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Integration of different pathways in early stage of Alzheimer's disease pathogenesis, from actin remodelling to Aβ formation

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TUTOR: Chiar.ma Prof.ssa Monica Di Luca

COORDINATORE: Chiar.mo Prof. Alberto Corsini

Silvia Cecilia Pelucchi

Matricola: R10639

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ABSTRACT (English):
Alzheimer disease (AD) pathogenesis is the result of an interplay of crossing pathways, as amyloid cascade and synaptic failure. It has been shown that Aβ is liberated from the amyloid precursor protein (APP) by BACE and γ-secretase activity. Alternatively, APP is cleaved within Aβ domain by ADAM10, which prevents Aβ formation. ADAM10 is a synaptic protein and works as sheddase towards several neuronal cell adhesion molecules. Therefore, ADAM10 activity can regulate not only Aβ generation, but also the synaptic morphology and the degree of functional synaptic connectivity. ADAM10 represents a potential pharmacological target for AD because the upregulation of its activity limits Aβ formation and affects synaptic function. Since ADAM10 synaptic localization/activity is controlled by the interactions with different protein partners, we aimed at identifying novel protein partners of ADAM10. To this, we performed a yeast two-hybrid screening, using ADAM10 C-terminal tail as a bait. We took advantage of several biochemical and imaging technique to analyze the role of such interaction.
The results revealed the cyclase-associated protein 2 (CAP2) as a new ADAM10 binding partner. CAP2 is a regulator of actin dynamics and, thereby, of spines morphology, and could be involved in the modulation of ADAM10 synaptic localization/activity. Here we confirmed ADAM10/CAP2 interaction and we identified the domains responsible for the association. Moreover, we defined the region of CAP2 involved in actin binding and we analyzed the effect of such domain on ADAM10 synaptic localization.
CAP2 can represent the crossing point between different aspects of AD pathogenesis, such as the amyloid cascade and actin-dependent spines shaping, thus taking part in a new cellular mechanism underlying synaptic dysfunction in AD.

ABSTRACT (Italian):
La malattia di Alzheimer (AD) è una malattia neurodegenerativa caratterizzata da un aumento di livelli di beta-amiloide (Aβ) e dalla sua deposizione in placche senili. In particolare, la forma solubile di Aβ svolge un ruolo importante nelle fasi iniziali di AD, portando a perdita delle sinapsi e conseguenti deficit cognitivi. Aβ deriva dalla proteina precursore (APP), che può essere sequenzialmente tagliata dalle proteasi BACE1 e γ-secretasi per produrre Aβ. Tuttavia, con un meccanismo mutualmente esclusivo rispetto al precedente, APP può essere processato da α-secretasi (ADAM10) e γ-secretasi; ADAM10 taglia APP all'interno del dominio Aβ, evitando così la generazione di Aβ. Inoltre, nei neuroni, ADAM10 è responsabile del taglio proteolitico di diverse molecole di adesione, come neuroligin-1, N-caderina, NCAM e Ephrin. Pertanto, in un quadro più ampio, ADAM10 ha un importante ruolo nel controllo della morfologia delle spine dendritiche e nella plasticità attività-dipendente.
Dato il ruolo chiave svolto da ADAM10 nella cascata dell'amiloide e nel controllo della morfologia delle spine dendritiche, ADAM10 può rappresentare un potenziale bersaglio farmacologico per prevenire la degenerazione sinaptica delle prime fasi della patologia di AD. A riguardo, è importante sapere che ADAM10 è in grado di agire sui suoi substrati solo quando è inserito correttamente nella membrana plasmatica. Di conseguenza il trasporto intracellulare di ADAM10 è di rilevante importanza per regolare l'attività dell'enzima stesso.
Alla luce di queste considerazioni, per identificare possibili proteine partner in grado di regolarne la localizzazione, abbiamo eseguito un yeast two-hybrid screening utilizzando la coda C-terminale di ADAM10 come esca. I risultati hanno rivelato CAP2 come nuovo partner di ADAM10.
CAP2 è una proteina poco descritta in letteratura, che svolge un ruolo chiave nella regolazione del citoscheletro di actina e nella trasduzione del segnale. Alla luce delle caratteristiche di CAP2, il nostro lavoro è stato volto allo studio e caratterizzazione di come questa proteina possa influenzare la localizzazione intracellulare di ADAM10 e di conseguenza la sua attività, agendo quindi indirettamente non solo sulla produzione di Aβ, ma anche, in generale, sul fisiologico rimodellamento delle spine dendritiche.

1. INTRODUCTION

1.1 DENDRITIC SPINES: THE LOCUS OF ALZHEIMER'S DISEASE

1.1.1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive and neurodegenerative disorder characterized by increased levels of amyloid β-peptides (Aβ) and their deposition in senile plaques and by the formation of the intracellular Neurofibrillary Tangles (NFTs), constituted of hyperphosphorylated Tau protein. ADAM10 prevents Aβ generation because it cleaves the Amyloid Precursor Protein (APP) within the Aβ domain. Aβ plays a central role in AD pathogenesis. Indeed, the "amyloid hypothesis" was first proposed from research conducted in the middle of the 1980s showing that senile plaques found in AD brain tissue were composed mainly of a sticky Aβ peptide[1]. This hypothesis was formalised by Hardy and Higgins (1992) who stated that Aβ "precipitates to form amyloid and, in turn, causes NFTs and cell death"[2]. Up to now, most investigators believe that the production and cerebral deposition of amyloid plaques composed of the 38 to 42 aa Aβ peptide is central to the development of AD[3]. According to the amyloid hypothesis, deposition and accumulation of Aβ in the brain is the primary factor driving AD pathogenesis[4]. In animal models Aβ deposition has also been observed prior to the tangle pathology[5]. Therefore, the basic biochemical formula for Aβ production was investigated in minute details to determine the aetiology of the disease.

The cloning of the gene encoding APP and its localization to chromosome 21[6,7], coupled with the earlier recognition that trisomy 21 (Downs syndrome) leads invariably to the neuropathology of AD[8], set the stage for the proposal that Aβ accumulation is the primary event in AD pathogenesis. In addition, the identification of mutations in the APP gene that cause hereditary cerebral haemorrhage with amyloidosis (Dutch type) showed that APP mutations could cause Aβ deposition, albeit largely outside the brain parenchyma[9]. Soon, the first genetic mutations causing AD were discovered in the APP gene[2,10 11]. The contemporaneous discovery that Aβ was a normal product of APP metabolism throughout life and could be measured in culture medium, cerebrospinal fluid, and plasma[12-14] allowed scientists to quickly establish the biochemical abnormalities caused by APP mutations. The majority of the mutations cluster at or very near the sites within APP that are normally cleaved by secretases. In accordance with

this, these mutations promote generation of Aβ by favouring proteolytic processing of APP by β or γ secretase or increase the relative production of Aβ42 compared to Aβ40 [15]. Furthermore, APP mutations internal to the Aβ sequence heighten the self-aggregation of Aβ into amyloid fibrils[16]. These exciting developments provided the genetic framework for the emerging amyloid hypothesis[2,4]. In the past years, bolstered particularly by the cloning of the γ-secretase components named Presenilins (PSs) [17,18] and the demonstration that AD-causing mutations in PS1 and PS2 also enhance the processing of APP to form Aβ, the amyloid hypothesis has become the focus of AD research. In addition to the cloning of PS1 and PS2 and the discovery that they alter APP metabolism[19-21] through a direct effect on the γ secretase protease[22,23], there have been four conceptually important observations that strongly support the amyloid hypothesis. First, mutations in the gene encoding the tau protein, the main component of NFTs, cause frontotemporal dementia with parkinsonism[24-26]. This neurodegenerative disorder is characterized by severe deposition of tau in NFTs in the brain, but no deposition of amyloid. The clear implication is that even the most severe consequences of tau alteration - profound NFTs formation leading to fatal neurodegeneration - are not sufficient to induce the amyloid plaques characteristic of AD. Thus, the NFTs of wild-type tau seen in AD brains are likely to have been deposited after changes in Aβ metabolism and initial plaque formation, rather than before[4]. Second, transgenic mice overexpressing both mutant human APP and mutant human tau undergo increased formation of tau-positive tangles (as compared with mice overexpressing tau alone), whereas the structure and number of their amyloid plaques are essentially unaltered[27]. This finding suggests that altered APP processing occurs before tau alterations in the pathogenic cascade of AD, a notion bolstered by the recent observation that in mouse hippocampal primary neuronal cultures, Aβ toxicity is tau dependent[28]. Third, crossing APP transgenic mice with ApoE–deficient mice markedly reduced cerebral Aβ deposition in the offspring[29], providing strong evidence that the pathogenic role of genetic variability at the human ApoE locus [30] is very likely to involve Aβ metabolism. And fourth, growing evidence indicates that genetic variability in Aβ catabolism and clearance may contribute to the risk of late-onset AD [31-34]. Taken together, these findings are consistent with the notion that cerebral Aβ accumulation is the primary mechanism of AD pathogenesis and that the rest of the disease process, including tau tangle formation, results from an imbalance between Aβ production and Aβ clearance.

As the above-described amyloid cascade hypothesis of AD, as initially formulated, proposed that the hallmark progressive deposition of insoluble fibrillar Aβ in plaques triggered neurodegeneration which, in turn, caused the insidious escalation of debilitating symptoms, including progression through the different stages of clinical dementia. Support for this proposal came from the discovery that application of fibril containing Aβ to cultured neurons was highly toxic in vitro and that intracerebral injection of fibril-containing Aβ caused a neurodegeneration-associated disruption of performance of cognitive tasks in animals [35,36]. However, the relatively poor correlation between the severity of clinical dementia at the time of death of patients with AD and either the magnitude of fibrillar Aβ load or the extent of neuron loss in the brain provided a major challenge for the original amyloid cascade hypothesis[37]. In fact, many studies demonstrated that the best statistical correlation occurs between measures of synaptic density and degree of dementia[38]. Data obtained by electron microscopy[39], immunocytochemical and biochemical analyses on synaptic marker proteins in AD biopsies and autopsies indicate that synaptic loss in the hippocampus and neocortex is an early event[40] and the major structural correlate to cognitive dysfunction[38,41]. Not NFTs, senile plaques, nor even neuronal loss show such a strong statistical correlation with dementia [42,43]. Moreover, the decrease in synapse number and density seems disproportionate to the loss of neuronal cell bodies[38,44], suggesting that pruning of synaptic endings may precede the demise of the neuron in the disease process. Furthermore, some changes in the brains of AD patients and APP transgenic mice suggest that synaptic function is compromised prior to the physical deterioration of neuronal structures[45-47]. This evidence, coupled with the fact that large fibrillar plaques present much less Aβ surface area to neuronal membranes than do a multitude of small oligomers that can diffuse into synaptic clefts, indicates that such soluble assembly forms are better candidates for inducing neuronal and/or synaptic dysfunction than plaques, per se. Indeed, human Aβ can exist in diverse assembly states, including monomers, dimers, trimers, tetramers, dodecamers, higher-order oligomers and protofibrils, as well as mature fibrils, which can form microscopically visible amyloid plaques in brain tissues[48]. Therefore, a new understanding of the amyloid cascade hypothesis proposes an alternative mechanism for memory loss based on the impact of small, soluble Aβ oligomers[4]. Indeed, different soluble molecular species that are generated at very early stages of the disease could be involved in synaptic failure and only at more advanced stages they are deposited in an aggregated form. It has, thus,

been suggested that soluble assembly states of Aβ peptides can cause cognitive problems by disrupting synaptic function in the absence of significant neurodegeneration. Therefore, current research investigates the relative importance of these various soluble Aβ assemblies in causing synaptic dysfunction and cognitive deficits.

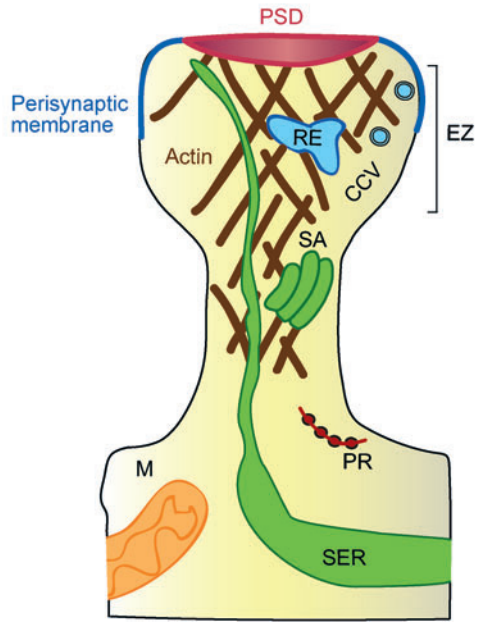
1.1.2 WHAT IS A DENDRITIC SPINE?

The synapse is the specialized junction that allows the communication between the neurons in the mammalian brain. In particular, the synapses that use glutamate as neurotransmitter are defined as excitatory synapses, and they are localized where the axon of a neuron contacts the dendrites of another neuronal cell, which receive synaptic inputs.

Most of the excitatory synapses are generally formed at the head of a protrusion, called dendritic spine, that is the structure specialized for synaptic transmission. Spines are typically small (less than 3 μm in length, from the dendritic attachment to the tip of the head), with a head (0.5–1.5 μm diameter) connected by a narrow neck (<0.5 mm diameter and ~1 μm long) to the dendritic shaft[49] . The geometry of the spines is highly variable from spine to spine. According the classification of Peters and Kaiserman-Abramof the spines can be categorized into three essential types: thin, mushroom and stubby spines. Thin spines are the most common and have a thin, long neck and a small bulbous head. Mushroom spines are those with a large head and are typically found in adult brains. Stubby spines are devoid of a neck[50,51] , and are particularly prominent during early postnatal development[52] , although they are still found in the adult[53]. In addition, dendritic filopodia have been observed and are longer that mature dentritic spines and normally they haven't a clear head[49]. Most of the excitatory synapses occur on the spine heads where it is possible to detect an electron-dense disc-like structure, named PSD (Post Synaptic Density),. The PSD is directly opposed to the presynaptic side. The presence of a prominent PSD, that can be revealed by Electron Microscopy, is a characteristic of the asymmetric synapses, that are typically glutamatergic; in contrast, the inhibitory and symmetric synapses occur mostly on dendritic shafts.

It has been demonstrated, over the years, that the neuronal activity regulates the morphology of the spines and, through this modification, controls the synaptic transmission and plasticity. Notably, the morphological features of the spines are correlated with the synaptic structure: the head volume (and also the total spine volume)

is correlated with the PSD area. The PSD is composed of multiple proteins that bind each other through specific domain-domain interactions, forming a mesh-like structure organized in consecutive layers. The shape and the size of the spines are dependent on the developmental stages and on the strength of the synapses[54]. The most relevant feature of this process is the presence in the young brain of the dendritic filopodia. Indeed, in the young brain there are many filopodia that partially disappear in the adult brain. Although the function of the filopodia is still unknown, it is has been shown that it is a very plastic structure that plays a role in the formation of connection among neurons, even if it is no clear yet if the spines derive directly from filopodia. Probably these structures mediate the process of spines formation even if they are just a transient structure. Likewise, the conversion of filopodia to spines is not likely to occur in mature neurons, when filopodia are rare and spines can form within minutes to hours, as shown in cycling female rats, during the recovery from hibernation as well as in living adult mice[55] (Fig. 1).



M. Sheng and C. C. Hoogenraad , Annu. Rev. Biochem-2007

Figure 1. Dendritic spine structure

1.1.3 SPINES COMPOSITION: THE ORGANELLES

The soma may not be able to provide enough proteins for the entire cell, and this may be due to "structural" limits of the biosynthetic capability of the soma. For this reason, the dendrites contain almost all of the cellular machinery necessary to synthesize proteins. Moreover, the transport of mRNA designated to the formation of synaptic proteins, and their localization and translation, is fundamental for the synaptic

composition[56]. The polyribosomes are found in the spines, therefore the spines must be able to synthesize proteins, even if it is known that the protein synthesis occurs mainly in the dendrite shafts[57]. In particular, since the production of many proteins is strictly activity dependent, the protein synthesis is differentially regulated in the spines depending on the association of the ribosome with the spines. Several spines contain the SER (Smooth Endoplasmic Reticulum), that is a continuous with the RER (Rough Endoplasmic Reticulum) of the dendritic tree and that can be extended through the neck to the head, sometimes even continuously[49]. The SER is involved in the intracellular trafficking and in the regulation of intracellular Ca^{2+} stores. The SER, especially in the hippocampus, is formed by specific vesicles and tubules, thus creating the "spine apparatus", a stack of SER cisternae and dense plates between them[49]. Larger spines usually contain the "spine apparatus" and the amount of cisternae is correlated with the size of the PSD[58].

Protein markers of Golgi membranes, including α-mannosidase II, giantin, and Rab6, have been found in the dendrites of some neurons[58-60]. The dendritic localization of these organelles suggests that "satellite" protein secretion can occur at sites far from the nucleus. What controls the flow of cargo that passes through several dendritic branch points to distal ends of dendrites? Golgi outposts situated at dendritic intersections engage in ongoing post-Golgi trafficking[61] and are properly positioned to regulate the identity or the amount of cargo that is trafficked to each branch. Despite the presence of functional Golgi outposts in some hippocampal dendrites, not all dendrites possess detectable Golgi. Moreover, even in those dendrites containing Golgi outposts, most ER-to-Golgi carriers originating in the dendrite are trafficked all the way back to the somatic Golgi[62]. Thus, dual modes of early secretory trafficking exist in dendrites. In fact, the major mode of ER-to-Golgi trafficking is directed long distances to the Golgi apparatus in the soma. This appears to be the exclusive mode of early secretory trafficking in those dendrites lacking Golgi outposts.

Also the mitochondria are present in the dendritic shafts, but not in the spines, and a recent study demonstrated that mitochondria mobility in dendrites is controlled by synaptic activity. Synaptic stimulation decreased mitochondrial mobility and increased the association of mitochondria with dendritic spines[63]. The high compartmentalization of dendrites properties leads to the correct spatial segregation and integration of the different pathways required for the structure of the synapse[62].

1.1.4 SPINES COMPOSITION: FROM RECEPTORS TO SCAFFOLDING PROTEINS

Hundreds of molecules have been described in the spines, and these are involved in many biochemical pathways. This molecular complexity is important to make functional the synaptic machinery and to create synapses independent from other synapses and from the rest of the neuron[49] (Yuste, 2010).

Glutamate receptors

Glutamate is the most important excitatory neurotransmitter in the brain. It is released from vesicles from pre-synaptic sites and interacts with its receptors on the post-synaptic site. The glutamate receptors are: ionotropic receptors (iGluR) and G-protein coupled receptors (mGluR). The first type of receptor give rise to fast postsynaptic response, while the metabotropic receptor produces slower postsynaptic effects. Several types of iGluRs have been identified: AMPARs (receptors activated by α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), NMDARs (receptors activated by N-methyl-D-aspartate), and kainate receptors (receptors activated by kainic acid)[64].
NMDARs are heteromeric assembly of different subunits that form a non-selective cation channel and allow the entry of Ca^{2+} in addition to monovalent cations, as Na^{+} and K^{+}. NMDARs are activated only when presynaptic glutamate release coincides with sufficient post-synaptic depolarization. Extracellular Mg^{2+} exerts a voltage-dependent block of the opened ion channel[65] and so it is necessary the relieve of the Mg^{2+} block of the ion channel[66]. Moreover, not only the glutamate, but also the binding of glycine appears to be necessary for receptor activation[67]. At hyperpolarized membrane potentials, more negative than -70 mV, the concentration of Mg^{2+} in the extracellular fluid is sufficient to virtually abolish ion flux through NMDARs channels even in the presence of glutamate and glycine. A positive change in transmembrane potential will increase the probability of the exit of Mg^{2+}. NMDARs are composed of multiple subunits (GluN1, GluN2A-D, GluN3) that assemble in heteropentameric or heterotetrameric structures. These structures exhibit distinct properties depending on the subunit composition. The various isoforms of NR2 subunits contain the glutamate-binding site[68] while glycine-binding site appears to be located on the NR1 subunit. NR1 is essential for NMDAR function and eight receptor variants can be generated by alternative splicing[69]. The GluN2A subunit confers a lower affinity for glutamate, distinct faster kinetics, greater channel open probability and more prominent Ca^{2+}-dependent desensitization compared to GluN2B. The GluN2D and GluN2C subunits are

characterized by low conductance openings and reduced sensitivity to Mg^{2+} block. NR3-containing receptors are more predominant in a narrow time window during development. The GluN3 containing receptors are considered a distinct class of nonconventional NMDARs since GluN3 shows limited sequence homology to GluN1 and GluN2 and since it confers unique properties to the channel. NMDARs have been shown to contain consensus sequences for phosphorylation by protein kinases[70]. CaMKII (Calcium/calmodulin-dependent protein kinase II) can associate to GluN1, GluN2A and GluN2B which can be phosphorylated also by other kinases as PKC (Protein Kinase C) or PKA (Protein Kinase A). In the brain, between 10 and 70% of NR1 and NR2 subunits seem to be phosphorylated by PKA or PKC[71] thus increasing the heterogeneity of NMDARs.

AMPARs are heterotetramers composed of the subunits GluA1-4 [72]. They are activated by glutamate and antagonized by 6-ciano-7-nitroquinoxaline-2,3-dione (CNQX) and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX). These receptors are the major mediator of fast glutamatergic excitatory synaptic transmission in the central nervous system (CNS). AMPARs can assume different role depending on their composition: AMPARs containing GluA2 subunits show lower permeability to Ca^{2+}, while receptors containing GluA1, GluA3 and GluA4 are permeable to Ca^{2+} [73]. The differences among the subunits are due to an alternative splicing[74]. All the subunits of AMPARs have phosphorylation sites on their C-terminal tail that regulate their function and that seem to be involved in the regulation of synaptic plasticity. GluA1 is one of the most abundantly expressed subunit of AMPARs in hippocampal and neocortical neurons[75]. Most of the phosphorylation on GluR1 occurs on serine and threonine[76], and tyrosine residues[77]. Ser831 and Ser845 are involved in the expression of LTP and LTD[78]. PKC and CaMKII mediate the phosphorylation on GluA1-Ser831[79], while GluA1-Ser845 is phosphorylated by protein kinase A (PKA). This phosphorylation can enhance currents through AMPARs[80]. GluR2 is a key subunit which renders AMPARs channel impermeable to Ca2+ and confers specific biophysical properties[81]. There are several serine phosphorylation sites (Ser863 and Ser880) on the C-terminal tail of GluA2. The kinase that act on the GluA2 is the PKC[82]. GRIP family stabilize AMPARs at the synaptic side (Dong et al., 1997) and as intracellular pools[83]. The phosphorylation of Ser880 give the AMPARs the capability to bind PICK-1 (Protein Interacting with C Kinase-1), that either promotes the endocytosis of the receptor[84] or allows its trafficking to the plasma membrane[83]. GluA3 and GluA4 are not highly expressed in the adult forebrain

structures compared to GluA1 and GluA2 subunits[85]. Expression of GluA4 in the hippocampus is limited during early post-natal period development and GluA4 can be inserted into synapses by spontaneous activity[86].

Scaffolding proteins: the MAGUK family

The Membrane Associated Guanylate Kinase (MAGUK) family is a super family of multi-domain proteins characterised by the presence of a shared set of structural domains. The MAGUK family includes the post-synaptic density 95 (PSD-95), chapsyn-110, synapse-associated protein 102 (SAP-102), and SAP-97. They are key protein in governing the localisation of the glutamate receptors at synapse and their function. According to their function, these proteins are present in the PSD, since the synaptogenesis (Boeckers et al., 1999). Their multi-domain structure is fundamental for their correct localisation and activity. They contain three PDZ (PSD-95/DLG/ZO1) domains, a Src homology 3 (SH3) region and a Guanylate Kinase (GK)-like sequence. They are all localized in the CNS[87], but each member is distributed differently in brain cell compartments. PSD-95 and PSD-93 are highly enriched in the PSD [88], where they interact with the cytoplasmic tail of NMDA receptor subunits, while SAP97 and SAP102 have been found in dendrites and axons and are abundant in the cytoplasm as well as at synapses and are involved in the trafficking of NMDARs and AMPARs respectively, SAP102 can associate with the exocytosis and regulate the delivery of NMDARs to the surface of the plasma membrane. Since these properties are related to the capability of MAGUK proteins to interact with other PSD components, it is important to understand the structural characteristics of their domains. PDZ domain scaffolds have been shown by genetic, electrophysiological and morphological studies to be essential for controlling the structure, strength and plasticity of synapses. SH3 modules regulates protein–protein interactions. SH3 ligands are composed of seven residues and contain PXXP sequences. The GK module, typical domain of the enzyme that catalyses the conversion of GMP to GDP, mediates protein–protein interactions independently of its predicted enzymatic activity[89]. Indeed, the MAGUK proteins are predicted to bind neither GMP nor ATP and are, therefore, assumed to be enzymatically inactive.

Adhesion molecules

The adhesion molecules are important for the formation and the development of the synapses, since they are involved in the formation of the dendritic spines[55]. Indeed,

they mediate the connection between an axon and a dendrite. However, they are expressed at mature synapse regulating plasticity and consequently contributing to a variety of cognitive functions, including learning and memory. For that reason, they are involved in the continual synapse remodelling. CAMs affect spine structural changes by influencing the actin cytoskeleton through molecular links or by engaging singling cascades. They can link in a stable way the presynaptic side to the postsynaptic side, giving the structure and stability to the synapse. They can nevertheless modulate the remodelling through their proteolytic cleavage disrupting the connection with the presynaptic side. Moreover, the CAM cleavage can also affect a variety of neurotransmitter receptor-CAM interactions to strongly influence the degree of functional synaptic connectivity. Different adhesion molecules, among which N-cadherin, Ephrins, Integrin, the adhesion molecules of neuronal cells (NCAM, neural cell adhesion molecule), Densin-180 and Neuroligin-1[49], are localised in spines. The adhesion molecules are bound to the actin cytoskeleton. The N-cadherin interact with the actin filament thought β-catenin and α-catenin [90]. The Integrin[91] and Densin-180 [92] are bound to the alpha-actinin while the Neuroligin-1 is bound to the actin filaments through PSD-95. In particular, the CAM capability to link these additional associated proteins results in a larger multi-molecular complexes. For example, at the postsynaptic side, PSD-95, a MAGUK scaffold protein, binds to neuroligins, NMDA receptors, and also TARPs that in turn link to AMPA receptors.

The CAMs are classified in homophilic or heterofilic, the homophilic CAM link interact with a partners across the two side of the membrane belonging to the same family, while the homofililic interaction occurs between CAMs belonging to different families. For example, the cadherin superfamily members belong to the homophilic class [93]. Among them, N-cadherin, is expressed at both excitatory and inhibitory synapses during early development but later becomes preferentially enriched at excitatory synapses[94]. This could reflect distinct roles played by N-cadherin first in synapse formation and subsequently in mature excitatory synapse function. The N-cadherin, through a morphological change in its conformation, is necessary for the activity-dependent enlargement of the spine. The N-cadherin principal role is the maintenance of the dendritic spine. Indeed, N-cadherin can modulate the spine morphology regulating the availability of the glutamate receptors[95]. N-cadherin is important for the maintain of the synaptic levels of AMPARs, because the N terminal domain of the GluA2 subunits of AMPARs can interact with the synaptic N-cadherin [96,97].

1.2 THE SPINOSKELETON: ACTIN IN ACTION IN THE SPINES

Actin is one of the most enriched protein in the synapses. Half of it is present in its glomerular form (G-actin) and half of it in the filamentous part (F-actin). The actin cytoskeleton formation process in the spine is highly dynamic and strictly regulated. The G-actin polymerizes to F-actin using an ATP-dependent hydrolysis that allows the formation of the filamentous actin. The filamentous actin is a polarized structure. It is possible to recognise a fast growing site on that structure (the barbed end, the side where the monomers are added) and the side where the actin depolymerize (the pointed end)[98].

The the actin bound to the ADP (ADP-actin) has the same affinity for both sides of the actin filament, while the monomer of actin bound to the ATP (ATP-actin) can bind the barbed-end because of the hydrolysis of ATP to ADP.

The cycle of polymerization and depolymerization of actin is influenced by the G-actin concentration inside the cell. When G-actin concentration is higher than 0,1 μM, an increase in polymerization is observed until G-actin concentration goes back to 0,1μM, called “critical concentration”. In the physiological conditions the concentration of the G-actin is between 50-200 μM. This high actin concentration is permitted by the presence of actin binding proteins that maintains the “critical concentration”. Indeed, a lot of proteins are able to sequester and bind G-actin, such as profilin, timosin β 4, the WASp (Wiskott-Aldrich Syndrome proteins) and the CAPs (Cyclase Associated Proteins) [99-102]. Anyway, the majority of the ABP (Actin Binding Proteins) are proteins necessary for the regulation of the actin filaments, such as the capping protein, the tropomodulin, the gelsolin and Cap Z [103,104]. They bind the terminal part of F-actin (barbed-end) preventing the binding of the G-actin. Another class of F-actin binding proteins are responsible for the severing, since they break the actin filament creating new barbed ends for the polymerization of actin.

The spinoskeleton represents the cytoskeleton of the spine. The principal component of the spinoskeleton is the F-actin that occupies the total volume underneath the PSD in the spine[105]. The actin gives to the spine stability and structure, influencing the shape and motility. The actin can assume different shapes: (1) the branched actin, that is mainly present in the distal part of the spine, and (2) the linear part, that is more equally distributed[106]. Only 5% of the actin in the spines is stable; in particular, the actin

filaments in the head of the spine are highly dynamic, giving rise to a phenomena called actin treadmilling [107,108], that occurs when one end of an actin filament grows in length (at the barbed end) while at the other end (the pointed end) there is a constant removal of the actin subunits.

The spinoskeleton is composed of two main parts: the core and the shell. The shell is the actin present in the terminal part of the spine that is highly dynamic (turnover rate: 10 sec). The other part is the core that represents the actin localized in the central part of the spine, where the turnover is slower, around 10 minutes[105].

It is clear that the core represents the structural part of the spine and is more stable. While the dynamic capability of the actin in the shell part can allow the elongation of actin filaments and can permit the nucleation of the new filaments relevant for the structural growth of the spine [105].

1.2.1 ACTIN BINDING PROTEINS

The ABPs regulate the physiology of actin giving to the actin the characteristic dynamism and stability. The most important and investigated in dendritic spines are:

Arp2/3 (actin-related protein 3)

It's a complex made of different subunits, among which Arp2, Arp3, ARPC1, ARPC2, ARPC3, ARPC4 and ARPC5. The Arp2/3 is the principal actin filaments nucleator[109]. It can bind the filamentous actin to both sides and allows the insertion and creation of an additional filament [110]. It is enriched in the PSD and its downregulation results in an impairment in the spine head formation[98,111]. It is activated by several proteins, such as Cortactin, Abi2, WAVE-1 (WASp-family verprolin homology protein-1), N-WASP (neural Wiskott-Aldrich syndrome protein) and Abp1. The deletion of such proteins is related to memory deficits [112-115] .

Profilin I and Profilin II

This class of protein is fundamental for the actin polymerization. They are responsible for the ADP to ATP nucleotide exchange on actin. These proteins catalyse actin polymerization in a concentration-dependent manner, it serves as a catalyst at lower concentrations and as inhibitor at higher levels[116] . The profilin II is the principal isoform in the mammalian brain[109], even if profilin I is also expressed [117].

ADF/cofilin

These proteins can promote the actin turnover (Fig. 2). In particular, they can bind G-actin and F-actin in a stoichiometric ratio 1:1, leading to a depolymerisation of the actin filaments from the pointed-end and, thereby, promoting the depolymerisation, but they can also create new barbed-ends. The cofilin, which exist in two isoforms, cofilin I and cofilin II, belongs to a highly conserved protein family, as the ADF (or destrin)[118]. The role of this complex is fundamental for continuous treadmilling of actin. In fact, since the actin monomers are fundamental for a fast reorganisation of the actin cytoskeleton, the complex ADF/cofilin promotes the depolymerisation of actin and creates a new pool of G-actin monomers available for other filaments formation [119]. The result of this activity is the correct maintenance for the morphology of the spine[111]. Indeed, the LIMK1 activation (LIM kinase 1), that inhibits the ADF/cofilin activity, leads to an altered morphology of the spine function[120].

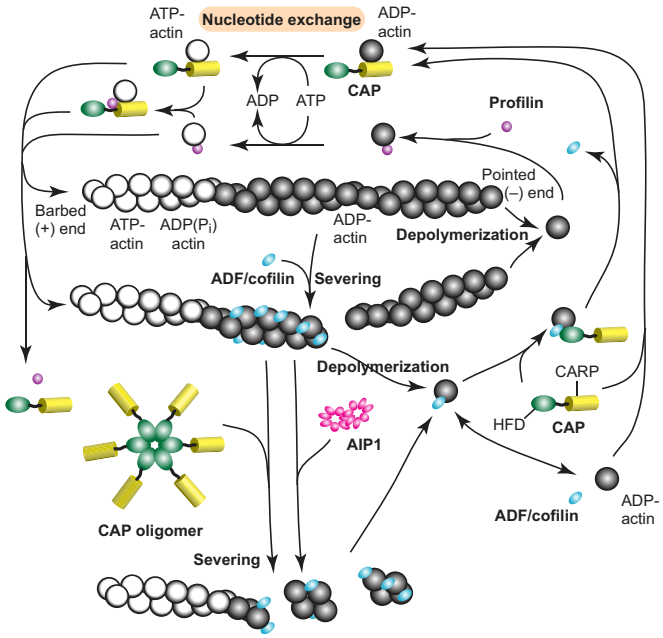


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Figure 2. Actin treadmilling

Eps8

Eps8 (epidermal growth factor receptor pathway substrate 8) is a capping protein. Capping proteins (CP) regulate actin polymerization by binding the barbed end of an actin filament, which blocks addition and loss of actin subunits[121]. The capping proteins are distributed in dendritic spine and their function is relevant to inhibit the filopodia

formation[122]. The actin-capping proteins control the organization of filopodia. They can cap the newly branched filaments created by the Arp2/3 complex, thus controlling the elongation of the filopodia and the concentration of the free G-actin monomers[123-125].

Myosin II and myosin IV

These proteins are complexes constituted by four light and two heavy chains and they regulate the actin filaments contraction[55]. The myosin II (in particular the isoform MyH10) is localized in the synapses and can regulate the morphology of the dendritic spines and the synaptic strength[55]. Also the isoform MyH7B can cooperate with MyH10 to control the correct morphology of the spine[109]. The myosin IV (the isoform Myo6) is involved in the internalisation of the AMPA receptor and the Myo6 KO shows a decrease in the number of synapses.

Drebrin A

Drebrin can bind the F-actin and promotes the polymerisation of actin[126] (Hayashi and Shirao, 1999). It is localized in postsynaptic terminals in adult brain and a decrease in drebrin levels has been shown in AD patients[127]. However, it is well known that is involved in spine formation in the early stage of the development regulating the F-actin polymerisation.

Rho family of GTPases

They are Ras protein. There are different components of the family, among which RhoA (Ras homolog gene family, member A), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (Cell division control protein 42 homolog). All the members of this family have been studied in neuronal cells since they are involved in the neuronal morphogenesis. In the dendritic spine the Rho activation is fundamental for the cofilin phosphorylation and therefore for the actin stabilisation of the spine[109]. While the Rac1 and Cdc42 activation leads to an enlargement of the head spine[111 115], promoting the formation of Arp2/3 complex.

1.2.2 CAP (Cyclase-Associated Protein)

CAP (Cyclase-Associated Protein), originally isolated from the Saccharomyces Cerevisiae, are components of the complex responsible for the Ras–cAMP signalling

activating the adenylate cyclase (AC) and are involved in the regulation of the cytoskeletal structure. Indeed, the yeast lacking of CAP is unable to grow on an enriched medium, are not resistant to the temperature in a minimal media and, moreover, present changes in cell morphology such as a shape rounder and larger than normal cells. Therefore, it has to be assumed a fundamental role of CAP in the cell growth. It should also be considered that not all the CAP physically interact with AC or regulate its function. For example the CAP proteins of Schizosaccaromyces pombe, which are structurally and functionally related to CAP of S. cerevisiae[128], are not closely involved in the RAS signalling. This suggests that CAP are bifunctional proteins, able to interact with the pathway of AC activation, but also directly with actin. In addition, the activities of Srv2/CAP in cell growth and cytoskeletal organization are conserved also in CAP homologues[129].

1.2.2.1 CAP STRUCTURE

CAPs are multidomain proteins of 450-550 amino acids and vertebrates have two CAP homolog forms, CAP1 and CAP2[130]. CAP is a predominantly hydrophilic protein, made up of α- helix, and of a β-sheets region located in the C-terminal region.

The most important structural and functional domains of the CAP protein of S. cerevisiae are:

1-the N-terminal domain, which interacts with AC and is required to activate the RAS protein;

2- the C-terminal domain, which is involved in cytoskeletal rearrangement[131,132]. Indeed, the deletion of the C-terminal domain leads to the expression of abnormal phenotypes: slow growth, altered cell morphology and actin dynamics.

3- the central domain rich in proline, between the N-terminal domains and C-terminal, responsible for the interactions with proteins containing SH3 domains.

N-terminal domain

This region contains tandem repetitions of a heptad motif alphaXXalphaXXX (where alpha represents a hydrophobic amino acid and X represents any amino acid), suggesting a coiled-coil structure[133]. The alpha-helical coiled coil is one of the principal subunit oligomerization motifs in proteins[134] and, in the yeast, is a highly conserved motif sufficient for the interaction with AC.

In yeast, this highly conserved motif interacts with AC. However, the amino-terminal domain of human CAP does not have the same function. Therefore, this structurally

conserved RLEXAXXRLE motif (where X represents non-conserved amino acid) has diverged functionally during evolution but may still be critical for CAP function in all organisms. Indeed, the coiled coil regions in other signalling proteins could interact with this motif in higher eukaryotes [129].

The structure of the N-terminal domain of CAP (residues 51-226) was solved by X-ray diffraction[135] showing that most of the N-terminal region is composed of a stable bundle of six antiparallel α-helices that are termed helical folded domain (HFD). The HFD binds to the ADF/cofilin-G-actin complex[136,137] and also interacts with ADF/cofilin to promote the severing of actin filaments[138].

Central domain

There are two proline rich regions in the central region of CAP: P1 and P2. P1 is highly conserved among different species, while P2 is less conserved.

Several of the CAP partners bind selectively P1 or P2. For example, the SH3 domain of Abl tyrosine kinase binds P1 of human CAP1, while Abp1 yeast protein binds to the P2 srv2 / CAP[139,140].

Recent experiments[141] with Drosophila CAP have detected a region just downstream from the SH3 binding domain that shows similarity to the verprolin homology domain (LKKAET), called WASP homology domain, found in a variety of actin binding proteins (e.g., thymosin, fimbrin, actinin and members of the WASp family). The WASp homology domain of CAP binds to G-actin without a clear preference between ATP-actin or ADP-actin, but it has a crucial role in actin nucleotide exchange.

C-terminal domain

Regarding the C-terminal domain, it has been demonstrated that the deletion of the last 27 aminoacids abolishes the binding of CAP to actin in S. cerevisiae[142] and humans[143]. Within this region there is a 7 a.a. stretch constituting the site E (X) 3PEQ: residues E, P and Q are present in all the analysed CAP proteins.

The C-terminal portion of CAP is mainly made up of β-sheets[144]. The 35 amino acids sequence in the C-terminal domain contains a dimerization motif.

1.2.2.2 ROLE OF CAP IN ACTIN DYNAMIC

CAP has a critical role in the RAS signalling pathway, but several studies suggest that this signalling function is related to a limited number of organisms, while its capability of maintaining the cytoskeleton structure appears to be conserved among eukaryotes[129].

The morphological abnormalities in cells lacking of CAP are associated with the lack of proper distribution of actin, the loss of normal bundles of actin and the formation of abnormal bundles[132]. In addition, the expression of a heterologous CAP protein in yeast cells lacking of CAP, can recover the phenotype linked to actin, but not the phenotype associated to RAS, suggesting that the actin regulation is the evolutionarily conserved role[128]. Similar genetic studies have shown that the regulatory function of the events related to actin has been preserved in the CAPs of Lentinus edodes (shiitake mushrooms) [145], rat [143] and human[146].

1.2.2.3 CAP AND G-ACTIN

Early biochemical studies demonstrated that CAP sequesters actin monomers preventing the nucleation of actin and the filament elongation[147]. However, recent studies have revealed that CAPs play more active roles in promoting the dynamics of actin filaments.

CAP binds to G-actin in a molar ratio 1: 1, and inhibits the spontaneous polymerization of G-actin in the F-actin. This activity has been demonstrated in vitro for the CAPs of S. cerevisiae[147], Dictyostelium[148], C. elegans (CAS-1 and DAC-2)[149], pork (ASP-56 / CAP1) and human (CAP2)[150].

The effect of CAP on actin is controversial. Freeman and collaborators[147] show that yeast CAP inhibits the incorporation of G-actin at both ends of the actin filament (to the pointed-end and to the barbed-end), while Mattila and colleagues[151] report that CAP selectively inhibits the incorporation of G-actin to the barbed-end. This discrepancy could be due to different ratios of CAP and G-actin used in these studies, since it was shown that human CAP1 accelerates the addition of G-actin to the barbed-end when it is present in sub-stoichiometric amounts respect to the G-actin, but inhibits the addition of the monomer when they are used in stoichiometric amounts[136].

At the actin steady state, when CAP is present in an equimolar concentration to G-actin, CAP sequesters G-actin [152]. In yeast, CAP locates in actin patches[140], while in Dictyostelium and mammalian cells is localized in the cell periphery [148,153]; therefore, in

these areas, the concentration of CAP may be high enough to sequester actin monomers.

CAP also increases the rate of nucleotides exchange on actin, and this can represent the most important effect on the actin polymerization. During the treadmilling of actin, exchange of actin-bound nucleotide occurs primarily at G-actin, whereas hydrolysis of ATP into ADP occurs primarily at F-actin. The newly depolymerized actin monomers are predominantly bound to ADP. When free ATP is present, ADP–G-actin can 'recharge' itself by rapidly exchanging ADP for ATP and, thus, maintaining the treadmilling cycle. However, when no free ATP is available, ADP–G-actin cannot recharge itself, thus preventing the treadmillingcycle[154-158]. However, different G-actin binding proteins, including thymosin β4 [159] and ADF / cofilin are able to inhibit this nucleotides exchange and, therefore, to prevent the process of "actin-recharging".

CAP is able to promote the exchange of the nucleotide bound to the G-actin[160,161] and so to increase the turnover of actin in the presence of thymosin β4[162], ADF / cofilin or profilin[163].

However, unlike the profilin[99], CAP binds to the ADP-G-actin with relatively high affinity and competes for it with ADF / cofilin[151].

In human CAP1 and yeast CAP, the HFD domain binds to ADF cofilin-actin complex, and increases the actin turnover mediated by ADF/cofilin [136,137]. These results were originally interpreted as evidence of an active involvement of the HFD domain in the dissociation of the complex ADF/cofilin-actin. However, a recent study shows that the HFD domain is not required for the recovery of ADF / cofilin and G-actin but, instead, has a severing function on the polymerized actin filament.

The sites necessary for monomer sequestering and nucleotide exchange activities of CAP reside in its C-terminal. The C-terminal half of CAP can bind to one molecule of G-actin[147], and WH2 and CARP bind independently to G-actin[150,151,164].

Although the C-terminal half of CAP is sufficient to promote the nucleotide exchange of ADP–G-actin, WH2 is necessary to facilitate nucleotide exchange when ADF/ cofilin is bound to ADP–G-actin[165].

Since ADF / cofilin and WH2 share the same binding site for the G-actin (Dominguez and Holmes, 2011), WH2 might be necessary to CAP to be able to compete with ADF/cofilin.

1.2.2.4 CAP AND F-ACTIN

CAP also interacts directly with the filaments of F-actin and promotes depolymerization of the filament. Recent observations have clearly shown that CAP induces cleavage of actin filaments[166]. For example mammalian CAP1 is able, alone, to sever actin filaments at acidic pH condition, but not at neutral pH, while the ADF / cofilin of mammal is able to “cut” actin filaments in basic pH, but not at neutral and acidic pH. However, when CAP1 and ADF / cofilin are combined, they promote the breakdown of actin filaments within a wide pH range.

In addition, yeast CAP promotes the severing of actin linked to ADF / cofilin, and this activity is mediated by the N-terminal portion of CAP containing the coiled-coil region and the HFD domain[138]. However, the mechanism by which the actin filaments are cleaved from CAP HFD domain remains unclear.

1.2.2.5 CAP2 (Cyclase-Associated Protein 2)

Higher eukaryotes have two homologs of CAP, CAP1 and CAP2, which share high homology. CAP1 is equally distributed in the organs of the mouse and is highly abundant, while CAP2 is expressed only in the skeletal muscle, heart, testis and brain. The N-terminal part of CAP2 is conserved through all the species, in particular the sequence LxxRLE/DxxxxRLE.

The best characterisation of CAP2 in mammalian cells, up to now, is made by Peche and colleagues, that generate a $CAP2^{gt/gt}$ knockout mice. This model show a particular phenotype:

1-Mutant mice showed a decrease in body weight and had a decreased percentage of survival (they survive not over 70 days in comparison to WT animals). Further, they developed dilated cardiomyopathy (DCM) associated with drastic reduction in basal heart rate and prolongations in atrial and ventricular conduction times. A further finding is that mortality due to DCM and atrial dilation is more evident in male than female animals. The authors demonstrated that this is due to the dishevelment of sarcomere. The lack of CAP2 leads to the loss of the G-sequestering capability of the protein and so to the filament-fragmenting activity. They demonstrated that CAP2 is present in the Z-line-banding pattern of the sarcomeres and regulates filament formation[150].

2-Kosmas and colleagues showed that the keratinocytes of mutant mice showed reduced velocity and a delay in scratch closure. They showed that CAP2 in murine and human skin is present in the nucleus, in the cytosol and in the cell periphery; moreover, they reported that interestingly, in human wounds, CAP2 was also expressed in

hyperproliferative epidermis and at the migrating tongue. CAP2$^{gt/gt}$ fibroblasts develop abnormal protrusions and more focal adhesions and show reduced velocity. The authors proposed a model according to which, the CAP2 knockout cells motility is affected by stabilization of focal adhesions and by disruption of cell polarity, since the depletion of CAP2 leads to an increase in focal adhesion in resting and in migrating cells[167].

3-Kumar and colleagues showed that CAP2 is highly express in different brain areas, such as in the olfactory bulb, cortex, hippocampus and cerebellum. Using cortical primary culture labelled with TRITC-phalloidin to stain F-actin, they observed that CAP2 colocalizes with F-actin. In particular, they showed that CAP2 also localizes in the dendritic shaft and presynaptic terminal with a synapsin I staining and in the excitatory postsynaptic sites, with a PSD-95 co-staining. Mutant mice showed a statistically significant increase in dendritic arbor complexity using a Sholl analysis respect to control. That results were confirmed by F/G actin ratio analysis, that shows an increase of the ratio in the mutant brain lysates. The authors found out that the induction of chemically long term potentiation (cLTP) results in a reduced surface density of GluA1 compared to the surface GluA1 in WT cortical neurons, which suggests that the synaptic plasticity is impaired in CAP2 mutant neurons. This was further analysed with a surface biotinylation assays, suggesting that CAP2 is important for the exocytosis of AMPA receptor. Since previous studies reported that CAP2 modulate the dissociation of ADP G-actin-cofilin complexes[136], they verified if CAP2 was able to bind cofilin. They confirmed the CAP2-n-cofilin interaction and found out that ablation of CAP2 in neuron results in the reduction of phosphorylated (inactive) cofilin and its accumulation in cytoplasmic aggregates cells[168].

A lot of studies reported that the gene of CAP2 is up-regulated in the early phases of Hepatocellular carcinoma (HCC)[169]. Shibata and co-workers have studied the expression of CAP2 and demonstrated its overexpression in multistage carcinogenesis of HCC. It was assumed that the stromal invasion capacity that occurs in the early months of HCC could be related to the actin binding activity of CAP2. The CAP2 involvement in other carcinomas remains unclear. Peche and collaborators[170] monitored mRNA levels in 47 different types of cancer. CAP2 was over up-regulated in renal cancer, brain, colon, bladder and thyroid, while it was down-regulated in breast cancer, suggesting that CAP2 is regulated in different ways according to the type and origin of the tumour.

1.3 SYNAPTIC PLASTICITY: HOW THE SYNAPSES STORE INFORMATION

Various factors can modify the strength of the transmission of the synapses. The synaptic activity is the capability of two neurons to communicate each other. Specific patterns of synaptic activity can modulate the strength of the synaptic transmission and, by this sophisticated way, the synapses store information in response to experience. The capability to modify neuronal circuit function generated by the experience is called synaptic plasticity. This term also includes the role in creating new circuits during the development and the capability to maintain the normal physiology of the neuronal circuits created[171].

1.3.1 SHORT-TERM PLASTICITY

The short-term plasticity is characterised by an increase in the probability of transmitter release, in response to the left over calcium concentration in the presynaptic side after short bursts of activity. The major function of the short-term plasticity is to modulate the filter capability of the synapses, inhibiting or enhancing the release probability of neurotransmitter[171].

In particular, the paired pulse facilitation /depression is a form of short-term plasticity. It is a facilitation or a depression of the synaptic transmission depending on the timing at which a second stimulus is delivered after a first one on the same synapse; it's a paired-pulse facilitation if the second stimulus occurs within an interval from the first of 20–500 ms, while the paired pulse depression occurs after a shorter interval (less than 20 ms)[172] [173]. Facilitation can be attributed to an increase of calcium concentration produced by the second stimulus leading to an increased probability to facilitate an additional release of neurotransmitter after the first stimulus. A lot of protein of the presynaptic compartment can contribute to promote the short-term plasticity, in particular the activation of protein kinases can modulate the activity of presynaptic phosphoprotein, for example synapsin[174]. On the other hand, a strong depletion of calcium in the presynaptic compartment can be generated after the first stimulus, contributing to create a depression. Indeed, paired-pulse depression is commonly observed in all synapses after short (less than 20 ms) interstimulus intervals, and it probably results from the inactivation of voltage-dependent sodium or calcium channels or from a

transient depletion of the release-ready pool of vesicles docked at the presynaptic terminal. Actually, we can point out that the depression, rather than facilitation, is strictly related to the transmitter release; in fact, in synapses where the probability of neurotransmitter release is high, the phenomena of depression may predominate[171]. Other forms of short-term plasticity are the post-tetanic potentiation (PTP) and the augmentation. The PTP occurs after a repetitive or tetanic stimulation of synapses with prolonged (approximately 200 ms to 5 s) trains of stimulation applied at high frequencies (10–200 Hz)[173]. On the other hand, repetitive activation leads to depression that can last for several seconds or even minutes[173]. Augmentation and PTP describe an enhancement of transmitter release lasting from seconds (augmentation) to several minutes (PTP)[171]. The concentration of Ca2+ in terminal boutons rises during PTP, suggesting that, like facilitation, it is a presynaptic process.

Moreover, the G protein–coupled receptors (GPCRs) play an important role in the modulation of the synaptic transmission, enhancing or depressing it. In addition, several neuromodulators can influence the presynaptic release: retrograde messengers that have been identified in specific cell types include dopamine, dynorphin, glutamate, GABA, nitric oxide, brain-derived neurotrophic factor (BDNF), and oxytocin[175-178]. Astrocytes and perisynaptic Schwann cells regulate the synapses because of their intimate association with them. They have different roles, such as the clearance of neurotransmitter, thus controlling the speed and the extent of such clearance (Bergles et al, 1999; Danbolt, 2001). The glia can act on the short-term plasticity releasing substances that can impact on the synaptic function, including extracellular messengers; in particular, glial cells have different receptors, including Ca^{2+}-permeable channels, that can control the internal stores of $[Ca^{2+}]_i$. The resulting increase in $[Ca^{2+}]_i$ can trigger vesicular release of substances from astrocytes, which can act on presynaptic terminals to regulate neurotransmitter release[173].

1.3.2 LONG-TERM PLASTICITY

1.3.2.1 LONG TERM POTENTIATION

LTP is a persistent increase in synaptic strength following a high-frequency stimulation of a chemical synapse. First it was described by Bliss and colleagues[179,180]. It has been reported that the repetitive activation of excitatory synapses in the hippocampus causes a potentiation of synaptic strength that could last for hours or even days. Nowadays, we know that there are different forms of LTP in several brain areas, in particular at the

glutamatergic synapses and the form of LTP that occurs in the hippocampus, between the Shaffer collateral (axons that derive from the cells in CA3) and the apical dendrites of CA1 pyramidal cells, is considered the prototypic system to explain the LTP that appears at the glutamatergic synapses. LTP is characterized by three basic properties: cooperativity, associativity and input-specificity. Cooperativity means that several presynaptic fibres must be activated to generate the trigger signal. Moreover, the LTP is associative, which means that strong activation of one set of synapses can facilitate LTP at an independent set of adjacent active synapses on the same cell if both sets of synapses are activated within a finite temporal window[181]. Finally, LTP is input-specific, because when the trigger signal is turned on, all the others stimuli can't be active. Many type of LTP have been described in the hippocampus, but the most described is NMDAR dependent. According to that, the induction of the LTP needs the increase in postsynaptic calcium concentration through NMDARs during a strong postsynaptic depolarisation of the cell[181]. In this process, the entry of the Ca^{2+} triggers the translocation of specific proteins to the synapse, including the AMPAR[182]. Practically the electrical stimulation, achieved with a high-frequency train of stimuli to the Schaffer collaterals, can generate, in the CA1 cells, excitatory postsynaptic potentials (EPSP). High-frequency stimulation protocols typically comprise delivery of one or several trains of pulses at 50–100 Hz for 1 sec[183], called tetanic stimulation. This strong stimulation that leads to a summation of the excitatory postsynaptic potentials (EPSPs) allows achieving a strong depolarisation of the postsynaptic cell, activating NMDARs. In the early phase of LTP there is a strong redistribution of AMPARs at the synapse, because of activity-dependent changes in AMPAR trafficking, and this events are required for the maintenance of LTP [181,184-186]. For example, the proteins of the TARP family control AMPAR distribution and insertion[187] [188,189]. The molecular mechanism by which the LTP is maintained is initiated by a sufficient depolarisation of the membrane that allows the Mg^{2+} removal and the influx of the Ca^{2+} ion through NMDAR. The calcium ions activate intracellular kinases and phosphatases, such as PKA, PKC and CAMKII.

In particular, CAMKII is activated by a structural change and it is recruited to the PSD, where it can phosphorylate several proteins such as AMPAR, NMDAR, PSD-95 [190]. The phosphorylation of Ser73 of PSD-95 can dissociated the PSD-95 from GluN2A while the phosphorylation of Ser295 can increase the stability of the association of the two proteins[191]. Also AMPARs are modified by several phosphorylations that impact on its trafficking and channel properties.

The late phase of LTP is more persistent (lasting > 8 hr) and requires transcription and synthesis of new proteins and, therefore, takes advantage of the protein synthetic machinery at synaptic sites on dendrites. During the late phase of LTP, the molecules that mediate the signalling to the nucleus are CAMKII, PKA, CAMKIV and MAPK, which in turn activate the key transcription factor CREB. However, the local synthesis apparatus can also be involeved in the generation of proteins that play a key role in the modifications triggered by LTP. Harris and co-workers showed that the percentage of spines containing polyribosomes increased 2 h after a tetanic stimulation[192]. As described, the synthesis and transport of new proteins enriches the composition of the spines. One of the most accredited hypothesis regarding the specificity of the new protein localisation is the capability of LTP to generate the so called “synaptic tag”, responsible for capturing the proteins and molecules only into selected spines[193]. The nature and composition of this synaptic tag is still unknown and object of debate, even if Morris and colleagues proposed anatomical changes and phosphorylation of receptors and kinases as possible tag candidates.

Eva Fifkovà described for the first time, by the EM microscopy, that the enlargement in the morphology of the spine was correlated to the LTP. This modification in spine morphology was found 2 min after tetanic stimulation and lasted up to 23 h[194]. Matsuzaki confirmed these data using photolysis of caged glutamate and imaged it by two-photon microscopy. He demonstrated that, in the hippocampus, LTP leads to an enlargement of the dendritic spines that is correlated with an increase in the synaptic electrical response. Moreover, it has demonstrated that the LTP can induce new spine formation[195]. Along with spine growth, morphological changes associated with LTP include enlargement of the PSD[196], the splitting of the single PSDs and spines into two functional synapses[197,198], the remodelling of spine actin cytoskeleton[199], redistribution of polyribosomes[200] and mitochondria[201].

In addition to the prototypic LTP described above, in the hippocampus another form of LTP has been described and occurs at the mossy fiber synapses, the synapses between the axons of dentate gyrus granule cells (i.e., mossy fibers) and the proximal apical dendrites of CA3 pyramidal cells[181]. As for the NMDARs mediated plasticity, this LTP can represent a paradigmatic example of forms of plasticity that occurs also in other areas of the brain. The trigger of mossy fiber LTP is an increase in intracellular calcium concentration in presynaptic terminals[202-204]. The involvement of the presynaptic kainate receptor[205-207] that leads to an increased probability of

neurotransmitter release is clear. Like NMDAR-dependent LTP, new protein synthesis appears to be required for maintaining mossy fibre LTP. However, it is unknown whether the increase in PKA activity following the induction of mossy fibre LTP is maintained for tens of minutes or hours. We also do not know whether long-lasting structural changes occur at the synapses that express mossy fibre LTP (Fig.3).

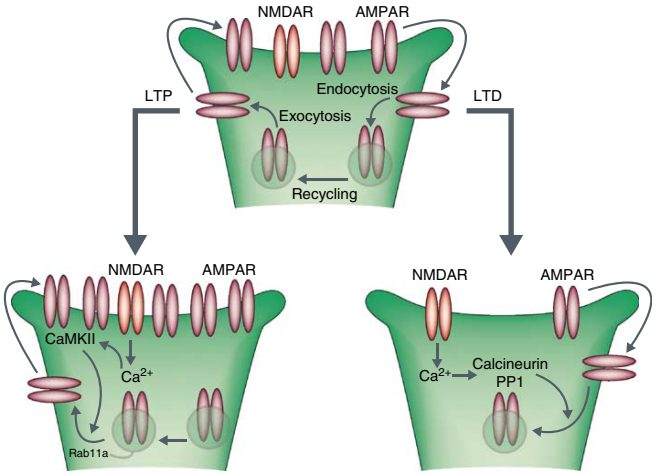
1.3.2.2 LONG TERM DEPRESSION

LTD (long term depression) has been considered as a parallel and opposite process to the more common LTP[55]. There are different forms of LTD through the brain, but, as I mentioned above, I will focus on the NMDAR-dependent LTD at excitatory synapses on hippocampal CA1 pyramidal cells[208].

We can induce LTD in neuronal cells applying a prolonged repetitive low frequency stimulation protocol; NMDAR-dependent LTD can also be induced by correctly timing the activation of presynaptic axons and the postsynaptic neuron[209] (STDP). As the LTP, also the LTD is input-specific depending on the postsynaptic concentration of calcium[210]. The difference between the two processes resides in the difference in the concentration of calcium: high concentration of calcium triggers LTP, low concentration of calcium triggers LTD. The low frequency stimulation that leads to LTD involves the activation of calcium/calmodulin-dependent phosphatase calcineurin (also known as protein phosphatase 2B), of PP1, and of a phosphoprotein inhibitor-1 which inhibits PP1 until calcineurin dephosphorylates it; it also inhibits and prevents the phosphorylation of several proteins, such as PKC and PKA. Consistent with a role for PKA, LTD is associated with selective dephosphorylation of Ser845 on GluR1, a PKA substrate site [211](Lee et al, 2000). This dephosphorylation event may contribute to the expression of LTD, as it decreases the AMPAR open-channel probability[212].

Indeed, LTP is associated with phosphorylation of GluR1-Ser831, a substrate of CAMKII and PKC. While LTD was found to be associated with selective dephosphorylation of GluR1-Ser845 without any change in GluR1-Ser831[208]. The LTD induction in the CA1 cells can also trigger an activation of metabotropic glutamate receptors. Probably the extrasynaptic group of mGluRs are responsible for activating this kind of LTD[213], including the mGluR1 and mGluR5 G-coupled protein receptors. In particular, it has been shown that the mGluR1 can activate the LTD in the ventral tegmental area (VTA), while the mGluR5 is involved in the hippocampus, cortex, and nucleus accumbens LTD induction[171]. In the cerebellum and in the VTA, the PKC mediates this kind of LTD[213], but probably it is not involved in the mGluR- mediated LTD

in the hippocampus. Other signalling proteins important for mGluR LTD include the MAPKs p38, ERK, and Jnk[214-216], tyrosine phosphatases[217], and phosphatidylinositol 3-kinase[218] but how these various enzymes might lead to a long-lasting decrease in synaptic strength is unknown[171]. It is not clear where the mGluR-mediated LTD takes place. It has been suggested that in slices prepared from neonatal (P8–P15) animals, mGluR LTD is independent from protein synthesis and is due to a presynaptic modification, whereas in older animals (P21–P35) mGluR LTD is mediated by a protein synthesis-dependent reduction in the postsynaptic levels of AMPARs[171]. In particular, in the hippocampus the mGlur5 receptors stimulates the initiation of the local synthesis thought the Homer scaffold protein[219] (Fig.3).



Citri and R.C. Malenka,Neuropsychopharmacology-2008

Figure 3. Model of spine remodelling during LTP and LTD

1.3.3 Other mechanisms for plasticity: tuning the synapses

Additional forms of synaptic plasticity that are not activity dependent have been identified, such as metaplasticity and homeostatic plasticity[220]. The homeostatic plasticity is the capability of neurons of regulating their own excitability relative to network activity, it uses a much slower timescale than LTP and is probably very important for the development. A mechanism, by which the homeostatic plasticity may allow local changes, is the synaptic scaling, which uses a set of calcium-dependent sensors that regulate receptor trafficking to increase or decrease the accumulation of glutamate receptors in the spine[221]. The metaplasticity consists in the ability of the neurons to modulate the activity-dependent synaptic plasticity[222], or in other words the LTP or LTD are regulated by a plasticity of the entire synaptic state. In fact, now we know that there is a threshold for a system to induce the synaptic plasticity, and this

limit is not a static property of synaptic connections but, instead, changes dynamically according to the recent history of synaptic activity [222].

1.3.4 SYNAPTIC PLASTICITY: THE BASIS OF MEMORY

According to Bear and Malenka (2004), the brain takes advantage of the neuronal capability to express long-lasting activity-dependent synaptic modifications as one of the key mechanisms by which experiences modify neural circuits behaviour. However, in the last years, the LTP phenomenon has been the object of intense investigation because is considered to be the basis of the process of memory formation. Indeed, since the hippocampus is a well-known locus for declarative memory[223], it is not surprising that in the last three decades a major effort aimed at demonstrating a role for hippocampal LTP in encoding new memories[224]. According to such hypothesis, the process of strengthening the synapses can increase the strength of circuits' connections thus leading to the storage of the new information in those circuits (i.e. in particular in the hippocampus, the declarative memory, the type of memory that, in humans, can be consciously recalled, is generated). During the last years, several correlations have been observed between defective hippocampal synaptic plasticity and defective hippocampal-dependent memory tasks upon perturbation of a number of proteins which have a role in synaptic plasticity, either pharmacologically, or through gene knockout [225].

1.3.5 SPINES ARE ELECTRICAL COMPARTMENTS

Spines are clearly electrical compartments separated from the parents' dendrite [54]. It is clear that during the APs (Action Potentials) the voltage of the spines is present also in the parents' shafts, whereas during excitatory postsynaptic potentials (EPSPs) spines must sustain a higher depolarization than dendritic shafts. Probably this occurs thanks to the particular shape of the spines: the most plausible hypothesis is that the action potential is amplified in the head of the spine and reduced as it invades the shafts by passing through the neck. There are still different hypothesis on how the electrical compartmentalisation is kept. In any case, it allows the spine to amplify the synaptic currents and reduce the EPSPs when they invade the dendrites thus preventing the dendrites saturation, especially if the neuron integrates a lot of inputs. Alternatively, this phenomenon can be interpreted as a mechanism through which spines could simply diminish the depolarization generated by each input, therefore more EPSPs can be integrated before the neuron fires an AP. Moreover, the electrical compartmentalization enables precise control of the synaptic strength. This process could occur by modifying

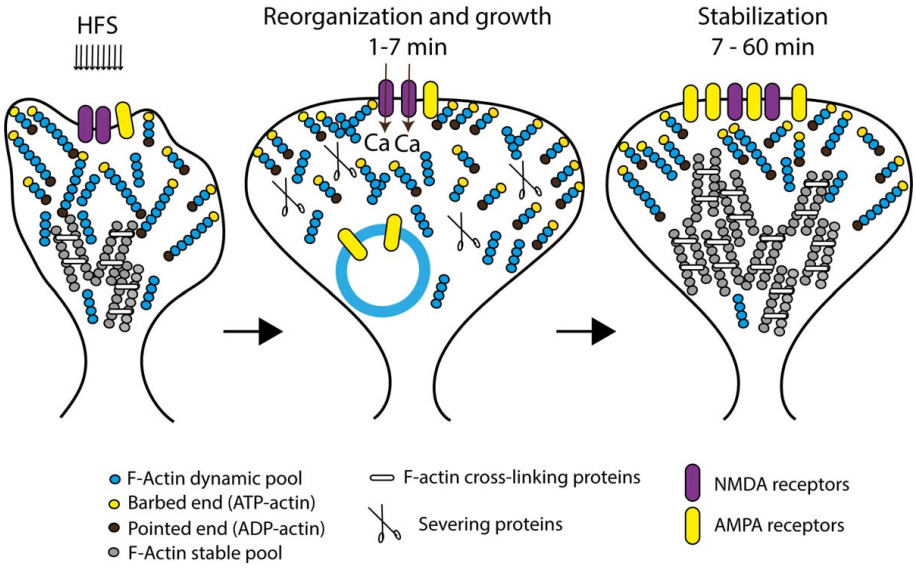
the amplification of EPSPs at the spine head, by altering the activation of spine conductance, or by altering the spine neck/dendritic shaft electrical coupling by either active or passive mechanisms. For this reason, after the LTP induction we can clearly see the modification of the morphology of the spine that allows the spines to be more responsive to the external signal [49]. A compelling possibility for a long-term maintenance mechanism of LTP is the structural remodelling of potentiated synapses [183]. Spines have a variety of shapes and sizes, and can undergo rapid shape changes that are influenced by activity[198]. Morphological changes, which have been reported to accompany LTP, include growth of new dendritic spines, enlargement of pre-existing spines and their PSDs, and the splitting of single PSDs and spines into two functional synapses [195,197,198]. An attractive model suggests that during LTP, recycling endosomes contribute to AMPAR subunits insertion in the synapse, as well as to lipids and constituents enrichment which enlarge the synapse[226,227]. At later time points, there is a concomitant increase in the presynaptic active zone, the size of which always closely matches to that of the PSD[226]. This presynaptic remodelling must involve post- and presynaptic protein interactions with cell adhesion molecules.

1.3.6 ACTIN ROLE IN PLASTICITY

It has been shown that the neuronal plasticity is strictly linked to the fast remodelling of the synaptic actin[228]. LTP induction moves the G and F actin ratio to an increase in F-actin levels leading to an increase of spine volume, while the LTD induction move the ratio toward an increase of the G-actin concentration leading to the shrinkage of the spine[199,229]. The link between the LTP and actin remodelling is demonstrated by the treatment with depolymerizing actin reagents[195,229]. Bosh and colleagues showed for the first time, the remodelling that occurs in the spine after the LTP stimulation. 20 seconds after the LTP induction there is a rapid increase of the actin levels in the spines. Moreover, there is also a change in the composition of the actin binding proteins that regulate the actin cytoskeleton into the spine. During the first minutes of LTP induction, the concentration of a lot of protein that can modify the actin cytoskeleton, such as debrin, CaMKIIβ e α-actinin, is decreased[230] . During this phase, that takes 1-7 minutes[194], the actin cytoskeleton is more unstable and susceptible to reorganization. During this phase the cofilin plays an important role in the remodelling of F-actin. First of all, upon the activation of the NMDAR the cofilin is translocated to the synapse. Since the actin concentration is high, the cofilin can severe the actin cytoskeleton, leading to

the formation of new barbed ends[231]. The severing is a phenomenon that allows Arp2/3 to enhance its function of branching, creating new filaments that are involved in the maintenance of spine expansion and in the proteins trafficking, such as the trafficking of AMPAR[232]. According to that, the pharmacological inhibition of the cofilin phosphorylation prevents the maintenance of spine enlargement, even if the first phase of the LTP is not blocked. Indeed, Bosch proposes the fundamental role for cofilin and others ABP for the maintenance of the structural remodelling of the actin cytoskeleton. During the second phase (7-60 min after the LTP induction) the actin concentration goes back to "normal" levels. In this phase all the changes that occured in the first phase are stabilized. According to that, the cofilin moves to the neck of the spine after its phosphorylation on Ser3. This is a key point, since if the cofilin phosphorylation is prevented, it is possible to assist to an excessive severing process that can lead to the spine shrinkage, fostering the depolymerisation of actin instead of polymerisation[233]. In the neck of the spine, the cofilin is dephosphorylated again in order to bind F-actin and create a stable structure, made of cofilin and F-actin, at the base of the spine head. This particular complex, in the neck, can decrease the normal movement of F-actin from the spine head[234]. Indeed, the complex cofilin-actin can stop this treadmilling leading to the enlargement of the spine at the spine head. Moreover, the importance of F actin is not only related to the remodelling, but after LTP induction it can capture and stabilize a lot of proteins, thus representing an anchoring for several molecules[235]. In the third phase (that correspond to L-LTP, late LTP), that occurs 60 minutes after induction, there is a stabilization of the spine composition. In particular, synaptic proteins, as the members of MAGUK family, reach a level that is proportional and closer to the new spine volume, thus the natural correlation between the volume and the PSD size is recovered [236] (Fig. 4).

As concern the structure of the spines, it has been demonstrated that the F-actin nucleation, mediated by WAVE complex, an activator of the Arp2/3 complex, occurs in the central structure of the spine, while the elongation occurs at the tip of finger-like protrusions. For that reason, the proteins involved in the branching of the already assembled filaments are localized in the central part of the PSD, while next to the membrane the filament elongator proteins are localized. The synaptic plasticity, modifying the distribution patterns of ABP, induces also the redistribution of branched F-actin regulators in spines, to create an enlargement also in the distal part of the spine [237].



I.Hlushchenko et al.,Cytoskeleton-2016

Figure 4. Actin remodelling during LTP

1.4 ADAM10: AN ENZYME INVOLVED IN SEVERAL SYNAPTOPATHIES

Neuronal signalling is an integrated process between the excitatory and inhibitory process. The signals that a neuron can receive could be excitatory or inhibitory, can be directed to the dendrites or to the cell body, and whether the neuron fires an action potential depends on the total input of all the synapses[238]. There are a lot of control mechanisms that maintain the balance between the excitatory and inhibitory synaptic transmission, the so-called E/I balance. Alteration in the E/I synapse balance has been proposed to be involved in the pathogenesis of many brain disorders, including autism and schizophrenia[238]. Moreover, it is known that the functional morphology of the spine is strictly linked to the synaptic function and therefore the deep comprehension of the mechanisms underlying the physiology of the synapse can help in the understanding of the basic mechanisms involved in the brain pathology. Spine morphology depends on rapid alteration in neuronal activity and glutamate receptor activation. Indeed, as described in the previous chapter, the induction of LTP causes enlargement of spine heads, whereas activity patterns that induce LTD cause spine head shrinkage[238].

A class of enzymes, such as the metalloproteases, responsible for the modulation of the synaptic morphology plays a specific role in the synaptic development and remodelling. For example, ADAM10 is involved in physiological processes such as the development to pathologies as AD. Indeed, the knockout mice for ADAM10 are characterized by prenatal lethality at 9.5 days of embryogenesis, with defects in the development of the central nervous system[239].

1.4.1 ADAM10 substrates

The proteases (also called peptidase or proteinase) are enzymes that performs proteolysis; proteases play a critical roles the brain having emerging roles in synaptic plasticity, memory, neurodegenerative disorders such as Alzheimer's, Parkinson's and prion diseases, ischemia and traumatic brain injury, inflammatory and infectious diseases, and tumour progression.

In the central nervous system (CNS), ADAM10 can cleave different proteins, among which: prion protein (PrP), N-Cadherin, neuroregulin, ephrins, L1 adhesion molecule, transmembrane chemokines, Notch and its ligand Delta and APP (Amyloid Precursor Protein).

The prion protein (PrP)

The cellular prion protein PrP is essential for the pathogenesis and transmission of prion diseases. Whereas the majority of PrP is bound to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor, a secreted form of the protein has been identified. PrP can be released into the medium by both protease and phospholipase-mediated mechanisms[240] (Parkin et al., 2004). PrP undergoes constitutive cleavage involving ADAM10 and phorbol ester-regulated proteolytic cleavage mediated by ADAM17 (Vincent, 2004). Therefore, activation of ADAM10 and ADAM17 could be considered a putative therapeutic strategy aiming at increasing normal PrP breakdown and, thereby, depleting cells of the putative toxic domain of PrP[241].

Adhesion molecules: N-Cadherin, γ-protocadherins and L1

Cadherins are Ca^{2+} dependent adhesion molecules expressed virtually by all cells that form solid tissue, during development as well as in adult life. N-Cadherin, the most abundant Cadherin in the CNS, belongs to type I classical Cadherins. The release of its extracellular domain, which contains the homophilic binding site, is functionally of major importance for the regulation of cell adhesion, cell migration and neurite outgrowth [242,243]. ADAM10 is responsible for the initial proteolytic cleavage of N-Cadherin, leading to the release of the extracellular soluble domain and the production of the membrane-bound carboxy-terminal fragment CTF1, which is cleaved by Y-secretase[244]. The enzymatic activity of ADAM10 on N-Cadherin may be significant for the coordinated interplay between cell-cell adhesion, cell detachment, cell proliferation and cell survival during embryogenic development, in wound healing and during tumour invasion. Other than N-Cadherin, at least two classes of cell-adhesion molecules are cleaved by ADAM10, γ-protocadherins (Pcdh-γ) and L1 adhesion molecule. Pcdh-γ are abundantly expressed in the nervous system. They are enriched at synapses and involved in synapse formation, specification and maintenance. Pcdh-γ C3 and Pcdh-γ B4 are constitutively cleaved within their ectodomains by ADAM10, thus inhibiting cell aggregation[245]. The immunoglobulin superfamily recognition molecule L1 promotes neuronal migration, neuronal survival and neurite outgrowth[246]. L1 undergoes constitutive cleavage at the cell surface which can be enhanced by stimulation with PMA, cholesterol depletion or NMDA-treatment (Maretzky et al., 2005; Mechtersheimer

et al., 2001). Constitutive and NMDA-induced shedding of L1 is mediated by ADAM10 while PMA stimulation or cholesterol depletion leads to ADAM17-mediated L1 cleavage[247]. ADAM10-mediated release was found to occur in intracellular vesicles that are subsequently released while cleavage of L1 in response to PMA occurred at the cell surface[248]. Soluble L1 has been shown to stimulate cellular migration, neurite outgrowth and recovery after spinal cord injury.

Ephrin family

Ephrins are neuronal guidance molecules that bind to receptor tyrosine kinases of the Eph family. Ephrin cleavage is fascinating. Ephrin ligands, presented on one cell surface, associate with their receptors on the surface of a juxtaposed cell, leading to cell-cell repulsion. Ephrin ligand can be proteolytically released from its membrane by a complex on the opposing cell composed of the ephrin receptor and ADAM10. While ADAM10 constitutively associates with EphA3, the formation of a functional EphA3/ephrin-A5 complex creates a new molecular recognition motif for the ADAM10 cysteine-rich domain that positions the proteinase domain for effective ephrin-A5 cleavage. Surprisingly, the cleavage occurs in trans, with ADAM10 and its substrate being on the membranes of opposing cells, suggesting a simple mechanism for regulating ADAM10-mediated ephrin proteolysis, which ensures that only Eph-bound ephrins are recognized and cleaved[249]. In particular, when the growth cone of a neuron that expresses Eph receptors encounters ephrin ligands on the surface of another cell, this event initiates bidirectional signalling cascades that regulate cell adhesion, axonal guidance and neuronal plasticity. The growth cone then overcomes these adhesive forces and breaks away from the ephrin surface by proteolytic shedding of ephrin. A cleavage-inhibiting mutation within ephrin A2 delays axon detachment, suggesting that shedding is critical for axon guidance in the CNS[250]. ADAM10 can cleave ephrin A5 bound to EphA3 and thus terminate binding via ephrin. Notably, the ephrin receptor EphB2 also undergoes calcium-influx and NMDAR-induced cleavage that is sensitive to ADAM10 inhibition[249]. Thus, ADAM10 can be regarded as promoter of axon guidance and extension in the CNS because of the cleavage of ephrins as well as of their receptors.

Notch

The receptor Notch and its ligand Delta 1 are required for neuroepithelial development during embryogenesis, but also for neuronal stem cell maintenance and self-renewal of the adult brain. ADAM10 cleaves the extracellular domain of Notch[239] which is then directly followed by an intramembranous cleavage of the remaining cell-associated Notch molecule via γ-secretase. This results in the generation of a cytoplasmic fragment which can translocate into the nucleus and can be a transcription factor[251]. Thus, ADAM10 is critically involved in the transcriptional signalling pathway of Notch and is required for its functions in neurogenesis, even in the adult CNS.

The Amyloid Precursor Protein (APP)

Although APP is one of the most studied proteins, the physiological function of APP itself or indeed APP-derived peptides has not been definitively elucidated. Most evidence suggests that APP has a trophic function promoting neurite outgrowth, neuronal migration and repair via interaction with extracellular matrix proteins[252,253]. Several reports indicate that the lack of APP or its overexpression affects the number of dendritic spines. APP knockout mice exhibit a baseline dendritic spine density that is approximately two-fold higher compared with wild-type controls because of a higher number of persistent spines[254]. Moreover, dendritic spine density is modulated in a dose-dependent manner by APP expression. In addition to a role in spine morphology, two reports showed that APP may contribute to postsynaptic mechanisms via regulation of the surface trafficking of excitatory NMDA receptors[255].

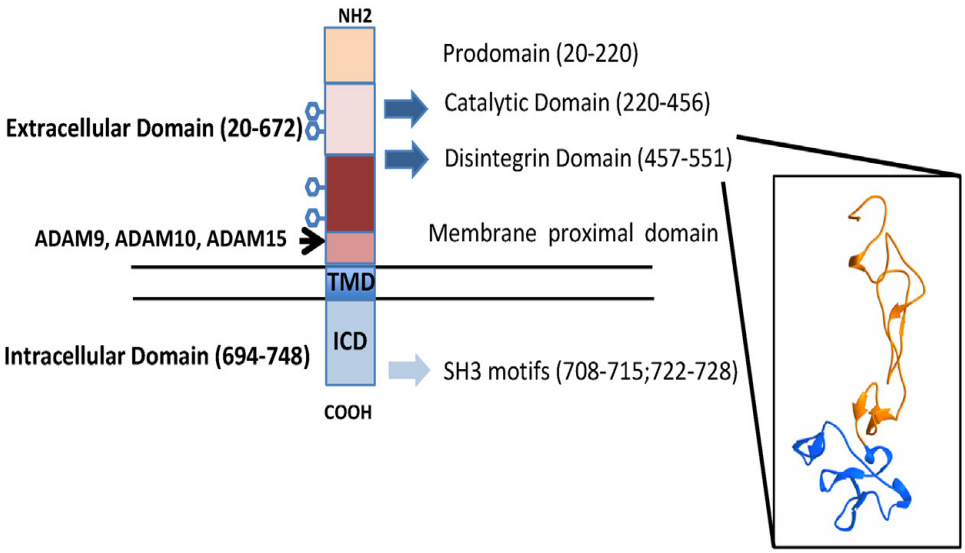
Generation of Amyloid peptide (Aβ) from APP is positioned at the beginning of a cascade that leads to Alzheimer's disease (AD). The shedding process is mediated by α- or β-secretases, while the cleavage of the membrane retained stubs is due to γ-secretase[3]. β- and γ-secretases are the principal players involved in Aβ production, while α-secretase cleavage on APP prevents Aβ deposition. Three members of the family of ADAMs have been shown to exert α-secretase activity on APP: ADAM10, ADAM17, which is also known as TACE, i.e., Tumor Necrosis Factor-alpha Converting Enzyme, and ADAM9, also referred to as MDC9, standing for metalloprotease/disintegrin/cysteine-rich protein[256,257]. Two recent studies finally demonstrated that the constitutively cleaving α-secretase activity in neurons is selectively mediated by ADAM10[258,259]. ADAM10-mediated non amyloidogenic

pathway on APP releases one soluble, neurotrophic fragment called sAPPα and one membrane associated stub, called CTF83, which can then be cleaved by the γ-secretase complex, liberating extracellular p3 and the amyloid precursor protein intracellular domain(AICD).

1.4.2 ADAM10 structure

ADAM10 is a 748 amino acid type I membrane glycoprotein, which is ubiquitously expressed[260]. Members of the ADAM family are characterized by a defined domain structure, including a signal sequence, a N-terminal prodomain, followed by a catalytic proteinase domain containing a zinc-binding motif, a disintegrin domain, a cysteine-rich sequence, a transmembrane domain, and a cytoplasmatic tail[260]. The nascent protein is not functional and is produced as a zymogene. ADAM10 is predominantly found as a proenzyme intracellularly in the Golgi, presumably in an inactive form[257]. After the cleavage of the signal sequence, ADAM10 enters the secretory pathway to be processed and thereby activated by the proprotein convertases furin or PC7[261], as demonstrated for the prodomains of several ADAMs[262-264]. Furin and PC7 are calcium-dependent endoproteases responsible for proteolytic cleavage of cellular and viral proteins transported via the constitutive secretory pathway. Cleavage occurs at the C-terminus of basic amino acid sequences, such as R–X–K/R–R and R–X–X–R. The furin was found predominantly in the trans-Golgi network (TGN), but also in clathrin-coated vesicles dispatched from the TGN, on the plasma membrane as an integral membrane protein and in the medium as an anchorless enzyme. ADAM10 prodomain exhibits a dual function: the separately expressed prodomain is capable of inactivating endogenous ADAM10 in cell cultures while overexpressed ADAM10 without its prodomain is inactive[261]. By contrast, coexpression of the prodomain in trans rescues the activity of the deletion mutant of ADAM10 lacking of the intracellular prodomain. In addition, the recombinant murine prodomain purified from Escherichia coli acts as a potent and selective competitive inhibitor in experiments performed in vitro[265]. This implicates that the prodomain of ADAM10 acts not only as a transient inhibitor, but also as an internal chaperone in the maturation of the enzyme. The catalytic domain of ADAM10 contains a typical zinc-binding consensus motif (HEXGHXX GXXHD); the mutation E384A compromises this motif and leads to a substantial decrease in sAPPα secretion[266,267]. Although the removal of the disintegrin domain of ADAM10 did not grossly affect shedding of APP in cell cultures, the cleavage of some substrates molecule is likely to be influenced by non-catalytic domains. For example, epidermal

growth factor cleavage is at least partially impaired in ADAM10 knockout cells overexpressing a cytoplasmic domain deletion mutant of ADAM10[268]. During transport through the secretory pathway, ADAM10 is complex N-glycosylated resulting in the active protease, which mediates proteolysis in the late compartments of the secretory pathway and at the plasma membrane. Cell surface biotinylation experiments demonstrated that the proteolytically activated form of ADAM10 is localized mainly in the plasma membrane[257] (Fig. 5).



P. Saftig, S.F. Lichtenthaler, Progress in Neurobiology-2015

Figure 5. ADAM10 structure

1.4.3 Regulation of ADAM10 activity: the role of intracellular trafficking

ADAM10, that is enriched in the PSD [269] can act on its substrate only when is correctly inserted into the plasma membrane. Therefore, the regulation of its trafficking can control its activity. Several proteins can modulate ADAM10 synaptic localization and, thereby, its activity.

SAP97, a member of the MAGUK family, can modulate ADAM10 trafficking fostering its insertion in the synaptic membrane[269]. SAP97 SH3 domain binds to the proline-rich sequences in the cytosolic domain of ADAM10, thereby driving the protease trafficking from dendritic Golgi outposts to the postsynaptic membrane and increasing its cleavage activity. This process is mediated by a previously uncharacterized protein kinase C phosphosite in SAP97 SRC homology 3 domain that modulates SAP97 association with ADAM10. Such mechanism is essential for ADAM10 trafficking from the Golgi outposts to the synapse, but does not affect ADAM10 transport from the endoplasmic reticulum[270].

The mechanism by which SAP97 mediates the ADAM10 trafficking, is regulated by LTD, one of the main paradigms of activity-dependent synaptic plasticity. Indeed, LTD boosts ADAM10 membrane insertion by fostering its SAP97-mediated forward trafficking to synaptic membrane[271]. Moreover, this mechanism is fostered by short-term activation of the NMDAR in primary neurons[269]. However, NMDAR can also affect ADAM10 expression, because its activation leads to upregulation of the genes encoding ADAM10 and β-catenin proteins. Inhibitors of Wnt/β-catenin signaling abolished the ADAM10 upregulation, while the activation of the Wnt/ β-catenin signaling pathway by recombinant Wnt3A stimulated ADAM10 expression. Moreover, ERK inhibitors blocked both the NMDAR and Wnt3A-induced ADAM10 upregulation. These data suggest that the NMDA receptors control ADAM10 expression via a Wnt/ MAPK signaling pathway[272].

In addition, the ADAM10 cytoplasmic tail contains a ER retention motif that regulates its intracellular localisation. Indeed, sequential deletion/mutagenesis on this arginine-rich sequence can alter the surface trafficking of the enzyme[273].

ADAM10 associates with different tetraspanins[274]. TSPAN12 is a partner of mature form of ADAM10 and promote the maturation of the protease[275]. Similarly, TSPAN15 also bound to ADAM10 in the ER. This complex was able to quickly pass through the secretory pathway, leading to more activated ADAM10 at the cell surface and increased ADAM10- mediated shedding events[276]. It is tempting to speculate that different TSPANs may modulate the cellular fate of ADAM10 (and other ADAMs), their substrates and also the function of the intramembrane cleaving proteases in a tightly coordinated fashion[277].

Another ADAM10 binding partner is the clathrin adaptor 2 (AP2), a heterotetrameric assembly that initiates the endocytosis process, interacts with an atypical AP2-binding motif (RQR) in ADAM10 C-terminal domain, and modifies its localisation. The LTP (long term potentiation) reduces the enzyme membrane levels by inducing AP2-mediated endocytosis[271].

1.4.4 ROLE OF ADAM10 IN ALZHEIMER'S DISEASE

As regards ADAM10 involvement in AD pathogenesis, ADAM10 protein levels were found to be significantly reduced in platelets of sporadic AD patients and a significant decrease in sAPPα levels was detected in platelets and cerebrospinal fluid (CSF) of AD patients [278]. In addition, it has been shown that α-secretase activity was reduced in temporal cortex homogenates from AD patients. In contrast, ADAM10 mRNA levels

were found to be two-fold increase in hippocampal and cerebellar sections of AD patients[279]. These results were obtained from the brains of severe AD patients and it is possible that, in later stages of the disease, ADAM10 expression is increased as a defense mechanism or as a secondary effect of inflammation and reactive gliosis.

As mentioned above, the correct spatial localization of ADAM10 in the postsynaptic membrane is pivotal for an efficient APP α-secretase cleavage (Fig. 6), thus the mechanisms regulating the trafficking of ADAM10 play a key role in the modulation of its activity. We know that SAP97 and AP2 can modulate the localisation of ADAM10 and thereby its activity. It has been shown that the ADAM10/SAP97 and ADAM10/AP2 associations are involved in AD pathogenesis. Indeed, ADAM10 synaptic levels and ADAM10/SAP97 association are reduced in the hippocampus of AD patients in the early stage of the disease[280]. Interfering with the ADAM10/SAP97 complex for 2 weeks by means of a cell-permeable peptide strategy in mice is sufficient to increase amyloid levels and leads to the reproduction of initial phases of sporadic AD[281]. Specifically, there is a significant reduction in PKC mediated phosphorylation of SAP97 site in AD hippocampi when compared with age-matched control patients, that leads to a decrease of the SAP97/ADAM10 association[270].

ADAM10/AP2 association has also a pathological relevance; in fact, it has been demonstrated a concomitant increase in ADAM10 association to AP2 in the hippocampus of AD patients[271]. Our results suggest that in early stages of the disease, the reduction of α-secretase synaptic localization and activity could be ascribed to a defect in ADAM10 exocytosis/endocytosis processes rather than to an alteration of its expression.

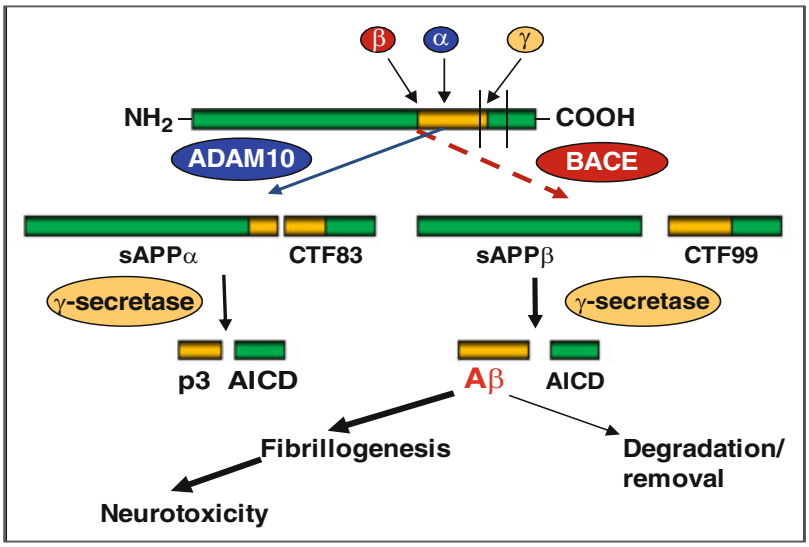


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Figure 6. Amyloid Cascade

1.4.5 ROLE OF ADAM10 IN OTHER CNS DISEASES

ADAM10 has a role in several brain disorders, ranging from Huntington disease to mental retardation.

For example, using conditional ADAM10 knockout mice (ADAM10 cKO or A10 cKO)[258], it has been shown that the depletion of the protease in neural precursors leads to posttranslational accumulation of PrPC in the early secretory pathway[282]. Altmeppen and colleagues demonstrated that the lacking of ADAM10 leads to an increase level of PrPC, increased prion conversion and a reduced time in the incubation of prion disease. In light of these observations, it is clear the fundamental role of ADAM10 in the PrPC metabolism and in neurodegenerative events.

Huntingtin is a large protein associated with the Huntington disease. The huntingtin protein (htt) inhibited ADAM10 activity and the shedding of its substrate N-cadherin, thus controlling neural adhesion during development. It is shown that the htt regulation of ADAM10 works through the modulation of the ADAM10-SAP97 complex [283].

Interestingly, the majority of substrates of ADAM10 are either directly or indirectly involved in autism spectrum disorder (ASD), schizophrenia (SCZ) and bipolar disorder (BD), through both functional and genetic association[284]. For example, ADAM10 is also involved in the Fragile X syndrome, caused by mutations or deletions of the Fragile X mental retardation protein (FMRP). ADAM10 levels were found to depend on FMRP that controls the ADAM10 mRNA translation presumably by binding to the G-quadruplex structure in the 5-UTR of the ADAM10 mRNA[285]

2. AIM

ADAM10 is one of the most important sheddase among the disintegrin and metalloproteinases family members. In neurons, ADAM10 is a component of the PSD, where it can act on many substrates, such as neuroligin-1, N-cadherin, NCAM and Ephrin. Thus ADAM10 has a crucial role in controlling synaptic adhesion molecules, spine morphology, and activity-dependent plasticity. Moreover, ADAM10 is the α-secretase, the enzyme that cleaves APP within the Aβ domain, thus preventing Aβ generation.

ADAM10 cleaves its substrate only when it is inserted at the plasma membrane and, therefore the intracellular trafficking of ADAM10 represents a mechanism capable of tuning its activity. In the last few years, the complex machinery responsible for ADAM10 activity regulation in the synapses has been described. According to this, ADAM10 forward trafficking to the synapse is mediated by SAP97. The phosphorylation of SAP97 by PKC allows the trafficking of ADAM10 to the synapse from Golgi outposts, whereas the clathrin adaptor AP2 mediates the removal of ADAM10 from the plasma membrane. Both SAP97 and AP2 interact with the cytoplasmic tail of ADAM10. Therefore, the C-terminal tail represents the locus responsible for the regulation of ADAM10 synaptic localization/activity.

In light of these considerations, my PhD project aimed to identify new protein partners of ADAM10 Cterminal domain capable of modulating ADAM10 synaptic localization. We performed a yeast two-hybrid screening of a brain cDNA library using the ADAM10 C-terminal tail as bait. Among the positive clones, we found a protein of particular interest, i.e. Cyclase-Associated Protein 2 (CAP2). CAPs are evolutionary highly conserved proteins involved in (i) processes orchestrating changes in actin cytoskeleton such as cell migration, movement and polarity, (ii) linking signalling pathways to elements of the cytoskeleton, (iii) vesicle trafficking and endocytosis1 Hubberstey AV, Mottillo EP. Cyclase-associated proteins: CAPacity for linking signal transduction and actin polymerization [129] are essential for maintaining the balance between G- and F-actin. CAPs are highly conserved proteins that have been described in yeast, plants, Dictyostelium, Drosophila and mammals. CAP deficiency results in defects in vesicle trafficking, endocytosis, and in an altered cell morphology and cell growth [286]. Two closely related homologs of CAP have been described in mammals. CAP1 is expressed in nearly all cells, whereas CAP2 expression is restricted to a limited number of tissues and is mainly found in brain, skin, skeletal and cardiac muscle [287].

Since at least one of the two CAP proteins is expressed in nearly all cells, it is likely that CAP1 and CAP2 complement each other in some cellular functions, but CAP2 may have unique roles, especially in neurons.

Given that, the main goal of my phD project were to understand the functional role of ADAM10/CAP2 interaction. In particular, the specific aims were:

1. to characterize CAP2 function in the brain,
2. to investigate the capability of CAP2 to modulate actin dynamic,
3. to determine CAP2 involvement in modulating ADAM10 synaptic localization
4. to unravel CAP2 role in the remodelling of dendritic spines.

3. MATERIALS AND METHODS

3.1 ANIMALS

Sprague–Dawley rats of 6 weeks, E18 embryos from Sprague–Dawley rats for primary hippocampal neuron cultures. All the experiments were approved by the OHSU Institutional Animal Care and Use Committee and by the Italian Health Ministry (#295/2012-A).

3.2. Y2H SCREENING

Y2H was conducted according to the manufacture's procedure guidelines using the Mate & Plate Library—Mouse Brain (normalized; cat #630488, TakaraBio/Clontech Europe, France). Briefly, the ADAM10(697-748) C-tail (bait) was cloned in the pGBKT7 plasmid and transformed in the AH109 haploid yeast strain (MATa). This was mated overnight with the Mouse Brain normalized library (prays) cloned in pGADT7 transformed in Y187 haploid yeast strain (MATa). Yeast were plated after 24 h on selective plate, allowing only the growth of diploid where a protein interaction between the bait and pray protein occurred (absence of Leucine, Adenine, Tryptophan and Histidine). Then the diploids were tested by a colorimetric assay (a-gal) to avoid the presence of false positive. Plasmids (twenty-one positive clones) were extracted from the yeast and sequenced.

3.3. PLASMIDS

EGFP CAP2 plasmid was a kind gift from Professor Angelika Noegel (University of Cologne). This plasmid was used to perform point mutation inserting a stop codon for the codon corresponding to aa 452 using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer's instructions.

Myc CAP2 plasmid was created using the restriction enzyme and it was used to perform point mutation inserting a stop codon for the codon corresponding to aa 452, C32G using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA),

following the manufacturer's instructions. Myc CAP2 plasmid was used also to create the deletion mutant Myc CAP2 165-476 using the the restriction enzyme SalHI and NotI. Glutathione S-transferase (GST)-CAP2 fusion protein contains CAP2 sequence and the deletion mutant GST CAP2-452Δ the restriction enzyme BamHI-HF and XhoI.
Glutathione S-transferase (GST)-ADAM10 C-terminal domain (Ct) fusion protein contains the cytoplasmic domain of ADAM10 (695–749)[269]. GFP was provided by Dr Maria Passafaro (CNR, Milan, Italy)

3.4. CELL CULTURES AND TRANSFECTIONS

COS7 cells were grown on 100 mm dishes and maintained in DMEM containing Glutamax (DMEM þ Glutamax, GIBCO) supplemented with 10% fetal bovine serum and penicillin–streptomycin (GIBCO). Cells were allowed to grow till confluence before passaging every 3–4 days using trypsin. The day before transfection, COS-7 cells were placed in a 12 wells multiwell (for imaging), then cells were transfected with 250–500 ng of plasmid DNA using the lipofectamine LTX method (Invitrogen). After 36 h, COS-7 cells were fixed for immunostaining/imaging. Hippocampal neuronal primary cultures were prepared from embryonic day 18–19 (E18-E19) rat hippocampi. Neurons were transfected at DIV10 using calcium-phosphate co-precipitation method with 2–4 μg of plasmid DNA. Neurons were treated at DIV15, fixed and then immunostained.

3.5. FLUORESCENT IMMUNOCYTOCHEMISTRY

Cells were fixed with 4% Paraformaldehyde (PFA)-4% sucrose in PBS solution at 4 °C and washed several times with PBS. Cells were either blocked with 5% BSA in PBS for 30 min at room temperature and then labelled with primary antibodies for surface labelling for 1 h at room temperature or permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature and then blocked with 5% BSA in PBS for 30 min at room temperature. Cells were then labelled with antibodies for intracellular epitopes for 1 h overnight at 4 °C. Cells were washed and then incubated with secondary antibodies for 1 h at room temperature. Cells were then washed in PBS and mounted on glass slides with Fluoromount mounting medium (Sigma Aldrich) or permeabilized for total labelling.

3.6 SUBCELLULAR FRACTIONATIONS

TIF, a fraction highly enriched in PSD proteins but absent of presynaptic markers63, was isolated from adult rat hippocampus. To this, samples were homogenized at 4 °C in an ice-cold buffer containing 0.32 M Sucrose, 1 mM HEPES, 1 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM MgCl in the presence of protease inhibitors (Complete, GE Healthcare) and phosphatase inhibitors (PhosSTOP, Roche Diagnostics GmbH), using a glass- Teflon homogenizer. Homogenates were then centrifuged at 1,000g for 5 min at 4 °C, to remove nuclear contamination and white matter. The supernatant was collected and centrifuged at 13,000g for 15 min at 4 °C. The resulting pellet (P2 crude membrane fraction) was resuspended in hypotonic buffer (1 mM HEPES with Complete). Resuspended P2 were then centrifuged at 100,000g for 1 h at 4 °C. Triton X-100 extraction of the resulting pellet was carried out at 4 °C for 20 min in an extraction buffer (1% Triton X-100, 75 mM KCl and Complete). After extraction, the samples were centrifuged at 100,000g for 1 h at 4 °C and the TIFs obtained were resuspended in 20 mM HEPES with Complete.

PSDs were isolated from rat hippocampus64. Rats (15 animals) were killed, hippocampi were dissected within 2 min and pooled. All hippocampi dissected in 42 min were discarded. Homogenization was carried out by 10 strokes in a glass-Teflon homogenizer (700 r.p.m.) in 4 ml/g of cold 0.32 M sucrose containing 1 mM HEPES, 1 mM MgCl2, 1 mM NaHCO2 and 0.1 mM PMSF (pH 7.4). The homogenized tissue was centrifuged at 1,000g for 10 min. The resulting supernatant was centrifuged at 13,000g for 15 min to obtain a fraction containing mitochondria and synaptosomes. The pellet was resuspended in 2.4 μl/g of 0.32 M sucrose containing 1 mM HEPES, 1 mM NaHCO3 and 0.1 mM PMSF, overlaid on a sucrose gradient (0.85–1.0–1.2 M), and centrifuged at 82,500g for 2 h. The fraction between 1.0 and 1.2 M sucrose was removed, diluted with an equal volume of 1% Triton X-100 in 0.32 M sucrose containing 1 mM HEPES, 15 min. This solution was spun down at 82,500g for 45 min. The pellet (Triton insoluble postsynaptic fraction, PSD1) was resuspended, layered on a sucrose gradient (1.0–1.5–2.1 M), and centrifuged at 100,000g at 4 °C for 2 h. The fraction between 1.5 and 2.1 M was removed and diluted with an equal volume of 1% Triton X-100 and 150 mM KCl. PSD2 were finally collected by centrifugation at 100,000g at 4 °C for 45 min and stored at 80 °C until processing. All purifications were performed in the presence of complete sets of protease and phosphatase inhibitors (Roche Diagnostics). Protein content of the samples was quantified by using Bio-Rad protein assay.

3.7 TRITON INSOLUBLE SYNAPTIC MEMBRANE (TIF)

To obtain the TIF fractions, samples of rat brain were homogenized at 4°C in an ice-cold buffer with protease inhibitors (Complete™, GE Healthcare, Mannheim, Germany), Ser/Thr and Tyr phosphatase inhibitors (Sigma-Aldrich), 0.32 M Sucrose, 1 mM Hepes, 1 mM NaF, 0.1 mM PMSF, 1 mM MgCl2 using a glass-teflon homogenizer. An aliquot of homogenate (Homo) was kept for Western Blot (WB) analysis. Homo were then centrifuged at 1000g for 5 min at 4°C, to remove nuclear contamination and white matter. The supernatant was collected and centrifuged at 13000g for 15 min at 4°C. The resulting pellet (crude membrane) was resuspended in resuspension buffer (1 mM Hepes with protease inhibitors (Complete™, GE Healthcare)) and then centrifuged at 100000g for 1 h at 4°C. Triton-X extraction of the resulting pellet was carried out at 4°C for 20 min in an extraction buffer (1% Triton-X, 75 mM KCl and protease inhibitors (Complete™, GE Healthcare)). After extraction, the samples were centrifuged at 100000g for 1 h at 4°C and the TIFs obtained were resuspended in 20 mM HEPES with protease inhibitors (Complete™, GE Healthcare). To get the soluble fraction (S2), samples of human brain were homogenized at 4°C in an ice-cold buffer with protease inhibitors (Complete™, GE Healthcare), Ser/Thr and Tyr phosphatase inhibitors (Sigma-Aldrich), 0.32 M Sucrose, 1 mM Hepes, 2 mM EDTA, 0.1 mM PMSF, 1 mM EGTA using a hand-held glass-teflon homogenizer. In this case, aliquots of Homo were kept to perform immunoprecipitation (ip) assay. Homo were then centrifuged at 1000g for 10 min at 4°C, to remove nuclear contamination and white matter. The supernatants were collected and centrifuged at 100000g for 1h at 4°C. The resulting pellet (crude membrane) was discarted and the supernatant obtained corresponds to the S2 fraction.

3.8. CO-IP ASSAY

A measure of 100 μg of proteins from rat hippocampus or HEK 293/COS 7 homogenate or 50 μg of proteins from rat hippocampus TIF were in RIA buffer containing 200 mM NaCl, 10 mM EDTA, 10 mM Na2HPO4, 0.5% NP-40, 0.1% SDS and protein A/G-agarose beads as pre-cleaning procedure. Primary antibodies were added leaving to incubate overnight at 4 °C on a wheel. Protein A/G-agarose beads were added and incubation was continued for 2 h, at room temperature on a wheel. Beads were collected by gravity or centrifugation (1200 rpm) and washed three times with RIA buffer

before adding sample buffer for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and the mixture was boiled for 10 min. Beads were pelleted by centrifugation, all supernatants were applied onto 7–8% SDS–PAGE gels.

3.9 PULL-DOWN ASSAY

GST fusion proteins were expressed in Escherichia coli and purified on glutathione agarose beads (Sigma Aldrich). A measure of 200 μg of brain homogenate proteins were incubated with 40 ml of GST alone and with of GST fusion proteins of the C-terminal domain of ADAM10 or GST CAP2-452Δ to a final volume of 1 ml in Tris-Buffered Saline (TBS, 10 mM Tris and 150 mM NaCl) for 2 h on the rotator at room temperature. After incubation, beads were washed four times with TBS and 0.1% Triton X-100.

3.10 NEURONAL CULTURE TREATMENT

To induce chemical LTP, hippocampal neuronal cultures at 14 DIV were first incubated in artificial cerebrospinal fluid (ACSF) for 30 minutes: 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 33 mM D-glucose, and 25 mM HEPES (pH 7.3; 320 mosM final), followed by stimulation with 50 μM forskolin, 0.1 μM rolipram, and 100 μM picrotoxin (Tocris) in ACSF (no MgCl2). After 16 minutes of stimulation, neurons were replaced in regular ACSF for 15 minutes and then subjected to TIF extraction or surface-expression assays[288,289]. TIF was isolated neurons as previously described [191].

3.11 INTERNALIZATION ASSAYS

To label surface TacADAM10-RAR, live COS7 cells were incubated with anti-Tac antibody for 45 minutes in medium at 4°C, while live neurons were labelled by incubation in ACSF with anti-Tac antibody for 30 minutes at 10°C. After brief washing in DMEM, COS7 cells were returned to 37°C for 10 minutes. Neurons were washed with cold ACSF, and internalization was allowed at 37°C in response to cLTP induction (16 minutes plus 15 minutes to allow internalization). A parallel set of controls was kept at 4–8°C to stop trafficking. Cells were then fixed with 4% PFA and 4% sucrose in PBS,

pH 7.4 and blocked with normal serum; remaining surface Tac chimeras were labeled with 488 secondary antibody (nonpermeabilized), and internalized receptors were labeled with 555-conjugated secondary antibody after Triton permeabilization. For COS7 internalization experiments, wide-field fluorescence images were acquired with a Zeiss ×40 objective and a CoolSnap CCD camera.

3.12 FRAP EXPERIMENT

Hippocampal neurons (DIV13) were transfected with GFP-Actin plus SH CAP2 or SCR as above. 48 hr later. After acquiring images from both channels, FRAP was performed only on the GFP-actin channel using the confocal LSM510 Meta system (Zeiss) with a 63X objective. Prebleach fluorescent signal was acquired using a 488 nm line argon laser. A circular Region of Interest (ROI, 2 μm diameter) on a selected dendritic spine head was photobleached by scanning with the 488 nm argon laser line at 100% laser power with pixel dwell time of 2.2 μs, using acquisition settings of 256 × 256.

3.13 ANTIBODIES

The following primary antibodies were used: Ant-CAP2 (Santa Cruz Biotechnology 1:100 WB and 1:200 ICC), Ant-CAP2 (Proteintech 1:1000 and 1:3000 WB and 1:100 ICC), Anti-Actin (Sigma-Aldrich 1:10000 WB), Anti-Actin (Sigma-Aldrich 1:3000 WB), Anti-Tubulin (Sigma-Aldrich 1:20000 WB), Anti-Myc (9E10) (Roche 1:1000 WB and ICC), Anti-Myc (Santa Cruz Biotechnology 1:200 WB and 1:500 ICC), Anti-GFP (Synaptic System 1:500 WB), Anti-GFP (Millipore 1:500 ICC), Anti-GFP (Aves 1:1400 ICC), Anti-Cofilin (Cell Signallin 1:1000 WB), Anti-MAP2 (Millipore 1:500 ICC), Anti-GFAP (Cell Signalling 1:200 ICC), Anti-PSD-95 (Neuromab 1:300 ICC and 1:1000 WB), Anti-Bassoon (Neuromab 1:300 ICC), Anti-Synaptophisin (Synaptic System 1:1000 WB) .The following secondary antibodies were used: goat anti-mouse-HRP (172–1,011) and goat anti-rabbit-HRP (172–1,019, Bio-Rad), goat anti-mouse-Alexa488 (A-11029), goat anti-mouse-Alexa555 (A-21422), goat anti-mouse-Alexa633 (A-21052), goat anti-rabbit-Alexa488 (A-11034) and goat anti-rabbit-Alexa555 (A-21429; Life Technologies).

4. RESULTS

4.1 CAP2 IS A NOVEL BINDING PARTNER OF ADAM10

To confirm the results of the two-hybrid screening, we carried out co-immunoprecipitation assays from homogenates of rat hippocampus. As shown in Fig. 1A, CAP2 is able to precipitate both ADAM10 and actin, but not ADAM22, another member of the ADAM family. No signal is detectable when the sample is precipitated without CAP2 antibody. Moreover, ADAM10 is not capable of precipitating CAP1, the protein homologous to CAP2, demonstrating the specificity of the complex ADAM10/CAP2. No signal is revealed when the sample is precipitated without ADAM10 antibody or in samples without homogenate.

These results were confirmed by colocalization analysis performed in COS7 cells transfected with ADAM10 and Myc CAP2. As shown in Fig. 1B, CAP2 expression modifies ADAM10 distribution pattern, enriching the enzyme in CAP2-clusters. Moreover, ADAM10 interaction with CAP2 was confirmed by proximity ligation assay (PLA). As shown in Fig. 1C, PLA signals were detected when the two antibodies recognizing ADAM10 and CAP2 were used, indicating that these two proteins are in close proximity (<40 nm) to each other. In order to identify the domain of ADAM10 cytoplasmic tail recognized by CAP2 we carried out pulldown assays. Fusion proteins of glutathione-S-transferase (GST) and GST bound to ADAM10 C-terminal domain (GST- ADAM10 Ct) were incubated with rat hippocampus homogenate. GST- ADAM10 Ct was able to specifically pull-down CAP2. Therefore, we tested a series of deletion mutants of GST-ADAM10 Ct, to identify the domain of ADAM10 responsible for the interaction with CAP2 (Fig. 1D). Truncation of the last 41 aa of ADAM10 tail (GST-708Δ) abolished binding to CAP2 (Fig. 1E), indicating that the ADAM10 most membrane proximal Pro Rich region is required for the interaction with CAP2.

On the other hand, we generated different deletion mutants of EGFP-CAP2 to define the CAP2 domain required for its interaction with ADAM10 (Fig. 1F). We deleted the last 22 aa (452Δ), the C-terminal domain and the Pro-rich region 2 (311Δ), the Pro-rich regions (232Δ)

HEK 293 cells stably transfected with ADAM10-HA were transfected with either EGFP CAP2 or EGFP CAP2 452Δ or EGFP CAP2 311Δ, or EGFP CAP2 232Δ. The cells extracts were immunoprecipitated with the HA antibody and the immunocomplex

probed for EGFP. Fig. 1G shows that the first 232 amino acids of CAP2 contains the ADAM10-binding site, revealing that ADAM10 interacts with the N-terminal part of CAP2. All together these data demonstrate that CAP2 is a novel protein partner of ADAM10 in neuronal cells, since CAP2 N-terminal domain is able to interact with ADAM10 most membrane-proximal sequence in its cytoplasmic domain.

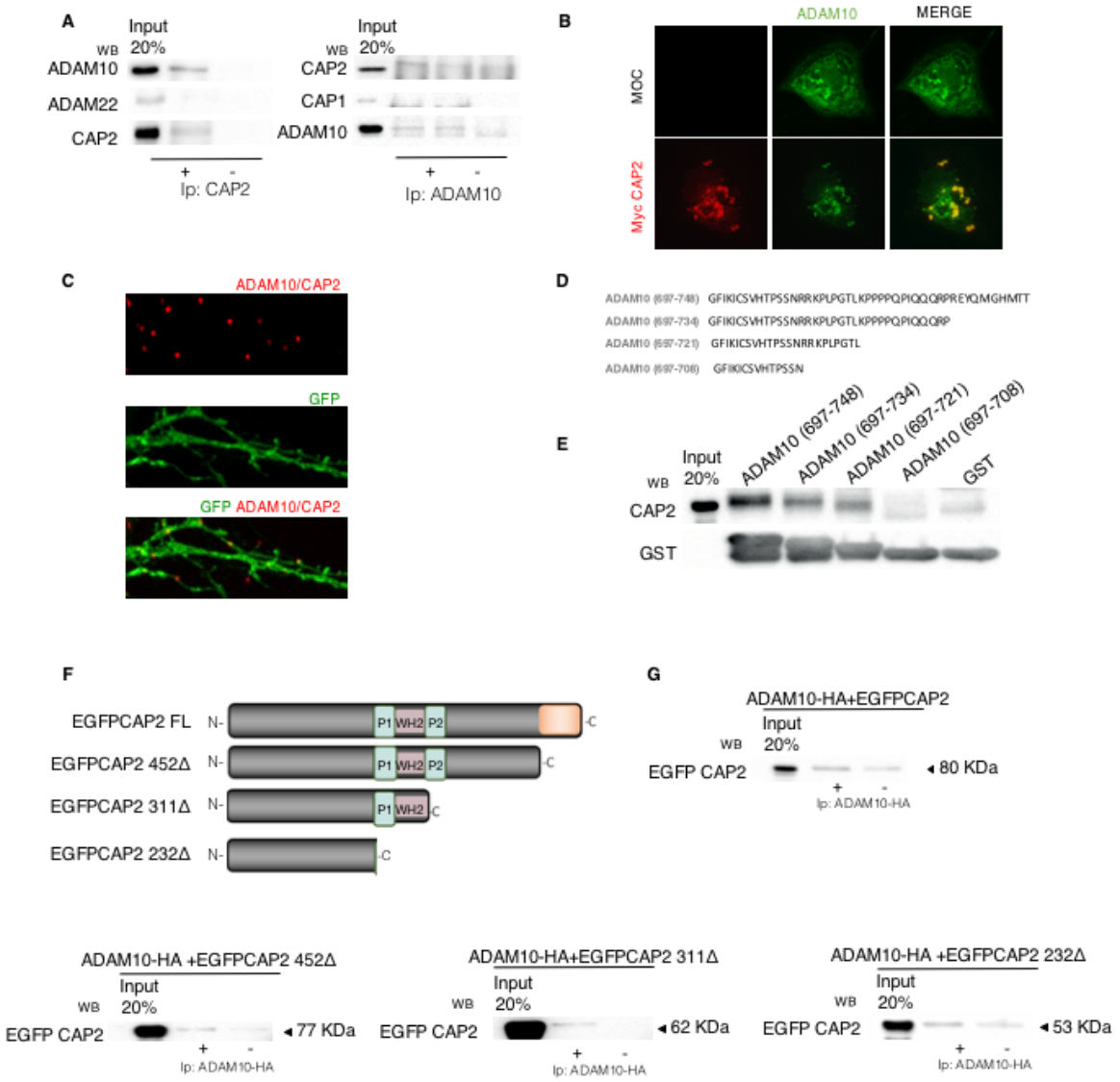
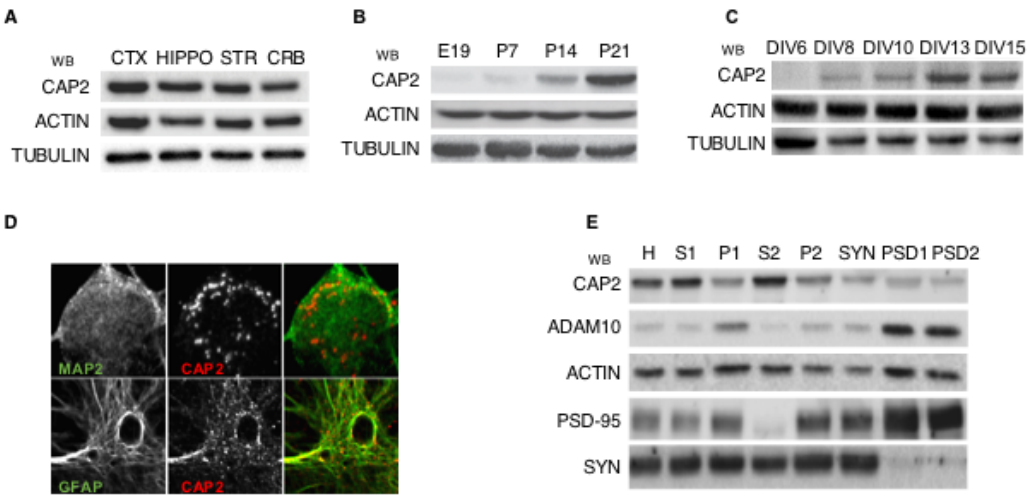


Figure 1. CAP2 is localized in the postsynaptic compartment but it is not enriched in the PSD.
A. Expression of ADAM10, CAP2, Actin in brain areas: HIPPO hippocampus, STR striatum, CTX cortex, CRB cerebellum. Tubulin was used as internal control.
B. Time course of expression of ADAM10, CAP2, Actin in brain homogenate of rats at E9, P7, P14 and P21 (E = embryonic day, P = post-natal). Tubulin was used as control.
C. Time course of expression of ADAM10, CAP2, Actin in hippocampal neurons at DIV6, 8, 10, 13, 15 (DIV= day in vitro). Tubulin was used as control.
D. Immunocytochemistry of CAP2, MAP2(neuronal marker) and endogenous GFAP (astrocytes marker).
E. Post-synaptic density purified from adult rat hippocampi, CAP2 is present all the fractions but not enriched in PSD as ADAM10. Legend: Homo, Homogenate; S1, Supernatant 1; P1, Pellet 1; S2, Supernatant 2; P2, Pellet 2; S3, Supernatant fraction; P3, microsomal fraction; PSD1; PSD2, post-synaptic density.

F. Representative staining of CAP2 and synaptic markers. After fixation, neurons were stained with post and pre synaptic markers, PSD-95 and Bassoon respectively, to evaluate the localisation of CAP2 at the synapse.
G. Immunocytochemistry of CAP2, phalloidin (F-actin marker) and endogenous PSD-95 in DIV15 primary hippocampal neurons.

4.2 CAP2 localization in the brain

Since a careful characterization of CAP2 function in the brain is still missing (Kumar et al., 2016), first we determined CAP2 expression profile in different brain areas and CAP2 localization at a subcellular level. Western Blot analyses revealed similar CAP2 protein levels in cortex, hippocampus, striatum and cerebellum. The analysis of CAP2 expression during development showed an increase from postnatal day 14 and, in hippocampal neuronal culture, from day in vitro 8 (Fig. 2A, B, C).
Immunostaining assays performed with a CAP2 antibody and markers for neuronal cells (MAP2) and astrocytes, as GFAP, demonstrated that CAP2 is expressed in both cells (Fig. 2D). To investigate CAP2 subcellular localization we took advantage of a biochemical fractionation approach to isolate excitatory PSDs from rat hippocampus. We found that CAP2 is present in all the fractions but is not enriched in the PSD, as ADAM10 (Fig. 2E). Focusing on the synaptic localisation of the protein, confocal analyses revealed that CAP2 co-localizes with PSD-95, a post-synaptic marker, but not with Bassoon, a presynaptic protein (Fig. 2F). Moreover, CAP2 showed a partial colocalization with the postsynaptic protein PSD- 95, while it perfectly colocalizes with phalloidin, a marker for F-actin, suggesting that CAP2 is localized in the region close to the PSD, where the actin cytoskeleton is present (Fig. 2G).



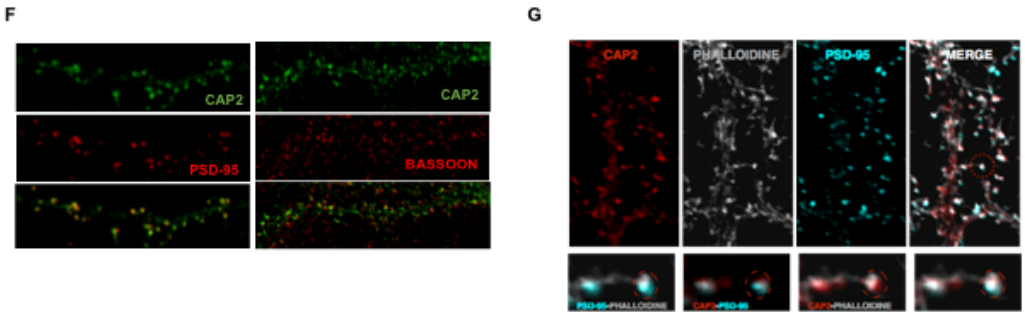


Figure 2. CAP2 is localized in the postsynaptic compartment but it is not enriched in the PSD.
A. Expression of ADAM10, CAP2, Actin in brain areas: HIPPO hippocampus, STR striatum, CTX cortex, CRB cerebellum. Tubulin was used as internal control.
B. Time course of expression of ADAM10, CAP2, Actin in brain homogenate of rats at E9, P7, P14 and P21 (E = embryonic day, P = post-natal). Tubulin was used as control.
C. Time course of expression of ADAM10, CAP2, Actin in hippocampal neurons at DIV6, 8, 10, 13, 15 (DIV= day in vitro). Tubulin was used as control.
D. Immunocytochemistry of CAP2, MAP2(neuronal marker) and endogenous GFAP (astrocytes marker).
E. Post-synaptic density purified from adult rat hippocampi, CAP2 is present all the fractions but not enriched in PSD as ADAM10. Legend: Homo, Homogenate; S1, Supernatant 1; P1, Pellet 1; S2, Supernatant 2; P2, Pellet 2; S3, Supernatant fraction; P3, microsomal fraction; PSD1; PSD2, post-synaptic density.
F. Representative staining of CAP2 and synaptic markers. After fixation, neurons were stained with post and pre synaptic markers, PSD-95 and Bassoon respectively, to evaluate the localisation of CAP2 at the synapse.
G. Immunocytochemistry of CAP2, phalloidin (F-actin marker) and endogenous PSD-95 in DIV15 primary hippocampal neurons.

4.3 CAP2 FORMS DIMERS

Since CAP2 staining appears punctuate (Fig. 1D) and it has been reported that the dimerization is an important characteristic of CAP1 (Hubberstey et al., 1996), we hypothesized that also CAP2 could form dimers. First of all, to test this hypothesis COS 7 cells were co-transfected with Myc CAP2 and EGFP CAP2. As shown in Fig. 3A, Myc CAP2 and EGFP CAP2 perfectly colocalize in perinuclear clusters. In addition, we performed immoprecipitation assays from the lysates of COS-7 cells transfected with Myc CAP2 and EGFP CAP2. The precipitation of Myc CAP2 implicates the co-precipitation of EGFP-CAP2, suggesting that CAP2 can associate in aggregates.

In order to identify the domain responsible for the self-association, we took advantage several deletion mutants of EGFP CAP2 (Fig. 1F) to performed a co-immunoprecipitation assays from lysates of COS 7 cells transfected with Myc CAP2 full length and the deletion mutants of EGFP CAP2. We found out that the deletion of the Proline rich regions of CAP2 and the WH2 domain did not interfere with the dimer formation, since the presence of the N-terminal (1-232 aa) was sufficient for the interaction with CAP2 full length (Fig. 3B). To narrow down the region responsible for

the dimerization we collaborated with Dr. Di Marino (University of Lugano), who carried out a computational analysis that predicted the first N-terminal 165 aa as the dimerization region (Fig. 3C).

We generated the mutant Myc CAP2 165-476, lacking of the predicted dimerization domain. When transfected in COS7 cells, Myc CAP2 165-476 displayed a diffuse pattern through the cell and no clusters were detected as in cells expressing Myc CAP2 FL and Myc CAP2 232 Δ (Fig. 3D).

To verify the presence of the dimer in physiological conditions, we loaded samples of total homogenate and Triton-Insoluble Fraction (TIF), which is enriched in postsynaptic proteins, onto a native-like condition gel. We detected a band corresponding to CAP2 monomer (53 kDa) and a band with an apparent molecular weight of 106 kDa that could correspond to a CAP2 dimer (Fig. 3E). Interestingly, the CAP2 dimer is enriched in the the TIF when compared with the total homogenate.

The CAP2 monomer and dimer are also detectable when we load lysates of Myc CAP2-transfected HEK293 cell onto a native-like. However, when we load homogenate of cells expressing the mutant Myc CAP2 165-476, lacking of the N-terminal domain, the band with an apparent molecular weight of 106 kDa is not revealed. Since the detection of such 106 kDa band depends on β-mecaptoethanol presence in the loading buffer we hypothesized that a disulfide bond could be responsible for the dimer formation. In the domain 1-165 aa there is only the cysteine 32, thus suggesting that this aa can be relevant for the dimerization. In fact, when we load the extracts of cells transfected with the mutant of MycCAP2 carrying the single point mutation 32C to G onto a native-like gel, the band at 106 kDa is not detectable (Fig. 3F).

However, both Myc CAP2 C32 to G and Myc CAP2 165-476 were able to co-precipitate with EGFP CAP2 full length, suggesting that the Cysteine 32 is not the only residue responsible for CAP2 self-association (Fig. 3G). Indeed, it has been shown that there are at least two binding sites within CAP that mediate its dimerization (Hubberstey et al., 2002). Hubberstey and colleagues reported that the amino-terminal domain of human CAP (aa 1–228) interacts with itself as well as with the carboxyl-terminal domain (amino acids 253–475). Likewise, the C-terminal domain of CAP2 interacts with itself and with the N-terminal region through different types of chemical bonds. Moreover, it is still unclear in the literature whether CAP proteins form dimers or higher order structures, with several possibilities, such as C-C or C-N or N-N binding (Hubberstey et al., 2002).

Finally, we verified whether the mutant Myc CAP2 C32 to G is still able to bind ADAM10. We performed coimmunoprecipitation assay in HEK 293 stably transfected with HA ADAM10 and transfected with either Myc CAP2 full-length or the mutant Myc CAP2 C32 to G. As shown in Fig. 2H, the mutant Myc CAP2 C32 to G is still able to bind ADAM10, demonstrating that such residue is not important for ADAM10/CAP2 association.

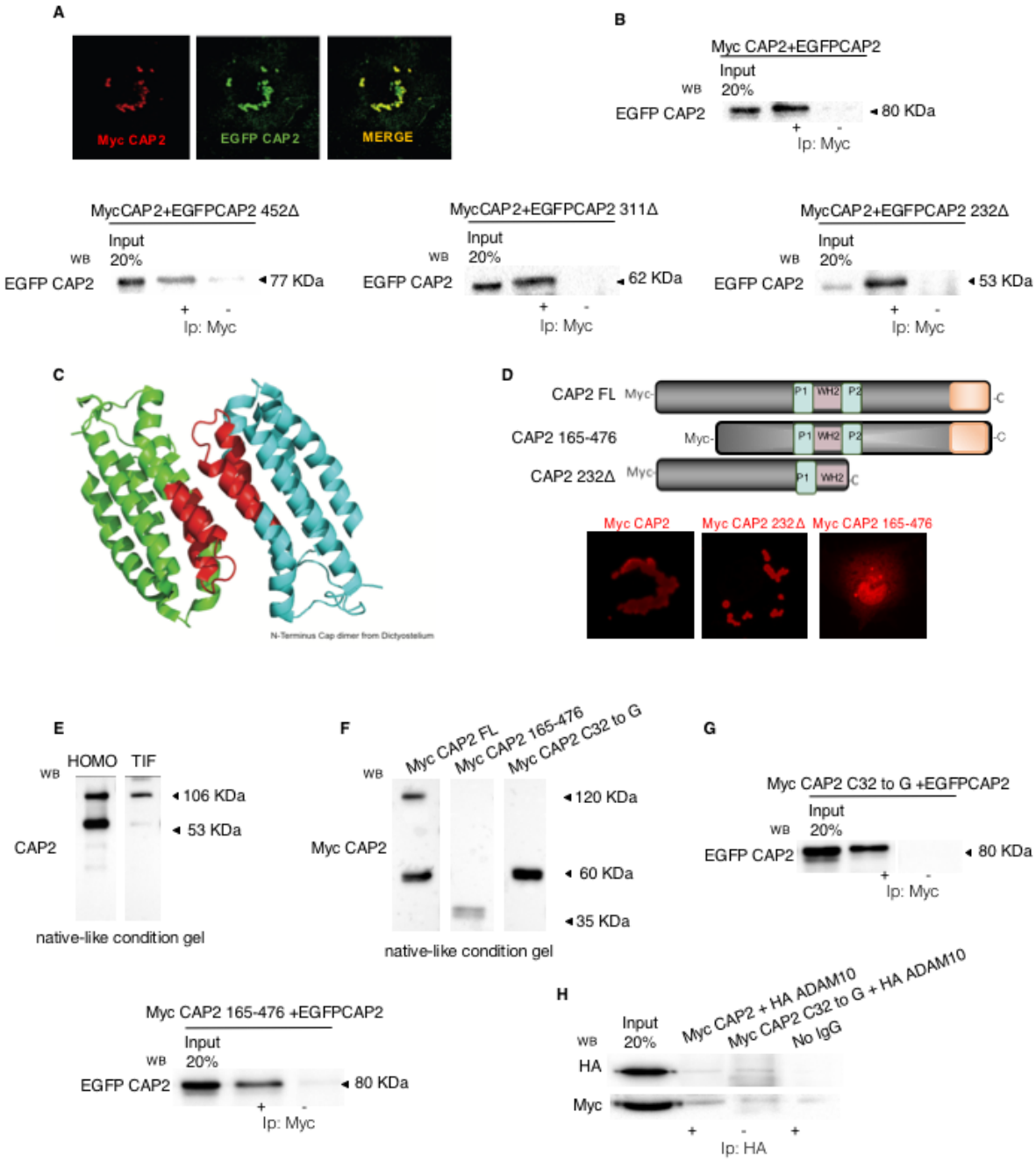


Figure 3. CAP2 is able to self-associate and forms dimers and cluster-like structures.
A. COS-7 cells transfected with Myc CAP2 and EGFP-CAP2 and stained with anti GFP and anti Myc antibodies; the analysis reveals that Myc CAP2 and EGFP-CAP2 colocalize in clusters.
B. Co-IP assays carried out from homogenate of HEK293 cells transfected with Myc CAP2 and different deletion mutants of EGFPCAP2. The sequence 1-232 of CAP2 is sufficient for CAP2 self-association.
C. Molecular modelling of N-terminal part of Cap of Dictyostelium.

D. Representative scheme of the different CAP2 mutant constructs transfected in COS7 cells. Confocal imaging of COS7 cells co-transfected with Myc CAP2 , Myc CAP2 232Δ and Myc CAP2 165-476 shows that the region 1-232 is sufficient for clusters formation while the deletion of the sequence 1-165 completely abolishes the presence of clusters .
E. Samples of rat brain homogenate and TIF were loaded onto a non-denaturating gel. Western blot analysis performed with CAP2 antibody showed a band corresponding to CAP2 (53 KDa) and another band at 106 KDa corresponding to a CAP2 dimer.
F. Homogenates of cells expressing different mutants of CAP2 were loaded onto a non-denaturating gel; WB analysis was performed with Myc antibody and revealed the different capability of the mutants to dimerize. The single point mutation 32C to G avoid the capability to form dimers onto a native-like condition gel.
G. Co-IP assays carried out from homogenate of HEK293 cells transfected with EGFP CAP2 and either Myc CAP2 C32 to G or Myc CAP2 165-476. The results show that this mutants are still able to interact with EGFP-CAP2.
H. Co-IP assays carried out from homogenate of HEK293 cells stable transfected with ADAM10-HA and Myc CAP2 C32 to G to evaluate its capability to bind ADAM10. This mutant is still able to associate to ADAM10.

4.4 CAPABILITY OF CAP2 TO MODULATE ACTIN DYNAMIC

CAP2 plays an important role in regulating the actin cytoskeleton and in signal transduction. It has been reported that in cardiomyocytes and keratinocytes, CAP2 is essential for normal actin organization because of the binding to G-actin, which regulates actin filament dynamics (Peche V.et al., 2007).

To verify it, we took advantage of COS7 cells as a quick and easy tool to analyse the actin dynamics. We transfected COS7 cells with Myc CAP2 and stained G-actin with DnaseI and F-actin with phalloidin. CAP2 full length colocalized with the monomeric actin but not with filamentous actin. The statistical analysis showed a significant increase in the G-actin/F-actin ratio in cells transfected with Myc CAP2 when compared to mock cells, suggesting that CAP2 mediates actin depolymerisation in COS7 cells (Fig. 4A). Moreover, we noticed that the actin dynamics was influenced by the aggregation status of CAP2.

We could recognize a population of cells in which CAP2 formed a single perinuclear structure (named polymeric structure) and other cells in which CAP2 was organized in small clusters scattered in the cytoplasm (called oligomeric structure). Interestingly, the G-actin/F-actin ratio was significantly higher in cells in which CAP2 was present as polymeric structure than in cells with CAP2 oligomeric structures, suggesting that CAP2 aggregation is important for actin dynamics. (Fig. 4B).

The next step was to investigate CAP2 directly in neurons. Immunostaining analyses revealed that CAP2 colocalized with F-actin. Indeed, CAP2/F-actin colocalization degree is significantly reduced by latrunculinA, a drug able to block actin polymerization,

thus indicating that CAP2 binds filamentous actin in neurons (Fig. 4C). Furthermore, we set up a biochemical assay to separate G-actin and F-actin. The results obtained confirmed the data of the immunostaining: CAP2 is enriched in the F-actin fraction. Moreover, we validated this assay by analysing the distribution of G-actin binding proteins, such as phosphorylated Cofilin, that is enriched in G-actin fraction, as expected (Fig. 4D).

Despite these unexpected results showing that CAP2 bids F-Actin and not G-actin in neuronal cells, we investigated the CAP2 role in actin polymerisation and we found out that CAP2 can mediate the depolymerisation of actin, since the overexpression of Myc CAP2 in neuronal cells leads to an increase in G-actin/F-actin ratio (Fig. 4E). According to these results, the transfection of CAP2 shRNA resulted in a decrease in G-actin/F-actin, indicating a shift towards F-actin (Fig. 4F).

The literature shows the complex role of CAP protein in actin dynamics. Indeed, several studies showed that CAP sequesters actin monomers, but also that CAP enhances the recharging of actin monomers promoting the severing of actin filaments in cooperation with ADF/cofilin. To clarify the mechanism by which CAP2 can modulate the actin dynamics in spines, we took advantage of fluorescence recovery after photobleaching (FRAP) experiments carried out in a single spine of hippocampal neuronal cells transfected with GFP actin, that was partially incorporated into the actin filaments, and either CAP2 shRNA or its scrambled control sequence (Koskinen et al,. 2012).

Actin filaments in spines normally turn over rapidly, due to treadmilling, while actin monomers exchange bidirectionally between spines and the adjacent dendritic shaft. If GFP-actin in a spine is photobleached, new fluorescent GFP-actin monomers normally diffuse into the spine and are incorporated into the barbed end of bleached filaments, predominantly in the juxtamembrane (‘shell’) region of the spine head (Hotulainen et al., 2009; Frost et al., 2010). Concomitantly, bleached actin molecules are severed from filament pointed ends (closer to the spine ‘core’) and exchange out of the spine, which recovers its fluorescence.

However, if turnover is impaired, bleached GFP-actin remains trapped within filaments and FRAP is attenuated. The fraction of actin filaments in the spine that are undergoing rapid turnover can thus be determined from the extent of FRAP (Star et al., 2002).

The recovery of GFP-actin fluorescence, after the spine photobleaching, in CAP2 knockdown neurons was significantly increased, compared to control neurons, indicating that is consistent with a significant decrease in the percentage of stable GFP-

actin in spines lacking of CAP2. In contrast to these marked effects on the pool of stable actin, the half-time of GFP-actin fluorescence recovery was not significantly different (Fig. 4G). Taken together, these data suggest that the predominant effect of loss of CAP2 on GFP-actin turnover is to increase the pool of mobile actin in spines, promoting the treadmilling of actin, thus leading to a faster rate of actin turnover.

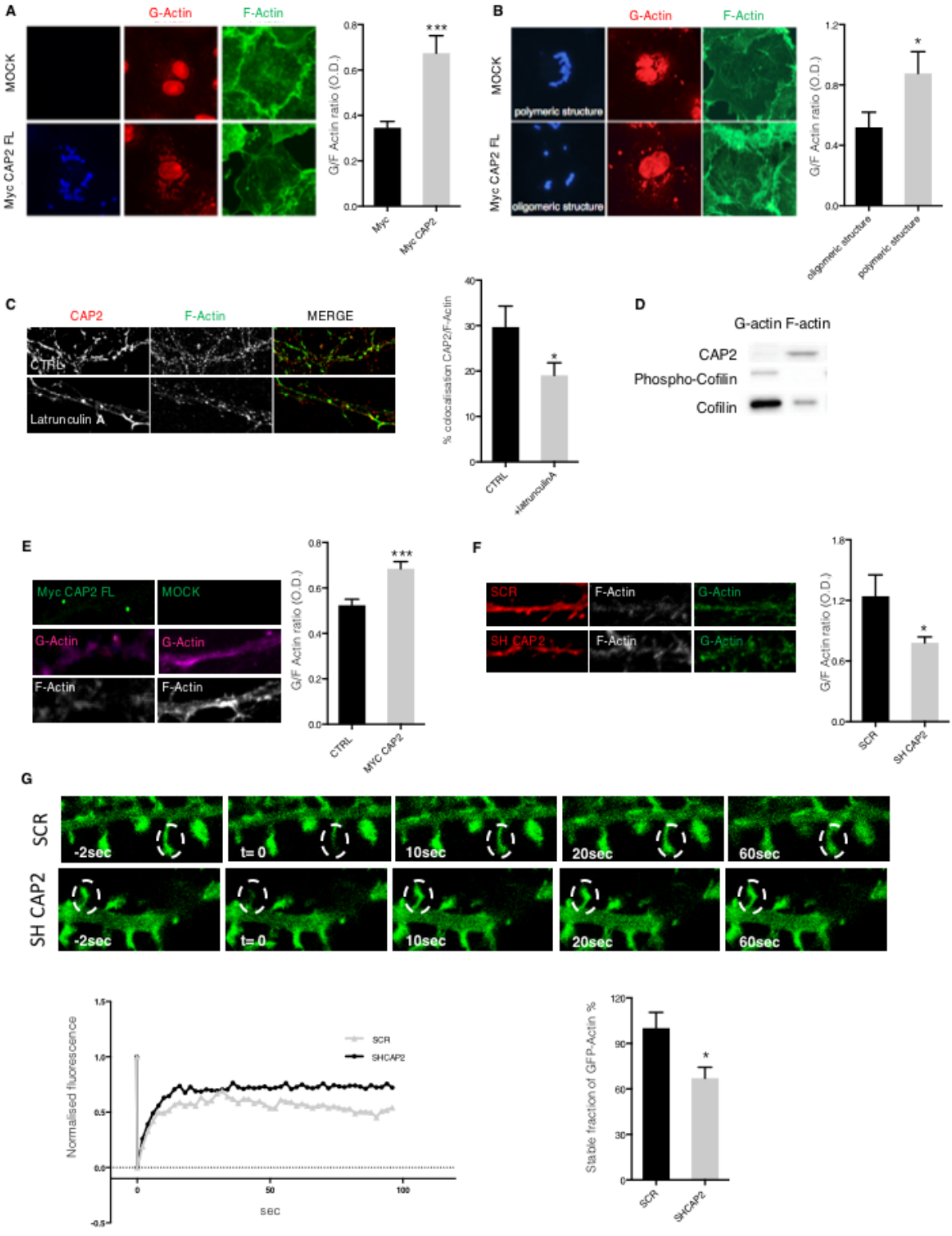


Figure 4. CAP2 affects actin dynamics in neurons and specifically in the spine.
A. COS 7 cells transfected with Myc CAP2 FL to evaluate the capability influence the polymerisation of actin. The ratio G-actin/Factin has been calculated by measuring the integrated density of DNAse (G-actin marker) and Phalloidin (F-actin marker) staining. Data are presented as mean ± s.e.m., n = 33–38, ***P< 0.001, unpaired Student's t-test.
B. G-actin/F-actin ratio was calculated in COS 7 cells transfected with Myc CAP2 FL presenting CAP2 as a single perinuclear structur(polymeric) e and in cells were CAP2 was aggregated in cluster localized in the cytoplasm (oligomeric). The capability of CAP2 to influence the G/F ratio is link to it's structure (oligomeric or polymeric structure). Data are presented as mean ± s.e.m., n = 17-15, *P< 0.05, unpaired Student's t-test.
C. Hippocampal neurons were treated with latrunculin A to evaluate the capability of CAP2 to bind F-actin. Immunostaining for CAP2 and F-actin (phalloidin) revealed that CAP2 colocalizes with F-actin, whereas the treatment with latrunculin A reduces the percentage of colocalisation. Data are presented as mean ± s.e.m., n = 17–15, *P< 0.05, unpaired Student's t-test.
D. Immunoblots of G-actin (G) and F-actin (F) fractions obtained from hippocampal neurons taking advantage of an Actin Polymerization Assay kit. CAP2 is present in the F-actin pellet, while Phospho Cofilin is present in the supernatant and Cofilin in both fraction, as expected.
E. Hippocampal neuron were transfected with Myc CAP2 to evaluate the capability of influencing the polymerisation of actin. As in COS-7 cells, CAP2 overexpression increases the G-actin/F-actin ratio. Data are presented as mean ± s.e.m., n = 19–29, ***P< 0.001, unpaired Student's t-test.
F. Hippocampal neuron were transfected with either SH CAP2 or the scrambled control sequence (SCR) and G-actin/F-actin ratio was assessed. The loss of CAp2 significantly decreases the G-actin/F-actin ratio. Data are presented as mean ± s.e.m., n = 22–22, *P< 0.05, unpaired Student's t-test.
G. The rate of actin turnover was analysed by FRAP in hippocampal neuron transfected with GFP actin and either SH CAP2 or SCR at DIV 11. The FRAP analysis was performed at DIV 13. FRAP curves (normalized to average pre-bleach
fluorescence), plotted from multiple single-spine ROIs for each condition. Histogram of the stable fraction of GFP-actin (mean ± SEM) calculated for each individual FRAP trace used to generate the pooled data

Since it was clear that CAP2 affects the actin dynamic, the next step was to identify the actin binding region in CAP2 sequence. First we demonstrated that CAP2 can associate with both actin and cofilin performing a coimmunoprecipitation assay from rat brain homogenate (Fig. 5A).

Then, we generated the fusion protein GST-CAP2 linking the GST to either CAP2 full-length or the deletion mutant GST-CAP2-452Δ lacking of the CAP2 C-terminal domain that has been reported to bind actin. Pull-down assays revealed that the GST-CAP2 452Δ mutant is not able to interact with actin, thus demonstrating that the last 22 aa of CAP2 are essential for actin binding (Fig. 5B). We confirmed these results carrying out a coimmunoprecipitation assay from the lysate of COS7 cells transfected with Myc CAP2 452Δ. This deletion mutant could not bind actin, but it is still able to interact with cofilin. In addition, we tested the binding capability of the Myc CAP2 C32 to G mutant and we verified that it can associate to both actin and cofilin (Fig. 5C). Moreover, when we loaded lysates of cells expressing the mutant Myc CAP2 452Δ onto a native-like gel, the 106 kDa band was detectable, suggesting that the lack of the last 22 aa does not affect CAP2 Cys32-dependent dimerization (Fig. 5D).

To unravel the effect of the loss of CAP2 actin binding domain on actin dynamics, we transfected either Myc CAP2 or Myc CAP2 452Δ in COS 7 cells and in hippocampal neurons. Surprisingly, the lack of the actin binding domain didn't revert the effect of CAP2 on the G-actin /F-actin ratio both in COS7 cells and in hippocampal neurons (Fig. 5E, F), indicating that this C-terminal domain is not important for the polymerization of actin.

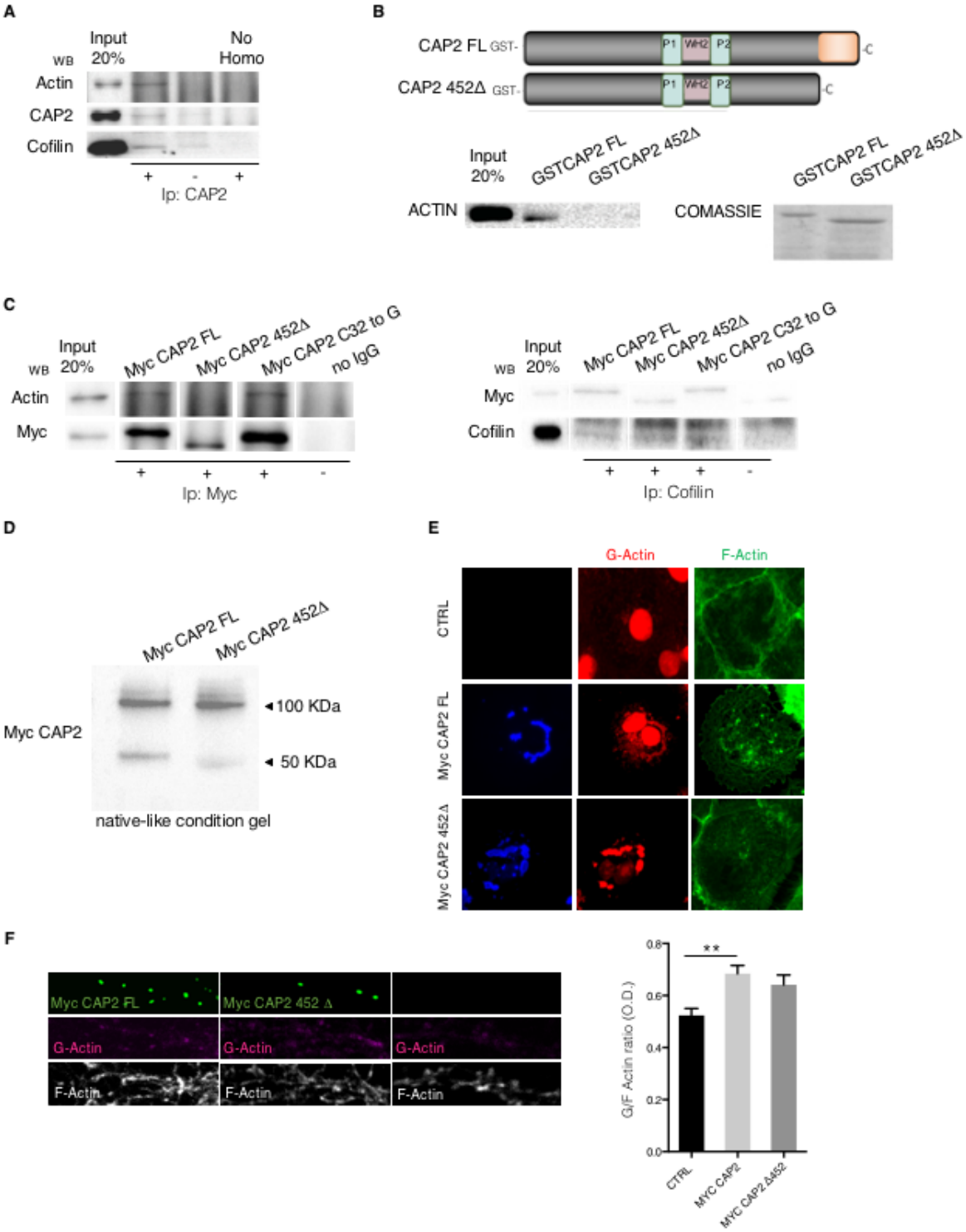
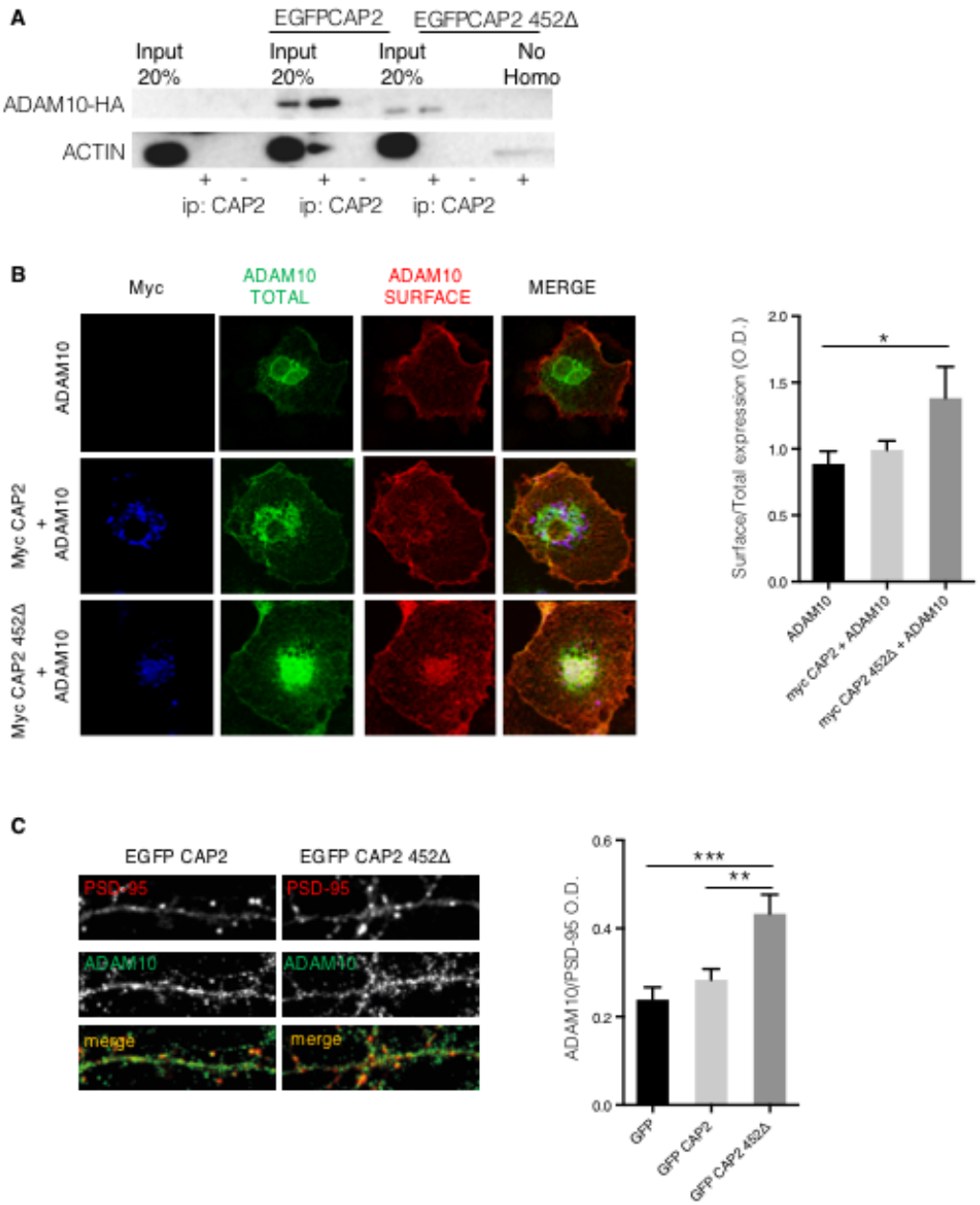


Figure 5. The actin binding domain of CAP2 is not relevant for the modulation of actin dynamics.
A. Rat brain homogenate was immunoprecipitated (IP) with CAP2 antibody and WB analysis was carried out with an anti actin and cofilin antibody. CAP2 specifically interacts with both actin and cofilin.
B. Actin interacts with the C-terminal tail of CAP2. GST CAP2 FL– full length, GST CAP2 Δ452 –(1-452) were incubated in a pull-down assay with rat brain homogenate. WB analysis was performed with ACTIN antibody. GST proteins were stained by Coomassie staining. The deletion of the last 24 amminoacids abolishes actin binding to CAP2, suggesting that this is the sequence responsible for the interaction.
C. Co-IP assays carried out from homogenate of HEK293 cells transfected with different mutants of Myc CAP2 to evaluate their capability to bind actin and cofilin.
D. The mutant Myc CAP2 Δ452 is able to create dimers, as shown by the presence of the band at 106 kDa in native-like gels.
E. COS 7 cells were transfected with Myc CAP2 FL and Myc CAP2 Δ452 to evaluate the capability of influencing the polymerisation of actin. Myc CAP2 induces the depolymerisation of actin, and the lack of the actin binding domain does not affect this property. Data are presented as mean±s.e.m., n= 33-38, ***P<0.001; n= 33-38, *P<0.05; one-way ANOVA followed by Bonferroni post-hoc test.
E. Hippocampal neuron were transfected with Myc CAP2 to evaluate the capability of influencing the polymerisation of actin. As in COS7 cells, CAP2 overexpression increases G-actin/F-actin ratio and the lack of the last 24 aa does not influence this effect, Data are presented as mean±s.e.m., n= 19;29; **P<0.005; one-way ANOVA followed by Bonferroni post-hoc test.

4.5 CAP2 ACTIVITY ON ADAM10 LOCALIZATION

Given the importance of the CAP family in protein trafficking and endocytosis, we evaluated that if CAP2 and, in particular, its actin binding domain played a relevant role in ADAM10 membrane localization. First, we performed coimmunoprecipitation experiments from the homogenate of ADAM10-HA stable transfected HEK293 cells transfected with either EGFP-CAP2 or EGFP CAP2 452Δ. As shown in Fig. 6A, we confirmed that EGFP CAP2 452Δ can't bind actin but it is still able to associate to ADAM10, indicating that ADAM10 and actin binding region did not overlap in CAP2.
Thus, we decided to evaluate the membrane localization of ADAM10 in COS7 cells transfected with ADAM10 and either with CAP2 full length (CAP2 FL) or the mutant lacking of the actin binding domain (CAP2 452Δ). The quantitative analysis showed that the mutant lacking of the actin binding domain significantly increases the surface localization of ADAM10 while the CAP2 FL didn't affect the membrane level of the protein (Fig. 6B). We confirmed this result in neuronal cells where the co-localization between ADAM10 and PSD-95 increased in neuron transfected with the mutant CAP2 452Δ while, again, the CAP2 FL didn't modulate the synaptic localization of ADAM10 (Fig. 6C).
Since the actin binding site of CAP2 affects the levels of surface level of ADAM10, we investigated which mechanism is responsible for the modulation of this process. We decided to evaluate two pathways that are fundamental for ADAM10 localization: the forward trafficking and the endocytic process of the protein (Marcello et al., 2013). First, we performed an internalization assay in COS7 cells transfected with TacADAM10-RAR

and either Myc CAP2 full length or the mutant Myc CAP2 452Δ. The mutant of CAP2 significantly impaired the endocytosis rate of ADAM10 while CAP2 full length didn't modulate ADAM10 internalization (Fig. 6D). The actin cytoskeleton plays an essential role in endocytosis. Actin assembly can create protrusions that encompass extracellular materials. Actin can also support the processes of invagination of a membrane segment into the cytoplasm, elongation of the invagination, scission of the new vesicle from the plasma membrane, and movement of the vesicle away from the membrane (Mooren et al., 2012). Therefore, these data suggest that CAP2 is involved in ADAM10 endocytosis, probably anchoring the protein to the actin cytoskeleton.



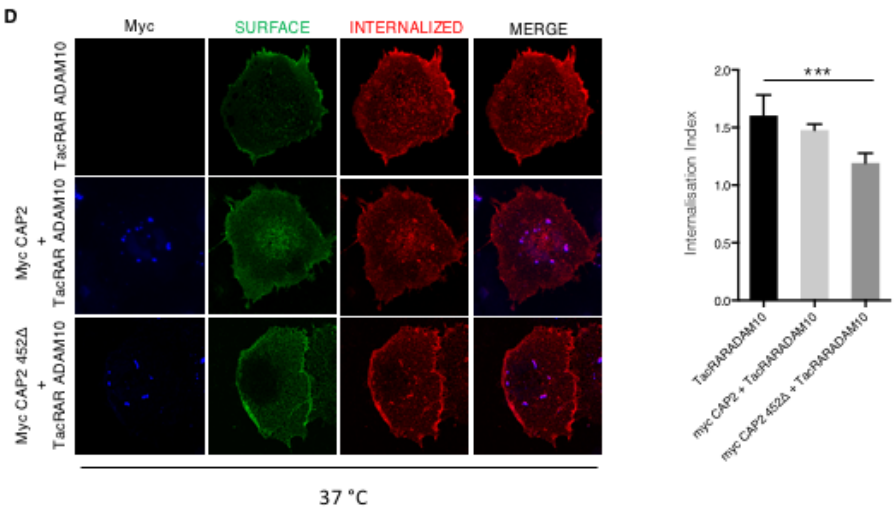


Figure 6. CAP2 actin binding domain impairs ADAM10 endocytosis.
A. Co-ip assay revealed the capability of CAP2 452D to bind ADAM10, even if it can't bind actin.
B. COS-7 cells were cotransfected with ADAM10 and either Myc CAP2 or the mutant Myc CAP2 Δ452, the mutant lacking of the actin binding domain. The expression of Δ452 significantly increases ADAM10 membrane localisation. Data are presented as mean±s.e.m., n= 16;13; *P<0.05; one-way ANOVA followed by Bonferroni post-hoc test.
C. Hippocampal neurons cotransfected with GFP, EGFPCAP2, or EGFPCAP2 Δ452. Cells were fixed, and stained for endogenous ADAM10 and PSD-95. The transefection of CAP2 does not affect ADAM10 synaptic localisation, while the expression of the mutant Δ452 significantly increases ADAM10 colocalisation with PSD-95. Data are presented as mean±s.e.m., n= 43;21; ***P<0.001; n= 46;21; **P<0.005; one-way ANOVA followed by Bonferroni post-hoc test.
D. Antibody uptake assays were performed on COS7 cells transfected with either TacADAM10-RAR and Myc-CAP2 or with the mutant Myc-CAP2 Δ452. Representative images of cells returned to 37°C to allow endocytosis. The expression of the mutant lacking of the actin-binding domain significantly impairs ADAM10 endocytosis. Data are presented as mean±s.e.m., n= 11;11; *P<0.05; one-way ANOVA followed by Bonferroni post-hoc test.

4.6 ADAM10/CAP2 COMPLEX IN SPINE REMODELLING

We hypothesized that CAP2 could affect spine morphology, since modulates actin pools in the spines, as demonstrated by FRAP experiments (see Fig. 3G), and can influence ADAM10 localization and, thereby, its synaptic activity. Indeed, ADAM10 is important for the spine morphology because works as sheddase towards a number of neuronal adhesion molecules.

To test this hypothesis, first we transfected hippocampal neuronal cells with CAP2 shRNA and the corresponding control. Our analysis revealed that there was no significant difference in the neck width, the head width and the density of spines, whereas we detected a significant difference in the length of the spines, suggesting that CAP2 is involved in the remodelling of the spine shape, which is deeply linked to the actin polymerization (Fig. 7A). The morphology of hippocampal neurons was also analysed by tracing the dendrites of neurons transfected with GFP and either CAP2

shRNA or the scrambled control sequence and we found out that the absence of CAP2 reduce the complexity of dendrites branching (Fig. 7B). We analysed the spine morphology also in neurons overexpressing either CAP2 full length or the mutant CAP2 452Δ. We used the GFP to fill the neuron and we noticed that CAP2 full length overexpression increased the spine head width, without affecting spine length and spines density. Notably, this effect was reverted by the lack of the last 22AA in the mutant CAP2 452Δ, thus suggesting that the actin binding site of CAP2 is essential for the spine remodelling related to CAP2 (Fig. 7C). We have shown that this actin binding site in CAP2 is not involved in the regulation of actin dynamics but has a role in the control of ADAM10 membrane levels. Therefore, the lack of the last 22 influence spine shape because it impairs ADAM10 endocytosis, thus increasing its synaptic levels and thereby its shedding activity towards neuronal adhesion molecules.

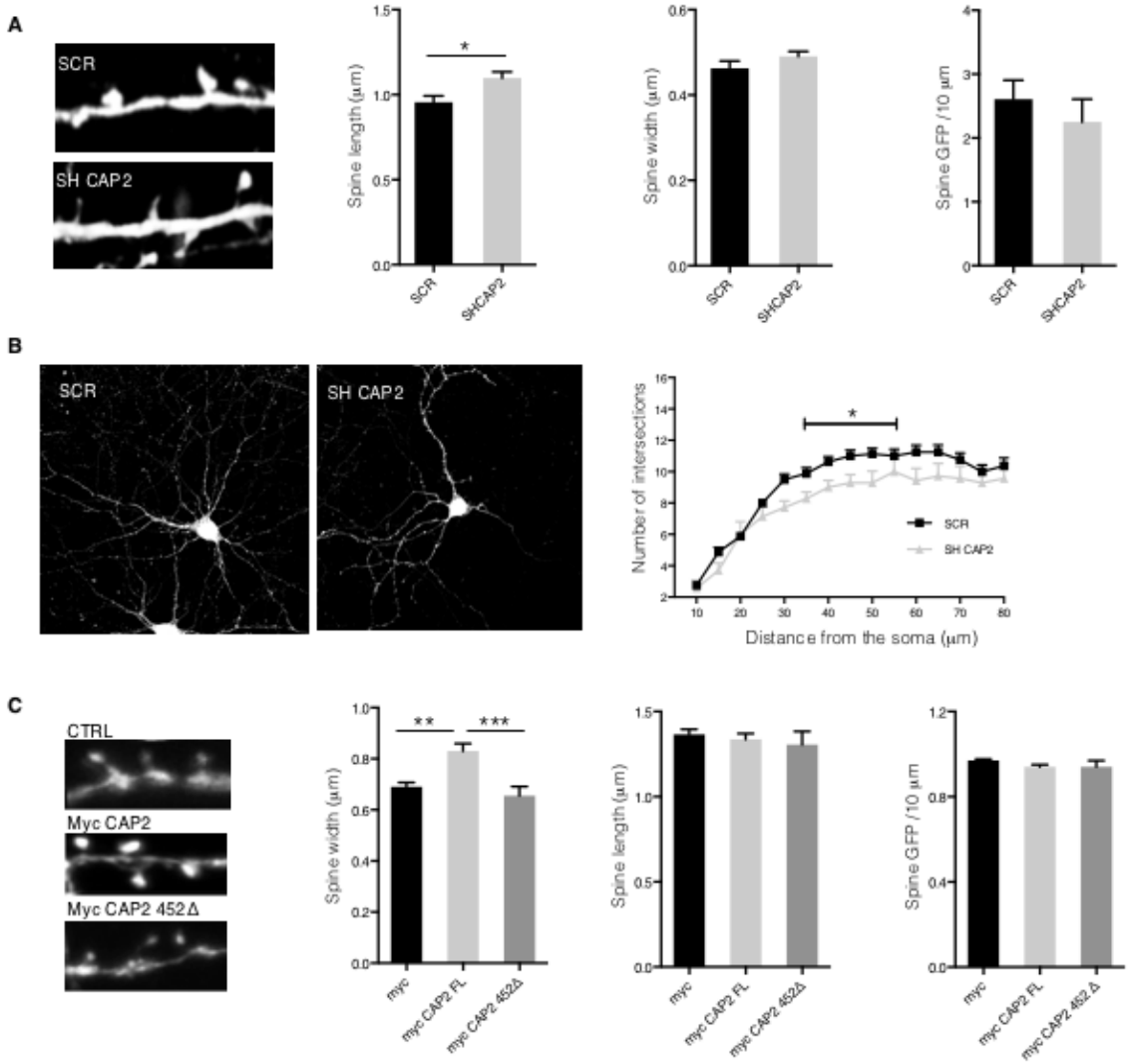


Figure 7. CAP2 modulates spine morphology.
E. The overexpression of CAP2 in hippocampal cultures significantly increases the width of dendritic spines without changing the length and the density; while the mutant 452D doesn't affect significantly the width parameter. Data are presented as mean±s.e.m., n= 10;10; **P<0.005; n= 10;10; ***P<0.001; one-way ANOVA followed by Bonferroni post-hoc test.
F. The silencing of CAP2 leads to a significant increase of the spine length. Data are presented as mean ± s.e.m., n = 7-7, *P< 0.05, unpaired Student's t-test.
G. Representative images of hippocampal neurons transfected with GFP and cotransfected with either SHCAP2 or SCR. Sholl analysis shows a decrease in dendrite branching in neurons lacking of CAP2

The importance of ADAM10 in spine remodelling is demonstrated by the synaptic plasticity-dependent modulation of ADAM10 synaptic localization/activity. It has been reported that LTD promotes ADAM10 synaptic membrane insertion and stimulated its activity. ADAM10 interaction with SAP97 is necessary for LTD-induced ADAM10 trafficking and required for LTD maintenance and LTD-induced changes in spine morphology. While in hippocampal neuronal cultures LTP induces ADAM10 endocytosis through AP2 association and decreases synaptic ADAM10 levels and activity (Marcello et al., 2013).

Therefore, we wondered whether activity-dependent synaptic plasticity modulates also CAP2 synaptic availability in the dendritic spines. To test this hypothesis, we induced chemical LTP (cLTP), using a highly validated protocol, in hippocampal cultures (Marcello et al, 2013). To induce LTP, we used a chemical stimulation protocol with forskolin plus rolipram for 16 minutes that results in prolonged NMDA receptor-dependent LTP (cLTP). 15 min after cLTP induction, TIF was purified from control and cLTP-treated hippocampal neurons, and TIF samples were loaded onto a native-like gel to reveal CAP2 monomer and dimer. cLTP stimulation caused a significant increase in CAP2 dimer levels in TIF (Fig. 8A). We analysed also the interaction of CAP2 and actin and coimmunoprecipitation assays demonstrated a significant decrease in the association between CAP2 and actin (Fig. 8B). Taken together these data demonstrate that LTP can modulate CAP2 properties promoting the C32-dependent dimerization in the synapses and inducing actin detachment from CAP2.

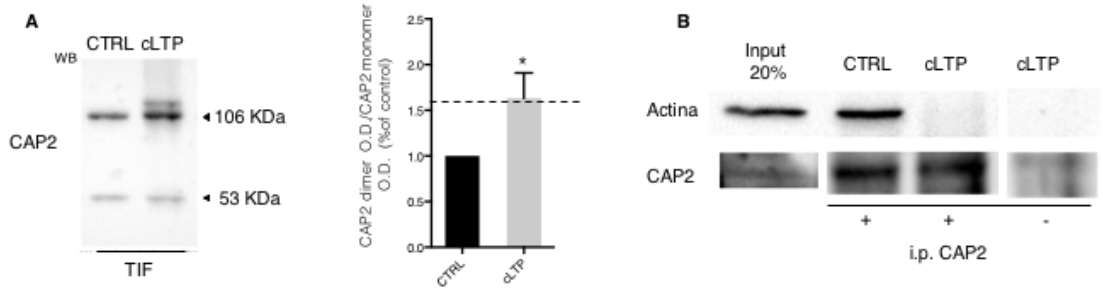


Figure 8. cLTP can modulate CAP2 properties.

A. Tif purification of control and cLTP-treated neurons. cLTP (15') promotes CAP2 dimerization in the TIF fraction. The ratio dimer/ monomeris expressed as percentage of control. Data are presented as mean ± s.e.m., n = 8, *P< 0.05, paired Student's t-test.
B. CAP2/Actin co-IP assays performed on tif of control and cLTP-treated neurons. cLTP (15') abolishes CAP2/Actin binding in the TIF fraction.

5. CONCLUSION

Several studies highlighted the key role of the disintegrin and metalloproteinase family member ADAM10 in health and disease, due to its shedding activity toward a number of functional membrane proteins such as APP and N-cadherin [271]

The proteins at the surface of cells play important functions for the cell-cell communication, intracellular signalling and they can control the synapse remodelling. The control of the cell surface protein indirectly regulates the shape of the spines and thereby their functionality. Indeed, spines undergo dynamic changes in their morphology, and this structural modifications correlate with the alterations in synaptic strength[228]. Therefore, ADAM10 activity can control the synaptic function through the cleavage of its substrates. On the other hand, ADAM10 undergoes a dynamic regulation of its activity by synaptic plasticity, that modulates its localisation. The synaptic localisation is crucial for ADAM10 synaptic activity because ADAM10 can act on its substrate only when it is inserted in the plasma membrane. Indeed, LTD promotes ADAM10 membrane insertion through the association with SAP97, whereas LTP reduces the enzyme membrane levels by inducing AP2-mediated endocytosis. In light of the above, ADAM10 delivery to the postsynaptic compartment seems to be critical for synaptic activity–induced spine remodelling. In fact, ADAM10 cleavage of synaptic adhesion molecules may allow the activated synapse to rapidly modulate the spine size during induction of activity-dependent synaptic plasticity [271].

Given the importance in the localisation of ADAM10 at the cell surface, the trafficking of the enzyme assumes a key role in the regulation of its activity. For this reason, we have performed a yeast two-hybrid screening of a brain cDNA library using the ADAM10 C-terminal tail as bait since this region represents the main biochemical locus for the regulation of the trafficking of the enzyme through protein-protein interaction. Among the positive clones, we found a protein of particular interest, i.e. CAP2. CAP proteins were originally described as Cyclase Associated Protein (hence CAP) from Saccharomyces cerevisiae [129]. CAPs are evolutionary highly conserved actin-binding proteins involved in changes in actin cytoskeleton and in vesicle trafficking/endocytosis, linking signalling pathways to elements of the cytoskeleton.

CAP2 is a multidomain protein expressed only in a limited number of tissues, including the brain, suggesting that CAP2 may have unique roles. CAP family has been described as an actin-monomer-binding protein that can sequester actin monomers and prevent them from polymerization in vitro [129]. Recent studies have revealed that CAP proteins

promote actin filament dynamics, cooperating with ADF/cofilin both in vitro and in vivo. First we confirmed the interaction between ADAM10 and CAP2 using different techniques, ranging from coimmunorpecipitation assays to PLA. The two proteins interact and this association is specific, since CAP1, the protein homologous to CAP2, does not bind ADAM10. Moreover, we identified the domains responsible for the association that are the most membrane proximal proline rich domain in ADAM10 tail and the N-terminal region of CAP2.

Given that, the first aim of our study was to characterize CAP2 in neuronal cells. We confirmed the presence of CAP2 in the brain and then we defined the localisation of the protein in the neuronal cell, showing that it is present in the excitatory neurons and it is localized in the postsynaptic compartment. However, CAP2 is not enriched in the postsynaptic density as ADAM10. Moreover, it has been reported in the literature that CAP2 can create aggregates through which it can regulate its activity (new). The real function of this aggregates and which domains of the protein are involved in the oligomerisation are not clear yet. It is known in the literature that Srv2/cyclase-associated protein (CAP) can create aggregates because of interactions between its C-terminal region (C) and the N terminal domain (N), but the interaction can occur also between C/C and N/N. We demonstrated that the Cysteine-32 is fundamental for the self-interaction in the N-terminal region. The biological role of this self-association is not clear yet, likely it can affect the localisation of the protein or it can optimize the CAP function. To clarify this issue, we analysed the major biological function of CAP2: the capability of binding actin and influencing its dynamics. Our results suggested that the CAP2 sequesters G-actin monomer in CAP2-clusters detectable in the cytoplasm and CAP2 overexpression triggers a shift of the G-actin/F-actin ratio towards G-actin, suggesting the induction of cytoskeleton depolymerisation. Interestingly, the rate of actin depolymerization depends on the aggregation form of CAP2. Indeed, the G-actin/F-actin ratio is higher in cells containing one single perinuclear structure compared to cells in which CAP2 is organized in cluseters. Even if CAP2 mainly binds G-actin in COS7 cells , we demonstrated that in neuronal cells CAP2 can bind F-actin. In neuronal cells, we observed that CAP2 overexpression can trigger the actin depolymerisation, while CAP2 downregulation reduces G-actin/F-actin ratio. To have more complete view of how CAP2 influences the actin dynamics in the spines, we took advantage of the FRAP analysis. We silenced CAP2 in hippocampal neuron overexpressing GFP-actin that was partially incorporated into the actin filaments [290]. The recovery of GFP-actin

fluorescence, after the spine bleaching, in CAP2 knockdown neurons was significantly increased, compared to control neurons, indicating that there was a significant decrease in the stable pool of GFP-actin in the spine. These results suggested that the absence of CAP2 increases the treadmilling of actin, leading to a faster rate of actin turnover. Thus CAP2 triggers a slower actin dynamic in the dendritic spine and, according to the literature, it can coordinate activities in the process of actin treadmilling catalysing different steps in actin turnover [137]. In support if this hypothesis, it has been recently described by Peche and colleagues that CAP2 binds cofilin in neuronal cells, thus suggesting that CAP2 takes part to the actin treadmilling cooperating with crucial steps of the depolymerisation dynamic. Probably CAP2 interacts with G and F actin mediating a complicated exchange of actin monomer, that involves the entire protein structure. Therefore, we aimed at identifying the actin binding in CAP2 sequence. In our hands, the sequence of CAP2 responsible for the actin binding is composed of the last 22 aa. The CAP2 mutant lacking of the last 22 aa was not able to bind actin. However, the loss of this domain did not revert the effect of CAP2 on G-actin/F-actin ratio in both COS7 cells and neuronal cells.

In light of the above, we assessed the effect of CAP2 on spine morphology: we found out that the silencing of the protein determines longer spines, as expected, since CAP2 downregulation decreases the stable pool of actin in the spines.

On the other hand, CAP2 overexpression leads to an increase in the width of the spine while. the transfection of the mutant lacking of the acting binding domain reverted the effect of CAP2 on spines. Since we have demonstrated that this actin binding domain does not affect actin dynamics, we wondered that the effect is mediated by ADAM10.

As previously reported, ADAM10 can modulate the morphology of the spines cleaving adhesion molecules, such as N-cadherin. Is CAP2 involved in the trafficking of ADAM10? We found out that CAP2 doesn't modulate the surface expression and the endocytosis of ADAM10 but, if we removed the actin binding domain of 22 aa in the C-terminus, we measured an increase in surface expression of ADAM10 and a decreased internalisation index. Therefore, this actin binding site in CAP2 is not involved in the regulation of actin dynamics but has a role in the control of ADAM10 membrane levels. Therefore, the lack of the last 22aa influences spine shape because it impairs ADAM10 endocytosis, thus increasing its synaptic levels and thereby its shedding activity towards neuronal adhesion molecules.

The importance of ADAM10 in spine remodelling is demonstrated by the synaptic plasticity-dependent modulation of ADAM10 synaptic localization/activity. Therefore, we wondered whether activity-dependent synaptic plasticity modulates also CAP2 synaptic availability in the dendritic spines. cLTP stimulation caused a significant increase in CAP2 dimer levels in TIF. We analysed also the interaction of CAP2 and actin and coimmunoprecipitation assays demonstrated a significant decrease in the association between CAP2 and actin. Taken together these data demonstrate that LTP can modulate CAP2 properties promoting the C32-dependent dimerization in the synapses and inducing actin detachment from CAP2.

The above-described pathways, i.e. ADAM10 cleavage of cell adhesion molecules, actin polymerization and spines' shaping, are required for stabilising synaptic contacts. In this framework, we hypothesized that the actin-binding protein CAP2, a newly identified partner of ADAM10, can be positioned at the crossroad of such pathways and can be implicated in brain pathologies, such as AD, in which there is an impairment of the localisation/activity of ADAM10. According to that, microarray analysis of hippocampal gene expression of AD patients, reported a downregulation of CAP2 gene. Moreover, the CAP2 gene is present at chromosome 6p22.3 in human. An interstitial 6p22-24 deletion syndrome of the short arm of chromosome 6 was reported where patients with this deletion have a variable phenotype including a developmental delay [168]. Since CAP2 could be involved in synaptic failure in both neurodegenerative and neurodevelopmental disorders, it represents a potential pharmacological target for several diseases.

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