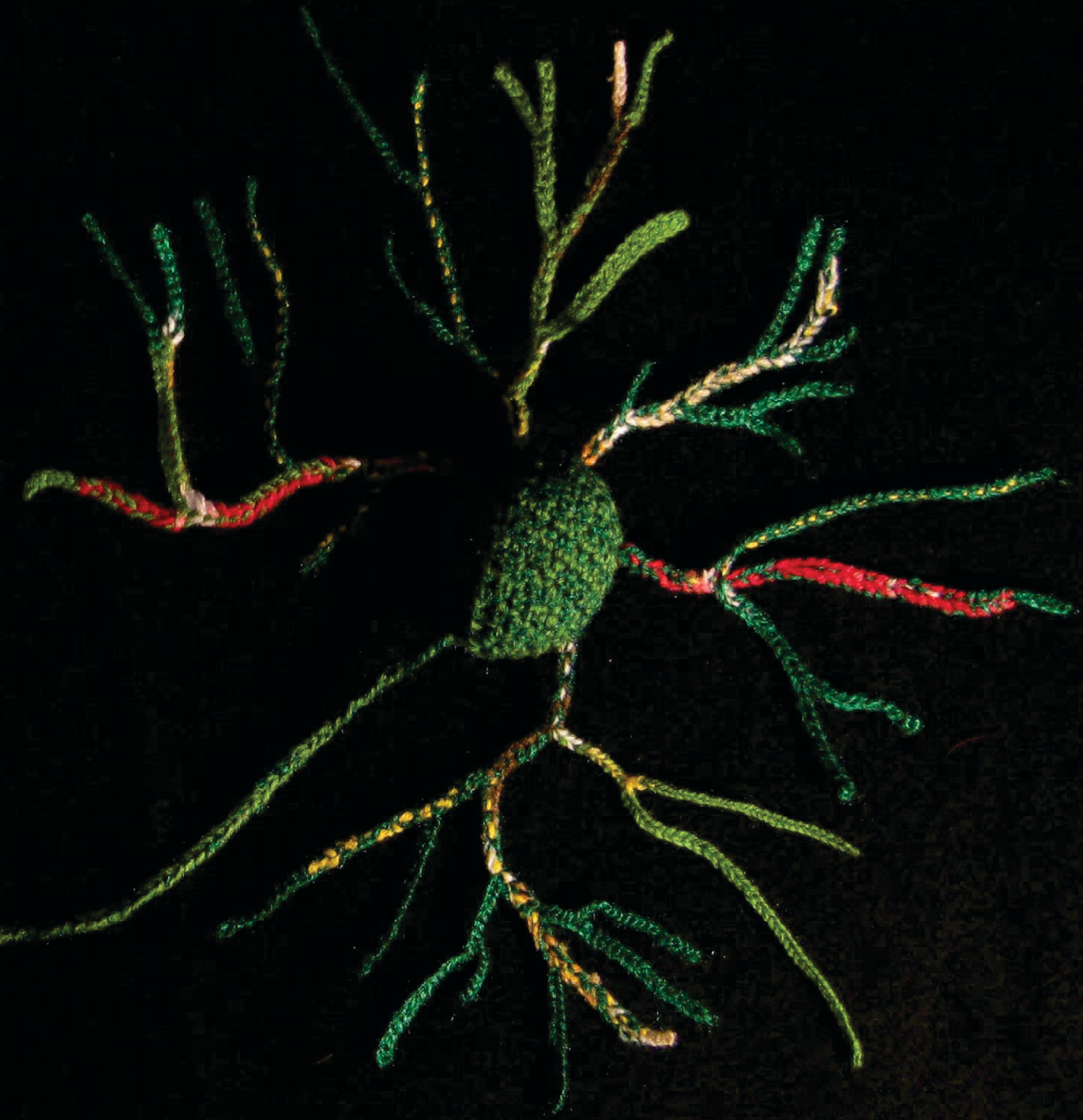
Regulation of Synapse Composition by Protein Acetylation: The Role of Acetylated Cortactin



Tatiana Andreia Forjaz Amaral Catarino

Universidade de Coimbra 2011

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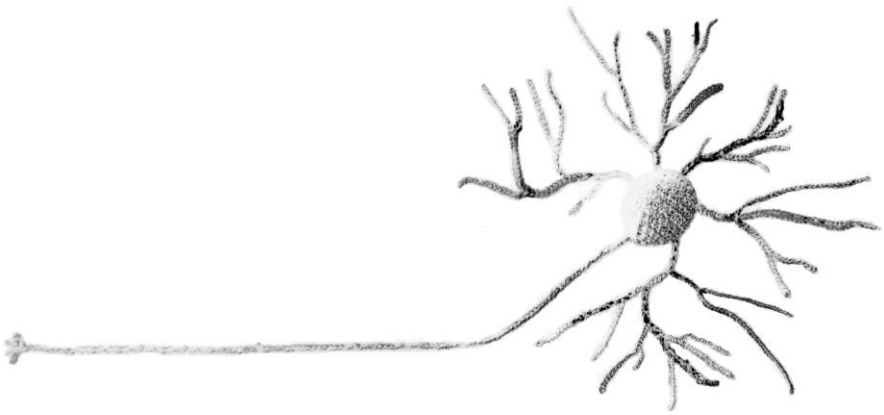

Dissertação apresentada à Faculdade de Ciências e Tecnologia, da Universidade de Coimbra, para a prestação de provas de Doutoramento em Biologia, na especialidade de Biologia Celular.

Tatiana Andreia Forjaz Amaral Catarino **Coimbra, 2011**

Ao Pivete e a todas as

pessoas que me fazem feliz!

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Abbreviations

ABP	Actin binding protein
ABR	Actin binding region
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	AMPA receptor
ANOVA	Analysis of variance
Arp2/3	Actin related protein 2 and 3
BDNF	Brain-derived neurotrophic factor
BCA	Bicinchoninic acid
CAM	Cell adhesion molecule
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CLAP	chymostatin, leupeptin, antipain and pepstatin
DIV	days *in vitro*
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DOC	deoxycholic acid
DTT	Dithiothreitol
EB3	microtubule-plus-end-binding protein 3
ECF	enhanced chemifluorescence
EM	Electron microscopy
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ER	Endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FMR1	fragile X mental retardation 1
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GST	glutathione S-transferase
HAT	histone acetyltransferase
HBSS	Hank's balanced salt solution
HEK	human embryonic kidney
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HDAC	histone deacetylase
HMw	High molecular weight
IFs	Intermediate filaments
IPSC	inhibitory postsynaptic current
JNK	c-Jun N-terminal kinase
kDa	kilodalton
KO	Knockout
LMw	Low molecular weight
LTP	long-term potentiation
LTD	long-term depression
mEPSCs	Miniature excitatory post-synaptic currents
mGluR	Metabotropic glutamate receptor
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
MAP 2	microtubule associated protein
mRNA	messenger RNA
MT	microtubules

Nck1	non-catalytic region of tyrosine kinase adaptor protein 1
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NPF	nucleation promoting factor
NTA	N-terminal acidic region
PAK	P21-activated kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	postsynaptic density 95-discs large-zona occludens 1
PICK1	protein interacting with C kinase
PI3-K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PSA	Polysialic acid
PSD	postsynaptic density
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
SAM	sterile alpha motif
SAP	synapse associated protein
SAPAP	synapse associated protein 90/postsynaptic density95-associated protein
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-poliacrylamide gel electrophoresis
SEM	standard error of the mean
SER	Smooth endoplasmatic reticulum
SH2	Src homology 2
SH3	Src homology 3
TARP	Transmembrane AMPAR-assocaited protein
Trk	tropomyosin-related kinase
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family Verprolin-homologous protein
WIP	WASP-interacting protein

Resumo

É aceite pela comunidade científica que alterações na expressão genética poderão estar envolvidas na plasticidade neuronal associada a processos de aprendizagem e memória. De facto, o aumento da acetilação nas caudas de histonas, induzido pelo uso da Trichostatina A (TSA), um inibidor das desacetilases de histonas (HDAC), facilita a potenciação de longa duração (LTP) no hipocampo. Portanto, a desacetilação mediada pelas HDAC funciona como um "interruptor" molecular intranuclear na modulação da actividade sináptica e alterações de longa duração dos circuitos neuronais. Embora o ciclo de acetilação/desacetilação dos resíduos de lisina nas histonas, mediado por acetil-transferases (HATs) e desacetilases, seja um factor obrigatório na regulação da transcrição, outros substratos para essas mesmas enzimas têm vindo a ser identificados ao longo dos últimos anos, sugerindo que a acetilação de proteínas pode modular um grande número de processos além da expressão genética. Neste trabalho abordámos alterações na composição das sinapses excitatórias desencadeadas por alterações do nível de acetilação das proteínas. Os nossos estudos demonstraram que o tratamento de neurónios do hipocampo mantidos em cultura durante 15 dias (15DIV) com TSA, um inibidor das desacetilases do tipo I e II, aumenta a intensidade da fluorescência, área e densidade dos agregados de PSD95, uma proteína pós-sináptica que funciona como regulador principal da força das sinapses excitatórias, e também da Shank, uma proteína âncora pós-sináptica, sem contudo alterar os níveis proteicos totais de qualquer destas proteínas. Por outro lado, a TSA teve um efeito oposto sobre os agregados de cortactina e p140Cap, proteínas relacionadas com o citoesqueleto, cuja área foi diminuída após tratamento com TSA. A intensidade, número, e área dos agregados de gefirina, proteína âncora das sinapses inibitórias e por isso usada como marcador deste tipo de sinapses, não foram alterados em neurónios tratados com TSA, indicando um efeito específico para acetilação de proteínas nas sinapses excitatórias.

Os efeitos da inibição das HDAC na plasticidade sináptica e memória, bem como nos componentes moleculares das sinapses excitatórias, podem potencialmente envolver variações no estado de acetilação de outras proteínas para além das histonas. Na verdade, além das histonas, as HDACs e HATs, também possuem outros substratos, sendo um desses substratos a cortactina (Zhang et al., 2007). A cortactina é uma proteína que interage com a F-actina facilitando a nucleação de novos filamentos laterais de actina a partir de filamentos pré-existentes. Nos neurónios, a cortactina encontra-se enriquecida nas espículas dendríticas, as estruturas pós-sinápticas das sinapses excitatórias, desempenhando um papel na sua morfogénese. A depleção da cortactina resulta na redução no número e tamanho das espículas dendríticas, enquanto que a sua sobre-expressão resulta no alongamento das espículas (Hering & Sheng, 2003). Estudos recentes mostraram que a acetilação da cortactina altera a sua capacidade para interagir com a F-actina, regulando a mobilidade celular em células cancerígenas (Zhang et al., 2007), mas a função da acetilação da cortactina em células neuronais é até agora desconhecida. Descobrimos que os níveis de acetilação da cortactina aumentam em neurónios tratados com TSA, e que a cortactina acetilada é redistribuída das espículas para o corpo da dendrite. Tendo em conta o papel da cortactina na morfogénese espicular, testámos se a acetilação da cortactina poderia influenciar a agregação dendrítica de PSD95. Para tal, sobre-expressámos cortactina tipo selvagem, ou mutantes que mimetizam a cortactina acetilada ou desacetilada, em neurónios do hipocampo, e descobrimos que a acetilação da cortactina tem um impacto na regulação da agregação de PSD95, independente da sua função como regulador da dinâmica da actina, uma vez que a sobre-expressão do mutante acetilado alterou a área e intensidade dos agregados de PSD95, mas não teve efeito sobre o número ou tamanho dos agregados de F-actina. Outra observação importante, foi a diminuição dos agregados de PSD95, resultante da depleção de cortactina em neurónios através de RNA de interferência (shRNA), a qual foi resgatada por uma forma insensível ao shRNA da cortactina tipo-selvagem e também por uma forma da cortactina que mimetiza o seu estado acetilado. Tendo em conta que as

modificações pós-traducionais funcionam em conjunto a diversos níveis, testámos se a acetilação da cortactina poderia de alguma forma influenciar a sua fosforilação, e descobrimos que a acetilação da cortactina está correlacionada com uma menor fosforilação na tirosina 421. Além disso, determinámos se a acetilação da cortactina está correlacionada com o nível de interação da cortactina com parceiros de interação conhecidos, como p140Cap e Shank1. Sabendo que o factor neurotrófico neuronal (BDNF), neurotrofina conhecida por desempenhar um papel na regulação da estrutura e função das sinapses glutamatérgicas promovendo, por exemplo, a agregação sináptica de PSD95 (Hu et al., 2011; Yoshii and Constantine-Paton, 2007) testámos se o mesmo poderia regular os níveis de acetilação da cortactina. Observámos que o BDNF promove a acetilação da cortactina em neurónios do hipocampo em cultura, e este efeito é dependente da activação das vias de sinalização MEK1/2 e PLCγ. Estas evidências sugerem que o BDNF pode regular a estrutura sináptica, alterando o nível de acetilação da cortactina.

Analisados em conjunto, os nossos dados sugerem que a acetilação das proteínas afecta as sinapses excitatórias e que a acetilação reversível da cortactina pode funcionar como um mecanismo atractivo na maturação das sinapses através de uma regulação indiscutivelmente única da agregação dendrítica de PSD95. No entanto, o ciclo de desacetilação/acetilação da cortactina pode também regular o desenvolvimento das espículas através duma possível via distinta, dependente da dinâmica da actina, relacionada com a sua função na promoção da ramificação e alongamento necessários para a formação dos filopodia e consequente “alargamento”. As nossas descobertas desvendam um papel inesperado para a acetilação da cortactina na regulação da agregação dendrítica de PSD95, a qual pode actuar em conjunto com o papel da cortactina no desenvolvimento da espícula dendrítica.

Abstract

Changes in gene expression are thought to be involved in neuronal plasticity associated with learning and memory. In effect, increased histone-tail acetylation induced by the use of the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) enhances induction of long term potentiation in the hippocampus. Therefore, HDAC-mediated deacetylation functions as an intranuclear molecular switch in the modulation of synaptic activity and long-lasting changes of neuronal circuits. Although acetylation/deacetylation of lysine residues on histones by histone acetyltransferases (HAT) and HDACs is an obligatory component of transcription regulation, other substrates of HATs and HDACs have lately been identified, suggesting that protein acetylation can modulate a myriad of processes besides gene expression. Here we addressed alterations in the composition of excitatory synapses triggered by changing the acetylation level of proteins. We show that treatment of hippocampal neurons in culture (15 DIV) with the inhibitor of types I and II histone deacetylase TSA increases the fluorescence intensity, area and density of the clusters for the excitatory postsynaptic protein PSD95, a major regulator of the strength of excitatory synapses, and for the postsynaptic scaffold protein Shank, without changing their total protein levels. Conversely, TSA had an opposite effect on cortactin and p140Cap, cytoskeleton-related proteins which cluster area was decreased by the TSA treatment. The intensity, number or area of the clusters for the inhibitory synaptic marker gephyrin were not altered in neurons treated with TSA, indicating a specific effect for protein acetylation at excitatory synapses.

The effects of HDAC inhibition on synaptic plasticity and memory and also on the molecular components of excitatory synapses may potentially involve variations in the acetylation status of proteins other than histones. In fact, in addition to histones, HDACs and HATs target nonhistone proteins, and one characterized nonhistone HDAC substrate is cortactin (Zhang et al., 2007). Cortactin is an F-actin-binding protein which facilitates the nucleation of actin

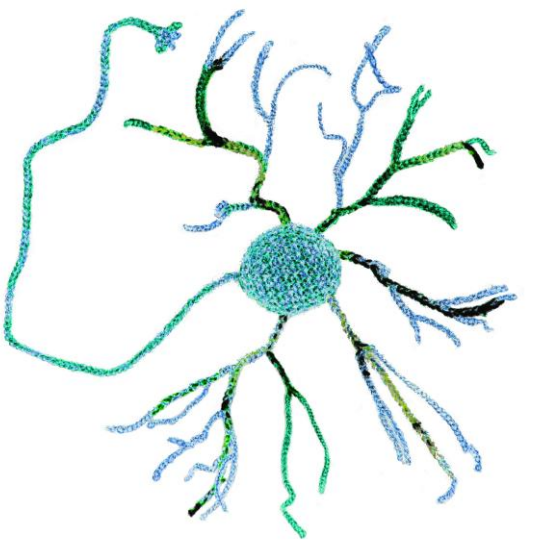
branches on the side of pre-existing filaments of actin. In neurons, cortactin is enriched in dendritic spines, the postsynaptic structures for excitatory synapses, and has a role in spine morphogenesis. Knockdown of cortactin results in depletion of dendritic spines, whereas overexpression of cortactin causes the elongation of spines (Hering & Sheng, 2003). Recent studies showed that cortactin acetylation changes its ability to bind F-actin, and regulates cellular motility in cancer cells (Zhang et al., 2007), but the function of cortactin acetylation in neuronal cells is so far unknown. We found that the cortactin acetylation levels are increased in neurons treated with TSA, and that acetylated cortactin is redistributed from spines to the dendritic shaft. Since cortactin plays a role in the morphogenesis of spines, we tested whether acetylation of cortactin influences the dendritic clustering of PSD95. We overexpressed wild-type cortactin, or the mimetic mutants for acetylated or deacetylated cortactin, in hippocampal neurons, and found that cortactin acetylation has an impact on regulating PSD95 clustering, independent from its function as a regulator of actin dynamics, since overexpression of the cortactin acetylation mutants altered the area and intensity of PSD95 clusters, but had no effect on the number or size of F-actin clusters. Importantly, depletion of cortactin in neurons using shRNA resulted in a decrease on the dendritic clustering of PSD95, which was rescued by a form of wild-type cortactin refractory to shRNA or by the mutant mimicking acetylated cortactin. Since posttranslational modifications work jointly at several levels, we tested whether cortactin acetylation affects its phosphorylation, and found that acetylation of cortactin is correlated with lower phosphorylation of cortactin at tyrosine 421. Additionally, we found that cortactin acetylation is correlated with decreased interaction with known interaction partners, such as p140Cap and Shank1. Since the neurotrophin BDNF (Brain-derived neurotrophic factor) is known to play a role in the regulation of the structure and function of glutamatergic synapses, for example by promoting the synaptic clustering of PSD95 (Hu et al., 2011; Yoshii and Constantine-Paton, 2007), we tested whether BDNF regulates cortactin acetylation. BDNF promotes the acetylation of cortactin in hippocampal neurons in culture, and this effect is dependent on activation of the

MEK1/2 and PLCγ signaling pathways. These evidences suggest that BDNF may regulate the synaptic structure by changing the acetylation level of cortactin.

Taken together our data suggest that protein acetylation affects excitatory synapses, and that reversible acetylation of cortactin may function as an attractive mechanism in synapse maturation through an undoubtedly unique regulation of the dendritic clustering of PSD95. Nevertheless, cortactin acetylation/deacetylation may also regulate spine development through a distinct possible pathway, dependent on actin assembly, which implicates its actin-branching and elongation activity for filopodia formation and outgrowth. Our findings unravel an unsuspected role for cortactin acetylation in the regulation of PSD95 dendritic clustering, which may work in concert with cortactin's role in spine development.

Chapter 1

Introduction



The Synapse and the Postsynaptic site

Glutamatergic Synapse

All our behaviors, thoughts, emotions, dreams, and memories are triggered by a hundred billion neurons interconnected into functional neuronal circuits, which constitute the human brain. At the cellular level, communication between neurons arises and is mediated via specialized cell junctions called synapses. These structures can transmit information through electrical or chemical signaling (Kandel et al., 2000). Electrical synapses provide rapid, bi-directional ionic conductivity between neurons, but offer a limited repertoire for signal transduction. On the other hand chemical synapses produce a mostly asymmetric transmission of information. When an action potential generated near the cell body arrives at the axon terminal, the opening of voltage-gated Ca^{2+} channels induces the release of neurotransmitters stored in the synaptic vesicles, and these neurotransmitters released into the synaptic cleft are ultimately detected by receptors on the postsynaptic cell. Therefore, variety in distinct presynaptic neurotransmitters and diverse postsynaptic specializations for downstream signaling, provide a vast combination for neuronal communication (Kandel et al., 2000).

Depending on the effect of presynaptic stimulation on the postsynaptic potential, synapses can be classified as excitatory or inhibitory. Activation of inhibitory synapses hyperpolarizes the postsynaptic membrane away from the threshold required for activation of an action potential (IPSP – inhibitory postsynaptic potential) (Hausser et al., 2000). Contrariwise, stimulation of excitatory synapses induces an excitatory postsynaptic potential that depolarizes the postsynaptic membranes towards the threshold potential (EPSP – excitatory postsynaptic potential) (reviewed in Sheng and Lin, 2001).

In the mammalian brain, the majority of synapses use glutamate as a neurotransmitter. Fusion of presynaptic vesicles promotes the release of this neurotransmitter which then transverses the synaptic cleft to bind to specific receptors on the postsynaptic membrane conducting to excitatory transmission. Clusters of these receptors are usually in direct apposition to the presynaptic active zone. The effects of glutamate are mediated by activation of ionotropic (Rosenmund et al., 1998) and metabotropic receptors (Ozawa et al., 1998), differing in their molecular, biochemical, pharmacological and physiological properties (Hollmann and Heinemann, 1994; Kew and Kemp, 2005). Ionotropic glutamate receptors (iGluRs) are a major class of heteromeric ligand-gated ion channels, which mediate the majority of the excitatory neurotransmission in the vertebrate central nervous system (CNS) and have been classified into three major subtypes according to their most selective agonist (Watkins et al., 1981): α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors . iGluRs couple the energy of agonist binding to the opening of a transmembrane ion pore, allowing influx of Na^+, K^+ or Ca^{2+} ions and thereby cause membrane depolarization and neuronal excitation to produce an electrical signal from the chemical stimulus. AMPA and kainate are voltage-independent ion channels permeable to Na^+ and K^+ and mediate the majority of fast excitatory postsynaptic transmission. The rapid flow of Na^+ and K^+ through activated AMPA receptor channels produces brief excitatory postsynaptic currents (EPSCs) and the summation of these EPSCs can conjoin in the bridging of neuronal membrane potential threshold and elicit potential firing (Etherington et al., 2001). The rapid positioning or removing of AMPA receptors from the postsynaptic membrane, which elicit synaptic changes, makes these receptors important for events of synaptic potentiation (Malinow and Malenka, 2002). The precise physiological role of kainate receptors remains the least understood of the glutamate receptors, especially in their proposed role in presynaptic signaling (Nicoll et al., 2000). NMDA receptors are ligand-gated ion channels that exhibit a strong voltage dependence owing to the blocking of the receptor channels at negative voltages by extracellular magnesium (Kauer et al., 1988; Watkins et al., 1981). Due to

this unique property, NMDA receptors contribute little to the postsynaptic response during low-frequency synaptic activity. Nevertheless, when the cell is depolarized, Mg2+ dissociates from its binding site within the NMDA receptors channel, allowing Ca^{2+} as well as Na^{+} to enter the dendritic spine (Malenka and Nicoll, 1999). Metabotropic glutamate receptors (mGluRs) are indirectly linked with ion-channels on the plasma membrane of the cell through signal transduction mechanisms, often G proteins. Hence, they are a type of G protein-coupled receptor. When a metabotropic receptor is activated, a series of intracellular events are triggered that also results in ion channel opening but must involve a range of second messengers, thus they mediate slower synaptic responses (occurring over seconds and minutes, rather than milliseconds as occurs for ionotropic glutamate receptors) (Sheng & Lin, 2001).

This neurochemical transmission of information is regulated by several interesting mechanisms. In fact, one fascinating aspect resides in understanding the role of the intense specialization found at postsynaptic sites and how the regulation of the components of this system correlates to neuronal communication, affecting neural circuits and behavior.

Dendritic Spines

Most excitatory synapses in the mammalian brain are formed at tiny dendritic protrusions, named dendritic spines (Bourne and Harris, 2008). Spines were first observed more than 100 years ago by the Spanish neuroscientist Ramon y Cajal (Cajal, 1888), and since then, many scientists and neuroscientists have been trying to understand their function. Spines are postsynaptic compartments composed of a complex biochemistry and cytoskeletal organization, which also harbor organelles and macromolecular complexes involved in protein synthesis, protein degradation, membrane trafficking and calcium signaling. Most mature spines have a club-like morphology, with variably-shaped bulbous tips (spine heads), ~0.5-2 µm in diameter, connected to the parent dendrite by thin stalks (spine necks) 0.04-1

μm long (Harris and Stevens, 1989). The spine neck is usually thinner than the spine head and can function as a molecular bottleneck to promote compartmentalization and biochemical isolation of the spine from the dendrite (Kennedy et al., 2005; Nimchinsky et al., 2002). The narrow neck of the spine creates a spatially isolated compartment where biochemical signals can rise and fall without spreading to neighboring synapses along the parent dendrite, thus allowing the isolation and/or amplification of incomplete signals. This "isolation" effect can also be expanded to the cellular signaling cascades occurring inside spines, since they are not homogeneously distributed. In fact, the current view of the biochemical processes involved in signal transduction is that molecular interactions arise within close proximity to sites of signal initiation (Eungdamrong and Iyengar, 2004) and progress through intracellular pathways whose components are at well-defined localization (Kennedy, 2000; Pawson and Scott, 1997).

Morphologically, some of the most commonly observed spines are either mushroom shaped with heads exceeding 0.6 microns in diameter or thin shaped with smaller heads (Harris et al., 1992). However, extensive electron microscopy studies of brain tissue have shown that individual spines can vary greatly in size and shape (Harris and Kater, 1994; Hering and Sheng, 2001) and that these different shapes can be found at the same time on the same dendrites (Spacek and Harris, 1998). For example, in addition to mushroom and thin shaped spines, there are sessile spines and stubby spines, that show no obvious constriction between the head and its attachment to the dendritic shaft, and also filopodia-like or branched spines, typically among the longest spines found in the brain (Fig. 1). Nevertheless, the real *in vivo* situation cannot be reflected by this static view, because the majority of the spines change their shape over periods of minutes or hours, at least in developing neurons (Parnass et al., 2000). In terms of density, spines range from 1 to 10 per 10 micrometer length of dendrite, depending on neuronal cell type and maturational stage. For example, pyramidal neurons in the visual cortex present 1 to 2 spines per 10 μm (Larkman, 1991), while striatal medium spiny neurons

display a spine density as high as 7 to 8 spines per 10 µm (Graveland et al., 1985).

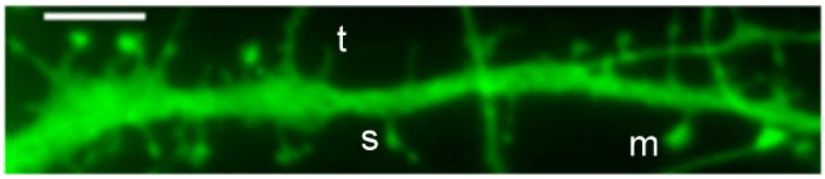


Fig. 1 – Dendritic branch from a 15 DIV hippocampal neuron filled with GFP. Dendritic spines of varied lengths and shapes can be observed. m) Mushroom spine, s) stubby spine, t) thin spine. Scale bar 5 µm.

Dendritic spines are different also in the intracellular composition, which mainly consists of postsynaptic density (PSD) facing the presynaptic button and a cytoskeletal structure formed almost exclusively by F-actin. The PSD occupies 10 % of the surface area of the spine and is probably the most complex spine organelle in which hundreds of components are associated with each other in a complex based on a series of protein-protein interactions (Sheng and Hoogenraad, 2007; Sheng and Sala, 2001). This structure contains glutamate receptors anchored to scaffolding proteins and serves as the major site for the local initiation of intracellular signaling (Kennedy, 2000). Other organelles are localized at dendritic spines. In many spines, the smooth endoplasmatic reticulum (SER) can be detected stretching from the dendritic shaft into to the spine neck (Spacek and Harris, 1997), and some pyramidal cell spines contain the spine apparatus, which is an organelle formed by two or more disks of SER separated by an electron-dense material (Westrum et al., 1980), shown to be required for some forms of synaptic plasticity (Deller et al., 2003). It has been observed that both SER and spine apparatus are usually associated with larger spines and are formally absent in small spines (Spacek and Harris, 1997). Since it is thought that SER plays a role in Ca^{2+} handling, functioning as a Ca^{2+} reservoir (Spacek and Harris, 1997; Verkhratsky, 2002),

differently sized spines may have different ways of controlling calcium homeostasis.

Larger spines are more likely to contain endosomal compartments (Cooney et al., 2002; Park et al., 2006). The endosome machinery is critical in sorting endocytic vesicles from the plasma membrane and selecting internalized proteins for either degradation in lysosomes or redirection to the membrane (Maxfield and McGraw, 2004). Among other roles, this system can aid in the rapid delivery of glutamate receptors to the postsynaptic membrane (Ehlers, 2000).

Spines are often associated with polyribosomes, so protein synthesis may be locally regulated in individual spines. Polyribosomes and protein translational machinery are found occasionally in spine heads or necks, but most commonly anchored at the base of the spines (Ostroff et al., 2002; Spacek, 1985; Steward and Schuman, 2001). In fact, local protein synthesis occurs locally in dendrites, although it is unclear how much can occur inside spines (Steward and Schuman, 2001). Conversely, protein degradation by the proteasome system has also been shown to be an active process in the postsynaptic metabolism, playing a role in activity dependent protein degradation (Ehlers, 2003).

Taken together these evidences suggest that individual spines represent partially autonomous compartments, having their own regulated membrane-trafficking events that shuttle components into and out of the spine membrane. When associated, these specialized molecular assemblies determine spine shape and, most importantly, enable the postsynaptic neuron to respond biochemically to glutamate or other transmembrane signals (reviewed in Hering and Sheng, 2001; Smart and Halpain, 2000; Zhang and Benson, 2001).

Spines are highly dynamic structures capable of changing rapidly in both number and morphology. In the mammalian brain, a critical phase occurs during early postnatal development in which a period of robust spine genesis is followed by pronounced pruning (Bourgeois et al., 1994; Huttenlocher and

Dabholkar, 1997; Markus and Petit, 1987). Recent studies have measured the turnover of dendritic spines *in vivo*, using two-photon microscopy of GFP-labeled dendrites in mouse cortex (Holtmaat et al., 2005; Zuo et al., 2005a). These studies found that a substantial fraction of thin spines appeared and disappeared over several days, whereas large spines were stable for months, and this fraction of stable spines increased during development and into adulthood. The convergent opinion is that the majority of mature spines are stable over months in the adult neocortex. Furthermore, sensorial manipulation and genetic mutations in animals are also known to affect spine density. In the binocular zone of the visual cortex, monocular deprivation during the critical period leads to reduced density and increased motility of dendritic spines (Mataga et al., 2004; Oray et al., 2004). However, it was recently reported that spine loss was prevented by long-term sensory deprivation, in primary somatosensory cortex (Zuo et al., 2005b). Another well-known factor that can perturb spine number and morphology is *in vivo* manipulation of neuronal genes. For example, animals with a disruption in the postsynaptic gene Shank 1 (SH3 and multiple ankyrin repeat domains protein 1) display reduced spine numbers in hippocampal neurons (Hung et al., 2008), while disruption of Fragile X mental retardation 1 (FMR1) gene leads to an increased spine density in mutant mice during the first postnatal weeks (Nimchinsky et al., 2001).

Moreover, alterations in spine density, morphology, and maturation strongly correlate with neuronal disorders, such as mental retardation, Fragile-X syndrome, Down's syndrome and epilepsy (Chechlacz and Gleeson, 2003; Ferrer and Gullotta, 1990; Grossman et al., 2006; Suetsugu and Mehraein, 1980; Swann et al., 2000). In advanced stages of Huntington's, Parkinson's and Alzheimer's disease, patients frequently display reduced levels of spines in discrete brain regions (Catala et al., 1988; el Hachimi and Foncin, 1990; Ferrante et al., 1991; McNeill et al., 1988), and abnormal spine density was also reported in schizophrenic patients (Garey et al., 1998; Glantz and Lewis, 2000). Changes in spine density where also seen in animal models of substance abuse, since chronic cocaine administration produced an increase in spine

density in the ventral striatum (Robinson and Kolb, 1997). Additionally, a large body of evidences has pointed to changes occurring in spine density and spine morphology during learning and memory formation (reviewed in Bhatt et al., 2009). It is now widely believed that information in the brain can be stored by strengthening or weakening existing synapses, as well as appearance and disappearance of dendritic spines, which subsequently leads to the formation and elimination of synapses. These functional and structural changes at spine and synapses are now considered to be the basis of learning and memory in the brain (Holtmaat and Svoboda, 2009; Kasai et al., 2010).

Morphological, biochemical and electrophysiological properties of dendritic spines may offer powerful insights into the understanding of neuronal circuitries during development, since these sub-cellular structures are the key sites of synaptic transmission for over 90% of excitatory synapses.

Postsynaptic Density

In the mammalian brain, excitatory neurotransmission is predominantly mediated by release of the neurotransmitter glutamate. At these excitatory synapses, receptors for glutamate are concentrated within a postsynaptic specialization termed the PSD, originally defined by electron microscopy (EM) as an accumulation of electron-dense material located on the cytoplasmic surface of the synaptic membrane (Fig. 2), immediately beneath the postsynaptic membrane (De Robertis and Bennett, 1955; Palay and Palade, 1955). Further understanding of the PSD was facilitated by its biochemical isolation, which confirmed the proteinaceous nature of this tight molecular network (Bloom and Aghajanian, 1966, 1968; Cotman and Taylor, 1972). Biochemical isolation of the PSD led to the idea that its composition of specific transmembrane and intracellular components reflects its unique structural and functional properties, which makes it a distinct structural and functional microdomain within neurons (Carlin et al., 1980). Another important contribution came from Gray through the detailed characterization of synapses as type I or

type II (assymetric or symmetric) according to the location of axonal enervation and PSD ultrastructural morphology (Gray, 1959).

Ultrastructurally, PSDs form a 30-nm- to 40-nm-thick protein meshwork with a diameter of a few hundred nanometers (Carlin et al., 1980). When isolated by differential centrifugation, PSDs appear as neuronal disc-shaped organelles that are relatively insoluble in non-ionic detergents (Ziff, 1997). Moreover, PSDs exhibit attached or juxtaposed filamentous structures that extend deeply into the spine cytoplasm, which can anchor the PSD to the underlying spinous cytoskeleton, or limit receptor mobility within the PSD (Landis and Reese, 1983; Landis et al., 1987; Pozzo Miller and Landis, 1993).

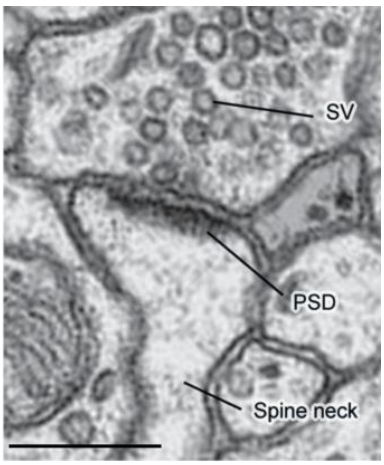


Fig. 2 – EM morphology of an excitatory synapse. The presynaptic terminal contains synaptic vesicles loaded with glutamate, facing the PSD located on the tip of the dendritic spine. The synaptic cleft separating pre- and postsynaptic membranes is 20-25 nm wide. SV: synaptic vesicles. Scale bar is ~400 nm. [Reproduced from (Sheng and Hoogenraad, 2007)].

The PSD contains a variety of molecules, which mediate physical linkage and/or functional communication with the presynaptic specialization as well as function in postsynaptic signaling. The extremely high concentration of ionotropic glutamate receptors, associated anchoring proteins, scaffolding proteins and signaling proteins maximizes the transduction of information

between the presynaptic and postsynaptic neuron. Classic approaches to identify the components of the PSD have utilized subcellular fractionation and differential centrifugation to purify PSD components (Carlin et al., 1980). In the early days, PSD proteins were separated by SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and two-dimensional gel electrophoresis, resulting in a few major and minor bands that were sequenced to identify abundant constituents such as Tubulin, actin and αCaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) (Cho et al., 1992; Kelly and Cotman, 1978; Kennedy et al., 1983; Walsh and Kuruc, 1992). Classic biochemical methods however, only allowed the identification of these and few other proteins. More recently, the combination of different procedures, including mass spectrometry (MS) methods, led to the identification and partial sequenciation of a large number of proteins in the PSD, which helped unravel the true molecular complexity of PSD preparations (Cheng et al., 2006; Collins et al., 2006; Husi et al., 2000; Yoshimura et al., 2004). Recent investigations have even measured the mass of a single postsynaptic density (1.1±0.36 GDa) and the relative or absolute amounts of single proteins within the one PSD, which is thought to number between several hundreds to over one thousand different proteins of varied stoichiometric levels (Chen et al., 2005; Kennedy, 2000; Sugiyama et al., 2005). The proteomic analysis also made possible the sorting and functional profiling of PSD proteins. In fact, identified proteins in the PSD serve a myriad of roles, from cell surface receptors to cytoplasmic signaling enzymes (including protein kinases and phosphatases), cytoskeleton associated and cell adhesion proteins and scaffolding proteins that hold them together (Collins et al., 2006; Peng et al., 2004) (Fig. 3).

Since the complex PSD protein assembly is the molecular basis for locally distinct but diverse intracellular events, it is not surprising that this intricate and rich protein network is also a highly organized structure composed of several partially overlapping protein complexes, regulated by intercommunication of these protein networks in selective protein-protein interactions. One example of these individual, tightly associated, multiprotein networks that organize within

the larger PSD ultrastructure are the complexes formed by neurotransmitter receptors and associated scaffolding or signaling molecules (Kim and Sheng, 2004; Kneussel, 2005). Proteomic approaches for GluR complexes have helped to identify individual protein networks such as NMDA receptor/MAGUK-associated signaling complex, AMPAR complex (ARC) and an mGluR complex (mGC) (Collins et al., 2006; Farr et al., 2004; Husi et al., 2000). The MASC complex, which is considered to be the largest, interacting with up to 186 identified proteins in order to form a 2000-kDa structure, is believed to strongly anchor NMDA receptors to the PSD (Husi et al., 2000). The mGC is another large complex and is known to assemble with 64 different proteins (Farr et al., 2004), whereas the ARC complex has been described as being much smaller with only 9 identified associated proteins until recently (Collins et al., 2006). However, a recent study unraveled new constituents of the macromolecular complex of long-form calcium-permeable AMPA receptors (Santos et al., 2010). The proteomic screening led to the identification of several interactors, most of which are novel AMPA receptor partners, namely, cytoskeleton proteins, motor proteins, RNA processing proteins which are part of neuronal RNA granules, and kinases, among others.

Better functional insight into postsynaptic molecular architecture requires an understanding of the stoichiometry and 3D structure of individual PSD components, since not all proteins represented in the PSD share the same absolute number of copies. Recently, thanks to several powerful approaches, quantitative information on the stoichiometry of proteins in the PSD has come to the forefront (Chen et al., 2005; Cheng et al., 2006; Sugiyama et al., 2005). One of the first identified PSD proteins, αCaMKII, was determined to be the most abundant protein in the PSD fraction, representing ~7.4% mass of the PSD (Cheng et al., 2006). The next most abundant protein among those measured was SynGAP, a postsynaptic RasGAP. Other highly abundant proteins in the adult forebrain PSD are the postsynaptic protein 95 (PSD95), which is a scaffold protein that binds to NMDAR subunits, synapse associated protein

90/postsynaptic density95-associated proteins 1-4 (SAPAP1-4) and SH3 and multiple ankyrin repeat domains protein 1-3 (Shank 1-3) (Cheng et al., 2006).

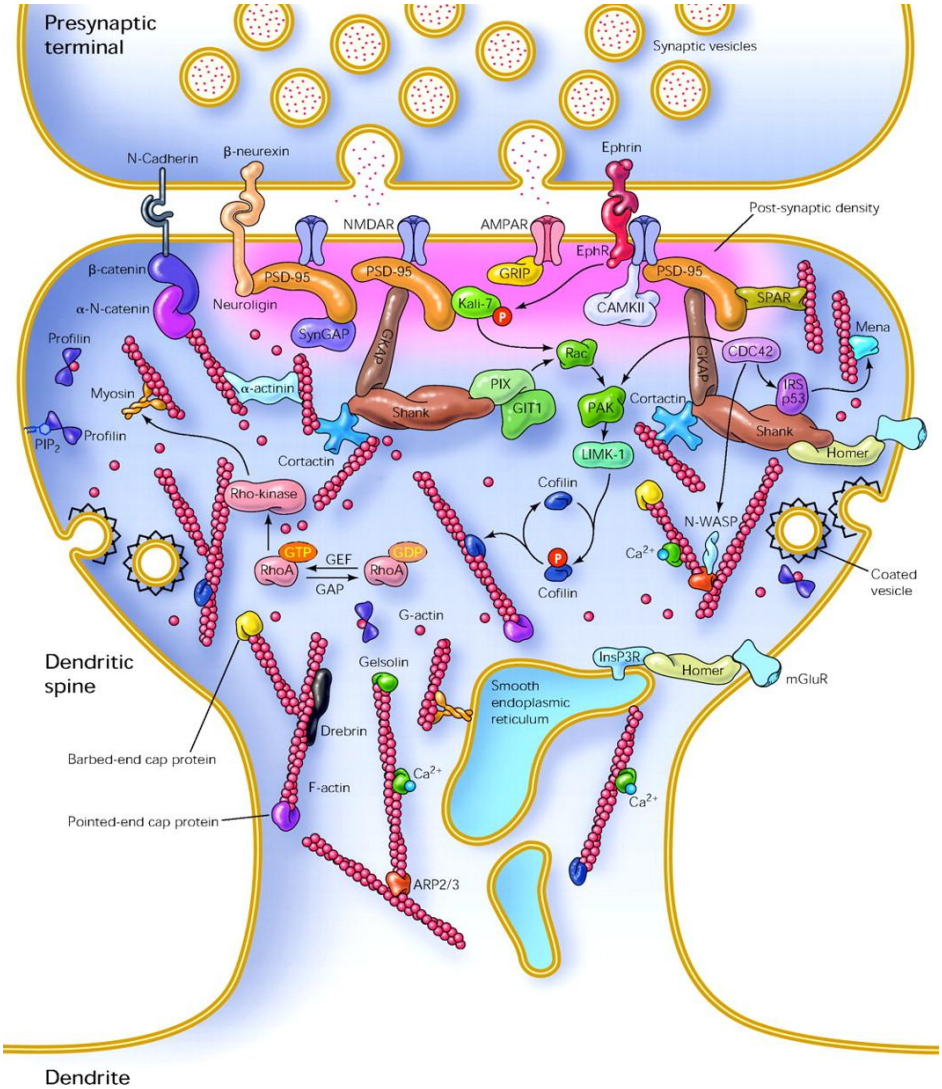


Fig. 3 – **Components of dendritic spines**. Schematic diagram of a mature mushroom-shaped spine showing the postsynaptic membrane containing the postsynaptic density, glutamate receptors, scaffold proteins, the actin filaments, and organelles. Subtypes of glutamate receptors are clustered at the postsynaptic site, within the postsynaptic density and connected to scaffold proteins such as PSD95. The actin network is spread in spine base, neck and head, exhibiting a continuous network of both straight and branched filaments. Actin filaments indirectly link up with neurotransmitter receptors and other transmembrane proteins that provide the main structural basis for spine shape, thus regulating spine shape and development. [Adapted from (Calabrese et al., 2006)].

The PSDs exhibit a striking and important feature, despite their molecular complexity, which is the capacity for dynamic change. The complex protein composition of the PSDs can change as a consequence of external stimuli, synaptic activity and normal development (Colledge et al., 2003; Lu et al., 2001; van Zundert et al., 2004). Dynamic regulation of abundance and activity of PSDs constituent proteins occurs through protein phosphorylation (Collins et al., 2005; Sheng and Kim, 2002; Trinidad et al., 2006), local protein translation (Schuman et al., 2006), the ubiquitin-proteasome system for protein degradation (Ehlers, 2003; Pak and Sheng, 2003) and redistribution of specific proteins to and away from the PSD (Dosemeci et al., 2001; Hu et al., 1998; Inoue and Okabe, 2003; Malinow and Malenka, 2002). In fact, changes in activity levels in a neuron culture model were show to produce a global remodeling in PSD composition through even altered expression, post-translational modifications or ubiquitination and activity dependent degradation of several PSD proteins (Colledge et al., 2003; Ehlers, 2003; Malinow and Malenka, 2002; Sheng and Kim, 2002). Similarly, during development, synapses change their functional properties and this is reflected in changes in the PSD. The most striking identified changes in PSD composition during development seem to represent a gain of one family member at the expense of another. During postnatal development synaptic levels of NMDA receptor subunits undergo a shift from a composition rich in NR1-NR2B to a predominantly NR1-NR2A receptor configuration (Sheng et al., 1994). Using immune-gold EM at several developmental time points to observe precise molecular localization and abundance, it was later determined that NR1 and NR2B subunits of the NMDAR predominate in young PSDs together with SAP102, and with postnatal development, NR2A, PSD95 and PSD93 increase while NR2B and SAP102 decline (Petralia et al., 2005; Sans et al., 2000). AMPARs and CamKII also accumulate greatly during development of PSDs, correlating with increased transmission strength during maturation of synapses (Petralia et al., 1999). A well-established developmental alteration at molecular level is the increased expression and inclusion of GluA2 (Glutamate receptor subunit 2) in functional AMPA receptors (Brill and Huguenard, 2008; Ho et al.,

2007; Kumar et al., 2002). Finally, the major PSD scaffold proteins and SynGAP levels also change during development, since they are present in PSDs at birth and generally increase in abundance with maturation (Petralia et al., 2005). Although it is possible that some of these major scaffolds exist in a preformed complex for incorporation into growing PSDs (Gerrow et al., 2006).

Scaffolding Proteins

The PSD is the most prominent spine microdomain in which glutamate receptors concentrate to mediate excitatory transmission. The PSD of excitatory synapses seems to be arranged in a clear-cut laminar hierarchical structure, and this hierarchy appears to be determined by the targeting and binding characteristics of the individual proteins (Valtschanoff and Weinberg, 2001; Zuber et al., 2005). GluRs span the plasma membrane and are concentrated within the PSD, organized as distinct nanometer-sized compartments. Immediately subcellular to GluRs within the PSD is a layer of scaffolding molecules that serve to anchor the receptors subunits and other postsynaptic transmembrane proteins. Both biochemical and imaging studies demonstrate that PSD scaffolding proteins (e.g. PSD95, GKAP, SAP97, Shank and Homer; 60-400 molecules of each per synapse) outnumber glutamate receptors (1-200 glutamate receptors per synapse) (Sheng and Hoogenraad, 2007). As a class these scaffolding proteins are characterized by the presence of one or more PDZ [**P**SD95-kDa/disks large (**D**lg)/zona occludens (**Z**O-1)] domains (Stuart et al., 2007). PDZ domains are found in a wide variety of eukaryotic proteins and display significant sequence variation, presumably underlying functional diversity and binding specificities (Sheng and Sala, 2001). The majority of known PDZ domains are 80-100-amino-acid domains that interact with specific short C-terminal sequences present in GluR (NMDAR and AMPAR) subunits or accessory proteins (Sheng and Sala, 2001; Tomita et al., 2003). However, PDZ domains are also known to heterodimerize or bind to certain internal β–hairpin structures (Brenman et al., 1996; Hillier et al., 1999). These scaffolding proteins are consisted of multiple domains that mediate protein-protein interactions.

Therefore, not only they anchor the C-termini of receptors and transmembrane proteins within the PSD, but are also capable of organizing large multiprotein complexes within the PSD, and these assembled protein units mediate specific functions related to the coassembled neurotransmitter receptors.

The PSD95 Family of Proteins

A group of membrane-associated guanylate kinase homologs (MAGUKs) has emerged as central organizers of multicomponent protein signaling complexes. MAGUKs form a superfamily of scaffolding proteins present in several organisms and appear to play various cellular roles, including fundamental roles in the transport, anchoring and signaling of specific subclasses of synaptic receptors and ion channels (Montgomery et al., 2004). In the mammalian brain, special consideration is given to a sub-family of MAGUKS, which is the commonly defined PSD95 family of proteins. This family comprises structurally similar proteins, namely SAP102 (synapse-associated protein 102), SAP97 (synapse-associated protein 97, PSD93 (postsynaptic density 93) and PSD95. These distinct proteins are encoded by unique genes and exhibit different expression patterns and functional properties. The best characterized of the synaptic PDZ proteins is PSD95, which was amongst the original proteins identified as being enriched in the PSD (Sampedro et al., 1981). Nonetheless, the molecular identity of PSD95 by protein sequencing and the recognition that it was highly homologous to *dlg* only came some years later (Cho et al., 1992).

Structurally, from the N-terminus to the C-terminus, the PSD95 family of proteins consists of an L27 domain, three PDZ domains (PDZ1, PDZ2 and PDZ3), a Src homology domain (SH3), and a guanylate kinase-like (Aoki et al.) domain, all of which mediate different protein-protein interactions. Yeast-two hybrid screening assays contributed to unravel the role of PSD95 as a central organizer of synaptic function by revealing numerous specific binding partners for PSD95. Some of the most noteworthy binding partners to the first two PDZ domains of PSD95 include the Shaker-type K^+ channels and NR2 subunits of

the NMDA receptor, both through C-terminal PDZ binding motifs (Kim et al., 1995; Kornau et al., 1995). Later, other members of the PSD95 family were included in these interactions; PDZ1 and PDZ2 from SAP97 were shown to interact, with equal affinity, with both NR2A and NR2B subunits, whereas all 3 PDZ domains in SAP102 were shown to bind to NR2B (Muller et al., 1996; Niethammer et al., 1996). PSD93 was also shown to interact and promote the clustering of both NMDA receptor subunits and Shaker K^+ channels in heterologous cells (Kim et al., 1996). Indeed, it was shown that PSD93 and PSD-95 can heteromultimerize with each other, being recruited into the same NMDA receptor and K^+ channel clusters. Other notable binding partners to the PDZ domains of the PSD95 family include the neuronal isoform of nitric oxide synthase (nNos), a Ca^{2+}/Calmodulin activated enzyme implicated in the regulation of neurotransmission and excitotoxicity, through a PDZ-PDZ interaction (Brenman et al., 1996); SynGAP to PDZ1-3 domains of PSD95 and SAP102 (Kim et al., 1998); and neuroligin to PDZ3 domain (Irie et al., 1997).

PDZ domains are also responsible for the regulation of AMPAR. Specifically, SAP97 directly interacts with the AMPAR subunit GluR1, and this interaction is involved in the trafficking of these receptors (Leonard et al., 1998). However, the interaction between PSD95 and GluR subunits is not direct. In fact, AMPARs are localized to synapses through direct binding of the first two PDZ domains of synaptic PSD-95 to the AMPAR regulatory protein, stargazin (Chen et al., 2000; Schnell et al., 2002; Tomita et al., 2004).

The two remaining conventional domains in PSD95 family are the SH3 and GK domains. The SH3 domains classically mediate protein-protein interactions by binding to proline-rich sequences (PXXP) (Mayer and Eck, 1995; Musacchio et al., 1994), but in MAGUKs, SH3 domains seem to have an atypical ligand binding specificity, distinct from classical SH3 domains. One of the most well known features is that SH3 domain interacts in an intramolecular manner with the GK region of PSD95 family proteins and this intramolecular interaction appears to be important for regulating the ion channel-clustering activity of

PSD95 (McGee and Bredt, 1999; Shin et al., 2000). In PSD95, both domains were shown to bind and promote clustering of kainate receptors (Garcia et al., 1998).

The GK domains of MAGUKs are highly homologous to the enzyme guanylate kinase, but catalytic activity has not been reported. Instead, the GK domains of certain MAGUKs have been characterized as protein-protein interaction interfaces, and have provided clearly established binding partners. In 1997, three independent research groups reported that GK domain of the PSD-95 family of MAGUKs interacts with the GKAP/SAPAP/DAP family of postsynaptic density proteins, which is of critical importance for the normal function of the PSD (Kim et al., 1997; Satoh et al., 1997; Takeuchi et al., 1997). The GK domain of PSD-95 also binds with high affinity to both microtubule-associated protein 1A (MAP1A) (Brenman et al., 1998) and brain-enriched guanylate kinase-associated protein (BEGAIN) (Deguchi et al., 1998). An additional partner to PSD95 GK domain is SPAR (spine-associated RapGAP), which regulates spine morphology and displays actin-reorganization activity (Pak et al., 2001). Thus, the GK domain of PSD-95 can interact directly with multiple postsynaptic proteins. Although this is not a comprehensive list of all known interactions to PSD95 family of proteins, this list of interactions serves to highlight the central role that PSD95 family of proteins may play at the PSD.

The functional importance of PSD95 as a synaptic scaffolding molecule was initially demonstrated by mutations of *Drosophila* homolog of PSD95, Dlg, which eliminates postsynaptic clustering of Shaker K^+ channels (Gramates and Budnik, 1999). Moreover, manipulation of the expression levels of PSD95 family of proteins has conceded several insights into the role that these proteins play at synapse and in the regulation of glutamate receptors. PSD-95 overexpression, in hippocampal neurons, causes an enhancement of postsynaptic clustering and activity of glutamate receptors and also increases the number and size of dendritic spines (El-Husseini et al., 2000). Conversely, acute knockdown of PSD-95 in brain slice cultures arrests the normal

development of synaptic structure and function that is driven by spontaneous activity, suggesting not only a decrease in synaptic AMPA receptors but also a decrease in total number of functional synapses (Ehrlich et al., 2007). Manipulations in SAP97, PSD93 and SAP102 protein levels also led to similar functional alterations (Elias et al., 2006; Schluter et al., 2006). Therefore, it is not surprising that PSD95 is considered a potent regulator of synaptic strength through its dominant role in controlling AMPA receptor numbers at synapses (Bats et al., 2007; Chen et al., 2000; Elias et al., 2006). Despite the lack of information on how PSD-95 actually organizes the molecular architecture of the PSD to support its functional properties, recent studies using electron microscopy tomography (Chen et al., 2011) revealed that the knockdown of PSD-95 leads to loss of entire patches of PSD material. This patchy loss correlates with loss of PSD-95-containing vertical filaments, horizontal elements associated with the vertical filaments, and putative AMPA receptor-type, but not NMDA receptor-type, structures (Chen et al., 2011). Additionally, the role of PSD95 family of proteins can be further explored by *in vivo* studies through analysis of mouse models for the deletion of these genes. SAP97 mutant mice are the less suitable to study, since the SAP97 null mice die in the neonatal period due to craniofacial abnormalities (Caruana and Bernstein, 2001; Zhou et al., 2008). SAP102 (Cuthbert et al., 2007), PSD93 (McGee et al., 2001) and PSD95 (Beique et al., 2006; Migaud et al., 1998) knockout animals display mild phenotypes, with no effect on AMPA receptor mediated transmission, which conflict with the results from *in vitro* and *ex vivo* experiments that credit a key role for PSD95 and PSD93 in a dramatic regulation of AMPA receptor mediated synaptic strength. The mild phenotypes displayed by these animals have to be interpreted with great caution owing to compensation that can occur due to synaptic specificity of each of these genes, since the expression patterns of the MAGUKs in the central nervous system mostly overlap (Elias and Nicoll, 2007). Comparing the effects of acute knockdown with the corresponding knockouts (and double knockouts) of these proteins (PSD93, PSD95 and SAP102), this work illustrated how synaptic specificity and developmental regulation of AMPAR is influenced by PSD95 and PSD93 in non-overlapping populations of

mature synapses. On the other hand, SAP-102 was shown to be the dominant MAGUK for AMPA receptor trafficking early in development as well as being able to partially compensate for the absence of both PSD-95 and PSD-93 in adults (Elias et al., 2006). Not only this work confirmed the difficulties encountered when observing neurobiological relevant alterations in models of genetic manipulations when closely related genes overlap in the same population of genes, but also reconciled the *in vivo* and *in vitro* observations on the role of PSD95 family of proteins in regulating synaptic glutamate receptors.

In addition to its role as organizer of intracellular signaling and anchor for the NMDA receptor at the synaptic plasma membrane, PSD95 also binds to other synaptic scaffolding molecules deeper within the PSD, through protein interactions. Yeast two-hybrid assays revealed the interaction between the GKAP/SAPAP family of proteins and the GK domain of PSD95 (described above). In turn, SAPAP proteins were used as the bait in yeast two-hybrid screens that permitted the identification of the Shank family of proteins, another family of core PSD proteins (Lim et al., 1999; Naisbitt et al., 1999; Tu et al., 1999). This family of proteins will be discussed in the next section.

The Shank Family of proteins

Shank and GKAP are probably two of the major scaffold proteins organizing the PSD. Shank is made of five domains that are likely involved in protein-protein interactions: ankyrin repeats, an SH3 domain, a PDZ domain, a Sterile Alpha Motif (Sampedro et al.) domain and a proline reach region. The Shank family contains three known members, Shank1, Shank2, and Shank3 (also called ProSAP, SSTRIP, cortBP, synamon, and Spank) and despite sharing essential identical domain structure, the long proline-rich regions (900-1000 residues) of Shanks share relatively low amino acid sequence identity as compared with the other recognizable domains (Lim et al., 1999). Binding to SAPAP proteins occurs through the PDZ domain of Shank to the C-terminal QTRL motif of SAPAP (Naisbitt et al., 1999). PSD95, SAPAP and Shank all enrich in biochemically isolated PSD samples, and also display a prominent

presence at the PSD when investigated by immune-gold EM (Naisbitt et al., 1999; Petralia et al., 2005). In fact, the interaction between these three proteins is well documented and supported by several lines of evidences, including the above mentioned yeast two-hybrid interaction screens, and also demonstrations that this complex co-clusters and co-precipitates in heterologous cells.

Shank proteins are associated with the NMDA receptor-PSD95 complex by their binding to GKAP, and they are also associated with type I metabotropic glutamate receptors (mGluRs) via an interaction with the EVH1 (Ena/VASAP homoly 1) domain of Homer in the proline rich-domain (Brakeman et al., 1997) (Fig. 3). Recent studies, using crystallographic analysis, found that Homer and Shank form a high-order polymerized complex, which recruits another postsynaptic protein (SAPAP), with a mesh like network forming a tetrameric structure (Hayashi et al., 2009). This Homer-Shank structure may serve as a structural framework and as an assembly platform for other PSD proteins. Therefore, Shank may bridge two different glutamate receptors (NMDA and mGluRs), regulating the interaction between glutamatergic ionotropic and metabotropic signaling (Ehlers, 1999; Tu et al., 1999).

Shank also acts as a molecular bridge linking multiple glutamate receptors subtypes to the postsynaptic cytoskeleton, since a number of actin regulatory molecules bind to both proline-rich or PDZ domains of the protein. Cortactin was found to bind to Shank 2 (originally named cortBP1 – cortactin binding protein 1) and later confirmed to also bind to Shank 1 and -3 through the proline-rich region (Du et al., 1998; Naisbitt et al., 1999). A further link to the actin cytoskeleton also occurs through the interaction between α-Fodrin with the ankyrin repeats (Bockers et al., 2001), IRSp53 (insulin receptor tyrosine kinase substrate p53) (Bockmann et al., 2002) and AbP1 (actin binding protein 1) with the proline-rich domain (Qualmann et al., 2004), and β-PIX (p21-activated kinase interacting exchange factor) with the PDZ domain of Shank (Park et al., 2003).

There is strong evidence for Shank proteins concerning the regulation of synaptogenesis and morphology of dendritic spines. In hippocampal neuron cultures, Shank1 overexpression was found to promote the maturation and enlargement of dendritic mushroom spines, although the effect was further potentiated by co-expression of Homer (Sala et al., 2001). Shank3 was also characterized as a modulator of dendritic spines. Knockdown of Shank3 reduces spine density in hippocampal neurons, whereas transgene expression of Shank 3 is sufficient to induce spine formation in aspiny cerebellar neuron. This dramatic effect was also observed using Shank3 mutation/deletion approaches. These manipulations promoted an increase of frequency and amplitude of miniature EPSCs, as well as increases in the number and size of synaptic contacts, demonstrating the ability of Shank3 to recruit functional glutamate receptors and inducing the formation of functional synapses (Roussignol et al., 2005).

Further components of the Shank master complexes are molecules that have the potential to alter the shape of the spines and PSDs by recruiting and/or regulating small GTPases within the PSD. Densin-180, which has been identified as a constituent of the postsynaptic densities in excitatory synapses, interacts with the SH3 domain and proline-rich region of Shank (Shank 1-3) proteins (Quitsch et al., 2005). In cultured hippocampal neurons, Densin-180 overexpression induces excessive branching of neuronal dendrites, but co-expression of Shank3 abrogates branch formation and targets Densin-180 into postsynaptic clusters instead, antagonizing the dendritic branching induced by Densin-180 overexpression. Furthermore, dynamin-2 (GTPase), which is specifically enriched in the postsynaptic densities of culture hippocampal neurons, interacts with at least two members of the Shank family of proteins (Okamoto et al., 2001). This interaction indicates a close association between the endocytic machinery and the PSD, providing another insight into the dynamics of this structure.

Another binding partner to Shank3, involved in spine and dendritic rearrangements, is the Abelson interacting protein 1 (Abi-1), an important component for controlled actin assembly. During early neuronal development, Abi-1 is present in neurites and growth cones, and at later stages, the protein is enriched in dendritic spines and in the PSD. Knockdown of Abi-1 in cultured neurons results in excessive dendrite branching, immature spine and synapse morphology and a reduction of synapses, whereas Abi-1 overexpression produced an opposite effect (Proepper et al., 2007). It is likely that during development Abi-1 is recruited to the PSD via the Shank3 interaction. The direct role Shank3 may play in dendritic branching is still unclear. Nevertheless, this protein has proven to be a good candidate for a role in the regulation of neuronal arbor complexity. In fact, crystallization of the SAM domain from Shank3 revealed that it can oligomerize and form large sheets of stacked fibers, possibly forming a platform for the construction of the PSD complex (Hayashi et al., 2009).

Each Shank form shows distinct tissue distribution of mRNA. Therefore, Shank proteins are differentially expressed in different regions and at different developmental stages of the rat brain (Lim et al., 1999). The perinatal expression of Shank1-3 is relatively low but rapidly increases during the first weeks of development, peaking at 3-4 weeks in the rat brain (Lim et al., 1999). Shank1 and Shank2 mRNAs are widely expressed early in postnatal brain development, whereas Shank3 expression increases during postnatal development, especially in the cerebellum and thalamus. Shank1 and Shank3 (but not Shank2) mRNAs are present in the molecular layers of the hippocampus (Bockers et al., 2004).

Analysis of mouse models for the deletion of genes can be used to study the *in vivo* role of Shank proteins. Genetic disruption of Shank1 in mice promotes the development of smaller dendritic spines and thinner PSDs, accompanied by reduced synaptic strength and reduced levels of PSD proteins such as SAPAP and Homer (Hung et al., 2008). At the behavior level, these

animals had increased anxiety-related behavior and impaired contextual fear memory. They also displayed enhanced performance in a spatial learning task; however, their long-term memory retention in this task was impaired (Hung et al., 2008). A recent study showed that striatal medium spiny neurons of mice genetically engineered with Shank3 gene deletions exhibited neuronal hypertrophy, reduced spine number and an abnormal PSD architecture (Peca et al., 2011). Behaviorally, these mice displayed self-injurious repetitive grooming and deficits in social interaction suggestive of an autist-like phenotype. These findings demonstrate a critical role for Shank3 in the normal development of neuronal connectivity.

In humans, the Shank genes have been associated with a form of mild mental retardation associated with severe expressive language delay and minor facial dysmorphisms called the 22q13.3 deletion syndrome (Phelan-McDermid Syndrome). This syndrome is widely regarded as an autism spectrum disorder, thought to be caused by the disruption of the Shank3 gene (Durand et al., 2007; Moessner et al., 2007), which is also thought to be responsible for other non-syndromic ASDs (Gauthier et al., 2009). This raises the possibility that, in fact, Shank plays a central role in the organization and function of excitatory synapses.

Actin Cytoskeleton

The cytoskeleton network is composed principally of three types of protein filaments - actin, microtubules, and intermediate filaments - that possess unique biophysical and biochemical properties. In dendritic spines, actin appears to be the major cytoskeletal element and its organization there reflects an elaboration of highly conserved mechanisms of actin regulation. Continual growth and depolymerization of filaments are likely to mediate most actin functions in the spine (Fig. 4). The overall flow within spines, rather than resulting from a single network centered at the synapse, likely results from multiple, potentially

independent filament networks with distinct sites of polymerization (Frost et al., 2010a).

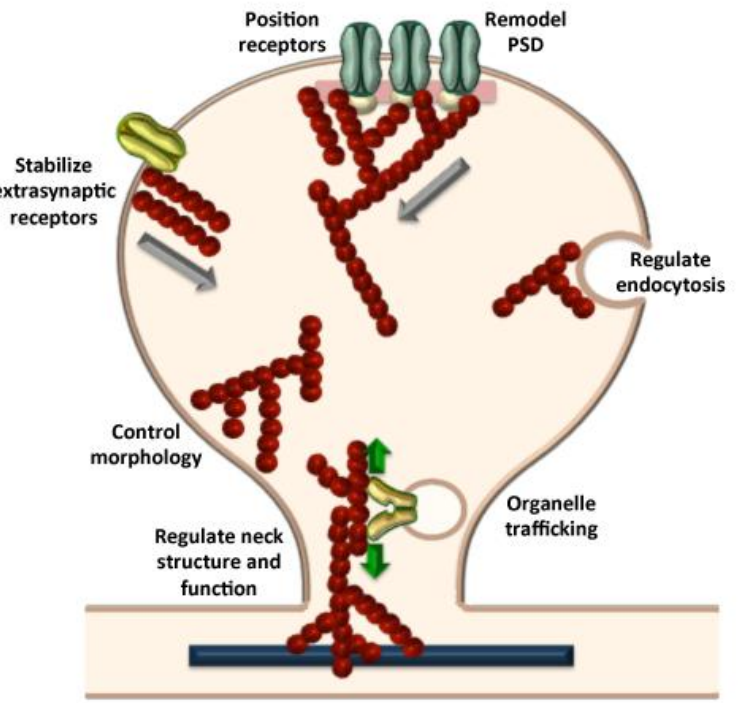


Fig. 4 - Actin cytoskeleton as a key organizer and coordinator of synapse function via numerous distributed mechanisms. Several known sites of actin regulation throughout individual spines, coordinately control PSD scaffolds and synapse function. Arrows indicate direction of actin flow (gray), and potential for cargo transport along filaments oriented in either direction (green). (Adapted from Frost et al., 2010a)

Actin filaments are closely, and apparently directly, associated with the PSD (Capani et al., 2001; Fifkova and Delay, 1982). Ultrastructural examination of dendritic spines from the rodent brain has revealed three prevalent forms of cytoskeletal filaments, all of which appear to consist of F-actin: a network of short filaments extending into the spine from the synaptic junction; a network present throughout the spine head; and filament forms (bundles) found in the

neck of the spine (Cohen et al., 1985; Fifkova and Delay, 1982; Landis and Reese, 1983; Matus et al., 1982). More recently, a study using photoactivable β-actin confirmed the existence of three distinct actin populations in spines, as well as the subspine localization and the kinetics of actin turnover (Honkura et al., 2008). This study showed that actin dynamics, and consequently spine function, can be regulated by neuronal activity, since they observed the formation of a more kinetically stable F-actin pool, in addition to the observed dynamic (at the tip of the spine) and stable pools, upon glutamate uncaging, which increases synaptic strength. A recent elegant study of the dendritic spine cytoskeletal organization has confirmed and extended these studies using electron microscopy to show that spines exhibit a continuous network of both branched and long, linear actin filaments (Korobova and Svitkina, 2010).

The functional role for actin in spines was revealed through pharmacological approaches that perturb actin dynamics. Inhibition of polymerization with lantruculin A destabilizes synapses in an age dependent manner (Zhang and Benson, 2001), and alters the number and localization of glutamate receptors and PSD proteins (Allison et al., 1998; Zhou et al., 2001). The synaptic localization is altered for scaffolding proteins such as GKAP, Shank, Homer 1c, but no effect is observed for the localization of PSD95 (Kuriu et al., 2006). A recent study showed that PSD-95 forms an enduring, spatially stable matrix within the PSD with extremely limited mixing of molecules over submicron distances (Blanpied et al., 2008). This suggests that the intermolecular binding of scaffold molecules, which constitute the foundation of the PSD architecture, is apparently not sufficient to maintain GKAP, Shank and Homer 1c in the absence of polymerizing actin. What these studies reveal is that the actin cytoskeleton can organize neurotransmitter receptors, signaling molecules and scaffolding proteins into functional subsets that are partially autonomous and spatially confined, consenting dispersal of molecules from PSD subcompartments, which in turn allows spines to modulate their shape, motility and function.

Regulation of Actin Dynamics

The molecular aspects of actin dynamics have to be considered in order to better understand the dynamic changes in dendritic spine morphology. Actin exists in two states within the cell: as globular or monomeric actin (G-actin) and as filamentous actin (F-actin), resulting from the polymerization of G-actin into double helical filaments. Actin assembly is determined by the available amount of unpolymerized G-actin and the G-actin critical concentration (Carlier, 1990), and occurs through three sequential steps: nucleation (rate limiting step), elongation, and ultimately steady state, with no net change in the amount of F-actin. Filamentous actin has a structural polarity, and the two ends lengthen and shorten at different rates (Woodrum et al., 1975). The remarkable feature of F-actin treadmilling, which results in continuous exchanges of actin molecules in F-actin, depends on critical monomer concentrations of both ends. At steady state, F-actin exhibits net polymerization at a fast growing end (the barbed or plus end) and simultaneous depolymerization at a slow growing end (the pointed or minus end), which results in continuous actin turnover in filaments. In migrating cells, the barbed ends push the plasma membrane and induce cell shape changes in the form of lamellipodia (sheet-like extension), which consist of short and highly branched actin filaments, or filopodia (rod-like extension), which contain long, unbranched actin filaments (Le Clainche and Carlier, 2008; Pollard and Borisy, 2003). The rapid turnover of F-actin has been demonstrated in dendritic spines by FRAP (fluorescence recovery after photobleaching) analysis, suggesting that most F-actin in dendritic spines undergo treadmilling (Star et al., 2002). Moreover, G-actin/F-actin equilibrium in dendritic spines is regulated in an activity-dependent manner. Measurements of fluorescence resonance energy transfer (FRET) between actin monomers revealed that synaptic stimulation rapidly changes the equilibrium between F-actin and G-actin and this regulation depends on the presence of a sequestered G-actin pool (Okamoto et al., 2004).

Several recent studies have shed light on where actin is polymerized and depolymerized in different regions of the spine. Using a barbed-end polymerization assay and FRAP analysis Hotulainen and colleagues showed that actin is capable of polymerizing not only at the spine tip, which confirmed a previous study (Honkura et al., 2008), but also at the base of the spine (Hotulainen et al., 2009). Another study using super-resolution optical imaging confirmed that the complex process of actin treadmilling takes place throughout the spine head and shaft (Tatavarty et al., 2009). These findings were recently expanded by Blanpied and colleagues, using single particle tracking photoactivated localization microscopy (sptPALM). Tracking the movements of individual actin molecules in different sub-regions of dendritic spines they found that actin filaments participate in retrograde flow from the periphery towards a central region of the spine (Frost et al., 2010b). Taken together these studies demonstrate the essential role for the complex and relatively dynamic actin filament array for the functional readout of dendritic spines.

The steady-state treadmilling of pure actin polymers is much slower *in vitro* than that of F-actin *in vivo* (Pollard, 1986; Zigmond, 1993). This is due to the presence of actin binding proteins (ABPs), which serve additional roles in regulating actin dynamics and are responsible for promoting actin polymerization and depolymerization.

Actin binding proteins in dendritic spines

A cohort of actin-binding proteins determines the characteristic of the organization of F-actin, such as the assembly of networks and straight or tangled bundles. In neurons, F-actin organizes various structures in a subcellular location-dependent manner, and each F-actin organization is accompanied by a certain set of actin-binding proteins. In fact, the interplay between many of these proteins is responsible for the growth or disassembly rate of actin, and ultimately the morphology of dendritic spines and development of synapses. In fact, these proteins can interfere with the clustering of postsynaptic proteins and with the regulation of neurotransmitter

receptor activities, creating functional synaptic connections. Many actin-binding proteins that regulate elongation, branching, and bundling or that trigger severing and depolymerization of actin filaments are found in dendritic spines (Ethell and Pasquale, 2005). These proteins can be organized according to their essential roles in the F-actin organization.

Actin Filaments Severing and Depolymerization

These proteins control F-actin length by severing F-actins and also by regulating the dynamics of treadmilling.

Gelsolin, which decreases F-actin length, has a variety of functions, such as binding and severing F-actin and capping fast-growing barbed ends (Harris and Weeds, 1984; Janmey et al., 1985), as well as nucleating new filament polymerization (Ditsch and Wegner, 1994). Gelsolin severing activity in combination with its capping activity leads to an increase in the number of pointed ends, promoting F-actin shortening. The actin severing and nucleating activities of gelsolin are inhibited by phosphatidylinositol 4,5-biphosphate (Janmey and Stossel, 1987), and are upregulated by micromolar concentrations of Ca^{2+}, which was shown to promote gelsolin severing activity, and to increase the efficiency of its capping activity (Janmey et al., 1985). Gelsolin is detected in cultured hippocampal neurons where it is implicated in plasticity (Petrucci et al., 1983). Gelsolin knockout mice exhibit increased Ca^{2+} influx following glutamate exposure and fail to display an NMDA receptor-dependent decrease in the actin turnover rate (Furukawa et al., 1997). In humans, mutations in the gelsolin gene were linked to Alzheimer's and Parkinson's diseases (Haltia et al., 1991), whereas gelsolin levels were implicated in Down syndrome (Ji et al., 2009). All these evidences support the idea that gelsolin mediates actin remodeling in dendritic spines.

Cofilin is a member of the ADF/Cofilin family of actin-depolymerizing proteins. It decreases F-actin length in a Ca^{2+}-independent and a pH-dependent manner (Bamburg et al., 1980; Nishida et al., 1984; Yonezawa et al., 1985).

ADF/Cofilin preferentially binds to ADP-actin subunits in a minor twisted conformation of F-actin, severing the filaments (Maciver et al., 1991). This increases the pool of G-actin monomers used by actin polymerizing factors (described below) and also creates free barbed ends that can nucleate filament growth. However, the depolymerization activity of ADF/cofilin is mainly derived from its ability to increase the rate of dissociation from the pointed end of F-actin (Carlier et al., 1997). Cofilin activity is regulated by phosphorylation, which inhibits its binding to F-actin, and also through other mechanisms that influence the actin-severing ability of cofilin [reviewed in (Pontrello and Ethell, 2009)]. Cofilin is expressed at high levels in the adult brain, being more abundant than ADF in mammalian neurons (Garvalov et al., 2007; Moriyama et al., 1990). Using EM, Cofilin was found to accumulate near the specific "shell" region of the spine that contains a dynamic F-actin pool, while it avoids the spine "core" region with a stable F-actin pool (Racz and Weinberg, 2006). Moreover, cofilin-1 knockdown in cultured hippocampal neurons was shown to decrease the number of mature dendritic spines and to induce the formation of abnormal protrusions-like branches (Hotulainen et al., 2009). Formation of cofilin-actin rods structures was detected in brains of subjects with Alzheimeir's disease (Bamburg et al., 2010; Bamburg and Bloom, 2009). It is possible that recruitment of a large number of cofilin molecules to actin rods in neurites depletes the cofilin pool in dendritic spines, affecting spine maintenance and synaptic plasticity in affected neurons. Together, these evidences support a role for cofilin in dendritic spine plasticity, due to the contribution of its actin-remodeling activity to dendritic spine morphology and also to its localization within spines.

Actin Polymerization and Elongation

Most of the proteins that participate in actin polymerization are considered cross-linking proteins, organizing F-actin into bundles or networks.

The Arp2/3 Complex enhances actin nucleation and causes the branching and cross-linking of F-actin. It nucleates branches from the sides of existing

actin filaments and caps the pointed ends, thus creating additional fast-growing barbed ends for further actin polymerization and elongation (Amann and Pollard, 2001; Mullins et al., 1997; Pantaloni et al., 2000), and ensuring that the newly produced filaments are short ad rigid. Spatially, Arp2/3 is positioned at the pointed end of the newly forming filament (Pollard, 2007). The nucleation activity and localization of the Arp2/3 complex is regulated by phosphorylation (LeClaire et al., 2008), which can be achieved by proteins such as MAPK-activated protein kinase 2 (MAPKPK2) and the p21-activated kinase (PAK) (Singh et al., 2003; Vadlamudi et al., 2004). Activation and regulation of this complex can also occur through interactions with several proteins such as proteins of the Wiskott-Aldrich Syndrome protein (WASP) family, Neural-WASP (N-WASP) Scar, the WASP-family verprolin-homologous protein (WAVE) (Takenawa and Suetsugu, 2007) and cortactin, which will be discussed in detail in a subsequent section. Concerning the balance between assembly and disassembly of actin filaments, some proteins work in cooperation with the Arp2/3 complex, like gelsolin and cortactin, while others compete against it, like ADF/cofilin. The Arp2/3 complex is sparsely distributed in the PSD of dendritic spines, concentrating in specific domains, which suggests that actin branching occurs in restricted spine regions (Racz and Weinberg, 2008). Knockdown of several regulatory proteins of the Arp2/3 complex in cultured hippocampal neurons, like N-WASP, WAVE and also Abp1, a protein structurally similar to cortactin that activates N-WASP or phosphorylates Arp2/3 complex directly, promoted a decrease in spine number and changes in spine morphology (Haeckel et al., 2008; Kim et al., 2006; Wegner et al., 2008). WAVE-1 knockout mice, as well as mice targeted with a WAVE-1 mutation, showed an impairment in synaptic plasticity and reduced learning and memory (Soderling et al., 2007; Soderling et al., 2003). Recently, knockdown of Arp2/3 complex in hippocampal neurons promoted a decrease in mushroom, thin, and stubby spines, as well as an overall decrease in protrusion density (Hotulainen et al., 2009). Taken together, these results suggest a tight regulation for the Arp2/3 complex in dendritic spines, which induces formation of mature dendritic spines and synapses, and regulates synaptic plasticity.

α-actinin promotes bundling and cross-linking of F-actin in a concentration-dependent manner (Grazi et al., 1991; Sjoblom et al., 2008). α-actinin activity depends on certain conditions and factors, such as the α-actinin/actin ratio, Ca^{2+} concentration, and binding partners (Wachsstock et al., 1993). Isotropic networks of F-actin are formed at low α-actinin concentrations, whereas at higher α-actinin concentrations formation of bundles of F-actin takes place. α-actinin is enriched at the PSD of excitatory synapses, and this localization depends on F-actin (Walikonis et al., 2000). In dendritic spines, the function of α-actinin to promote both actin filament elongation and branching depends on its interacting proteins. Interaction of α-actinin with SPAR (Spine Associated Rap GTPase) has been shown to promote mature dendritic spines, *i.e.,* dendritic spines with large heads (Hoe et al., 2009). Conversely, overexpression of α-actinin in cultured hippocampal neurons led to an increase of both the length and number of dendritic filopodia-like (immature) extensions (Nakagawa et al., 2004). α-actinin is thought to modulate NMDA receptor function, since it interacts with the NMDA receptor in dendritic spines (Dunah et al., 2000). By cross-linking F-actin, α-actinin anchors the NMDA receptor to the postsynaptic membrane, and regulates NMDA receptor activity (Wyszynski et al., 1998). Using mouse models, it was shown that α-actinin can be linked to neural pathologies such as Alzheimer's and Huntington's disease. Decreases in postsynaptic α-actinin were observed in both cases, and these differences may be due to the cross-linking activity of α-actinin leading the formation of neuronal inclusions (Galloway et al., 1987; Luthi-Carter et al., 2003). Therefore, α-actinin is also in the pole position for modulating synaptic plasticity, due to its actin branching and elongation activity.

Debrin is considered a F-actin side-binding protein, altering its configuration from kinky to straight at the filament level, thus strongly modifying the structural property of F-actin (Ishikawa et al., 1994). These so-called side-binding proteins sometimes prevent filaments from interacting with other actin-binding proteins, such as α-actinin, fascin, gelsolin, cofilin, and myosin, regulating many interactions of F-actin. Of all isoforms, debrin A is neuron-specific (Shirao and

Obata, 1986), and was found to accumulate in the head of mushroom dendritic spines, specifically at postsynaptic sites of excitatory synapses, and is barely detected in presynaptic terminals, neuronal cell bodies or axons (Aoki et al., 2005; Hayashi et al., 1996). Overexpression of debrin A in immature neurons induces accumulation of F-actin and PSD95 at postsynaptic sites (Mizui et al., 2005), whereas its knockdown decreases spine density, width and also filopodia, and disrupts PSD95 postsynaptic localization (Takahashi et al., 2006; Takahashi et al., 2003). These findings suggest that debrin A is implicated in the development of dendritic spines and synapses. Debrin A localization was shown to change upon AMPA and NMDA receptor activation, suggesting that it is regulated by synaptic activity (Takahashi et al., 2009). NMDA receptor activation induces a loss of debrin A from dendritic spines, whereas AMPA receptor activation induces debrin clustering at postsynaptic sites. This reinforces the idea that debrin A is involved in postsynaptic differentiation during spine development, and also in spine remodeling. Debrin A was also implicated in learning and memory and the regulation of dendritic spine plasticity (Hayashi and Shirao, 1999). Furthermore, isoforms of debrin are markedly reduced in hippocampi of Alzheimer's patients, as well as in frontal temporal cortex of Down syndrome's patients (Harigaya et al., 1996; Shim and Lubec, 2002). Taken together, these observations support the significance of debrin in normal synapse development and synaptic plasticity.

Profilin is a multifunctional G-actin-binding protein. It has opposite effects: profilin can inhibit actin polymerization and elongation by sequestering actin monomers, decreasing their free concentration available to form F-actin polymers (Carlsson et al., 1977; Tobacman and Korn, 1982), but it also can promote actin polymerization by binding the barbed ends of actin filaments and directly elongating them, and also by catalyzing the exchange of ADP for ATP on G-actin, since ATP-bound actin has a higher efficiency of assembly into filaments (Frieden and Patane, 1985; Pring et al., 1992; Tilney et al., 1983). Profilin activity can be regulated by phosphorylation, which increases its association with G-actin, inhibiting actin polymerization (Ackermann and Matus,

2003), and also through interaction with PIP_2, which dissociates the profilin-actin complex leading to release of free G-actin monomers and polymerization (Lassing and Lindberg, 1988). Profilin is detected at synaptic sites in the adult cerebellar cortex (Faivre-Sarrailh et al., 1993). In fact, of both profilin subtypes, profilin II is the one targeted to spine heads in an activity-dependent manner, stabilizing spine morphology (Ackermann and Matus, 2003). This suggests that profilin may be involved in the regulation of the dendritic spine during experience-dependent plasticity. Furthermore, Profilin II has been suggested to mediate cytoskeletal changes related with diseases such as Huntington's, Miller-Dieker syndrome and fragile X mental retardation (Pontrello and Ethell, 2009). Due to remarkably diverse functions, profilin controls actin dynamics underlying formation, stabilization or remodeling of dendritic spines.

Neurabin-I and Neurabin-II (Spinophilin) are structurally related and display actin cross-linking and bundling activity. Neurabin-I was originally identified as a neuronal F-actin binding protein, and has been shown to accumulate in dendritic spines and the lamellipodia of the growth cone of immature neurons (Nakanishi et al., 1997). On the other hand, despite being ubiquitous, Spinophilin is expressed most abundantly in the brain, where it localizes in the PSDs (Satoh et al., 1998). Spinophilin phosphorylation by protein kinase A (PKA) or CaMKII regulates not only its activity, but also its expression and specific localization within dendritic spines, by reducing the affinity of spinophilin for F-actin (Grossman et al., 2004; Hsieh-Wilson et al., 2003). Spinophilin binds to a multitude of partners (Sarrouilhe et al., 2006), including protein phosphatase-1 (PP1), which is highly abundant in dendritic spines. Spinophilin allows PP1 to desphosphorylate its substrates, such as AMPA and NMDA, by participating in PP1 targeting to the postsynaptic membrane (Allen et al., 1997). Despite being structurally similar and localizing in dendritic spines, where they share some partners, Neurabins I and II have variable effects on dendritic spine morphology (Pontrello and Ethell, 2009).

Actin-based Molecular motor: Myosins

Actin motor proteins are actin-binding proteins, which are responsible for moving organelles along F-actin as well as for moving the F-actin filaments themselves. Myosin is an ATP-driven, actin-based molecular motor that influences actin dynamics (Sellers, 2000). There are various types of myosins, such as myosins I, II, V, and VI, which have been observed in neurons (Bridgman, 2004). The myosin II, V and VI isoforms are found in dendritic spines, where they are suggested to regulate dendritic spine shape, since they contribute to the formation of mushroom and stubby spines, and synaptic plasticity (Morales and Fifkova, 1989). The interaction between actin and myosin, specifically myosin II, is inhibited by debrin, and this was concluded due to differences in the immunoreactivity of both proteins in various dendritic domains (Hayashi et al., 1996). Myosin motors can also interact with NMDA receptor subunits, and controlling the trafficking of the GluA1 subunit of AMPA receptors to dendritic spines, in a Ca^{2+}-dependent manner. Increasing evidences suggest that myosins, at least the isoforms II, V and VI, execute important roles in promoting mature dendritic spine morphology and regulating synaptic plasticity (Pontrello and Ethell, 2009).

Actin cytoskeleton and spine formation (morphogenesis)

During synaptogenesis, a transitional stage in spine formation has to follow the initial contact between an axon and a dendrite, and this process involves the conversion of a dynamic filopodium to a stable mushroom spine (Arikkath and Reichardt, 2008; Craig et al., 2006; Yoshihara et al., 2009). Filopodia initiation, elongation, and spine head formation are controlled by specific mechanisms of actin regulation. Dendritic filopodia are most prevalent in young neurons undergoing active synaptogenesis, which suggests a major role in synapse formation. However, the molecular mechanisms involved in this initial step of dendritic filopodia formation are currently unknown. It has been shown

that these structures initiate from preexisting patches of branched actin or small lamellipodia, from the dendritic shafts, and these actin-rich sites may subsequently become the filopodial base (Andersen et al., 2005; Korobova and Svitkina, 2010). In fact, Korobova and colleagues showed that dendritic filopodia have network-like cytoskeletal organization, which is unusual for highly elongated membrane protrusions, where a tight actin filament bundle is considered to be obligatory (Chhabra and Higgs, 2007). The structural organization of dendritic filopodia suggests potential mechanisms of their differentiation into spines, since their network-like organization makes them more plastic, which allows frequent changes of direction (Portera-Cailliau et al., 2003).

Several possible mechanisms may be involved in the initiation and elongation process, which can be random or signal-induced [reviewed in (Hotulainen and Hoogenraad, 2010)]. It has been shown that glutamate released from presynaptic sites influences filopodia initiation and elongation. There is possibly a contribution from membrane-deforming proteins, such as IRSp53 (insulin receptor tyrosine kinase substrate protein 53) (Choi et al., 2005; Mattila and Lappalainen, 2008), which can induce and support tubular membrane protrusions (Saarikangas et al., 2009; Yang et al., 2009). Enhanced polymerization of actin filaments assisted by formin mDia2 or Ena/VASP-induced anti-capping of filaments generated by the Arp2/3 complex may also contribute to initiate filament elongation (Hotulainen et al., 2009). Moreover, and surprisingly, some actin structures frequently reside directly on the microtubule array and these filaments seem to branch off of a microtubule in the neurite suggesting involvement of a microtubule-associated actin filament nucleator(s) or actin–microtubule cross-linkers, which remain to be identified (Korobova and Svitkina, 2010).

The dendritic filopodium, due to the plastic network-like organization of its cytoskeleton, performs a wide range of movements searching for an axon. Once the axonal contact is made and an appropriate signal is received, a

dendritic filopodia is formed, spine motility gradually decreases and the spine structure is stabilized, which requires the assembly of both pre- and postsynaptic components (Arikkath and Reichardt, 2008; Craig et al., 2006; Yoshihara et al., 2009). The filopodium-to-spine transformation occurs as swelling of the filopodial tip and shortening, which has been observed by imaging techniques (Marrs et al., 2001). The swelling, or spine head growth, may occur through Arp2/3 complex-dependent extensive actin filament branching at the filopodial tip, which would drive the head expansion (Fig. 5). In fact, Hotulainen and colleagues proposed the attractive mechanism of a switch from formin mDia2 (mammalian *diaphanous*-related)-based actin polymerization, which promote formation of unbranched actin filaments, to Arp 2/3 complex-based actin polymerization leading to the initiation of spine head growth (Hotulainen et al., 2009). In support to this model, other studies showed that a complex with WAVE and Arp2/3 inhibits formin mDia2-induced filopodia formation in fibroblast cells (Beli et al., 2008). The filopodia shortening during spine maturation was proposed to involve myosin II (Ryu et al., 2006). Accordingly, myosin II, as well as actin filaments of mixed polarity were detected in the shafts of dendritic filopodia (Korobova and Svitkina, 2010). Myosin II-dependent contractility within the head and the neck can modulate the shape of the spine to fit the requirements of synaptic transmission.

Newly formed spines are usually thin and elongated and generally possess a small spine head. Out of three structural domains of a mushroom spine, the base is a previously unrecognized compartment whose structure ranges from an elaborate mixture of branched and linear filaments to a few converging linear filaments. Surprisingly, the spine neck is supported by a longitudinally stretched network of branched and linear filaments of different lengths, which are only roughly aligned with each other (Korobova and Svitkina, 2010). In fact, the presence of long bundled filaments has been revealed in other studies (Korobova and Svitkina, 2008; Svitkina et al., 2003; Yang et al., 2007).

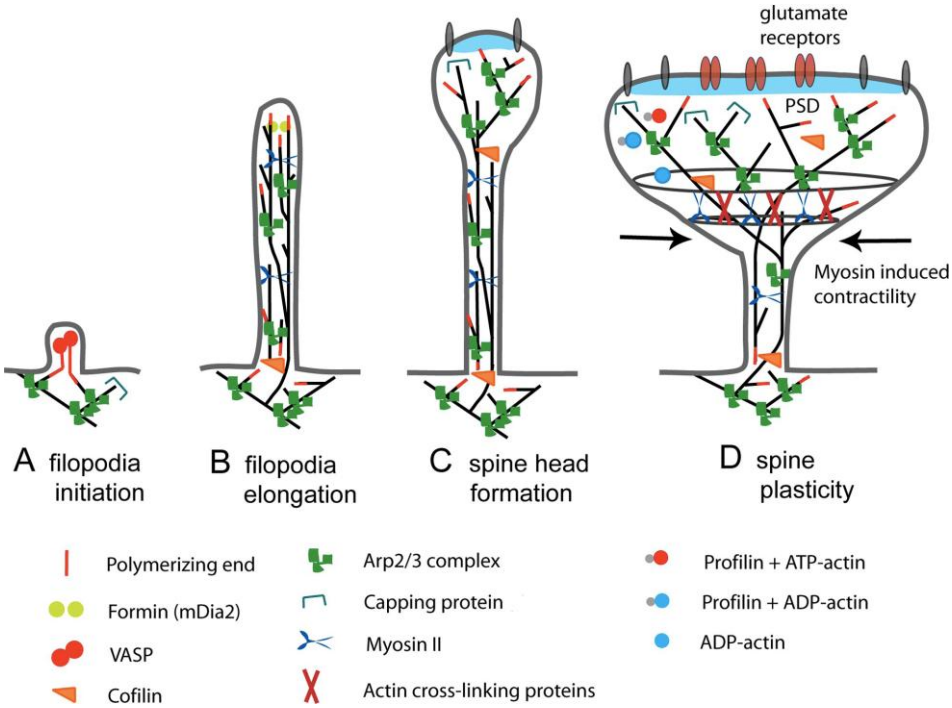


Fig. 5 – **Schematic of actin regulatory mechanisms during spine formation**. A) Dendritic filopodium initiation and its elongation start spine development. B) Filopodia elongation is promoted by actin filament polymerization, in which myosin and Ena/VASP participate. C) Spine head begins to form and enlarge due to extensive actin branching leaded by the large Arp 2/3 complex at the filopodium tip. Proper length of actin filaments is controlled by ADF/cofilin function, which also replenishes the cytoplasmic actin monomer pool. D) Mature dendritic spine with dynamic properties. Despite maintaining its overall morphology, spine head shape can be further modulated by the combined activity of different actin-binding regulatory proteins, during synaptic plasticity. (Adapted from Hotulainen and Hoogenraad, 2010)

The spine head undergoes constant actin-dependent shape changes, probably regulated by synaptic stimulation (Bourne and Harris, 2008; Tada and Sheng, 2006). Dynamic actin-dependent processes are frequently associated with extensively branched actin networks nucleated by the Arp2/3 complex in the distal regions of the spines (Goley and Welch, 2006; Pollard and Borisy, 2003). Spine head formation not only requires actin filament polymerization, but a three-dimensional organization of actin filaments is also vital. Mechanisms for size and shape remodeling, like protrusion and retraction, cooperate in

generating a proper shape, which may explain the presence of myosin II in spines (Morales and Fifkova, 1989; Ryu et al., 2006). Both activation and inhibition of myosin II-induced contractility is an important process in dendritic spine morphogenesis (Zhang et al., 2005). Furthermore, proper morphology and stabilization of spines can also be achieved by regulation of cofilin activity (Hotulainen et al., 2009). Moreover, actin cross-linking proteins such as CaMKIIβ, neurabin I, and debrinA can also be important for spine head modification and stabilization (Ivanov et al., 2009; Okamoto et al., 2007; Terry-Lorenzo et al., 2005).

In conclusion, a likely sequence of cytoskeletal reorganization events underlying the spine morphogenesis is suggested by the complex cytoskeletal organization and molecular composition of dendritic spines and dendritic filopodia.

Actin Remodeling and Synaptic plasticity

Synaptic plasticity is associated with a rapid and persistent reorganization of the spine actin cytoskeleton (Cingolani and Goda, 2008). The synapse is a highly dynamic structure that can rapidly respond and adapt to different intrinsic or extrinsic cues, via regulation of the actin cytoskeleton. The strength of a synapse is defined by the change in transmembrane potential resulting from activation of the postsynaptic neurotransmitter receptors and activity-dependent changes in synaptic strength are called synaptic plasticity (Malinow and Malenka, 2002). Synaptic plasticity is the cellular basis of learning and memory and is exemplified by two extensively characterized models involving changes that last for hours or longer: long-term potentiation (LTP), which enhances synaptic transmission, and long-term depression (LTD), which decreases synaptic transmission (Bliss and Lomo, 1973). The most common approach to induce these two forms of plasticity is to apply high frequency or low frequency stimulation to induce LTP or LTD, respectively (Malenka and Bear, 2004). Long-term potentiation (LTP) and long-term depression (LTD) occur as a result of correlated or uncorrelated activity of two coupled neurons and are controlled by

glutamate receptors (Malenka and Bear, 2004). In many synapses, LTP is dependent on the activation of postsynaptic NMDA receptors, upon which calcium flows through the NMDAR channel into the postsynaptic cell and transduces information into biochemical signals. This includes activation of several protein kinases such as CaMKII, PKA, PKC, and protein phosphatases, that in turn regulate the phosphorylation and trafficking of AMPAR to the plasma membrane, increasing the number o AMPAR on synaptic membranes, thereby potentiating synaptic transmission (Esteban, 2003; Malinow and Malenka, 2002; Sheng and Kim, 2002; Shepherd and Huganir, 2007). In contrast, long-term depression (LTD), which is the weakening of a neuronal synapse that lasts from hours to days, is also thought to result from changes in postsynaptic receptor density, since AMPA receptors are rapidly internalized in response to LTD-inducing stimuli (Beattie et al., 2000; Carroll et al., 1999; Ehlers, 2000). During LTP and LTD, synapses undergo structural remodeling. LTP-inducing stimuli causes growth of new dendritic spines, enlargement of pre-existing spines and their associated postsynaptic densities (PSDs), and the splitting of single PSDs and spines into two functional synapses (Abraham and Williams, 2003; Yuste and Bonhoeffer, 2001), whereas, LTD-inducing stimulation is associated with shrinkage and/or retraction of spines (Nagerl et al., 2004; Okamoto et al., 2004; Zhou et al., 2004). Therefore, this structural remodeling links the regulation of underlying actin dynamics and reorganization to synaptic plasticity.

Actin rearrangements drive the formation and loss of dendritic filopodia and spines as well as their morphological plasticity (Matus, 2000). The activity-dependent change of actin-binding proteins is a highly possible mechanism of F-actin reorganization. In fact, F-actin and several ABPs are regulated within dendritic spines in an activity dependent manner. Through imaging techniques it was shown that LTP inducing stimulation increases actin polymerization in spines of hippocampal neurons (Fukazawa et al., 2003; Okamoto et al., 2004), In addition, a FRET-based approach also showed that induction of LTP results in a higher F-actin/G-actin ratio followed by the enlargement of spine heads, whereas LTD inducing stimulation shifts the actin equilibrium toward G-actin,

resulting in depolymerization of actin and spine shrinkage (Okamoto et al., 2004). Disruption of the actin cytoskeleton by actin depolymerizing agents affects AMPAR-mediated basal synaptic transmission, as well as the maintenance of the early and late stages of LTP (Kim and Lisman, 1999; Krucker et al., 2000). These findings suggest a close relationship among synaptic activity, spine size, and F-actin/G-actin ratios in spines (Lin et al., 2005). Furthermore, some actin binding proteins, such as profilin (Ackermann and Matus, 2003) and cofilin (Fukazawa et al., 2003), accumulate in dendritic spines, and others, such as cortactin (Hering and Sheng, 2003), debrin (Sekino et al., 2006), and SPAR (Pak and Sheng, 2003), disappear from dendritic spines after NMDA receptor activation, implicating these proteins in the activity-dependent regulation of spine morphogenesis.

NMDA and AMPA-type glutamate receptors regulate the actin signaling pathways in spines. The major signaling hot spots in actin cytoskeleton regulation are small GTPases of the Rho and Ras families, which regulate the ABPs that control actin dynamic (Ethell and Pasquale, 2005; Tada and Sheng, 2006). RhoA, Rac1, and Cdc42 are ubiquitously expressed but present at high levels in neurons having profound influence on dendritic spine morphogenesis (Hall, 1998; Nakayama et al., 2000). Constitutively active Rac1 causes a reduction in the size of the dendritic spines but increases their density, in parallel with increasing the number of synapses (Nakayama et al., 2000), whereas Cdc42 induces spine head enlargement in dendritic spines (Irie and Yamaguchi, 2002; Wegner et al., 2008). RhoA activation has been shown to be necessary for expression of LTP via cofilin inactivation in dendritic spines (Rex et al., 2009). Thereby, RhoA is important to inhibit cofilin activity, which will result in actin filament and spine stabilization. On the other hand, Rac and Cdc42 regulate spine head formation, mainly by activating Arp2/3 complex-induced nucleation and inhibiting actin depolymerization via cofilin.

The Ras family of GTPases and their downstream MAP kinase signaling pathways, as well as many of the Rho and Ras GTPase activators (GEFs –

guanine nucleotide exchange factors) and inhibitors (GAPS – GTPase activating proteins), also regulate spine morphogenesis and neuronal functioning (Kennedy et al., 2005). Deletion of synGAP (synaptic Ras GAP), which is a major postsynaptic inhibitor of Ras signaling abundantly enriched in the PSD, has been shown to accelerate spine development and to cause an increase in the number of mushroom spines on hippocampal neurons (Vazquez et al., 2004). On the other hand, Rap GTPases appear to play opposing roles in synaptic plasticity. In fact, Rap1 and Rap2 mediate LTD and depotentiation, respectively, whereas Ras promotes LTP (Zhu et al., 2005). Also, the RapGAP SPAR (spine associated Rap GAP) in contrast to SynGAP, promotes the growth of spines (Pak and Sheng, 2003; Pak et al., 2001).

Various receptor tyrosine kinases, such as members of the Trk (Menna et al., 2009) and Eph/ephrin families (Schubert and Dotti, 2007), as well as synaptic adhesion molecules (Yoshihara et al., 2009), have been shown to be important in regulating actin in spines. Members of the Src family of non-receptor tyrosine kinases were also found in dendritic spines and implicated in spine reorganization, most likely through controlling actin polymerization (Morita et al., 2006).

Formation of protrusions from the spine head is stimulated by glutamate and blocked by AMPA receptor antagonists, suggesting that AMPAR activation may play a part in spine morphogenesis (Richards et al., 2005). Interestingly, AMPAR themselves, specifically the N-terminal domain which extends far enough from the postsynaptic membrane potentially to interact with presynaptic membrane, may induce spine enlargement, although the mechanism is unknown (Nakagawa et al., 2005; Passafaro et al., 2003).

Taken together, these findings suggest that many of the mechanisms that affect the development, maintenance and plasticity of excitatory synapses, which are the most regulated and heterogeneous of all neuronal structures, may influence the number and shape of dendritic spines.

Cortactin

Cortactin was discovered over two decades ago, and was initially characterized as both an F-actin binding protein and a v-Src substrate (Kanner et al., 1990; Wu et al., 1991), which suggested that it was one key molecule involved in cortical actin regulation, possibly linking structural (cytoskeletal) organization and signal transduction. This idea was further supported when cortactin was recognized as binding partner of the Arp2/3 complex, potentiating is function (Uruno et al., 2001; Weaver et al., 2002; Weed et al., 2000). In fact, the multi-domain protein cortactin was shown to play an essential role in many actin-based cellular processes such as cell migration an invasion (Yamaguchi and Condeelis, 2007), axon guidance (Knoll and Drescher, 2004), neuronal morphogenesis (Gray et al., 2005; Martinez et al., 2003), endocytosis (Cosen-Binker and Kapus, 2006) and tumor cell metastasis (Li et al., 2001), including cancer progression and invasion (Ayala et al., 2006; Buday and Downward, 2007; Weaver, 2006, 2008; Yamaguchi and Condeelis, 2007). One can say that cortactin is always present when changes in the cortical actin skeleton initiate, modify, or accompany major membrane events.

As a nearly ubiquitous protein, cortactin is also present in the brain. Cortactin is enriched in growth cones of developing neurons (Du et al., 1998) and also in dendritic spines, where it colocalizes with F-actin. Two pools of cortactin were identified in EM studies: a large pool in the actin core within the dendritic spine, which is implicated in regulating its shape, and a smaller pool near the PSD that may be involved in the regulation of synaptic function (Racz and Weinberg, 2004). Indeed, cortactin plays a key role in the morphogenesis of dendritic spines as suggested by the findings that its overexpression leads to spine elongation, whereas its down-regulation results in loss of spines (Hering and Sheng, 2003).

Cortactin Structure

Cortactin is a 63-65kDa protein, which migrates as an 80/85kDa doublet in SDS-PAGE gels, when purified from most cell types as well as from recombinant cell-free sources (Huang et al., 1997a; Wu and Parsons, 1993; Wu et al., 1991). These two bands, the 80 kDa and 85 kDa, are likely to represent different conformational isomers of cortactin (Evans et al., 2011). Analytical ultracentrifugation and electron microscopy experiments of full-length cortactin revealed a monomeric rod-shaped molecule between 220 and 290 Å long and 20 Å wide (Weaver et al., 2002), which may represent the “open” form of the protein, opposing the partially globular conformation of the folded protein (Cowieson et al., 2008).

The domain structure of cortactin gives clues to its function and potential mode of action. The cortactin protein is composed of ~550 amino acids “divided” in five discrete regions (Fig 6): the N-terminal acidic (NTA) region, the actin-binding repeats region, the helical region, the proline-rich region, and the SH3 domain (Weed and Parsons, 2001). The N-terminal acidic region incorporates the conserved DDW region that is responsible for interaction with Arp3 and subsequent activation of the Arp2/3 complex (Higgs and Pollard, 2001; Weed et al., 2000). Following the NTA region, there are six-and-a-half repeats of a 32-amino-acid motif that together bind to F-actin (ABR – actin binding region), with maximal binding activity centered on the fourth repeat. These repeats have also been reported to bundle actin into filaments (Weed and Parsons, 2001). Thereby this half of the molecule is responsible for coupling cortactin to structural elements of the cytoskeleton. The COOH-terminal half of cortactin can be regarded as the regulatory segment of the protein, since it is composed of an α-helical domain comprising a site of calpain cleavage (Perrin et al., 2006), followed by a proline-rich region that contains many sites of serine, threonine, and tyrosine phosphorylation (PST), and finally a Src homology 3 domain. The PST region is targeted by kinases that transduce signals from cell surface receptors, such as p21-activated kinase

(PAK) (Vidal et al., 2002) and the MAP kinase ERK (Campbell et al., 1999; Martinez-Quiles et al., 2004), implicated in serine phosphorylation, and also members of the Src kinase family, like v-Src, c-Src, and Fyn, that phosphorylate tyrosine residues (Huang et al., 1997a; Kapus et al., 2000; Kapus et al., 1999; Wu and Parsons, 1993). Other non-receptor tyrosine kinases such as Fer (Kapus et al., 2000; Kim and Wong, 1998) and Syk (Gallet et al., 1999) also phosphorylate cortactin. The C-terminal SH3 domain of cortactin binds to several proteins, including modulators of actin polymerization, through their proline-rich binding sequences, thus linking cortactin-mediated actin remodeling to various specific loci and processes. These partners include the Arp2/3 activating proteins N-WASP (Arp2/3-stimulating Wiscott-Aldrich protein) (Mizutani et al., 2002) and WIP (WASP-interacting protein) (Kinley et al., 2003), the endocytic GTPase dynamin 2 (McNiven et al., 2000) and several PSD scaffolding proteins such as dynamin 3 (Gray, 2005) and proteins of the Shank family (Naisbitt et al., 1999). This plethora of interactions indicates that cortactin is in fact used as a link between the array of processes where these proteins interfere and cytoskeleton remodeling.

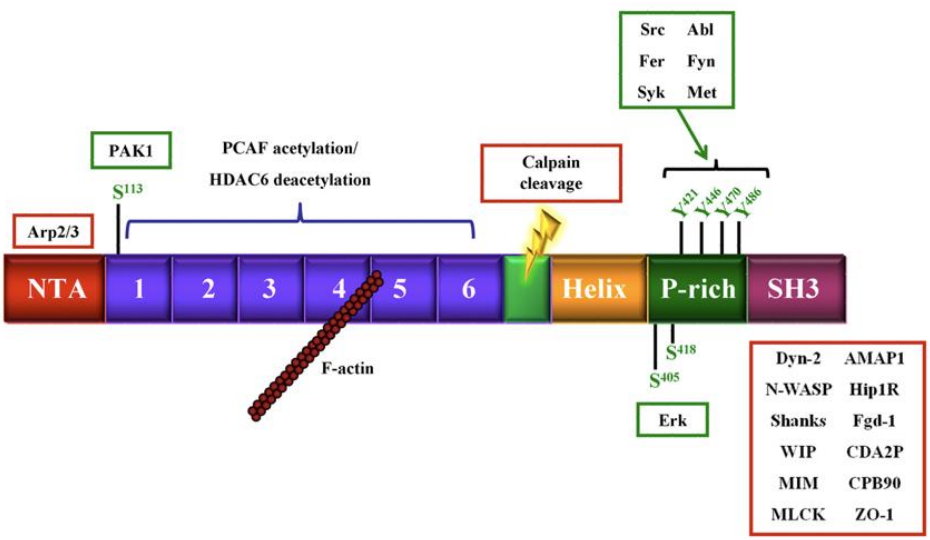


Fig. 6 – **Schematic representation of the domain structure and interactions of cortactin**. The various functional domains are shown, and described in the text. The different outlined proteins represent kinases known to phosphorylate cortactin at the indicated sites (green), and also SH3 binding partners (red). [Adapted from (Ammer and Weed, 2008)]

Cortactin molecular interactions

The association of cortactin with the Arp2/3 complex, and its activation, was the breakthrough to understand the basic function of cortactin. The NTA region is sufficient to directly bind the Arp2/3 complex, and mutation of both acidic residues, or of the tryptophan residue, in the DDW motif abolishes Arp2/3 binding (Uruno et al., 2001; Weed et al., 2000). As it has been previously described, the activation of the seven protein complex – Arp2/3 complex - initiates the nucleation and formation of "daughter" filaments in a 70-degree angle, on the side of "mother" actin filament (Higgs and Pollard, 2001). Arp2/3 is activated by nucleation promoting factors (NPFs), and cortactin, along with members of WASP superfamily, is the most important. Generally, NPFs are proteins that directly bind to and activate, due to conformational changes, *de novo* nucleation and elongation functions of Arp2/3 complex (Welch and Mullins, 2002), Cortactin functions as an NPF for Arp2/3, since it stabilizes the active conformation of the Arp 2/3 complex through its ability to bridge Arp3 with other proteins of the complex, facilitating the addition of actin monomers to the Arp2 and Arp3 subunits on the side of the mother filament (Pollard, 2007; Weaver et al., 2002). *In vitro*, cortactin is a substantially weaker activator of F-actin assembly than N-WASP; nevertheless, cortactin possesses a unique property since it simultaneous binds to F-actin, thus coupling the Arp2/3-dependent actin polymerization to an existing actin filament (Fig.7).

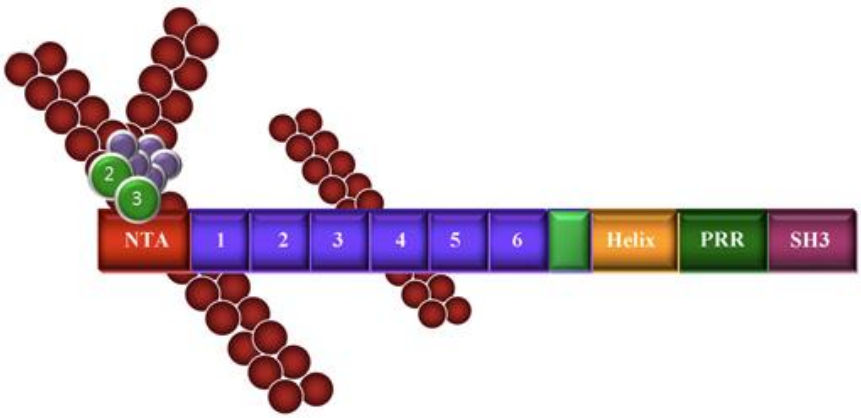


Fig. 7 – **Cortactin-mediated activation of Arp2/3 complex**. Cortactin functions as an NPF for Arp2/3 complex (consists of seven subunits, including Arp2, Arp3, and five other actin related proteins, represented by the green and purple circles), binding the Arp2/3 through its NTA domain and F-actin through the fourth tandem repeat, resulting in direct activation Arp2/3 actin nucleation activity. [Adapted from (Ammer and Weed, 2008)]

In fact, cortactin has a 20-fold higher affinity for F-actin than the Arp2/3 complex (Uruno et al., 2001). Contrariwise, cortactin has a much higher affinity for Arp2/3 once it has been associated with an actin filament (Uruno et al., 2003). Furthermore, cortactin preferentially binds to F-actin filaments containing ATP or ATP/ADP-Pi, demonstrating a higher affinity for newly polymerized actin filaments (Bryce et al., 2005). Thereby the NPF function of cortactin requires both the NTA and ABR regions of the protein, demonstrating the importance of interaction with both F-actin and Arp2/3 to efficient actin polymerization, and additional stabilization of newly formed branches (Weaver et al., 2001).

The WASP family of proteins, including WASP and N-WASP, and the related WAVE/Scar proteins also function as NPFs for Arp2/3 (Pollard, 2007). These proteins bind to F-actin through a basic region, and not an ABR like cortactin (Kelly et al., 2006; Suetsugu et al., 2003). These proteins can bind the Arp2/3 complex and, unlike cortactin, WASP, N-WASP and WAVE/Scar bind G-actin monomers through a Wasp Homology-2 (WH2)-central-acidic (WCA) region (Marchand et al., 2001) (Fig. 8). Despite the importance of F-actin

binding for WASP-family protein function, the binding of G-actin to the WCA region is extremely significant since it results in dramatic enhancement of NPF activity by placing actin monomers in close proximity to activated Arp2/3 (Machesky et al., 1999).

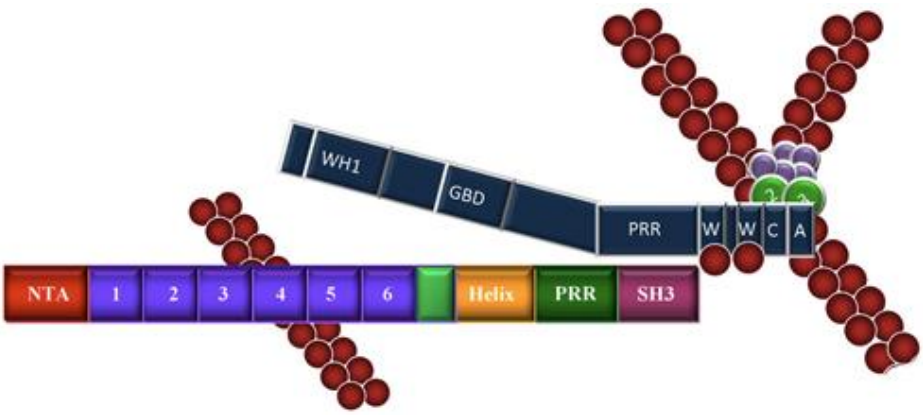


Fig. 8 – **N-WASP-mediated activation of Arp2/3 complex**. Cortactin facilitates N-WASp mediated Arp2/3 activation through binding of its SH3 domain to the PRR region of N-WASp, thereby disrupting N-WASp autoinhibition The WCA domain of N-WASp binds to and activates Arp2/3, and also binds ATP-loaded G-actin. [Adapted from (Ammer and Weed, 2008)].

Cortactin can stimulate Arp2/3 through a number of potential mechanisms. First, it directly binds to Arp3, inducing a conformational change. N-WASP-cortactin-Arp2/3 may form a ternary complex since the binding site on Arp3 for both proteins only partially overlap, resulting in enhanced NPF activity. In fact, the binding of the cortactin NTA domain displaces the N-WASP WCA domain from the Arp3 subunit, but not from the other subunits involved in the interaction between N-WASP and Arp2/3 complex (Weaver et al., 2002). Therefore, though cortactin-mediated Arp2/3 activation is weaker than that of N-WASP, both proteins synergize in activating Arp2/3, enhancing N-WASP mediated actin nucleation (Weaver et al., 2001). In turn, the activation of Arp2/3 by N-WASP and cortactin may occur in a sequential manner: after Arp2/3 is activated by N-WASP, cortactin effectively binds and displaces N-WASP, since it has higher

binding affinity for activated Arp2/3 complex, presumably stabilizing the Arp2/3/cortactin/F-actin complex (Uruno et al., 2003). Cortactin may promote actin-nucleation activity, through a second mechanism, via the SH3 domain. The SH3 domain of cortactin binds directly to a proline-rich region on N-WASP, liberating N-WASP from its auto-inhibited state, resulting in N-WASP-mediated Arp2/3 nucleation activity (Martinez-Quiles et al., 2004; Mizutani et al., 2002). WASP-interacting protein (WIP) also binds to the SH3 domain of cortactin and enhances cortactin-mediated Arp2/3 activation (Kinley et al., 2003). When the cortactin-WIP complex is associated with actin filaments, maximal Arp2/3 activity is achieved, suggesting a novel role for cortactin in linking WIP to pre-existing filaments. Stabilization of actin branch points and inhibition of filament depolymerization is also achieved by cortactin-WIP interaction, since WIP also inhibits the depolymerization of actin filaments (Martinez-Quiles et al., 2001). Collectively these studies suggest cortactin and N-WASP are intimately intertwined in regulating Arp2/3 activity responsible for cortical actin assembly.

Topical mapping analysis determined that the repeat region was responsible for F-actin binding, and deletion mapping of these repeats indicated that the fourth repeat is required for optimal F-actin branching (Weed et al., 2000). These repeats have also been reported to bundle actin into filaments (Weed and Parsons, 2001). Three naturally-occurring cortactin splice isoforms have been identified that lack one or two of these repeats. These isoforms bind F-actin, although F-actin bundling activity is reduced in the 5.5-repeat isoform (lacks the 6^{th} ABR) as compared with the 6.5-repeat isoform, and absent in the 4.5-repeat isoform (lacks both 5^{th} and 6^{th} ABRs). In addition, these splice variants retain the ability to activate Arp2/3-mediated actin nucleation (van Rossum et al., 2003). The importance of the fourth repeat in maintaining the actin binding activity of the protein is supported by these studies. Recent elegant studies combining a variety of techniques, demonstrated that cortactin adopts a globular conformation, thereby bridging distant parts of the molecule into close proximity, like the helical domain residing next to the actin-binding region (Cowieson et al., 2008). The deduced conformation has important

implications regarding how cortactin can interact with F-actin. In addition these authors showed that cortactin can assemble fully polymerized actin filaments into sheet-like structures, thereby bundling F-actin into parallel arrays.

Discovering that cortactin interacts with members of the Shank family was a major step to understand the role of cortactin at the synapse (Du et al., 1998; Naisbitt et al., 1999). The SH3 domain of cortactin binds to the proline-rich region of Shank, forming a multiprotein bridge between excitatory receptors and the cytoskeleton, since Shank is linked to both ionotropic and metabotropic glutamate receptors through various adaptor proteins (PSD95, GKAP, and Homer respectively) located under the membrane (Ehlers, 1999) (Fig. 9).

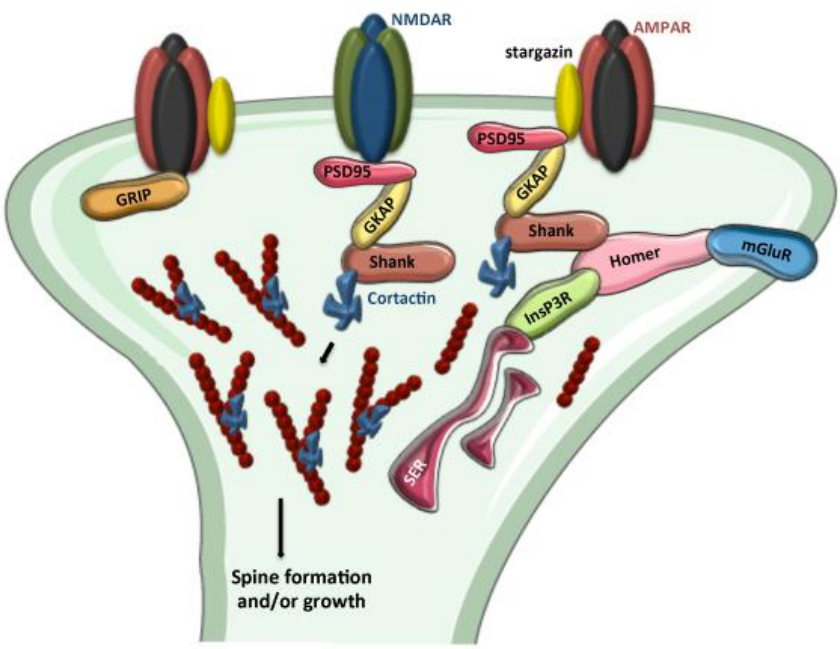


Fig. 9 - Cortactin as a key organizer and coordinator of cytoskeleton remodeling and membrane dynamics. Cortactin is a dynamic regulator of the excitatory synapse, where it links the adaptor protein Shank to the cytoskeleton. Shank is coupled to metabotropic receptors, through Homer and to ionotropic glutamate receptors through the postsynaptic density protein-95 (PSD) and the guanylate kinase-associated protein (GKAP). Cortactin (via its tyrosine phosphorylation) regulates the stability of the postsynaptic density.

Cortactin interaction with the large GTPase dynamin 3, specifically the Dyn3baa variant, appears to be critical for dendritic spine maturation, since it promotes the formation of immature dendritic filopodia in cultured neurons. Disruption of the complex cortactin-dyn3baa leads to formation of mature dendritic spines with PSDs, whereas the presence of the complex enables the formation of immature dendritic filopodia (Gray et al., 2005). These findings provide evidence that, in developing neurons, an enhanced interaction between cortactin and a specific Dyn3 splice variant modulate actin-membrane dynamics, thus regulating the morphogenesis of dendritic spines, through the ability of cortactin to use its actin-branching and elongation activity for filopodia formation and outgrowth.

In addition, cortactin regulates spine morphology by mediating the interaction between actin and microtubules. Microtubules are highly dynamic and participate intimately with actin filaments in many processes, such as polarization, migration, and areas of cellular growth or reorganization during cell division (Rodriguez et al., 2003; Siegrist and Doe, 2007), serving as a primary spatial regulator of cell shape in non-neuronal cells. MTs were thought not to play any role in dendritic spines directly, since stable MTs are confined to the dendritic shaft and do not branch off into spines (Matus, 2000). Modern advances in high-resolution live-cell microscopy have now shown that, while stable microtubules that express microtubule-associated protein 2 (MAP2) are concentrated in dendritic shafts, dynamic microtubules do in fact enter dendritic spines and affect actin dynamics (Jaworski et al., 2009). By entering dendritic spines, dynamic microtubules may induce a signaling cascade affecting actin dynamics through the microtubule plus-end tracking protein EB3. This protein may act through a p140Cap-Src pathway to promote cortactin activity, leading to Arp2/3 complex activation and spine head growth. These authors showed that p140Cap is in fact an abundant PSD protein in spines that interacts with cortactin. Additionally, they found that p140Cap or cortactin overexpression reversed the effects of EB3 knockdown, which induces a loss of F-actin in dendritic protrusions, as well as a reduction of mature mushroom spines and an

increase in filopodia like structures (Jaworski et al., 2009). Therefore, cortactin arrangement and activity may be indirectly regulated by its interaction with dynamic microtubules, and this interaction might serve as a signaling device to locally reorganize the actin cytoskeleton, thus shaping dendritic spines.

Cortactin interacts directly with several other actin-associated proteins that aid in its ability to alternatively regulate cortical actin dynamics. Cortactin is in fact involved in multiple actin-based cellular processes, exerting differential effects on cortical actin cytoskeleton organization and assembly. These interactions can shed light into how cortactin regulates actin polymerization and organization [reviewed in (Ammer and Weed, 2008)].

Cortactin Post-translational Modifications

Protein phosphorylation serves as a key switch for many cellular signaling events. Phophorylation of cortactin can modify the interaction with other proteins that promote F-actin assembly, having a major impact in actin organization. In the case of tyrosine phosphorylation, initial mapping studies identified the major Src phosphorylation sites on murine cortactin (Huang et al., 1998; Huang et al., 1997a). Cortactin tyrosine phosphorylation may serve a variety of functions: it can provide binding sites for specific signaling proteins with SH2 domains, such as Src family kinases and Nck (Okamura and Resh, 1995), regulating the cellular functions performed by cortactin; or it may alter the conformational state of the protein, since the sites of Src phosphorylation reside within the PRD, which may act as an axis separating the repeat region and SH3 domain. In fact, enhanced cortactin function by the binding of select SH3-ligands, such as MLCK (myosin light chain kinase) (Dudek et al., 2002), CD2AP (adaptor protein) (Lynch et al., 2003), and dynamin 2 (Zhu et al., 2007), can be regulated by tyrosine phosphorylation. The conformational change also regulates the cellular level of cortactin, since the phosphorylated form is preferentially degraded by calpain proteases (Perrin et al., 2006). Tyrosine phosphorylation of cortactin by Src occurs at tyrosine residues 421, 466 and 482 through a progressive manner with initial phosphorylation at tyrosine 421

followed by 466, and this “triple” phosphorylation has known biochemical and cellular processes consequences with regards to actin dynamics (Head et al., 2003). Phosphorylation of these tyrosine residues attenuates cortactin ability to cross-link F-actin *in vitro* (Huang et al., 1997a), and also inhibits its activation on N-WASP (Martinez-Quiles et al., 2004). This suggests a negative regulatory role, which may regulate the flexibility and/or turnover of actin networks. However, cortactin tyrosine phosphorylation is associated with and appears to be necessary for many positive functions as well. The majority of reports indicate that high levels of tyrosine phosphorylation correlate with elevated cell migration and cancer metastasis (Bourguignon et al., 2001; Huang et al., 1998; Huang et al., 2003; Li et al., 2001; Liu et al., 1999), as well as formation and turnover of structures associated with invasive potential (Ayala et al., 2008; Luxenburg et al., 2006; Tehrani et al., 2006). In fact, additional work now points to a positive effect for Src-mediated cortactin phosphorylation on Arp2/3-mediated actin polymerization through Nck and N-WASP, since trimeric phosphocortactin/Nck1/N-WASP (or WIP) complexes enhance Arp2/3 nucleation activity (Tehrani et al., 2007). Therefore, the binding of Nck1 to phosphorylated cortactin, in turn interacting with N-WASP or WIP, provides an indirect link to Arp2/3 regulation.

Additionally to tyrosine phosphorylation, cortactin is also a substrate for several serine/threonine kinases. The extracellular signal-regulated kinase (Erk) targets cortactin at serine 405 and serine 418 and their phosphorylation facilitates cortactin binding to N-WASP for actin polymerization, through activation of Arp2/3 actin nucleation (Martinez-Quiles et al., 2004). This binding between cortactin and N-WASP is inhibited by Src-mediated tyrosine phosphorylation, possibly due to the absence of Nck, resulting in decreased Arp2/3 nucleation [reviewed in (Lua and Low, 2005)]. Increases in phosphorylation on these residues are accompanied by a mobility shift of cortactin upon SDS/PAGE, from p80 to p85, potentially due to the disruption of intramolecular interactions between the cortactin amino-terminal residues and the SH3 domain (Campbell et al., 1999; Martinez-Quiles et al., 2004). A second

serine/threonine kinase, p21-activated kinase (PAK), has been implicated in cortactin regulation (Vidal et al., 2002). Cortactin association with PAK is related with cortactin translocation, however it remains unclear whether this is indeed due to phosphorylation *per se*. Phosphorylation of S405 and S418 is also required for efficient invadipodia formation and extra cellular matrix degradation (Ayala et al., 2008). Both tyrosine and serine phospho-mimetic forms of cortactin promote lamellipodia protrusion and cell migration, in living cells; however, serine phosphorylation of cortactin preferentially promotes actin assembly, which suggests that Src-mediated tyrosine phosphorylation of cortactin may contribute to these dynamic processes in a way distinct from actin assembly. In fact, it has been shown that cortactin mutants mimicking serine phosphorylation predominantly affect actin polymerization, whereas mutation of cortactin tyrosine residues results in alterations of focal adhesion turnover (Kruchten et al., 2008). Observation of actin comet tails indicated that serine phosphorylation of cortactin preferentially promotes actin assembly in cells, while tyrosine phosphorylation by Src increases focal adhesions turnover, regulating their sizes and dynamics. Consequently, to function properly, cortactin has to be dynamically recycled through a continuous series of phosphorylation and dephosphorylation events.

In neurons, cortactin localization can be regulated by synaptic activity. In fact, cortactin is regulated by NMDA receptor activity and brain-derived neurotrophic factor (BDNF), and these regulations occur through phosphorylation. Activation of Src kinases, by NMDAR activation, induces cortactin phosphorylation and depletion from the postsynaptic sites. On the other hand, activation of a MAP kinase, by BDNF application, stimulates redistribution of cortactin from the dendritic shaft to spines (Iki et al., 2005). Serine phosphorylation of cortactin dissociates intramolecular associations and facilitates SH3 domain-dependent association at the postsynaptic sites, whereas tyrosine phosphorylation not only reduces its ability to initiate actin polymerization, but also results in dissociation of cortactin from the postsynaptic actin cytoskeleton, probably due to reduced affinity of the cortactin SH3 domain

for Shank. This shift in cortactin localization, paralleled by the control exerted over cortactin activity, may play an important role in spine development, synaptogenesis and also activity dependent spine remodeling.

Beside phosphorylation, cortactin activity can be further regulated by other post-translational modifications. A recently identified regulatory mechanism that governs cortactin binding to F-actin involves the histone deacetylase HDAC6, and depends on cortactin regulation by acetylation (Zhang et al., 2007). In fact, cortactin was identified as a substrate for the histone acetyltransferase PCAF (p300/CBP-associated factor). In a subsequent study, Zhang and colleagues also showed that SIRT1 (a class III histone deacetylase) binds to and deacetylates cortactin, whereas the histone acetyltransferase p300 acetylates cortactin (Zhang et al., 2008). Cortactin is acetylated within its ABR domain, where eight lysine residues are located and postulated to form two positively "charged patches" that facilitate the favorable interaction of cortactin with F-actin. PCAF and p300 acetylate these lysine residues, neutralizing the "patches" and inhibiting binding of the ABR to F-actin. Conversely, deacetylation reverses this process and restores the ability of cortactin to bind F-actin. Cortactin regulation by acetylation also has physiological consequences, mainly by influencing actin-dependent cell motility and migration. Therefore, reversible acetylation status of cortactin provides another distinct signaling mechanism that regulates F-actin binding activity.

Protein Acetylation in Synaptic Plasticity

Memory consolidation and an *in vitro* analog, long-term potentiation (LTP), require a cascade of signaling events that include activation of NMDA receptors, protein kinases and transcription factors; events that ultimately lead to changes in gene transcription. The pivotal role of phosphorylation in synaptic plasticity and memory has been extensively recognized using several different model systems (Bliss and Collingridge, 1993; Lynch, 2004; Raymond, 2007). However, another posttranslational modification of proteins, acetylation, has

recently emerged as having an important role in both synaptic plasticity and memory. Specifically, acetylation refers to the process of introducing an acetyl group from acetyl-coenzyme A to the lysine residue of a protein, and is determined by the relative activities of acetyltransferases and deacetylases. Acetylation/deacetylation is emerging as a significant post-translational regulatory mechanism, analogous to phosphorylation by the action of kinases or dephosphorylation by the action of phosphatases, in the sense that it is reversible. Several proteins, such as transcription factors, effector proteins, molecular chaperones, and cytoskeletal proteins, are regulated by acetylation, suggesting this modification regulates various processes including protein stability, protein-protein interactions and gene transcription (Kouzarides, 2000; Spange et al., 2009).

Recent evidence indicates that regulation of chromatin structure, especially histone-tail acetylation, serves as an additional level of control for regulating gene expression, implicated in synaptic plasticity and learning behavior. In particular, memory formation has been shown to be associated with histone acetylation (Alarcon et al., 2004; Guan et al., 2002; Korzus et al., 2004; Levenson et al., 2004; Vecsey et al., 2007). Histone acetylation relaxes chromatin structure, changing the accessibility of DNA to the transcriptional machinery and in general promoting gene transcription (Lunyak et al., 2002; Turner, 2002; Varga-Weisz and Becker, 1998). Core histones are primarily targeted by histone deacetylases (HDACs) and histone acetyltranferases (HACs); acetylation correlates with transcriptional activity, whereas deacetylation correlates with gene silencing (Kouzarides, 2007). Mammalian HDACs have been classified into three classes: class I (HDACs 1, 2, 3 & 8) that localizes to the nucleus; class II (HDACs 4, 5, 6, 7, 9 & 10), found in both the nucleus and the cytoplasm; class III (Sirt1 - Sirt7), forms a structurally distinct class of NAD-dependent enzymes found in both the nucleus and the cytoplasm. Classes I and II HDACs are inhibited by trichostatin A.

The effect of histone acetylation in long-term potentiation has been widely studied since the late phases of LTP require the synthesis of new proteins and transcription. Levenson and colleagues investigated the effects of HDAC inhibitors (HDACis), which modulate the acetylation levels of histones, on LTP, and found that TSA and sodium butyrate (HDACis) enhance LTP in the hippocampus, without affecting basal signal transmission (Levenson et al., 2004). These findings were somehow confirmed by another study by Vecsey and colleagues. These authors found TSA-induced enhancement of LTP dependent upon CREB (cAMP response element-binding protein) and CBP (CREB-binding protein), proteins that play critical roles in synaptic plasticity and memory (Vecsey et al., 2007) [reviewed in (Sharma, 2010)].

Histone acetylation has also been associated with memory consolidation, a process which has been shown to require new protein and RNA synthesis along with several signaling molecules. In fact, several different studies have pointed out the important role of histone acetylation in memory formation, demonstrating that inhibition of HDACs facilitates not only LTP, but also memory (Sharma, 2010). This fact suggests that HDACs may be considered as memory suppressor genes, since they act to limit LTP and memory. A recent study substantiated this idea; Guan and colleagues showed that HDAC2 deficiency enhanced memory formation and increased synapse number, whereas HDAC2, but not HDAC1, overexpression impaired LTP and memory and also decreased dendritic spine density and synapse number (Guan et al., 2009).

Histones are not the only substrates of the so-called HDACs. In fact, there has been a rapid proliferation in the description of new non-histone targets of HDACs and also HATs. Of these, transcription factors, like C/EBP beta (Cesena et al., 2007) and CREB (Lu et al., 2003), comprise the largest class of new targets. The substrates for these enzymes extend to cytoskeletal proteins, such as α-tubulin (Haggarty et al., 2003; Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003) and cortactin (Zhang et al., 2007; Zhang et al., 2008),

molecular chaperones (Bali et al., 2005; Kovacs et al., 2005), and also acetyltransferases themselves (Spange et al., 2009). These substrates open yet another exciting new field of discovery in the role of the dynamic acetylation and deacetylation on cellular function. Indeed, a possibility is raised concerning the facilitatory effects of deacetylases inhibition described above in synaptic plasticity and memory, which may potentially involve, at least in some cases, changes in the acetylation status of other proteins in addition to histones.

Objectives of the present study

The reversible acetylation of lysine residues is an important post-translational modification for the regulation of histones, transcription factors, chaperones, and microtubules, *i.e*, it regulates many cellular processes, especially gene regulation activities such as transcription, DNA replication and damage repair. Synaptic plasticity and memory have been shown to be regulated by protein acetylation, but surprisingly the impact of protein acetylation on the molecular composition of synapses has never been addressed. Therefore, we explored the effects of protein acetylation, enhanced by HDACs inhibitors, on the synaptic localization and expression levels of scaffold proteins for excitatory and inhibitory synapses, as well as of cytoskeletal proteins, in cultured rat hippocampal neurons. The results presented in this thesis point to a selective role for protein acetylation in the clustering of excitatory postsynaptic scaffold proteins.

A recent study by Zhang and colleagues (Zhang et al. 2007) described cortactin as a new target for reversible acetylation in cancer cells. These authors found that the histone deacetylase HDAC6 associates with and deacetylates cortactin *in vitro* and *in vivo*, and demonstrated that hyperacetylation of cortactin prevents its translocation to the cell periphery, blocks its association with F-actin and impairs the motility of cancer cells. The role of cortactin acetylation on its targeting to dendritic spines, its interactions with postsynaptic proteins, as well as the changes in cortactin acetylation induced by alterations in synaptic activity, have never been addressed. Since the activity of cortactin is modulated upon acetylation, especially its association with F-actin, it is important to determine how acetylation can modulate cortactin function in dendritic spines. The goal of the present study was to contribute to a better understanding of the function of cortactin acetylation on hippocampal neurons, by characterizing its localization and describing how it regulates the rearrangement/redistribution of the important postsynaptic player PSD95. The following specific objectives were pursued:

1. We examined the localization and function of cortactin under acetylation regulation in hippocampal neurons. Our studies were based on the novel observation that cortactin acetylation levels regulate the clustering of the postsynaptic scaffolding protein PSD95, in the dendrites of hippocampal neurons.

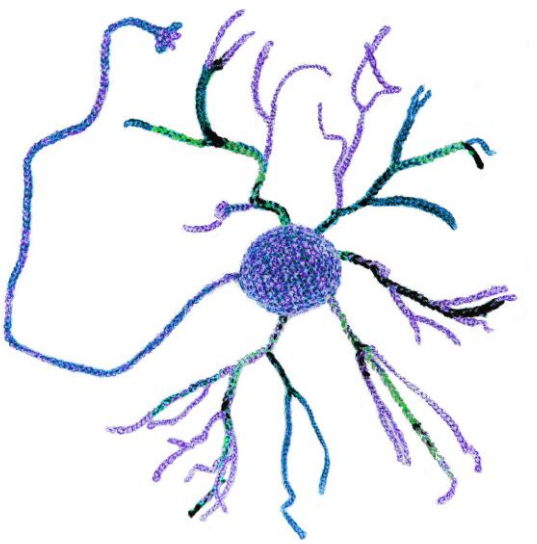
2. We investigated possible consequences of cortactin acetylation in terms of cortactin interaction with synaptic binding partners as well as cortactin phosphorylation at tyrosine residues.

3. We determined how synaptic activity regulates cortactin acetylation. We tested whether treatments of hippocampal neurons in culture with BDNF or glutamate, which change the cellular localization of cortactin, have an effect on the acetylation state of cortactin.

Our findings provide the first evidence that cortactin acetylation, which can be regulated by BDNF and glutamate levels, regulates the dendritic clustering of PSD95 in hippocampal neurons, and affects tyrosine phosphorylation of cortactin as well as its intracellular interactions. These studies reveal an unsuspected role for cortactin acetylation in the regulation of excitatory synapses.

Chapter 2

Materials and Methods



Materials

Dulbecco's modified Eagle's medium (DMEM), Trichostatin A, as well as the protease inhibitors chymostatin, leupeptin, antipain and pepstatin (CLAP, stock solution 1mg/ml in dimethyl sulfoxide – DMSO) were purchased from Sigma-Aldrich Química S.A. (Sintra, Portugal). Fetal bovine serum (FBS), horse serum (HS), trypsin and gentamycin were purchased from Gibco, as part of Invitrogen Life Technologies (Barcelone, Spain). The QIAGEN Plasmid midi, maxi and mini kits were obtained from QIAGEN (QIAGEN GmbH, Hilden, Germany). OptiMEM was purchased from Invitrogen Life Technologies (Barcelone, Spain). QuikChange II XL-site-directed mutagenesis kit was purchased from Stratagene (Cambridge, UK). Protein A Sepharose CL-4B and the ECF immunodetection substrate were obtained from GE Healthcare (Carnaxide, Portugal). The BCA assay kit, EZ-link Sulfo-NHS-SS-biotin and UltraLink Plus Immobilized Neutravidin Gel were purchased from Pierce, as part of Thermo Fisher Scientific (Rockford, Illinois, USA). All other reagents were from Sigma (Sintra, Portugal) or from Merck (Darmstadt, Germany).

Antibodies

Primary Antibodies	Application (dilution)	Source
Acetylated cortactin	ICC (1:200) WB (1:300)	kind gift from Dr. Xiaohong Zhang
Acetylated tubulin	WB (1:2000)	Sigma (Sintra, Portugal)
Acti-Stain 555 Fluorescent Phaloidin	ICC (follow manufacturers instruction)	Cytoskeleton, Inc. (Denver, USA)
Actin	WB (1:5000)	Sigma (Sintra, Portugal)
Cortactin	ICC (1:500) WB (1:750)	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)
Cortactin pY421	WB (1:200)	Biosource-Invitrogen (Leiden, The Netherlands)

Flag monoclonal	ICC (1:500) WB (1:1000)	Sigma (Sintra, Portugal)
Flag polyclonal	ICC (1:500) WB (1:1000)	Sigma (Sintra, Portugal)
Gephyrin	ICC (1:1000) WB (1:1000)	Synaptic Systems (Goettingen, Germany)
GluA1 N-terminal	ICC (1:200)	kind gift from Dr. Andrew Irving
GluA1 N-terminal	ICC (1:10) WB (1:1000)	Merck (Darmstadt, Germany)
MAP2	ICC (1:5000)	Abcam (Cambridge, UK)
Pan-Shank (1,2 & 3)	WB (1:200)	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)
PSD95	ICC (1:200)	Affinity BioReagents (Golden, CO)
PSD95	WB (1:2000)	Cell Signaling Technology (Danvers, Massachusetts, USA)
Shank1	ICC (1:200)	UC Davis/NIH NeuroMab Facility (University of California, USA)
SNIP/140Cap	ICC (1:200)	Cell Signalling Technology (Danvers, Massachusetts, USA)
Synaptophysin	WB (1:10000)	Abcam (Cambridge, UK)
Tubulin	WB (1:200000)	Sigma (Sintra, Portugal)
VGAT N-terminal	ICC (1:750)	Synaptic Systems (Goettingen, Germany)
VGLUT1	ICC (1:100000)	Millipore (MA, USA)
VGLUT1	WB (1:5000)	Synaptic Systems (Goettingen, Germany)
GFP	ICC (1:500) WB (1:1000)	MBL International (MA, USA)
Secondary Antibodies		
AMCA-conjugated anti-chicken	ICC (1:200)	Jackson ImmunoResearch (Pennsylvania, USA)

Alexa 647-conjugated anti-guinea pig	ICC (1:1000)	Molecular Probes (Leiden, The Netherlands)
Alexa 647-conjugated anti-mouse	ICC (1:500)	Molecular Probes (Leiden, The Netherlands)
Texas Red-conjugated anti-mouse	ICC (1:200)	Molecular Probes (Leiden, The Netherlands)
Alexa 488-conjugated anti-rabbit	ICC (1:1000)	Molecular Probes (Leiden, The Netherlands)
Alexa 594-conjugated anti-rabbit	ICC (1:200)	Molecular Probes (Leiden, The Netherlands)
Alexa 568-conjugated anti-sheep	ICC (1:500)	Molecular Probes (Leiden, The Netherlands)
Alkaline phosphatase-conjugated anti-mouse	WB (1:20000)	GE Healthcare (Carnaxide, Portugal)
Alkaline phosphatase-conjugated anti-rabbit	WB (1:20000)	GE Healthcare (Carnaxide, Portugal)

ICC – Immunocytochemistry
WB – Western Blot

Constructs and primers for transfection of neurons and HEK293FT Cells

Cortactin-FLAG constructs were a kind gift from Dr. Zhang (University of South Florida, Tampa, FL, USA). Cortactin shRNA resistant constructs were prepared with the QuikChange II XL-site-directed mutagenesis kit (Stratagene), using cortactin constructs (kind gift from Xiaohong Zhang and Edward Seto from University of South Florida, Tampa, FL, USA) as template and the primers 5′ tcc aag cat tgc tca caa gtt gac tca gtc t 3′ and 5’ gac tga gtc aac ttg tga gca atg ctt gga 3’. For the generation of the shortinterfering RNA construct, pll3.7shRNA, the following DNA oligonucleotides, 5’ gatccccgcactgctcacaagtggacttcaagagagtccacttgtgagcagtgctttttggaaa-3’ and 5_’agcttttccaaaaagcactgctcacaagtggactctcttgaagtccacttgtgagcagtgcggg- 3’ (corresponding to nucleotides 331-348 of the rat and mouse cortactin sequence with the first nucleotide of the start codon counted as nucleotide 1) were annealed and subcloned into the *XhoI* and *HpaI* sites of the pll3.7 vector.

Methods

HEK 293FT cells cultures

HEK 293FT cells were maintained at 37ºC in a humidified incubator of 5% CO_2/95% air and diluted 1:5 every three days. Cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% Geneticin, 1% 200mM L-Glutamine (100x), 1% 10 mM MEM Non-Essential Amino Acids (100x) (GIBCO Invitrogen), 1% 100mM MEM Sodium Pyruvate (100x) and 44 mM $NaHCO_3$, pH 7.2. HEK 293FT cells were transiently transfected with 10-11 μg of DNA, using calcium phosphate method.

Hippocampal cultures (high density cultures)

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, 37 ºC; GIBCO Invitrogen), in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampal cells were then washed with 10% fetal bovine serum prepared in HBSS, to stop trypsin activity, and then washed once with HBSS to remove serum and avoid glia growth. Finally, hippocampal cells were transferred to Neurobasal medium (GIBCO Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin, the cells were mechanically dissociated and then plated in 6 well plates ($8,9x10^4$ cells/cm^2), coated with poly-D-lysine (0.1 mg/mL). The cultures were maintained in a humidified incubator of 5% CO_2/95% air, at 37 °C, for seven or fourteen days.

Preparation of hippocampal culture extracts

Hippocampal cultures were washed twice with ice-cold PBS, and once more with PBS buffer supplemented with 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF), CLAP (1

µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml pepstatin; Sigma). The cells were then lysed with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5) supplemented with 50 mM sodium fluoride (NaF), 1.5 mM sodium ortovanadate (Na_3VO_4) and the cocktail of protease inhibitors. After centrifugation at 16,100xg for 10 min at 4ºC, protein in the supernatants was quantified using the Bicinchoninic acid (BCA) assay kit (Singh et al.), and the samples were denaturated with 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM Na_3VO_4, and 0.01% bromophenol blue). Extracts used for VGLUT1 analysis were not subjected to a boiling step to avoid VGLUT aggregation.

Hippocampal cultures (low density cultures - Banker cultures)

Cultures were prepared from hippocampal neurons using previously described methods (Goslin et al., 1998). Briefly, hippocampi were dissected from E18 rat embryos and dissociated using trypsin (0.25%) and trituration. Neurons were plated at a final density of 1-5 x 10^4 cells/dish on poly-D-lysine-coated coverslips in 60 mm culture dishes in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid). After 2-4 hr, coverslips were flipped over an astroglial feeder layer in Neurobasal medium supplemented with B27 supplement (1:50 dilution), 25 µM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The neurons grew face down over the feeder layer but were kept separate from the glia by wax dots on the neuronal side of the coverslips. To prevent the overgrowth of the glia, neuron cultures were treated with 5 µM cytosine arabinoside after 3 days *in vitro* (Meyers et al.). Cultures were maintained in a humidified incubator with 5% CO_2/95% air, at 37 °C, for up to 3 weeks, feeding the cells once per week by replacing one-third of the medium per dish.

Synaptoneurosomes Preparation

Synaptoneurosomes (SNSs) were prepared as previously described with slight modifications (Yin et al., 2002). Briefly, 6-8 hippocampi were dissected from

adult Wistar rats and the tissue was minced with scissors and homogenized with a glass homogenizer in a buffer containing 0.32 M sucrose, 10 mM HEPES-Tris pH 7.4 and 0.1 mM EGTA. After centrifugation for 3 min at 1000x g, the supernatant was collected and passed initially through nylon membranes (150 and 50 µm, VWR) and finally through an 8 µm pore size filter (Millipore, MA). The flow-through was centrifuged for 15 min at 10,000x g, and the pellet was resuspended in incubation buffer (in mM: 8 KCl, 3 $CaCl_2$, 5 Na_2HPO_4, 2 $MgCl_2$, 33 Tris, 72 NaCl, 100 sucrose). All the procedure was done at 4°C.

Subcellular fractionation of rat hippocampus

The procedure for purification of postsynaptic density fractions (PSDs) was adapted from Peça et al., 2011. Two hundred mg of hippocampi, dissected from adult Wistar rats were collected and homogenized in a motor driven glass teflon homogenizer (30 stokes, 900rpm) in HBS (0.32 M sucrose, 4 mM HEPES, pH 7.4) containing protease and phosphatase inhibitors (0.2 mM PMSF, 0.1 mM Na_3VO_4, 50 mM NaF, 1µg/ml CLAP), as described above. The culture homogenate was centrifuged at 900×g for 15min to obtain the non-nuclear fraction (S1). The resultant supernatant was centrifuged at 18.000×g for 15min to yield the crude synaptosomal pellet (P2). P2 was resuspended in HBS and centrifuged at 18.000×g for 15min to yield the washed crude synaptosomal fraction. This fraction was submitted to hypo-osmotic shock by resuspending the pellet in HEPES buffer (4 mM HEPES, pH 7.4, plus protease and phosphatase inhibitors) and incubated for 1 hour with orbital rotation at 4°C. The lysate was centrifuged at 25.000×g for 20min to yield supernatant (crude synaptical vesicle fraction – S3) and a pellet (lysed synaptosomal membrane fraction), which was resuspended in HBS (without Na_3VO_4) and placed on top of a continuous sucrose gradient (0.8 M, 1 M, 1.2 M). The tube was filled with 0.16 M sucrose solution and spun at 150.000×g for 2h in a swinging bucket rotor (Beckman Optima™ L-100 XP). Synaptic plasma membranes (SPM) were recovered between the 1.0 M and 1.2 M layers, diluted to 0.32 M sucrose, and centrifuged at 150.000×g for 30 min. SPMs were resuspended in HEPES/EDTA

(HE) buffer (50 mM HEPES, 2 mM EDTA, pH 7.4) containing protease and phosphatase inhibitors and washed with 0.5% Triton X-100 for 15 min with orbital rotation at 4°C, followed by 20 min centrifugation at 200.000×g for 20 min. PSD pellet was resuspended in HE with 0.5% SDS. Homogenate, S1 and P2, S3, PSD fractions were collected (50µl) throughout the fractionation procedure and kept at -80ºC. All experimental procedures and centrifugations were performed on ice or at 4°C.

Neuron transfection with the calcium phosphate protocol.

Constructs were recombinantly expressed in primary cultures of hippocampal neurons using a calcium phosphate transfection protocol adapted from Jiang and collaborators (Jiang et al., 2004). Plasmid DNAs (4µg per coverslip) were diluted in Tris-EDTA transfection buffer (10 mM Tris-HCl and 2.5 mM EDTA, pH 7.3). A $CaCl_2$ solution (2.5 M in 10 mM HEPES) was then added, drop-wise, to the DNA solution to give a final concentration of 250 mM $CaCl_2$. This was then added to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, and 42 mM HEPES, pH 7.2). The DNA solution was added, a small fraction at a time ($1/8^{th}$), to the HEPES-buffered transfection solution. This was then vortexed gently for 2 to 3 s, and the precipitate was allowed to develop at room temperature for 30 min, protected from light, and vortexed every 5 min. Then, 100 µl of precipitate was added, drop-wise, to each coverslip, and the cultures were incubated with the precipitate for 1 to 3 h in the presence of kynurenic acid (2 mM). Each coverslip was transferred to a fresh well of the 12-well plate containing 1 ml of culture medium with kynurenic acid (2 mM), slighltly acidified with HCl (~5 mM final concentration), and the plate was returned to a 37°C/5% CO_2/95% O_2 incubator for 10 to 15 min. Each coverslip was then transferred to the original dish containing the conditioned medium. The cells were then returned to a 37°C and 5% CO_2/95% incubator to allow expression of the transfected constructs.

HEK 293FT transfection with the calcium phosphate protocol.

For each well of a 6-well multiwell culture plate (MW6), 250 µl 2X HBS were aliquoted into a sterile 1.5 ml microfuge tube. In a separate tube, 10 µg of DNA, 37.5 µl of 2 M CaCl2 and enough distilled water to bring the total volume to 250 µl, were also aliquoted. The $CaCl_2$/DNA mix was added to the HBS slowly with a P1000 pipette, mixing gently during the addition. The mixture was pippeted directly to the cells by dropping slowly and evenly into medium, trying to cover as much of the well as possible. Without mixing, the multiwell was simply carried to the incubator and placed at 37°C/5% CO_2 for 5 hrs. The medium was removed and cells were washed once with warm PBS, then fresh, warm complete medium was added and the incubation was resumed for 48 hrs, prior to assaying.

Gel electrophoresis and western-blot

Samples were resolved by SDS-PAGE in 7.5% polyacrylamide gels. For western blot analysis, proteins were transferred onto a PVDF membrane (Millipore, Madrid, Spain) by electroblotting (40V, overnight, at 4ºC). The membranes were blocked for 1 hour with 5% (w/v) BSA or skim milk and 0.1% Tween 20 in TBS [(20 mM Tris, 137 mM NaCl, pH 7.6 (TBS-T)], and probed during 1 hour, at room temperature, or overnight, at 4ºC, with the primary antibody. Following several washes with TBS-T, the membranes were incubated for 1 hour, at room temperature, with alkaline phosphatase-conjugated IgG secondary antibody (anti-mouse or anti-rabbit, depending on the primary antibody host-species). The membranes were then washed again and immunostaining was visualized by the enhanced chemifluorescence method (ECF) on a Storm 860 Gel and Blot Imaging System (GE Healthcare, Carnaxide, Portugal).

Immunoprecipitation assays

Co-immunoprecipitaton assays were performed using HEK 293FT lysates transfected with the constructs of interest. Nine hundred µg of protein from HEK

293FT lysates were solubilized in Immunoprecipitation Buffer [IPB: 10 mM Tris (pH 7.0), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1% Triton X-100] and protease inhibitors (0.2 mM PMSF, 100 mM DTT, 1 μg/ml each of chymostatin, pepstatin, antipain, and leupeptin or Complete mini protease inhibitor mixture – Roche Diagnostics)]. The samples were sonicated with a probe sonicator, on ice, for 30 s. The insoluble material was removed by centrifuging the sample at 13 000×g during 10 min, at 4ºC. At this point the supernatant was collected and 50 µl were removed for analysis by Western blot (input). The supernatant was transferred to a tube containing 30 µl of a 50% slurry of protein A sepharose beads suspended in IPB. The tube was rotated at 4ºC, for 1 hour; this step preabsorbs any protein that may stick non-specifically to the protein A sepharose beads. After a 5 min centrifugation step the sedimented sepharose beads were discarded. The supernatant was incubated with 3 µg of anti-FLAG antibody. This incubation step was performed at 4ºC, overnight. The tubes were then incubated with 100 µl of a 50% slurry of protein A sepharose beads and rotated for 2h at 4ºC. Five steps of washing were performed in order to avoid non-specific bindings: 2x IPB+1% Triton, 3x IPB+1% Triton+ 500 mM NaCl and 2x IPB. The proteins were eluted by boiling the beads in 50 μl of sample buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue) for 5 min. Proteins were separated by SDS-PAGE in 7.5% polyacrylamide gel, followed by western-blot for the proteins of interest.

Immunocytochemistry

Neurons were fixed for 10 min in 4% sucrose/4%paraformaldehyde in PBS, and permeabilized with PBS + 0.25% Triton X-100 for 5 min, at 4ºC. The neurons were then incubated in 10% BSA in PBS for 30 min at 37ºC to block nonspecific staining, and incubated in appropriate primary antibody diluted in 3% BSA in PBS (2h, 37ºC). After washing 6 times in PBS, cells were incubated in secondary antibody diluted in 3% BSA in PBS (45 min, 37ºC). The coverslips were mounted using florescent mounting medium from DAKO (Glostrup,

Denmark). For labeling surface GluA1-containing receptors, live neurons were incubated for 30 min at room temperature with the GluA1 N-terminal antibody diluted in PBS, after which the cells were briefly rinsed in PBS and were then fixed and probed as described above. Imaging was performed on a Zeiss Axiovert 200 M microscope, using a 63× 1.4 NA oil objective.

Microscopy and quantitative fluorescence analysis

Images were quantified using image analysis software (ImageJ). For quantitation, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The protein (the ones studied in this work) signals were analyzed after thresholds were set, such that recognizable clusters were included in the analysis. Synaptic proteins were selected by colocalization with VGLUT1. Regions around thresholded puncta were overlaid as a mask in the VGLUT1 channel, and colocalization was determined. For quantifying the protein signals in transfected neurons, fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons. Measurements were performed in three or four independent preparations, and at least 9 cells per condition were analyzed for each preparation. Statistical analysis was performed using unpaired student t-test or One way ANOVA followed by the Dunnett's test.

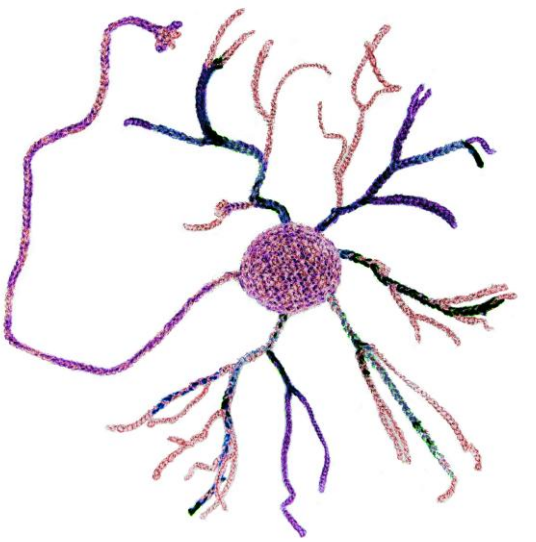
For analysis of F-actin organization experimental and control samples were encoded for blind analysis; hippocampal neurons, labeled with Alexa555-coupled phalloidin, were chosen by the GFP channel. F-actin clusters (branched F-actin) were defined operationally as 0.3-3.0 μm^2 F-actin-enriched puncta along dendrites (with an average pixel intensity at least 50% above that in the adjacent dendritic region). Six to 11 neurons were selected for each experimental group, and three to four proximal dendrites per each neuron were analyzed.

Statistical Analysis

The immunoreactivity obtained in each experimental condition was calculated as a percentage of the control. Data are presented as mean ± SEM of at least three different experiments, performed in independent preparations. Statistical analysis of the results was performed using unpaired student t-test or one-way ANOVA analysis followed by either Dunnett's or Bonferroni post test: n.s. non significant, ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Chapter 3

Regulation of Synapse Composition by Protein Acetylation



Introduction

It is widely known that posttranslational modifications (PTM) of proteins regulate various aspects of cellular processes. In fact, the critical role of phosphorylation in synaptic plasticity and memory has been established using a variety of different model systems (Sharma, 2010). In synaptic terminals, protein phosphorylation is the primary PTM that contributes to the control of the activity and localization of synaptic proteins. More recently, acetylation, which is another posttranslational modification of proteins, has been attributed an important role in these processes.

Modification by acetylation regulates various processes including protein stability, protein-protein interaction and gene transcription (Kouzarides, 2000; Spange et al., 2009) and several proteins can be altered by this modification. The critical role of transcription factors in synaptic plasticity and memory in different model systems has been recognized for a long time, and it recently became clear that histone acetylation is important in these processes (Barrett and Wood, 2008; Borrelli et al., 2008; Graff and Mansuy, 2008; Levenson and Sweatt, 2005; Reul and Chandramohan, 2007; Roth and Sweatt, 2009; Sweatt, 2009)

Learning and memory in wild-type mice as well as in mouse models of neurodegeneration is facilitated by increased histone-tail acetylation induced by histone deacetylases inhibitors (HDACis). Fischer A. *et al*. (2007) showed that a non-selective HDAC inhibitor (sodium butyrate) promotes the retrieval of long-term memory and reinstates learning ability in mice even after massive memory loss. Another structurally distinct HDAC inhibitor, Thricostatin A, enhanced LTP in the hippocampus, without affecting basal synaptic transmission (Levenson et al., 2004). HDACis were reported to enhance memory formation and synapse plasticity through CREB-CBP-dependent transcriptional activation (Vecsey et al., 2007).

Conversely, it has been shown that neuron specific overexpression of HDAC2, but not that of HDAC1, decreases dendritic spine density, synapse number, synaptic plasticity and memory formation (Guan et al., 2009). These authors suggested that HDAC2 suppresses the expression of synaptic remodeling and plasticity genes.

In addition to histones, acetylation of other proteins may also be important for memory formation. In fact, histones are not the only substrates of HDACs and several other proteins including transcription factors, transport proteins, α-tubulin and acetyltransferases are acetyl-proteins themselves (Kouzarides, 2000; Spange et al., 2009; Yang and Seto, 2008). Some proteins which play important roles in synaptic plasticity and memory, such as C/EBP and CREB, are acetyl-proteins and their activity is modulated by acetylation (Cesena et al., 2007; Lu et al., 2003). Therefore, it is to be expected that changes in the acetylation status of other proteins, in addition to histones, may potentially be involved in the facilitatory effects of deacetylase inhibition in synaptic plasticity and memory.

Despite the evidences indicating a role for protein acetylation in synapse number (Guan et al., 2009), synaptic plasticity and memory (Chwang et al., 2007; Fischer et al., 2007; Fontan-Lozano et al., 2008; Guan et al., 2009; Levenson et al., 2004; Miller et al., 2008; Stefanko et al., 2009; Vecsey et al., 2007; Yeh et al., 2004), the impact of protein acetylation on the molecular composition of synapses has never been addressed. Therefore, in order to explore the effects of protein acetylation, enhanced by HDACs inhibitors, on synapses, we used immunocytochemical methods to characterize the localization of several synaptic proteins in rat hippocampal neurons, a model where the protein localization relative to synapses is very amenable to study.

Results

Acetylation affects scaffold proteins of excitatory synapses

Several studies showed that acetylation plays important roles in LTP, and that enhancing acetylation by inhibiting HDACs facilitates LTP in the hippocampus and amygdala (Sharma, 2010). Additionally, Levenson et al. (2004) investigated the effects of HDAC inhibitors on LTP and found that Thricostatin A (TSA), which increases the acetylation status of histones, enhanced LTP in the hippocampus.

To assess the effect of protein acetylation on excitatory synapses, we performed quantitative immunofluorescence analysis of the expression of synaptic proteins in hippocampal neurons at 15 DIV. At first we looked at the postsynaptic density-95 (PSD95), a scaffolding protein that has been identified as a marker for synaptic strength. It has been shown that overexpression of PSD-95 promotes synaptic maturation (El-Husseini et al., 2000), while knockdown of PSD-95 results in decreased synaptic strength and spine density (Ehrlich et al., 2007). PSD-95 remains mobile in mature neurons, and can transit in and out of spines on the order of minutes (Marrs et al., 2001). In fact, a pool of dendritic PSD95 molecules is shared and redistributed among neighboring spines through diffusion (Gray et al., 2006; Tsuriel et al., 2006).

Hippocampal neurons cultured at low-density were treated with TSA, an inhibitor of types I and II histone deacetylases, or vehicle (ethanol at 99%) for 12 hours. After fixation, neurons were stained with an antibody against PSD95 (postsynaptic marker), to visualize excitatory synapses and an antibody against MAP2, a somatodendritic marker. Compared with neurons treated with vehicle, neurons treated with TSA showed a significant increase in the fluorescence intensity of PSD95 clusters (Figures 10A). The area and the density of PSD95 clusters were also increased in TSA treated neurons (Fig. 10B).

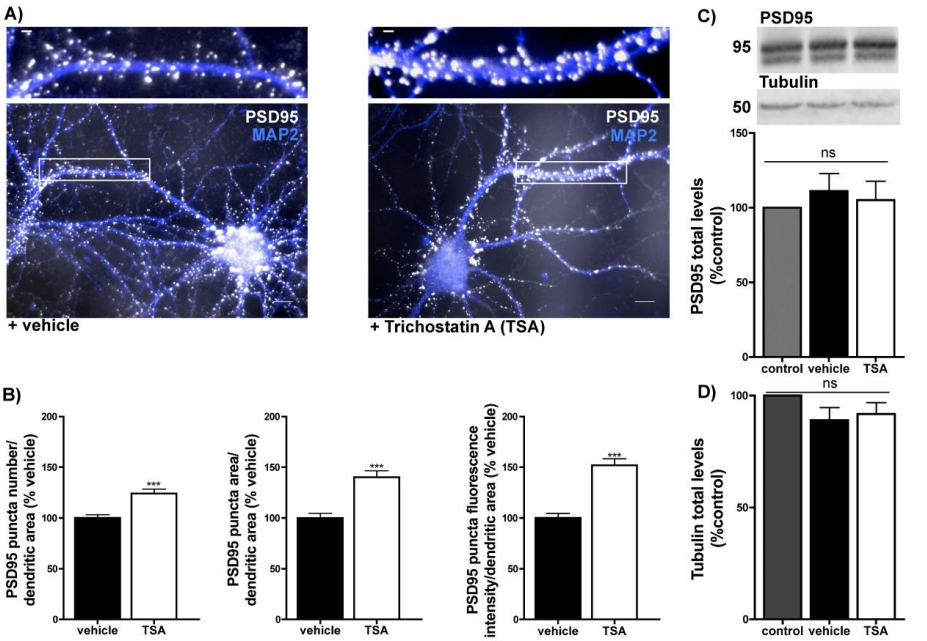


Fig. 10 – Incubation of cultured hippocampal neurons with the HDACI/II inhibitor trichostatin A (TSA) leads to an increase in the number, area and intensity of PSD95 clusters. A) Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. After fixation, neurons were stained for PSD95 and for the somatodendritic marker MAP2. (Scalebar: 10µm, insert: 2µm). B) Neurons were analysed for PSD95 cluster fluorescence intensity, area and number, per dendritic area. Results are presented as % of vehicle-treated control cells, and are averaged from four independent experiments (n≥81 cells). Errorbars, ± S.E.M. Significance, ***p<0,001 relative to control neurons (unpaired student t-test). C, D) TSA treatment does not change PSD95 or Tubulin expression levels**.** Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Western blot was performed using an anti-PSD95 (C) or an anti-Tubulin antibody (D). Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of five experiments (for PSD95) or nine experiments (Tubulin) performed in independent preparations, and are expressed as a percentage of PSD95 or Tubulin expression levels in control conditions. Data were statistically analysed with Graphpad software, using One-way ANOVA. p>0.05.

The effect of TSA stimulus on the clustering of PSD95 could be explained by an effect at the protein expression levels. So, in order to investigate potential effects of TSA on PSD95 expression levels we assessed PSD95 expression levels by western blot (Fig. 10C) and observed that the TSA treatment did not

alter total PSD95 protein levels. Tubulin expression levels were also assessed, in order to use it as a loading control, and no differences were observed (Fig. 10D). These observations suggest that protein acetylation is correlated with the accumulation of PSD95 at synapses, and this accumulation is due to redistribution of the protein and not to increased synthesis of PSD95.

Hippocampal neurons treated with TSA or vehicle for 12 hours were also stained with an antibody against VGLUT1 (vesicular glutamate transporter) (Fig. 11A), a presynaptic marker of excitatory synapses. When comparing treatments, no differences were observed concerning density, area or intensity of VGLUT1 clusters (Fig. 11B). VGLUT1 protein levels were also assessed by western blot and no differences were observed (Fig 11C).

To further investigate the effect of TSA on excitatory synapses, we performed quantitative immunofluorescence analysis of the expression of Shank1, a scaffold protein, using 15 DIV hippocampal neurons. Shank proteins have multiple binding partners that include both membrane-associated proteins and cytoskeletal proteins, and are therefore thought to integrate the submembranous molecular assembly and the cytoskeletal polymers at the postsynaptic interface (Okabe, 2007). In fact, it was shown that overexpression of Shank has a profound effect on the morphology of synapses in a culture system (Sala et al., 2001).

Low density cultured neurons were submitted to the treatments with TSA, and neurons were stained with an antibody against Shank1, an antibody against VGLUT1, used as a presynaptic marker of excitatory synapses, and an antibody against MAP2 (Fig 12A). TSA-treated neurons showed a significant increase in the fluorescence intensity of total dendritic Shank1 clusters, as well as in the density and area of those clusters (Fig. 12B). The intensity, area and density of Shank1 clusters that colocalized with VGLUT1 (synaptic clusters) were also increased in TSA treated neurons (Fig. 12C).

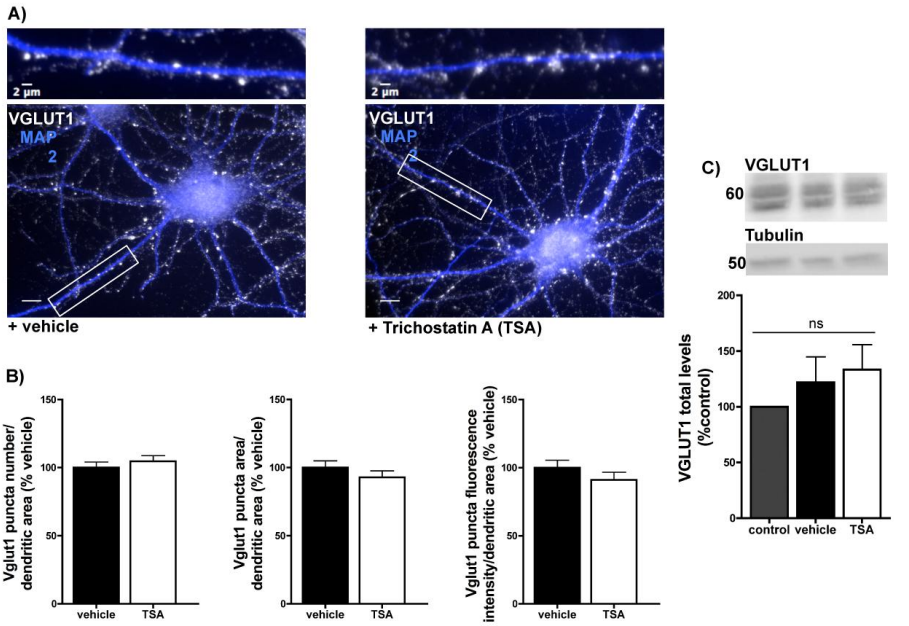


Fig. 11 – TSA treatment in hippocampal neurons in culture has no effect on the number, area and intensity of VGLUT1 clusters. A) Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. After fixation, neurons were stained for VGLUT1 and for the somatodendritic marker MAP2. (Scalebar: 10µm, insert: 2µm) B) Neurons were analysed for VGLUT1 cluster fluorescence intensity, area and number, per dendritic area.Results are presented as % of vehicle-treated control cells, and are averaged from three independent experiments (n≥64 cells). Errorbars, ± S.E.M. (unpaired student t-test).. C) TSA treatment does not change VGLUT1 total expression levels. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Western blot was performed using an anti-VGLUT1 antibody. Staining for Tubulin was used for normalization of VGLUT1 values. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of three experiments performed in independent preparations, and are expressed as a percentage of VGLUT1 expression levels in control conditions. Data were statistically analysed with Graphpad software, using One-way ANOVA. p>0.05.

To determine if the increased clustering of Shank1 was due to changes in the expression level of Shank1, we assessed Shank1 total levels by western blot and observed that Shank 1 protein levels were not altered by the TSA treatments (Fig. 12D). As for PSD95, these observations suggest that protein acetylation, promoted by inhibition of HDACs, is correlated with the

accumulation of Shank1 at synapses, and this accumulation is due to redistribution of Shank1 and not to changes in the expression level of Shank1.

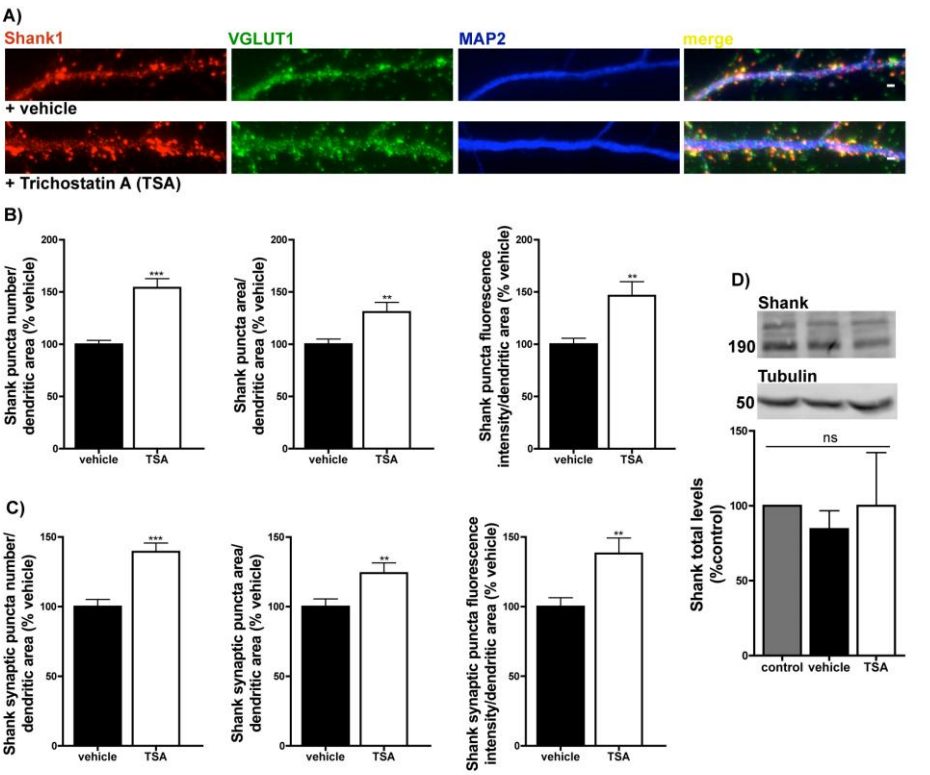


Fig. 12 – Incubation of hippocampal neurons with TSA leads to an increase in the number, area and intensity of total and synaptic Shank1 clusters. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. After fixation, neurons were stained for Shank1, VGLUT1 and for the somatodendritic marker MAP2. (Scale bar: 2µm) Neurons were analysed for total (B) and synaptic (C) Shank1 cluster fluorescence intensity, area and number, per dendritic area. Synaptic Shank1 is defined as Shank1 signal that overlaps with VGLUT1. Results are presented as % of vehicle control cells, and are averaged from three independent experiments (n≥61 cells). Errorbars, ± S.E.M. Significance, **p<0,01 ***p<0,001 relative to control neurons (unpaired student t-test).. D) TSA treatment does not change Shank expression levels. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Western blot was performed using an anti-PanShank (for detection of Shank 1, 2 and 3) antibody. Staining for Tubulin was used for normalization of Shank expression level. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of three experiments performed in independent preparations, and are expressed as a percentage of Shank expression levels in control conditions. Data were statistically analysed with Graphpad software, using One-way ANOVA. p>0.05.

Since TSA treatment had a significant effect on the clustering of scaffold proteins such as PSD95 and Shank1, we then investigated whether this treatment could interfere with the clustering of receptors at the membrane (surface receptors). Quantitative immunofluorescence analysis was performed for the expression of synaptic cell surface GluA1 in hippocampal neurons at 15 DIV. Neurons were live-stained with an antibody against the N-terminal extracellular region of GluA1. After fixation, neurons were stained with an antibody against VGLUT1 and an antibody against MAP2 (Fig. 13A). TSA treatment had no effect in the fluorescence intensity of total dendritic GluA1 clusters, or in density or area of total GluA1 clusters (Fig. 13B). The area, density and fluorescence intensity of GluA1 synaptic clusters were also unaltered in TSA treated neurons (Fig. 13C).

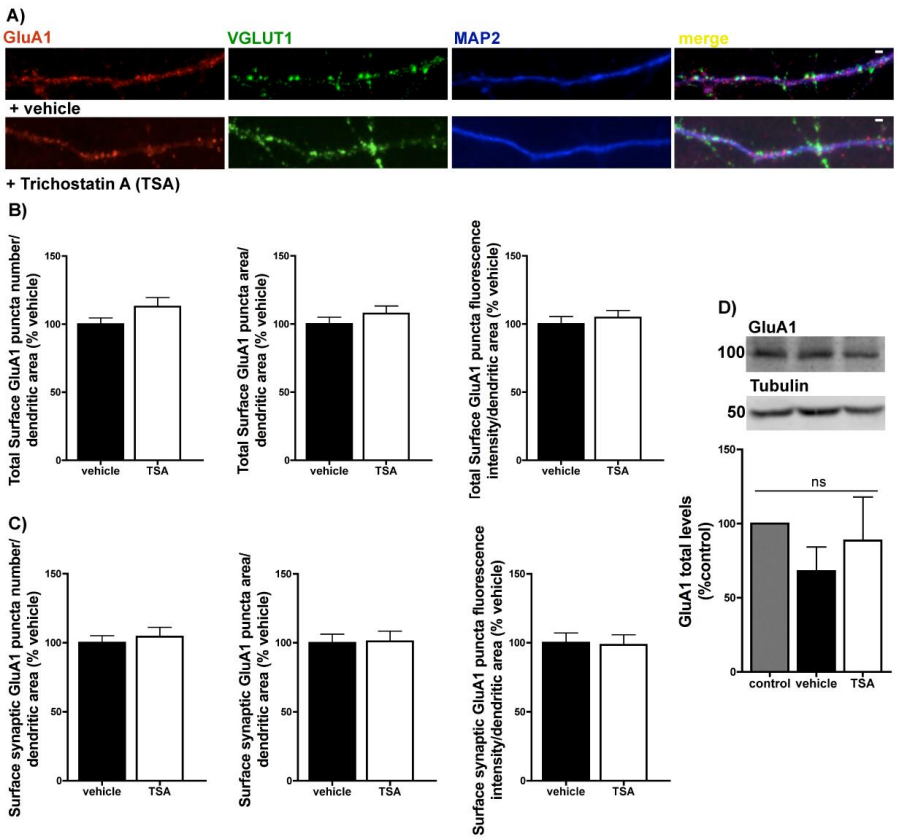


Fig. 13 – TSA treatment of hippocampal neurons does not change the number, area or intensity of surface GluA1 clusters. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Neurons were live-stained for surface GluA1 and, after fixation, for VGLUT1 and the somatodendritic marker MAP2. (Scale bar: 2μm). Neurons were analysed for total (B) and synaptic (C) surface GluA1 cluster fluorescence intensity, area and number, per dendritic area. Synaptic GluA1 is defined as GluA1 signal that overlaps with VGLUT1. Results are presented as % of vehicle control cells, and are averaged from three independent experiments (n≥63 cells). Errorbars, ± S.E.M. (unpaired student t-test). D) TSA treatment does not change GluA1 expression levels. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Western blot was performed using an anti-GluA1 antibody. Staining for Tubulin was used for normalization of GluA1 values. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of three experiments performed in independent preparations, and are expressed as a percentage of GluA1 expression levels in control conditions. Data were statistically analysed with Graphpad software, using One-way ANOVA. $p>0.05$.

GluA1 protein levels were also assessed by western blot and no differences were observed in neurons treated with TSA compared with vehicle-treated or control neurons (Fig. 13D).

Acetylation effect on inhibitory synapses

In order to understand if the TSA effect is specific for excitatory synapses, we tested whether this treatment could affect specific proteins of inhibitory synapses. Hippocampal neurons (15 DIV) were submitted to TSA and vehicle treatments and were stained after fixation using a specific antibody for Gephyrin, a protein that is a component of the postsynaptic protein network of inhibitory synapses, and VGAT, a presynaptic marker of GABAergic neurons (highly concentrated in the nerve endings of GABAergic neurons) (Fig. 14A). The TSA treatment had no effect in the density, area or fluorescence intensity of total dendritic Gephyrin clusters (Fig. 14B), or of Gephyrin clusters colocalized with VGAT (Fig. 14C). Gephyrin protein levels were also assessed by western blot and no differences were observed (Fig. 14D). The results obtained for proteins of inhibitory synapses suggest that protein acetylation effect is specific for excitatory synapses.

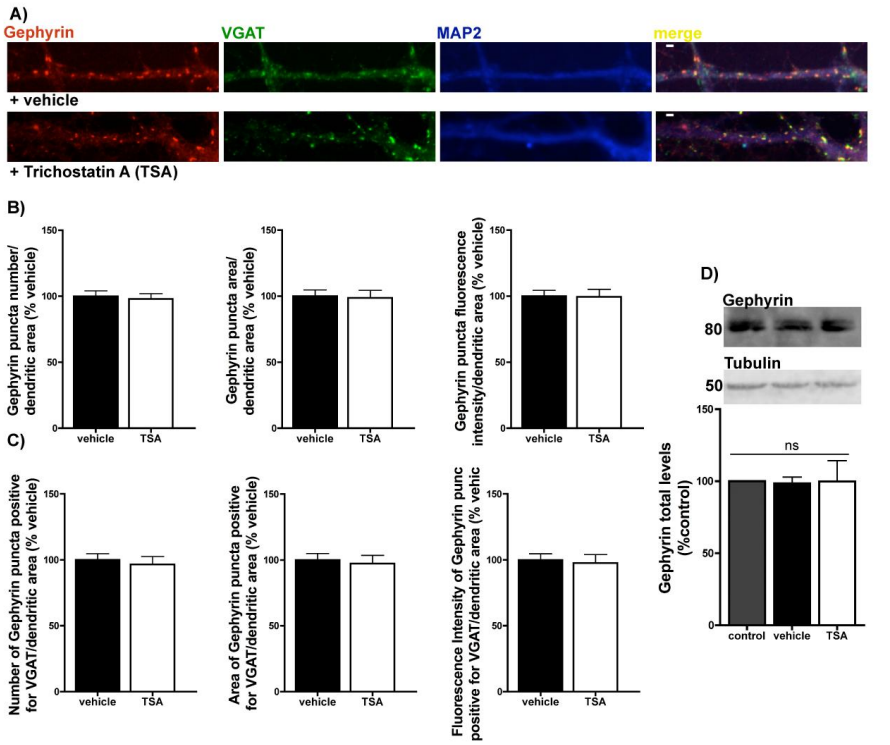


Fig. 14 - TSA has no effect on the number, area or intensity of Gephyrin clusters. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Neurons were stained for gephyrin, VGAT and for the somatodendritic marker MAP2. (Scale bar: 2µm). Neurons were analysed for total (B) and synaptic (C) gephyrin clusters fluorescence intensity, area and number, per dendritic length. Synaptic gephyrin is defined as gephyrin signal that overlaps with VGAT. Results are presented as % of vehicle treated control cells, and are averaged from three independent experiments (n≥61 cells). Errorbars, ± S.E.M. (unpaired student t-test). D) TSA treatment does not change gephyrin expression levels. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Western blot was performed using an anti-gephyrin antibody. Staining for Tubulin was used for normalization of gephyrin values. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of three experiments performed in independent preparations, and are expressed as a percentage of gephyrin expression levels in control conditions. Data were statistically analysed with Graphpad software, using One-way ANOVA. $p>0.05$.

Acetylation affects actin-related synaptic proteins

Having looked at scaffold proteins of the PSD and at AMPA receptors, we then sought to dissect if TSA treatment could have any effect on proteins that interact directly or indirectly with the actin cytoskeleton, since it is a major component of dendritic spines. We focused on Cortactin and p140Cap. Cortactin, which is an activator of the Arp2/3 actin nucleation machinery, is enriched in dendritic spines, where it co-localizes and interacts with F-actin and also with the PSD scaffold Shank. It has been shown that RNAi knockdown of cortactin results in depletion of dendritic spines, whereas overexpression of cortactin causes elongation of spines (Hering and Sheng, 2003), implicating cortactin in spine morphogenesis. Cortactin can regulate spine morphology by mediating the interaction between actin and microtubules. In fact, recent work showed that dynamic microtubules can enter dendritic spines and affect actin dynamics (Jaworski et al., 2009). These authors uncovered p140Cap (SNAP-25 interacting protein) as an abundant PSD protein in spines, and also as a binding partner for the microtubule plus-end tracking protein EB3 in hippocampal neurons. They also found that p140Cap interacts with cortactin, suggesting that the association between EB3-bound microtubule ends and p140Cap regulates cortactin function, leading to Arp2/3 activation and spine head growth (Jaworski et al., 2009).

Quantitative immunofluorescence analysis was performed for expression levels of both p140Cap (actin indirect interactor) and cortactin (direct proteic partner of F-actin). Hippocampal neurons were used at 15 DIV and TSA and vehicle treatments were applied. After fixation neurons were stained with the specific antibodies. Neurons were stained with an antibody against p140Cap, an antibody against VGLUT1 and an antibody against MAP2 (Fig. 15A). TSA treatment did not affect the fluorescence intensity of total dendritic p140Cap clusters, or their density or area (Fig. 15B). The density of p140Cap synaptic clusters was also unaltered in TSA treated neurons, but the area and the fluorescence intensity of those clusters showed a significant decrease (Fig.

15C). Total p140Cap protein levels were also assessed by western blot and no differences were observed (Fig 15D).

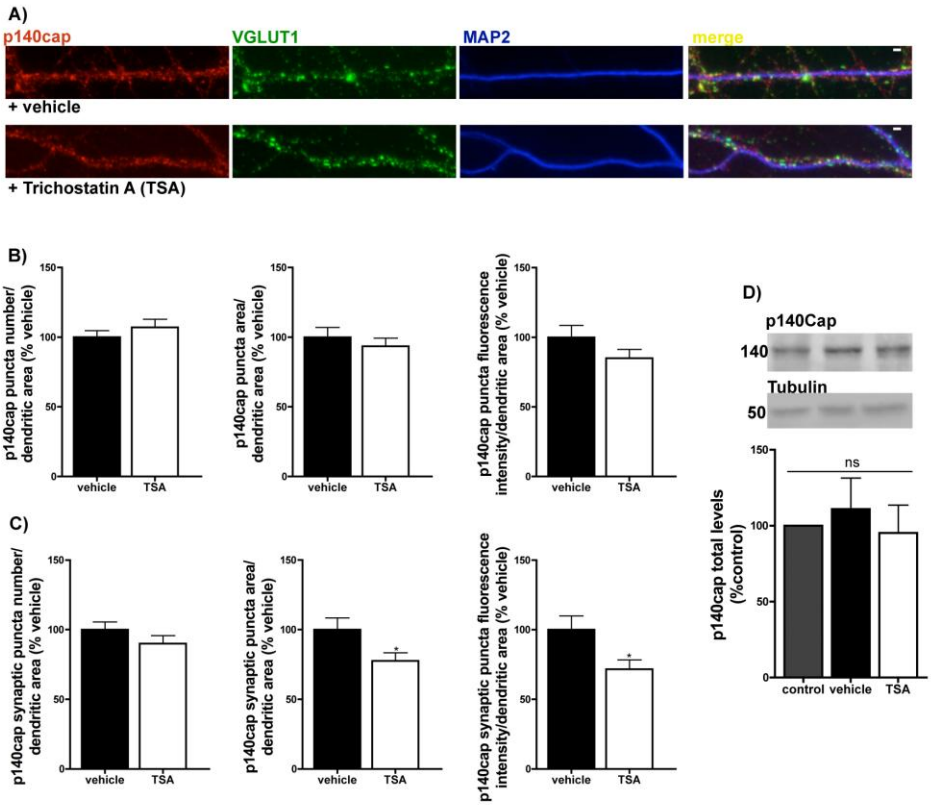


Fig. 15 – TSA treatment of hippocampal neurons leads to a decrease in the area and intensity of synaptic p140Cap clusters. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. After fixation, neurons were stained for p140Cap, VGLUT1 and for the somatodendritic marker MAP2. (Scale bar: 2µm). Neurons were analysed for total (B) and synaptic (C) p140cap cluster fluorescence intensity, area and number, per dendritic area. Synaptic p140cap is defined as p140cap signal that overlaps with VGLUT1 Results are presented as % of vehicle control cells, and are averaged from three independent experiments (n≥63 cells). Errorbars, ± S.E.M. Significance, *p<0,05 relative to control neurons (unpaired student t-test). D) TSA treatment does not change p140Cap expression levels. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Western blot was performed using an anti-p140Cap antibody. Staining for Tubulin was used for normalization of p140Cap values. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of three experiments performed in independent preparations, and are expressed as a percentage of p140cap expression levels in control conditions. Data were statistically analysed with Graphpad software, using One-way ANOVA. p>0.05.

Neurons were also stained with an antibody against cortactin, a protein that has been identified as a target for reversible acetylation (Zhang et al., 2007), an antibody against VGLUT1 and an antibody against MAP2 (Fig 16A).

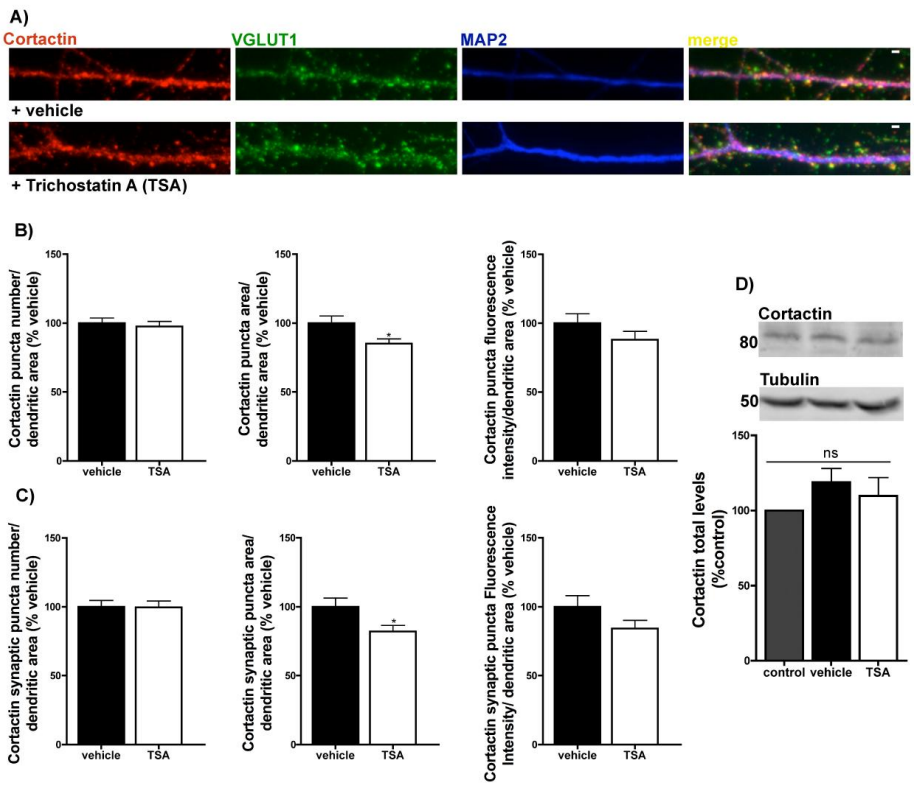


Fig. 16 - TSA leads to a decrease in the area of synaptic cortactin clusters. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. After fixation, neurons were stained for cortactin, VGLUT1 and for the somatodendritic marker MAP2. (Scale bar: 2µm). Neurons were analysed for total (B) and synaptic (C) cortactin cluster fluorescence intensity, area and number, per dendritic area. Synaptic cortactin is defined as cortactin signal that overlaps with VGLUT1. Results are presented as % of vehicle control cells, and are averaged from four independent experiments (n≥81 cells). Errorbars, ± S.E.M. Significance, *p<0,05 relative to control neurons (unpaired student t-test). D) TSA treatment does not change cortactin expression levels. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400ng/ml) for 12hr. Western blot was performed using an anti-cortactin antibody. Staining for Tubulin was used for normalization of Cortactin values. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of three experiments performed in independent preparations, and are expressed as a percentage of cortactin expression levels in control conditions. Data were statistically analysed with Graphpad software, using One-way ANOVA. p>0.05.

We observed that TSA treatment did not have an effect in the density or fluorescence intensity of total or synaptic cortactin clusters (Fig. 16B and C). On the other hand, the area of both total and synaptic cortactin clusters decreased (Fig. 16B and C). Cortactin expression levels were also analyzed by western blot and no differences were detected (Fig. 16D).

Taken together, these observations suggest that protein acetylation correlates with a decrease in the area of clusters of two cytoskeleton related proteins at synapses, probably due their redistribution from dendritic spines to shafts.

Discussion

In this chapter we studied the effects of inhibiting types I and II HDACs with TSA on the localization of synaptic proteins. We found that TSA treatment of hippocampal neurons led to a dramatic increase in density, area and intensity of PSD95 and Shank1 clusters. Conversely, TSA treatment resulted in decreased cluster area of the cytoskeleton associated proteins cortactin and p140Cap. VGLUT1 and GluA1 clustering was not affected by inhibiting HDACs.

Accumulation of PSD constituent proteins by dynamic regulation of abundance and activity may occur through: A) increase in the expression levels by local protein translation; B) localized trafficking and/or redistribution to and away from the PSD; C) decrease in their degradation process through the ubiquitin-proteasome system. Therefore, the changes in the clustering observed for some scaffold proteins could be explained by any of these processes.

It is known that HDAC inhibitors, like TSA, modulate the acetylation levels of histones and thereby affect transcription. Since expression levels of the proteins that we tested were not altered by TSA treatment, we suggest that transcription of the genes that encode them is not being affected by TSA. The observed effect may be due to acetylation and regulation of non-histone proteins, which in turn interfere with activity and localization of these synaptic proteins. Alternatively, the acetylation of histone tails, which regulates chromatin structure and interferes with gene transcription, may regulate the expression levels of specific proteins which in turn have an impact on the localization of PSD95 and Shank1 and also p140Cap and Cortactin.

Protein clustering or accumulation could also be explained by a decrease in the degradation process. For example Hung et al. (2010) showed that the protein TRIM3, present in PSD fractions from rat brain, stimulates ubiquitination and proteasome-dependent degradation of GKAP, and induces the loss of GKAP and associated scaffold Shank1 from postsynaptic sites. Suppression of endogenous TRIM3 by RNA interference (RNAi) results in increased

accumulation of GKAP and Shank1 at synapses, as well as enlargement of dendritic spine heads (Hung et al., 2010). It was also shown that PSD95 interacts with and is ubiquitinated by the E3 ligase Mdm2, meaning that PSD95 is regulated by the ubiquitin-proteasome pathway (Colledge et al., 2003).

In fact, some molecules involved in protein degradation have been identified as targets of HDAC6. Bali et al., (2005) showed that HDAC6 is an HSP90 deacetylase and targeted inhibition of HDAC6 leads to acetylation of HSP90 and disruption of its chaperone function, resulting in polyubiquitylation and depletion of pro-growth and pro-survival HSP90 client proteins including Bcr-Abl. HDAC6 is also capable of interacting with ubiquitin (Boyault et al., 2006). These authors demonstrated that HDAC6-interacting chaperone p97/VCP dissociates the HDAC6–ubiquitin complexes and counteracts the ability of HDAC6 to promote the accumulation of polyubiquitinated proteins.

However, and since we observed that the expression levels of the proteins were maintained after TSA stimulation, the idea that clustering of synaptic proteins could be happening by their increased production and/or impaired degradation can be put aside. Nevertheless, a localized effect on either protein synthesis or degradation could be occurring without being noticed when assessing the total levels of the synaptic proteins.

Results from previous studies have shown that HDACs associate with and regulate the acetylation of several non-histone proteins. One of the most extensively studied and best characterized non-histone HDAC substrates is the cytoplasmic protein α-tubulin (Haggarty et al., 2003; Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003). Zhang et al. (2003) showed that HDAC6 associates with and deacetylates α-tubulin *in vitro* and *in vivo*, and that overexpression of HDAC6 in mammalian cells leads to tubulin hypoacectylation, whereas inhibition of HDAC6 leads to hyperacetylation of tubulin and microtubules. Consistent with this, Hubbert et al. (2002) found that 3T3 cells stably overexpressing HDAC6 have an increased motility, probably due to the deacetylation of microtubules.

In neurons, it has been shown that an increase of α-tubulin acetylation induced by pharmacological inhibition of histone deacetylase 6 (HDAC6) corrected the axonal transport defects caused by HSPB1 (heat shock protein beta-1) mutations and rescued the Charcot-Marie-Tooth (CMT) phenotype of symptomatic mutant HSPB1 mice (d'Ydewalle et al., 2011). Other studies showed that pharmacological treatments (TSA) that increase microtubule acetylation caused a redirection of kinesin-1 transport of JIP1 (c-Jun N-terminal kinase-interacting protein 1) to nearly all neurite tips *in vivo*, suggesting that microtubule PTMs are important markers of distinct microtubule populations and that they act to control motor-protein trafficking (Reed et al., 2006). In addition to the recruitment of kinesin-1, MT acetylation also leads to the recruitment of the retrograde motor dynein. Thus, MT acetylation stimulates not only anterograde but also retrograde transport, suggesting a general role for MT acetylation in the stimulation of intracellular dynamics through the recruitment of both anterograde and retrograde motors (Dompierre et al., 2007). For example, the molecular mechanism by which the PSD95-based protein complex is trafficked to the postsynaptic site presumably involves specific motor proteins. A direct interaction between the PSD95-associated protein guanylate kinase domain-associated protein (GKAP) and dynein light chain (DLC), a light chain subunit shared by myosin-V (an actin-based motor) and cytoplasmic dynein (a microtubule-based motor) has been demonstrated (Naisbitt et al., 2000). This PSD95-GKAP complex may also exert a functional and structural role in Shank assembly, targeting and stability to synapses (Romorini et al., 2004). One can presume that MT acetylation may be involved in the synaptic targeting of synaptic proteins such as PSD95 and Shank1.

Zhang et al. (2007) also identified cortactin, an F-actin binding protein, as a HDAC6 substrate. Their findings suggested that, in addition to its role in microtubule-dependent cell motility, HDAC6 influences actin-dependent cell motility by altering the acetylation status of cortactin, which, in turn, changes the F-actin binding activity of cortactin. So acetylation modulates the activity of

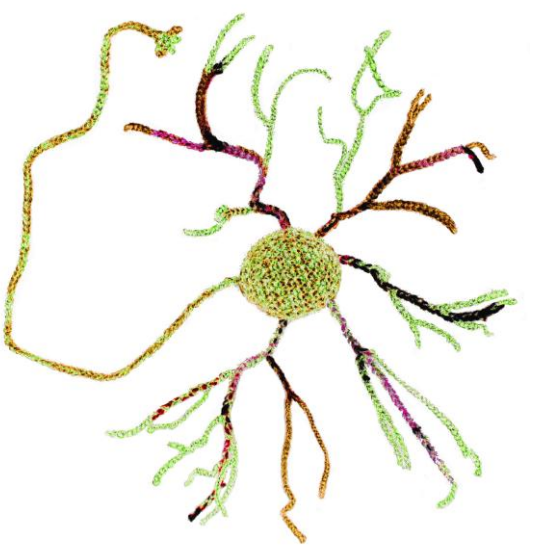
cortactin. These authors also identified SIRT1 as a cortactin deacetylase and p300 as a cortactin acetyl-transferase (Zhang et al., 2008).

Filamentous (F)-actin in the postsynaptic cytoplasm regulates rapid turnover of individual PSD scaffolding molecules, which plays an essential role in rapid alterations in the PSD size and composition. Kuriu et al. (2006) demonstrated that four major scaffolding molecules, PSD95, GKAP, Shank, and PSD-Zip45, show distinct instability in total molecular content per synapse. Acute pharmacological disruption of F-actin rapidly eliminated the dynamic fraction of GKAP, Shank, and PSD-Zip45, without changing synaptic localization of PSD95 and inhibition of F-actin dynamics prevented activity-dependent redistribution of all three scaffolds (Kuriu et al., 2006). These authors also assessed involvement of glutamate receptors in the regulation of PSD dynamics and found that genetic manipulations eliminating either NMDA receptors or metabotropic glutamate receptors did not primarily influence mobility of their binding scaffolds.

Cortactin not only interacts with F-actin but also binds to Shank proteins (Du et al., 1998; Naisbitt et al., 1999) (including Shank1), and in turn, Shank interacts, through SAPAP, with PSD95 (Naisbitt et al., 1999). The effect of HDACs inhibition with TSA on PSD95 and/or Shank1 clustering in neurons may be partially the result of changes in cortactin interaction with F-actin and scaffold proteins in hyperacetylation conditions, due to HDAC6 inhibition. In addition, acetylation can be responsible for cortactin redistribution, since we observed that the area of cortactin clusters was diminished after TSA treatment. Considering these evidences, in the next chapter we address the hypothesis that cortactin acetylation may play a role in regulating the dendritic clustering of PSD95 in hippocampal neurons.

Chapter 4

Cortactin Acetylation Regulates PSD95 Dendritic Clustering



Introduction

Cell motility and directional migration are essential for a wide range of cellular processes. These complicated processes require dynamic regulation of the actin cytoskeleton, utilizing a network of signaling proteins that coordinate regulated changes in the actin architecture. The adaptor protein cortactin is one of the key molecules involved in the actin regulation (Ammer and Weed, 2008). Cortactin, which is highly enriched in spines where it colocalizes with F-actin (Hering and Sheng, 2003; Racz and Weinberg, 2004), has been identified as a central player in a number of neuron-specific functions including dendritic spine morphogenesis (synaptogenesis) as verified by the observation that its downregulation resulted in spine depletion, whereas its overexpression caused spine elongation (Hering and Sheng, 2003). The findings of Gray et al., (2005) also demonstrated a critical role for the interaction between cortactin and dynamin-3 in dendritic spine maturation. These authors showed that disruption (or dissociation) of this complex leads to formation of mature dendritic spines with postsynaptic proteins, whereas its presence facilitates the formation of immature dendritic filopodia (Gray et al., 2005).

Discovering that cortactin interacts with members of the Shank family, scaffold proteins of the postsynaptic density, was an important step to understand the role of cortactin at the synapse (Du et al., 1998; Naisbitt et al., 1999). The binding of Shank, which is linked to both ionotropic and metabotropic receptors through various adaptor proteins (Fig.9), to cortactin, forms a multiprotein bridge between the cytoskeleton and excitatory receptors (reviewed in Cosen-Binker and Kapus, 2006). Furthermore, cortactin distribution and therefore postsynaptic cytoskeleton organization, is dynamically regulated by synaptic transmission. In fact, some fascinating findings suggested that movement of cortactin might be important for activity-dependent remodeling of the spine cytoskeleton. Iki et al., (2005) demonstrated that overstimulation of NMDA receptors in hippocampal neurons removed cortactin and thereby collapsed dendritic spines, an effect due to Src family-mediated

tyrosine phosphorylation of cortactin, whereas brain-derived neurotrophic factor (BDNF) triggered ERK-mediated serine phosphorylation of cortactin, concomitant with its translocation to spines, therefore solidifying synaptic transmission. Thus, cortactin may play a role in synaptic plasticity and long term potentiation, due to its regulation of ion channels (through Shank) and dynamic modification of synapses.

Recently, cortactin has also been implicated in the regulation of spine morphology and synaptic plasticity by dynamic microtubules. The general idea is that actin is essential for dendritic spine structure and function. Interestingly, new studies revealed that microtubules also enter dendritic spines in both hippocampal and cortical neurons (Jaworski et al., 2009) and this microtubule invasion into spines is associated with transient morphological changes, such as formation of spine head protrusions and spine growth. Additionally, these studies showed that cortactin associates with microtubule plus-end binding protein EB3, through p140Cap, and this interaction appears to be required for EB3-mediated spine expansion. These results raise the possibility that cortactin-p140Cap-EB3 interaction can serve as a link between dynamic microtubules and the actin cytoskeleton in dendritic spines, which leads to spine changes and synaptic plasticity.

The phosphorylation of cortactin and resulting functional consequences has been an intense area of study for many years. Tyrosine phosphorylation of cortactin at specific residues was hypothesized to lead to a conformational change in the protein (Huang et al., 1997b; Perrin et al., 2006), possibly affecting the ability of cortactin to bind to and cross-link actin filaments. In addition to tyrosine phosphorylation, cortactin is also a substrate for several serine/threonine kinases, and several serine residues were identified as phosphorylation targets (Campbell et al., 1999; Webb et al., 2006), with functional consequences (Martinez-Quiles et al., 2004). Recent studies demonstrated that cortactin is also a target for acetylation. Zhang and colleagues (2006) showed that cortactin is a substrate for HDAC6 and identified

p300-CBP-associated factor (PCAF) as a potential cortactin acetyl-transferase (Zhang et al., 2008). Cortactin acetylation has functional consequences: cortactin deacetylation enhances the ability of cortactin to bind F-actin by modulating a “charge patch” in its repeat region, whereas acetylation of cortactin ablates the interaction between cortactin and F-actin, resulting in decreased cell migration (Zhang et al., 2007). These evidences suggest that cortactin activity can be regulated by both phosphorylation and acetylation. In fact, since cortactin influences actin organization through interactions with several proteins that promote F-actin assembly, these interactions can be regulated, not only by cortactin phosphorylation, but also acetylation.

In this chapter we examine how acetylation regulates cortactin localization and function in hippocampal neurons. Our studies are focused on how cortactin acetylation regulates the clustering of the postsynaptic scaffolding protein PSD95, a marker for synaptic strength, in the dendrites of cultured hippocampal neurons. Furthermore, we addressed the effects of cortactin acetylation on its tyrosine phosphorylation, as well as on its interactions with multiple synaptic binding partners. Finally, we characterized the effect of BDNF, which promotes an increase on PSD-95 in dendritic spines (Yoshii &Constantine-Paton, 2007), on the acetylation of cortactin. Our data support a function for cortactin acetylating in the regulation of the clustering of PSD95.

Results

Acetylation affects synaptic localization of cortactin

In order to test whether neuronal cortactin is regulated by acetylation, we assessed the protein levels of acetylated cortactin in hippocampal neurons cultured at high-density (8.9 x 10^4 cells/cm2) and treated with the classes I and II HDAC inhibitor Thricostatin A (TSA), or vehicle, for 12 hours. Protein levels were assessed by western blot analysis using an antibody against acetylated cortactin (a kind gift of Xiaohong Zhang and Edward Seto) and an antibody for total cortactin. Neuronal treatment with TSA led to an increase on the acetylation level of cortactin (Fig. 17).

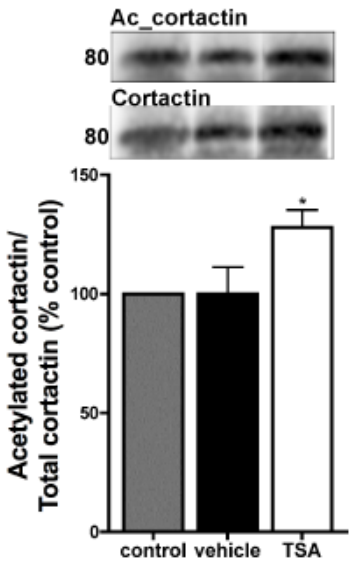


Fig. 17 – Treatment with the classes I and II HDAC inhibitor TSA promotes cortactin acetylation in hippocampal neurons. Hippocampal neurons at 15 DIV were treated with vehicle or TSA (400 ng/ml) for 12hr. Western blot was performed using an anti-acetylated cortactin and an anti-cortactin antibodies. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of ten experiments performed in ten independent preparations, and are expressed as a percentage of cortactin acetylation in control conditions. Data were statistically analysed with Graphpad software, using One-way ANOVA, followed by Dunnett's Multiple Comparison Test. *p<0.05.

Cortactin has been described to concentrate with F-actin to dendritic spines of hippocampal neurons (Hering and Sheng, 2003). In the previous chapter we described that TSA treatment, which leads to an overall increase on protein acetylation, decreased the area of synaptic clusters of cortactin (Fig. 16). Since cortactin is a substrate for HDAC6 (inhibited by TSA) (Zhang et al., 2007), and as a consequence, is more acetylated when HDACs are inhibited (Fig. 17), we used immunocytochemical methods to characterize the localization of acetylated cortactin in rat hippocampal neurons in culture (15 DIV). Hippocampal neurons 15 DIV were double-stained with antibodies for the postsynaptic marker PSD95 and for cortactin (Fig 18 A). Cortactin was found to strongly colocalize with PSD95 wich is in accordance with previous studies, proving that it is highly enriched synaptically in cultured rat hippocampal neurons.

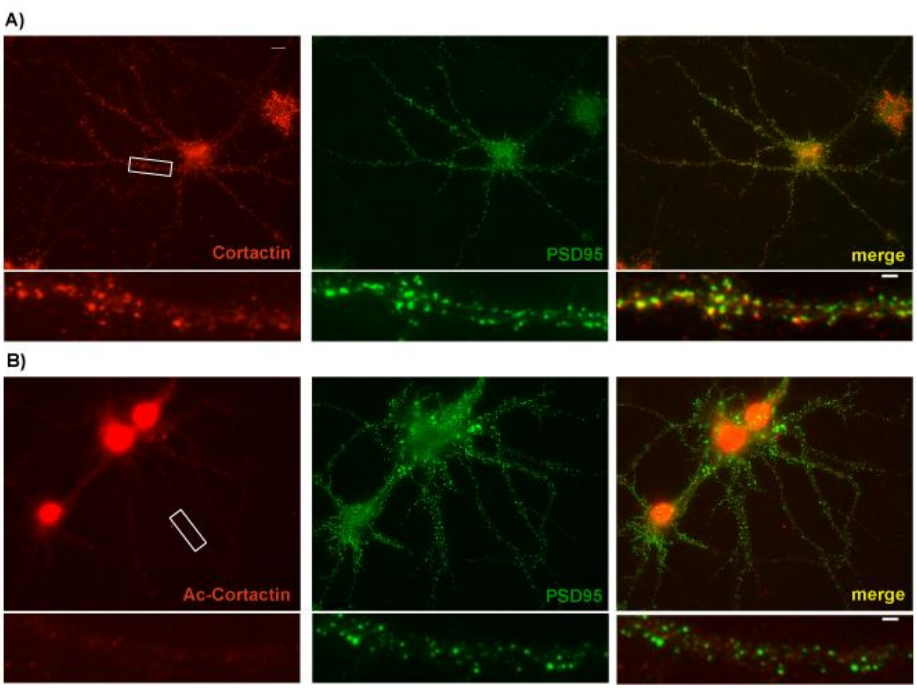


Fig. 18 – Acetylated cortactin is less abundant in synapses. Hippocampal neurons at 15 DIV were stained with an antibody for cortactin (A), or an antibody for acetylated cortactin (B). Synaptic localization was determined by colocalization with the excitatory postsynaptic marker PSD95. Scalebar: 2 µm.

Additionaly, hippocampal neurons were stained using antibodies for PSD95 and acetylated cortactin; acetylated cortactin immunoreactivity is absent from dendritic spines, and concentrates in the cell bodies of neurons and in dendritic shafts (Fig. 18 B).

Immunolabelling of cultured hippocampal neurons for acetylated cortactin demonstrated that the protein is distributed to shafts of dendrites but is not accumulated at synaptic sites, since it shows little colocalization with the postsynaptic protein PSD95. To further evaluate the subcellular localization of acetylated cortactin, we biochemically isolated postsynaptic densities (PSDs) from hippocampi of adult rats. We first used western blotting to assess the level of enrichment of PSD95 in PSD fractions, and the presence of the presynaptic marker synaptophysin in these fractions (Fig. 19IA). Western blot analysis indeed showed that synaptophysin is enriched in synaptosomes and crude synaptic vesicles fraction and not in the PSDs, whereas PSD95 is enriched in the PSD preparations and is absent in the crude synaptic vesicles fraction (Fig. 19IA). Taken together these evidences indicate that the subsynaptic fractions obtained display the expected differential expression of post- and presynaptic proteins. When compared with total cortactin, acetylated cortactin is less enriched in the synaptic fractions, since we observed smaller amounts in the crude synaptosomes and reduced amounts in the isolated PSDs (Fig. 19IB), and these results were substantiated by normalizing the levels of total or acetylated cortactin to actin levels in each fraction (Fig. 19IC). Additionally, to confirm the subcellular localization of acetylated cortactin, we used a preparation of synaptic bodies, termed “synaptoneurosomes”, which contains the presynaptic (synaptosome) and postsynaptic (neurosome) vesicularized components. Reports suggest that synaptoneurosomes are physiologically active synapses and can be useful in studying synaptic events (Titulaer and Ghijsen, 1997). Using western blot to assess the level of acetylated cortactin in synaptoneurosome fractions (Fig. 19IIA), we found that acetylated cortactin is less abundant in synaptoneurosomes than in total hippocampal homogenates (Fig. 19IIB). We also used western blotting to assess the level of enrichment of

PSD95 and of the presynaptic marker VGLUT1 in synaptoneurosome fractions (Fig. 19 IIA), in order to validate the synaptoneurosomes preparation. Both proteins are enriched in the synaptoneurosome fractions.

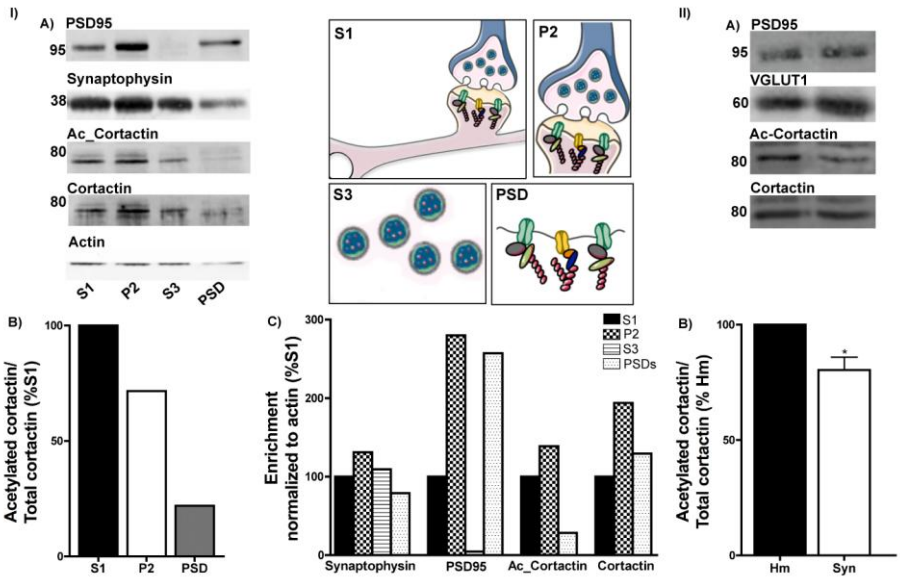


Fig. 19 – Acetylated cortactin is less abundant in synaptic fractions. I) PSD preparations from rat hippocampus. A) Synaptic profile of non-nuclear fraction (S1), crude synaptosomes (P2), crude synaptical vesicle fraction (S3) and postsynaptic densities (PSDs) isolated from rat hippocampus. Western blot was performed using the following antibodies: anti-PSD95, anti-synaptophysin, anti-acetylated cortactin antibody, anti-cortactin and anti-actin. B) Quantitative analysis was performed with ImageQuant. Data represent one experiment, and is expressed as a percentage of cortactin acetylation in the S1 fraction. Equal amounts of protein (9 µg) were applied to each lane. C) The plot represents protein enrichment in each fraction, normalized to actin levels, and is expressed as a percentage of the S1 fraction. II) Synaptoneuromose preparations from rat hippocampus A) Western blot profiles of total homogenate (Hm) and synaptoneurosome fractions (Stefanovic et al.) from rat hippocampus were performed using an anti-PSD95, anti-VGLUT1, anti-acetylated cortactin antibody and an anti-cortactin antibody. B) Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of five experiments performed in independent preparations, and are expressed as a percentage of cortactin acetylation in total homogenate condition. Data were statistically analysed with Graphpad software, using paired student t-test. *p<0.05.

Taken together, these data suggest that acetylation of cortactin triggers its redistribution from spines to shafts of dendrites.

Acetylation of cortactin: role on PSD95 clustering

It has been shown that cortactin plays a role in the morphogenesis of spines. Hering & Sheng (2003) observed that knockdown of cortactin by short-interfering RNA (siRNA) results in depletion of dendritic spines in hippocampal neurons, whereas overexpression of cortactin caused elongation of spines. Correlating with this loss of spines, these authors also observed that the density of F-actin and PSD95 clusters on dendrites of cortactin–deficient neurons were diminished greatly. This effect of cortactin depletion on PSD95 clustering can be an indirect effect of the collapse of spines, or a specific effect that results from the loss of cortactin. Having observed that neuronal cortactin is a substrate for acetylation, we tested whether acetylation of cortactin can influence the clustering of PSD95, a scaffolding protein which is a member of the membrane-associated guanylate kinase (MAGUK) family, and which clusters at the synapse NMDA receptors as well as complexes of AMPA receptors with TARPs.

To perform these experiments, we resorted to specific acetylation mimetic forms of cortactin. Zhang et al. (2007) identified the repeat region of cortactin as the target for acetylation. Additionally, these authors identified eleven lysine residues within the protein targeted for acetylation, and eight of the targeted lysine residues are located in this repeat region. Therefore, in order to mimic the non-acetylated form of cortactin we used a charge preserving cortactin mutant in which all of the nine repeat-region lysines were mutated to arginine (9KR), and which is able to efficiently bind to F-actin. To mimic the acetylated form of cortactin we used a charge-neutralizing cortactin mutant in which all nine of the repeat-region lysine residues were mutated to glutamine (9KQ) and which, therefore, is not able to bind to F-actin (kind gifts of Xiaohong Zhang and Edward Seto).

Hippocampal neurons cultured at low-density were transfected at 7 DIV with GFP together with wild-type FLAG-tagged cortactin, or the FLAG-tagged mimetic mutants for acetylated or deacetylated cortactin. At 15 DIV, when

neurons have established mature synapses, the preparations were fixed and neurons were triple-stained with antibodies against PSD95, the FLAG epitope and MAP2 (Fig. 20A). Transfected neurons were identified by GFP fluorescence, and the FLAG signal was observed for confirmation of overexpression of the cortactin constructs. Quantitative analysis of the PSD95 signal showed that in neurons transfected with the mutant that mimics the deacetylated form of cortactin (9KR) the fluorescence intensity and area of PSD95 clusters are decreased (Fig. 20B), whereas no changes relatively to control cells (transfected with the empty vector) were observed in neurons expressing wild-type cortactin or the mutant form of cortactin that mimics the acetylated protein (9KQ). No significant difference was detected in the number of PSD95 puncta observed in the neurites of cells transfected with deacetylated mimetic mutant (Fig 20B). Conversely, we found that the number of PSD95 puncta was increased in hippocampal neurons expressing wild-type cortactin (Fig. 20B). These data suggest that acetylation of cortactin may be required for the clustering of PSD95 in synapses.

In the experiments reported in Fig. 20, we overexpressed wild-type cortactin or the acetylation mutants for cortactin in hippocampal neurons expressing endogenous cortactin. Therefore, interpretation of the observed effects is confounded by the functional role of endogenous cortactin, which is likely a combination of acetylated and non-acetylated cortactin. To further assess the effect of cortactin acetylation on excitatory synapses we used a short-hairpin RNA (shRNA) sequence against cortactin to knockdown the expression of endogenous cortactin. We used a lentiviral vector, pLentiLox3.7(CMV)EGFP (pLL3.7), which was engineered by introducing the mouse U6 promoter upstream of a cytomegalovirus promoter-based GFP expression cassette to create a vector that simultaneously produces cortactin shRNA and GFP, allowing us to easily identify shRNA transfected neurons. The sequence used was as follows: rat cortactin shRNA, 5′-CACTGCTCACAAGTGGAC -3′ (Hering and Sheng, 2003). Hippocampal neurons in culture were transfected at 12 DIV with cortactin-shRNA and

analyzed at 15 DIV for cortactin expression (Fig. 21). The cortactin-shRNA construct decreased the expression of cortactin to a great extent, specially at dendrites and spines, even though some expression of cortactin is still detected in the cell body.

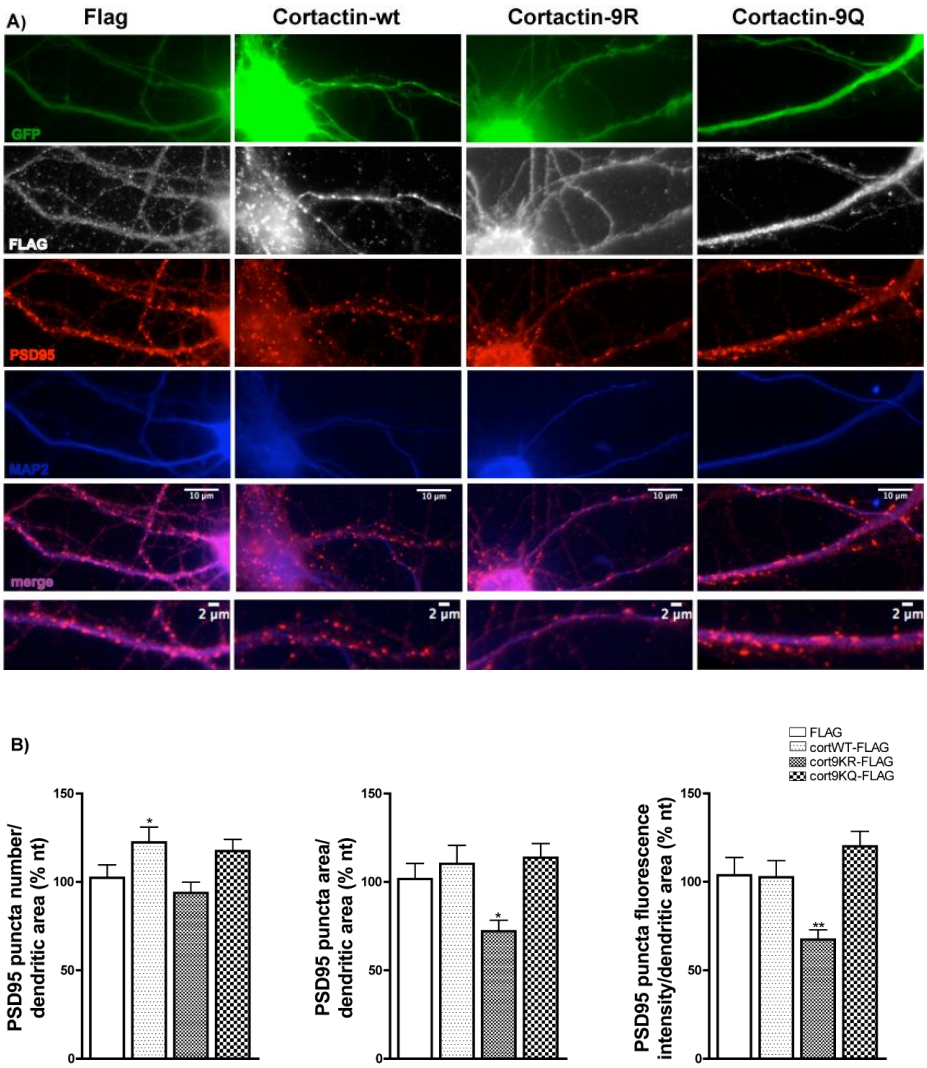


Fig. 20 - Acetylation of cortactin may be required for PSD95 clustering. A) Hippocampal neurons were transfected at 7 DIV with GFP along with FLAG, cortactinWT-FLAG, cortactin9KR-FLAG or cortactin9KQ-FLAG. Neurons (15 DIV) were stained for FLAG, MAP2 and for the postsynaptic marker PSD95. Transfected neurons, identified by GFP fluorescence, were analysed for PSD95 cluster fluorescence intensity, number and area, per dendritic area. B) Results are presented as % of control cells (transfected with empty vector), and are averaged from four independent experiments (n≥29 cells). Errorbars, ± S.E.M. Significance, *p<0.05, **p<0,01, relative to non-transfected neurons (One-way ANOVA followed by Dunnett's Multiple Comparison Test). Scalebar: 10 µm, insert: 2 µm.

The clustering of PSD95 was evaluated in control neurons and in neurons expressing cortactin-shRNA (Fig. 22A). In agreement with what was previously suggested by the study of Hering & Sheng (2003), the knockdown of cortactin in hippocampal neurons at this age results in a decrease in the density (70.29 ± 29.9 % of GFP) and size (62.16 ± 29 % of GFP) of PSD95 clusters (Fig. 22B).

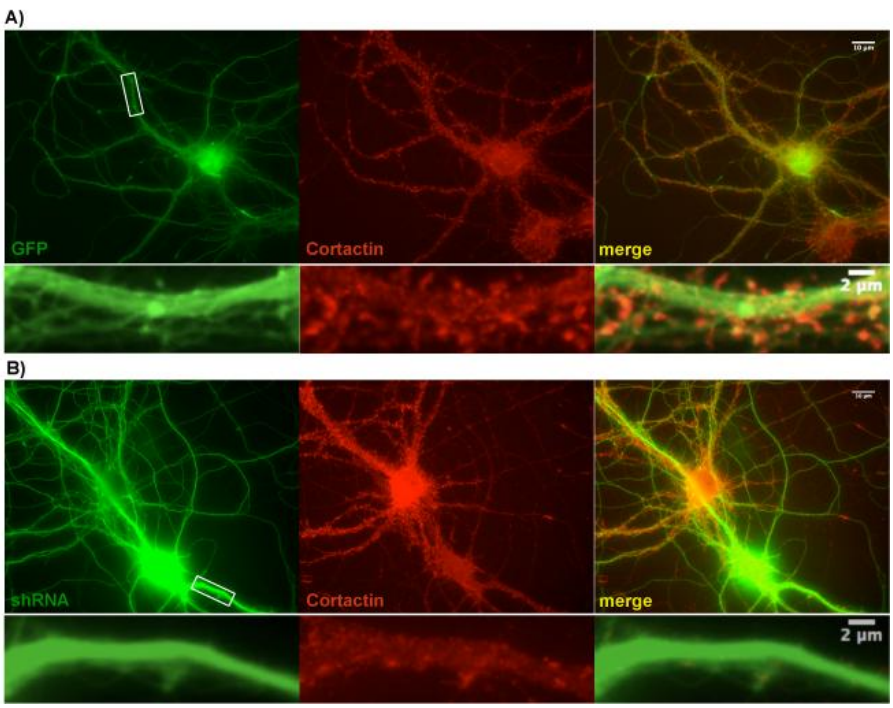


Fig. 21 – shRNA inhibition of cortactin expression. Hippocampal neurons at 15 DIV, transfected with pLL3.7EGFP (A) or pLL3.7EGFP-cortactin-shRNA (B) at 12 DIV, were stained with an antibody for cortactin. Hippocampal neurons transfected with cortactin-shRNA showed a strong decrease in the staining intensity for cortactin. Scalebar: 10 µm, insert:2 µm

In order to exclude the contribution of off-target effects of the cortactin-shRNA, a rescue construct was generated with silent mutations in the cortactin region targeted by the cortactin-shRNA. The mRNAs rescue experiments are usually used to demonstrate the specificity of a knockdown. In these experiments, an shRNA is co-administered with a construct that encodes the protein which the shRNA putatively knocked down. This rescue construct should not be targeted by the shRNA. In fact, in neurons co-transfected with the cortactin-shRNA plasmid and the cortactin construct refractory to cortactin-shRNA-mediated knockdown (Cortactin-wt*) the levels of PSD95 were recovered (Fig. 22B). In these neurons, the density, area and fluorescence intensity of PSD95 clusters were rescued to the levels observed in neurons transfected with the empty pLentiLox 3.7 vector (Fig. 22B). These results indicate that the defects observed for PSD95 clustering with cortactin-shRNA are specifically due to the loss of cortactin.

Knowing that cortactin knockdown causes loss of PSD95 clusters, and considering the effect of cortactin acetylation mutants on PSD95 clustering (Fig. 20), we tested the ability of the acetylation mutants of cortactin to rescue the decrease on PSD95 clustering observed upon loss of endogenous cortactin. For this purpose, we introduced silent mutations in the acetylation mutant constructs of cortactin to render them insensitive to the cortactin-shRNA, and co-expressed them with the cortactin-shRNA plasmid in cultured hippocampal neurons. Transfected neurons, identified by the GFP fluorescence, were evaluated for PSD95 clustering. Hippocampal neurons cultured at low-density were transfected at 12 DIV (and analyzed at 15 DIV) with the cortactin-shRNA construct together with the cortactin-shRNA-resistant mimetic mutants for acetylated (Cortactin-9KQ*) or deacetylated (Cortactin-9KR*) cortactin. After fixation, neurons were stained with an antibody against PSD95, an antibody against the FLAG epitope (to check for co-expression of the cortactin constructs) and also an antibody against MAP2 (Fig. 22A).

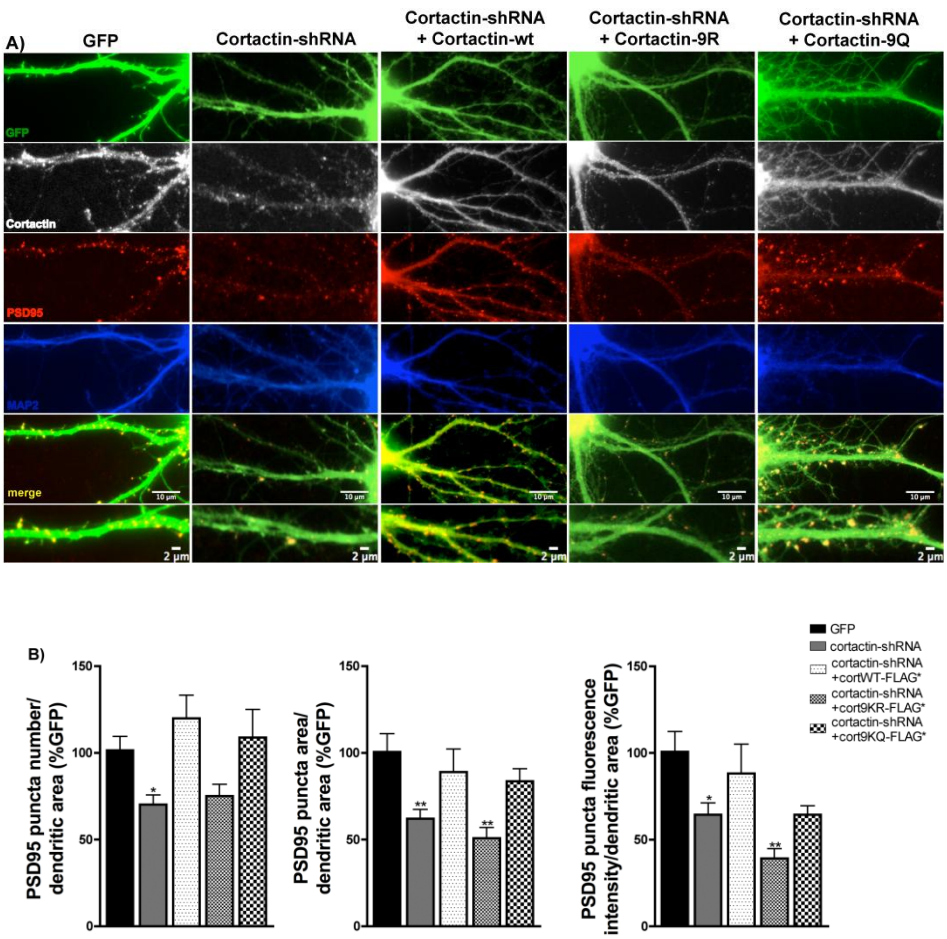


Fig. 22 – Effects of cortactin shRNA on the number, area and fluorescence intensity of PSD95 clusters, in hippocampal neurons. A) Hippocampal neurons were transfected at 12 DIV with pLentiLox3.7, with the cortactin-shRNA construct or co-transfected with the cortactin-shRNA construct along with constructs encoding shRNA resistant cortactinWT-FLAG*, cortactin9KR-FLAG* or cortactin9KQ-FLAG*. After fixation neurons were stained for cortactin or the FLAG epitope, MAP2 and for the postynaptic marker PSD95. Transfected neurons at 15 DIV, identified by GFP fluorescence, were analysed for PSD95 cluster number, area and fluorescence intensity. B) Results are presented as % of GFP-expressing control cells, and are averaged from two independent experiments (n≥20 cells). Errorbars, ± S.E.M. Significance, *p<0.05, **p<0,01, relative to GFP-transfected neurons (One-way ANOVA followed by Dunnett's Multiple Comparison Test). Scalebar: 10 µm, insert: 2 µm.

Expression in hippocampal neurons of the acetylated cortactin mimetic mutant rescued the cortactin-shRNA-mediated decrease on the area and density of PSD95 clusters (but not their fluorescence intensity) (Fig. 22B). On the other hand, expression of deacetylated mimetic form of cortactin could not rescue the cortactin-shRNA-mediated decrease on PSD95 clustering. These observations indicate that the acetylation of cortactin is important for its effect on PSD95 clustering.

Expression of wild type cortactin or of the acetylated cortactin mimetic mutant rescue the PSD95 clustering phenotype produced by cortactin-shRNA, whereas the deaceylated cortactin mimetic mutant is uncapable of such achievement. These evidences unravel an important function of cortactin acetylation in synapse maturation. As mentioned previously, dendrites of cortactin-deficient neurons not only showed a decrease in the density of protrusions and of PSD95 clusters, but also on the density of F-actin clusters (Hering & Sheng, 2003). Therefore, we sought to investigate if acetylation of cortactin could also promote changes in the density and size of F-actin clusters.

Hippocampal neurons cultured at low-density were transfected at 7 DIV with GFP together with cortactin constructs, including the mimetic forms for deacetylated and acetylated cortactin (9KR and 9KQ, respectively). After fixation at 15 DIV, neurons were stained with antibodies against the FLAG epitope and MAP2 and with Alexa 555 conjugated-phalloidin (Fig. 23A). Phalloidin binds to F-actin and is therefore a very useful tool for investigating the distribution of F-actin in cells. Surprisingly, analysis of F-actin clusters showed no significant difference in the number of clusters observed in the neurites of cells transfected with any of the cortactin constructs (Fig. 23B and C). The same experiment should be performed using an antibody against VGLUT1, to serve as a presynaptic marker. Colocalization of F-actin with VGLUT1 could be very useful to understand the effect of cortactin acetylation in synaptic F-actin clusters.

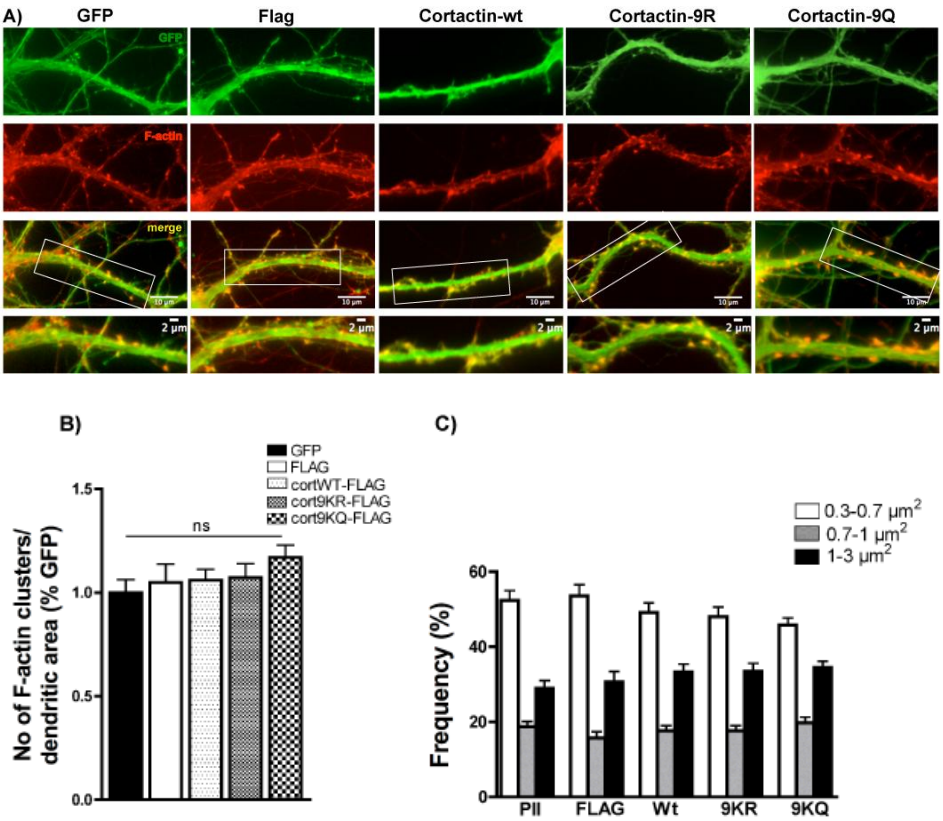


Fig. 23 – Acetylation of cortactin does not change the number or size of dendritic F-actin clusters in hippocampal neurons A) Images of 15 DIV hippocampal neurons expressing GFP alone, or GFP along with different cortactin constructs. Hippocampal neurons were transfected at 7 DIV with GFP alone, or GFP along with FLAG, cortactinWT-FLAG, cortactin9KR-FLAG or cortactin9KQ-FLAG. Dendritic spine morphology was observed with GFP fluorescence (green). Polymerized F-actin was detected in dendrites with rhodamine-coupled phalloidin (red). B) Transfected neurons, identified by GFP fluorescence, were analysed for F-actin cluster number. Data represent the average number of F-actin clusters per dendritic area. Results are presented as % of GFP-transfected control cells, and are averaged from three independent experiments (n≥32 cells). C) Analysis of size distribution for dendritic F-actin cluster. Errorbars, ± S.E.M. (One-way ANOVA). (scalebar: 10 µm, insert: 2µm).

We then tested whether cortactin acetylation has an impact on the synaptic localization of AMPA receptors. We resorted to quantitative immunofluorescence analysis of the expression of synaptic cell surface GluA1 AMPA receptor subunit in hippocampal neurons cultured at low-density and transfected at 7 DIV with GFP together with wild type cortactin, or the mimetic mutants for acetylated (9KQ) or deacetylated (9KR) cortactin. Neurons were live-stained at 15 DIV with an antibody against the N-terminal extracellular region of GluA1. After fixation, neurons were stained with an antibody against the presynaptic marker for excitatory synapses VGLUT1 (Fig. 24A).

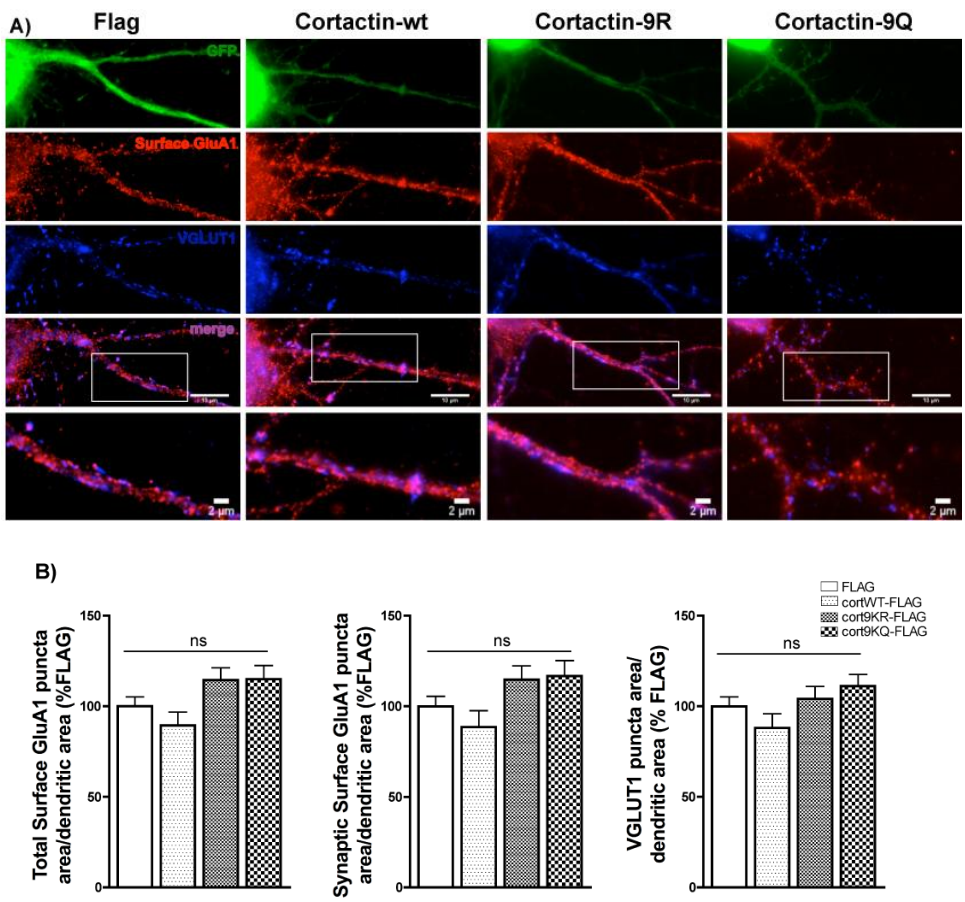


Fig. 24 - Acetylation of cortactin does not change the number, area or intensity of surface GluA1 clusters in hippocampal neurons. A) Hippocampal neurons were transfected at 7 DIV with GFP along with FLAG, cortactinWT-FLAG, cortactin9KR-FLAG or cortactin9KQ-FLAG. Neurons were live-stained for surface GluA1 and, after fixation, for MAP2 and for the presynaptic marker VGLUT1. Transfected neurons, identified by GFP fluorescence, were analysed for total and synaptic surface GluA1, as well as total VGLUT1 cluster fluorescence intensity, area and number, per dendritic area. Synaptic GluA1 is defined as GluA1 signal that overlaps with. B) Results are presented as % of control cells, transfected with the empty vector (FLAG), and are averaged from five independent experiments (n≥27 cells). Errorbars, ± S.E.M. (One-way ANOVA). (scalebar: 10 µm, insert: 2 µm).

Analysis of GluA1 surface expression showed no significant differences in the number, area or fluorescence intensity of total or synaptic (VGLUT1 co-localized) GluA1 clusters observed in the neurites of cells transfected with the cortactin constructs, compared to cells transfected with the empty vector (Flag) (Fig. 24B). VGLUT1 expression was also assessed, and no differences were observed concerning any of the analyzed parameters (Fig. 24B). These results suggest that, despite altering PSD95 clustering at synapses, acetylation of cortactin does not have an effect on surface GluA1 levels.

For many years, the phosphorylation of cortactin and the resulting functional consequences have been an intense area of study. Tyrosine (Y) phosphorylation of cortactin has been widely studied, and several residues have been identified as targets of phosphorylation. Phosphorylation of Y421, Y466 and Y482 has known biochemical and cellular consequences with regards to actin dynamics, possibly affecting the ability of cortactin to bind to and cross-link actin filaments (Ammer and Weed, 2008). Considering these evidences, we hypothesized that the acetylation status of cortactin could regulate cortactin Y421 phosphorylation, since acetylation also interferes with the ability of cortactin to interact with F-actin (Zhang et al., 2007) In order to test this hypothesis, HEK 293 FT cells were transfected with the different FLAG-tagged constructs of cortactin, wild type and acetylation mimetic forms, and the levels of cortactin phosphorylated at Y421 were assessed by western blot. Notably, cortactinY421 phosphorylation levels are much higher in the deacetylated

cortactin mimetic mutant when compared with the acetylated cortactin mimetic mutant (Fig. 25).

These results strongly suggest that cortactin acetylation regulates its phosphorylation at tyrosine residues, which has consequences with regard to actin dynamics (reviewed in Ammer and Weed, 2008). Tyrosine phosphorylation of cortactin has been shown to increase cell motility and invasion *in vivo*, but has been reported to have both positive and negative effects on actin polymerization *in vitro* (Martinez-Quiles et al., 2004; Tehrani et al., 2007). A recent study showed that tyrosine phosphorylation of cortactin promotes formation of lamellipodial protrusions and cell migration through an effect on cell adhesion (Kruchten et al., 2008), This study suggests that cortactin phosphorylated at tyrosine residues increases the turnover of focal adhesions by promoting focal adhesion disassembly. Interestingly, this study raises the hypothesis for a function of cortactin other than its role as an actin nucleation promoting factor.

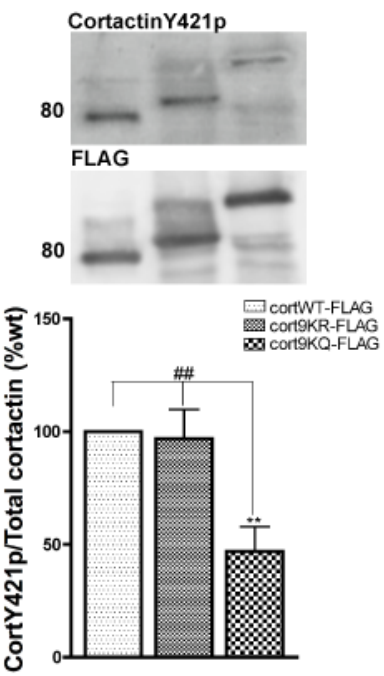


Fig. 25 - Cortactin acetylation regulates its phosphorylation at Tyrosine 421. Wild-type FLAG-tagged cortactin or the FLAG-tagged cortactin acetylation mutant constructs were transfected in HEK 293FT cells. Cortactin phosphorylation levels were assessed by Western blot using an antibody against phosphorylated cortactin at Tyrosine 421, and normalized to total cortactin levels using an anti-FLAG epitope antibody. Quantitative analysis was performed with ImageQuant. Results are presented as % of wt cortactin, and are averaged from seven independent experiments. Errorbars, ± S.E.M. Statistical significance was determined by One-Way ANOVA, followed by the Bonferroni's and Dunnett's Multiple Comparison Tests. ** indicates a significant difference from the control with $p<0.01$ (Dunnett's post-hoc test) and ## indicates a significant difference from control and cort9KR-FLAG with $p<0.01$ (Bonferroni's post-hoc test).

Deacetylated cortactin associates with Shank1 and p140Cap in an heterologous system

Given the evidences that cortactin phosphorylation can regulate its interactions with SH3 binding proteins, and our observation that cortactin acetylation regulates its phosphorylation state, we tested whether acetylation of cortactin interferes with its binding to synaptic proteins. In order to study the interaction between cortactin in its acetylated and deacetylated forms and known synaptic interactors we performed co-immunoprecipitation experiments in an heterologous system. It has been showed that cortactin interacts through its SH3 domain with the Shank family of proteins that are localized to the PSD of excitatory synapses (Naisbitt et al., 1999), and through this interaction cortactin may stabilize postsynaptic clusters of glutamate receptors during synaptogenesis. Another known cortactin interactor is p140Cap, a regulator of the Src tyrosine kinase (Di Stefano et al., 2007). p140Cap also interacts with (growing) microtubule plus-end, through EB3 tracking protein and was shown to be an abundant PSD protein (Jaworski et al., 2009). The current idea is that association between EB3-bound microtubule ends and p140 controls Src kinase activity and regulates cortactin function, which could lead to Arp2/3 complex activation and spine head growth (Hotulainen and Hoogenraad, 2010). Considering the two different types of cortactin interaction (to scaffold proteins and to cytoskeleton related proteins), we were interested in determining if

cortactin modification by acetylation could interfere with the binding to either Shank1 or p140Cap proteins. HEK 293 FT cells were co-transfected with the FLAG-tagged wild type or acetylation mimetic forms of cortactin, along with GFP-tagged Shank1 (a kind gift from Carlo Sala) or GFP-tagged p140Cap (a kind gift from Casper C. Hoogenraad). Immunoprecipitation of FLAG-tagged constructs using a specific antibody to the FLAG epitope resulted in the co-precipitation of both Shank1 and p140Cap, as assessed by western blot for GFP (Fig. 26). Notably, both p140Cap and Shank1 coimmunoprecipitated to higher levels with the deacetylated cortactin mimetic mutant than with the wild-type cortactin or with the mutant that mimics acetylated cortactin. These results confirm that cortactin interacts with both Shank 1 and p140Cap, and suggest that these interactions are modulated by the acetylation state of cortactin.

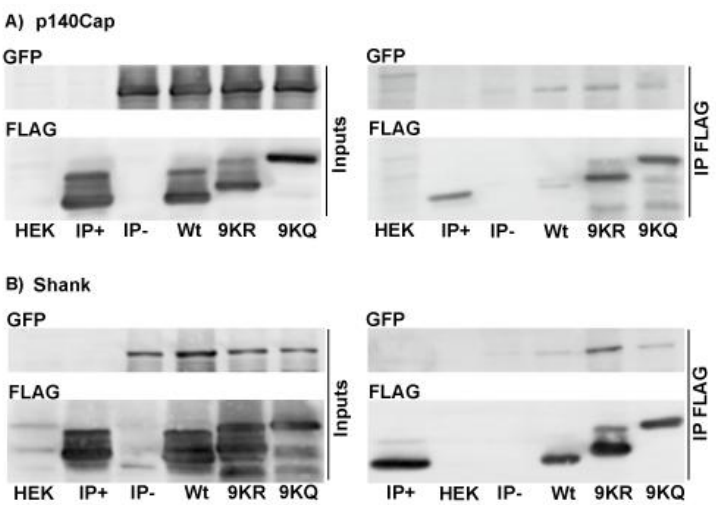


Fig. 26 - Deacetylated cortactin associates with Shank1 and p140Cap in HEK 293FT cells. Cortactin acetylation mutant constructs were co-transfected with p140Cap-GFP (A) or Shank-GFP (B) in HEK 293FT cells. Cortactin constructs were immunoprecipitated using a specific antibody against their tag (FLAG), as indicated. Co-immunoprecipitations were assessed by Western blot. (HEK: non transfected cells; IP+: cells transfected with cortactinwt-FLAG only; IP-: Cells transfected with p140Cap-GFP (A), or Shank1-GFP (B) only; Wt: cells transfected with GFP tagged construct plus cortactinWt-FLAG; 9KR: cells transfected with GFP tagged construct plus cortactin9KR-FLAG; 9KQ: cells transfected with GFP tagged construct plus cortactin9KQ-FLAG. The efficiency of immunoprecipitation was assessed by probing the immunoprecipitated samples for the FLAG epitope (lower panels), whereas the coimmunoprecipitation was assessed using an anti-GFP antibody (upper panels).

Effect of BDNF on cortactin acetylation

There are no described stimuli in neurons, or in other cell types, that regulate acetylation of cortactin. The neurotrophin BDNF plays a key role in the regulation of the structure and function of the glutamatergic synapses (Carvalho et al., 2008). BDNF is secreted at glutamatergic synapses in response to activity (Pang et al., 2004), increases synaptic AMPA and NMDA receptors (Caldeira et al., 2007), induces the transport of PSD95 to dendrites (Yoshii and Constantine-Paton, 2007), and increases PSD95 in dendritic spines (Hu et al., 2011). BDNF has also been shown to promote cortactin redistribution in neurons, an effect dependent on MAP kinase activation and cortactin phosphorylation (Iki et al., 2005). Therefore, we tested whether BDNF could also change the acetylation state of cortactin in hippocampal neurons.

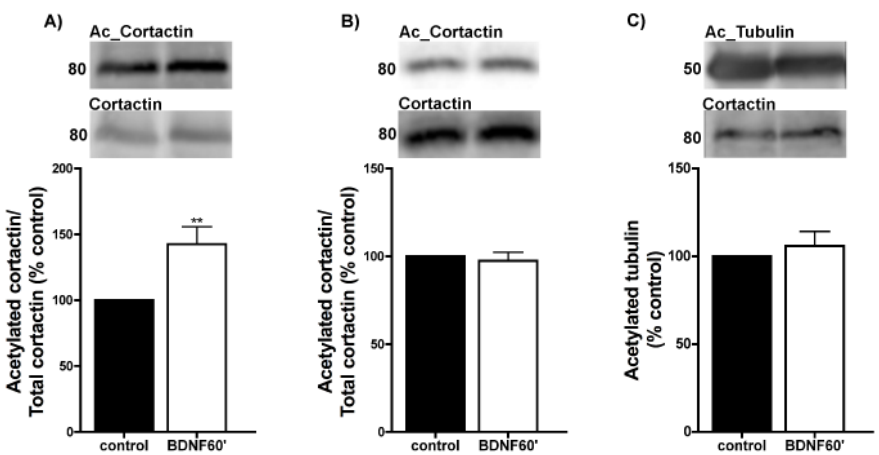


Fig. 27 – Acetylation of cortactin is increased in response to BDNF treatment whereas tubulin acetylation is not affected. 15 DIV neurons (A) and 7 DIV neurons (B) were incubated with 100 ng/ml BDNF for 60 min. Western blot was performed using an anti-acetylated cortactin antibody, and an anti-cortactin antibody. C) Hippocampal neurons (15 DIV) were treated with BDNF (100 ng/ml) for 60 min. Western blot was performed using an anti-acetylated tubulin antibody, and an anti-cortactin antibody. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of several experiments (n≥5) performed in independent preparations, and are expressed as a percentage of cortactin and tubulin acetylation in control conditions. Data were statistically analysed with Graphpad software, using paired student t-test. **p<0.01.

The acetylation of cortactin was assessed by western blot in control hippocampal neuron extracts as well as in extracts of hippocampal neurons treated with 100 ng/ml BDNF for 60 minutes. Neuron treatment with BDNF led to increased acetylation of cortactin in relatively mature hippocampal neurons (15 DIV) (Fig. 27A), whereas the same treatment did not significantly alter the acetylation level of cortactin in developing neurons (7 DIV) (Fig. 27B). To determine the specificity of the BDNF effect on cortactin, we assessed the acetylation levels of tubulin, another cytoskeleton protein targeted for acetylation. We observed that acetylation of tubulin is not increased in response to BDNF stimulation (Fig. 27C).

Iki and colleagues (2005) observed that cortactin localization within neurons is not only regulated by BDNF, but also by NMDA receptor activity. NMDA receptor activation induced cortactin redistribution from dendritic spines to the shaft, which was mediated by cortactin phosphorylation by Src-receptor tyrosine kinases in hippocampal cultures. Previous studies have also reported that NMDA receptor activation induces loss of cortactin from spines. Hering and Sheng (2003) showed that glutamate excitation induces the specific translocation of cortactin from postsynaptic sites in dendritic spines, and this glutamate-induced dispersal of cortactin is mediated by NMDA receptors. Therefore, we tested whether glutamate could also promote acetylation of cortactin in hippocampal neurons.

Western blot was used to assess the acetylation of cortactin in control hippocampal neuron extracts as well as in extracts of hippocampal neurons treated with 100 µM glutamate for 15 minutes. The levels of acetylated cortactin were increased in relatively mature hippocampal neurons (15 DIV) treated with glutamate (Fig. 28A), whereas the acetylation levels of tubulin were not altered in response to glutamate treatment (Fig. 28B), showing the specificity of the glutamate effect on cortactin.

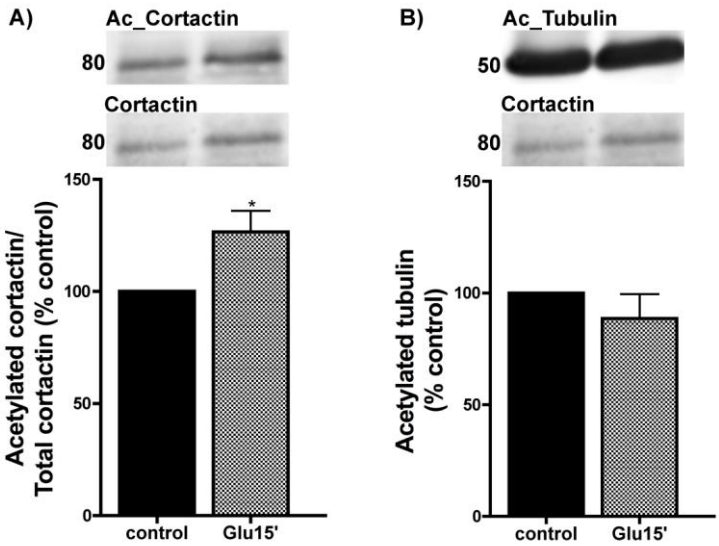


Fig. 28 – Acetylation of cortactin is increased in response to glutamate treatment. 15 DIV neurons were incubated with glutamate (100 µM) for 15 min. A) Western blot was performed using an anti-acetylated cortactin antibody, and an anti-cortactin antibody. B) Western blot was performed using an anti-acetylated tubulin antibody, and an anti-cortactin antibody. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of seven experiments performed in independent preparations, and are expressed as a percentage of cortactin (A) and tubulin (B) acetylation in control conditions. Data were statistically analysed with Graphpad software, using paired student t-test. *p<0.05.

BDNF has been shown to promote the translocation of cortactin to dendritic spines, through an effect dependent on ERK activity (Iki et al. 2005). In order to compare the effect of BDNF treatment on cortactin acetylation vs phosphorylation, we assessed the levels of phosphorylated cortactin at tyrosine 421, since it has already been shown that tyrosine 421 (along with tyrosines 466 and 481) is phosphorylated by Src family kinases (Huang et al., 1998). Hippocapampal neurons in culture at 15 DIV were treated with BDNF or H_2O_2. Treatment of neuronal cultures with H_2O_2 activates Src family tyrosine kinases directly (Martinez et al., 2003), inducing a dramatic increase in tyrosine phosphorylation of cortactin (Iki et al., 2005). So we used it as a positive control for Src induced tyrosine phosphorylation (Fig. 29).

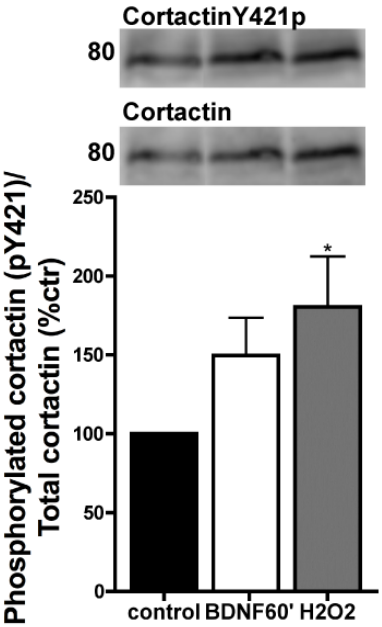


Fig. 29 – Phosphorylation of cortactin at tyrosine 421 is increased in response to BDNF treatment. 15 DIV neurons were incubated with 100 ng/ml BDNF for 60 min, or 100 µM H_2O_2 for 60 min. Western blot was performed using an antibody against phosphorylated cortactin at tyrosine 421, and an antibody against cortactin. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of thirteen experiments performed in independent preparations, and are expressed as a percentage of cortactinY421 phosphorylation in control conditions. Data were statistically analysed with Graphpad software, using One-Way ANOVA, followed by Dunnett's Multiple Comparison Test . *p<0.05.

Western blot analysis shows that in fact H_2O_2 significantly increases the levels of phosphorylated cortactinY421. BDNF treatment also leads to an increase in the Y421 phosphorylated levels, although this effect is not statistically significant, even though thirteen independent experiments were performed (Fig. 29). Considering these evidences, BDNF promotes both acetylation and phosphorylation of cortactin, but the effect is more pronounced when considering acetylation, potentially because cortactin acetylation negatively regulates its tyrosine phosphorylation.

Having observed that BDNF treatment leads to acetylation of cortactin, we then investigated how BDNF regulates cortactin acetylation. We focused on the various signalling pathways activated by activation of the TrkB receptors by BDNF (namely PLCγ, PI3K and MEK), and acetylation of cortactin was evaluated, in 15 DIV cultured hippocampal neurons, in the presence or absence of specific inhibitors of each pathway: U0126 for the MEK1/2 pathway, LY29400 for the PI_3K pathway, and U73122 for the PLCγ pathway (Fig. 30).

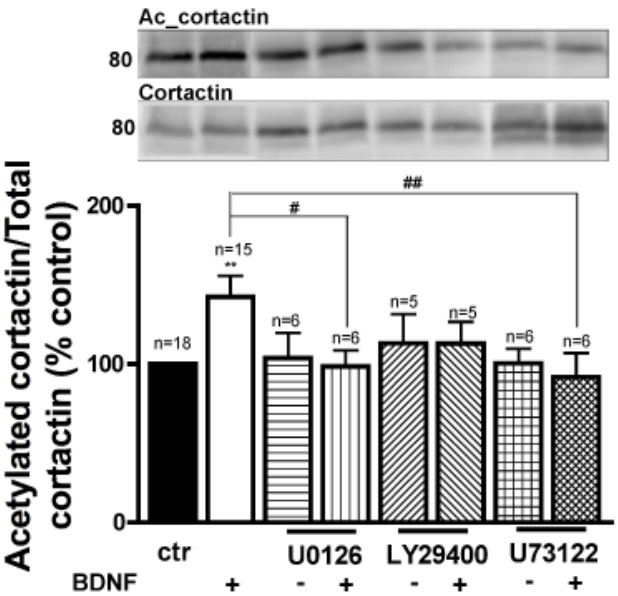


Fig. 30 – BDNF promotes the acetylation of cortactin and this effect is dependent on the MEK1/2 and PLCγ pathways. 15 DIV hippocampal neurons were incubated with or without 100 ng/ml BDNF for 60 min, in the presence or absence of specific inhibitors (200 nM) of BDNF activated intracellular signaling pathways. When present, the inhibitor was preincubated 30 min before stimulation with BDNF. Western blot was performed using an anti-acetylated cortactin antibody, and an anti-cortactin antibody. Quantitative analysis was performed with ImageQuant. Data are presented as average ± S.E.M. of several experiments performed in independent preparations, and are expressed as a percentage of cortactin acetylation in control conditions. Statistical significance was determined by One-Way ANOVA, followed by the Bonferroni's and Dunnett's Multiple Comparison Tests. ** indicates a significant difference from the control with $p<0.01$ (Dunnett's post-hoc test); # and ## indicate a significant difference from BDNF60' with $p<0.05$ and $p<0.01$, respectively (Bonferroni's post-hoc test).

We found that BDNF promotes the acetylation of cortactin through a complex effect, dependent on the MEK1/2 and PLCγ pathways (Fig. 30). The LY29400 compound used to inhibit the PI_3K pathway by itself increased the acetylation level of cortactin; therefore, our results concerning the involvement of this pathway in the observed effect are inconclusive. These evidences suggest that BDNF possibly regulates the synaptic structure at least partially by changing the acetylation level of cortactin.

Discussion

Several conclusions about the relationship between cortactin acetylation and synaptic clustering of PSD95 can be drawn from the results presented in this chapter:

1) Cortactin is acetylated in neurons, and acetylated cortactin is redistributed from dendritic spines to dendrites and the cell body. BDNF promotes cortactin acetylation in hippocampal neurons 15 DIV.

2) Cortactin acetylation regulates PSD95 clustering, but not the size and density of F-actin clusters in cultured hippocampal neurons.

3) Acetylated cortactin can rescue the decrease on PSD95 clustering mediated by loss of cortactin, which is not rescued by the deacetylated form of cortactin.

4) Acetylation of cortactin is correlated with lower phosphorylation at tyrosine 421 and with decreased interaction with p140Cap and Shank1 interaction partners.

We confirmed that cortactin is in fact a substrate for acetylation/deacetylation in neurons. Using the specific HDAC inhibitor TSA, we determined that the levels of actetylated cortactin in relatively mature hippocampal neurons were increased. Zhang et al. (2007) showed that inhibition of HDAC6 activity led to cortactin hyperacetylation, and consequently changed the F-actin binding activity of cortactin, influencing actin-dependent cell motility. These authors also identified SIRT1 (a class III histone deacetylase) as a cortactin deacetylase and p300 as a cortactin acetyl-transferase, in ovarian cancer cells (Zhang et al., 2008).

Taken together our results indicate that cortactin acetylation has an impact on regulating PSD95 clustering. The effect of cortactin acetylation on PSD95 may be independent from the function of cortactin as a regulator of actin dynamics, since the size and number of F-actin clusters was not changed by overexpression of the cortactin acetylation mutants that changed the

fluorescence intensity and area of PSD95 clusters. A previous study showed that the synaptic clustering of PSD-95 is unaffected by actin depolymerization, suggesting that PSD-95 is a core scaffolding component and that its localization at synapses is independent of the actin cytoskeleton (Allison et al., 2000). This study suggests that F-actin is necessary for the initial formation or transport of the synaptic structure, but not for the maintenance of PSD95 at the postsynaptic density. Once a synapse has formed, actin dynamics is involved in mediating activity-dependent changes in spine morphology, rather than in the localization of core synaptic proteins. In agreement with this model, our data suggest that cortactin acetylation may affect PSD95 synaptic clustering independently of its effects on F-actin polymerization.

When the spine head is forming, dynamic actin assembly is required. A function for cortactin at this stage is supported by the data showing that knockdown of cortactin dramatically decreases spine density in cultured neurons (Hering & Sheng 2003). We propose that deacetylated cortactin binds to F-actin and interacts with the Arp2/3 complex, and the large Arp2/3-nucleated branched actin filament network leads to enlargement of spine head. During spine maturation, in order for the assembly of postsynaptic components to occur, spine motility must gradually decrease so that the spine structure can stabilize. Cortactin acetylation may play a role at this stage, since in its acetylated form cortactin is not able to bind to F-actin, and will therefore not act as a bridge to couple Arp2/3-dependent actin polymerization to an existing actin filament. Therefore, the continuous dynamic branching of F-actin may be slowed down by cortactin acetylation, leading to a stabilized actin structure, and a stabilized mature spine head (Fig. 31). Indeed, overexpression of wild-type cortactin in hippocampal neurons promoted longitudinal spine growth and resulted in longer immature spines, as a result of enhanced actin polymerization (Hering & Sheng, 2003). In parallel, it has been shown that while the activity of the actin-severing protein cofilin is important in mediating dendritic spine remodelling, its suppression by phosphorylation plays an important role in the stabilization of mature dendritic spines (Shi et al., 2009). These evidences

support the idea that F-actin maintenance in mature spines may depend on mechanisms that slow down F-actin turnover.

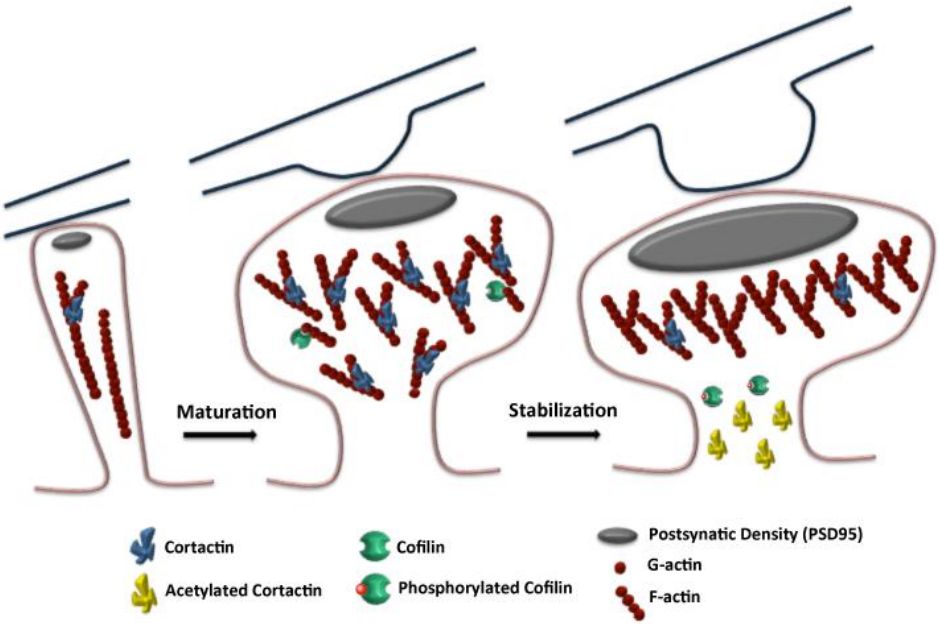


Fig. 31 – Cortactin de/acetylation regulatory mechanisms during spine development and plasticity. Spine development starts with the initiation of the dendritic filopodium and its elongation. During synaptogenesis, dendrites are bristled with many diffuse-type filopodia where actin filaments are elongated from the tip of filopodia by different actin binding proteins. In addition to tip polymerization, actin filaments in dendritic filopodia elongate from the base. Extensive actin branching occurs at the fillopodium tip and the spine head begins to form. The mechanism of actin assembly is now increased and the large Arp2/3-nucleated branched actin filament network leads to enlargement of the spine head. We propose that deacetylated cortactin induces actin branching. ADF/cofilins depolymerize pointed ends of actin filaments to replenish the actin monomer pool, and create free barbed ends that can nucleate filament growth, controlling the proper length of actin filaments. Mature spines spines are still dynamic but mantain their overall morpholy, through the stabilization of the actin filament network. Suppression of cofilin activity by phosphorylation is known to be involved in the stabilization of mature dendritic spines. We propose that acetylation of cortactin occurs in order to aid in the stabilization of the F-actin structure in the spine head. During LTP, the activity of all actin-binding proteins, including cortactin, is modulated in order to promote the dynamic changes in the spine head morphology.

In the present study we investigated whether expression of wild-type cortactin or the acetylation mutants of cortactin influenced the density or area of F-actin

clusters in hippocampal neurons. We could not detect changes in the total number of F-actin clusters per dendritic length, or in the size of F-actin clusters (Fig. 23 A and B) induced by overexpression of these proteins in neurons expressing endogenous cortactin, but our data do not exclude that cortactin acetylation may have an impact on the spine morphology, namely on the width or length of spines, or on spine motility.

The localization of acetylated cortactin in neurons

Immunocytochemical studies in mature cultures of rat hippocampal neurons confirmed that cortactin is concentrated in dendritic spines, whereas acetylated cortactin was found to be distributed throughout dendrites, and cell bodies (Fig. 18). These observations for cortactin localization are in agreement with previous immunocytochemical studies which showed that cortactin is enriched in spines in hippocampal neurons in culture (Hering and Sheng, 2003). Racz & Weinberg (2004) obtained a similar result through immunogold electron microscopy, proving that cortactin concentrates within the spine core. Our analysis of biochemically purified synaptoneurosomes from adult rat brain showed that cortactin is present in the synaptoneurosomal fractions. On the other hand, acetylated cortactin was decreased in the synaptoneurosomal fraction, compared to the total homogenate from adult rat brain (Fig. 19). Accordingly, the fraction of cortactin that is acetylated is lower in synaptosomes or in the isolated PSDs than in the total extract from rat hippocampus (Fig. 19). In agreement with our data, it has been reported that acetylation of cortactin inhibits its Rac-mediated translocation to the cell periphery and its presence in membrane ruffles in transfected NIH 373 cells (Zhang et al., 2007).

Ultrastructural studies suggested the presence of two distinct pools of cortactin within the spine (Racz and Weinberg, 2004). These authors revealed the existence of a small synaptic pool and a larger core pool (only indirectly coupled to synaptic activity) of cortactin, and proposed that these two pools may have distinct functions. The core pool is situated to mediate changes in

spine shape, whereas the synaptic pool might help to modify the composition or shape of the PSD in response to specific patterns of activity (Desmond and Levy, 1986; Geinisman et al., 2000; Harris et al., 2003; Malinow and Malenka, 2002; Marrone and Petit, 2002). Acetylation of cortactin in neurons may be exerting different effects depending on which "pool" cortactin is being targeted to acetylation/deacetylation.

We found that cortactin acetylation is increased by neuronal treatment with BDNF at 15 DIV, whereas no effect was of BDNF was detected in younger (7DIV) neurons (Fig. 27). BDNF has been previously found to trigger ERK-mediated serine phorphorylation of cortactin in 12-16 DIV neurons, concomitant with cortactin translocation from the dendritic shaft to spines. This effect of BDNF on cortactin redistribution was not observed in more mature hippocampal neurons (20 DIV), indicating that BDNF may have different effects depending on the maturation stage of the culture (Iki et al 2005). In our culture conditions, where neurons are cultured on inverted coverslips on top of a glia cell feeder layer, at 15 DIV BDNF promotes the acetylation of cortactin, which we find to lead to the relocalization of cortactin away from synapses.

Our findings are in accordance with the well known increase in PSD95 within spines after BDNF treatment (Yoshii and Constantine-Paton, 2007). These authors showed that when BDNF is applied to cultured visual cortical neurons, the size of PSD95 puncta in spines and the overall amount of PSD95 in dendrites are increased within 60 min. We saw that a 60 min BDNF treatment promotes cortactin acetylation, and we also observed that inhibition of HDAC6 activity, by TSA, leads to clustering of PSD95 in dendritic spines (Chapter 3, Fig. 1). It is therefore possible to speculate that cortactin acetylation plays a role in the BDNF-triggered accumulation of PSD95 in dendritic spines. Furthermore, Yoshii and Constantine-Paton sowed that PI3K, Akt and an intact Golgi apparatus are essential for TrkB-dependent increases in PSD95 at synapses. We found that the BDNF effect on cortactin acetylation may also require activation of the MEK1/2 or PLCγ pathways. BDNF may induce PSD95 accumulation through different post-translational modifications of cortactin,

depending on which BDNF signaling pathway is activated, and also on the targeted pool (synaptic or core) of cortactin.

In addition, we found that cortactin acetylation is increased by neuronal treatment with glutamate at 15 DIV (Fig. 28). NMDA receptor stimulation was described to induce cortactin redistribution from the spine to the shaft (Hering & Sheng 2003) and this effect was attributed to Src-mediated phosphorylation of cortactin at tyrosine residues, since it was blocked by a Src inhibitor (Iki et al., 2005). Alongside, glutamate-induced cortactin acetylation may be involved in cortactin translocation from spines to the shafts of dendrites. Our studies identify cortactin acetylation as another cortactin modification that can impact on its cellular localization in neurons. Moreover, our data show that cortactin acetylation is negatively correlated to its tyrosine phosphorylation, suggesting that the acetylation-mediated redistribution of cortactin is not secondary to the tyrosine phosphorylation-mediated relocalization of cortaction to dendritic shafts.

Cortactin acetylation vs. tyrosine phosphorylation

Protein post-translational modifications tend to work in concert at several levels on a given protein, and it would be important to test whether acetylation affects phosphorylation or vice-versa. Interestingly, we found that the cortactin mutant that mimics deacetylation is more phosphorylated at Y421 than the acetylated cortactin mutant (Fig. 25). This suggests that acetylation of cortactin at the tandem repeats region may affect its phosphorylation at tyrosine residues downstream the tandem repeats domain of the protein. The role of tyrosine phosphorylation in the regulation of cortactin function, localization and interaction with other proteins is far from being fully understood, and contradictory evidences have been reported [reviewed in (Ammer and Weed, 2008)]. An early study showed that the N-terminal domain of cortactin is essential for Src-targeted phosphorylation at the C terminus (Head et al., 2003). Considering that acetylation primarily occurrs within the N-terminal domain of

cortactin, it is possible to speculate that acetylation in the N-terminal domain may influence Src-induced phosphorylation at the C terminus, including phosphorylation at Y421, or vice versa (maybe due to conformational changes). It has been shown that tyrosine phosphorylation of cortactin by Src, which occurs at tyrosine residues 421, 466 and 482 (Head et al., 2003) in a progressive manner, attenuates its ability to cross-link F-actin *in vitro* (Huang et al., 1997a) and also inhibits its activation of N-WASp (neuronal Wiskott–Aldrich Syndrome protein), consequently inhibiting actin branching through Arp2/3 (Martinez-Quiles et al., 2004). Our finding that the deacetylated cortactin form, which binds F-actin, is more phosphorylated at Y421 suggests that phosphorylation of this form of cortactin can regulate its ability to bind to F-actin. Additional work now points to a positive effect for Src-mediated cortactin phosphorylation on Arp2/3 mediated actin polymerization through Nck (non-catalytic region of tyrosine kinase adaptor protein) and N-WASp (Tehrani et al., 2007). These authors showed that Nck1 binds to Src-phosphorylated cortactin through its SH2 domain, with the Nck1/phosphocortactin complex in turn interacting with either WIP (WASp interacting protein) or N-WASp through its SH3 domain, and this trimeric complex enhances Arp2/3 nucleation activity. The binding of Nck1 to phosphorylated cortactin therefore provides an indirect link to Arp2/3 complex regulation, as well as involving cortactin, possibly cortactin acetylation, in an additional layer of complexity in regulating actin nucleation (Ammer and Weed, 2008).

Cortactin acetylation and cortactin interaction partners

Biochemical evidences demonstrated that the deacetylation mimetic mutant of cortactin not only interacts with Shank1 but also with p140Cap (Fig. 26). Confirmation that the interaction of cortactin with both Shank1 and p140Cap occurs was obtained in immunoprecipitation assays performed in HEK 293FT cells. These results are also in accordance with the previous described theory of the cortactin “pools” in the spine (Racz and Weinberg, 2004). The interaction between deacetylated cortactin (able to bind to F-actin) and p140Cap may

occur in the mentioned “core pool”, situated to mediate changes in spine shape. The localization of this “pool” of cortactin is compatible with the suggested link between actin cytoskeleton and dynamic microtubules, since these authors showed that dynamic MTs in fact enter dendritic spine (Jaworski et al., 2009). By transiently targeting dendritic spines, the function microtubules serve, along with actin cytoskeleton, is likely to involve transport of essential proteins into and out of the spine (reviewed in Dent et al., 2011). This could explain the results obtained in the previous chapter, concerning the clustering of some scaffold proteins; when acetylated, cortactin may not be able to interact with p140Cap, thus disrupting the interaction between actin and microtubules, which in turn could result in the accumulation of scaffold proteins in spines. On the other hand, Shank1 can bind to deacetylated cortactin present in the “synaptic pool”, and may modify the composition or shape the PSD in response to synaptic activity. Sheng et al. (2001) showed that overexpression of Shank promotes maturation of dendritic spines and the enlargement of spine heads via its ability to recruit Homer to postsynaptic sites. Since Shank may play a role at the interface between the PSD and the postsynaptic cytoplasm and cytoskeleton (Sala et al., 2001), one could say that the enlargement of spine head effect of Shank overexpression could be facilitated by its interaction with de/acetylated cortactin.

Recent work showed that the increase in PSD95 within spines after BDNF treatment is dependent on MT invasions of dendritic spines (Hu et al., 2011). We found that p140Cap, which interacts with MT through EB3 protein, binds to the deacetylated mutant to a large extent. Additionally, we also determined that the deacetylation mimetic form of cortactin is more phosphorylated at Y421. Possibly, when acetylation occurs in the actin-binding region (ABR) of cortactin, it induces conformational changes that impair tyrosine or serine/threonine phosphorylation, in the PRD (proline rich domain), inhibiting some of the SH3 interactions. On the other hand, the deacetylation mimetic mutant, since it cannot be acetylated, may be a perfect target for tyrosine phosphorylation at Y421 due to its conformational sate. In fact, some work has been done

concerning the conformational changes of cortactin and its regulation by serine/threonine and tyrosine phosphorylation (Campbell et al., 1999; Lua and Low, 2005; Martinez-Quiles et al., 2004; van Damme et al., 1997). It has been proposed that non-phosphorylated cortactin exists in the 80 kDa “closed” form with the carboxyl-terminal SH3 domain binding back upon the proline-rich cortactin domain, blocking the ability of the SH3 domain binding interface to interact with other ligands. ERK1/2 phosphorylation in turn results in displacing the SH3-proline-rich homotypic cortactin interaction, rendering cortactin in an “open” 85kDa state where the SH3 domain can bind N-WASp and other cortactin SH3 binding proteins (reviewed in Evans et al., 2011).

Undisputedly, the reversible acetylation status of cortactin provides a unique mechanism that regulates its F-actin binding activity. We have now revealed an important role for cortactin acetylation in regulating synapse maturation. What may be necessary for proper cortactin function is not so much a net change in serine or tyrosine phosphorylation or de/acetylation in the ABR of cortactin, but a continuous cycle of these processes allowing the dynamic regulation and recycling of the protein.

Chapter 5

General Conclusions

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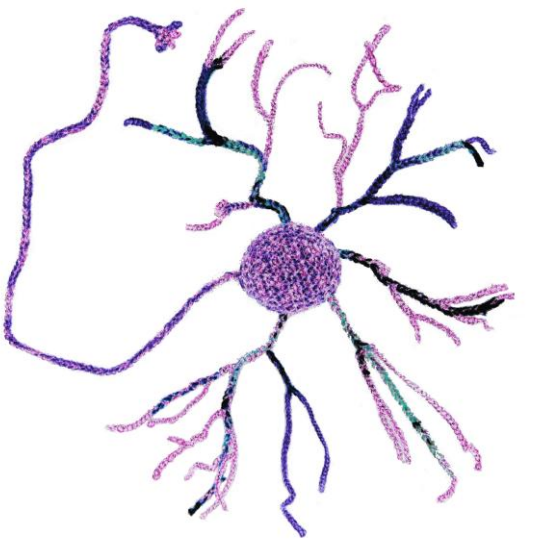
Future Directions



How global changes in protein acetylation change synapse composition

This work sought to describe the effects of protein acetylation, enhanced by HDACs inhibitors, on synapses, using immunocytochemical methods to characterize the localization of several synaptic proteins in rat hippocampal neurons. Treatment of hippocampal neurons with the types I and II histone deacetylase inhibitor Thricostatin A (TSA) increases density, area and intensity of the dendritic clusters for excitatory synapse scaffold proteins such as PSD95 and Shank1, whereas the postsynaptic inhibitory synapse protein gephyrin was unaffected. Cytoskeleton associated proteins such as cortactin and p140Cap show decreased cluster areas, after the same treatment. Given the regulatory role for protein acetylation at the level of the cytoskeleton, it would be important to explore the effect of TSA treatment on synaptic F-actin, in order to conclude if the observed clustering effects are accompanied by changes in actin dynamics within dendritic spines. For this purpose, we plan to detect polymerized F-actin with Alexa 555-coupled phalloidin, and co-localize it with a presynaptic marker; this will enable us to investigate the effects of protein acetylation on the synaptic cytoskeleton. Ideally, the study should also focus on the effect of protein acetylation on the morphology of dendritic spines. Quantitative analysis of spine morphologies (morphometric analysis) are usually performed using hippocampal neurons whose basal dendritic tree is completely filled, e.g. GFP filled dendrites. The area, major and minor axis of the head of spines and the length of the necks (usually measured from the point of attachment of the dendrite to the beginning of the spine head) in each portion of the analyzed dendrites are determined with the aid of suitable imaging software. This technical approach would provide the first evidence for whether protein acetylation regulates actin reorganization, accompanied, or not, by marked changes in spine morphology, in dendritic spines.

Cortactin acetylation & PSD95 clustering

After the validation of cortactin as a target for TSA induced acetylation in neurons, our efforts concentrated in exploring the physiological role for acetylation of cortactin in the clustering of PSD95, particularly in the synaptic region. Analysis of PSD95 expression in mature hippocampal neurons showed a significant decrease in the average area and fluorescence intensity of the PSD95 clusters observed in the neurites of cells transfected with the deacetylation mimetic mutant of cortactin. On the other hand, the cortactin mutant that mimics acetylated cortactin rescued the cortactin knockdown-induced decrease on PSD95 clustering, similarly to wild-type cortactin, whereas the deacetylation mimetic mutant did not. These results suggest that cortactin acetylation promotes PSD95 clustering at synapses. In hippocampal neurons in culture, de/acetylation of cortactin did not have an effect on surface GluA1 or F-actin levels. We propose that when the functional deacetylation mimetic mutant of cortactin is overexpressed, and since it is able to bind to and cross-link F-actin, extensive actin branching keeps occurring at the spine head, preventing the gradual decrease of spine motility necessary to stabilize the spine structure. This could explain the differences observed in the area and fluorescence intensity of PSD95 clusters. On the other hand, overexpression of the acetylation mimetic mutant of cortactin has the opposite effect. This mutant is not able to bind to F-actin, which can prevent a continuous branching led by the interaction between cortactin, Arp2/3 complex and F-actin, leading to a stabilized actin structure in spines. Since cortactin binds to Shank, and indirectly Shank interacts with PSD95 (Shank-PSD95-GKAP complex), the acetylation of cortactin, its removal from spines and a decrease on its interaction with Shank may be leading to a clustering of PSD95 in spines. In order to determine whether cortactin acetylation and its contribution to PSD95 clustering are implicated in the TSA-induced clustering of PSD95 observed by us, hippocampal neurons, transfected with the cortactin acetylation mutants, will be submitted to TSA treatment. If the effect of TSA on PSD95 clustering is partially mediated by an increase on cortactin acetylation, we expect the

expression of the acetylated cortactin mimetic mutant to occlude at least partially the TSA effect. These data would reveal whether cortactin acetylation is a central player in the TSA effect on PSD95 clustering, or whether other crucial mechanisms are involved.

Cortactin acetylation & actin dynamics

To a certain extent, our data suggest that the effect of cortactin acetylation on PSD95 may be independent from the function of cortactin as a regulator of actin dynamics, since the size and number of F-actin clusters were not changed by overexpression of the cortactin acetylation mutants. However, we aim to further investigate a possible role for cortactin acetylation in regulating actin dynamics. For this purpose, three different experiments are proposed. On one hand, changes in the synaptic clusters of F-actin should be investigated, by detecting colocalization of polymerized F-actin with a presynaptic marker, in hippocampal neurons transfected with cortactin acetylation mutants. In the present study, the effect of the overexpression of the cortactin acetylation mutants was tested on the total F-actin clusters, and an effect on synaptic clusters may be diluted in the total population of F-actin aggregates. On the other hand, it would be important to test whether the morphology of spines, largely dictated by the actin cytoskeleton, is changed in neurons expressing the acetylated/deacetylated mimetic mutants of cortactin. Finally, actin tagged with a fluorescent protein could be used to look at the motility of spines. Transfection of neurons with actin fused to mEos2, which fluoresces green until exposed to UV light that irreversible converts its emission to red (Frost et al., 2010b) would help to track polymerized actin molecules by eye over consecutive frames, allowing measurement of orientation and dynamics of filaments within the small confines of dendritic spines.

The cellular localization of acetylated cortactin

Immunocytochemical studies in cultures of rat hippocampal neurons indicate that both cortactin and acetylated cortactin distribute to dendrites.

Cortactin is co-localized with a synaptic marker, whereas acetylated cortactin is less abundant in dendritic spines. Additionally to the immunolabeling methods, these observations were consolidated by rat brain/hippocampi subcellular fractionation techniques. Based on these results, and considering the effect observed in the area of cortactin clusters after TSA treatment, we propose that cortactin acetylation triggers its redistribution from spines to the shafts of dendrites. Moreover, we found that cortactin acetylation is increased by neuronal treatment with BDNF and glutamate, suggesting that synaptic activity may regulate cortactin localization within neurons, through cortactin acetylation, in a similar way to what happens with cortactin phosphorylation regulation and localization through synaptic activity. In fact, activation of Src kinases induces cortactin phosphorylation and cortactin depletion from the postsynaptic sites, whereas MAP kinase activation promotes redistribution from shafts to the spines (Iki et al., 2005). We propose that cortactin localization within neurons may be regulated by synaptic activity, not only through phosphorylation, but also acetylation. This control possibly has a critical role in spine development and synaptogenesis, as well as synaptic plasticity.

BDNF, cortactin acetylation and PSD95 clustering

Another speculation arises from our findings, concerning the importance of cortactin acetylation in the accumulation of PSD95 in dendritic spines. Since we found that BDNF treatment promotes cortactin acetylation, and inhibition of HDACs activity, by TSA, leads to clustering of PSD95 in dendritic spine, we propose that cortactin acetylation is part of the mechanism through which BDNF induces PSD95 accumulation in spines. To further test this possibility, we intend to use fluorescence microscopy to monitor PSD95 levels and clustering in dendritic spines of hippocampal neurons transfected with cortactin acetylation mutants and stimulated with BDNF. According to the results obtained and described in this work, we expect that transfection of hippocampal neurons with the deacetylated cortactin mutant, submitted to a 60’ BDNF treatment, will prevent the characteristic PSD95 clustering on dendritic spines triggered by

BDNF (Yoshii and Constantine-Paton, 2007), whereas we expect neuron transfection with the acetylated cortactin mutant to partially occlude the effect of BDNF on PSD95 application, since the expression of the mutant itself promotes PSD95 clustering, as we have observed.

It was proposed that the mobility of PSD95 occurs through diffusion and is primarily regulated by PSD95 interactions with proteins in the spine head, such as CaMKII and Shank (Gray et al., 2006). Recently, a novel mechanism whereby MT invasions regulate PSD95 in the spine head has been proposed, and BDNF treatment has been shown to promote a robust increase of PSD95 in spines invaded by MTs (Hu et al., 2011). Since this accumulation effect does not seem to depend on the direct interaction between polymerizing MT tips and PSD95, another intriguing possibility emerges. Possibly, F-actin cytoskeleton, along with MTs, may be delivering unknown cargo that causes PSD95 to be captured or retained in the spine head. To study this hypothesis, we plan to perform fluorescence microscopy experiments using hippocampal neurons treated with BDNF and labelled with Alexa55-coulped phaloidin, focusing on the quantification of F-actin staining colocalized with VGLUT1 (presynaptic marker), to identify F-actin clusters localized in active synapses. Analysis of F-actin organization after BDNF treatment may be useful to better understand the possible role of actin assembly/disassembly, and consequently cortactin post-translational modifications, in the characteristic PSD95 clustering in dendritic spines.

Cortactin acetylation, phosphorylation & binding partners

Our biochemical evidences suggest that the interaction between cortactin and two known interactors, Shank1 and p140Cap, is mediated by the acetylation status of cortactin. Additionally, we found that acetylated cortactin presents lower levels of tyrosine phosphorylation (Y421). This suggests that acetylation of cortactin at the tandem repeats region may affect its phosphorylation at tyrosine residues downstream the tandem repeats domain of the protein, consequently interfering with SH3-dependent interactions of

cortactin. Apparently, both post-translational modifications of cortactin, which work in concert at several levels, may affect each other concerning the regulation of cortactin function, localization and interaction with other proteins.

How cortactin impacts actin dynamics during vertebrate development still remains unknown, in part due to the lack of transgenic knock-out rodent models. Nevertheless, unveiling the role of additional post-translational modifications, such as phosphorylation and acetylation, in the regulation of cortactin's activity, may help to better understand the impact of cortactin in the regulation and formation of actin-rich motility structures, as well as the interplay between cortactin and different actin-regulating factors under an array of physiological and pathological conditions. In fact, cortactin has been described to have precise roles in tumor progression and other disease types, and these roles are just beginning to be identified. Since cortactin is widely involved in the regulation of spine dynamics including variations in the shape and number of spines, a challenge for future research will be to understand the implication of cortactin post-translational modifications in processes of learning and memory and neurodegenerative diseases, as well as some forms of mental retardation and autistic spectrum disorders, which are largely associated with dendritic spine dynamics abnormalities. Indeed, evidence is accumulating that cortactin impacts on complex functions such as learning and sleep (Davis et al., 2006; Meighan et al., 2006), clearly demonstrating an important neuronal role for cortactin that is only starting to be identified.

Chapter 6

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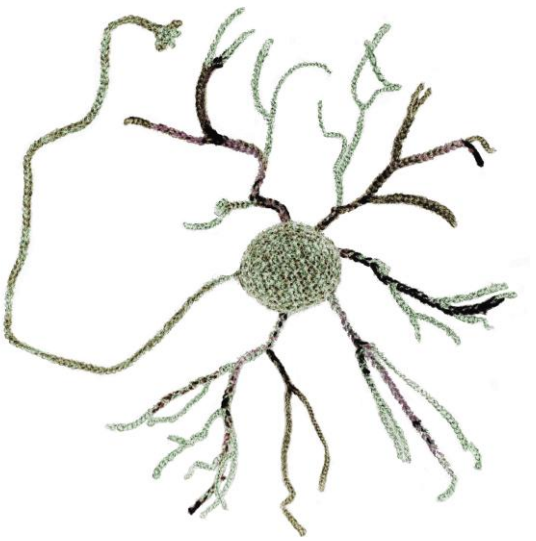

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