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Studies on Hemodynamics and Coagulation in Neuroanesthesia
NOVEL MOLECULAR MECHANISMS OF DENDRITIC SPINE DEVELOPMENT

Olaya Llano

Dissertationes Scholae Doctoralis ad Sanitatem Investigandam Universitatis Helsinkiensis

Neuroscience Center

and

Division of Biochemistry and Biotechnology

Department of Biosciences

Faculty of Biological and Environmental Sciences

and

Integrative Life Sciences Doctoral Program

Academic Dissertation

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences, University of Helsinki.

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University of Helsinki, Finland

To the memory of my brother, Demian.
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>βPix</td>
<td>p21-activated kinase interacting exchange factor</td>
</tr>
<tr>
<td>ABP</td>
<td>actin-binding protein</td>
</tr>
<tr>
<td>ADF</td>
<td>actin-depolymerizing factor</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMPA(R)</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor)</td>
</tr>
<tr>
<td>Arf</td>
<td>ADP ribosylation factors</td>
</tr>
<tr>
<td>Arp</td>
<td>actin-related protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AVC</td>
<td>amorphous vesicular clumps</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>CaMK</td>
<td>calmodulin-dependent kinase</td>
</tr>
<tr>
<td>CCC</td>
<td>cation-chloride cotransporter</td>
</tr>
<tr>
<td>CKB</td>
<td>brain-type creatine kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTD</td>
<td>carboxyl-terminus domain (C-terminal)</td>
</tr>
<tr>
<td>DH</td>
<td>Db1 homology</td>
</tr>
<tr>
<td>DIV</td>
<td>days in vitro</td>
</tr>
<tr>
<td>DN</td>
<td>dominant negative</td>
</tr>
<tr>
<td>EGF</td>
<td>epithelial growth factor</td>
</tr>
<tr>
<td>Egr</td>
<td>early growth response</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ERM</td>
<td>ezrin, radixin, moesin</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FBE</td>
<td>free-barbed end assay</td>
</tr>
<tr>
<td>FH</td>
<td>formin homology</td>
</tr>
<tr>
<td>Fn</td>
<td>fibronectin</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>G-actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GB</td>
<td>GIT1-binding domain</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GIT1</td>
<td>G-protein-coupled receptor-kinase</td>
</tr>
<tr>
<td>GlyR</td>
<td>glycine receptor</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IDD</td>
<td>intelectual developmental disorder</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IgSF</td>
<td>immunoglobulin superfamily</td>
</tr>
<tr>
<td>KAR</td>
<td>Kainate receptor</td>
</tr>
<tr>
<td>KCC</td>
<td>K⁺-Cl⁻ cotransporter</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>LFA</td>
<td>lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM domain kinase</td>
</tr>
<tr>
<td>LNS</td>
<td>Laminin-alpha, Neurexin and Sex hormone-binding globulin</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAGUK</td>
<td>membrane associated guanylate kinase</td>
</tr>
<tr>
<td>mDia2</td>
<td>mammalian diaphanous-2</td>
</tr>
<tr>
<td>mEPSCs</td>
<td>miniature excitatory post-synaptic currents</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NAKCC</td>
<td>Na⁺-K⁺-2Cl⁻ cotransporter</td>
</tr>
<tr>
<td>NMDA(R)</td>
<td>N-methyl-D-aspartate (receptor)</td>
</tr>
<tr>
<td>NRSE</td>
<td>neuron-restrictive silencing element</td>
</tr>
<tr>
<td>NTD</td>
<td>amino-terminus domain (N-terminal)</td>
</tr>
<tr>
<td>N-WASP</td>
<td>neuronal Wiskott–Aldrich Syndrome protein</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PDZ</td>
<td>post synaptic density protein (PDS95), Drosophila disc large (Dlg1) and zona occludens-1 protein</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PTV</td>
<td>Piccolo-Bassoon transport vesicles</td>
</tr>
<tr>
<td>PD</td>
<td>quantum dots</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-Related C3 Botulinum Toxin Substrate1</td>
</tr>
<tr>
<td>Rif</td>
<td>Ras homolog family member F (filipodia)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROs</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SA</td>
<td>spine apparatus</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SER</td>
<td>smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>shRNA</td>
<td>short-hairpin RNA</td>
</tr>
<tr>
<td>sRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SSC</td>
<td>somatosensory cortex</td>
</tr>
<tr>
<td>SV</td>
<td>synaptic vesicle</td>
</tr>
<tr>
<td>SYNPO</td>
<td>synaptopodin</td>
</tr>
<tr>
<td>TARP</td>
<td>transmembrane AMPA receptor regulatory protein</td>
</tr>
<tr>
<td>TLN (TLCN)</td>
<td>telencephalin</td>
</tr>
<tr>
<td>USF</td>
<td>upstream stimulation factor</td>
</tr>
<tr>
<td>WASp</td>
<td>Wiskott–Aldrich Syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP-family verprolin-homologous protein</td>
</tr>
<tr>
<td>WH</td>
<td>WASp homology</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>Y2H</td>
<td>yeast two-hybrid</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
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ABSTRACT

Dendritic spines are the main site of reception of glutamatergic -excitatory- neurotransmission in the central nervous system. According to the current view on neuronal function, dendritic spines play a pivotal role in the formation of synaptic networks for memory storage. Consequently, dendritic spines are crucial for cognitive processes, e.g. learning. Numerous disorders such as intellectual developmental disorders, schizophrenia and cognitive impairment are associated with functional and structural abnormalities of dendritic spines. The main objectives of this project were to identify molecular regulators of the structure and function of dendritic spines and to characterise novel mechanisms leading to dendritic spine development and synapse formation.

Actin is the most abundant protein in dendritic spines. Rearrangements of the actin cytoskeleton are responsible for the morphological changes of dendritic spines, making actin a major player in the regulation of glutamatergic synaptogenesis. Increasing evidence shows that dendritic filopodia are crucial in the formation of dendritic spines. Often filopodia act as precursors of mature dendritic spines. While filopodial protrusions in other cell types have been widely studied, the molecular mechanisms regulating the emergence and maintenance of dendritic filopodia are poorly understood. In this thesis work, we show that the polymerizing factor mDia2 promotes initiation and polymerization of actin in the filopodial tip. We also describe a novel observation of filopodial root polymerization. Spine maturation is accompanied by expansion of the spine head. We propose here that the actin polymerizing factor Arp2/3 complex takes active part in the branched actin polymerization during spine head expansion. Spine heads are dynamic structures, with long protrusions often visible on their surface. Our results demonstrate that the actin depolymerizing factor cofilin-1 has a double function in the regulation of dendritic spine actin dynamics. On one hand cofilin-1 replenishes the actin monomer pool, and on the other hand it shapes the spines by severing the actin filaments and therefore controls actin filament length.

The maturation of synaptic networks is strictly dependent on the synchronous development of both inhibitory and excitatory transmission. Within this context the formation of and stabilization of dendritic spines is an important step in the maturation of glutamatergic transmission. However, in terms of functional maturation, chloride regulatory proteins, such as the K-Cl cotransporter KCC2, are crucial regulators of GABAergic -inhibitory- transmission. Interestingly, previous studies have identified KCC2 as an important agent required for the maturation of dendritic spines and consequently glutamatergic transmission. The mechanism how KCC2 exerts its chloride-extrusion independent effect on dendritic spines and excitatory synapses remained obscure. In this thesis work we have identified the molecular interaction between the potassium-chloride cotransporter KCC2 and the guanine nucleotide exchange factor βPix. Importantly, KCC2 inhibits the action of βPix towards the GTPase Rac1, a major regulator of the actin cytoskeleton in dendritic spines. The inhibition of βPix by KCC2 leads to decreased cofilin-1 inactivation and subsequent reduction in...
the fraction of actin that is stable. This novel molecular pathway leads to the regulation of glutamatergic synaptogenesis and spine formation by KCC2 via βPix.

Synaptic cell adhesion molecules orchestrate trans-synaptic recognition as well as specification of glutamatergic synapses. Fine-tuning of synaptic networks requires a delicate balance between positive and negative signalling mechanisms that regulate dendritic spine formation. The intercellular adhesion molecule ICAM-5 negatively regulates the maturation of dendritic spines. We have found that ICAM-5 binds to pre-synaptic β1 integrins in filopodia and immature dendritic spines, preventing spine maturation. We have characterized the molecular mechanisms leading to the diminished interaction of ICAM-5 and β1 integrins during spine maturation. Moreover, genetic manipulation of ICAM5 affected the morphology and function of dendritic spines.

The results included in this thesis work contribute to the deeper understanding of the molecular mechanisms regulating the development of dendritic spines. We have studied molecules that control all steps of these processes, from filopodia formation to mature spine regulation; encompassing structural and functional synaptogenesis.
REVIEW OF THE LITERATURE

1. Introduction

The central nervous system is dynamic and always at work: continuously receiving, perceiving and analysing information whilst simultaneously making decisions. All of our conscious and unconscious bodily functions depend on the central nervous system. Unsurprisingly, the CNS possesses remarkable functional and structural complexity. On average, the human brain is composed of $10^{11}$ neurons and several times as many glial cells.

The site at which one neuron confers its information to another neuron is the synapse. Synapses are more than mere cell-to-cell contacts; they are the structure where neuronal communication occurs. Thus, synapses are the fundamental building blocks of neuronal networks. Typically, neurons in the CNS form up to ten thousand synapses. Neurons continuously take into account the information received from diverse inputs and create messages of their own in a phenomenon known as synaptic integration. The efficacy of synaptic connections is not a set parameter, but it has the capacity to vary as a consequence of the ongoing and past activity. This capacity for change is called synaptic plasticity.

The study of the molecular mechanisms underlying synaptic transmission is of great importance in modern neurobiology since these processes are responsible for neuronal integration and plasticity.

2. Synapses

The structure of synapses in the CNS consists of two major compartments, a presynaptic structure (axon terminal of the afferent neuron) and a postsynaptic specialization (dendrite or neuronal soma of the efferent neuron). The space existing in between those compartments is the synaptic cleft. In the process of chemical neurotransmission, the synaptic cleft forms the extracellular space that the secreted chemical compounds (neurotransmitters) need to travel in order to convey the signals from the presynaptic to the postsynaptic neuron.

Neurotransmitters released from the presynaptic neurons will bind their receptors and will elicit the opening or closing of ion channels existing on the membrane of the postsynaptic cell. As a result, the electric potential of the membrane of the receiving cell will change. This postsynaptic response will be of excitatory or inhibitory nature depending on the type of channel that is coupled to the receptor as well as on the concentration of permeant ions on both sides of the membrane. Excitatory potentials (i.e. depolarising) increase the likelihood of an action potential occurring in the postsynaptic cell and inhibitory potentials (i.e. hyperpolarising) decrease this probability. Most fast excitatory neurotransmission in the central nervous system of vertebrates relies on the action of the neurotransmitter L-glutamate on ligand gated (ionotropic) receptors (reviewed in (Meldrum, 2000)).
The ultrastructural features of synapses have long been the object of study. In the 1950’s Gray used electron microscope micrographs to classify synapses and he defined two types of synapses according to the characteristics of the presynaptic and postsynaptic sites. These two types are distinguished also by the location. Type I synapses are made onto dendritic spines and dendrite shafts while type II synapses are mainly found on dendritic shafts and bodies (E. G. Gray, 1959). Subsequently, Colonnier coined the terms asymmetric and symmetric synapse in order to classify synapses. Colonnier visualized aldehyde-fixed tissue and then he defined asymmetric synapses as the ones including presynaptic partners (axons) containing round or spherical vesicles and the postsynaptic side that is thickened. Symmetric synapses, on the other hand, do not show an electro-dense postsynaptic structure but the apposed membranes at the synapse site are more parallel than the surrounding (non-synaptic) membrane (Colonnier, 1968). Later, the terms were found to be virtually synonymous and are used indistinctly. In my thesis work, I have concentrated in the study of asymmetrical synapses. The term “synapse” will refer hereafter to type I asymmetrical synapses, and type II synapses will be properly noted.

The term synaptogenesis refers to the process by which nascent synapses are formed. Synaptogenesis is occurring continuously in the CNS. However, during the period of brain development, there is a major peak in formation of new synapses. The rate decreases later in life. During embryonic and early postnatal brain development, synaptogenesis takes place in parallel to neuronal differentiation and circuitry maturation, giving rise to the mature CNS. In visual cortex of rats, the density of asymmetric synapses increases continuously during the three weeks following birth. The density of synapses peaks at around day 20, when it achieves a mean value close to that of adult animals (Blue & Parnavelas, 1983). In rat hippocampus, the process is highly similar (J C Fiala, Feinberg, Popov, & Harris, 1998). This rapid synaptogenesis in the cortex of mammals is followed by a great loss of synapses and spines, through a process named synaptic pruning. The rate of loss declines in early adulthood and the number of synapses is then rather stable until age related processes produce a second decline in synapse number (Bhatt, Zhang, & Gan, 2009; Markus & Petit, 1987; Rakic, Bourgeois, Eckenhoff, Zecevic, & Goldman-Rakic, 1986).

3. Dendritic spines

Dendritic spines (hereafter commonly referred to only as spines) are small, bulbous protrusions that jut from the dendrites in neurons. Spines show high heterogeneity in their shape. However, they commonly possess a head around 1μm in diameter and a thin neck, which ranges between 0.5 to several micrometres in length and that connects them to the shaft. Spines were first described by Santiago Ramón y Cajal at the end of the 19th century (Ramón y Cajal, 1888) and were extensively studied by him during his whole life. Ramón y Cajal used the then recently discovered Golgi staining to observe the brains of birds and strongly opposed the views of his contemporaries who claimed that spines were mere artefacts of the technique. Later, he used other staining methods and investigated spines and their morphologies in diverse vertebrates. He published a
compendium of his results named “Histology of the Nervous System of Man and Vertebrates” (Ramón y Cajal, 1899). Nowadays we know that spines are present in neurons of many organisms, from annelids to primates, and are especially numerous in vertebrates. Spines are very abundant in several cell types as cerebellar Purkinje cells and pyramidal neurons in the cortex, while other cells, such as aspiny striatal neurons in the basal ganglia for example, are devoid of spines (Theriault & Landis, 1987).

Spines receive most of the synapses produced in the vertebrate CNS and they are the major site of excitatory neurotransmission. Nonetheless, spines are far away from being mere reception sites; they regulate signal transduction at the postsynaptic membrane and represent the smallest processing unit in the brain (for recent reviews see (Bellot et al., 2014; Shigeo Okabe, 2012; Sala, Cambianica, & Rossi, 2008; Rafael Yuste, 2011)). Spine formation and synaptogenesis are overlapping phenomena. During development of the monkey visual striatal cortex, the frequency of spines increases continuously and peaks at 8 weeks of age, declining thereafter (Boothe, Greenough, Lund, & Wrege, 1979; Duan, 2003). In rodents, the time frames are different but the same general tendency is conserved. In primary sensory cortex, spine density is continuously increasing from postnatal day 7 to 24 (Cruz-Martin, Crespo, & Portera-Cailliau, 2010), after which spine elimination is greater than formation, provoking an overall spine loss (A. J. G. D. Holtmaat et al., 2005; Zuo, Yang, Kwon, & Gan, 2005). Remarkably, direct observations have confirmed the relationship between spinogenesis and synapse formation. Localized glutamatergic stimulation in mouse developing cortex during the period of high rate of synapse formation is sufficient to promote de novo spine growth (Kozorovitskiy, Saunders, Johnson, Lowell, & Sabatini, 2012; Kwon & Sabatini, 2011). In adulthood, spine formation occurs in connection with experience during learning and memory formation. Newly formed spines can be life-lasting, although the fraction of stable spines elicited by novel experience is relatively small and the process is cellspecific (A. J. G. D. Holtmaat et al., 2005; A. Holtmaat, Wilbrecht, Knott, Welker, & Svoboda, 2006; Xu et al., 2009; G. Yang, Pan, & Gan, 2009). These observations let us speculate that those long-lasting spines might be the physical substrate for memory storage.

According to the prevalent view on memory formation and learning, information in the brain is stored through plastic changes in the brain connectivity. Importantly, these changes have been described as strengthening or weakening of synapses, also known as functional plasticity, and corresponding expansion and shrinkage of spine size, named structural plasticity (Kasai, Matsuzaki, Noguchi, Yasumatsu, & Nakahara, 2003; R Yuste & Bonhoeffer, 2001), reviewed in (Sala & Segal, 2014). Therefore, dendritic spines are a pivotal component of the cellular machinery for memory formation and learning.

In the last years, different hypotheses have appeared to explain the existence of dendritic spines and why excitatory axons use them as a preferred site of connection. Especially, considering that aspiny neurons exist and synapses are also made directly onto dendritic shafts, such is the case of symmetric synapses. These hypotheses can be divided into three main groups. In the first group, hypothesis implying that dendritic
spines have emerged to increase the connectivity of the neurons, making the neuronal matrix more distributed. The distribution of spines along the shafts together with the straightness of axons increases the diversity of presynaptic partners while avoiding “double hits” with the same axon. The second group of hypotheses propose that spines work to electrically isolate the inputs to avoid electric interference and shunting from one synapse to the adjacent ones. Indeed, experimental data suggest that the spine neck might generate increased resistance. Finally, the third group postulates that spines are biochemical compartments that can isolate ions, such as calcium (due to diffusional restriction and local extrusion mechanisms) and proteins (due to physical segregation). The two last mechanisms would make input-specific plasticity possible (Reviewed in (K. F. H. Lee, Soares, & Béïque, 2012)). Recently, an integrative view of these mechanisms has aroused and postulated that all three roles of dendritic spines contribute to achieving a distributed circuit that would allow widespread connectivity (Rafael Yuste, 2011).

Accumulating evidence has related dendritic spine abnormalities to diverse neurological disorders such as cognitive impairment, schizophrenia and other intellectual developmental disorders (IDDs). Notably, in fragile X-syndrome, autism-spectrum disorders and schizophrenia patients, the density of dendritic spines is increased, while in Down’s syndrome patients, there is decreased spine density. In many cases, this phenotype is accompanied by synaptic dysfunction, perturbed spine maturation and impaired dendritic tree arborisation. Other disorders like epilepsy and Alzheimer disease (AD) show hallmarks such as spine loss or degeneration. Importantly, after many studies using animal models of these disorders, numerous proteins that accumulate at dendritic spines and are essential for their structure and function have been identified, pinpointing the importance of these small dendritic protuberances in the functioning of the entire CNS (reviewed in Bellot et al., 2014; John C Fiala, Spacek, & Harris, 2002; Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011; Svitkina et al., 2010).

3.1. Structure of dendritic spines

Classification of dendritic spines was initially based on the information from anatomical studies on fixed brain tissue by means of Golgi staining and EM. This traditional segregation of spines included three types: 1) mushroom spines, which show a sturdy neck and a wide bulbous head (>0.6 μm in diameter, sometimes over 1μm); 2) thin spines, with long necks and small bulbous heads (<0.6 μm in diameter), and 3) stubby spines, which have no distinct neck, so the diameter of the neck and the spine head are of the same magnitude (Peters & Kaiserman-Abramof, 1970). However, a fourth category is commonly accepted to include dendritic filopodia. Dendritic filopodia are long protrusions, which show no distinct neck and differ from filopodia present in growth cones (Sekino, Kojima, & Shirao, 2007) and they have been proposed as precursors of dendritic spines (Figure 1. and read below). In addition to these spine morphologies, further observations have described cup-shaped spines, branched spines and others (Sala et al., 2008). Recent live imaging studies
have demonstrated that, far away from stable, dendritic spines are highly dynamic and change from one type to another at a high rate, especially during the periods of intense synaptogenesis (Honkura, Matsuzaki, Noguchi, Ellis-Davies, & Kasai, 2008; Hotulainen & Hoogenraad, 2010).

The basic structure of dendritic spines contains the post-synaptic density (PSD; Palade & Palay, 1954), a network of actin cytoskeleton ((Matus, 2000); will be extensively reviewed below) and distinct organelles (Figure 2 and read below).

3.1.1. The post-synaptic density

The post-synaptic density (PSD) is a highly specialized structure that occurs mostly in dendritic spines. It consists of an electron-dense structure found on the membrane of the spine and apposed to the presynaptic terminal. It is commonly disk-shaped and up to 0.5 μm in length and 60 nm in width (Harris, Jensen, & Tsao, 1992). Importantly, this highly packed structure contains glutamate receptors, both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartic acid (NMDA) type, which accumulate there (D. Cheng et al., 2006).

The PSD and its components are held in place by abundant scaffolding proteins like PSD-95 and Homer. These proteins serve a double role as sustaining elements and promoters of the framework of the PSD as they interact simultaneously with membrane proteins and the cytoskeleton beneath them. Importantly, PSD-95, a member of the membrane associated guanylate kinase (MAGUK) family (Cho, Hunt, & Kennedy, 1992), binds to NMDAR through their NR2 subunit (Kornau, Schenker, Kennedy, & Seeburg, 1995) and indirectly to AMPAR, through their auxiliary regulatory proteins, transmembrane AMPA receptor regulatory proteins (TARPs) (Tomita et al., 2005). Among other synaptic molecules, Homer proteins interact with group I metabotropic glutamate receptors (mGluRs) (Brakeman et al., 1997) and the scaffolding protein Shank, which also binds PSD-95 (Tu et al., 1999). The clustering of proteins promoted by scaffolding proteins facilitates signal transduction in postsynaptic sites (Shigeo Okabe, 2012; Shiraishi-Yamaguchi & Furuichi, 2007).
morphology of the PSD is highly plastic and responds to synaptic activity (Bourne & Harris, 2008; Shigeo Okabe, 2012). Interestingly, the size of the PSD positively correlates with spine size, AMPA and NMDA receptor content and even number of presynaptic vesicles. Thus, size of the PSD and dendritic spine size are strongly related to the sensitivity to glutamate and synaptic strength of the given synapse. Trans-synaptic mechanisms coordinate changes in those components during synaptic development and plasticity (Harris et al., 1992; Harris & Stevens, 1989; Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004; Takumi, Ramírez-León, Laake, Rinvik, & Ottersen, 1999).

3.1.2. Organelles found in spines

The smooth endoplasmic reticulum (SER) is an organelle that consists of membranous tubules which form a continuum with the nuclear membrane. The main functions of the SER are the synthesis of lipids, the maintenance of calcium homeostasis and the detoxification reactions in order to avoid cell damage from water-insoluble substances (Pavelka and Roth 2015). The SER is commonly found in mature spines in the hippocampal and cortical cells and in all Purkinje neurons at the cerebellum (Nimchinsky, Sabatini, & Svoboda, 2002; Spacek & Harris, 1997). In mature spines the SER can develop and become the spine apparatus (SA), a structure first observed by Gray in the 50's and extensively studied thereafter (Gray, 1959). The SA consists of multiple membrane-bound sacs interspaced by plaques of electron-dense material. This continuation of the SER might even reach the PSD. The SA functions as a direct site of delivery of proteins to the synapse and as calcium storing and regulating site, of great importance especially during synaptic activity. The F-actin interacting protein synaptodin (SYNPO) is crucial for the formation of the SA (Deller et al., 2003). SYNPO is already present in spines at early stages (Czarnecki, Haas, Bas Orth, Deller, & Frotscher, 2005). In this context it is interesting that SYNPO also controls Ca\(^{2+}\) handling in spines (Eduard Korkotian & Segal, 2011; Segal, Vlachos, & Korkotian, 2010), because calcium release from internal stores elicited by neurotransmitters is crucial to the stabilization of dendritic protrusions (Lohmann, Finski, & Bonhoeffer, 2005; Lohmann, Myhr, & Wong, 2002).
Recent studies have found increasing evidence of local synthesis of proteins in spines. Polyribosomes can be found at the base of dendritic spines especially during synaptic activity (Ostroff, Fiala, Allwardt, & Harris, ...
Moreover, the mechanisms by which certain mRNAs are targeted specifically to spines and how transcription occurs locally are finally starting to be disentangled. Dendritic targeting by means of intronic sequences such as ID elements, a class of short interspersed repetitive elements (SINE) transposons, is a common phenomenon and plays an important role in the biology of the neuron (Buckley et al., 2011; Miyashiro, Bell, Sul, & Eberwine, 2009).

Other organelles present in spines are proteasomes, which degrade proteins *in situ*; and vesicular components that confer the mechanism by which the new cellular membrane is acquired during spine remodelling and growth. These vesicular components are endosomes, clathrin-coated vesicles and large amorphous vesicular clumps (AVC) (Bourne & Harris, 2008). Mitochondria are transiently present in spines during periods of active synaptogenesis and remodelling in order to provide energy and possibly to buffer Ca^{2+}. However, mitochondria are more abundant in dendritic shafts, close to spines (Z. Li, Okamoto, Hayashi, & Sheng, 2004).

### 3.2. Development of dendritic spines and synapses

The formation of new spines occurs during development and in some forms of adult plasticity, such as during the regeneration following trauma. Four main phenomena occur during spine maturation: the density of spines increases, the mean length of the protrusions decreases, the proportion of filopodia is dramatically reduced and there is a general decrease in motility (Sala et al., 2008). Spine elimination can be considered the fifth phenomenon, also of great importance for the maturation of neuronal networks, (G. Yang et al., 2009; Zuo et al., 2005). Dendritic filopodia have been identified as precursors of spines as they are abundant in early stages of development (J C Fiala et al., 1998; Papa, Bundman, Greenberger, & Segal, 1995; Ziv & Smith, 1996). Filopodia can be devoid of synapse or they might bear nascent synapses, which can also be multiple (J C Fiala et al., 1998). Filopodia are highly motile (Dailey & Smith, 1996; Dunaevsky, Tashiro, Majewska, Mason, & Yuste, 1999; E Korkotian & Segal, 2001) and tend to be transient protrusions (Ziv & Smith, 1996). By means of their flexibility, filopodia actively probe the extracellular environment and seek out synaptic partners (Dunaevsky et al., 1999). When filopodia contacts an appropriate axon terminal, it retracts towards the dendritic shaft and starts developing into a mature spine, forming a fully developed synapse (Dailey & Smith, 1996; J C Fiala et al., 1998; Ziv & Smith, 1996). Indeed, spine motility is reduced and there are fewer transitions between categories in more mature neurons (Dailey & Smith, 1996; De Roo, Klauser, Mendez, Poglia, & Muller, 2008; Dunaevsky et al., 1999; Parnass, Tashiro, & Yuste, 2000). In the intellectual developmental disorder Fragile-X syndrome, spines show a delay in the reduction of their turnover, highlighting the importance of this process in network maturation (Cruz-Martín et al., 2010). Interestingly, filopodial selection of presynaptic partners occurs at an early stage. Indeed, dendritic filopodia that contact with a population of GABAergic neurons are only transient and do never stabilise (Lohmann & Bonhoeffer,
Moreover, transient calcium rises are observed in filopodia upon axonal contact and these are occurring in the presence of glutamate receptor antagonists. The frequency of these transients will determine whether the synapse will stabilize or be eliminated (Lohmann & Bonhoeffer, 2008). The proportion of contacts that become stabilized has been estimated around 10-20% irrespectively of the developmental stage of the neurons (De Roo et al., 2008).

Nevertheless, not all dendritic spines derive from filopodia. In some cases, the pre-formed PSD is present on the dendritic shaft and the membrane extends from the shaft to form a new spine (Harris et al., 1992). One possibility is that these preformed shaft synapses might be remnants of a previously existing filopodia that retracts and disappears into the shaft. Later on, those synapses either re-emerge as spines or are eliminated later in development (Bourne & Harris, 2008; Harris, 1999). With these data we can conclude that the specific microenvironment and the dynamic state of a given synapse are crucial to determine the mechanism of spine morphogenesis.

In contrast to what happens in later stages of synapse development, neuronal activity may have relatively small influence on the first phase of synapse formation. Notably, pharmacological blockage of synaptic transmission affected spine density in neurons around three weeks of age but not younger ones (postnatal days 6-7) (Kirov, Petrak, Fiala, & Harris, 2004). Interestingly, the presence of glutamate receptor antagonists had no effect either in the stabilization of newly formed synapses or in the calcium transients occurring in the filopodia immediately after axonal contact (Gomperts, Carroll, Malenka, & Nicoll, 2000; Lohmann & Bonhoeffer, 2008). Notably, excitatory synaptogenesis still occurs in the absence of synaptic vesicle release (Harms & Craig, 2005; Varoqueaux et al., 2002; Verhage et al., 2000). Indeed, mutant mice lacking the presynaptic protein Munc13, isoforms 1 and 2, which are the two isoforms responsible for the priming of synaptic vesicles in the hippocampus, show no spontaneous or evoked release of neurotransmitters GABA and glutamate while the number and ultrastructure of their synapses is unaffected (Varoqueaux et al., 2002). However, detailed quantification of synapse number in Munc18 KO embryos showed that the number of synapses and their morphology is irregular (Bouwman et al., 2004). In conclusion, glutamate release is not the only determinant for excitatory synapse formation.

Membrane bound cell adhesion molecules (CAMs) regulate initial contacts in nascent synapses. They also have been shown to trigger synapse formation (Biederer et al., 2002; Fu, Washbourne, Ortinski, & Vicini, 2003; Scheiffele, Fan, Choih, Fetter, & Serafini, 2000) as they function as signal transducing molecules. Due to the asymmetric nature of the excitatory synapse, the regulation on both sides of the synaptic cleft is governed by different mechanisms. Well known molecules such as presynaptic β-neurexin and its postsynaptic partner neuroligin, mediate the asymmetry of the synapse due to their heterophilic binding. Other CAMs like cadherines, synaptic cell adhesion molecules (SynCAMs) and neuronal cell adhesion molecule (NCAM), to name a few, contribute to partner recognition and to the first steps of establishment of...
the synaptic contact through their homophilic binding and subsequent signalling (Biederer et al., 2002; Kleene & Schachner, 2004; Obata et al., 1995; Washbourne et al., 2004). There is still much room for knowledge on how these cell-cell interactions differentially attune synaptic specificity and how asymmetry is accomplished in order to produce the existing huge variety of synaptic strengths and connections.

Stabilization of spines requires the assembly of pre- and post-synaptic elements and delivery of molecular synaptic components. The assembly process can be fairly rapid. Functional pre- and postsynaptic components are recruited to the synaptic site within 30-45 min. after the first synaptic contact (Friedman et al. 2000). Yet, the order and timing of the events can vary (Friedman, Bresler, Garner, & Ziv, 2000; Nägerl, Köstinger, Anderson, Martin, & Bonhoeffer, 2007; S Okabe, Miwa, & Okado, 2001). Interestingly, the delivery of presynaptic molecular components takes place in pre-packed vesicles and not through local recruitment of single proteins. Dense core vesicles, named Piccolo-Bassoon transport vesicles (PTVs), containing key presynaptic scaffold proteins Piccolo and Bassoon together with N-cadherin and syntaxin accumulate early after synaptic contact in axonal processes. These vesicles travel along the axons and where they stop, a new synapse will start to form (Bury & Sabo, 2011; Shapira et al., 2003; Zhai et al., 2001). Further recruitment of synaptic components occurs via small clear-cored pleomorphic vesicles called synaptic vesicle protein transport vesicles (STVs) (Ahmari, Buchanan, & Smith, 2000).

On the postsynaptic side, PSD-95 is crucial to the assembling mechanism as evidenced by the loss of excitatory synapses and spines caused by siRNA against the scaffolding protein (Ingrid Ehrlich, Klein, Rumpel, & Malinow, 2007; Nakagawa et al., 2004). Moreover, PSD95 is the first molecule observed in the postsynaptic specialization appearing as early as 20 min. after synaptic contact (S Okabe, Miwa, et al., 2001). Importantly, PSD-95 clustering, spine morphogenesis and presynaptic assembly are correlated in time and space (Marrs, Green, & Dailey, 2001; S Okabe, Miwa, et al., 2001). Recruitment of postsynaptic components was initially proposed to occur via pre-packed clusters. Nevertheless, the trafficking of scaffolding proteins like Shank and PSD-95 seems to be dependent on a diffusible cytoplasmic pool rather than pre-assembled packs (T Bresler et al., 2001; Tal Bresler et al., 2004; Gray, Weimer, Bureau, & Svoboda, 2006; Kanaka et al., 2001; Kuriu, Inoue, Bito, Sobue, & Okabe, 2006; S Okabe, Urushido, Konno, Okado, & Sobue, 2001).
In order to stabilise dendritic spines, insertion of glutamatergic receptors is an important step. At early stages of development of synapses the ratio AMPAR: NMDAR is low. NMDAR trafficking to postsynaptic sites is very rapid and independent of that of PSD-95. Indeed, NMDAR are delivered to the nascent synapse in transporting packets and they can either precede or be concurrent with the transport of PSD-95 (Washbourne, Bennett, & McAllister, 2002). These transporting packets contain the scaffolding protein SAP-102 (Washbourne, Liu, Jones, & McAllister, 2004). Targeting of NMDAR to the nascent synapses is dependent on the combination of trafficking of the receptors by lateral diffusion and direct insertion of NMDAR from a cytoplasmic pool (Lau & Zukin, 2007; Washbourne, Liu, et al., 2004). It is important to note here that pharmacological block of NMDAR fails to affect emergence and density of spines during development (Alvarez, Ridenour, & Sabatini, 2007; Kirov et al., 2004). However, depletion of NMDAR by siRNA results in increased spine motility and eventual elimination (Alvarez et al., 2007).

AMPAR are trafficked by stargazin and related transmembrane AMPA receptor regulatory proteins (TARPs) which facilitate their insertion in the PSD and also regulate channel properties of the receptors (Chen et al., 2000; Tomita et al., 2005). Even so, the mechanisms for delivery are similar to those for NMDAR: both local insertion and lateral diffusion of AMPAR occur in spines (Borgdorff & Choquet, 2002; Passafaro, Piëch, & Sheng, 2001).

AMPAR are found in developing synapses and their recruitment is independent of NMDAR signalling (Groc & Choquet, 2006). In the presence of NMDAR antagonists, AMPAR are recruited to synapses both in vivo and in vitro (Colonnese, Shi, & Constantine-Paton, 2003; Friedman et al., 2000; Gomperts et al., 2000; Liao, Zhang, O’Brien, Ehlers, & Huganir, 1999; Rao & Craig, 1997). Additionally, mice lacking the NR1 subunit of NMDAR, which is normally present in all functional NMDAR subunit compositions, still show AMPAR-dependent currents (Tsien, Huerta, & Tonegawa, 1996). Thus, NMDAR activation is not needed for AMPAR recruitment to synapses in the period of synaptogenesis but seems to be important for insertion of calcium permeable AMPAR later, when synapses are mature and the mechanisms required for synapse plasticity are dependent on long-term potentiation (LTP) and depression (LTD) (Hall & Ghosh, 2008; H.-K. Lee & Kirkwood, 2011; Malenka & Bear, 2004).

The final steps of maturation require, from the presynaptic side, enlargement of axonal boutons and subsequent increase in synaptic vesicles (SV) density (Santos, Li, & Voglmaier, 2009). On the postsynaptic site, in addition to morphological changes discussed above (including spine head expansion and PSD size increase), the functional maturation of synapses requires changes in the composition of the PSD. Maturation is profoundly marked by redistribution of the subunits that form ionotropic glutamatergic receptors as well as a switch in the weight of the NMDAR-induced currents towards AMPAR-induced currents (Hall & Ghosh, 2008). There is a reduction in the NR2B-containing NMDAR and the scaffolding protein SAP102 and an
increase in NR2A-containing NMDAR, AMPARs and PSD95 as well as important signalling molecules like calmodulin-dependent kinase II (CaMKII) (Petralia, Sans, Wang, & Wenthold, 2005).

4. Regulation of spine development by the actin cytoskeleton

Actin is often the most abundant protein inside eukaryotic cells. The actin cytoskeleton confers mechanical stability to the cells while enabling a myriad of physiological processes, from cell motility to process extension and vesicle transport. Actin exists in two forms in cells: as a monomer (G-actin; globular actin) and as a filament (F-actin, also called microfilaments). In order to be added to a microfilament, an actin monomer needs to be bound to ATP and a Mg\(^{2+}\) ion. Addition (polymerization) and loss (depolymerization) of actin monomers occurs at both ends of the filament and the assembly of actin monomers into filaments entails the hydrolysis of ATP into ADP + Pi (Lodish et al., 2000). In 1976, Wegner proposed a theory by which the chemical state of the bound nucleotide determines the rates of subunit addition and removal of actin. The theory states that actin bound to ADP has lower affinity for the other subunits in the filament that actin bound to ATP, and therefore the ADP-actin tends to be lost from the filament. The “treadmilling theory” explains the polarity of actin filaments by which actin monomers are added at a higher rate from the plus or barbed end and they are lost at a higher rate from the minus or pointed end. This theory is widely accepted and it stands up to date (A. Wegner, 1976).

Actin filaments consist of two strands that interlace in a helicoid conformation and both strands have the same polarity. This structure provides mechanical strength and also allows subunit association only at the ends of the polymer, which will allow filament growth necessary for cell polarity and motility. Actin then is arranged at a higher level, where many filaments are put together. The two most common arrangements of actin filaments in a cell are bundles and networks. In these conformations, microfilaments are held together by actin cross-linking proteins (Lodish et al., 2000).
Actin cross-linking proteins are just an example of members of a big functional group of actin-binding proteins (ABPs). These molecules assist the actin cytoskeleton by catalysing many crucial processes such as nucleation for rapid assembly of the filament, polymerization, capping, depolymerization and severing, among other processes (Lodish et al., 2000).

4.1. Actin in dendritic spines

Actin is the main cytoskeletal protein in dendritic spines (Matus, Ackermann, Pehling, Byers, & Fujiwara, 1982). The actin cytoskeleton is responsible for several crucial functions in dendritic spines. As in other cellular compartments, actin controls general spine morphology. Moreover, the actin cytoskeleton serves to stabilize synaptic and extra-synaptic receptors, remodels the PSD as well as regulates spine neck structure and function. Importantly, actin in spines regulates organelle trafficking and endocytosis (reviewed in (Frost, Kerr, Lu, & Blanpied, 2010)). The specific characteristics of the actin cytoskeleton in dendritic spines and filopodia are unique in any given moment thanks to the existence of a set of actin-regulating proteins, or ABPs, in these compartments. The role of some ABPs regulating the actin cytoskeleton in dendritic spines is discussed in detail later (see section 5.1.).
Immature dendritic filopodia which lack contact with a presynaptic axon have already rich actin content. Remodelling of the actin cytoskeleton is therefore needed for the appropriate synaptic contact formation (Korobova & Svitkina, 2010; Shigeo Okabe, 2012). A recent comprehensive study by Korobova and Svitkina (Korobova & Svitkina, 2010) has greatly contributed to our understanding of the organization of the actin cytoskeleton in dendritic spines and filopodia. Korobova and colleagues used correlative light and electron microscopy to show that, unlike previously thought, the actin cytoskeleton in dendritic filopodia- as well as in the necks of dendritic spines- has a network of branched and linear actin filaments with high content of the actin-branching Arp2/3 complex. The functional relevance of this actin conformation is not clear but it might contribute to the dynamicity of dendritic filopodia, since it would allow fast spine head expansion (Korobova & Svitkina, 2010 and see Figure 4. A, B). Several studies have proposed that at least two distinct pools of actin filaments exist in

![Figure 5. Comparison of actin organization in fibroblast cells and dendritic spines. A. In conventional filopodia from fibroblast cells, actin filaments are elongated from the tip of filopodia by mDia2, Ena/VASP, and myosin X. Polymerized actin filaments are bundled by fascin. In dendritic filopodia (neurons), mDia2 elongates actin filaments from the tip of filopodia. The functions of Ena/VASP and myosin X have not yet been studied in dendritic spines. In addition to tip polymerization, actin filaments of dendritic filopodia elongate from base. Fascin is absent from dendritic filopodia. B. In lamellipodium from fibroblast cells, actin filaments are nucleated by Arp2/3 complex. Actin barbed ends are capped by capping protein to maintain filaments short. ADF/cofilins depolymerize pointed ends of actin filaments to replenish the actin monomer pool. Profilins change the ADP to ATP and transport ATP-actin monomers to the free barbed ends. In dendritic spine heads (neurons), functions of Arp2/3 complex and cofilin resemble those in lamellipodium. The function of capping protein has not been investigated. Profilins localize to dendritic spines in an activity-dependent manner.](https://example.com/figure5.png)

dendritic spines and they display different roles. The first pool is composed of stable actin filaments and serves as an anchor to the post-synaptic receptors present in the PSD thanks to the interaction of actin with multiple scaffolding proteins. Importantly, acute pharmacological disassembly of the actin filaments affects the mobility of several, but not all, PSD scaffolding proteins (Kuriu et al., 2006; Qualmann, Boeckers, Jeromin, Gundelfinger, & Kessels, 2004). The second actin pool present in dendritic spines consists of more labile actin filaments that contribute to neurotransmitter receptor mobility within and out of dendritic spines. Two important studies have contributed to this knowledge, both in hippocampal neurons. Upon actin depolymerization by latrunculin A, a drug that binds to actin monomers with high affinity and therefore inhibits polymerization and promotes depolymerization (Spector, Braet, Shochet, & Bubb, 1999; Spector, Shochet, Kashman, & Groweiss, 1983), the content of AMPA and NMDA receptor in spines is decreased (Allison, Gelfand, Spector, & Craig, 1998). In the second study, treatment of the cultures with glutamate provoked AMPAR internalization. Notably, jasplakinolide (an actin polymerization promoter and stabilizer of actin filaments (Crews, Manes, & Boehler, 1986; Spector et al., 1999; Zabriskie et al., 1986) blocked the glutamate-induced internalization of AMPAR and spine loss (Halpain, Hipolito, & Saffer, 1998). Moreover, dynamic actin filaments contribute to morphological remodelling taking place in filopodia and dendritic spines during development and synaptic plasticity. Therefore, actin cytoskeleton rearrangements are the hub where functional and morphological plasticity meet each other (reviewed in (Asrar & Jia, 2013; Dent, Merriam, & Hu, 2011)).

Dendritic filopodia are different from “conventional” filopodia present in other cell types. They receive the name of filopodia due to seemingly similar external structure to non-neuronal-cell filopodia and only recently it has been made obvious that they are different cell structures. Not only the function of dendritic filopodia is highly specific and relevant only in the context of neuronal synapses, but also their regulation and components are very different from the ones in other filopodial structures (See 3.1. and figure 5). Unlike in dendritic filopodia, the distribution of actin filaments in “conventional” filopodia is parallel and along the longitudinal axis of the structure. The actin filaments of “conventional” filopodia form a bundle maintained by specific ABPs through protein-protein interactions, also the molecular machinery regulating the actin cytoskeleton in dendritic filopodia is substantially different to the one in “conventional” filopodia. Interestingly, the modulation of the actin cytoskeleton in dendritic filopodia is mediated largely by molecules that are characteristic of lamellipodial structures which are not found in “conventional” filopodia (Figure 5 and 6) (for a recent review see Hotulainen & Hoogenraad, 2010).
The treadmilling of actin filaments in developing spines has been shown to be rapid and capable of modulation by synaptic activity. In their study, Star and colleagues applied the Fluorescence Recovery After Photobleaching (FRAP) technique to show that the high rate of actin turnover in spines is not compatible with the previous idea of a large stable actin pool but rather with a dynamic arrangement of rapid treadmilling filaments (Star, Kwiatkowski, & Murthy, 2002). Later, Honkura et al. continued to study the actin cytoskeleton in spines of hippocampal neurons by means of two-photon photo activation. In 2004 they concluded that the actin at the tip of the spine has diverse dynamics from the one at the base of the spine. The treadmilling at the tip is fast and it generates an expansive force, while at the base, the size of the stable actin pool is dependent on the spine volume. They went on to stimulate the spines by glutamate uncaging and they observed that a third pool of actin was formed. In some occasions, this third pool of actin was released to the dendritic shaft. Only when the neck of the spine was able to retain it, the enlargement of the spine head took place (Honkura, Matsuzaki, Noguchi, Ellis-Davies, & Kasai, 2008). Following this line of studies, Tatavarty and colleagues used the single particle tracking technology to visualize actin movement with high resolution in dendritic protrusions of hippocampal neurons. They used this technique combined with stochastic modelling and drug application and they observed the kinematic movements of actin. Kinematic dynamics of actin cytoskeleton are due to physical movements of the whole actin filament and need to be distinguished from kinetic dynamics which are due to actin-turnover. They observed that the kinematic dynamics of actin in spines are highly heterogeneous, thus not consistent with a model where the actin filaments are highly polarized and along the spine longitudinal axis. The authors demonstrate that the moving molecules observed can be attributed mainly to two phenomena: 1. elongation of the F-actin filament from the barbed...
end and 2. nucleation and/or growth of nearby filaments that can push the labelled filament, causing it to move. Therefore, the authors concluded that the actin cytoskeleton in spines is weakly polarized, i.e. the spine actin cytoskeleton consists of short filaments and they are not distributed parallel to each other (Tatavarty, Kim, Rodionov, & Yu, 2009). Importantly, these conclusions are in line with the physical evidence laid out by Svitkina and colleagues and visualized by platinum replica electron microscopy, showing not parallel distribution of actin filaments in spines (Korobova & Svitkina, 2010). In a subsequent study, Tatavarty and colleagues measured kinematic retrograde actin flow in different spine types, which is indicative of physical motion of the actin filaments. The authors showed that the retrograde flow was fast in dendritic filopodia and rather slow in the neck of spines. These and other observations made in this study led the authors to conclude that the polarization of actin filaments is high in filopodia and low in spine necks (Tatavarty, Das, & Yu, 2012).

The link among actin cytoskeleton regulation, synaptic connectivity and higher cognitive functions is nowadays better delineated (Cingolani & Goda, 2008). Several signalling pathways that regulate synaptic activity converge at actin remodelling by ABPs (Cingolani & Goda, 2008; Ethell & Pasquale, 2005; Okamoto, Nagai, Miyawaki, & Hayashi, 2004; Tada & Sheng, 2006) and it has been shown that regulation of these ABPs affects memory formation and learning (Y. Huang, Wang, & Yung, 2013; Pontrello et al., 2012). Moreover, disruption of the actin cytoskeleton or inhibition of actin polymerization results in memory loss (Fonseca, 2012; Honkura et al., 2008; Krucker, Siggins, & Halpain, 2000). Thus, the functional relevance of the actin cytoskeleton regulation in spines is clear. These conclusions prompt further studies to unveil the role of the actin cytoskeleton regulation in the mechanisms of memory formation and retrieval.

### 4.2. Actin-Binding Proteins

Actin-binding proteins (ABPs) are the “gardeners” of the actin cytoskeleton; they modulate the microfilaments at any given moment. The ABPs present in a cellular structure will determine the characteristics of the F-actin organization. In dendritic spines, ABPs have a variety of functions. The actin cytoskeleton has a dual function: as a stable structural component and as a dynamic network. Therefore, ABPs anchor the cytoskeleton to the scaffolding proteins in the PSD and also rearrange the cytoskeleton and change the morphology of dendritic spines. Dynamic remodelling of the actin cytoskeleton occurs during development, in response to synaptic inputs and to other extracellular signals (Cingolani & Goda, 2008; Hotulainen & Hoogenraad, 2010; Sekino et al., 2007).

#### 4.2.1. Proteins regulating actin filament nucleation and polymerization

Even when the assembly of actin onto filaments is thermodynamically favourable in physiological conditions, spontaneous nucleation of actin monomers is highly inefficient in cells. Pure G-actin subunits are unable to nucleate a new filament because actin oligomers are unstable. Additional mechanisms are required. Thus,
actin nucleating factors help stabilizing the newly formed actin filaments providing a seed that is resistant to depolymerization. Subsequently, actin polymerizing factors catalyse the process of subunit addition so the net balance between polymerization and depolymerization is positive and the filament grows from the plus (or barbed) end (Lodish et al., 2000).

One major actin filament nucleating factor present in lamellipodia, among other structures, is the Arp2/3 complex. This complex of 7 subunits (two actin-related proteins at the core, Arp2 and Arp3, as well as ARPC1-5) is enriched in spine heads (Rácz & Weinberg, 2008), where it promotes branching of the actin filament. Arp2/3 complex binds to the side of an existing filament and the daughter filament will grow at a 70° angle in respect to the original filament (Goley & Welch, 2006).

Notably, the Arp2/3 complex has a central role in regulating spine development as knockdown of Arp3 reduces the spine density in forebrain (A. M. Wegner et al., 2008). In addition, postnatal depletion of Arp2/3 leads to asymmetric structural synaptic plasticity followed by spine loss and progressive behavioural deficits in mice (I. H. Kim et al., 2013). The Arp2/3 complex is a primary downstream target of N-WASP and WAVE1 in spines (Takenawa & Suetsugu, 2007). Due to the low catalytic activity of Arp2/3 complex on its own, the interaction of WASp or WAVE proteins is needed to form a nucleation trimer that drives actin polymerization. While the V domain of WASp/WAVE interacts with an actin monomer, the Arp2 and 3 work as “pseudoactin” and the polymerization in Y shape is facilitated (Kurisu & Takenawa, 2009). Interestingly, also the F-actin-interacting protein cortactin is an activator of Arp2/3 complex and it is enriched at the PSD, directed there by its interaction with Shank. Depletion of cortactin reduces spine density, demonstrating its role in spinal actin cytoskeletal changes (Hering & Sheng, 2003; Iki, Inoue, Bito, & Okabe, 2005).

Profilin is another important regulator of actin polymerization and it operates together with WAVE/WASp proteins. This small protein contains interaction surfaces for both actin monomers and polyproline sequences (Witke, 2004). Profilin promotes rapid assembly of filaments by allowing monomer addition to the barbed ends of the filament while inhibiting monomer addition to pointed ends. Interestingly, profilin also suppresses spontaneous filament assembly. Filaments with free barbed ends will grow with the help of profilin until capped by capping proteins (Schafer, Jennings, & Cooper, 1996). The main isoform of profilin in the brain is profilin II. Interestingly, not all spines contain profilin in basal conditions, but upon synaptic stimulation the ABP is trafficked to active spines. Its importance in spine stabilization is highlighted by the fact that interference of synaptic targeting of profilin causes spine destabilization (Ackermann & Matus, 2003).

Profilin interacts also with the formin family of proteins. Formins are large multidomain proteins that nucleate actin filaments and promote their growth via a distinct mechanism, known as “tip nucleation model”. In contrast to Arp2/3 complex, formins do not nucleate branched but linear actin filaments. Also, formins do not contain actin-like domains. Therefore, they promote assembly of microfilaments in structures
such as actin cables, filopodia, stress fibres, and actin-rich cell adhesions (reviewed in Goode and Eck 2007). The formin homology 2 (FH2) domain of formins nucleates the actin filaments, so that the profilin-actin complexes are inserted between the FH2 domain and the plus end. Profilin remains bound to the barbed end protecting it from the action of capping proteins (Pruyne et al., 2002). The FH1 domain is able to recruit profilin-actin complexes, stimulating the process of filament growth (Chang, Drubin, & Nurse, 1997; Evangelista et al., 1997). Mammalian Diaphanous-2 (mDia2) is a member of the formin family of actin polymerizing proteins. It is implicated in the formation of canonical filopodia in assorted cell types and therefore in cell locomotion (Goode & Eck, 2007). mDia2 is activated and targeted to the plasma membrane by small GTPase Rif (Gorelik, Yang, Kameswaran, Dominguez, & Svitkina, 2011).

4.2.2. Proteins depolymerizing actin filaments
Actin depolymerizing factors (ADF)/cofilins are present in all cell types and they sever and depolymerize actin filaments, allowing the free G-actin pool to have an adequate size. These proteins are key regulators of the actin filament length and treadmilling rate, and bind both actin monomers and filaments. Cofilin-1 has a much higher affinity for ADP-actin than for ATP-actin, and therefore it binds preferentially to the side of the actin cytoskeleton towards the pointed end of the filament, twisting it and causing filament instability, which will lead to filament disassembly. Cofilin can also sequester actin monomers, impeding their addition to the filament (Carlier, 1997). There are two isoforms of ADF/cofilins present in the CNS of vertebrates: cofilin-1 (non-muscular or n-cofilin) and ADF (or destrin). Cofilin-2 (m-cofilin) is the main isoform in the muscle tissue. Cofilin-1 is the main isoform responsible for actin depolymerization and severing in the brain. In situ hybridization and western blotting analysis reveal a general distribution of both isoforms throughout the CNS, at all developmental stages analyzed (E12-P10) with a ratio of ADF:cofilin-1 up to 1:6. Despite the colocalization of the two isoforms, mice lacking ADF (ADF KO mice) show no alterations in brain morphology and general development while conditional cofilin-1 KO mice had highly reduced survival rate. The few surviving cofilin-1 conditional KO mice had a severe phenotype including ataxia and epileptic seizures. Detailed investigation of the brain anatomy revealed defects in radial and tangential neuronal migration and concomitant cell division abnormalities. Moreover, cell morphology is aberrant in neurons of cofilin-1 conditional KO mice (Bellenchi et al., 2007). Importantly, genetic ablation of cofilin-1 exclusively in principal neurons in the mouse cortex revealed the importance of this depolymerizing protein for synaptic plasticity and non-associative learning (Rust et al., 2010). In spines, cofilin localizes to the shell (cortical area) and within the postsynaptic density (Racz & Weinberg, 2006).

LIM kinases (LIMK) are known to phosphorylate cofilin-1 at Ser3, inhibiting its actin-binding activity and thus the capacity of disassembling F-actin. LIMK appear to be necessary for normal spine development and synaptic connectivity (Reviewed in (Meng, Zhang, Tregoubov, Falls, & Jia, 2003)). Moreover, ADF/cofilin-1 phosphorylation is related to the formation of long-term potentiation (LTP), increased spine head size
(Fedulov et al., 2007) and, more recently to AMPAR trafficking to spines (Gu et al., 2010). On the other hand, dephosphorylation of ADF/cofilin-1 is linked to long-term depression (LTD) and spine shrinkage (Zhou, Homma, & Poo, 2004). Notably, in patients with the intellectual disability and developmental disorder Williams’ syndrome, there is a mutation in LIMK-1 and the regulation of the cofilin-1 pathway is also affected (Frangiskakis et al., 1996). In addition to LIMK, several molecular pathways regulate cofilin-1 phosphorylation state. Phosphatases slingshot and chronophin are highly specific for cofilin-1. Moreover, cofilin-1 is regulated by oxidative stress, pH and phosphoinositides, among others (reviewed in Bernstein & Bamburg, 2010).

4.2.2. Rho-family of small GTPases

The Rho GTPase family members, a subfamily of Ras superfamily of small GTPases, are important regulators of many critical steps during neural development from neuronal migration, axon growth and guidance to dendritic arborisation and synaptic formation (Tolias, Duman, & Um, 2011). Rho GTPases are critical regulators of actin cytoskeleton remodelling in dendritic spines. Importantly, small GTPases convey the signal from the membrane anchored receptor molecules to actin binding proteins. In most cases they do so through GTPase effector molecules (Penzes & Cahill, 2012).

All GTPases are active when bound to GTP and inactive when bound to GDP. The exchange of GDP for GTP is catalysed by guanine nucleotide exchange factors (GEFs) and the inverse process is facilitated by GTPase activating proteins (GAPs), which promote the hydrolysis of GTP into GDP, inactivating the GTPase. Moreover, the GTPase activity is controlled by guanine nucleotide dissociation inhibitors (GDIs) that can bind directly to certain GTPases and localize them in the cytoplasm, away from the membrane and therefore from their activating molecules and targets (for a review see (Heasman & Ridley, 2008)).

Several GTPases are involved in spine morphogenesis. So far, the most extensively studied are the Rho family of small GTPases including RhoA, Rac1 and Cdc42. These GTPases have been shown to have antagonistic roles in spine formation: while Rac1 and Cdc42 have been shown to promote spine formation, RhoA inhibits spine formation (Nakayama, Harms, & Luo, 2000; Tashiro, Minden, & Yuste, 2000). Cdc42 is the canonical GTPase responsible for filopodia formation in non-neuronal cell types (Kozma, Ahmed, Best, & Lim, 1995; Nobes & Hall, 1995). In neurons, depletion of Cdc42 by means of RNA interference (RNAi) as well as overexpression of dominant negative forms of Cdc42 reduced spinogenesis (Irie & Yamaguchi, 2002; A. M. Wegner et al., 2008). Interestingly, overexpression of dominant negative Cdc42 caused the dendritic protrusions to show increased length and filopodia-like morphology (Irie & Yamaguchi, 2002).

There is an increasing amount of evidence showing that Rac1 has a positive role on spine morphogenesis by stimulating F-actin polymerization and consequent spine stabilization. Several studies have overexpressed mutant forms of Rac1 incapable of interacting with GEFs in dissociated hippocampal neurons and slices with similar results. The neurons show dramatic reduction in spine density and impaired synapse formation...
(Nakayama et al., 2000; Penzes et al., 2003; Zhang, Webb, Asmussen, & Horwitz, 2003). Strikingly, studies in which constitutively active Rac1 was expressed also show impaired synaptogenesis with aberrant spine morphology (Luo et al., 1996; Nakayama et al., 2000; a Tashiro et al., 2000; A. Tashiro & Yuste, 2004). Therefore, fine tuning of GTPases like Rac1 is extremely important. The dynamic range in which Rac1 promotes spine stabilization and synapse formation is narrow and tightly regulated. In their work from 2010, Hayashi-Takagi and colleagues propose a molecular mechanism to explain why constitutively active Rac1 reduces the size of spines. They elegantly demonstrate that DISC1 (disrupted in schizophrenia 1), by regulating the spatial availability of Rac1 to interact with its GEFs (kalyrin-7 in this case) in response to NMDA receptor activation, acts as an important regulator of spine head enlargement in both cortical cultures and rat brain in vivo (Hayashi-Takagi et al., 2010).

The effects of RhoA on actin are, at least partially, mediated by the formin mDia. RhoA stimulates the nucleating function of the formin by direct binding to it (Ridley, 2006). RhoA can also affect the actin cytoskeleton in neurons through Rho-associated kinases (ROCKs) (Nakayama, Harms, & Luo, 2000; Schmandke, Schmandke, & Strittmatter, 2007). In addition to negatively regulating the GTPases Cdc42 and Rac1, ROCK1 can activate LIM-kinase (Hirose et al., 1998). Importantly, ROCK inhibitors increase spine density (Kang, Guo, & Huganir, 2009).

Other members of the family of Rho small GTPases have received substantially less attention than those presented above. One example is the Rif GTPase (RhoF), an understudied member of the Rho family. Rif has low homology to other family members (Ellis & Mellor, 2000). The effect of Rif GTPase is mediated through the members of the formin family of actin nucleating proteins, concretely by Diaphanous-related formin-3 (DRF3/mDia2) (Gorelik et al., 2011; Pellegrin & Mellor, 2005). Interestingly, in non-neuronal cells, Rif and mDia2 control filopodia formation through an alternative pathway to that of Cdc42 and Rac1 (Goh et al., 2011; Pellegrin & Mellor, 2005). A recent study has shown a direct interaction of Rif with its effector protein mDia1. Moreover, the authors have followed the dynamics of filopodia induced by Rif in neuroblastoma-derived cell line by time lapse microscopy. Rif expressed together with GFP-actin produced shorter and more numerous filopodial-like protrusions in these cells than only GFP-actin (Goh et al., 2011).

Ras, the prototypical member of the superfamily of Ras family of small GTPases, is also involved in spine development, although its role has received considerably less attention than Rac1 and Cdc42. Ras has been shown to promote spinogenesis. Mice expressing constitutively active Ras have increased spine density in pyramidal neurons (Arendt et al., 2004). One very interesting characteristic of the Ras GTPase is that it might be part of the facilitation mechanism by which the spines that are in close proximity to an active spine have greater possibility to get enhanced themselves. Indeed, inducing spine-specific plasticity -by single spine glutamate uncaging- led to the activation of Ras in a portion of the dendrite. Activated Ras spread and
invaded neighbouring spines by diffusion. As a consequence, the neighbouring spines needed only sub-threshold synaptic stimulation to increase their volume (Harvey, Yasuda, Zhong, & Svoboda, 2008).

4.2.3. Downstream effectors of small GTPases: regulators of ABPs

One of the most studied effectors of the GTPases Rac1 and Cdc42 is the p21-interacting protein (PAK) (Masner et al. 1994). The GTPases activate PAK by releasing it from its intramolecular autoinhibition (Knaus et al. 1998, Tu and Wingler 1999). Members of the group I of PAK molecules, including PAK1-3, can be regulated by Rac1 and Cdc42 with different efficiencies, with Cdc42 being a broader activator of PAKs than Rac1 (Kreis et al. 2007). Upon PAK activation by the small GTPases, the interaction with LIM-kinase and its subsequent activation is facilitated (Edwards 1999). The kinase, in turn, phosphorylates cofilin-1, causing its deactivation (Arber et al., 1998; N. Yang et al., 1998). Also other small GTPases regulate cofilin activity although the mechanism is not clearly established. For example, deletion of the Ras-inactivating protein Syn-GAP in mice leads to impaired NMDAR-mediated cofilin regulation and excess in mushroom spines in adult mice (Carlisle, Manzerra, Marcora, & Kennedy, 2008).

In order to study the role of LIMK 1 and 2 in the regulation of cofilin-1 phosphorylation, Meng and colleagues created a transgenic mice line devoid of LIMK1. The mice showed impaired synaptic function and abnormal spine morphology. Moreover, at the cellular level, they observed enhanced LTP in these mice, which could explain their altered spatial learning and fear responses (Meng et al., 2002). Thereafter, the investigators generated LIMK2 KO mice, which showed no differences with the WT mice in terms of cofilin-1 phosphorylation, gross CNS anatomy or synaptic plasticity. However, double KO mice LIMK 1/2 were more severely impaired than the LIMK2 KOs, with enhanced LTP (similarly to LIMK1 KO) and more pronounced phospho-cofilin-1 deficit (Meng et al., 2004).

Another well characterized pathway through which Rac1 and Cdc42 regulate the actin cytoskeleton is by direct activation of the Wiskott-Aldrich syndrome protein (WASP) family, which includes WASP and neural-WASP (N-WASP). These GTPase effectors contain a Cdc42/Rac interactive binding region (CRIB domain) and a WH1 domain (Egile et al., 1999). Intramolecular interactions maintain WASPs inhibited in resting conditions, until the signal comes through Rac1 or Cdc42 and the GTPases relieve their inhibition (S. Kim et al., 2000; Rohatgi, Ho, & Kirschner, 2000). In turn, WASPs interact with the nucleating protein Arp2/3 to promote actin filament assembly and polymerization.

WAVE-1 protein is yet another target of Rac1/Cdc42. The structure of WAVE-1 is similar to that of WASP proteins but is missing the GTPase CRIB binding domain (H Miki, Suetsugu, & Takenawa, 1998). Unlike WASPs, WAVE-1 is constitutively active, although its activity is normally inhibited by a protein complex. Rac1 relieves WAVE-1 inhibition by interacting with and disassembling its inhibitory complex (Eden, Rohatgi, Podtelejnikov,
Mann, & Kirschner, 2002). WAVE-1 is located in mature spines and is important for spine maintenance and maturation through the activation of the Arp2/3 complex (Y. Kim et al., 2006; Soderling et al., 2007).

The VCA region (verprolin homology, cofilin homology domain, acidic region) is located at the C-terminus of WASP and WAVE proteins. The V region binds to actin monomers, while the CA region binds to the Arp2/3 complex (Hiroaki Miki & Takenawa, 2003). Similarly to other cellular structures, the VCA region is crucial for actin polymerization in spines. Indeed, expression of a mutant N-WASP constructs lacking this region, as well as dominant-negative N-WASP, results in reduced spine density (A. M. Wegner et al., 2008).

Gelsolin is another ABP controlled by Rac1 and involved in actin regulation in spines. Rac1 negatively regulates actin-gelsolin interaction in non-neuronal cells (Arcaro, 1998). Gelsolin severs actin filaments in a calcium-dependent manner (Furukawa et al., 1997; Kinosian et al., 1998) and it also caps the barbed ends of the filaments to avoid addition of actin subunits (H. E. Harris & Weeds, 1984). Additionally, dissociation of gelsolin from actin stimulates Arp2/3 complex activity (Falet et al., 2002). Importantly, genetic ablation of gelsolin in neurons does not affect actin dynamics at basal conditions but, in response to electric stimulation, gelsolin stabilizes actin in synapses (Star et al., 2002).

4.2.4. Guanine nucleotide exchange factors

As GTPase regulators, guanine nucleotide exchange factors are key molecules that link the signals from the membrane receptors to the small GTPases. GEFs (together with GAPs) allow the context specific regulation of GTPases due to several interesting characteristics. First, GEFs show large variety. There are over three times more GEFs than GTPases. This characteristic allows the diversification of the signals arriving to the GTPases. Also, GEFs are developmentally strictly regulated. Their expression pattern is narrower than that of the GTPases. Not only is their subcellular distribution tightly regulated, but the GEFs sometimes also have intramolecular inhibitory interactions working as intrinsic activity regulators, conferring even higher specificity to their signalling. Certain GEFs can be promiscuous while others have been shown so far to activate one particular GTPase. Finally, GEFs exhibit different domains by which they can integrate the signals arriving to the GTPases at the same time as they work as scaffolding proteins (Penzes, Cahill, Jones, & Srivastava, 2008; Tolias et al., 2011).

GEFs regulate their GTPases by catalysing the exchange of GDP for GTP, they take advantage of the high intracellular ratio of GTP:GDP. When the GEF binds physically its preferred GTPase, it destabilizes the interaction of the GTPase with GDP, favouring a nucleotide-free and Mg$^{2+}$-free intermediate that is unstable and will easily be charged with GTP and Mg$^{2+}$. The Dbl homology (DH) domain present in all GEFs is responsible for this catalysing activity of the protein and it seems to be sufficient for the exchange (Rossman, Der, & Sondek, 2005; Schmidt & Hall, 2002).
Every guanine nucleotide exchange factor possesses a combination of multiple functional domains that makes it unique. Apart from the DH, other domains are commonly present in these large proteins. For example, the pleckstrin homology domain (PH) serves the protein to bind certain forms of phosphatidylinositol lipids in the plasma membrane, where the GEF will interact with the GTPases. The PH domain is present in all GEFs responsible for actin cytoskeleton regulation in dendritic spines and it is often in tandem with the DH domain. Some of the GEFs, like Kalirin and Trio show more than one tandem DH-PH. Another feature present in many GEFs is the Src homology (SH3) domain, commonly occurring in proteins that belong to signaling pathways regulating the cytoskeleton. The SH3 domain will serve as a docking station, mediating the specific assembly of protein complexes, typically via proline-rich areas of their interacting proteins. PDZ binding motives are also common in GEFs implicated in regulation of post-synaptic structures. As mentioned before, PDZ (PSD-95, Drosophila disc large tumour suppressor (Dlg1), and zonula occludens-1 protein (zo-1))-binding motives allow proteins to interact with scaffolding proteins at the PSD. Other domains appearing in GEFs can include coiled-coil regions, spectrin-like repeats, protein kinase conserved regions, etc. (Tolias et al., 2011)

4.2.4.1 The guanine nucleotide exchange factor βPix

βPix (p21-activated kinase interacting exchange factor, p85cool, ARGHEF7) is a GEF for the GTPases Rac1 and Cdc42 (E Manser et al., 1998). Thus, βPix stimulates Rho-dependent signals that are regulated upstream by extracellular stimuli through G protein-coupled receptors and others.

**Figure 7. Schematic representation of the GEF βPix, neuronal-specific b isoform.**

N-terminus- SH3 domain, DH: Dbl homology, GBD: GIT1 binding domain, Pro: proline-rich stretch, CC: coiled-coil, PDZb: PDZ-binding domain- C-terminus.

4.2.4.1.1. Expression

Immunoblot analysis detects four to five isoforms of βPix, a-d. While βPixa is ubiquitously expressed, βPixb and c are restricted to CNS and testis, and βPixd is enriched in the neural tissue. Finally, βPixbL is a splice variant of the b isoform (S. Kim et al., 2000; T. Kim & Park, 2001; Oh et al., 1997; Rhee, Yang, Lee, & Park, 2004). The neuron-specific isoforms βPix, b and c, as well as βPix d, are expressed at high levels in the brain during development. In adult tissue, βPix b, c and d are still highly present in the hippocampus and cerebellum, while their expression is moderated to low in cortex (S. Kim et al., 2000; T. Kim & Park, 2001).
Alike all GEFs, the main βPix isoform present in neurons, βPix b, consists of a unique combination of functional domains that will determine its precise function. Those include, from N- to C-termini: an SH3 domain, a DH-PH tandem, a GIT1-binding domain (GBD), a proline-rich segment and finally, a coiled-coil region, immediately followed by the terminal PDZ-binding domain (Figure 7) (Feng, Albeck, Cerione, & Yang, 2002; Tolias et al., 2011).

4.2.4.1.2. Regulation and function

βPix binds selectively to p21-activated kinases, PAK1 and 3, and activates them. The interaction occurs by means of the SH3 domain of βPix and the non-canonical proline-rich domain of PAK 1/3 (Bagrodia, Taylor, Jordon, Van Aelst, & Cerione, 1998; E Manser et al., 1998). The binding of βPix and PAK is negatively regulated by auto-phosphorylation of PAKs (Zhao, Manser, Loo, & Lim, 2000). Although PAKs have been shown to act both upstream (Obermeier et al., 1998) and downstream of Rac1 and Cdc42 GTPases (Edward Manser, Leung, Salihuddin, Zhao, & Lim, 1994; Martin, Bollag, McCormick, & Abo, 1995), they are widely regarded as important effectors of the cellular signalling of Rac1/Cdc42. Interestingly, activated PAKs can also phosphorylate βPix at Thr526, increasing its GEF activity as well as its membrane localization and creating a positive feedback loop (Shin et al., 2002). βPix binds Rac1 in a nucleotide-independent manner. The interaction is sufficient for Rac1 recruitment to membrane ruffles and focal adhesions (ten Klooster, Jaffer, Chernoff, & Hordijk, 2006). Therefore, these molecules form a signalling complex that contains both an upstream regulator and a downstream effector of the GTPases Cdc42 and Rac1. PAK has two main phosphorylation substrates which convey the signal towards the actin cytoskeleton. These are myosin regulatory light chain kinase (MLCK) and LIM kinases (UMK). Upon phosphorylation by PAK1, the ability of MLCK to activate the light chain of myosin is hindered (Sanders, Matsumura, Bokoch, & de Lanerolle, 1999). In the case of LIM kinases, their phosphorylation by PAK1 promotes, in turn, phosphorylation of coflin-1 and subsequent deactivation of the actin-depolymerizing protein which results in higher actin filament stability (Arber et al., 1998; N. Yang et al., 1998).

The ADP-ribosylation factor-GAP (Arf-GAP) and scaffolding protein GIT1 are important components of the βPix/PAK/Rac1 signalling complex. Since GIT1 is a G protein-coupled receptor kinase-associated protein, activation of the receptor promotes membrane recruitment of the whole complex (R T Premont et al., 1998; Richard T Premont et al., 2004). The interaction of GIT1 with paxillin (Bagrodia et al., 1999; Turner et al., 1999) links the complex to integrin-dependent specialized actin structures situated beneath the cell membrane, denominated focal contacts (Oh et al., 1997). Interestingly, GIT1 and βPix promote focal contact disassembly (Zhao et al., 2000).
Figure 8. Regulation of βPIX. The GEF βPIX is regulated in postsynaptic areas by upstream membrane receptors and PSD proteins. In turn, βPIX interacts with and activates Rho small GTPases Rac1 and Cdc42. Here only the Rac1 pathway is highlighted. In addition, βPIX binds to the actin-pathway effectors PAK1 and N-WASP and regulates their activity.

βPix in synapses
Synaptic targeting of βPix is regulated by postsynaptic proteins. In 2003, Eunhye Park and colleagues reported a novel interaction between the C-terminal domain of βPix and the PDZ domain of the postsynaptic scaffolding protein Shank, which plays an important role in organizing the PSD. The authors showed that βPix colocalizes with Shank and synaptophysin in cultured hippocampal neurons, but is also present in other parts of the neurons, including axons. Remarkably, when the authors overexpressed Shank1B in hippocampal neurons, the colocalization of endogenous βPix with Shank, as well as the immunostaining density of βPix at the synaptic area, increased. Accordingly, Shank was able to recruit PAK to synaptic sites (E. Park et al., 2003). More recently, the crystal structure of the interplay between the PDZ-binding domain of βPix and the PDZ domain of Shank was solved, determining the stoichiometry of the complex formation. The C-terminal coiled-coiled domain, situated upstream of the PDZ-binding domain, allows trimerization of βPix. The βPix trimer associates with a single Shank PDZ domain (Im et al., 2010).

As discussed previously, dynamic rearrangement of the actin cytoskeleton in spines is greatly mediated by the localized activation of Rac1 and the spine shape is maintained by a balanced activation between Rac1 and Rho (A. Tashiro et al., 2000; A. Tashiro & Yuste, 2004). The first evidence of the implication of βPix in these processes came from the group of Alan Horwitz (2003). Zhang and colleagues studied the role of the Arf-GAP GIT1 in synapse formation. Using deletion mutants, they defined a minimum synaptic localization domain within the protein. Ectopic overexpression of the synaptic localization domain reduced the number of mushroom shaped spines and increased the population of long, thin dendritic protrusions. Interestingly, this overexpression was able to diminish localization of βPix in the spines, indicating that the synaptic localization of βPix partly depends on GIT1 and that precise targeting of βPix at these postsynaptic sites is crucial for proper spine formation. Moreover, neurons expressing mutations in the Dbl-homology (DH) domain of βPix that abolished its GEF activity showed a significant decrease in both dendritic spines and protrusions. The authors proposed that the spine phenotype is due to mislocalized activation of Rac1 outside of the spines (Zhang et al., 2003). In their publication of 2005 (Zhang, Webb, Asmussen, Niu, & Horwitz, 2005), they went on to demonstrate this hypothesis using a Förster Resonance Energy Transfer (FRET) probe for Rac1. The probe contains the Rac1 p21-binding domain, YFP and CFP in the same cDNA and is denominated Raichu-Rac1. Interestingly, when expressing the Raichu probe together with wild type βPix, overall FRET efficiency increased, indicating that βPix is able to activate Rac1 in cultured cells. Interestingly, βPix to Raichu probe overexpression at a ratio 5:1 provoked not only higher FRET efficiency throughout dendritic protrusions but also at the dendritic shafts, as opposed to just the base of dendritic protrusions in control cultures. When dominant negative βPix was expressed, the overall FRET efficiency, as well as the number of dendritic spines, was decreased. These results suggested that βPix regulates Rac1 activation, and locally regulated Rac1 activation is necessary for spine formation. In the same study, they knocked down adaptor protein GIT1 to show its role in spine morphology and they used PAK1 and PAK3 mutants to demonstrate their ability to
Induce the formation of dendritic spines as well as long, thin dendritic protrusions. These effects of PAK1 and PAK3 relied on their kinase activity that phosphorylates myosin II regulatory light chain (MLC). Therefore, the authors propose a mechanism to regulate dendritic spine and synapse formation by means of the signalling complex: GIT1, βPix, Rac1 and PAK1/3, which is able to induce actin cytoskeleton remodelling through modulating MLC activity.

Some years later, the group of Premont generated a mouse line for GIT1 that lacked the protein throughout the nervous system. The lack of GIT1 caused premature death to many of the animals but some of them survived and had a normal lifespan and fertility. The authors showed that the mice lacking GIT1 had impaired responses to fear conditioning, as mice would not freeze upon presentation of second conditioned stimulus. The GIT1 KOs showed no difference in behavioural tests aimed to measure anxiety response and depression (Schmalzigaug et al., 2009). These results pointed towards a regulation of memory formation by GIT1. Following this line of thought, the group of Berk, generated a new global GIT1 KO mouse line. They studied the gross anatomy of the brain as well as hippocampus and found no difference between the KOs and the WT littermates. However, at the cellular level, they observed an important reduction of the dendritic length and spine density in hippocampal neurons of the GIT1 KO mice. The functional relevance of these results is highlighted by the fact that the GIT1 KO mice have reduced adaptation to new environments (Menon et al., 2010). In conclusion, the phenotype of GIT1 KO mice could be, at least partially, due to impaired synaptic localization of βPix.

In an attempt to elucidate possible cascades upstream of the signalling complex GIT1/βPix/PAK/Rac1 in neurons, the group of T.R. Soderling studied how a local calcium increase might activate this pathway, leading to actin remodelling in dendritic spines during synaptic signalling. They show that calmodulin-kinase-kinase beta (CaMKKβ) and calmodulin-kinase I (CaMKI) respond to NMDA-mediated Ca^{2+} increase in neurons. CaMKI phosphorylates βPix at its residue Ser516, which increases its GEF activity toward Rac1. Mutations of the residue that rendered βPix incapable of phosphorylation at this site (S516A) provoked a reduced number of spines in cultured neurons as did another construct lacking a functional GEF domain (DH mutant). Similarly, siRNA for βPix provoked a decrease in the number of spines that could be restored by expressing resistant forms of WT βPix but not S516A βPix. Interestingly, knocking down the expression of the GEF had an effect on spine length, with the average length increasing, while the width of the spine head was not altered. Finally, glutamatergic transmission was assessed on cultured neurons. Transfection with S516A βPix significantly reduced the frequency of miniature excitatory postsynaptic currents (mEPSCs) by 60% while their amplitude was only slightly decreased (Saneyoshi et al., 2008).

More recently, a study has found a new interacting partner for the neuron-specific isoform βPixb. Neuronal specific βPixb binds N-WASP (neuronal Wiskott–Aldrich syndrome protein) in cells and brain tissue through the proline-rich domain of βPix and the WH1 domain of N-WASP. N-WASP is a well characterized activator of
the actin-nucleating complex Arp2/3. The authors showed that, in a GEF-dependent manner, βPixb regulates dendritic spine formation and they proposed a mechanism through direct binding to N-WASP (J. Park, Kim, Park, Park, & Chang, 2012).

5. Regulation of spine development by cell adhesion molecules (CAMs)

Synapses are sites of asymmetric cell to cell communication and, as such, their structure is largely controlled by adhesion molecules. A large number of these proteins, commonly denominated synaptically localized adhesion molecules (SAMs), have been identified so far and their roles in synapse formation and maintenance go beyond the first contacts between neurons. Importantly, interactions between adhesion molecules regulate target recognition, synapse formation, dendritic spine morphology; they modify receptor function and synaptic plasticity. Notably, SAMs are nowadays understood as signal transducing proteins rather than mere structural components (Dalva, McClelland, & Kayser, 2007; Washbourne, Dityatev, et al., 2004; Yamagata, Sanes, & Weiner, 2003).

Most SAMs are transmembrane proteins. They possess an extracellular domain through which they bind their partners across the synaptic cleft. Synaptic CAMs commonly span the plasma membrane one single time. Their cytoplasmic domains bind adaptor proteins that organize the post-synaptic structure and, importantly, convey signals towards the cell. This characteristic confers SAMs the ability to act as bidirectional molecules. SAMs can modify the synaptic structure and function through their cytoplasmic portion while conveying signals across the synaptic cleft through interaction with their ligands.

The most relevant synaptic CAMs include integrins, immunoglobulin superfamily proteins (IgSF), such as SymCAM, neurexins, neuroligins and cadherins. The extracellular components of these proteins contain diverse cell adhesion domains including immunoglobulin (Ig-) domains (homo- or heterophilic binding), fibronectin III domains (commonly occurring in Ig-containing proteins), cadherin domains (whose interactions are Ca$^{2+}$-dependent and often homophilic), sex hormone-binding protein domains (laminin G-like domains, which also have a conserved Ca$^{2+}$-binding site), EGF-like domains and leucin-rich repeats (LRRs) (Missler, Südhof, & Biederer, 2012).

Regulation of the actin cytoskeleton in dendritic spines and subsequent spine morphogenesis by adhesion molecules represents a crucial mechanism to reshape spines in response to contact with the presynaptic cells, the extracellular matrix (ECM) as well as other cell types. N-cadherin is the best studied CAM in relation to dendritic spine development. It is localized to the surrounding of synapses (Uchida, Honjo, Johnson, Wheelock, & Takeichi, 1996) and it plays a broad regulatory role in synapse development, rather than affecting synapse density (Jüngling et al., 2006). Neuronal cadherin (N-cadherin) regulates spine morphogenesis by its phosphorylation-dependent binding to β-catenin. A specific tyrosine needs to be dephosphorylated by tyrosine phosphatases in order to localize from the dendritic shaft into the spines. After
binding N-cadherin, β-catenin interacts and modifies the actin cytoskeleton (Dunah et al., 2005; Murase, Mosser, & Schuman, 2002).

Along with classical CAMs, tyrosine kinase receptors affect synapse formation. Such is the case of EphB receptor tyrosine kinases which, upon binding to their pre-synaptic partners, ephrin ligands, produce a postsynaptic cascade recruiting other molecules like RhoGTPases Rac1 and Cdc42 and result in the remodelling of the actin cytoskeleton and the postsynaptic spines (Lai & Ip, 2009). Indeed, reverse signalling through the transmembrane ligands for EphR, ephrinB triggers a signal involving the SH2 and SH3 domain-containing protein Grb4 and the G protein-coupled receptor GIT1. In turn, these adaptor proteins recruit GEFs, i.e. βPix, that convey the signal towards small GTPases Rac1 and Cdc42. Disruption of this pathway negatively impacts actin cytoskeleton regulation and, consequently, spine morphogenesis and synapse formation (Segura, Essmann, Weinges, & Acker-Palmer, 2007).

Accumulating evidence pinpoints the relevance of the functions of SAMs. Notably, numerous studies have reported mutations in the genes encoding neuroligins 1-4 in patients with autism-spectrum disorders. Additionally, mutations in genes taking part of the neurexin-neuroligin complexes, such as Shank3, have been found in autistic patients. The importance of some of these mutations to the development of the disorder has been confirmed by the generation of mice models that replicated the phenotype of the illness (reviewed in (Südhof, 2008)).

5.2.1. ICAM-5

The intercellular adhesion molecule-5 (ICAM-5) or telencephalin (TLN or TLCN) is a forebrain-specific member of the ICAM family that belong to the Ig-super family (Ig-SF). The five members of the ICAM family are numbered 1-5 and they have important roles in mediating cell-to-cell adhesion or cell-to-extracellular matrix adhesion and signalling. Of the five, ICAM-5 is the only brain-specific member and it is expressed exclusively in neurons. ICAM 1-4 are membrane proteins expressed by immune system-related cells, such as leucocytes, platelets and endothelial cells, and therefore they are important components of the immune response (Gahmberg, Tolvanen, & Kotovuori, 1997).

All members of the ICAM family possess multiple Ig-domains in the extracellular portion. In the case of ICAM-5, there are nine Ig-domains that interact in a homo- or heterophilic fashion 

in trans, across the synaptic cleft, with molecules such as presenilin and two integrins, LFA-1 and β1 integrin. Through the cytoplasmic domain, ICAM-5 interacts with ERM (ezrin, radixin, moesin) proteins (Furutani et al., 2007) and α-actinin (Nyman-Huttunen, Tian, Ning, & Gahmberg, 2006).
The intercellular adhesion molecule is important for neuronal development and it serves its role due to four unique characteristics. First of all, ICAM-5 is expressed solely in the brain; more precisely, its expression is telencephalon-specific. Secondly, ICAM-5 is not present in all neurons, but only in spiny neurons. In the rodent olfactory bulb, it is expressed in granule cells but not in tuft cells, mitral neurons or principal neurons (Murakami, Tada, Mori, Oka, & Katsumaru, 1991). In the hippocampus, ICAM-5 is expressed by pyramidal neurons but absent from GABAergic inhibitory neurons (Benson, Yoshihara, & Mori, 1998). Thirdly, axons are devoid of ICAM-5. The molecule contains a 17 amino acid long sequence in the C-terminal tail that has been found responsible for its dendrite-compartment specificity (Benson et al., 1998; Mitsui, Saito, Hayashi, Mori, & Yoshihara, 2005). Finally, the developmental expression pattern of ICAM-5 parallels the dendritic development and synaptogenesis in telencephalon (Imamura, Mori, Oka, & Watanabe, 1990; Mori, Fujita, Watanabe, Obata, & Hayashi, 1987). Indeed, in rodents, ICAM-5 starts expressing upon birth and its levels increase during the early postnatal weeks, reaching levels that will be stable during adulthood (Yoshihara et al., 1994).

The above mentioned characteristics make the molecule ICAM-5 an interesting candidate as regulator of dendritic outgrowth and synaptogenesis. Through the cytoplasmic region, ICAM-5 interacts with α-actinin, which links it to the actin cytoskeleton. This interaction has been shown to promote neurite outgrowth in activated Paju cells (Nyman-Huttunen et al., 2006). Importantly, ICAM-5 and α-actinin colocalize in neuronal soma and dendritic shafts (Furutani et al., 2007). In addition, two studies showed that chimeric ICAM-5-Fc protein in young hippocampal neurons produces neurite outgrowth and arborisation (Tamada, Yoshihara, & Mori, 1998; Li Tian et al., 2000).

In regard to the development of dendritic spines and synaptogenesis, it is important to note that ICAM-5 is highly localized to dendritic filopodia. The sequence of ICAM-5 determines the targeting to these dendritic
protrusions (Ohgomori, Nanao, Morita, & Ikekita, 2012). In 2006, the groups of Mori and Yoshihara showed that ICAM-5 facilitates filopodia formation and maintenance and it slows maturation of dendritic spines (Matsuno et al., 2006). In 2007, the same research groups went on to describe yet another connection between ICAM-5 and the actin cytoskeleton, mediated by members of the ERM (ezrin, radixin, moesin) family of proteins. In this study, they showed that active forms of ERM proteins colocalize with ICAM-5 in filopodia and that RNA-mediated knockdown of ERM proteins leads to decreased density of filopodia in hippocampal neurons (Furutani et al., 2007). A very recent study has performed ultrastructural localization of ICAM-5 in developing visual cortex by transmission electron microscopy. The study confirms that the abundance of ICAM-5 in dendritic spines at P14 is higher than later, at P28. This developmental shift in localization fails to occur in matrix metalloprotease-9-deficient mice (MMP-9 KOs), indicating the relevance of the cleavage of ICAM-5 by MMP-9 in the process (Kelly, Tremblay, Gahmberg, Tian, & Majewska, 2014). ICAM-5 binds to α-actinin and this cytoskeletal anchorage is important for ICAM-5-mediated neurite outgrowth (Nyman-Huttunen et al., 2006). Upon ICAM-5 cleavage, the interaction with α-actinin is lost, promoting the clustering of α-actinin and triggering actin cytoskeleton reorganization (Ning, Paetau, Nyman-Huttunen, Tian, & Gahmberg, 2015; Li Tian et al., 2007). Moreover, the activation of NMDA receptors leads to increased interaction of GluN1 receptor subunit to α-actinin. Thus, NMDA receptor signalling promotes a change in binding partner for α-actinin, from ICAM-5 to GluN1. This change in interaction partner promotes actin cytoskeleton rearrangements leading to spine maturation (Ning et al., 2015).

Additionally to its role in neuronal development, ICAM-5 is crucial for immunity in the brain and its action can have opposite outcomes depending on the domains of the protein involved. On one hand, full-length ICAM-5 expressed by neurons binds to the integrins present in T-lymphocytes and presents the T-cells to the surrounding glial cells, activating their immune response. This immune response might lead to either inactivation or apoptosis of the cell (Li Tian, Yoshihara, Mizuno, Mori, & Gahmberg, 1997; Li Tian et al., 2000). On the other hand, when ICAM-5 is cleaved by matrix metalloproteases MMP-2 and -9, the soluble extracellular portion, sICAM-5, can bind to T-cells and its binding has a totally different outcome; it suppresses T-cell activation. Therefore, sICAM-5 can act as an anti-inflammatory factor and it contributes to the immune privilege of the brain (Li Tian et al., 2008).

Although sICAM-5 can work as a suppressor of the inflammation, abnormally high levels of MMPs have been related to several CNS immune disorders and they impair synaptic plasticity. Importantly, elevated levels of ICAM-5 in the cerebrospinal fluid and serum have been found in patients of diseases such as acute encephalitis, ischemia and temporal lobe epilepsy (Borusiak, Gerner, Brandt, Kilgannon, & Rieckmann, 2005; Guo et al., 2000; Lindsberg et al., 2002; Rieckmann, Turner, Kilgannon, & Steinhoff, 1998).

6. Regulation of spine development by membrane proteins
6.1. KCC2

The potassium chloride cotransporter 2 (KCC2) belongs to the non Na\(^+\)-dependent branch of the solute carrier 12 (SLC12) family of cation-chloride cotransporters (CCCs). The CCC family consists of nine members in mammals, encoded by the genes Slc12a1-9. The family contains two Na\(^+-K\(^+\)-2Cl\(^-\) cotransporters (NKCCs isoforms NKCC1 and NKCC2), four K\(^+\)-Cl\(^-\) cotransporters (KCCs isoforms KCC1-4) and one Na\(^+\)-Cl\(^-\) cotransporter (NCC). KCC2 together with eight other members of the family have been described as plasmalemmal ion transporters (Gamba, 2005; Mercado, Mount, & Gamba, 2004; John A Payne, Rivera, Voipio, & Kaila, 2003). The two remaining cotransporters, CIP and CCC9, are less studied and the transported ions are still unknown (Caron, Rousseau, Gagnon, & Isenring, 2000; Hewett et al., 2002). NKCC1, NKCC2 and NCC generally take up Cl\(^-\) into the cell by means of the energy stored in the electrochemical potential of Na\(^+\) while KCC1-KCC4 use the transmembrane K\(^+\) gradient to transport Cl\(^-\) from the cytoplasm. Thus, CCCs are secondary active cotransporters. The direction of the transport is determined by the electrochemical gradient for every ion in any given moment and location (Blaesse, Airaksinen, Rivera, & Kaila, 2009; Kaila, 1994; J A Payne, 1997). So far, the information available on the tertiary structure of mammalian CCCs is greatly based on hydropathy analyses. In the case of NKCC1 the structure was further confirmed experimentally (T Gerelsaikhan & Turner, 2000; Tudevdagva Gerelsaikhan, Parvin, & Turner, 2006). The hydropathy analyses predict a twelve transmembrane domain structure with both N- and C-terminal ends being cytoplasmic for all CCCs but CCC9. In 2009, the group of Dutzler unveiled the X-ray structure of the C-terminal domain of a faraway relative of mammalian CCCs present in the archaea Mathanosarcina acetivorans. The structure is distantly related to universal stress proteins. Interestingly, the isolated protein formed dimers when in solution and the dimerization happened in the context of the full-length transporter as well. These data are consistent with the previously proposed dimeric structure of functional eukaryotic CCCs (Warmuth, Zimmermann, & Dutzler, 2009). In the case of KCC2, there has been much debate on the functional multimeric structure of the cotransporter. So far, technical challenges have

![Schematic representation of KCC2](image.png)

**Figure 10. Schematic representation of KCC2.** The putative structure of KCC2 consists of 12 transmembrane domains (TM). The predicted N- and C-terminus are situated in the intracellular side of the plasma membrane. Note the glycosylation sites in the large extracellular loop.
undermined the capacity to disentangle the issue due to the tendency of KCC2 to form high molecular mass aggregates that are resistant to the detergent SDS (reviewed in (Medina et al., 2014)).

Alternative splicing of the exon1 of KCC2 produces two isoforms, KCC2a and KCC2b. In 2007, Uvarov et al found an alternative N-terminal isoform of KCC2 and they noted it as KCC2a, as the alternative exon1 for KCC2a isoform is upstream of the one present in KCC2b. Exon1a and exon1b encode 17 and 40 amino acids, respectively (Uvarov et al., 2007). This finding denoted the fact that many of studies before 2007 had used primers, antibodies or probes to detect KCC2 based on the C-terminal part of KCC2 and were, therefore, recognizing both isoforms. Thus, unless otherwise noted, the term KCC2 will refer to both KCC2a and b in this thesis work. Importantly, KCC2a and KCC2b have distinct phosphorylation patterns and thus, the isoforms show different regulation. Additionally, the two isoforms can form heterodimers in vivo in neonatal rodent brain (Uvarov et al., 2009).

6.1.1. Expression

The expression of the KCC2 gene (SLC12A5) is very specific, being restricted to neurons of the CNS. In mature CNS, KCC2 is abundantly expressed in most brain areas. These include cortex (and hippocampus), cerebellum, olfactory bulb, retina, thalamus, brainstem and spinal cord (Kanaka et al., 2001; J. A. Payne, Stevenson, & Donaldson, 1996). Importantly, KCC2 is not expressed in the peripheral nervous system (PNS) (Kanaka et al., 2001; A. Okabe et al., 2003; J. A. Payne et al., 1996; Stein, Hermans-Borgmeyer, Jentsch, & Hübner, 2004) or in the choroid plexus (Kanaka et al., 2001). However, not all neurons in the CNS express KCC2. Primary sensory neurons in the dorsal root ganglion and the mesencephalic trigeminal nucleus, vasopressin-positive neurons in the thalamus (Barthó, Payne, Freund, & Acsády, 2004; Kanaka et al., 2001), as well as dopaminergic neurons in substantia nigra (Gulácsi et al., 2003), are devoid of KCC2. At the subcellular level, KCC2 is localized in a punctate fashion at the plasma membrane of neuronal soma and dendrites, including spines (Gulyás, Sík, Payne, Kaila, & Freund, 2001; Szabadics et al., 2006; Williams, Sharp, Kumari, Wilson, & Payne, 1999; Zhu, Lovinger, & Delpire, 2005). Interestingly, the cotransporter is absent from the distal part of axons, with axon initial segment being its most distal location, where the density of KCC2 immunoreactivity drops substantially (Szabadics et al., 2006). Some recent studies have shown expression of KCC2 protein in foetal human lens epithelial cell line (Lauf, Di Fulvio, Srivastava, Sharma, & Adragna, 2012; Lauf, Misri, Chimote, & Adragna, 2008), chicken cardiomyocytes (Antrobus, Lytle, & Payne, 2012) and cancer cells (Wei et al., 2011) suggesting that the regulation of KCC2 expression outside the CNS can be hijacked.

During development, the expression of KCC2 mRNA is strongly upregulated and it parallels neuronal maturation in a caudal-rostral fashion. The protein expression concordantly follows the expression of mRNA (Balakrishnan et al., 2003; Ben-Ari, 2002; Blaesse et al., 2009; Clayton, Owens, Wolff, & Smith, 1998; Gamba, 2005; Gulyás et al., 2001; Horn, Ringstedt, Blaesse, Kaila, & Herlenius, 2010; C. a Hübner et al., 2001; H. U,
Tornberg, Kaila, Airaksinen, & Rivera, 2002; Lu, Karadsheh, & Delpire, 1999; Ludwig, Li, Saarma, Kaila, & Rivera, 2003; Mercado et al., 2004; Claudio Rivera, Voipio, & Kaila, 2005; Stein et al., 2004; Vu, Payne, & Copenhagen, 2000). The developmental upregulation of KCC2 has been extensively studied in several mammalian models including human (Dzhala et al., 2005). According to in situ hybridization studies, the first KCC2 transcripts detected in mice are present in the ventral horn of the spinal cord and in the immature brainstem by embryonic day 10.5 (E10.5). At E15.5, the expression of KCC2 is detected in the spinal cord and the medulla. At birth, the levels of KCC2 expression in the spinal cord are in the range of adult levels. In the case of rats, the expression starts two days later, on average (C. A. Hübner, Hentschke, Jacobs, & Hermans-Borgmeyer, 2004; H. Li et al., 2002; Stein et al., 2004; Wang et al., 2002). However, one more recent study has detected KCC2 immunoreactivity in the marginal zone of the posterior neural tube and in a small number of neural crest cells of developing mice already at E9.5 (Horn et al., 2010). The upregulation of KCC2 in more rostral areas of the brain like cortex and hippocampus, as well as in cerebellar granule and molecular layers, occurs postnatally and in a steep fashion. Once the adult levels of KCC2 are reached, the expression stays stable (Clayton et al., 1998; C Rivera et al., 1999). In keeping with mRNA expression, the protein levels of KCC2 in the whole brain are four times higher in adult than in new born rats. It is important to note that the profile of KCC2 expression follows the development of individual neurons as well as brain areas, and it is correlated with synaptogenesis (Gulyás et al., 2001; C Takayama & Inoue, 2006; Chitoshi Takayama & Inoue, 2007).

In comparative studies, the group of Airaksinen have shown that the expression of the two isoforms of KCC2 protein, KCC2a and KCC2b, is similar in embryonic and neonatal brainstem of mice (Uvarov et al., 2009). While KCC2b undergoes a strong upregulation during the first postnatal weeks in mouse cortex, the expression of KCC2a does not increase in these areas. On the contrary, the immunoreactivity for KCC2a disappears in some tissues such as thalamus and cerebellar Purkinje cells. In mature mice brain, KCC2a isoform is found in the basal forebrain, hypothalamus, brainstem and spinal cord. At the subcellular level, both KCC2a and KCC2b are found in dendritic compartment and they colocalize partly. The authors suggest that the two isoforms have distinct roles in the mature brain (Markkanen et al., 2014).
6.1.2. Function

In the adult mammalian CNS, fast inhibitory neurotransmission relies in the action the neurotransmitter GABA in its postsynaptic ionotropic receptors, GABA\(_A\)R and the neurotransmitter glycine acting through ionotropic GlyR. While GABA\(_A\)R is more abundant in the brain, GlyR prevails in the brainstem and spinal cord (Kuhse, Betz, & Kirsch, 1995). Both GABA\(_A\)R and GlyR are gated ion channels that are selectively permeable to the chloride ion and, in the case of GABA\(_A\)R, to some extent to HCO\(_3^-\). The electrochemical gradient of chloride across the neuronal membrane determines the direction of ion flux upon binding of the amino acid neurotransmitter to its receptors. In other words, GABA\(_A\)R reversal potential is determined by the differential distribution of Cl\(^-\) and HCO\(_3^-\) across the neuronal membrane (Kaila, 1994).

Two members of the CCCs family, NKCC1 and KCC2, are the main regulators of equilibrium potential of chloride in neurons (Ben-Ari, 2002; Blaesse, Airaksinen, Rivera, & Kaila, 2009; Mercado, Mount, & Gamba, 2004; Claudio Rivera, Voipio, & Kaila, 2005). In immature neurons, the chloride concentration is higher than expected by passive distribution due to the expression of NKCC1 (Delpire, 2000; Plotkin et al., 1997). Therefore, GABA\(_A\)R reversal potential in young neurons is more depolarized than the typical resting membrane potential, which provokes high neuronal excitability and in certain cases even spiking (Ben-Ari, Gaiarsa, Tyzio, & Khazipov, 2007; Ben-Ari, 2002; Serafini, Valeviev, Barker, & Poulter, 1995). Later in development (see above), KCC2 starts its upregulation in neurons and the shift of GABA\(_A\)R mediated responses commences. As a result, the responses that once were depolarizing will turn hyperpolarizing, in a process that entails neuronal maturation (C Rivera et al., 1999; Claudio Rivera et al., 2005) reviewed in (Blaesse et al., 2009; Kaila, Price, Payne, Puskarjov, & Voipio, 2014). Confirmation of these phenomena, i.e. the developmental decrease of intracellular chloride concentration and the concomitant shift in GABA\(_A\)R reversal potential towards more...
negative values, have been observed in many areas of the CNS such as cortex including hippocampus, cerebellum, hypothalamus and spinal cord (Ben-Ari, 2002; Blaesse, Airaksinen, Rivera, & Kaila, 2009; Mercado, Mount, & Gamba, 2004; Claudio Rivera, Voipio, & Kaila, 2005). In the case of GlyR, its reversal potential undergoes a similar developmental shift in the auditory brainstem (I Ehrlich, Lohrke, & Friauf, 1999). However, a few neurons make an exception to this mechanism: photoreceptors (Vu et al., 2000), horizontal retinal cells (Perlman & Normann, 1990) olfactory neurons (Kaneko, Putzier, Frings, Kaupp, & Gensch, 2004) and neurons in the dorsal suprachiasmatic nucleus (Albus, Vansteensel, Michel, Block, & Meijer, 2005).

**Morphogenic role of KCC2**

Upon the discovery made by Gulyás et. al in 2001 when they found KCC2 localized in the vicinity of excitatory synapses at dendritic spines in rat hippocampal neurons, a new perspective on the functionality of KCC2 was necessary. At that time, Gulyás and colleagues hypothesized that KCC2 may provide a chloride extrusion mechanism in order to maintain water and ionic homeostasis at spines, structures with small volume and high levels of excitation (Gulyás et al., 2001).

In 2007 new discoveries expanded these views, giving an alternative explanation to the presence of KCC2 near glutamatergic synaptic sites. Li et al. showed that DIV14 neurons of mice lacking KCC2 throughout development (KCC2 -/- or KCC2 KO) have aberrant spines that are longer and more commonly branched than in WT. This spine phenotype was also observed recently by Puskarjov and colleagues (Puskarjov et al., 2014). The formation of these aberrant protrusions in KCC2 KO neurons was not attributable to general hyperexcitability of the network as an identical phenotype was observed under the effect of the Na⁺-channel blocker TTX. Moreover, functional blockers of GABAA,R produced no effect on the morphology of spines. These results were confirmed *in vivo* by use of slices of brain from mice with reduced expression of KCC2. Importantly, the number of functional synapses was significantly reduced in KCC2 KO cultured neurons as shown by immunostainings and electrophysiological recordings of mEPSCs. Thus, glutamatergic synaptic connectivity is impaired in KCC2 -/- neurons. Importantly, the overexpression of mutant KCC2 construct which lacked the N-terminal domain (KCC2-ΔNTD), and is incapable of chloride transport, could rescue the phenotype just as well as WT KCC2 constructs. These findings gave rise to a new view on the functionality of the cotransporter KCC2. In order to elucidate the mechanisms behind this novel role, the authors studied potential binding partners of KCC2 and found the interaction of the cotransporter with the actin- and spectrin-binding protein 4.1N (Hoover & Bryant, 2000; Lin et al., 2009; Shen, Liang, Walensky, & Huganir, 2000). The interaction is mediated by the C-terminal domain of KCC2 and the FERM (4.1 protein, ezrin, radixin, moesin) domain of 4.1N (H. Li et al., 2007).

Genetic downregulation of KCC2 by shRNA in more mature hippocampal neurons also affects excitatory neurotransmission. The group of Lévi and Poncer utilized overexpression of shRNA against KCC2 for 10 days in
neurons that had been cultured for 14 days (DIV14-24). They showed reduced amplitude of mEPSCs and decreased number of GluR1-containing clusters by expression of shRNA against KCC2 (Gauvain et al., 2011). These effects were mimicked by overexpression of the C-terminal domain of KCC2, the domain of KCC2 that interacts with 4.1N (H. Li et al., 2007). Pharmacological inhibition of the activity of KCC2 was not able to evoke the same effect in excitatory transmission, suggesting that the structural role of KCC2 and not its capacity to transport chloride ions is important for the mechanism. Notably, the morphology of dendritic spines was affected by expression of shRNA against KCC2 in mature neurons as the spine heads were significantly enlarged. In an attempt to clarify the molecular processes behind these effects, the authors used quantum dots to track the movements of GluR1-containing AMPAR and showed that knocking down KCC2 increases the mobility of what presumably are extrasynaptic receptors (Gauvain et al., 2011). Later on, the same research group was able to demonstrate that KCC2 has a preference for synaptic sites. By combining the overexpression of synaptic markers with quantum dots (QD) in cultured hippocampal neurons, Chamma et al (2013) observed that the dwelling time of KCC2 molecules is longer in synaptic than in extrasynaptic areas. Despite the fact that KCC2 immunoreactivity overlaps with inhibitory synaptic markers at the same degree than with excitatory ones, KCC2 escaped more easily from inhibitory synapses than from excitatory, suggesting that the mechanisms regulating the presence of KCC2 are different in the two types of synapses. Indeed, the dwell time of KCC2 in excitatory synapses is directly dependent on its C-terminal domain, actin cytoskeleton and 4.1N. In the case of inhibitory synapses, KCC2 dwell time was unaffected by shRNA against 4.1N, latrunculin treatment and KCC2 C-terminal domain overexpression (Chamma et al., 2013). On the other hand, in vivo selective genetic ablation of KCC2 cerebellar cells of mice produced no phenotype in terms of density of synapses- inhibitory and excitatory- density of spines of Purkinje cells or spine length. These results indicate that the mechanisms controlling synapse and spine formation in these cells might be substantially diverse from the ones taking place in the cortex (Seja et al., 2012).

In 2010, Horn et al. suggested that the morphogenic role of KCC2 might be of importance prior to the development of dendritic spines. As mentioned earlier (see KCC2 expression), Horn and colleagues detected KCC2 protein at the posterior part of the neural tube already at E9.5, well preceding the developmental shift in GABAergic inhibition. In their study, premature ectopic expression of KCC2 in mice altered normal development of neural structures and reduced neuron differentiation (Horn et al., 2010). Notably, ectopic expression of a KCC2 variant deficient in chloride transport (KCC2-C568A, (Reynolds et al., 2008)) had milder consequences in the mice phenotype than WT-KCC2. The authors attribute this phenomenon to the reduced interaction of KCC2-C568A with 4.1N (Horn et al., 2010). The phenotype of embryos electroporated with the variant of KCC2 (KCC2-ΔNTD, H. Li et al., 2007), incapable of chloride transport but with the C-terminal domain intact, had a more severe phenotype, similar to WT KCC2 expressing ones. Thus, the authors suggest that the developmental defects of the embryos are not dependent on the function of KCC2 as a chloride
cotransporter but they rather are mediated through direct structural interaction with the postsynaptic protein 4.1N and the actin cytoskeleton (Horn et al., 2010). It is important to note here that premature expression of KCC2 had been previously used in the retinotectal system of Xenopus laevis, (Akerman & Cline, 2006), rat neurons (Cancedda, Fiumelli, Chen, & Poo, 2007) and globally in fertilized zebrafish (Danio rerio) embryos (Reynolds et al., 2008). In all three cases, the authors reported abnormalities in the development of excitatory neurotransmission. Concretely, they found a pronounced reduction in the total neurite length and branch number (Cancedda et al., 2007), a perturbed axonal growth in zebra fish (Reynolds et al., 2008) and a reduced AMPA-mediated glutamatergic neurotransmission in the tectum of the tadpoles (Akerman & Cline, 2006). The authors attributed the phenomena to the premature closure of GABAergic depolarization. Nevertheless, the results do not exclude the plausible involvement of the morphogenic role of KCC2. More recently, in utero coelectroporation of WT-KCC2 with GFP in mice at E17.5 was used to analyse dendritic architecture and function of neurons from somatosensory cortex (SSC). In layers 2/3 of SSC at all ages studied (P10, 15 and 90) the gross dendritic arbour anatomy was unaltered by premature expression of KCC2 (Fiumelli et al., 2013). However, the neurons that had elevated KCC2 expression showed concomitant increased spine density and those spines had larger spine heads. Electrophysiological recordings demonstrated an increased frequency with unaffected amplitude of mEPSCs produced by premature expression of KCC2. Moreover, when the constructs electroporated were KCC2-ΔNTD and KCC2-CTD (H. Li et al., 2007), the spine density and spine head were enlarged as by WT-KCC2. In the case of KCC2-C568A overexpression, the spine density and spine head were not significantly different from neurons that expressed EGFP only (Fiumelli et al., 2013). As discussed above, in the previous work by Cancedda, Fiumelli and colleagues, in utero electroporation of WT-KCC2 led to seemingly opposite effects, although the expression of the mutant KCC2-C568A produced no differences when compared to EGFP-expressing neurons in both cases (Cancedda et al., 2007). In their work of 2013, Fiumelli and colleagues attribute this discrepancy to the use of bicistronic plasmids, where the expression of the marker (EGFP) is downstream of an internal ribosomal entry site (IRES), in the 2007 study. Poor expression of EGFP in case of IRES constructs could explain biases in the visualization of the whole dendritic arborisation; although it is difficult to see why this would affect only the constructs containing WT-KCC2 and not the ones with the point mutated KCC2-C568A (Cancedda et al., 2007; Fiumelli et al., 2013). Recent data by Puskarjov et al. are in concordance with the work of Fiumelli et al 2013. In utero electroporation of WT human KCC2 produced a significant increase in the spine density of neurons in layers 2/3 of SSC in P6-7 rats. Importantly, electroporation of a point mutant KCC2, KCC2-R952H, had no effect on the number of spines. KCC2-R952H was identified as a mutation that co-segregated with increased susceptibility to febrile seizures in the patients of this genetic study. The point mutation decreased significantly the chloride extrusion function of KCC2 and its surface expression. Moreover, overexpression of KCC2-R952H was unable to rescue the length of spines in dissociated cultured KCC2 -/- neurons, as opposed to WT-KCC2. It is thus difficult to conclude whether the morphogenic spine
phenotype produced by the variant KCC2-R952H is related to the chloride transport capability of KCC2 or independent of it (Puskarjov et al., 2014).

The structural role of KCC2 is not exclusive of neuronal cell types. Human cervical cancer cells (SiHa cells) express substantial levels of KCC2 and in these cells KCC2 promotes carcinogenesis by increasing insulin-like growth factor-1 (IGF-1)-induced cell migration. Overexpressing WT-KCC2 in SiHa cells reduced total cell area and promoted cell migration and invasion. Interestingly, manipulation of the expression of KCC2 had dramatic effects on the actin organization in SiHa cells as shown by immunostaining of actin. Overexpression of KCC2 induced cortical actin formation and knockdown of KCC2 generated very densely packed F-actin filaments closely resembling stress fibres. The distribution of tubulin was not affected by these manipulations. Moreover, overexpressing the point mutant KCC2-Y1087D, unable to transport chloride ions (Strange et al 2000), produced comparable results to WT-KCC2, indicating that the reported increase in cell invasion and reduced cell area are independent of the chloride transport activity of KCC2. In addition, the levels of KCC2 expression correlated to those of focal adhesion kinase (FAK), indicating that KCC2 is related to the formation of focal adhesions in cancer cells. Focal adhesions are actin structures that are intrinsically crucial to cell migration and invasion. Therefore, the authors conclude that the functional interaction of KCC2 with the actin cytoskeleton is responsible for its role in carcinogenesis. Interestingly, the cancer profiling database Oncomine, where gene expression reports from human cancer studies are compiled, showed increased levels of KCC2 expression in many, but not all, cancer cells from epithelial origin such as breast cancer, cervical cancer, colorectal cancer, lung cancer etc. in relation to normal expression (Wei et al., 2011).

In conclusion, KCC2 plays a fundamental role in morphogenesis of neurons independently of its chloride transport capacities but the specific mechanisms will need further scrutiny.

6.1.3. Regulation

KCC2 is a neuron specific protein with strict regulation of both expression and function. This regulation takes place at different levels: transcriptional and postranscriptional (i.e. by multimerization, membrane expression, subcellular targeting, protein interaction, phosphorylation state and degradation).

The expression of the SLC12A5 gene is regulated by transcription factors. Up to 10 putative transcription factors binding sites have been identified in the promoter region and the first exon. These sites are highly conserved among CCCs (Uvarov, Ludwig, Markkanen, Rivera, & Airaksinen, 2006). The functional relevance has been shown for the transcription factor Egr4 (Ludwig, Uvarov, Soni, et al., 2011; Uvarov et al., 2006), the stimulating factors USF1 and USF2 (Markkanen, Uvarov, & Airaksinen, 2008), and two neuron-restrictive silencing elements (NRSE) (Karadsheh & Delpire, 2001; Uvarov, Pruunsild, Timmusk, & Airaksinen, 2005; Yeo et al., 2005). In addition to the regulation of transcription of DNA into mRNA, the stability of the KCC2 mRNA
is also regulated by microRNAs. Specifically, microRNA-92 binds to mRNA of KCC2 and it leads to its degradation. Interestingly, microRNA-92 is developmentally downregulated (Barbato et al., 2010).

Neurotrophic factors are key molecules throughout the development of the CNS; they regulate neuronal differentiation, survival, neurite outgrowth, synaptic formation and maturation (E. J. Huang & Reichardt, 2003; Lewin & Barde, 1996). The regulation of KCC2 by the brain-derived neurotrophic factor (BDNF) is well documented and the involvement of BDNF in the developmental upregulation of KCC2 has been extensively studied (Aguado et al., 2003; Ludwig, Uvarov, Pellegrino, et al., 2011; Ludwig, Uvarov, Soni, et al., 2011; Yeo et al., 2005). However, the direction of the effect of BDNF on KCC2 depends on the maturation state of neurons, as exposure of mature neurons to BDNF decreases KCC2 mRNA and protein (Claudio Rivera et al., 2002, 2004; Shulga et al., 2008). Along with BDNF, at least two more neurotrophic factors and one growth factor have been implicated in the regulation of KCC2, nerve growth factor (NGF, (Lagostena et al., 2010)) and insulin-like growth factor-1 (IGF-1; (Kelsch et al., 2001)) and neurturin (Ludwig, Uvarov, Pellegrino, et al., 2011).

The interaction of KCC2 with other proteins can modulate the activity of KCC2 by affecting its subcellular distribution (scaffolding, stabilizing proteins) and the availability in protein complexes or by allosteric modulation of KCC2. Of major relevance is the possible interaction of KCC2 with GABA\(_\text{A}\)R (Y. Huang et al., 2013) that would confirm the previous observations that KCC2 colocalizes with GABA\(_\text{A}\)R and gephyrin in inhibitory synapses (Gulyás et al., 2001; Tyagarajan & Fritschy, 2010). In excitatory synapses, the interaction between KCC2 and 4.1N is crucial for spine development and protein stability (see chapter “morphogenic role of KCC2”). Other interacting partners of KCC2 such as brain-type creatine kinase (CKB) and Na\(^+\)-K\(^+\)-ATPase regulate the cotransport activity of KCC2 (Ikeda et al., 2004; Inoue, Ueno, & Fukuda, 2004). Recent findings show that KCC2 interacts with kainate type glutamate receptors (KARs). KARs are required for oligomerization and membrane localization of KCC2. Accordingly, genetic deletion of KARs impairs the chloride extrusion function of KCC2 and leads to diminished synaptic inhibition in hippocampal neurons (Mahadevan et al., 2014). Previous data showing the interaction with Neto2 add to these new findings, as Neto2 is an accessory molecule for KARs and it modulates their activity and their synaptic localization (Copits, Robbins, Frausto, & Swanson, 2011; Tang, Ivakine, Mahadevan, Salter, & McInnes, 2012). These data, together with the ability of KCC2 to regulate glutamatergic transmission independently of the effects mediated by GABAergic depolarization (see chapter “Morphogenic role of KCC2”) highlight the plausible role of KCC2 as a key synchronizing molecule for the development of excitatory and inhibitory neurotransmission.

A high number of putative phosphorylation sites are present in KCC2. Many of them are situated in the C-terminal cytosolic part of KCC2, but there are also putative sites in the N-terminal cytosolic part and even in the predicted transmembrane region. These phosphorylatable residues include serine, threonine and tyrosine. Accumulating evidence points towards phosphorylation as the basis for rapid control of the activity of KCC2 as a chloride transporter, its presence at the plasma membrane and even its localization to
specialized compartments in the plasma membrane called lipid rafts. It is important to mention here that only around 20% of the total KCC2 present in hippocampal neurons of juvenile rats (P19-22) is localized at the plasma membrane (Ahmad, Coleman, Kaila, & Blaesse, 2011).
AIMS OF THE STUDY

The general aim of this thesis work was to study the molecular pathways regulating morphological changes in spines during development. Moreover, we have concentrated on the consequent changes on glutamatergic synaptic connectivity by modulation of those molecular pathways. More specifically, we studied the role of synaptic molecules and characterize their action regarding:

1) Direct regulation of the actin cytoskeleton by ABPs like cofilin, mDia2 and Arp2/3 in developing neurons. Observation of actin polymerization in dendritic filopodia and spines.

2) Molecular mechanisms that regulate the course of development of dendritic spines. Study of the regulation of dendritic development by the pathways of RhoGTPases Rac1, Cdc42 and Rif; the cell adhesion molecules ICAM-5 and β1 integrin; as well as KCC2 and βPix.

3) Regulation of synaptic maturation by the pathways aforementioned.
MATERIALS AND METHODS

1. Dissociated neuronal cultures

In all the studies presented in the current thesis work (I, II and III), we have used dissociated neuronal cultures as model. Murine embryonic cells (E17) were dissociated as described in Banker and Goslin, 1998 (Banker, 1998). This model was chosen as appropriated to our purposes due to several reasons. First of all, dissociated neurons remarkably retain their qualities as within the brain. Importantly, by the fourth day in vitro (DIV4) dissociated neurons in culture outwardly expand their neurites and start establishing connections with neighbouring cells. Eventually, as they do in vivo, these cultured neurons form networks. The whole process is easily observable with the use of rather basic light microscopy. Secondly, neuronal cultures are very versatile and allow numerous genetic and pharmacological manipulations. Thirdly, neuronal cultures can be plated directly on glass cover-slips, making it very easy to stain with antibodies and subsequently observe them under the microscope.

Notably, in study II, we explored the phenotype of neurons from KCC2 KO mouse. These mice lacking both isoforms of KCC2, a and b, die soon after birth due to motor deficits and respiratory failure (C. a Hübner et al., 2001), making it impossible to study neuronal development postnatally. Dissociated KCC2 KO neurons can be extracted at embryonic stages and plated in culture, where they will develop their observable phenotype. This way we can easily perform genetic and pharmacological modifications and compare them with neurons from their WT litter-mates.

Dendritic spines of cultured neurons show the same morphology as the ones observed in vivo (Ebrahimi & Okabe, 2014; Papa et al., 1995) therefore they constitute a suitable model for our studies. Another important feature of cultured neurons is the feasibility to repeat the experiments in a relatively large numbers, which allows us to have statistically significant differences and to draw conclusions from them. Indeed, cultured neurons show high reproducibility.

On the other hand, spines in cultured neurons might have a slightly different 3D distribution along the dendrite as they tend to be situated on the sides of the dendrite and not all around it in a helicoidal fashion (Rafael Yuste, 2011). Also, the motility and turnover of dendritic spines might be different in the whole animal brain than in cultured paradigm. However, this issue has been little studied and there are no clear conclusions yet. Importantly, the experiments performed for these studies always included a control culture to compare with. Thus, the results presented here are readily conclusive.

Neuronal cultures were prepared as follows:

Standard dissociated hippocampal and cortical cultures were prepared from embryonic day 17 (E17) mice and cultured for up to 3 weeks in vitro. We used the protocol described in Culturing Nerve Cells, Second
Edition (Banker, 1998), with minor modifications. Briefly, a pregnant mouse was anaesthetized in a CO2 chamber and killed by cervical dislocation, embryos were removed, and hippocampi were dissected. Cells were dissociated by enzymatic treatment (trypsin) and plated on poly-DL-ornithine-coated cover glasses \(10^5\) cells/cm\(^2\) in Neurobasal medium containing B27 supplement and 0.5 mM L-glutamine. Before plating the medium was preincubated on astroglial culture for 24 hours. Neuronal cultures were fed once a week by changing half of the medium. Astroglial cultures were prepared according to Culturing Nerve Cells, Second Edition (Banker, 1998) and maintained in DMEM supplemented with 10% of foetal bovine serum, penicillin and streptomycin (named complete DMEM hereafter). All animal experiments were approved by the local ethics committee for animal research at the University of Helsinki.

### 1.1. Transfection of neurons

One day before transfection half of the medium of cultured neurons was changed to fresh glia-conditioned medium. For neurons older than 10 days \textit{in vitro} one hour before transfection culture medium was substituted with 10 mM MgCl\(_2\) in Neurobasal medium. Neurons were transfected with 0.5 \(\mu\)g of plasmid DNA per 1.6 cm\(^2\) well using Lipofectamine 2000 according to the manufacturer’s protocol. Culture medium was returned to the cells 4-6 hours after transfection.

### 2. Cultured cell lines

Immortalized cell lines constitute a useful tool to study certain cell processes as they are easily transfected and maintained. One advantage of the use of cell lines is that they express large amount of proteins that can be analyzed by biochemical methods such of western blots. Also, HEK293T cells used in the study II, do not expressed endogenously significant amounts of KCC2b or \(\beta\)Pix and can be used as a model to express those proteins in a cell context but in a controlled fashion.

HEK293T cells were cultured in complete DMEM. The number of cell passages was limited to 20 post thaw in order to avoid accumulation of genetic mutations in them.

Cultured HEK293T cells were transfected using JetPei reagent (Polyplus-transfection) or Calcium Phosphate Transfection Kit according to provider instructions.

### 3. Imaging techniques:

#### 3.1. Microscope setups

Confocal images were acquired with Leica TCS SP5 microscope equipped with the software LAS AF and lasers OPSL 488 nm/270mW, DPSS 561 nm/20mW and HeNe 633 nm/12mW. For fixed samples, the objective Leica HCX PL APO 63x / 1.3 GLYC CORR CS (21° C) was used. Live cell microscopy (FRAP and motility analysis, see below) was carried with HCX APO L 63x/0.90 W objective. Neurons were maintained at 37°C and 5% CO2 in a
custom chamber (The Box, Life Imaging Services) in Neurobasal medium containing B27 supplement and 0.5 mM L-glutamine throughout the imaging session.

Fluorescent wide-field images in study II were acquired with Zeiss AxioImager.M1 microscope equipped with AxioCam HR camera and AxioVision 4 software. The neurons were imaged with 40x objective ECPlan-Neofluar/0.75/Ph2. The Colibri LED system with 470 and 555nm modules was used and the beam combiners were BC490, BC425 (for GFP) and BC565 (for Alexa568).

3.2. FRAP

The fluorescence recovery after photobleaching technique has been previously used to determine the turnover of GFP-actin in dendritic spines (Koskinen & Hotulainen, 2014; Star et al., 2002; Zheng, Petralia, Wang, & Kachar, 2011). Since GFP and related proteins are not efficient reactive oxygen species (ROS) generators (Rajfur, Roy, Otey, Romer, & Jacobson, 2002), the bleaching of spines and the subsequent imaging of the recovery of fluorescence is a safe technique that should not significantly damage dendritic spines or change their dynamics.

Fluorescence recovery of the GFP-actin intensity after photobleaching was measured using Leica Confocal Software as described previously (Koskinen, Bertling, & Hotulainen, 2012) with slight modifications. To visualize dendritic spines a 63×/ 0.90 NA dipping water objective and 6 times digital zoom were used. The settings for the Leica TCS SP5 microscope were as follows: format 256 × 256, speed 700 Hz (unidirectional), 2-line averaging, and pinhole 2.0 AU (airy unit). The frame of 41 x 41 µm, including the region of interest (ROI, the whole spine), was imaged five times before bleaching, followed by photo-bleaching that was achieved with one scan (total bleach time 1.5 s, total laser power ~2.2 mW) of the ROI. Imaging of the area was resumed immediately after the photo-bleaching. A total of 66 frames were scanned during 152 s (26 frames at 0.78 s/ frame, 20 frames at 2 s/ frame, followed by 20 frames at 5 s/ frame). The intensity of the bleached area was normalized to a neighbouring non-bleached dendritic area to diminish error caused by photobleaching during the monitoring period. The pre-bleach value was normalized to 1.0.

3.3. FRET

Förster resonance energy transfer (FRET) is a reliable technique to assess the proximity of two fluorophores in living cells. Here, the technique is used in study II to analyse the activation state of Rac1 by βPix. The probe used here was developed by the group of Matsuda M and is called Raichu-Rac1. Raichu-Rac1 uses Rac1 as a sensor and the region of the protein PAK as the ligand region. Whenever Raichu-Rac1 binds GTP, the effector region of Rac1 binds to PAK, which will put these regions in closer proximity and therefore increase FRET from CFP to YFP. The technique allows us to assess the balance between GEFs and GAPs at the membrane of living cells, where they are anchored. See Methods Fig.1 (Nakamura, Kurokawa, Kiyokawa, & Matsuda, 2006).
Dissociated hippocampal neurons were analyzed two days after transfection. Before imaging neurons were preincubated in Neurobasal without B27 for 2h at 37ºC, 5%CO₂.

In case of the young neurons (DIV 7-9) fluorescence from Raichu-Rac1 was imaged and measured using an inverted Olympus microscope IX71 controlled by CellIR software and equipped with oil immersion objective (UApO/340 40x/1.35). Cells were illuminated with a 150W Xenon lamp (used at 40% of full power) through D436/20 (CFP) or HQ500/20 (YFP) excitation filters. Signal was collected using 480/40 (CFP) or 535/30 (YFP) emission filters. The exposure time was 1000 ms. Neurons were transferred into HEPES buffered extracellular solution and incubated 30 min at room temperature prior to imaging in the same solution.

In case of the mature neurons (DIV 13-14) fluorescence from Raichu-Rac1 was imaged using confocal microscope Zeiss LSM 710/Axio controlled by ZEN 2011 software and equipped with water immersion objective (Zeiss 63x/1.0), CO₂ and temperature control. The imaging was performed in culture medium at 5% CO₂ and 37ºC. Dendrites of transfected neurons were illuminated with Argon Laser at 458 nm (CFP and FRET) and 514 nm (YFP) using 0.5-10% of full laser power. Emission was collected at 461-519 nm (CFP) and 519-621 nm (FRET and YFP alone). The pinhole was fully opened. Scanning was performed in XY mode using 6x digital zoom that resulted in a pixel XY size of 68x68 nm.

For both young and mature neurons FRET signal was calculated as reported elsewhere (van Rheenen et al., 2004). Briefly, after background subtraction FRET image was generated using three images: \( M_{\text{Donor}} \) (CFP excitation and CFP emission filters), \( M_{\text{indirectAcceptor}} \) (CFP excitation YFP emission filters), and \( M_{\text{directAcceptor}} \) (YFP excitation and YFP emission filters), using the following equation:

\[
FRET = \frac{(M_{\text{indirectAcceptor}} - M_{\text{Donor}} \cdot \beta - M_{\text{directAcceptor}} \cdot (\gamma - \alpha \beta))}{1 - \beta \delta}
\]
Coefficients $\alpha, \beta, \delta, \gamma$ were obtained by independent control experiments analyzing cells expressing CFP or YFP as described in (van Rheenen et al., 2004). After generation of FRET image, intensity of FRET signal was calculated using mask obtained from a corresponding YFP image and covering neuronal cell bodies and dendrites.

3.4. Barbed end assay

The free barbed end assay was used in study I in order to identify the sites of active actin polymerization in cultured neurons. The barbed end assay was performed as described previously (Symons & Mitchison, 1991). Live cells were permeabilized and shortly incubated in a buffer containing fluorescently-labelled actin monomers together with ATP. Fluorescent monomers will be added to the barbed ends of actin filaments due to actin polymerization. Subsequently, the cells were fixed and stained with Alexa Fluor-phalloidin in order to visualize the whole actin filament.

3.5. Immunostaining

3.5.1. Immunocytochemistry.

This technique has been used in all the studies included in the thesis (study I, II and III). Neurons were fixed with 4% paraformaldehyde in PBS at room temperature and permeabilized with 0.5% Triton X-100. Cells were blocked with 10% goat/donkey serum, 0.2% Triton X-100 in PBS at room temperature and then incubated at 4ºC overnight in 5% goat/donkey serum containing the appropriate primary antibodies. Species-specific secondary antibodies were used at 1:1000 dilution. Cover glasses were mounted in Prolong Gold mounting media.

3.5.2. Immunohistochemistry.

Brain sections were stained in study II. Paraffin coronal sections of the hippocampi of an adult mouse (P30) expressing YFP under Thy1 promoter (B6.Cg-Tg(Thy1-YFPH)2Jrs/J mouse) were co-stained for KCC2b and $\beta$Pix as described previously (Ludwig, Uvarov, Soni, et al., 2011) with some modifications. Concisely, deparaffinized sections were washed in SDS solution and subsequently masked epitopes were retrieved by heating the samples in a microwave oven in a solution containing 1mM EDTA, 1mM sodium citrate and 2mM Tris buffer pH 8.5. In order to avoid desiccation of the samples during the microwave treatment, more solution was added frequently. The samples were let to cool down at RT in the same solution and then washed with TBST and blocked with 5% BSA in TBST. Next, tissues were incubated with the suitable primary antibodies in 2% BSA, 0.2% Triton X-100 in TBST overnight at 4ºC. To further reveal YFP, mouse anti-GFP antibodies were applied to the incubation solution. Species-specific secondary antibodies were used at 1:1000 dilution. Cover glasses were mounted in Prolong Gold mounting media.
3.6. Image analysis

In order to analyse effectively the images in my studies, I needed to develop specific protocols in image analysis software such as Imaris (Bitplane), AutoquantX and Image-Pro (Media Cybernetics), NeuronIQ (HMRI), NeuronStudio (CNIC) and ImageJ (NIH).

3.6.1. Motility analysis

In order to study the motility of spines in study II, confocal images of neuronal cultures were collected in time-lapse every 60s and later analysed in 4 dimensions (x, y, z, t) using Imaris 6 software. In order to analyse the protrusive motility, spots were placed at the tip of every spine and the total displacement of the dendritic spines was measured using the track length measurements in ImarisXT module.

3.6.2. Colocalization analysis

In study II, 3D (x, y, z) image stacks were blindly deconvoluted in AutoQuantX2 Deconvolution Software using the 3D deconvolution protocol and the number of iterations was set to 10. Subsequently, the deconvoluted 3D images were analyzed in Imaris 6 software. The colocalization coefficient was determined numerically in ImageJ by means of the colocalization plugin JACoP (Bolte & Cordelières, 2006). In brief, the suitable colocalization threshold was determined by Costes’ automatic thresholding paradigm. Subsequently, the threshold was used to determine Pearson’s coefficient of colocalization.

In study III, in order to quantify the correlation of β1 integrins with the EGFP-labeled spines, colocalized clusters were defined as >50% area of spine heads overlapping with β1 integrin staining, while <50% was considered juxtaposed. Images were processed with Photoshop and ImageJ.

3.6.3. Spine characterization

The morphology of spines was analysed in every study in a case-specific fashion, depending on the quality of images and software available at the moment. It is important to note that in the scientific community there is no standardization in the classification of dendritic protuberances in neuronal cultures.

In study I, dendritic protrusions were analysed as follows: protrusion density and length were measured by NeuronIQ software from Nyqvist sampled confocal stacks taken with a 63× objective lens according to (J. Cheng et al., 2007), except that 1-pixel-wide Gaussian filtering in Imaris 6.0 (Bitplane) was used instead of median filtering. The densities of dendritic protrusions with different morphologies were counted from the same confocal stacks. The spines were classified according to this criteria: filopodia, thin protrusions without a distinguishable head; thin spines, thin protrusions with a distinguishable head, typically long neck, and small head with a width <0.75 μm; mushroom spines, typically short neck and large head with width >0.75
μm; and stubby spines, no distinct neck. The head widths of thin and mushroom spines were measured with ImageJ.

In study II, the morphology of spines was analysed and the spines were subsequently divided into categories according to their morphological parameters. For that, 3D (x, y, z) confocal images were blindly deconvoluted in AutoQuantX2 Deconvolution software (Media Cybernetics). The distribution of KCC2 and βPix in different spines was assessed in Imaris 6 software (Bitplane) by building surfaces around the dendritic spines of hippocampal neurons expressing eGFP. Next, the intensity of KCC2 and βPix inside the spine surfaces was measured and normalized to the total intensity of KCC2 and βPix in the whole image (dendritic shaft and spines). Finally, spines were classified as mushroom when they showed a distinguished head and their width was greater than half of their length, stubby when their length was inferior to 1.2 μm and they showed no head or when their length was lesser than 1.2 μm, half of their length was bigger than their width and they had a distinguished head, thin when they possessed a distinguished head, their length was greater than 1.2 μm and half of their length was superior to their width, filopodia when they did not show a clearly distinguishable head and their length was above 1.2 μm.

In study III, dendritic protrusions were categorized by the following criteria: mushroom spine: length <3 μm and with an enlarged head; thin spine: length >3μm and with an enlarged head; filopodia length 3–10 μm, without an enlarged head. The analyses were performed in ImageJ.

Importantly, observed dendritic protrusions can have different measures depending on the fixation methods used, the age of the cultures and the culturing density, among other causes. The criteria need to be case-dependent and relative to the specific culture parameters in order to fulfil their purpose. Despite the seemingly different criteria used to classify dendritic spines, there is a general consensus that has been used. The consensus describes filopodia as relatively long protrusions with no distinguishable head, thin spines as relatively long processes with a small although distinguishable head, mushroom spines as relatively short spines with a clearly enlarged head and stubby spines as relatively short protuberances with no distinguishable head and neck.

3.6.4. Quantification of synapses

In study I, the synapses were detected by partial colocalization of anti vGLUT1 (labels the presynaptic sites of synapses) antibody staining with GFP fluorescence or myc tag staining of the transfected cell. The synapses from deconvoluted confocal image stacks were counted using the Bitplane Imaris suite software. A surface was rendered comprehending the GFP/myc volume, and the V-GLUT-1–positive spots that were close enough were counted using the spots near surface function in the ImarisXT module as synaptic terminals. The maximum distance between GFP/myc and presynaptic terminals was set to 0.68 μm.
In study II, blindly deconvoluted confocal stacks (AutoquantX2) were analyzed in 3D (x, y, z) in Imaris 6 software. In short, the synapses were detected by partial colocalization of the pre-synaptic marker anti-vGLUT1 antibody staining with post-synaptic anti-PSD95. A surface was rendered comprehending the GFP/myc volume, and the colocalization spots that were situated at <0.4μm from the rendered surface were counted using the spots near surface function in the ImarisXT module as synaptic terminals.

4. Biochemical techniques

4.1. Western blotting

Western immunoblotting has been extensively utilized in all the studies shown here (study I, II and III). This is due to the great value of this technique when analysing the expression levels of proteins in cells. Neuronal or HEK293T cultures were rinsed in PBS, scraped and homogenized in ice-cold RIPA lysis buffer with protease- and phosphatase-inhibitor cocktails. Protein concentrations were determined using DC Protein Assay kit or BCA assay kit. Samples were separated using SDS-PAGE and transferred onto Hybond ECL nitrocellulose membrane. Blots were probed with the appropriate primary antibodies and HRP-conjugated secondary antibodies, developed with ECL-plus and visualized with Luminescent image analyser LAS-3000. Band densities were analysed with AIDA imaging software (Raytest).

4.2. Co-immunoprecipitation (coIP) assay

Co-Immunoprecipitation has been used in the presented studies in several occasions. The protocols were optimized in a case-specific manner. In all co-immunoprecipitations, great care was put to maintain the samples at a stable temperature of 4ºC, at which enzymatic processes are stopped. Moreover, the coIP buffers always contained commercial cocktails of phosphatase and protease inhibitors in order to avoid degradation of the target proteins.

4.2.1 CoIP in HEK293T cells

In study II, co-Immunoprecipitation of proteins expressed in HEK293T cells was performed 40h after transfection as follows: cells were washed twice with cold PBS and lysed in cold TNE buffer (1% Nonidet P-40, 140mM NaCl, 5mM EDTA, 50mM Tris-HCl pH 8.0). Cells were collected using a cell scraper and the lysate was centrifuged at 12000g in order to remove particulate matter. Cell lysate containing around 1mg of total protein was pre-cleared with G-sepharose previously blocked rotating during 1h. Polyclonal antibodies against pan βPix or KCC2b were coupled to their antigen by adding 3-5μg of antibodies to the cleared lysate and rotating overnight. The immune complexes were precipitated by adding blocked sepharose to the tubes and incubating for 2-4h with rotation. Beads were washed with TNE buffer, last wash with PBS. Elution of the
immune complexes was done by heating the samples at 95º for 5min. and the samples were processed for western blotting.

4.3. CoIP assay in whole brain homogenates

Co-immunoprecipitation of proteins from tissue is a very demanding technique. The protocol used in order to co-immunoprecipitate two proteins can vary depending on the nature of the proteins (their molecular weight, their abundance, their biochemical properties such as hydrophobicity, etc.) and also depending on the nature of the interaction of the two proteins, that is always context specific. For that reason, in the presented studies, we have used two different protocols.

In study II, native-membrane fractions and immunoprecipitations were prepared similarly to previously described (Mahadevan et al., 2014) with slight modifications. One adult male NMRI mouse brain (P40-60) was manually homogenized in PBS using a glass-Teflon homogenizer. The homogenate was centrifuged at 900g for 10 min and the pellet was resuspended in 5ml ice-cold lysis buffer by pipetting. The homogenate was subsequently centrifuged for 30 minutes at 25000g. The native membrane fractions were recomposed in 4Xw/v solubilization buffer (50mM TrisHCl pH 7.5, 50 mM NaCl, 0.05 mM EDTA, 1.5% C12E9). The sample was let to solubilize for 2 hours on a rotating wheel, followed by a centrifugation at 25000g for 1 hour. Immunocomplexes of the membrane fractions were attained by incubation of 4-5 mg of membrane fraction homogenate with pan anti- βPIX (SH3 domain) for 3 hours in a rotating wheel. Subsequently, the immunocomplexes were precipitated by incubation with G-sepharose beads for 1 hour with rotation. Next, the beads were washed twice with solubilization buffer containing detergent (1.5% C12E9) and once with solubilization buffer without detergent. Elution of proteins was performed as described before for the coIP in HEK293T cells. The eluates were subsequently analyzed by Western blotting with antibodies against KCC2b, pan- βPIX (SH3 domain), PSD95 and PAK1/2/3.

In study III, the co-immunoprecipitation of endogenous proteins was performed with a slightly different protocol than in study II. Two adult mouse forebrains were homogenized in 10 volumes of ice-cold homogenization buffer (1% Triton X-100 in phosphate buffered saline (PBS) and centrifuged at 100,000g for 1 h. The supernatant was pre-cleared with Protein G Sepharose for 1 h, and divided into 1 ml aliquots, which were left untreated or incubated with 2 μg/ml antibodies against ICAM-5, β1 and α5 subunits respectively, overnight. Then, protein G Sepharose was added and the samples were incubated with rotation for an additional hour. The precipitates were washed with ice-cold homogenization buffer three times and resuspended in sample loading buffer.

4.4. GLISA
In order to study the activation state of Rac1, in study II we used the commercial kit GLISA (Small GTPase Activation Assay, Cytoskeleton Inc.). This assay is based on the binding of activated Rac1 to an immobilized domain of PAK protein that binds only GTP-bound Rac1. Then, the amount of activated Rac1 is analyzed by a specific antibody. The protocol used was as follows: HEK293T cells expressing respective constructs were serum-starved overnight. Cells were lysed and snap frozen in liquid nitrogen. After fast thawing, binding buffer was added to the cell lysate and subsequently incubated on a Rac1-GTP affinity plate coated with Rac1-GTP-binding protein. The plate was placed on an orbital plate shaker at 300 rpm for 30 min. Cell lysis and incubation were carried out in a cold cabinet at 4ºC. After washes, anti-Rac1 antibody (1:500) was added to the wells. Then the primary antibody was washed away and the HRP-linked secondary antibodies (1:500) were added to the wells. Both primary and secondary antibodies were incubated on the orbital shaker at 300 rpm for 45 minutes at RT. Thereafter, the signal was developed with HRP-detection reagents. The luminescence was measured by means of VICTOR multi-label plate reader spectrophotometer (Perkin Elmer).

5. Statistical analysis

All statistical analyses were performed using Prism software (Raytest). The statistical significance was measured using t-test or non-parametric tests when two populations were compared and by analysis of variance (ANOVA), followed by adequate post-hoc tests in case the number of populations was over two.
RESULTS and DISCUSSION

1. Molecular mechanisms regulating actin dynamics in dendritic protrusions (I)

Actin is the most abundant protein in dendritic spines (Matus et al., 1982). The dynamic actin cytoskeleton sustains and modifies the morphology of dendritic filopodia and spines throughout their development (Calabrese, Wilson, & Halpain, 2006; Cingolani & Goda, 2008; Tada & Sheng, 2006). Thus, the study of actin dynamics and the molecules controlling them in dendritic protrusions can produce a major insight into regulation of synaptic development.

We used cultured hippocampal neurons as model. In an attempt to study dendritic protrusions in depth, we performed manipulations of actin-regulating proteins in the cells and studied the consequence on spine morphology. We quantified the density (referred to as “general density of spines” hereafter) and length of spines and filopodia. Subsequently, we segregated the protrusions according to their morphology and quantified them separately. Spine morphology analyses were performed at DIV12-14. Additionally, we directly assessed actin dynamics by GFP-actin fluorescent recovery after photo bleaching (FRAP) and identified the sites of actin polymerization by free-barbed (FBE) end assay at different stages of spinogenesis. Finally, we studied the correlation between actin cytoskeleton regulation and synaptic activity by the use of synaptic markers and electrophysiology.

1.1. Actin dynamics in filopodia and spines

Actin monomers are added to a new filament preferentially at the free plus (barbed) ends, which are commonly localized against the plasma membrane. In order to identify the sites of actin polymerization in dendritic filopodia we marked free barbed ends with fluorescently labelled actin monomers. Surprisingly, we observed that polymerization occurred not only at the tip of the dendritic filopodia but also close to dendritic shafts, at the root of the protrusion (I, Fig. 1 A, B). Quantifications revealed that about 20% of the filopodia had new actin monomers incorporated at the root of the protrusion. Below 20% had free barbed ends in both the root and the tip of the protrusion. The rest of the filopodia (around 60%) showed sites of actin polymerization exclusively at the tip (I, Fig. 1 C). To confirm these results, we performed FRAP analysis using GFP-actin expression in filopodia from DIV9 neurons. Indeed, we observed actin turnover in filopodia occurring at the tip of the protrusions in most of the cases and also near the dendritic shafts in certain cases (I, Fig. 1 D, E). Importantly, this was the first time that the base polymerization in dendritic filopodia was observed. This observation contradicts the previous assumptions on the polarity of actin filaments in dendritic filopodia. The presence of barbed ends at the base of filopodia points towards a multipolar orientation of actin filaments in these protrusions.
The GTPase Cdc42 is the main player of the canonical pathway of filopodia formation in non-neuronal cells (Kozma et al., 1995; Catherine D Nobes & Hall, 1995). Interestingly, Cdc42 has been previously shown to regulate spine morphogenesis (Irie & Yamaguchi, 2002). Here we observed that overexpression of both constitutively active (CA)-Cdc42 and dominant negative (DN)-Cdc42 in neurons reduced the general density of spines (I, Table IV). More precisely, CA-Cdc42 produced a reduction in the density of filopodia while it increased that of stubby and mushroom spines (I, Tables I, II). The head width of mushroom spines was also increased when compared to control neurons (I, Table III). Thus, Cdc42 seems to have a negative role on filopodia formation and a positive role in spine head expansion. The result coincides with the observations by Irie and Yamaguchi, when they overexpressed dominant negative Cdc42 and observed increased length and filopodia-like morphology of spines (Irie & Yamaguchi, 2002). It is interesting to point out that “conventional” filopodia rely on Cdc42 activation for their formation. Taken together, these results remark the different regulation of dendritic filopodia and conventional filopodia.

The small GTPase Rif forms part of an alternative pathway of filopodia formation in fibroblasts. In this cell type, Rif promotes filopodia elongation through its effector mDia2 (Faix & Rottner, 2006). To our knowledge, there are no previous studies on the role of Rif in spinogenesis. Therefore, we overexpressed CA- and DN-Rif and observed that inactivation of Rif reduces the general spine density (I, Fig. 2 A, B). Importantly, neurons expressing DN-Rif had decreased number of thin spines (I, Fig. 2 C) and increased head width in thin and mushroom spines (I, Table III). CA-Rif had no effect in any of the studied parameters (I, Fig. 2 D, E). In order to further understand the pathway downstream of Rif, we then overexpressed activated mDia2 together with DN-Rif. The result was a reduction of the general density of spines as well as their length (I, Fig. 3 A-C). Moreover, closer examination revealed that the number of thin spines was greatly reduced and that of stubby was slightly reduced (I, Fig. 3 D). In addition, knock-down of mDia2 by means of siRNA, reduced the general density and length of spines (I, Fig. 3 F) and specifically, it affected the density of thin spines (I, Fig. 3 G). siRNA against mDia2 produced increased head width in mushroom and thin spines (I, Table III). Notably, our results show that Rif and mDia2 are involved in spinogenesis and seem to negatively regulate spine head expansion. However, the phenotype of spines in neurons expressing inactive Rif and that of neurons with reduced mDia2 was not identical (I, Fig. 3 E) and also activated mDia2 was unable to effectively restore the phenotype after DN-Rif (I, Fig. 3 A-C). Thus the results underline the possibility that mDia2 is not the only effector of Rif in dendritic spines.

A subsequent study in 2011 explored the effect of Rif on neuroblastoma-derived cells (N1E115 cells). First of all they showed the direct interaction of Rif with the formin mDia1 by FRET studies. Moreover, the authors showed that Rif was able to induce actin dynamics and filopodia formation in N1E115 cells. When overexpressing Rif together with actin-GFP in N1E115 cells, the authors observed an increase in the density of filopodia with a concomitant reduction of the length of the protrusions (Goh et al., 2011). Together, all this
data suggest a role of Rif GTPase in filopodia formation, independent of Cdc42. Cdc42 is the main Rho GTPase involved in filopodia formation in non-neuronal cells (Nobes & Hall, 1995). The concrete mechanisms of Rif GTPase and its effectors, as well as the possible crosstalk with the classical pathway of Cdc42, in dendritic filopodia formation will need further scrutiny in the future. Moreover, other less studied GTPases have been shown to produce filopodia in non-neuronal cells. Such is the case for TC10, RhoT and Cdc42-like protein named WRCH1 which have not yet been scrutinized for their relationship to dendritic filopodia formation (reviewed in (Mattila & Lappalainen, 2008)).

1.2. Molecular mechanisms regulating actin dynamics in dendritic spines: spine head expansion

We next performed experiments towards the understanding of mechanisms regulating spine head formation and expansion. First of all, we identified the sites of actin polymerization in dendritic spine heads by free barbed end (FBE) assay as we did for filopodia (I, Fig. 1A, B). This visualization revealed competent barbed ends localized at the surface of the spine heads (I, Fig. 4A-C). The proportion of mature spines displaying root-polymerization was greatly reduced compared with dendritic filopodia (I, Fig. 1B, C; and Fig. 4C, D). We confirmed these observations by FRAP analysis (I, Fig. 4E). We can hypothesise that the regulation of actin filaments changes during the transition from dendritic filopodia to mature spines and that mature spines concentrate their polymerization sites to the spine head in order to produce the morphing concomitant to synaptic plasticity (Dunaevsky, Tashiro, Majewska, Mason, & Yuste, 1999; Tashiro & Yuste, 2004) rather than elongation of the dendritic protrusions, like in filopodia.

The ABP Arp2/3 complex promotes nucleation of branched actin networks and it has long been studied for its role in regulating actin during spine morphogenesis. However, its exact role within the spines has not been determined. We confirmed the presence of Arp2/3 complex in spine heads (I, Suppl. Fig. 4B) and proceeded to examine its function by silencing the expression of the p34 subunit of the complex by siRNA (I, Fig. 5A). Importantly, in siRNA-expressing neurons the general density of spines was diminished, while their length was increased (I, Fig. 5B). The spine-types that suffered significant depletion were thin and stubby (I, Fig. 5C). Thus, we confirm that Arp2/3 complex is an important actin-nucleating protein in dendritic spines, as the deficiency of the complex affects the general number of spines as well as their length. This effect of p34 siRNA can be interpreted as delaying spine maturation.

In an attempt to elucidate the possible cross-talk between the independent molecular pathways of Rif GTPase and its effector mDia2 with Cdc42 GTPase and its effector Arp2/3 complex, we tested the hypothesis that Rif and Arp2/3 complex had antagonizing roles on the actin cytoskeleton. Thus, we used the DN-Rif together with the Scar-1 WA-fragment to disrupt the localization of Arp2/3 complex (I, Fig. 5D). The Scar-1 WA is a fragment of the C-terminus of Scar-1 protein that has been previously shown to interact with Arp2/3
complex (Machesky & Insall, 1998). Importantly, overexpression of WA-fragment in neurons produced a similar phenotype to siRNA against Arp2/3 components (I, Fig. S4, E–G). When expressed together, WA-fragment and DN-Rif resulted in reduction of spine density (I, Fig. 5 E), with reduced thin and mushroom spines (I, Fig. 5 F). As mentioned before, DN-Rif expression had caused reduction in thin spines and siRNA against the downstream effector of Rif, mDia2, had produced increased head width of mushroom and thin spines (I, Fig. 2, 3 and Table III). Thus, Arp2/3 promotes spine head expansion and it can override Rif, suggesting a possible cross-talk between the two pathways.

1.3. The role of actin depolymerization in spine morphogenesis

Actin depolymerization is of crucial importance to the regulation of actin dynamics. ADF/cofilins are the main depolymerizing agents in cells (Bamburg, McGough, & Ono, 1999). First, we utilised western blot to analyse the expression of cofilin-1 in hippocampal cells and found that it was expressed throughout the development of spines (I, Suppl. Fig. 5 A). Then we determined the subcellular localization of cofilin-1 and confirmed previous data showing that cofilin-1 is enriched in dendritic spine heads (Racz & Weinberg, 2006); high cofilin-1 levels were found in shafts as well (Suppl. Fig. 5 A).

In order to better characterise the role of cofilin-1 in the regulation of the actin cytoskeleton dynamics in developing spines we used siRNA to deplete cofilin-1 in neurons and performed GFP-actin FRAP experiments in them. We expected that, as in fibroblasts (Hotulainen, Paunola, Vartiainen, & Lappalainen, 2005), knockdown of cofilin-1 would reduce the rate of actin turnover in dendritic spines. Indeed, the first order rate constant in siRNA-expressing spines was three times lower than in WT spines. Also, the stable actin pool was larger in cofilin-1 knock-down spines as their final recovery was substantially less than in WT (I, Fig. 6 D, E). Interestingly, the spine analysis of cofilin-1 siRNA-expressing neurons revealed increased length of spines and reduced number of thin spines (I, Fig. 6B, C). Importantly, the spines from siRNA-expressing neurons were branched (I, Fig. 6F). Quantification showed a significant increase in branched spines in cofilin-1 siRNA sample (I, Fig. 6C). Taken together, these data demonstrate a prominent effect of cofilin-1 as regulator of actin dynamics. Cofilin-1 increases actin dynamics as its depletion results in slow turnover rate and larger stable pool. Also, cofilin-1 is crucial for actin remodelling in spines, since its reduction provoked aberrant morphology of spines. Importantly, accumulated evidence highlight the importance of a tight regulation of cofilin-1 in relation to spine morphogenesis. Recent data suggest that both spine growth and shrinkage correspond to low cofilin-1 activity, although the precise outcome depends on the mechanism of inhibition. Spine shrinkage is related to low barbed-end production due to low cofilin-1 activity and spine growth corresponds to LIMK activity, cofilin-1 phosphorylation and inactivation, which leads to activation of phospholipase D-1 to promote actin polymerization, respectively (Calabrese, Saffin, & Halpain, 2014). Taken together, the results underscore that regulation of actin dynamics by cofilin-1 in spine remodelling is a crucial
process, but much work is still needed in order to determine the precise mechanisms underlying these phenomenon.

1.4. Synaptogenesis is regulated by actin dynamics

Actin dynamics are pivotal for synaptogenesis (Ethell & Pasquale, 2005). To assess the involvement of cofilin-1, Arp2/3 complex and Rif-mDia2, which we described as crucial regulators of spine morphology, we negatively manipulated these pathways in neurons. Subsequently, we performed immunostaining in the cultured neurons with vGLUT-1 as a pre-synaptic marker. We quantified synaptic contacts and observed that neurons in which the localization of the Arp2/3 complex had been disrupted by expression of the WA-fragment had less pre-synaptic contacts (I, Fig. 7 A, B).

Next, we analysed synaptic function in neurons by means of electrophysiological recordings. The analyses showed an increased inter-event interval of miniature excitatory post-synaptic currents (mEPSCs) in neurons expressing inactive Rif or p34-siRNA. Depletion of cofilin-1 by siRNA had no effect in the inter-event intervals of mEPSCs (I, Fig. 7 C-E). Importantly, none of the mentioned manipulations affected the amplitude of the events (I, Fig. 7 E). Taken together, these data show that actin cytoskeleton dynamics during spine development are important regulators of synaptic efficacy.

It is important to note that the number of spines and synapses do not always correlate, as there can be a shift in the number of synapses formed onto the dendritic shafts. With the method used here, we have not aimed to distinguish between synapses formed on spines and those formed onto shafts (I, Fig. 7 A, B).

This work allowed us to build a working model for spine development (I, Fig. 8). The development of dendritic spines starts by initiation of a dendritic filopodium that becomes later elongated thanks to the action of the actin polymerizing protein mDia2 at the tip of the filopodium, driven by the GTPase Rif. If the spine receives the necessary synaptic input and becomes stabilized, the spine head starts its expansion with the help of the actin nucleating Arp2/3 complex, which promotes branching of the actin filaments. The mechanisms regulating the step from filopodia elongation and spine head expansion are so far unclear and should be a subject for further study. The final maturation occurs by shortening of the spine neck and growth of the spine head. Actin remodelling through the action of the depolymerizing factor cofilin-1 takes place continuously during the whole process to ensure maintenance of the actin monomer pool, formation of polymerizing barbed-ends and shortening of unnecessary long actin fibres (I, Figure 8).
2. KCC2 regulates actin cytoskeleton and synapse formation through βPix (II)

The potassium chloride cotransporter KCC2 regulates chloride extrusion in mature neurons. Therefore, KCC2 is crucial for fast GABAergic and glycinergic inhibition in adult CNS (reviewed in (Chamma, Chevy, Poncer, & Lévi, 2012; Uvarov, Llano, Ludwig, Airaksinen, & Rivera, 2013)). In addition, KCC2 has been shown to be involved in the regulation of dendritic spine formation in a chloride-extrusion independent manner (Fiumelli et al., 2013; Horn et al., 2010; H. Li et al., 2007). Several publications have proposed that the main mechanism by which KCC2 controls morphogenesis could be by regulation of the actin cytoskeleton. However, the authors do not show a direct link (Horn et al., 2010; Wei et al., 2011), and therefore we analysed the mechanisms leading to the actin cytoskeleton remodelling in dendritic spines by KCC2.

2.1. Deficiency of KCC2 leads to increased actin stability and elevated levels of phosphorylated cofilin-1

First, we examined KCC2 deficient neurons in dissociated culture (KCC2 KO neurons). We used the FRAP technique to determine the turnover of GFP-actin in dendritic spines of DIV15-17 hippocampal neurons lacking KCC2. Interestingly, spines of KCC2 KO neurons showed much lower recovery of fluorescence through actin turnover than spines of WT littermates (II, Fig. 1). Lower recovery of fluorescence in FRAP indicates a larger stable actin pool and reduced actin turnover. Overexpression of KCC2 or mutants of KCC2, which are incapable of chloride transport (KCC2-C568A (Reynolds et al., 2008) and KCC2-Y1087D (Strange, Singer, Morrison, & Delpire, 2000) improved actin turnover to a great extent, showing that KCC2 is responsible for the phenomenon. These results also show that the function of KCC2 as an actin turnover-regulator is independent of chloride transport (II, Fig. 1). In an attempt to clarify which molecule could mediate the effect of KCC2 in the turnover of actin, we quantified the levels of phosphorylated, and thus inactive, cofilin-1 (p-cofilin-1) by western blotting. We observed that KCC2 deficient neurons have elevated levels of p-cofilin-1 when compared to WT neurons (II, Fig. 2 a). To clarify whether elevated p-cofilin-1 is an indirect effect of KCC2-deficiency, we overexpressed the mutants incapable of chloride transport, KCC2-C568A and KCC2-Y1087D, in KCC2 KO neurons and measured the level of p-cofilin-1 by immunostaining. Both constructs were able to reduce the levels of p-cofilin-1, indicating that the effect of KCC2 in cofilin phosphorylation is not dependent on the chloride extrusion activity of the transporter (II, Fig. 2 b). Moreover, we asked whether the lack of KCC2 affects the motility of dendritic spines. We measured protrusive motility of DIV21 KCC2 KO neurons by means of live confocal imaging. Indeed, spines of KCC2 KO neurons revealed a reduction in protrusive motility when compared to WT littermates (II, Fig. 7d). Taken together, this group of experiments show that the control of the actin turnover and phosphorylation state of cofilin-1 in cultured neurons is independent of the role of KCC2 as chloride extruder, as both mutant constructs KCC2-C568A and Y1087D
behave similarly to WT-KCC2 in these experiments (II, Fig. 1 and Fig. 2). Importantly, enhanced phosphorylation in Ser3 and consequent deactivation of cofilin-1 (Mizuno, 2013) observed in KCC2 KO mice would lead to decreased actin depolymerization and this could explain the aberrantly long and branched spine phenotype of neurons of KCC2 KO mice (H. Li et al., 2007). Indeed, in study I we showed that reduction of cofilin-1 by siRNA in neurons leads to decreased actin turnover and to abnormally branched spine phenotype, resembling KCC2 deficiency (I, Fig. 6). Moreover, reduced cofilin-1 activity and the subsequent stabilization of actin filaments can lead to the decreased motility found in spines of neurons expressing KCC2-shRNA (II, Fig. 7d).

2.2. KCC2 interacts and is colocalized with βPixb in neurons

Actin dynamics are regulated by small GTPases of the Rho superfamily, which work as molecular switches. The function of Rho GTPases is tightly regulated by GEFs and GAPs. By means of Yeast two-Hybrid (Y2H) assay, we identified the β isoform of the GEF βPix as a potential interacting partner of KCC2 (II, Supp. Fig. 2 a). We then confirmed the interaction between KCC2 and βPixb in adult rat brain by co-immunoprecipitation (II, Fig. 3 a). In order to better characterise the interaction, we performed co-immunoprecipitation assays in HEK293T cells overexpressing the two proteins (II, Fig. 3 b). Interestingly, when we overexpressed βPixb together with the C-terminal domain and treated the lysates with anti-βPix antibodies, the C-terminal domain of KCC2 was co-precipitated (II, Fig. 3 b, right). Thus, KCC2 and βPix interact through the C-terminal domain of KCC2. It is relevant to note here that the actin and spectrin-binding protein 4.1N also binds to KCC2 through the C-terminal domain of the cotransporter (H. Li et al., 2007) (see section 6.1.2 Morphogenic role of KCC2). In an attempt to elucidate the concrete area of interaction between KCC2 and βPix, we generated a construct in which we mutated two proline residues present at a proline-rich stretch in the C-terminal domain of KCC2. Of note, this region is conserved among KCCs (KCC1-4) and in the case of KCC3, it has been shown to be responsible for the direct interaction between KCC3 and the SH3 domain of the GEF Vav2 (Salin-Cantegrel et al., 2013). However, in the case of KCC2, the mutation of the proline residues into glutamine and alanine did not affect the ability of KCC2 to interact with βPix (Supp. Fig. 2b), and therefore the exact interacting residues await further studies.

In order to study localization of KCC2 and βPix in vivo, we performed double immunostaining of brain sections of mice expressing YFP in a sparse subpopulation of neurons (II, Fig. 5a). We observed colocation of the KCC2 and βPix in spines, dendritic shafts as well as neuronal soma. Next, we analysed the localization of the two proteins in different spine types. Both KCC2 and βPix were highly enriched in mushroom spines while their presence was low in filopodial protrusions (II, Fig. 5b, c). This data, together with the co-immunoprecipitation data, suggest that the interaction between KCC2 and βPix could have a functional
relevance in neurons and more specifically in the development of spines through the regulation of their morphology.

2.3. KCC2 inhibits βPixb

As βPix is a GTP exchange factor for Rac1 and Cdc42 (E Manser et al., 1998), we assessed the influence of KCC2 on the activity of βPix by GLISA (small GTPase activation assay) in HEK293T cells. By use of this method, we detected the level of Rac1 activation. We observed that expression of βPixb increased Rac1 activity, as expected. When KCC2 and βPixb were expressed together, the βPixb-induced activation of Rac1 was fully abolished (Fig. 3c). βPix interacts with PAK1 (Bagrodia et al., 1998), and they belong to the signalling cascade βPix/Rac1/PAK/cofilin-1, where cofilin-1 is phosphorylated and thus inactivated by LIMK (Saneyoshi & Hayashi, 2012; Saneyoshi et al., 2008). Therefore, we next measured the p-cofilin-1 levels of HEK293T cells overexpressing KCC2 and βPix and observed that, when both proteins were present in the cells, phosphorylation of cofilin-1 was significantly diminished (II, Fig. 3 d). Taken together, these results demonstrate that KCC2 interacts with βPix and inhibits its GEF activity towards Rac1 GTPase, which results in reduced cofilin-1 phosphorylation.

The next step was to analyse whether KCC2 could influence the activity of βPix in neuronal cells. For that we performed Förster resonance energy transfer (FRET) measurements with Raichu-Rac1 (a probe that responds to activation of Rac1 with increased FRET signal (see materials and methods, Itoh et al., 2002)) in neurons (II, Fig. 4). Indeed, overexpression of KCC2-C568A repressed endo- and exogenously expressed βPix to the same extent; at a level that was comparable with expressing dominant-negative βPix (βPix-DHm; (Saneyoshi et al., 2008) (II, Fig. 4b). This important result highlights the functional relevance of the interaction between KCC2 and βPix since the cotransporter is able to inhibit the activity of βPix in the context of neurons and could explain the mechanism by which KCC2 regulates the activity of cofilin-1.

2.4. Regulation of synaptogenesis by KCC2 and βPix

Our results above show that KCC2 inhibits βPixb-induced activation of Rac1. To study, if this regulatory interaction has consequences on synaptogenesis, we next analysed the activity of Rac1 in DIV13-14 neurons with reduced levels of KCC2 (neurons expressing shRNA against KCC2, Pellegrino et al., 2011). As expected, the dendrites of KCC2-shRNA expressing neurons showed increased intensity of FRET activity, indicating that Rac1 is over-activated. In neurons, the activity of βPixb is stimulated by phosphorylation on the Ser516 residue of the protein by CaMKI. Overexpression of the mutant βPixb-Ser516Ala (βPix-S516A) has been previously shown to reduce the number of synapses, similarly to expression of siRNA against βPixb (Saneyoshi et al., 2008). Importantly, when we overexpressed the mutant βPix-S516A in cells with reduced
KCC2 expression, the level of Rac1 activation was significantly reduced (II, Fig. 6a, b). These results show that endogenous KCC2 represses the activity of endogenous βPix towards Rac1 in dendrites.

Finally, we addressed the functional relationship between KCC2 and βPix during synaptogenesis. For that, we analysed several synaptic parameters in DIV13-14 cultured hippocampal neurons expressing shRNA against KCC2 with or without the inactive βPix-S516A. Neurons with diminished levels of KCC2 had reduced number of dendritic spines and synapses (II, Fig. 7a); while their spine-heads were enlarged (II, Fig. 7b), as reported previously (Gauvain et al., 2011). The expression of the βPix mutant βPix-S516A reduced the number of synapses and dendritic spines (Fig. 7a) and it also increased the inter-event interval of mEPSCs (II, Fig. 7c). These effects of βPix were in concordance with previous studies (Saneyoshi et al., 2008). Notably, the spine head diameter was smaller in βPix-S516A-expressing neurons (II, Fig. 7b) than in control or in KCC2-shRNA expressing neurons. Most importantly, addition of the mutant βPix-S516A on KCC2-shRNA-expressing neurons had no additive effect on KCC2-shRNA phenotype for any of the studied parameters (II, Fig. 7a, b, c). Hence, KCC2 regulates spine and synapse formation through βPix.

The data presented here let us draw a working model for the regulation of the actin cytoskeleton in dendritic spines by the functional interaction between KCC2 and βPix. In our model (II, Fig. 8, left), KCC2 is expressed in neurons and it is localized to the plasma membrane during synaptogenesis under physiological conditions. There, it interacts with βPix through the C-terminal domain of KCC2 and negatively regulates the function of βPix. In this scenario, the presence of KCC2 ensures physiological levels of Rac1 activity and cofilin-1 phosphorylation. In the event of low KCC2-expression levels (II, Fig. 8, right), activation of βPix is excessive and it over-activates Rac1. As a consequence, cofilin-1 is mostly in its phosphorylated form, causing the balance in actin treadmilling to shift towards actin stabilization. Reduced turn-over of actin leads to decreased spine motility and aberrant spine morphology, resulting in impaired synaptogenesis.

One can hypothesise that the mechanisms described here in the context of synapto- and spinogenesis are possibly applicable to other cellular processes. KCC2 is expressed in cancer cells, where it promotes cell invasion and affects cell morphology. In these cells, KCC2 is present at focal adhesions and is correlated with FAK expression (Wei et al., 2011). In this context, it is interesting to note that βPix recruits Rac1 to focal adhesions (ten Klooster et al., 2006) and that it negatively regulates maturation of these actin structures (Zhao et al., 2000). Moreover, βPix contributes to cell migration by activating Rac1 and promoting the turnover of nascent focal adhesions (Kuo, Han, Hsiao, Yates, & Waterman, 2011). It is plausible to think that KCC2 might negatively regulate βPix in order to create cycles of Rac1 activation/inactivation that will lead to lamellipodial protrusion during cell migration.

A very recent study has shown the molecular complex formed by GIT1/βPix and GABA_A receptors. The authors claim that GIT1 and βPix are essential for the surface expression of GABA_AR through Rac1 activation
and the effector PAK. Importantly, manipulations of the actin cytoskeleton disrupt inhibitory synapse integrity and GABA$_A$R surface expression. Electrophysiological recordings show that inhibitory synaptic transmission is affected by overexpression of shRNA against GIT1 and βPix as well as by overexpressing DN-PAK (Smith et al., 2014). In this context, it is interesting that also KCC2 has been proposed to interact with GABA$_A$R (Y. Huang et al., 2013) and to regulate inhibitory neurotransmission (see section 6.1.2 KCC2 function). Therefore the latest publication of Smith et al. reinforces the idea that inhibitory and excitatory synapses share the main players regulating their morphology and function (Smith et al., 2014). However, the precise molecular mechanisms taking place in the two types of synapses are likely not identical. As demonstrated, for instance, by the difference in dwell time of KCC2 in excitatory and inhibitory synapses (Chamma et al., 2013). It is nevertheless plausible to think that KCC2 might act as a synchronizing factor between excitatory and inhibitory neurotransmission through regulation of βPix and Rac1.

3. ICAM-5 and β1 integrins interact trans-synaptically to regulate synaptogenesis (III)

Intercellular adhesion molecule-5 (ICAM-5) is involved in dendritic spine remodelling as it negatively regulates morphological and functional spine maturation (Barkat, Polley, & Hensch, 2011; Matsuno et al., 2006; L Tian et al., 2000). ICAMs act as ligands for integrins from surrounding cells. Integrins are vastly known for their role in regulation of axonal growth and guidance, as well as neurite outgrowth, synapse formation and plasticity (reviewed in (McGeachie, Cingolani, & Goda, 2011)). β1 integrin is widely expressed in CNS and it has been previously reported to bind the ectodomain of ICAM-5 (Conant et al., 2011). However, the functional relevance of the interaction remained unclear. We performed the following experiments in an attempt to clarify the synaptic localization of these interacting proteins as well as further characterize their functional role in the maturation of dendritic spines.

3.1. Genetic ablation of ICAM-5 promotes synapse efficacy

ICAM-5 has been found to prevent spine maturation by slowing down the filopodia-to-spine transition (Yoshihara, Roo, & Muller, 2009). To confirm these findings in vivo, we performed electrophysiological recordings of ICAM-5 KO neurons and found that the frequency of mEPSCs was higher in ICAM-5 KO than in WT, supporting the knowledge that ICAM-5 negatively regulates functional synapse formation (III, Fig. 1 C-F).

3.2. ICAM-5 and β1 integrin interact through the extracellular domain

In order to corroborate previous data, we performed co-immunoprecipitation (co-IP) from mouse brain homogenates and observed that by using antibodies against β1 integrin, we precipitated ICAM-5 and vice versa (III, Fig. 3 A and B). In Paju cells, we characterized the interaction further. Deletion of the cytoplasmic-tail domain of ICAM-5 did not affect the interaction (III, Fig. 3C), indicating that the binding site is within the
extracellular domain. We then mapped the binding sites in ICAM-5 to the two first Ig-like domains of the CAM with cell adhesion assays (Ill, Fig. 4A, B and Table I). Finally, enzyme-linked immunosorbent assays (ELISA) confirmed the direct interaction between β1 integrin and ICAM-5 (Ill, Fig. 3D). With this, we corroborated the previous results where the ectodomain of ICAM-5 had been shown to interact with β1 integrin in a neuronal-derived cell line as well as in hippocampal lysates (Conant et al., 2011). We also further defined the interaction sites on both proteins.

3.3. β1 integrins are preferentially pre-synaptic and ICAM-5 post-synaptic

In order to study the distribution of β1 integrins and ICAM-5 in neurons, we stained DIV15 hippocampal neurons with specific antibodies against either of the studied proteins together with synapsin I (pre-synaptic marker) and PSD-95 (post-synaptic marker). Colocalization analysis showed that the integrin is colocalized in a higher degree with the pre-synaptic marker synapsin I than with the post-synaptic PSD-95 (Ill, Fig. 5 A, C). On the other hand, ICAM-5 colocalizes preferentially with PSD-95 (Ill, Fig. 5 B, D). Decisively, immuno-EM (Ill, Suppl. Fig. 3) and synaptosomal fractionation (Ill, Fig. 5) further confirmed the pre-synaptic presence of β1 integrins.

3.4. β1 integrins and ICAM-5 act together to prevent spine maturation

To elucidate at which stage of dendritic spine development does the interaction between ICAM-5 and β1 integrins take place, we transfected hippocampal neurons in dissociated culture and fixed them at DIV15 and 22, followed by immunostaining for β1 integrin. β1 integrin was weakly localized at the tips of filopodia, while localizing inside the tip of more mature, mushroom spines. In the case of thin spines, β1 integrins were not localized inside the spine head but juxtaposed to the head (Ill, Fig. 6 A, B). Subsequently, we analysed the colocalization of β1 integrins with ICAM-5. The two molecules colocalized at the tip of filopodia and thin spines. More mature spines were devoid of ICAM-5 and still contained β1 integrins (Ill, Fig. 6C).

By use of activating and blocking antibodies, we addressed the issue whether ICAM-5 and β1 integrins acted together to delay spine maturation. Interestingly, blocking antibodies for either ICAM-5 or β1 integrins led to an increase in the density of mushroom spines, while the density of immature spines was decreased. On the other hand, by delivering an antibody that activates β1 integrins, the density of filopodia increased, while that of spines was reduced (Ill, Fig. 2 A, B). Moreover, overexpressing shRNA against β1 integrins in pre-synaptic neurons reduced the number of immature spines and increased that of mature spines in contacting post-synaptic dendrites (Ill, Fig. 8 C-E). These data point towards a coordinated regulation of spine maturation by ICAM-5 and β1 integrins where the interaction of the two molecules prevents spine maturation. According to this model, disruption of the interaction between ICAM-5 and β1 integrins is necessary for the progression of spine maturation.
3.5. Interaction with β1 integrins inhibits ICAM-5 cleavage

The ectodomain of ICAM-5 is cleaved and subsequently released to the intercellular space in a phenomenon called shedding. Shedding has been found responsible for spine maturation (Li Tian et al., 2007). Thus, we analysed whether the interaction of ICAM-5 and β1 integrin affected ICAM-5 shedding. We detected the presence of ICAM-5 ectodomain in media from cultured neurons treated with antibodies by immunoblot. Notably, antibodies that blocked the interaction between ICAM-5 and β1 integrin significantly enhanced the cleavage of ICAM-5. On the contrary, the β1 integrin-activating antibodies treatment attenuated the shedding (III, Fig.7). Since the soluble fragment of ICAM-5 has been found to have immunosuppressive effects, the interaction between ICAM-5 and β1 integrin could have further consequences on the brain homeostasis beyond regulating spine maturation (Gahmberg et al., 2014; Li Tian et al., 2008). Moreover, since the soluble ectodomain of ICAM-5 produces integrin-dependent phosphorylation of cofilin-1 in neurons (Conant et al., 2011), the interaction with β1 integrin could represent the mechanism by which ICAM-5 delays spine maturation. As seen in our previous studies (I and II), the phosphorylation state of cofilin-1 regulates actin dynamics and therefore, spine morphology changes. Actin dynamics and morphological maturation of spines are key steps in the regulation of synaptic connectivity.

In conclusion, we demonstrate here that ICAM-5 and β1 integrins interact in neurons and work together to prevent spine maturation by inhibiting the extracellular cleavage of ICAM-5.
CONCLUDING REMARKS

Dendritic spine development is a tightly regulated process. The importance of such stringency in the regulation of the phenomenon is quite obvious. This is true not only in the light of memory formation and learning, but also when taking into account extensive observations of the correlations between intellectual and behavioural disabilities and abnormal spine formation.

The results presented in this thesis work are small engine pieces that contribute to the great machinery of molecules regulating brain function. By use of dissociated neuronal cultures, we have studied dendritic spine development in detail. More concretely, we can divide the findings in three main categories:

1) Direct regulation of the actin cytoskeleton.

By direct observation of the sites of polymerization in filopodia and spines, we have identified where polymerization occurs at the subcellular level. The data suggest that polymerization does not only occur at the tip of the protrusions but also at the base. FRAP studies have allowed us to pinpoint cofilin-1 and KCC2 as direct and indirect, respectively, regulators of actin turnover in spines. In addition, the results broaden our knowledge on actin turnover as well as actin filament distribution in normal physiological conditions.

We have further characterized the role of actin-regulating proteins Arp2/3 complex in spine head expansion; mDia2 in dendritic protrusion elongation, and cofilin-1 in actin depolymerization, severing of actin filaments and spine shaping. In the case of mDia2, we identified, for the first time, the molecule as a regulator of dendritic filopodia and therefore prompt the appearance of further studies to clarify its precise role in filopodia and spine development.

2) Molecular mechanisms that regulate the course of development of dendritic spines.

The data presented here contribute to refining our view on the molecular pathways of the GTPases Rif and Rac1 and their effectors mDia2 and Arp2/3 complex; and their contribution to spine elongation and head expansion, respectively. We corroborate that, as in other cell types, these pathways are present in dendritic spines and are relevant to the regulation of the actin cytoskeleton in them.

In addition, we describe a novel interaction between the cotransporter KCC2 and the guanine-nucleotide exchange factor βPix. The inhibition of βPix by KCC2 and subsequent Rac1 de-activation provides a mechanism of control of spine formation.

Furthermore, the presented results provide insight to the mechanism by which ICAM-5 negatively regulates spine maturation. Trans-synaptic interaction of ICAM-5 with β1 integrin prevents ICAM-5 cleavage by MMPs, delaying filopodia-to-spine transition through regulation of cofilin-1.

3) Regulation of synaptic maturation.
Finally, the functional relevance of the mechanisms described above at the network level is studied here by electrical recordings of synaptic activity. We have identified Arp2/3 complex, Rif, KCC2 and βPix as positive regulators of synaptic efficacy, while ICAM5 and β1 integrin have an opposite effect. Clarification of the individual role of these molecules in the development of synaptic connectivity is a necessary step towards our understanding of the brain development and function.
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Olaya Llano
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