 mM HEPES-KOH (pH 7.4), 100 mM KCl, 2mM MgCl<sub>2</sub> and 50  $\mu$ M CaCl<sub>2</sub> buffer. Fluorescence emission spectra of Badan-CaM were acquired with 385nm excitation wavelength. Titrations of 10 nM HiLyte™-Fluor555-A $\beta$ 1-42 with unlabeled CaM were performed in a “low calcium buffer” containing 50 mM HEPES-KOH (pH 7.4), 100 mM KCl, 2mM MgCl<sub>2</sub> and 20 mM EGTA buffer, and the fluorescence emission spectra were acquired with excitation wavelength of 525 nm.

The fluorescence intensity data were corrected for volume changes during titrations, which were always <3%. No inner filter corrections were needed in titration data, because the increase of absorbance at excitation and emission wavelengths were always <0.002. Each experiment was performed in quadruplicate.

## 2.3. Calcium titration assays

For calcium titration assays 150 mM HEPES-KOH (pH 7.1), 100 mM KCl, 2mM MgCl<sub>2</sub> and 250  $\mu$ M EGTA buffer was used. Afterwards, 10 nM of Badan-CaM(T110C) were added. To test the effect of  $\beta$ -amyloid peptides, Badan-CaM(T110C) was preincubated with either A $\beta$ 1-42 or A $\beta$ 25-35 at 37°C for 30 minutes at an equimolar ratio. Subsequently, CaCl<sub>2</sub> was added at increasing amounts and fluorescence was

measured with an excitation wavelength of 385 nm and the fluorescence emission spectra were acquired from 400 nm to 650 nm, using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). Fluorescence measurements were performed at 25°C in 1 cm quartz cell with both excitation and emission slits of 10 nm. After subtracting the weak buffer fluorescence spectrum, the fluorescence emission spectra were corrected for volume changes. Each experiment was performed in quadruplicate.

#### 2.4. Thioflavin T aggregation assays

The assay was performed as described in Maezawa *et al.* [17]. Briefly, a 35  $\mu$ l of a 0.043  $\mu$ M A $\beta$ 1-42 solution was mixed with 115  $\mu$ l of Thioflavin T (ThT) solution (5  $\mu$ M in 50 mM glycine-NaOH at pH 8.5) giving an A $\beta$ 1-42 final concentration of 10  $\mu$ g/ml. When indicated, A $\beta$ 1-42 was previously aged at 37°C for 72h, with or without the addition of CaM at equimolar ratio. After mixing with ThT, fluorescence emission at 490 nm, using an excitation wavelength of 440 nm [18], was recorded by a Varioskan Flash fluorescence spectrophotometer (Thermo Scientific).

#### 2.5. Docking simulations

Modeling of protein–peptide interactions was performed using the CABS-dock web server (<http://biocomp.chem.uw.edu.pl/CABSdock>) [19; 20]. PDB files for both calmodulin conformations were obtained from Uniprot (<http://www.uniprot.org/>).

#### 2.6. Statistical analysis

Non-linear regression fits, statistical analysis and plotting of titration data were done using OriginPro 8 software.

Differences of A $\beta$ 1-42 fibrillation among treatments were assessed by analysis of variance with Turkey HSD (Honest Significant Difference) multiple comparison test using SigmaStat 3.10 (Systat). Significant difference was accepted at the  $p < 0.05$  levels.

### 3. Results

To quantify and analyze the interaction between CaM and A $\beta$ 1-42 peptide, which is one of the major hallmarks of AD, we have used fluorescent Badan-CaM derivatives and fluorescence spectroscopy measurements. Titration of the fluorescence of 10 nM BadanCaM-110 with A $\beta$ 1-42 in a standard buffer 50 mM HEPES-KOH (pH 7.4), 100 mM KCl, 2 mM MgCl<sub>2</sub> and 50  $\mu$ M CaCl<sub>2</sub> showed that nanomolar concentrations of A $\beta$  elicited a large quenching of Badan-CaM fluorescence (Figure 1A). The dependence of quenching data upon A $\beta$ 1-42 concentration can be fit well to a *one-site binding* equation (Figure 1A, insert). A dissociation constant of  $0.98 \pm 0.11$  nM was calculated for A $\beta$  dissociation from the A $\beta$ -CaM complex. As this A $\beta$  concentration is within the intracellular range reported for non-fibrillar amyloid [21; 22; 23], this result unraveled that CaM is a novel intracellular high affinity target for A $\beta$ . Furthermore, CaM at a 1:1 molar ratio with A $\beta$ 1-42 peptide efficiently protected against amyloid fibrils development as shown in Figure 1B.

On these grounds, we decided to experimentally assess whether the A $\beta$ 25-35 peptide, which contains the neurotoxic domain of the full-length A $\beta$ 1-42, is directly involved in the interaction with CaM. The results of the BadanCaM(T110C) fluorescence titration with A $\beta$ 25-35 are shown in the Figure 1C, and like those obtained with A $\beta$ 1-42, these data can be fit to the *one-site binding* equation (Figure 1C, insert), yielding a dissociation constant of  $0.66 \pm 0.07$  nM for A $\beta$ 25-35 dissociation from the A $\beta$ -CaM complex. Therefore, A $\beta$ 25-35 binds to CaM even more strongly than the full A $\beta$ 1-42 peptide, pointing out that the toxic domain of A $\beta$  plays a leading role in the interaction of this peptide with CaM.



The CaM conformation largely changes upon saturation with calcium [7; 8]. Results of Figure 1 were obtained under experimental conditions where CaM was completely saturated with  $\text{Ca}^{2+}$ , thus showing  $A\beta$  interaction with  $\text{Ca}^{2+}/\text{CaM}$ . Considering that BadanCaM(T110C) fluorescence is strongly dependent on the saturation of CaM by calcium (Figure 2A), we used this property to experimentally evaluate the possibility that interaction of CaM with  $A\beta$ 1-42 and  $A\beta$ 25-35 peptides could impair the affinity of CaM for calcium ions. However, fluorescence data (Figure 2A) pointed out that these peptides produced at most only a slight shift in calmodulin's calcium saturation curves, yielding average  $\text{Ca}^{2+}$  dissociation constants of  $0.32\pm 0.01 \mu\text{M}$  and  $0.38\pm 0.01 \mu\text{M}$  in the presence of  $A\beta$ 25-35 and  $A\beta$ 1-42, respectively. These values were only slightly higher than the average  $0.30\pm 0.01 \mu\text{M}$   $\text{Ca}^{2+}$  dissociation constant obtained for CaM in this buffer. Nevertheless, owing to the position of Badan near calcium binding at sites 3 and 4 of CaM, solely with these data we cannot exclude the possibility that BadanCaM(T110C) fluorescence could be differentially affected by calcium binding to sites 1-2 or 3-4. Because of the functional relevance of this point, to further assess whether  $A\beta$ 1-42 altered the extent of calcium binding to CaM, we measured the drop of free calcium using the calcium indicator Fura-2 after addition of CaM both in the absence and in the presence of  $A\beta$ 1-42. Results are shown in Figure 2B and proved that  $A\beta$ 1-42 did not reduce the extent of calcium binding to CaM.

To evaluate the dissociation constant of  $A\beta$ 1-42 from CaM in the absence of calcium, we cannot use BadanCaM(T110C) because of its low fluorescence when calcium concentration falls in the nanomolar range (Figure 2A). Therefore, we selected a fluorescence tagged HiLyte<sup>TM</sup>-Fluor555- $A\beta$ 1-42, whose fluorescence emission at 560-580 is not significantly altered by changes of calcium concentration up to  $50 \mu\text{M}$ . First, as shown in Figure 3A, we confirmed that titration with HiLyte<sup>TM</sup>-Fluor555- $A\beta$ 1-42 elicited a extent of quenching of BadanCaM(T110C) fluorescence saturated by calcium similar to that measured with  $A\beta$ 1-42 in Figure 1A. Moreover, using non-linear regression the data fit well to a *one-site binding* equation and yielded a dissociation constant of  $0.97\pm 0.09 \text{ nM}$   $A\beta$ 1-42 (Figure 3B), a value which is the same obtained from the titration of BadanCaM(T110C) with unlabeled  $A\beta$ 1-42. These results

allowed us to conclude that HiLyte<sup>TM</sup>-Fluor555 labeling of A $\beta$ 1-42 does not alter A $\beta$ -CaM interaction. Then, we performed the titration of the fluorescence of HiLyte<sup>TM</sup>-Fluor555-A $\beta$ 1-42 with unlabeled CaM in 50 mM HEPES-KOH (pH 7.4), 100 mM KCl, 2mM MgCl<sub>2</sub> and 20 mM EGTA, i.e. with the closed apo-(Ca<sup>2+</sup> free)CaM conformation (Figure 3C). The data are fit by nonlinear regression to a *one-site binding* equation and yielded a dissociation constant of 22.1 $\pm$ 1.7 nM for CaM (Figure 3D). Since BadanCaM(T110C) and HiLyte<sup>TM</sup>-Fluor555-A $\beta$ 1-42 concentrations were fixed (10 nM) during A $\beta$  and CaM titrations, the k<sub>d</sub> value obtained from Figure 3D pointed out that the close conformation of apo-CaM has more than 20-fold lower affinity for A $\beta$  than the open 4Ca<sup>2+</sup>-CaM conformation.

#### 4. Discussion

This work has demonstrated that neurotoxic A $\beta$  peptides, A $\beta$ 1-42 and A $\beta$ 25-35, have a relatively high affinity for CaM with a calculated dissociation constant  $\leq$ 1 nM for the A $\beta$ /CaM complex at 25°C, and physiological pH and ionic strength. This result showed that CaM is a novel intracellular high affinity target for A $\beta$  because the concentration of non-fibrillar A $\beta$  peptides is within the nanomolar range in the brain [21; 22; 23]. It is also to be recalled here that critical concentration values in the submicromolar range have been reported for induction of A $\beta$ 1-42 fibrillization [24; 25]. Moreover, the dissociation constant of A $\beta$ -CaM complex obtained in this work indicates that the affinity of CaM for A $\beta$  is approximately 20-fold higher than for human recombinant apo-E3 and -E4, a major risk factor for late onset Alzheimer's disease and also a well-accepted cellular target for A $\beta$  with a dissociation constant value of 20 nM [26]. A binding affinity in the low nanomolar range has been calculated using surface plasmon resonance for A $\beta$ 1-42 interaction with the major intracellular target tau [27]. Noteworthy, the A $\beta$ 1-42 dissociation constant from A $\beta$ -CaM complex is nearly identical to the reported dissociation constant of A $\beta$  from PrP<sup>c</sup> [28], a cellular prion protein that has been proposed to mediate A $\beta$ -induced synaptic dysfunction in the mouse brain [25], and also from glycogen synthase kinase 3 $\alpha$  (GSK3 $\alpha$ ), a kinase that mediates hyperphosphorylation of tau and that it is stimulated *in vitro* by A $\beta$ 1-42 [29].

The 25-35 domain of A $\beta$ 1-42 binds to CaM even strongly than the full A $\beta$ 1-42 peptide, pointing out that CaM interaction is expected to antagonize, at least partially, the binding of A $\beta$  to other recognized intracellular targets which mediate its neurotoxicity. Moreover, since the A $\beta$ 25-35 segment forms a structural loop that protrudes out of the core structure in A $\beta$  oligomers [30; 31], CaM is expected to have a similar affinity for A $\beta$  monomers and oligomers. In addition, CaM at a molar ratio 1:1 with A $\beta$ 1-42 peptide efficiently protected against amyloid fibrils development. Taken all together these results suggest a relevant, and up to now overlooked, direct neuroprotective role of CaM against A $\beta$  neurotoxicity in the brain. Currently, *in vivo* experiments are being undertaken to experimentally address this hypothesis in neuronal cultures (*manuscript in preparation*).

In contrast to the functional impairment of A $\beta$ , our results allowed to conclude that the interaction of A $\beta$  with CaM does not alter calcium binding to CaM, despite that the closed conformation of apo-CaM has more than 20-fold lower affinity for A $\beta$  than the open 4Ca<sup>2+</sup>-CaM conformation.

Docking simulations online using the program CABS Dock yielded two putative sites at the central connection helical domain of 4Ca<sup>2+</sup>-CaM for A $\beta$ 25-35 interaction, being one located close to the N-terminus domain containing calcium binding sites 1 and 2, and the other located near the C-terminus domain containing the calcium binding sites 3 and 4. The lack of significant fluorescence quenching of BadanCaM(T34C) by A $\beta$ 1-42 (*data not shown*), while it induced approximately 40% quenching of the fluorescence of BadanCaM(T110C), indicated that A $\beta$  binds to a site located at or close to the CaM C-terminal domain and further apart from the protein N-terminus (Figure 4A). Taking into account that the central helical domain of Ca<sup>2+</sup>/CaM folds over the target domain of cellular signaling proteins linked to Alzheimer's disease modulated by calmodulin [8; 9; 11] and the major role of calmodulin-binding proteins in neuronal plasticity and activity [7; 8], it is likely that the interaction between A $\beta$  and Ca<sup>2+</sup>/CaM will have major functional consequences for brain physiology. Indeed, in a previous work we have shown that calmodulin antagonizes amyloid  $\beta$  peptides-mediated inhibition of brain plasma membrane Ca<sup>2+</sup>-

ATPase [15]. Moreover, BadanCaM derivatives have been shown to be useful to monitor conformational changes not only in CaM, but also those in the CaM-CaM binding proteins interface.

Docking simulations for A $\beta$ 25-35-apoCaM complex showed that most-probable docking conformations were completely different from those aforementioned for Ca<sup>2+</sup> saturation conditions (Figure 4B). Since apo-CaM has been reported to co-immunoprecipitate with APP, and to promote ADAM10-mediated proteolysis of APP through the non-amyloidogenic pathway resulting in sAPP $\alpha$  production [32] (thereby antagonizing the stimulation of BACE1-amyloidogenic pathway by CaM), binding of A $\beta$  to apo-CaM can be seen as a feed-back inhibition mechanism to modulate the production of neurotoxic A $\beta$  peptides.

In summary, this work shows a novel and high affinity interaction between calmodulin and the amyloid  $\beta$  peptide, involving the potent neurotoxic 25-35 domain of A $\beta$  which is calcium-dependent, as it is modulated by the conformational change induced by calcium binding to CaM. Since the affinity of A $\beta$ 1-42 and A $\beta$ 25-35 for Ca<sup>2+</sup>/CaM is among the higher, if not the highest, reported until now for A $\beta$  intracellular or extracellular protein partners, our results open new perspectives to further understand the neurotoxic effect of A $\beta$  in different neural cells and also to address the potential of calmodulin and calmodulin-derived peptides as therapeutic agents in AD.

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**References**

- [1] S.W. Scheff, D.A. Price, F.A. Schmitt, S.T. DeKosky, and E.J. Mufson, Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. *Neurology* 68 (2007) 1501-1508.
- [2] G.M. Shankar, S. Li, T.H. Mehta, A. Garcia-Munoz, N.E. Shepardson, I. Smith, F.M. Brett, M.A. Farrell, M.J. Rowan, C.A. Lemere, C.M. Regan, D.M. Walsh, B.L. Sabatini, and D.J. Selkoe, Amyloid- $\beta$  protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med* 14 (2008) 837-842.
- [3] E. Marcello, R. Epis, and M. Di Luca, Amyloid flirting with synaptic failure: Towards a comprehensive view of Alzheimer's disease pathogenesis. *European Journal of Pharmacology* 585 (2008) 109-118.
- [4] F.M. LaFerla, Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. *Nat Rev Neurosci* 3 (2002) 862-872.
- [5] I. Bezprozvanny, and M.P. Mattson, Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends in Neurosciences* 31 (2008) 454-463.
- [6] A. Demuro, I. Parker, and G.E. Stutzmann, Calcium Signaling and Amyloid Toxicity in Alzheimer Disease. *Journal of Biological Chemistry* 285 (2010) 12463-12468.
- [7] D. Chin, and A.R. Means, Calmodulin: a prototypical calcium sensor. *Trends in Cell Biology* 10 (2000) 322-328.
- [8] S.W. Vetter, and E. Leclerc, Novel aspects of calmodulin target recognition and activation. *European Journal of Biochemistry* 270 (2003) 404-414.
- [9] D.H. O'Day, K. Eshak, and M.A. Myre, Calmodulin Binding Proteins and Alzheimer's Disease. *Journal of Alzheimer's Disease* 46 (2015) 553-569.
- [10] D.R.C. McLachlan, L. Wong, C. Bergeron, and K.G. Baimbridge, Calmodulin and calbindin D28K in Alzheimer Disease. *Alzheimer Disease & Associated Disorders* 1 (1987) 171-179.

- [11] D.H. O'Day, and M.A. Myre, Calmodulin-binding domains in Alzheimer's disease proteins: extending the calcium hypothesis. *Biochemical and Biophysical Research Communications* 320 (2004) 1051-1054.
- [12] H. Tidow, and P. Nissen, Structural diversity of calmodulin binding to its target sites. *FEBS Journal* 280 (2013) 5551-5565.
- [13] R. Padilla, R.B. Maccioni, and J. Ávila, Calmodulin binds to a tubulin binding site of the microtubule-associated protein tau. *Molecular and Cellular Biochemistry* 97 (1990) 35-41.
- [14] D.-Y. Yu, L. Tong, G.-J. Song, W.-L. Lin, L.-Q. Zhang, W. Bai, H. Gong, Y.-X. Yin, and Q. Wei, Tau binds both subunits of calcineurin, and binding is impaired by calmodulin. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1783 (2008) 2255-2261.
- [15] M. Berrocal, M.R. Sepulveda, M. Vazquez-Hernandez, and A.M. Mata, Calmodulin antagonizes amyloid- $\beta$  peptides-mediated inhibition of brain plasma membrane  $\text{Ca}^{2+}$ -ATPase *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1822 (2012) 961-969.
- [16] K. Török, A. Tzortzopoulos, Z. Grabarek, S.L. Best, and R. Thorogate, Dual Effect of ATP in the Activation Mechanism of Brain  $\text{Ca}^{2+}$ /Calmodulin-Dependent Protein Kinase II by  $\text{Ca}^{2+}$ /Calmodulin. *Biochemistry* 40 (2001) 14878-14890.
- [17] I. Maezawa, H.-S. Hong, R. Liu, C.-Y. Wu, R.H. Cheng, M.-P. Kung, H.F. Kung, K.S. Lam, S. Oddo, F.M. LaFerla, and L.-W. Jin, Congo red and thioflavin-T analogs detect  $\text{A}\beta$  oligomers. *Journal of Neurochemistry* 104 (2008) 457-468.
- [18] N.D. Younan, and J.H. Viles, A Comparison of Three Fluorophores for the Detection of Amyloid Fibers and Prefibrillar Oligomeric Assemblies. ThT (Thioflavin T); ANS (1-Anilinonaphthalene-8-sulfonic Acid); and bisANS (4,4'-Dianilino-1,1'-binaphthyl-5,5'-disulfonic Acid). *Biochemistry* 54 (2015) 4297-4306.
- [19] M. Blaszczyk, M. Kurcinski, M. Kouza, L. Wieteska, A. Debinski, A. Kolinski, and S. Kmiecik, Modeling of protein-peptide interactions using the CABS-dock web server for binding site search and flexible docking. *Methods* 93 (2016) 72-83.

- [20] M. Kurcinski, M. Jamroz, M. Blaszczyk, A. Kolinski, and S. Kmiecik, CABS-dock web server for the flexible docking of peptides to proteins without prior knowledge of the binding site. *Nucleic Acids Research* 43 (2015) W419-W424.
- [21] M.B. Podlisny, D.M. Walsh, P. Amarante, B.L. Ostaszewski, E.R. Stimson, J.E. Maggio, D.B. Teplow, and D.J. Selkoe, Oligomerization of Endogenous and Synthetic Amyloid b-Protein at Nanomolar Levels in Cell Culture and Stabilization of Monomer by Congo Red. *Biochemistry* 37 (1998) 3602-3611.
- [22] D.J. Selkoe, Alzheimer's Disease: Genes, Proteins, and Therapy. *Physiological Reviews* 81 (2001) 741-766.
- [23] J.P. Cleary, D.M. Walsh, J.J. Hofmeister, G.M. Shankar, M.A. Kuskowski, D.J. Selkoe, and K.H. Ashe, Natural oligomers of the amyloid-b protein specifically disrupt cognitive function. *Nat Neurosci* 8 (2005) 79-84.
- [24] E. Hellstrand, B. Boland, D.M. Walsh, and S. Linse, Amyloid b-Protein Aggregation Produces Highly Reproducible Kinetic Data and Occurs by a Two-Phase Process. *ACS Chemical Neuroscience* 1 (2009) 13-18.
- [25] I.W. Hamley, The Amyloid Beta Peptide: A Chemist's Perspective. Role in Alzheimer's and Fibrillization. *Chemical Reviews* 112 (2012) 5147-5192.
- [26] A.A. Golabek, C. Soto, T. Vogel, and T. Wisniewski, The Interaction between Apolipoprotein E and Alzheimers Amyloid b-Peptide Is Dependent on b-Peptide Conformation. *Journal of Biological Chemistry* 271 (1996) 10602-10606.
- [27] J.-P. Guo, T. Arai, J. Miklossy, and P.L. McGeer, Ab and tau form soluble complexes that may promote self aggregation of both into the insoluble forms observed in Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America* 103 (2006) 1953-1958.

- [28] J. Lauren, D.A. Gimbel, H.B. Nygaard, J.W. Gilbert, and S.M. Strittmatter, Cellular prion protein mediates impairment of synaptic plasticity by amyloid-[bgr] oligomers. *Nature* 457 (2009) 1128-1132.
- [29] C.J. Dunning, G. McGauran, K. Willén, G.K. Gouras, D.J. O'Connell, and S. Linse, Direct High Affinity Interaction between Ab42 and GSK3 $\alpha$  Stimulates Hyperphosphorylation of Tau. A New Molecular Link in Alzheimer's Disease? *ACS Chemical Neuroscience* 7 (2016) 161-170.
- [30] A. Rauk, Why is the amyloid beta peptide of Alzheimer's disease neurotoxic? *Dalton Transactions* (2008) 1273-1282.
- [31] J. Zheng, H. Jang, B. Ma, C.-J. Tsai, and R. Nussinov, Modeling the Alzheimer Ab<sub>17-42</sub> Fibril Architecture: Tight Intermolecular Sheet-Sheet Association and Intramolecular Hydrated Cavities. *Biophysical Journal* 93 (2007) 3046-3057.
- [32] I. Canobbio, S. Catricalà, C. Balduini, and M. Torti, Calmodulin regulates the non-amyloidogenic metabolism of amyloid precursor protein in platelets. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1813 (2011) 500-506.
- [33] S.T. DeKosky, and S.W. Scheff, Synapse loss in frontal cortex biopsies in Alzheimer's disease: Correlation with cognitive severity. *Annals of Neurology* 27 (1990) 457-464.

### Abbreviations

The abbreviations used are: A $\beta$ , amyloid  $\beta$  peptide; APP, amyloid  $\beta$  precursor protein; AD, Alzheimer's Disease; CaM, apo-CaM, calcium-free calmodulin; a.u., arbitrary units; BACE1, beta-site A $\beta$ PP cleaving enzyme 1; Badan, 6-bromoacetyl-2-dimethylaminonaphthalene; CaM, calmodulin; Ca<sup>2+</sup>/CaM, calcium-saturated calmodulin; EGTA, ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ThT, Thioflavin T.



### Figure legends

**FIGURE 1. Calmodulin-A $\beta$  interaction in the presence of calcium.** **A)** Sequential fluorescence emission spectra of 10 nM BadanCaM(T110C) were acquired as indicated in the Materials and Methods in the absence (bold trace) and presence of indicated concentrations of A $\beta$ 1-42 (thin line traces from top to bottom). Insert: Non-linear regression analysis of fluorescence quenching (excitation and emission wavelengths: 385 and 487nm, respectively) fit to *one binding site* equation ( $R^2 = 0.992$ ). **B)** Formation of A $\beta$ 1-42 (A $\beta$ ) fibrils, after incubation of 10  $\mu$ g A $\beta$ /ml during 72h at 37 °C (A $\beta$  72h), were monitored by the increase of ThT fluorescence with respect to non-incubated A $\beta$  (A $\beta$ ), as indicated in the Materials and Methods, in the absence and presence of CaM at a molar ratio 1:1 (\*  $p < 0.05$ ). **C)** Sequential fluorescence emission spectra of 10 nM BadanCaM(T110C) were acquired as indicated in the Materials and Methods in the absence (bold trace) and presence (thin line traces from top to bottom) of indicated concentrations of A $\beta$ 25-35. Insert: Non-linear regression analysis of fluorescence quenching (excitation and emission wavelengths: 385 and 487nm, respectively) fit to *one binding site* equation ( $R^2 = 0.994$ ).

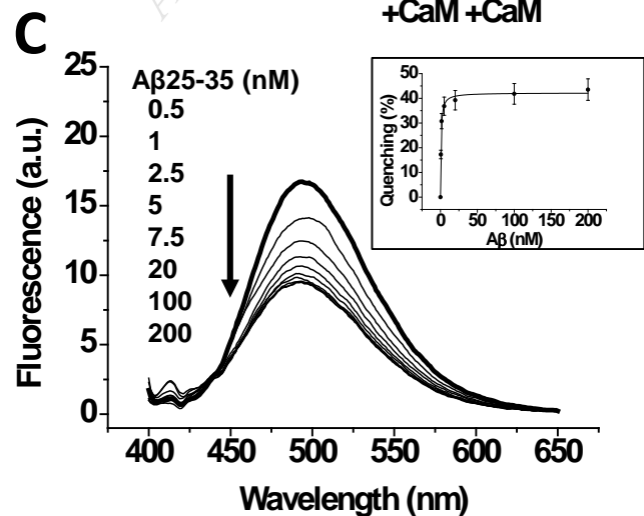
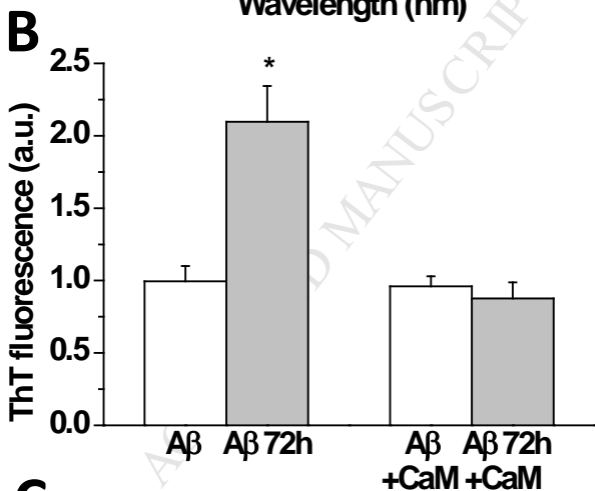
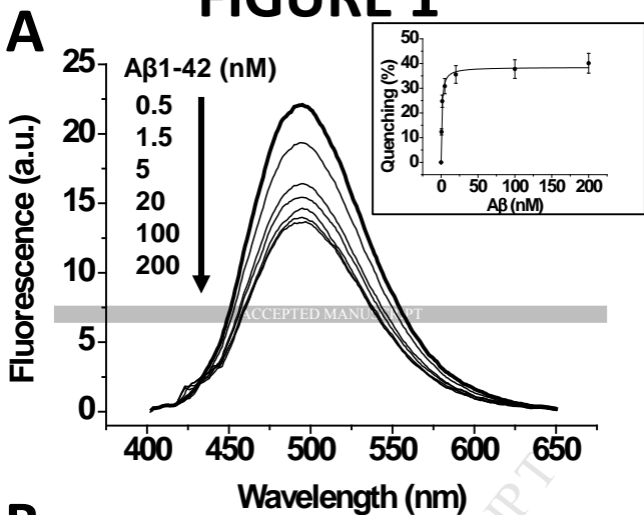
**FIGURE 2. Effect of A $\beta$  on calcium binding to calmodulin.** **A)** Calcium dependence of the fluorescence of BadanCaM(T110C) in the absence or presence of A $\beta$ 1-42 and A $\beta$ 25-35. ■, BadanCaM(T110C) ( $R^2 = 0.990$ ); ●, +A $\beta$ 1-42 ( $R^2 = 0.996$ ); ▲, +A $\beta$ 25-35) ( $R^2 = 0.993$ ). Excitation and emission wavelengths: 385 and 487nm, respectively. **B)** Free calcium measurements using the calcium indicator Fura-2 before and after addition of 2  $\mu$ M of CaM (▲), A $\beta$ 1-42 (■), or CaM+A $\beta$ 1-42 (●).

**FIGURE 3. Calcium modulates calmodulin-A $\beta$  interaction.** **A)** Quenching of 10 nM BadanCaM(T110C) fluorescence upon titration with HiLyte<sup>TM</sup>-Fluor555-A $\beta$ 1-42 (A $\beta^*$ ). Sequential fluorescence emission spectra of 10 nM BadanCaM(T110C) were acquired in the presence of 50 $\mu$ M calcium buffer as indicated in the Materials and Methods in the absence (bold trace) and presence of

indicated concentrations of HiLyte<sup>TM</sup>-Fluor555-A $\beta$ 1-42 (thin line traces from top to bottom). **B)** The non-linear regression analysis of A $\beta$ \* quenching titration data (excitation and emission wavelengths: 385 and 487nm, respectively) fit to a *one binding site* equation ( $R^2 = 0.991$ ). **C)** Enhancement of the fluorescence of 10 nM HiLyte<sup>TM</sup>-Fluor555-A $\beta$ 1-42 upon titration with unlabeled CaM in “low calcium buffer”, see Materials and Methods. Bold trace: fluorescence spectra of 10 nM HiLyte<sup>TM</sup>-Fluor555-A $\beta$ 1-42 in the absence of CaM. **D)** The non-linear regression analysis of the increase of HiLyte<sup>TM</sup>-Fluor555-A $\beta$ 1-42 fluorescence induced by CaM (excitation and emission wavelengths: 525 and 573nm, respectively) fit to a *one binding site* equation ( $R^2 = 0.997$ ).

**FIGURE 4. Potential CaM binding sites for A $\beta$ 25-35 given by selected PDB-model outcomes of simulations performed with the CABS Dock web server.** Putative binding site of A $\beta$ 25-35 (light red) to CaM (light blue) in the conformations adopted in the presence (A, Ca<sup>2+</sup>/CaM) and in the absence (B, apo-CaM) of calcium. The approximate positions of Badan in the fluorescent derivatives of CaM used in this work are highlighted as circles and labeled as T34C and T110C.

# FIGURE 1



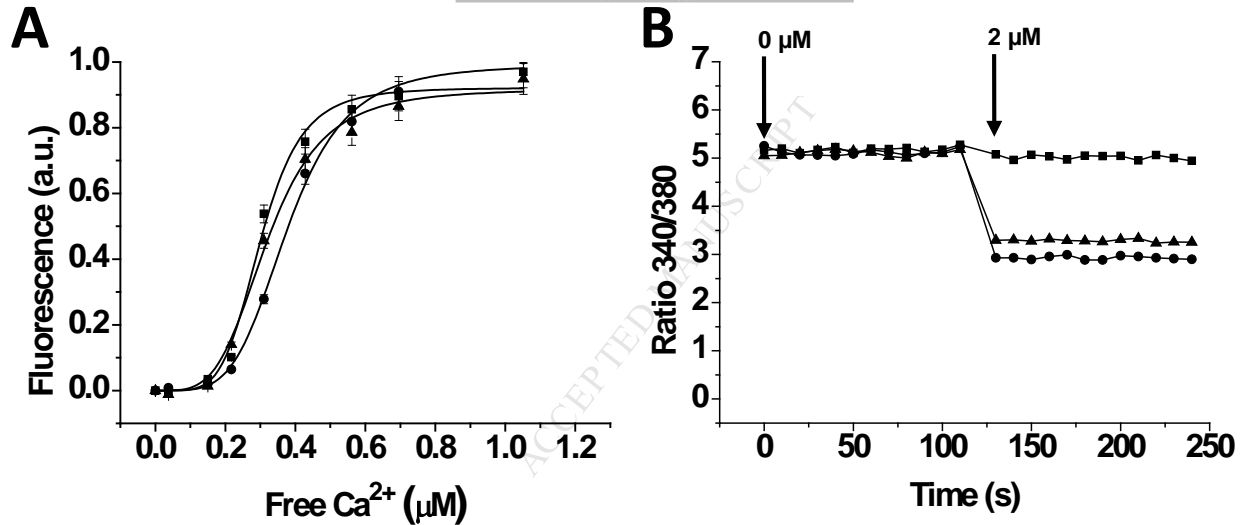
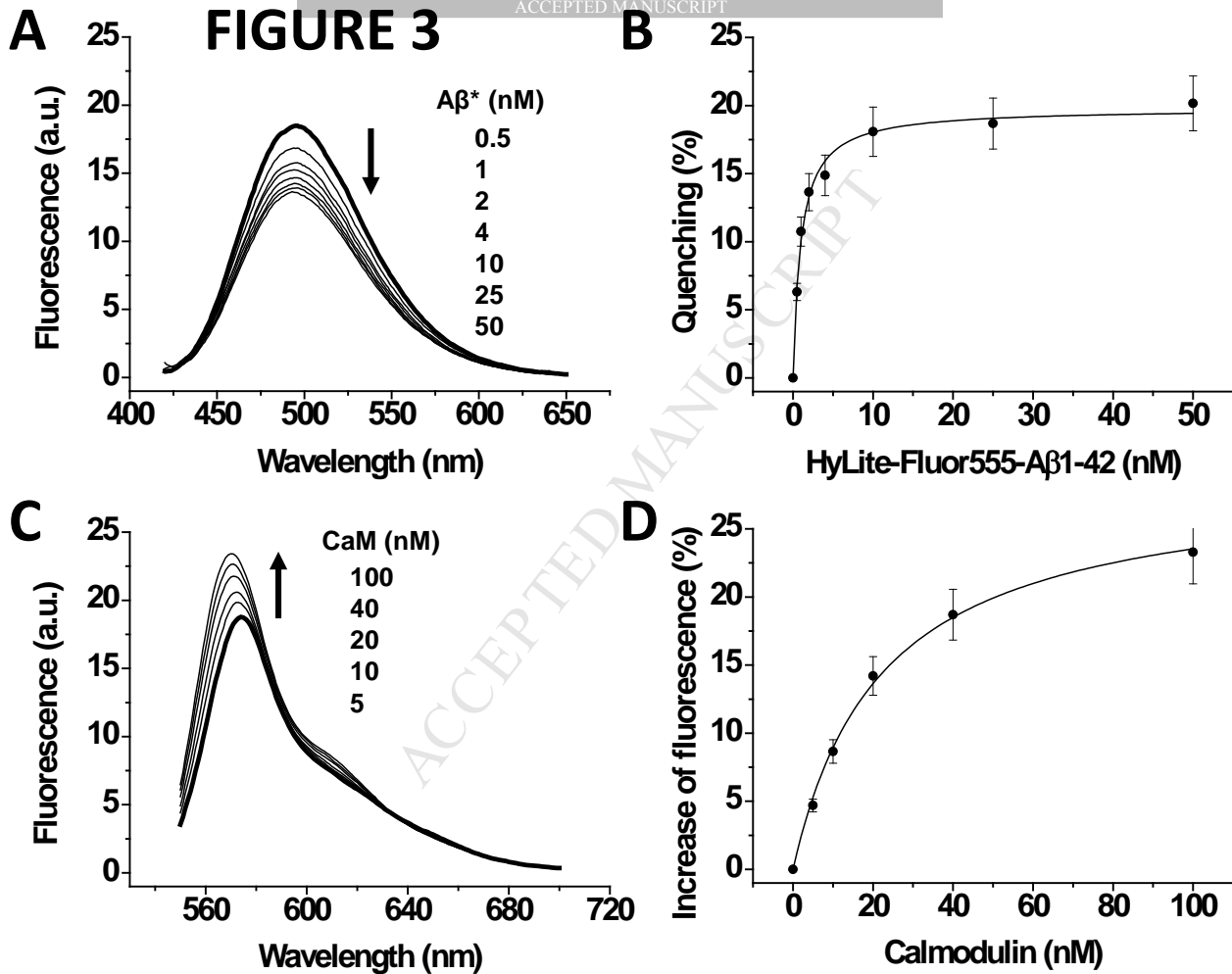
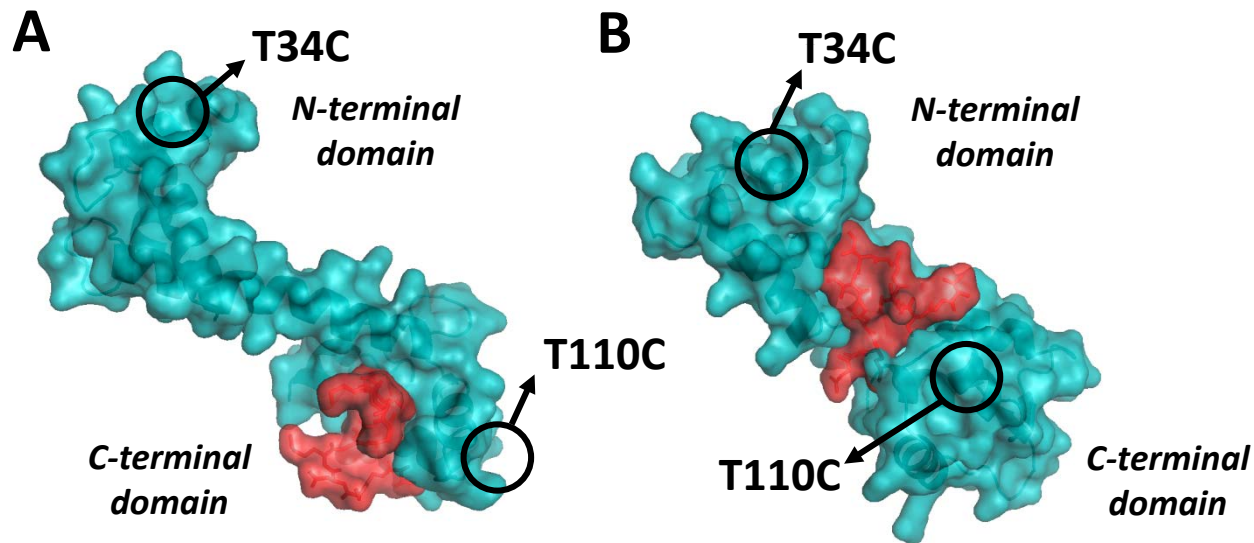


FIGURE 2



**FIGURE 4**

## Highlights:

- $A\beta$  binds with high affinity to calmodulin through the  $A\beta_{25-35}$  domain.
- $A\beta$  has higher affinity for  $Ca^{2+}$ -saturated calmodulin.
- Calmodulin is one of the cellular targets with highest affinity for  $A\beta$ .
- $A\beta$ -Calmodulin interaction slows down  $A\beta$  fibrillation.