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ACTIN CAPPING PROTEIN IN DENDRITIC SPINE DEVELOPMENT

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2013

ACTIN CAPPING PROTEIN IN DENDRITIC SPINE DEVELOPMENT

By

Yanjie Fan

B.S., Huazhong University of Science and Technology, 2007

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An Abstract of

A dissertation submitted to the Faculty of
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Abstract

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Dendritic spines are the tiny membrane protrusions from dendritic processes and they serve as the postsynaptic platform for most excitatory synapses in the mammalian brain. The development of dendritic spines is a key step in forming synaptic contacts and establishing the neuronal circuitry. Actin is a major structural component of dendritic spines and plays a crucial role in spine development. However, the detailed mechanisms that regulate the actin structure and dynamics in spines remain to be elucidated. Our study has identified that the actin capping protein (CP), a regulator of actin filament elongation, plays an essential role in spine development and synapse formation. We found that CP expression in hippocampus was elevated at and after the stage of extensive synapse formation. Moreover, CP was found to gradually localize to dendritic protrusions during development and become highly accumulated in mature dendritic spines. These observations suggest a potential role of CP in spine formation. Utilizing a loss-of function approach, we found CP knockdown in cultured hippocampal neurons resulted in a marked decline in spine density and a concomitant increase of filopodia-like thin dendritic protrusions. The spines which were able to form in CP knockdown cells exhibited an altered morphology, highlighted by multiple thin filopodia-like protrusions emerging from the spine head. Functionally, CP knockdown reduced the number of synapses as evidenced by a reduction in the density of paired pre- and postsynaptic markers. Electrophysiological studies also showed that the frequency of miniature excitatory postsynaptic currents was markedly reduced in CP knockdown neurons. These findings indicate that CP is indispensible for the spine morphogenesis and synaptic formation- likely involved in the conversion of dendritic filopodia to spines. Further experiments suggest that CP knockdown resulted in the instability of dendritic filopodia, as well as a reduction in the clustering of postsynaptic scaffolding proteins in dendritic protrusions. Based on these results, we propose a model in which CP gradually accumulates in dendritic protrusions during synaptic development to stabilize them and facilitate the recruitment of postsynaptic scaffolding proteins. This process enables the morphological differentiation and postsynaptic receptor clustering that are needed for the formation of functional synapses.

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Chapter 1 Introduction

This dissertation investigates the role of actin capping protein, a cytoskeletal regulator, in dendritic spine development and synapse formation. Synapse is the primary site for neuronal communication, and dendritic spine serves as the postsynaptic platform of most excitatory transmission in the mammalian brain. Actin cytoskeleton is the major structural support in dendritic spines. However, the mechanism of actin regulation during dendritic spine development remains to be elucidated. Actin capping protein (CP), the barbed-end capper of actin filaments, is concentrated in dendritic spines. We hypothesize that CP regulates the morphogenesis of dendritic spines and synapse formation. To understand whether and how CP contributes to the spine/synapse formation process, the foundation of synapse and actin cytoskeleton will be introduced in this chapter.

First, the structure of synapse and dendritic spines, as well as the structure-function correlation of dendritic spines, are reviewed in section 1.1 and 1.2. The cytoskeletal control of dendritic spine structure and function is summarized in section 1.3, followed by an introduction of CP and its regulation of cellular processes in section 1.4. In the end, current understanding of actin regulatory mechanisms underlying dendritic spine formation is reviewed in 1.5. This chapter provides an overview of the cytoskeletal control of dendritic spines and synapses, and how CP could potentially contribute to this regulation.

1.1 Synapses

Brain is the central governor of human thoughts and behaviors. It is a superior neural network that can perceive, integrate and discriminate numerous environmental stimuli and generate appropriate responses. The brain functions through 100 billion of neurons and the intricate connections. The neuron is the basic node in this network. Although more than one thousand neuronal types have been identified, majority of neurons share a similar structure including the cell body, axon and dendrites. The organization, pattern and strength of

interconnections between neurons mediate the superior complexity and diversity of neuronal network (Kandel, Schwartz, & Jessell, 2000; Sherrington, 1947).

Neurons connect through a specialized junction called synapse, the name of which originated from the Greek word “clasp”. Synapse is the primary site for neuronal communication. There are two types of synapses - electrical or chemical synapses, based on the mediators of signal transduction. Electrical synapses function by direct current flow through gap junctions between neurons, allowing fast propagation of electrical signal to interconnected neurons and generating synchronous firing (Makowski, Caspar, Phillips, & Goodenough, 1984). Chemical synapses employ chemical neurotransmitters to mediate the communication between neurons. Neurotransmitters are released from the presynaptic neuron, and then bind to corresponding receptors on the postsynaptic membrane of another neuron or receptor organ to accomplish the signal transduction (Ungar & Irwin, 1968). Compared with the simple and transient depolarizing signal spread by electrical synapses, chemical synapses enable the directional flow of information and allow neuronal communication with a great diversity of signaling. Chemical synapses can also mediate long-lasting changes of neuronal connections. These properties make chemical synapses the ideal storage sites of experience-dependent modulation. The organization and strength of chemical synapses serve as the foundation of neuronal activities that produce a great complexity of behaviors, like perception, movement, learning and memory (Kandel et al., 2000).

The chemical synapse is structurally composed of presynaptic axonal terminal, postsynaptic dendritic specialization, and a synaptic cleft (20-40nm) between them (Figure 1-1). Synaptic vesicles are filled with neurotransmitters and cluster at the active zone of presynaptic terminals. During synaptic transmission, the presynaptic terminal is depolarized by action potential, and a subsequent influx of calcium into the terminal is mediated by voltage-gated ion channels. The increase of calcium concentration triggers the fusion of vesicles to the presynaptic

membrane and the release of neurotransmitters into the synaptic cleft. Neurotransmitters bind to corresponding receptors on the postsynaptic surface membrane, which directly generates currents through ion channels or activates signaling cascades in the postsynaptic neuron (Hall, 1972).

Chemical synapses can be categorized as excitatory or inhibitory, based on the action of neurotransmitters generated at the postsynaptic membrane. Whether excitatory or inhibitory potentials will be generated is highly dependent on the ion channels coupled to the receptors on the postsynaptic membrane. Most excitatory synapses in the vertebrate brain are mediated by glutamate transmitters, with AMPARs, NMDARs or metabotropic GluRs on the postsynaptic membrane. Other common transmitters of excitatory synapses include acetylcholine, catecholamine (dopamine, epinephrine and norepinephrine), serotonin and histamine. However, "excitatory" transmitters can mediate inhibitory potentials as well. For example, glutamate can be the transmitter for certain inhibitory synapses in auditory system through activation of metabotropic glutamate receptors. GABA and glycine are the most common neurotransmitters in inhibitory synapses, although GABA-mediated synaptic transmission can be excitatory at the early stage of brain development (Kandel et al., 2000). Excitatory and inhibitory synapses have differential distribution in neurons. Excitatory synapses are primarily located on the dendrites, and for glutamatergic synapses in the central nervous system, a large portion reside on the tiny protrusions named dendritic spines. Inhibitory synapses are usually located on the cell body and the proximal section of dendrites. An average neuron integrates thousands of axonal inputs through synaptic connections, and all neurons have a heterogeneous combination of excitatory and inhibitory synapses (Gray, 1969). While excitatory synapses mediate depolarizing potentials that are the driving force for the firing of neuronal circuitry, inhibitory synapses are essential for the stability of neuronal network. A well maintained balance between excitatory and inhibitory inputs is crucial for normal functions of the brain (Mody, Otis, Staley, & Kohr, 1992). This is

exemplified by the imbalance of excitation and inhibition in a large number of neurological disorders, like epilepsy, autism and schizophrenia (Dichter & Ayala, 1987; Yizhar et al., 2011).

Synapses can be dynamically modulated by environmental stimuli and experience. Synaptic plasticity represents an important type of modulation of the synaptic strength by specific patterns of activity, which is considered to be the basis of learning and memory. The best studied paradigms of synaptic plasticity are long-term potentiation (LTP) and depression (LTD), which represent a persistent increase of synaptic strength following high-frequency of stimulation or persistent weakening of synaptic strength following low-frequency of stimulation, respectively. The expression of LTP and LTD involve multiple aspects of modification on the synapse, including the change of neurotransmitters released from presynaptic terminals, altered sensitivity or number of receptors on the postsynaptic membrane, and sometimes the formation or elimination of synapses (Alkon, Sakakibara, Naito, Heldman, & Lederhendler, 1986). Together these modifications control the enhancement or weakening of synaptic connections in neuronal circuits.

Synaptic function and efficacy are the foundation of many brain activities, including learning and memory. Brain development is a process of establishing synaptic contacts, wiring and modifying neuronal circuitry. This complex wired network is subjected to change throughout life. One way that experience is thought to alter our thinking and behavior is by the modulation of synaptic efficacy and rearrangement of synapses at different orders of circuitry. Understanding the molecular mechanisms of synapse is the first step to understand the function and organization of the nervous system. Over the past century, tremendous efforts have been put in this area to reveal the synaptic structure and mechanisms of synaptic modifications, which have greatly advanced our understanding of the brain and complex behaviors.

1.2 Postsynaptic compartment - dendritic spines

One of the key features of chemical synapses is the existence of a specialized postsynaptic structure that contains concentrated neurotransmitter receptors for effective neurotransmission. In excitatory synapses of the vertebrate brain, this postsynaptic structure is hosted by dendritic spines, the tiny membrane protrusions bulging from dendritic shafts. Such an arrangement is thought to be advantageous in the biochemical and electrical isolation of signaling from different synaptic inputs. Understanding the structure and molecular components of spines is the basis of functional interpretation of synaptic activity. Due to the extremely small size of spines, characterization of spine structure is traditionally done by electron microscopy. In recent years, with the improvement of labeling methods and imaging techniques, the ultrastructure of dendritic spine begins to be resolved. Fluorescent microscopy, especially newly developed super-resolution imaging, provides complementary visualization of structural components in dendritic spines. The size and shape of dendritic spines have been well characterized. The majority of the key molecular components and organelles in the spine are identified as well, including postsynaptic receptors on the membrane, scaffolding and signaling proteins, cytoskeleton, and organelles like mitochondria, smooth endoplasmic reticulum and polyribosomes.

1.2.1 Size and shape of dendritic spines

The typical dendritic spine has a head about 0.001~1 μm^3 and a thin neck that connects to the dendritic shaft. Based on 3D reconstruction of dendritic spines from serial-section EM, the dimension of dendritic spines varies greatly among brain regions and can be quite different even in same dendritic region. For example, the average size of spines in the hippocampal region is ~0.054μm^3, while the average spine in cerebellum Purkinje cells is about twice as large with a dimension around 0.12μm^3. Even in the same region of hippocampal CA1, the size of spines in

different pyramidal cell layers can be quite different, ranging from 0.003 to 0.56 μm^3. The spine size in hippocampal CA1 neurons does not follow normal distribution. Instead, the typical population of spines in CA1 region is made up of many small spines together with some exceptionally large spines, which are considered to have organelles like polyribosomes (Harris & Weinberg, 2012).

The shape of dendritic spines can be categorized as stubby, thin and mushroom shape (Figure 1-1). Stubby spines reside on dendritic shaft with a short neck and indistinguishable head. Thin spines are typically defined as protrusions with a thin neck and a small head expansion, while mushroom spines have a bulbous head on a constricted neck. There is another form of dendritic protrusion named filopodia, which are thin and long protrusions from dendritic shafts (1-10μm). Filopodia are considered to be the precursor of dendritic spines, and are called protospines or immature spines in some literature (Yoshihara, De Roo, & Muller, 2009). The categorization of spine morphology is quite arbitrary. Therefore it is important to note the definition of spine category in specific literature. In primary cultured hippocampal neurons, a large percentage of spines exhibit mushroom or thin shape when they are mature (Figure 1-1).

1.2.2 Postsynaptic receptors

Postsynaptic receptors mediate synaptic transmission by responding to the neurotransmitters released by presynaptic terminals. One major excitatory neurotransmitter in the central nervous system is glutamate, and the primary receptors for glutamate on the postsynaptic surface are AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) type receptors, NMDA (N-methyl-D-aspartate) type receptors and mGluRs (metabotropic glutamate receptors). AMPA and NMDA receptors are glutamate-gated ion channels, and both of them are permeable to Na^+ and K^+. NMDA receptors are unique in their high permeability of Ca^{2+} and a distinct

voltage-dependent Mg^{2+} block mechanism. The activation of NMDA receptors requires the removal of magnesium block by depolarized membrane potential. mGluRs are coupled to G-proteins, and their activation by glutamate is linked to the downstream signaling cascades by phospholipase C or adenylyl cyclases (Ozawa, Kamiya, & Tsuzuki, 1998).

AMPARs are the main group of receptors mediating rapid excitatory postsynaptic currents (EPSCs). The average number of AMPARs in each PSD is estimated to be ~15 based on quantitative Mass Spectrometry approaches, though individual possession of AMPARs can be quite heterogeneous among PSDs. EM results suggest AMPARs per synapse in hippocampal CA3 pyramidal neuron ranges from <3 to 140. This is at least consistent in scale with electrophysiological recordings based on the minimal EPSC mediated by opening of AMPARs (Nusser et al., 1998). With similar methods, the average number of NMDARs is estimated to be ~20 (Sheng & Hoogenraad, 2007). NMDARs and AMPARs are not homogeneously distributed at the synaptic site. NMDARs are more centrally localized with highest concentration in the middle of PSDs, while AMPARs are denser at the periphery (Kharazia & Weinberg, 1997). The peripheral distribution of AMPARs may facilitate lateral mobility and the exchange with extrasynaptic AMPARs (Choquet & Triller, 2003). Another type of glutamate receptors, mGluRs, usually concentrate outside the edge of the PSD (Lujan, Nusser, Roberts, Shigemoto, & Somogyi, 1996).

The property and abundance of postsynaptic receptors directly correlate with synaptic strength. Modification and trafficking of receptors are crucial aspects of postsynaptic modulation during synaptic plasticity. The conductance and synaptic localization of AMPARs and NMDARs can be regulated by phosphorylation and dephosphorylation through kinases and phosphatases, like CaM kinase, PKC and calcineurin. Following synaptic stimulation, AMPARs are activated by glutamate released from presynaptic terminals, and the current influx through AMPARs provides the initial drive for postsynaptic depolarization, which relieves the magnesium block of

NMDARs. Subsequent calcium influx mediated by NMDARs triggers signaling cascades that are crucial for synaptic plasticity, including the classic cAMP-PKA-CREB pathway. These pathways further transduce the signals to the regulation of gene expression and the modification of synapses. For example, AMPARs are phosphorylated and added to the synaptic membrane during LTP, while they are dephosphorylated and removed from synaptic sites during LTD. These modifications of AMPARs directly affect their response to synaptic activation afterwards and can mediate long-term alteration of synaptic strength (Ozawa et al., 1998).

Besides receptors for neurotransmitters, receptor tyrosine kinases (RTKs), which are activated by protein ligands like growth factors and cytokines, reside on the postsynaptic surface as well. Ephrin receptors, with multiple protein binding sites, regulate the formation of excitatory synapse and synaptic plasticity through diverse protein-protein interactions and signaling pathways. The processes influenced by Ephrin receptors include the recruitment, localization and function of NMDARs in the synapse, the contact between pre- and postsynaptic membrane and the morphogenesis of dendritic spines (Hruska & Dalva, 2012). Tyrosine receptor kinase type B (TrkB) is another type of RTKs on the postsynaptic membrane. TrkB responds to the neurotrophin BDNF and mediates neurotrophin-dependent regulation of dendritic morphology and spine growth (McAllister, Lo, & Katz, 1995; Shimada, Mason, & Morrison, 1998) . A prominent role of BDNF/TrkB signaling in synaptic plasticity was discovered as well in the past decade (Nagappan, Woo, & Lu, 2008). Deficiency in either Ephrin receptors or TrkB results in severe defects of synapse formation and plasticity (Shen & Cowan, 2010).

1.2.3 Postsynaptic density of dendritic spines

One characteristic of dendritic spines is the presence of a prominent postsynaptic density (PSD), a thickening of postsynaptic membrane under EM that extends ~35-50nm into the

cytoplasm from the postsynaptic membrane (Fig 1-1). Inhibitory synapses using GABA or Glycine as neurotransmitters lack a prominent postsynaptic thickening thus appear symmetric to presynaptic terminals. The molecular composition of the PSD has been identified by biochemical analysis. The spatial organization of molecules within the PSD has also been revealed with improved EM technique and more recently super-resolution imaging (Dani, Huang, Bergan, Dulac, & Zhuang, 2010; J. E. Lisman & Harris, 1993).

The biochemical components of the PSD have been determined by traditional gel analysis, and more recent mass spectrometry methods. First, synaptosomes were obtained by differential centrifugation and gradient sedimentation of brain lysate, and PSDs were purified after detergent extraction since they cannot be solubilized by nonionic detergent. Purified PSDs were then subjected to biochemical analysis. Identified proteins include cell surface receptors, scaffolding molecules, signaling enzymes and cytoskeleton regulatory proteins. Sheng and Hoogenraad summarized proteins identified in the PSD fraction from a quantitative perspective (Sheng & Hoogenraad, 2007). Following is a summary of each category of proteins, with its abundance and spatial organization in the PSDs based on current evidences (Figure 1-2).

Scaffolding proteins anchor the postsynaptic receptors to the PSD. The best known scaffold in the PSD is the PSD-95 family of MAGUK proteins, including PSD-95, PSD-93, SAP102, and SAP97. All these proteins contain several PDZ domains, an SH3 domain and a guanylate kinase domain plus multiple protein binding sites. PSD-95, the best studied scaffold in this family and much more abundant than its relatives, is often used as a postsynaptic marker. PSD-95 binds to the NR2 subunit of NMDA receptors and plays an important role in anchoring NMDARs (Niethammer, Kim, & Sheng, 1996). There is also evidence suggesting PSD-95 involvement in AMPARs trafficking and incorporation into synapses (Ehrlich & Malinow, 2004).With improved EM and newly developed super-resolution imaging technique, the position of PSD-95 scaffolds has been mapped at high resolution and shown to be around10-20nm

underneath the plasma membrane, right beneath the postsynaptic receptors. A little further into the cytoplasmic end of the PSD are Shank and Homer scaffolds, which are linked to PSD-95 by GKAP/SAPAP (guanylate kinase-associated protein/synapse-associated protein-associated protein) (Dani et al., 2010). Shank is a family of scaffolding proteins with multiple domains for protein-protein interaction, including ankyrin repeats, an SH3 domain, a PDZ domain, a sterile alpha motif (SAM) domain, and a proline-rich region (Lim et al., 1999). Three members of shank family, shank 1, 2, and 3, show differential spatiotemporal distribution in the brain, though all three proteins are quite abundant in adult hippocampal regions (Bockers et al., 2004). Shank binds to the PSD-95-associated protein GKAP via the PDZ domain, and connects to the PSD-95/NMDARs complex at the exterior of the PSD. Shank also binds to cortactin via proline-rich domain and tethers the PSD/NMDARs/GKAP complex to actin cytoskeleton (Naisbitt et al., 1999). Shank is considered to play a central role in the structural and functional organization of dendritic spines (Sala et al., 2001). It works synergistically with another scaffold Homer, by forming a mesh-like matrix structure, to promote dendritic spine maturation (M. K. Hayashi et al., 2009). Homer family protein is associated with mGluRs, so shank-homer scaffolds bridge different glutamate receptor complex together in the PSD (as shown in Figure 1-2). Based on quantitative biochemistry and EM, the average PSD contains ~300 copies of PSD-95 (far more abundant than NMDARs, ~20 folds), ~150 GKAP/SAPAP family proteins, ~150 shank and ~60 Homer (Sheng & Hoogenraad, 2007). All together, these scaffolds serve as the structural core of the PSD.

Besides structural components, signaling molecules are abundant constituents in the PSD. One main category of signaling proteins are protein kinases/phosphatases, and the other category is GTPases. Calcium/calmodulin-dependent protein kinase II (CaMKII) is the most abundant kinase (>1% of total proteins) in the PSD of rat forebrain (Feng, Raghavachari, & Lisman, 2011). CaMKII usually localizes at the cytoplasmic side the PSD (~40 nm beneath the plasma surface)

(Dani et al., 2010), and forms a tower shape structure protruding out from the PSD towards the cytosol (Figure 1-2). CaMKII is activated by Ca^{2+}/Calmodulin and thought to mediate the calcium-initiated biochemical cascades in the neuron. Through its kinase activity, CaMKII regulates synGAP, Kalirin-7 and β-PIX, which are important GTPase regulators (Okamoto, Bosch, & Hayashi, 2009). These downstream signaling pathways converge on RhoA or Ras GTPases and eventually regulate actin cytoskeleton. The binding partners and downstream substrates of CaMKII also include subunits of AMPARs and NMDARs (Fox, 2003), and the interaction between CaMKII and AMPARs is thought to be important for regulation of AMPARs channel conductance and trafficking during synaptic plasticity (J. Lisman, Yasuda, & Raghavachari, 2012). In addition, CaMKII binds to F-actin and is involved in the size control of dendritic spines, which functions independent of its kinase activity (Pi et al., 2010). CaMKII has been extensively studied in synaptic plasticity including both long-term potentiation (LTP) and depression (LTD). Results indicate that activation of CaMKII is sufficient to trigger LTP, and also necessary for induction and expression of LTP as well as learning behaviors (J. Lisman et al., 2012). CaMKIIα knockout mice were the first animal model showing deficits in learning and memory behaviors (Coultrap & Bayer, 2012). More recently, spatio-temporal pattern of CaMKII distribution upon synaptic activation is further studied with high-resolution imaging of CaMKII biosensors. CaMKII activation is synapse-specific and largely restricted to the stimulated spine. The activation of CaMKII also shows a temporal pattern that suggests different roles in early and late phase of LTP (Lee, Escobedo-Lozoya, Szatmari, & Yasuda, 2009; J. Lisman et al., 2012). Other kinases in the PSD include ErB4 and the tyrosine kinase interactor liprin-α. There are also abundant phosphotases in the PSD as well, including protein phosphatase-1, spinophilin and neurabin (Harris & Weinberg, 2012).

Another main category of signaling molecules in the PSD is the GTPase family together with its upstream regulators and downstream effectors. GTPases are a group of small signaling

molecules that are active when they are in GTP-bound state and inactive when bound to GDP. Their direct regulators are guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs facilitate the exchange of GDP for GTP thus switch the GTPase to active state, while GAPs stimulate the GTPase to hydrolyze GTP and switch to the inactive state. GEFs and GTPases respond to higher levels of signaling from synaptic surface receptors, like NMDARs, AMPARs, ephrin receptors and other adhesion receptors. They function as intermediators of upstream signaling to GTPases, and GTPases further transduce the signaling to numerous downstream effectors. One major category of Ras-like GTPase is the Rho family, which includes Rac1, cdc42, and RhoA. Rac1 GEFs include Kalirin-7, β-PIX, GEFT and Tiam1, and downstream effectors include Pak1, N-WASP and WAVE-1, which transduce the signaling to a broad network of actin regulatory proteins, like LIM kinases, gelsolin, profilin, cortactin and Arp2/3. Some GEFs of Rac1, like β-PIX and GEFT, regulate cdc42. The downstream effectors of Rac1 and cdc42 overlap, including Pak1, WAVE-1 and N-WASP. Therefore, signaling pathways through different GTPases form a complex network. Most of the pathways eventually converge on actin regulatory machinery (Penzes & Cahill, 2012). One abundant protein in GTPase signaling is SynGAP, which is a GAP for Ras signaling. It is the second most abundant protein in the PSD, slightly less than CaMKII (Sheng & Hoogenraad, 2007). SynGAP binds to PSD95 and locates very close to the plasma side of PSD, unlike many other GTPase regulators which distribute more diffusively in the cytoplasm of dendritic spines (Figure 1-2).

1.2.4 Organelles in the dendritic spines

Endosomal compartments were found in a subset of dendritic spines of hippocampal neurons, based on reconstruction of serial sections by EM. The proteins involved in endocytosis, like clathrin, AP-2 and dynamin, are all found in the dendritic spine. Interestingly, these proteins

were found to localize laterally to the PSD, which is the putative endocytic zone (Cooney, Hurlburt, Selig, Harris, & Fiala, 2002).

Besides endosomal compartments, another type of membrane-bound organelle is the smooth endoplasmic reticulum (SER), called spine apparatus sometimes. SER is only found in a subset of the largest spines in hippocampal neurons, but found more frequently in cerebellar neurons (Harris & Weinberg, 2012). These SER are considered as the outpost of Golgi and may serve as a local store of calcium in dendrites and spines. Proteins related to vesicle trafficking are also found on the membrane of SER. Together with endosomal compartments, SER may serve as the membrane source to support vesicle trafficking and expansion of dendritic spine surface during synaptic plasticity (Bourne & Harris, 2012).

Polyribosomes are also found in dendritic spines and non-uniformly distribute along the dendritic shaft. The presence of polyribosomes provides the machinery for local protein synthesis. Differential distribution may reflect the variance in protein turnover and the requirement of local protein synthesis. Polyribosome can be free floating or bound with SER. The entry of free polyribosomes to dendritic spines is activity-dependent, as shown by increased presence of polyribosomes in large spines after synaptic stimulation (Harris & Weinberg, 2012).

Another organelle found in dendritic spines is mitochondria. Mitochondria presence in dendritic shaft is quite common but the invasion into dendritic protrusions is relatively rare, found in less than 10% of filopodia and spines even during the peak time of mitochondria invasion. The presence of mitochondria in dendritic protrusions decreases along development and the fraction of protrusions containing mitochondria drops to ~4% in aged culture. The invasion of mitochondria into dendritic spine is also activity-dependent. A redistribution of mitochondria from dendritic shaft to spines was observed after repetitive stimulation of neurons. Mitochondria

may serve as a local pool of calcium buffer and source of energy (Li, Okamoto, Hayashi, & Sheng, 2004).

1.2.5 Morphology and function correlation of dendritic spines

As early as spines were first depicted by Cajal's drawing, the heterogeneity of shape and size of dendritic spines was noticed. Detailed categorization and description of spine shape and size were achieved later by electron microscopy. These EM studies confirmed the heterogeneity of spine size and shape, which exists even between nearby spines in the same dendritic region (Harris & Stevens, 1989). Since then, an intriguing aspect of spine study is to decipher if the spine morphology has any meaningful implication of the underlying synaptic strength. This "form and function" hypothesis is part of the larger efforts in finding structural correlates of learning in the central nervous system. Extensive studies have been conducted to scrutinize if spine morphology or density can be the "readout" of synaptic strength especially during synaptic plasticity and learning process.

Intuitively, larger spines correlate with expanded synaptic surface for neuronal contacts, and accordingly provide more space for anchoring postsynaptic receptors and increase the capacity of synaptic transmission. Spine formation creates new sites for synaptic communication. Therefore, spine enlargement and new spine formation are conceived to associate with the enhancement of synaptic transmission. To obtain experimental evidence for this hypothesis, three questions need to be addressed: 1.Do larger spines always accompany with stronger synaptic transmission? 2. Does spine enlargement or new spine formation associate with synaptic potentiation process, like LTP? 3. Is there any evidence suggesting that the alteration of spine morphology or density in vivo accompanies the learning process or other environmental stimulation?

The correlation between spine volume and strength of synaptic contacts has been examined by both EM and electrophysiology. Earlier work by Harris, Lisman and Weinberg groups has shown that spine volume positively correlates with PSD area, as well as the number of AMPARs based on immunogold staining and EM. Moreover, the number of loaded presynaptic vesicles at the active zone of axonal terminal also correlates well with the size of postsynaptic dendritic spine (J. E. Lisman & Harris, 1993). This is a strong indication of size-function correlation of synaptic contacts. A subsequent study by Kasai examined the synaptic transmission by electrophysiology through recordings of microEPSCs, which are spikes evoked by single synaptic activation. They found that smaller synaptic potentiation was evoked when stimulating smaller dendritic spines by glutamate uncaging at single spine level (Matsuzaki et al., 2001). A complementary study by high-resolution calcium imaging showed smaller calcium influx mediated by AMPARs in smaller spines, implicating the amount of AMPARs positively correlates with spine volume (Matsuzaki et al., 2001). Altogether, the correlation between spine size and synaptic transmission, especially AMPAR-mediated synaptic currents, has been consistently reported by multiple studies, at least in populations at baseline level.

Compared with the size-function correlation at steady state, the evidences regarding morphological change of dendritic spine during synaptic plasticity is much less consistent, and sometimes contradictory to each other. In early EM studies, almost all the possible directions of spine number and morphological change have been reported under similar LTP or LTD stimulations. Enlargement of spine heads and shortening of spine necks were found in some studies after dentate gyrus LTP (Fifkova & Anderson, 1981). However, unaffected spine head volume and length of neck was reported as well, even though both groups studied LTP in the dentate gyrus region (Trommald & Hulleberg, 1997). In the hippocampl CA1 LTP, there are reports of very small changes of spine volume and number (Hosokawa, Rusakov, Bliss, & Fine, 1995), and also reports of the increase of new spines or filopodia after LTP (Chang & Greenough,

1984). One critical factor contributing to these discrepancies is that the pre-existing functional state of spines being studied cannot be determined, so there are different potentials for possible changes by synaptic stimulation. EM study also highly depends on sample numbers and statistics, due to the large variations of spine morphology and density even in the same brain regions or different dendritic regions of the same neuron. A third reason is that the stimulation method, like tetanic LTP protocol, only potentiates a subgroup of dendritic spines, so the results can be easily biased depending on which loci were chosen for analysis. These limitations are partially overcome by live imaging studies especially when combined with electrophysiology. By comparing the same dendritic region before and after stimulation, the variance of spine morphology and density is largely eliminated, and the temporal sequence of spine morphology or density change can be analyzed. Kasai group did substantial work on the function-structure correlation of dendritic spines since the early 2000s. These studies generate more consistent results that spine enlargements are associated with LTP based on single spine stimulation protocol, and the increase of dendritic spine numbers is observed by electrical and chemical stimulating protocols. They also found that smaller spines have larger capacity of enlargement during LTP (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004). Inconsistencies of reported results still exist even based on live imaging studies. These inconsistencies may originate from the variation of pre-existing intrinsic states that grant spines with different potentials to plastic changes (Kasai, Fukuda, Watanabe, Hayashi-Takagi, & Noguchi, 2010).

Besides those studies with in vitro stimulation protocols to induce LTP, evidences also point to in vivo changes of spine morphology and density with natural stimuli, like learning or environmental exposure (R. Yuste & Bonhoeffer, 2001). In general, it is consistent that stimulations that activate neuronal network produce more spines. The evidence includes visual stimulation increased spine density in primary visual cortex of mice (Parnavelas, Globus, & Kaups, 1973), while light deprivation caused the reduction of spine numbers (Freire, 1978).

Morphological alteration of spines was also observed after the change of environmental context, like exposure to enriched environment or social isolation(Connor & Diamond, 1982). This phenomenon was even observed in squirrels, which have a drastic decline of spine density during hibernation and recover to normal density after the arousal from hibernation (Popov, Bocharova, & Bragin, 1992). It is also shown that stimulation and learning could alter spine morphology and density in the barrel cortex of mice (Wilbrecht, Holtmaat, Wright, Fox, & Svoboda, 2010).

All the evidence mentioned above suggests a tight correlation of spine morphology/density and function. However, it is by no means any causal relationship, when it comes to whether the spine structural alteration results in the change of synaptic efficacy. As Yuste and Bonhoeffer pointed out in their 2002 review, it still takes time and evidence to show that "spine structure is necessary or sufficient for spine function" (R. Yuste & Bonhoeffer, 2001). Further understanding of this correlation would benefit from evidence regarding whether functional plasticity without structural change can be observed and persist, and also whether structural change alone is adequate for functional plasticity. In recent years, there are reports about the segregation of structural alteration and the functional expression of LTP or LTD. It was observed in cerebellar Purkinje neurons that LTD based on electrophysiological recording is not associated with the shrinkage of dendritic spines (Sdrulla & Linden, 2007). A series of work by Zhou provided evidence that there is a dissociation of the functional and structural plasticity of dendritic spines in hippocampal neurons as well (X. B. Wang, Yang, & Zhou, 2007; Zhou, Homma, & Poo, 2004). They found the functional expression of LTD is dependent on protein phosphatase 1 (PP1) signaling, while spine shrinkage depends on signaling through calcineurin, suggesting different downstream pathways are involved in functional expression of LTD and spine shrinkage. In a later study, they combined the electrophysiological recording with two-photon live imaging to show that the trafficking of AMPARs can occur independently without spine morphological change in either LTP or LTD (Yang, Wang, Frerking, & Zhou, 2008a,

2008b). However, it is still not conclusive if synaptic potentiation or depression can persist in these studies, given that those manipulations to abolish morphological alteration of spines are involved in multiple aspects of synaptic modification. While it is generally accepted that spine morphological change is correlated with functional alteration under physiological conditions, there is still much to explore regarding the true functional meaning of spine morphology.

1.3 Cytoskeleton in dendritic spines

Cytoskeleton provides the architectural support for the unique geometry of dendritic spines. As the correlation between structure and function of dendritic spines has been gradually appreciated, how spine structure is regulated becomes a key question in synaptic biology. Actin is enriched in dendritic spines and serves as the major cytoskeletal element. The dynamics of actin networks is closely related to almost all the aspects of spine function, including the organization of PSD, receptor trafficking and the alteration of spine morphology in response to synaptic activity. In recent years, microtubule invasion into dendritic spines was reported and considered to play a functional role in spine formation and plasticity as well. Cytoskeleton is the convergent target of multiple signaling pathways, and disruption of cytoskeleton is accompanied by defects of spine formation and plasticity. The regulation of cytoskeleton has been an extensively studied area of synaptic biology.

1.3.1 Actin cytoskeleton in dendritic spines

Actin filaments (F-actin) are assembled from monomeric G-actin. At steady state, F-actin preferentially polymerizes at one end of actin filament (+end or the barbed end) and depolymerizes to G-actin at the other end (-end or the pointed end). The difference in

polymerization and depolymerization determines the length and turnover rate of the actin filament. Actin filaments are interlinked with each other through many actin binding proteins to form networks. Together these networks with different dynamic properties constitute the architecture of the cell.

Actin is enriched in dendritic spines. The initial evidence for the enrichment of actin in dendritic spines was reported in the 1980s. When actin antibody was used to probe the isolated PSDs based on biochemical approaches, actin was found to be a prominent component in the PSD. The same antibody was used in immunohistochemistry to study the distribution of actin in brain tissues. High concentration of actin was found in dendritic spines, compared with other parts of the neuron like the cytoplasm and dendritic shafts. They also confirmed this finding with immunogold staining of actin by EM (Matus, Ackermann, Pehling, Byers, & Fujiwara, 1982). Following this initial report, numerous studies with different labeling methods unambiguously showed that dendritic spines are one of the major sites with high concentration of actin in the neuron (Hotulainen & Hoogenraad, 2010a).

The organization of actin cytoskeleton and its dynamic properties have been revealed in an unprecedented detail in the past decade. There are a few really elegant studies from platinum replica EM, photoactivation of actin and single particle tracking with PALM (photoactivated localization microscopy). These studies have revealed an extremely complicated but highly organized actin cytoskeleton in dendritic spines.

The Svitkina laboratory provided the molecular architecture of actin cytoskeleton in dendritic spines by platinum replica electron microscopy (Korobova & Svitkina, 2010). As shown in Figure 1-3, the branched network of actin filaments is featured in spine heads of cultured hippocampal neurons. Moreover, they found this continuous network of branched actin, instead of bundled structure, is the major architecture of spine neck, base and filopodia. Arp2/3 and CP

are two actin regulatory proteins featured in branched network, nucleating filaments and keeping filaments short respectively. Both Arp2/3 and CP are highly enriched in dendritic spines. The branched network of actin is contrasted to the actin bundles in traditional filopodia, which is featured by linear arrays of actin filaments with the same orientation of barbed ends and bundled by Fascin. Based on these observations, Svitkina proposed a model that dendritic spines, as well as filopodia, contain a network of actin filaments composed of branches and linear arrays with mixed polarity that are continuous throughout the spine base, neck and head (shown in Figure 1-3).

The dynamic properties of actin cytoskeleton and subregional differences of actin networks in the spine were investigated by live-cell imaging studies. The rapid turnover of actin in dendritic spine was reported based on Fluorescent Recovery After Photobleaching (FRAP). The majority of actin in dendritic spines was found to be quite dynamic, with a half life around 44 seconds (Star, Kwiatkowski, & Murthy, 2002). This FRAP study was followed by the Kasai group to investigate the dynamics of actin cytoskeleton at different subdomains of dendritic spines. In this elegant study, the authors used two-photon laser to selectively illuminate a small pool of actin by point photoactivation of GFP-tagged actin in different subspine regions, and then trace the dynamics of this illuminated pool of actin (Honkura, Matsuzaki, Noguchi, Ellis-Davies, & Kasai, 2008). Besides the fast diffusive G-actin, they found two distinct pools of actin filaments in dendritic spines. The pool at the tip of spine head showed a very high turnover rate, with an average half-life ~40 seconds. The second pool is relatively stable and localized at the base of the spine, with an average half life ~17min. For both pools of actin, variations of turnover rate exist among different spines, possibly due to difference of spine volume and stability. They also showed a nice retrograde flow of illuminated actin from the tip towards the base of dendritic spines, which may reflect treadmilling of actin filaments at the spine tip to generate an expansive force towards the plasma membrane. There is a third pool of actin filaments that is formed

immediately following synaptic stimulation. This third pool of F-actin is relatively stable with a half life between 2-15min, and this pool is considered as "the enlargement pool" to provide actin filaments for structural expansion of spine head. Notably, most spines in the Kasai study are mature mushroom shaped spines. The dynamics of actin filaments in premature form of spines, like the filopodia, was characterized by Hotulainen et al. using similar FRAP approach and barbed end assay (Hotulainen et al., 2009a). They found the polymerization of actin filaments happens at both the base and the tip of dendritic filopodia, which is consistent with the EM finding that mixed arrays of actin filaments with different orientations exist in the filopodia.

Investigation of actin dynamics within dendritic spines was elevated to a new level with the development of super-resolution imaging in past few years. Tatavarty et al. utilized photoactivation localization microscopy (PALM)-based single molecule tracking technique to study F-actin movements in cultured hippocampal neurons, with a resolution at 30nm. They found a quite heterogeneous pool of dynamic F-actin in dendritic spines, with different velocity of flow at both retrograde and anterograde directions. Their results suggest that actin filaments were arranged as a mixed array of short filaments in spines (Tatavarty, Kim, Rodionov, & Yu, 2009). Single particle tracking PALM was used to further analyze the flow of actin filaments at sub-regions of dendritic spines (Frost, Shroff, Kong, Betzig, & Blanpied, 2010). The Blanpied laboratory confirmed the existence of a highly heterogeneous flow of actin filaments within dendritic spines. They showed the retrograde flow of actin filaments from the periphery towards the core region of the spine with high resolution. Interestingly, they observed a differential turnover rate of actin filaments at the PSD region and the perisynaptic endocytic zone. The actin filaments near the PSD are particularly dynamic, which may indicate a requirement of dynamic actin for receptor insertion to the synaptic area; while the F-actin network in the perisynaptic region is relatively stable, which may reflect a requirement of relatively stable actin filaments for

clathrin-mediated endocytosis. Based on their findings, a few known sites of actin subdomains were summarized in Figure 1-3 (Frost, Kerr, Lu, & Blanpied, 2010).

1.3.2 Regulators of actin cytoskeleton in dendritic spines

With "local" actin dynamics exist in different subdomains of dendritic spines, many categories of actin regulatory proteins are present in dendritic spines as well. A nice review by Hotulainen and Hoogenraad summarized the major categories of actin regulatory proteins in dendritic spines (Hotulainen & Hoogenraad, 2010a). Those actin binding proteins function in different aspects of actin remodeling, including nucleating, severing actin filaments, promoting polymerization or depolymerization, barbed ends capping, cross-linking and ADP/ATP exchanging. The main nucleators in dendritic spines are Arp2/3 complex, which promote the formation of branched actin filaments (Wegner et al., 2008). Many activators of Arp2/3 complex are accumulated in spines as well, including cortactin, N-WASP, WAVE-1 and Abp1 (Haeckel, Ahuja, Gundelfinger, Qualmann, & Kessels, 2008; Hering & Sheng, 2003a; Soderling et al., 2007). ADF/cofilin severs actin filaments and promotes depolymerization, which is a key regulator of actin turnover in dendritic spines (Sarmiere & Bamburg, 2004). Ena/VASP family proteins bind to the plus ends of actin and promote elongation of actin filaments, while its competitor, capping protein restricts the elongation of actin filaments and promotes the formation of branched network with short filaments (W. H. Lin, Nebhan, Anderson, & Webb, 2010). Cross-linking proteins include α-actinin, neurabin 1 and drebrin, which bundle and stabilize actin filaments (Hoe, Lee, & Pak, 2009). Profilin, exchanger of ADP/ATP, is also present in dendritic spines (Ackermann & Matus, 2003). Profilin provides actin monomers that are readily incorporated into actin filaments by catalyzing the exchange of ADP to ATP. Actin-based motor proteins, like myosin II and myosin VI, were also found in dendritic spines (Ryu et al., 2006).

Gelsolin, severing actin filaments and capping the barbed ends, was reported to stabilize actin filaments during synaptic plasticity (Cingolani & Goda, 2008). Many of the proteins mentioned above are indispensible for normal synaptic function, and knockdown of those proteins results in alteration of spine morphology or density (Hotulainen & Hoogenraad, 2010a).

Besides those regulators directly binding to actin, the actin regulation machinery includes multiple signaling pathways, particularly those involving Rho and Ras family small GTPase. These regulatory elements are brought together by scaffolding proteins to form "micro" signaling complex and transduce environmental stimuli into the remodeling of actin cytoskeleton in dendritic spines. The upstream of signaling pathways usually start with the surface receptors, like NMDARs, AMPARs, or neuronal adhesion receptors N-cadherin, neuroligin and beta-neurexin. Tada and Sheng reviewed recent progress in understanding the regulatory pathways of actin dynamics in dendritic spines (Tada & Sheng, 2006). They summarized two major groups of signaling pathways that GTPases are involved – one promotes spine growth and stabilization, and the other promotes spine shrinkage or destabilization.

The Rho family GTPase members include Rac, cdc42 and RhoA. Rac and cdc42 promote the growth and stability of dendritic spines, while RhoA inhibits the growth and enlargement of dendritic spines. The GEFs of Rac1, which switch GTPase to active state by exchanging GDP for GTP, are well characterized. These GEFs include α-PIX, Tiam1 and kalirin, which promote spine morphogenesis by increasing Rac1 activity. Tiam1 responds to NMDAR-dependent CaMKII activation and also EphB signaling (Tolias et al., 2005). α-PIX and Kalirin mediate CaM Kinase dependent spine growth (Xie et al., 2007). The best characterized effectors of Rac1 and cdc42 signaling in the neurons are the family of p21-activaed kinases (Pak). Both Rac1 and cdc42 could activate Pak1. Multiple evidences showed that inhibition of PAK activity could disrupt the spine formation and maturation process (Boda et al., 2004). Suppression of PAK3 in organotypic

hippocampal slice culture resulted in loss of mature spine synapses, and PAK3 knockout mice exhibited impaired long-term synaptic plasticity and defects in memory tasks (M. L. Hayashi et al., 2004) . The eventual targets of PAK are LIM kinases, which phosphorylate and inactivate cofilin to regulate the severing of actin filaments. Except the PAK-dependent pathways, Rac1 and cdc42 could also directly activate the WASP and WAVE family proteins to promote spine maturation and spine head enlargement through actin nucleator Arp2/3 (Hering & Sheng, 2003b). RhoA effectors include mDia, a plus end binding protein of actin filaments that prevents the termination of actin polymerization. RhoA also regulates LIM kinase and cofilin activity through ROCK1 (Meng, Zhang, Tregoubov, Falls, & Jia, 2003). In all, actin regulators like cofilin, Arp2/3 and mDia are convergent targets of multiple pathways, and they translate the upstream messages into reorganization of actin cytoskeleton thus control the morphological change of dendritic spines during development and plasticity.

Scaffold proteins are involved in the Rho GTPase signaling as well. IRSp53, a scaffold protein, connects Rac1 to WAVE2 and facilitates the activation of Arp2/3. IRSp53 is enriched in the PSD and is associated with the larger scaffolds composed of Shank and PSD95 family. By bringing those signaling machinery near the PSD, IRSp53 hold the key regulators of actin near the edge of spine surface (Sawallisch et al., 2009). IRSp53 also directly binds to the capping protein Eps8 (Disanza et al., 2006), which may be part of the mechanism of accumulating Eps8 at the spine head and exerting capping effect on actin filaments near the PSD. Many RacGEFs contain PDZ-interacting motifs, including kalirin, Tiam1 and STEF. These motifs can mediate the interaction of RacGEFs to PSD-95 and may be important for their targeting and enrichment at synaptic sites.

The Ras family of GTPase is another important pathway in the formation and morphological control of dendritic spines. They function mainly through its downstream MAP

kinase. The Ras signaling promotes dendritic spine growth, as shown that overexpression of Ras could increase spine density as shown in transgenic mouse model (Arendt et al., 2004). One of the most abundant scaffold proteins in the PSD, synGAP, is actually a RasGAP. SynGAP deactivates Ras and inhibits the spine growth mediated by Ras signaling (Vazquez, Chen, Sokolova, Knuesel, & Kennedy, 2004).

1.3.3 Actin cytoskeleton in synaptic plasticity

One of the most intriguing aspects of synaptic biology is the interplay between structure and function. How the morphological alteration correlates with synaptic efficacy is a fundamental question in the field of dendritic spine research. The best modeled change of synaptic efficacy is LTP and LTD. It is well documented that spine growth/enlargement accompanies LTP, while spine shrinkage/loss is associated with LTD. Actin cytoskeleton, as the main support of spine architecture, is thought to mediate the morphological reorganization of spines during synaptic plasticity. Besides regulating structural alteration of dendritic spines, actin is also involved in anchoring of postsynaptic receptors at the synaptic area, exocytosis and endocytosis, and trafficking of receptors and organelles in and out of dendritic spines. These implicate essential roles of actin in multiple processes that are critical for the expression of synaptic efficacy.

The morphological change of dendritic spines during synaptic plasticity is dependent on the reorganization of actin cytoskeleton. With FRET-based technique, actin polymerization and depolymerization in dendritic spines were visualized based on the ratio of G-actin and F-actin. In organotypic slice culture of hippocampus, it was reported that actin equilibrium shifted towards F-actin after LTP induction by tetanic stimulation. On the other hand, actin equilibrium shifted towards G-actin in dendritic spines following LTD induction (Okamoto, Nagai, Miyawaki, & Hayashi, 2004). Several other studies reported similar findings of actin polymerization in LTP

with different induction protocols and analysis methods. Theta burst stimulation protocol induced rapid actin polymerization in dendritic spines of hippocampal slices based on phalloidin labeling of F-actin (B. Lin et al., 2005). In another study, an in vivo model of dentate gyrus LTP was used. The authors induced LTP in dentate gyrus of free moving animals, and then used laser capture microdissection to get brain tissues and analyzed the actin content of dendritic spines in the potentiated region. They observed persistent increase of F-actin content in those spines (Fukazawa et al., 2003). Moreover, they found that preventing actin polymerization by treating neurons with latrunculin A could block the late phase of LTP. These findings suggest that actin polymerization is required for the expression of LTP. However, treating neurons with actin polymerization drugs alone is not sufficient for the expression of synaptic enhancement (Okamoto et al., 2004). This hints subpopulations of actin dynamics may exist in dendritic spines, and actin may be involved in processes other than the structural alteration.

Postsynaptic receptor density and properties are directly correlated to the functional expression of synaptic efficacy. The integrity of actin near the PSD area is critical for anchoring of postsynaptic receptors and scaffolds. Drug treatments that depolymerize F-actin could disperse both AMPARs and NMDARs from the postsynaptic surface. About half of the PSD scaffolding components, including PSD95, shank and GKAP, were lost within minutes of treatment by actin depolymerizing drugs (Allison, Gelfand, Spector, & Craig, 1998). This F-actin-dependent anchoring of receptors is not limited to excitatory synapses in dendritic spines. Gephyrin, which is an adaptor for inhibitory GABA and Glycine receptors, could be dispersed from the synaptic area as well following the treatment of actin depolymerization drugs (Kirsch & Betz, 1995).

Besides providing an anchor for postsynaptic receptors and scaffolds, actin is thought to play a more active role in the exocytosis, endocytosis and trafficking of receptors. Our own studies found that disruption of actin dynamics by both Latrunculin A (prevents actin polymerization) and Jaspakinolide (prevents actin depolymerization) could block AMPARs

addition to spine surface following chemical LTP induction in cultured hippocampal neurons. This suggests both actin polymerization and depolymerization are required for AMPARs exocytosis or trafficking to the synaptic area. Moreover, manipulating the activity of cofilin, which severs actin filament and increases the dynamics of actin, could affect the AMPARs addition to synaptic surface after LTP induction and eventually affect synaptic efficacy based on electrophysiological studies (Gu et al., 2010). It is reported that after LTP induction, AMPARs are delivered at the perisynaptic sites and then incorporated into synaptic area via lateral diffusion (Yang et al., 2008a). In both synaptic and peri-synaptic region, the mobility of AMPARs on the plasma membrane highly depends on local actin dynamics (Kerr & Blanpied, 2012). Therefore, the incorporation of AMPARs into the synapse may involve multiple fine-tuned actin networks. Actin is also known to play an important role in endocytosis from studies on various cell types. In dendritic spines, clathrin-dependent endocytosis happens in the extra-synaptic endocytic zone (Frost, Shroff, et al., 2010). AMPAR endocytosis is coupled with synaptic depression. Following LTD induction, the inhibition of actin depolymerization could facilitate the endocytosis of AMPARs (Zhou, Xiao, & Nicoll, 2001). Manipulation of actin dynamics by cofilin could alter the AMPARs internalization as well (Frost, Kerr, et al., 2010). Besides those proteins affecting the organization of actin filaments, motor proteins like myosin are also involved in the intracellular trafficking of vesicles. Myosin walks towards or from the barbed ends of actin filaments, which affects the in and out of the cargos associated with them. Myosin VI is required for AMPAR endocytosis in neurons (Osterweil, Wells, & Mooseker, 2005), and Myosin Vb is required for the recycling of endsome and exocytosis of AMPARs (Z. Wang et al., 2008).

With more investigation of actin in morphological alteration and receptor trafficking, an emerging view is that these two processes are mediated by temporally or spatially segregated actin dynamics. Our own research found a temporal sequence of cofilin activation and inactivation during LTP, which underlies AMPA receptor trafficking and spine enlargement

respectively (Gu et al., 2010). In a previous study, Zhou laboratory also found a narrow time window of actin disruption that would affect receptor trafficking but not the spine enlargement following LTP (Yang et al., 2008a), suggesting a temporal segregation of actin dynamics mediating these two processes. They also found the receptor endocytosis and spine shrinkage during LTD are mediated by different signaling pathways (Zhou et al., 2004). These lead to the hypothesis that the events triggering changes of spine morphology are distinct from those regulating receptors trafficking, and actin cytoskeleton mediates these simultaneous events through spatially segregated actin networks at different subregions. Frost et al. proposed a model of "network of networks" which is consisted of different populations of actin in spines – those near the PSD area, lateral mobility, endocytic zone, and the neck region (Frost, Kerr, et al., 2010), as shown in Figure 1-3. These networks have different properties like turnover time and filament length, thus different resistance to the perturbation of actin dynamics, which may explain why certain aspect of synaptic plasticity is affected while others are preserved under the same treatment condition.

Actin cytoskeleton is involved in the trafficking of organelles into dendritic spines. Transportation of endosomes into dendritic spines is a myosin-dependent process (Z. Wang et al., 2008), which is affected by the length, orientation and dynamics of actin filaments. Mitochondria and mRNA granules transportation into dendritic spines are also affected by the assembly and branching of actin filaments (Bramham & Wells, 2007; MacAskill & Kittler, 2010).

1.3.3 Microtubule in dendritic spines

Traditionally dendritic spines are considered to be devoid of microtubules. Microtubules are rarely seen in dendritic spines by light microscopy with standard sample preparation methods, although they are highly concentrated in dendritic shaft. Early observation of microtubules in

dendritic spines was reported in 1980s. With enhanced technique of microtubules preservation, EM studies showed that microtubules could enter dendritic spines and even penetrate to the proximity of the PSD in hippocampal neurons (Westrum, Jones, Gray, & Barron, 1980). In the subsequent studies by Harris laboratory, microtubules were found in a group of large spines in hippocampal CA3 neurons from freshly cut brain slices. However, they failed to detect the presence of microtubules in spines from the hippocampi samples after fixation (Chicurel & Harris, 1992). These inconsistence called the earlier findings into the question whether microtubules entry into dendritic spines was an experimental artifact during sample preparation, which may not happen under physiological conditions.

The puzzle regarding microtubule presence in dendritic spines was brought back into attention when major evidence supporting microtubule entry into dendritic spines were reported in 2008 from three independent groups. Zheng laboratory documented the presence of microtubules in spines with confocal imaging of both live and fixed hippocampal neuronal culture. For live cell imaging, they used GFP-tagged tubulin and carefully compared the fluorescence with a volume marker to assure the presence of polymerized microtubule instead of diffusive GFP-tubulin. For fixed samples, a special fixation and staining protocol was used to stabilize and preserve microtubules, which is critical for the detection of dynamic microtubule structure. They detected microtubule entry into ~4% of mushroom spines (Gu, Firestein, & Zheng, 2008). The other two groups reported similar findings based on cortical neuronal culture and hippocampal slices (Hu, Viesselmann, Nam, Merriam, & Dent, 2008; Mitsuyama et al., 2008). Although the percentage of spines containing microtubule is low at any given time, a much larger percentage of spines are visited by microtubules based on long-term recording, and it is postulated that most dendritic spines are targeted by microtubule over the time course of a day (Dent, Merriam, & Hu, 2011). Each entry of microtubule is transient, averaging about 3 minutes (Hu et al., 2008). EB3, a microtubule plus-end tracking proteins (+TIPs), is also present in

dendritic spines. All of these studies utilized a fixation buffer that contains microtubule stabilization and preservation reagents, which were not included in the traditional staining protocol. This may be the crucial difference from earlier EM studies. Although these studies provide compelling evidence of microtubule entry into dendritic spines, in vivo imaging of dendrites in live mouse brain may provide the ultimate evidence about the presence of microtubule in spines under physiological conditions.

The next question is whether microtubule entry has any functional significance on the spine dynamics and synaptic function. All three studies reported activity-dependent nature of microtubule invasion and its association with spine dynamics. Microtubule entry into spines was increased three fold after exposure to KCl, a classic method of stimulating neuronal activity in hippocampal cultures. This increase of invasion could be blocked by TTX, which abolishes action potential-driven neuronal activity. Moreover, the invasion of microtubules to spines is accompanied by spine growth and enlargement based on time-lapse imaging (Hu et al., 2008). Consistent findings were reported in CA1 neurons of hippocampal slices after strong tetanic stimulation (Mitsuyama et al., 2008). The functional effect of microtubules on spine structure and dynamics has been studied by loss-of-function approach. Knockdown of EB3, which is a regulator of plus end microtubule, results in decreased spine density. Pharmacological intervention of microtubule dynamics also alters spine density and plasticity. BDNF treatment of neurons for duration of days could potentiate spine growth in primary cultured neurons. With a low dose of microtubule drugs, baseline spine density can be maintained. However, the increase of spine density by BDNF treatment is further potentiated by including Taxol in the culture medium, which stabilizes the dynamic microtubule. On the other hand, the effect of BDNF on spine growth can be completely blocked by nocodazole, a drug disrupting microtubule polymerization (Gu et al., 2008). In another line of study, pharmacological inhibition of

microtubule dynamics by nocodazole significantly suppressed LTP in schaffer collateral-CA1 pathway of mouse hippocampal slices (Jaworski et al., 2009).

Even though it is widely accepted that microtubule invasion plays a role in dendritic spine structure and synaptic plasticity, the precise function of microtubule invasion in spines remains unsolved. It is hypothesized that microtubules serve as tracks for the delivery of cellular organelles or vesicular components. The transient nature of microtubule invasion suggests that the cargos delivered may not be those vesicles constantly transporting in and out of spines, like AMPARs. More likely, those cargos or organelles can be sustained for a while in the spines once delivered. One possible cargo is mitochondria, which is found in about 9% of spines, and the entry of mitochondria into spines is also activity-dependent. Other possible cargos transported by microtubules include smooth endoplasmic reticulum (SER), polyribosomes, and even mRNAs associated with local protein synthesis machinery.

An intriguing aspect of cytoskeleton in spines is the crosstalk between microtubule and actin cytoskeleton. Hoogenraad group showed that EB3, the +TIPs of microtubules, modulates spine morphology through the interaction with actin filaments. They found the spine loss by EB3 knockdown was blocked by pharmacological manipulation of actin dynamics. p140Cap, a binding partner of EB3, interacts with F-actin binding protein cortactin. They proposed that p140Cap is a potential mediator of the talk between microtubule and actin since it binds both EB3 and cortactin. The accumulation of p140Cap is dependent on the interaction with EB3, so the dynamic entry of EB3 into spines may determine the localization of p140cap, and then affect cortactin activity to regulate actin networks in the spine. This way, a “talk” between microtubules and actin network is accomplished, and this interaction may be important for the spine morphological change during synaptic plasticity (Jaworski et al., 2009).

1.4 Capping protein and its regulation

Capping protein (CP) is one of the actin regulatory proteins in dendritic spines. CP binds to the barbed ends of actin filaments, and prevents the polymerization and depolymerization of actin filaments. It is a molecule that has been extensively studied in vitro and in various cell types but not much investigated in neurons. Most of the research focuses on the regulation of CP-actin interaction and the coordination between CP and other actin binding molecules. The function of CP in the nervous system remains largely unexplored. A recent study found CP regulates axonal growth cone morphology and neurite growth (Davis et al., 2009), and another study based on EM showed the presence of CP in dendritic spines (Korobova & Svitkina, 2010). These implicate potential involvement of CP in synaptic function. In this section, current understanding of CP structure and its regulation of cellular morphology is reviewed, which serves as the basis of our investigation on how CP contributes to the morphogenesis of dendritic spines and synapses.

1.4.1 Structure, isoforms and localization of capping protein

Capping protein, also named "CapZ" or "CP", is the protein that binds to actin filaments and "caps" the barbed ends, preventing the polymerization and depolymerization. It was initially isolated and discovered in the muscle cells of chick embryos (Maruyama & Obinata, 1965). CP only functions when a heterodimer is formed with an α subunit of 32-36kDa and a β subunit of 28-32kDa. CP is stable and functional when both subunits co-exist, but is inactive and subjects to degradation when only one subunit exists. CP is found in almost all the eukaryotic organisms and is highly conserved. In vertebrates, there are two isoforms for each subunit existing in somatic cells and one additional isoform, α3 and β3, specifically expressed in sperm cells. The α1 and α2 subunits in vertebrates are coded by different genes, while β1 and β2 are coded by the same gene with alternative splicing. The sequence similarity between α and β subunit is very low. However,

when comparing the sequence similarity across species for each individual subunit, both of them are highly conserved. This suggests these two subunits may have distinct functions (Cooper & Sept, 2008; Wear & Cooper, 2004).

The crystal structure of CP has been resolved. Although the α and β subunits of CP have low sequence similarity, there is a striking resemblance of their structure. Together they form a heterodimer with high symmetry as shown in Figure 1-4. The shape of CP heterodimer is like a mushroom, with a stalk formed by the N-termini of two subunits, and the central part of the mushroom held together by interaction of β sheet structure from each subunit (Yamashita, Maeda, & Maeda, 2003). The cap side of the mushroom faces the interaction sites with actin filaments. One model of CP binding to actin filaments is named "wobbling" model – first, the C-terminus of α subunit binds to one actin monomer at the barbed end. In a second step, the more flexible C-terminus of β subunit, like a tentacle, attaches to the actin filaments. The mobility of the β subunit tentacle makes the whole heterodimer of CP "wobble". If some molecules bind to the β subunit tentacle or block the site of tentacle-actin interaction, the binding between CP and actin can be inhibited, and the dissociation of actin and CP, named "uncapping", is favored (Cooper & Sept, 2008; Narita, Takeda, Yamashita, & Maeda, 2006).

CP is present in almost all the cell types and tissues of vertebrates. In different cell and tissue types, the ratio of different α isoforms varies. This variation in ratio also happens with isoforms of β subunit. It is not very clear how different isoforms of α or β subunit differ in function. However, it is known that they do have distinct distribution even in the same cell type. For example, in the sarcomere of striated muscle, the β1 isoform localizes specifically at the Z-disc while β2 isoform is enriched at the intercalated discs (Schafer, Korshunova, Schroer, & Cooper, 1994). Moreover, they are not able to substitute each other functionally. Replacing one isoform with another in the heart tissues of mice resulted in severe phenotypes – replacing β1

resulted in juvenile lethality, and replacing β2 showed abnormal intercalated disc in mice (Hart & Cooper, 1999). This suggests they have distinct functions in the cell.

CP is present in the mammalian brain. While both α1 and α2 isoforms are abundantly expressed in the brain, only β2 isoform of β subunit can be detected by western blot in the brain lysate (Hart, Korshunova, & Cooper, 1997). Allen Brain Institute profiles the spatial distribution of both α and β subunits of CP based on mRNA measurement and in situ analysis. For both subunits, robust signal was detected across the whole brain region. Structures with particularly high expressions of β subunit are isocortex and hippocampus based on the quantification offered, as shown in Figure 1-4. For α subunit, only α1 isoform data is available. The structures with highest α1 are hypothalamus, pallidum and pons.

1.4.2 Regulation of the CP-actin binding

Activity of capping protein has been extensively studied due to its importance of regulating actin dynamics. However, no phosphorylation or other posttranslational modification of CP has been reported yet. Based on the primary sequence of CP protein, there are a few serine and lysine sites in both α and β subunits, which could be potential sites of phosphorylation or acetylation (based on data from HPRD). However, no enzymes or substrates of these sites have been reported. It is considered that CP-actin binding is regulated by the availability of the binding interface. For example, the actin binding sites of CP can be blocked by inhibitory molecules, and local availability of barbed ends can be regulated by antagonists of CP.

The binding between barbed ends of actin filaments and CP is at sub-nanomolar affinity. This means CP could robustly bind to free barbed ends if no inhibitory molecules or other antagonizing proteins are present. Two early identified proteins that bind to CP and inhibit its

interaction with actin barbed ends are CARMIL and V-1. These two proteins appear to function differently – CARMIL exhibits potent uncapping activity, which means it can remove CP from barbed ends when CP is already on the actin filament; while V-1 (also named myotrophin) has no uncapping activity and works by binding to free CP and inhibiting CP from adding on to actin filaments (Cooper & Sept, 2008). Most studies regarding these two proteins are within cardiac cells, and their function in the nervous system is poorly studied yet although they are expressed in brain tissues.

CD2AP is a CP-binding protein gaining more attention recently. CD2AP together with its family member CIN85 (Cbl-interacting protein) can bind CP and inhibit capping activity. Structural analysis revealed a common motif in CP binding proteins that could bind CP and inhibit its interaction with actin filaments. This shared CP-binding motif is LXHXTXXRPK(6X)P (Hernandez-Valladares et al., 2010). CD2AP is a membrane scaffold that recruits CP, cortactin and other signaling molecules to the close proximity of the plasma membrane, thus facilitate the actin assembly into branched network in the lamellipodia (Zhao et al., 2013). This CD2AP and CP interaction is mediated by Rac1 and plays an important role in maintaining the contacts between epithelial cells (van Duijn, Anthony, Hensbergen, Deelder, & Hordijk, 2010). CIN85 is abundantly expressed in the brain and localized in the postsynaptic compartments of striatal neurons. It is possible that CIN85-CP interaction is involved in forming and maintaining synaptic contacts similarly as in epithelial junctions. Actually CIN85 is shown to regulate dopamine receptor endocytosis, and knockout mice of CIN85 exhibit hyperactivity (Shimokawa et al., 2010). These suggest CIN85 could influence synaptic function.

Another key regulator of CP-actin binding is phosphatidylinositol 4,5-bisphosphate (PIP2), a common second messenger in signaling transduction. PIP2 could result in rapid and efficient dissociation of CP from actin barbed ends (Schafer, Jennings, & Cooper, 1996). PIP2 binds to the C-terminus of α subunit, which interferes the CP-actin binding. Mutation of those

residues at the predicted binding site could weaken the binding between PIP2 and CP (K. Kim et al., 2007). PIP2 could remove CP from actin filaments under physiological conditions. For example, CP in the platelet is removed by PIP2 following thrombin receptor activation, which leads to rapid polymerization of actin filaments in the platelet (Barkalow, Witke, Kwiatkowski, & Hartwig, 1996). This rapid growth and spread of actin filaments is considered to be important for blood clot formation. PIP2 is also highly enriched in the plasma membrane of postsynaptic surface. Notably, the concentration of PIP2 in dendritic spines shows rapid change following synaptic stimulation (Horne & Dell'Acqua, 2007). One interesting postulation is that PIP2 could transduce the upstream signaling to the structural reorganization of spines during synaptic plasticity by regulating the CP-actin interaction.

Another important way of CP-actin regulation is through actin binding proteins that antagonize CP at the barbed ends of actin filaments. The major competitors of CP at actin barbed ends are formin and Ena/VASP family proteins. Formin remains on actin barbed ends and allows the incorporation of actin monomers into the filament. The anti-capping activity of formin is largely through the inhibition of CP-actin binding, and formin could also accelerate actin elongation (Mellor, 2010). Ena/VASP is another antagonist of CP at barbed ends of actin filaments. Ena/VASP promotes elongation of actin filaments after binding to the barbed ends (Kwiatkowski, Gertler, & Loureiro, 2003). Both formin and Ena/VASP are localized at the tip of filopodia and considered to play important roles in regulating the motility and morphogenesis of filopodia (Cooper & Sept, 2008). Their function in spine morphogenesis will be introduced with more details in the following section.

1.4.3 CP in cellular processes

CP-dependent actin regulation is well characterized in lamellipodia formation. Borisy laboratory presented strong evidences that CP is required for the formation of lamellipodia (Mejillano et al., 2004). They found dramatic loss of lamellipodia after CP knockdown in melanoma cell line, which is accompanied by explosive formation of filopodia. They also found this phenotype switched to ruffling of lamellipodia when Ena/VASP was depleted as well. Based on these results, they proposed a model that CP binding to actin filaments is favored during lamellipodia formation, while Ena/VASP, the elongating factor of actin filaments, is favored at barbed ends during filopodia formation. This model is further supported by a fluorescent speckle microscopy study that tracked the localization of CP and other actin regulatory proteins in Drosophila S2 cells (Iwasa & Mullins, 2007). The authors found that lamellipodia formation highly depends on the presence of both CP and Arp2/3. Wear and Cooper proposed a model of CP regulation of actin assembly at the cell edge (Wear & Cooper, 2004). As shown in Figure 1-6, Arp2/3 mediated lamellipodia formation requires active CP. While Arp2/3 keeps generating new branches, CP is required to cap the ends of those relatively older actin filaments in the back. This way, actin filaments can be kept short and remain branched. At the same time, actin monomers can be directed only to barbed ends at the cellular edge, providing the protrusive force for plasma membrane expansion. When CP is inhibited by PIP2 or the antagonist like Ena/VASP, the branched network cannot be formed. Instead, the elongation mechanism dominates, and thin protrusions may be formed where actin filaments keep elongating. Those actin filaments may form a bundle with fascin, and a complex of elongating factors occupy the barbed ends at the tip of filopodia as shown in Figure 1-6 (Cooper & Sept, 2008). Only when actin monomers can be incorporated at multiple ends of short branches, instead of being continuously added on a few filaments to elongate, a protrusive force can be generated for the expansion of plasma membrane.

The role of CP in regulating cellular morphology is investigated based on in vivo models as well. The loss-of-function mutations of CPβ are lethal in Drosophila, resulting in death at the early larval stage. In heterozygous adult Drosophila, there are defects in the structure of bristles with abnormal actin bundles formed in the surface (Hopmann, Cooper, & Miller, 1996). In more recent studies, mutations of CPα were found to result in defects of eye development in a genetic screen of Drosophila (Janody et al., 2004), and the same group also found that both α and β subunits of CP could result in malformation of wings (Janody & Treisman, 2006). These implicate CP plays an important role in the pattern formation and morphogenesis during development.

Several other cellular processes that require CP include endocytosis and intracellular trafficking, but the precise function of CP remains poorly understood in these processes. It is reported that the actin patches beneath the membrane, which is critical for endocytosis, require proper CP regulation. Loss-of-function of CP in yeast resulted in the failure of endocytosis (K. Kim et al., 2006), and CP was shown to be important for the vesicle scission and release. CP interacts with synaptic vesicle protein NAP-22 (Odagaki, Kumanogoh, Nakamura, & Maekawa, 2009), and is a component of dynactin complex based on biochemical studies (Cooper & Sept, 2008). These implicate a potential role of CP in vesicle endocytosis and trafficking, though this area has not been explored much yet.

1.4.4 CP function in the nervous system

Even though CP is abundantly expressed in the nervous system, CP function in the brain was not well studied previously. Current knowledge about CP in the brain is mostly from proteomic screening of samples with neurological diseases. Both α and β subunits of CP were significantly reduced in the fetal brain of Down Syndromes (Gulesserian, Kim, Fountoulakis, &

Lubec, 2002). In the postmortem hippocampi of alzheimer's disease, there was a significant increase of CP β2 expression (Kao et al., 2010). In a rat model of dementia, a reduction of CP was reported in the hippocampus (Kitanishi et al., 2010). Those results, though sometimes contradictive, hint that CP may be important for cognitive functions.

The only study of CP in neurons at cellular level is about CP in the formation of axonal growth cone (Davis et al., 2009). The authors found defects in the formation of lamellipodia of axonal growth cone after CP knockdown, based on RNAi approaches in primary cultured hippocampal neurons. Instead of forming a flat lamellipodia at the edge, a fork-structure was formed, which affected the mobility of axonal growth cone. Further investigations revealed that, surprisingly, an interaction between CP and tubulin mediated this process. Loss of CP resulted in the overgrowth of microtubule into the periphery which disrupted the proper formation of axonal growth cone. This leads to the postulation that CP is not only an actin regulator, but also mediates the crosstalk between actin filaments and microtubules. One study supports this postulation is from the screening of hippocampal protein interactions after spatial learning – an increased CP interaction with tubulin was observed in the brain of mice after learning tasks (Nelson, Backlund, & Alkon, 2004), though how this interaction affects synaptic efficacy is not known yet.

1.5 Mechanisms of dendritic spine development

Synaptogenesis is a key process to establish neural circuitry. The development of the postsynaptic platform, spinogenesis, is an important part of this wiring process. Spinogenesis in hippocampal neurons starts with the initial contact of dendrites and axons. This interaction between dendrites and axons triggers the subsequent stabilization of dendritic protrusions and the recruitment of synaptic components, which eventually serve as the functional domain of dendritic spines. The process of spinogenesis involves multiple steps of morphological remodeling, regulated by complex molecular cascades. In this section, recent progress in understanding the dendritic spine formation is summarized, with emphasis on the aspect of morphological remodeling and the underlying molecular mechanisms of cytoskeletal regulation.

1.5.1 Three models of dendritic spine formation

Spinogenesis is thought to be one critical step in the wiring of neural circuitry during brain development. Though the importance of this process is greatly appreciated, it remains unclear how spines develop and what triggers the emergence of spines. There are controversial evidence and opinions regarding the spinogenesis process. Key questions that have been debated for almost a decade include – Does axonal input trigger the dendritic protrusion to make contact? Or does the spine form autonomously and later attract axonal terminals? Are filopodia the precursors of dendritic spines?

In 2004, Yuste and Bonhoeffer had an insightful review of three different models of spine formation based on literature at that time (Rafael Yuste & Bonhoeffer, 2004), as shown in Figure 1-7. All these three models have gained evidence to support from different laboratories. The

authors think these models quite likely don't exclude each other. The variation of findings and interpretations is largely due to the difference of methodology and brain regions examined.

The first one proposed by Sotelo is based on the study of Purkinje cells in the cerebellum. It provides a model that spines can be formed on Purkinje cells without the axonal input from granule fibers. The main evidence came from mutations of granule cells or surgical operations that largely destroyed the axons in mouse cerebellum (Sotelo, 1977). Purkinje cells of those mice, surprisingly, developed quite normal spines, at least in morphology and density. Moreover, "naked" spines on Purkinje cells, without axonal innervations, were observed in early postnatal cerebellum. These suggest there are intrinsic mechanisms that govern spine morphogenesis independent of axonal input, at least in the Purkinje cells. So far, no evidence has been found to support this model in pyramidal neurons yet.

The second model is proposed by Miller/Peters – axonal terminals make the initial contact with the surface of dendrites and forms a shaft synapse; later, the shaft synapse is "pulled out" by the axonal terminal and outgrows to a spine. In this model, axonal induction and "pulling out" of dendrites play a central role in spinogenesis. The main evidence is from ultrastructural analysis of pyramidal neurons of mouse cortex based on EM. In the first two postnatal weeks of mouse cortex, many synapses reside on the shaft; while in the third postnatal week (P21), most synapses reside on spines (Miller & Peters, 1981). The postulation is that the pre-existing synapse on the shaft extends out to become spines. However, this model suffers from lacking evidence support from live imaging studies. Although the shift of synapses from the shaft to spines is generally agreed, it is not demonstrated convincingly that the synapses on spines were indeed the same group of synapses on the shaft. It cannot rule out the possibility that the shaft synapses are completely turned over and new spines grow without carrying the preexisting shaft synapses.

The third model is the filopodia model – based on pyramidal neurons in the hippocampus and cerebral cortex. In this model, filopodia are considered to be the precursors of spines. They arise from the dendrites and serve as the exploratory structure to find axonal terminals. Once they make contacts, filopodia undergo a transformation of both morphology and protein composition to become spines. The main evidence comes from hippocampal neurons in cultures and slices. Most filopodia are dynamic and transient. When the filopodia are in contact with axonal terminals, based on labeling of synaptic markers, they are stabilized and the thin and long protrusions are shorten to become "spine" like structure. The authors propose that the "grabbing" of an axon by filopodia is the triggering event that stabilizes filopodia and initiates the transformation to spines.

Almost 10 years have elapsed since that review and more investigations have been conducted regarding the three models. The Sotelo model is still valid in Purkinje cells. In pyramidal neurons, more evidence in the past few years endorse the filopodia-spine model, while the Miller/Peters model has not gained support from live imaging studies yet. One strong evidence supporting the filopodia model came in 2005. Gan laboratory reported the direct transformation of filopodia to dendritic spines based on in vivo two-photon imaging of layer 5 pyramidal neurons in the live mouse brain. In both young and adult mice, they observed that filopodia underwent morphological change and formed spines with bulbous heads. These spines can persist at least days over their recording. Although a large percentage of these spines were eliminated eventually, it may be a selective process of spine formation. Similar observations were reported from other groups based on pyramidal neurons as well (Cruz-Martin, Crespo, & Portera-Cailliau, 2010; Zuo, Lin, Chang, & Gan, 2005). However, for the Miller/Peter model, no direct evidence about axons "pull out" shaft synapses has been reported in either in vitro or in vivo imaging studies.

Notably, Sabatini group reported de novo formation of spines on dendrites based on uncaging of glutamate (Kwon & Sabatini, 2011). They used an artificial way to mimic the vesicle release of glutamate from axonal terminals, and observed the production of spines that are functionally and morphologically compatible with existing spines. In their observation, spines with bulbous heads can be formed without the transformation from filopodia. This appears to contradict the filopodia hypothesis that filopodia are necessary precursors of spines. However, to what extent the glutamate uncaging could mimic the endogenous release of glutamate from axonal terminals is unclear. One possibility is that the artificial induction method represents a model of spine formation under intense synaptic stimulation in a compressed time scale (only a few minutes), instead of hours as in vivo. Moreover, the endogenous signaling cues from axons for synaptic contacts are likely to be a mixture of secreted proteins and molecules instead of pure glutamate. Though this artificially-induced spine formation skips the filopodia step, it is still widely accepted that filopodia transformation to spines is one major mechanism, if not exclusive, of spinogenesis in hippocampal neurons.

1.5.2 Extrinsic and intrinsic factors regulating dendritic spine formation

Extracellular cues and their receptors on the dendritic surface play an important role in spine formation. Two main categories of signaling molecules involved in spinogenesis are BDNF and EphB (Shen & Cowan, 2010). Both were shown to be required for the synapse formation in hippocampal neurons. TrkB-deficient mice exhibited substantial loss of dendritic spines in the CA1 region of hippocampus (Luikart et al., 2005). It is thought that BDNF/TrkB signaling promotes the filopodia motility thus increases the chance of filopodia to encounter an axonal terminal. BDNF/TrkB signaling is mediated by PI3K and Rac GEF Tiam1, eventually alters actin dynamics and filopodia motility (Luikart et al., 2008). EphB2 deficiency also results in dramatic

loss of synaptic density in cultured hippocampal neurons (Henkemeyer, Itkis, Ngo, Hickmott, & Ethell, 2003). It is thought that EphB2 is required for filopodia motility and also for stabilizing the contact at the surface of axon and dendrite. Overexpression of the extracellular domain of EphB2 could promote the spine formation and maturation, suggesting EphB2 may stabilize synaptic contacts via adhesion mechanisms (Kayser, Nolt, & Dalva, 2008).

Neuronal activity is essential for synaptic formation and neural wiring. Inhibiting glutamate transmission by blocking either AMPARs or NMDARs halts the spine formation process. Also, treatment of neurons with TTX, which blocks the activity-dependent release of glutamate, dramatically reduced spine density (Riccio & Matthews, 1985). This suggests neuronal activity is critical for proper spine formation and density. Notably, antagonists of both AMPARs and NMDARs reduce the density and motility of filopodia, but they don't affect the stabilization of filopodia contact with axonal terminals (Yoshihara et al., 2009). This suggests glutamate may function primarily in filopodia initiation, while the stabilization of filopodia may require other signaling molecules or recognition of surface receptors. Besides the role as recipient of the excitatory transmitter, AMPARs and NMDARs also bind directly to scaffolding or actin regulatory proteins, including PSD95, CaMKII and light chain of myosin (Hotulainen & Hoogenraad, 2010a). The enrichment of those proteins in dendritic protrusions is an important feature of the spine formation as well.

Although synapse is a unique type of cellular junction, adhesion molecules play a similarly important role in the development and maturation of synaptic contacts as in many other cellular junctions. Cadherins are enriched in the perisynaptic region of spine surface, and is indispensible for normal morphology and maturation of spines. Blockade of cadherins resulted in the loss of mushroom shape spines and increased filopodia-shaped protrusions (Togashi et al., 2002). Neurons lacking the α-catenin, cytosplasmic partner of cadherin, exhibited extremely high motility of dendritic spines and extensions of filopodia from the spine head (Abe, Chisaka, Van

Roy, & Takeichi, 2004). These suggest cadherins and catenins are required to maintain stable contacts and the maturation of spines (Takeichi & Abe, 2005). Besides cadherin family proteins, neuroligin, neurexin and SynCAM are important cell adhesion molecules involved in spine formation as well.

1.5.3 Regulation of actin cytoskeleton during dendritic spine formation

Actin cytoskeleton provides the structural support for the initiation of synaptic contacts and the eventual spine morphogenesis. Current understanding about actin reorganization during the spine formation process largely focuses on the filopodia model. Hotulainen and Hoogenraad reviewed recent evidences about the actin regulatory mechanisms at different steps of the spine formation – the initiation of filopodia, elongation of filopodia and formation of the spine head, as shown in Figure 1-8 (Hotulainen & Hoogenraad, 2010a).

The initial step is the protrusion of filopodia from dendritic shafts (Figure 1-8-A). There are a few mechanisms that possibly co-exist in this process. The generation of an actin base at the dendritic shaft is thought to involve anti-capping mechanism. Several anti-capping factors identified in spine formation are formin family proteins mDia2, and Ena/VASP family. Both of them could antagonize the capping at barbed ends and promote the polymerization of uncapped actin branches (Hotulainen et al., 2009b). Another hypothesis is that myosin X moves laterally and brings together barbed ends to the potential site of filopodia initiation (Tokuo, Mabuchi, & Ikebe, 2007). All of these actin reorganizations at the base of filopodia are considered to be under the influence of glutamate (Tashiro, Dunaevsky, Blazeski, Mason, & Yuste, 2003). However, whether the generation of filopodia is solely a glutamate-induced process, or there are pre-patterned organization of actin patches that serve as designated filopodia sites and respond to

axonal release of glutamate, remains unclear. The exact mechanisms translating glutamate release to actin organization also remain to be addressed.

The second step is the elongation of filopodia (Figure 1-8-B). Filopodia from dendritic shafts elongate in a formin and Ena/VASP dependent way, both are elongating factors of actin filaments. Dendritic filopodia differ from traditional filopodia in the organization of actin filaments – instead of forming a stiff bundled structure, dendritic filopodia are devoid of fascin, which bundles the parallel and uniform actin filaments to an array. Dendritic filopodia are formed with mix polarity even though the majority of filaments elongate towards the tip of filopodia.

Next step is the spine head formation, a key process for the transformation of filopodia to dendritic spines (Figure 1-8-C). Most filopodia are transient, and only those filopodia maintaining contacts with presynaptic terminals can be stabilized and recruit clusters of synaptic components. Multiple evidence support that Arp2/3-dependent nucleation and branching of actin filaments plays a central role in the transformation of filopodia to spines. The key question is what signals trigger the conversion of formin or Ena/VASP-dominant elongation machinery to Arp2/3-dominant branched network formation. This question remains unanswered. One interesting hypothesis is proposed by Hoogenraad – based on their finding that dynamic microtubules can interact with actin cytoskeleton through the signaling cascades mediated by plus-end binding protein EB3. EB3 binds to p140Cap, which interacts with cortactin. Cortactin is an activator of Arp2/3 and could lead to subsequent spine head growth. This hypothesis needs support showing the temporal sequence of microtubule invasion into filopodia precedes the spine head expansion. In other cell types, it is shown that increased Arp2/3 activity could antagonize mDia-mediated actin elongation, which may be the intrinsic coordination between actin regulators to facilitate the switch from actin elongation to branching. Besides Arp2/3, it is well documented that neuronal adhesion molecules, the cadherin-catenin complex also plays a role in stabilizing the filopodia structure and facilitates spine head growth. Knockdown of β-catenin resulted in immature spine

morphology without affecting spine density, suggesting it is involved in the spine head formation process (Arikkath & Reichardt, 2008). Myosin II is also involved in the morphogenesis of spine head, possibly through its contractility to control the geometry of spine neck.

In all, actin regulation is highly coordinated during spine formation, involving major reorganization of the cytoskeleton in response to both intracellular and extracellular signaling. Dissecting the mechanisms of how actin regulation is fine tuned in spine formation is an intriguing area of synaptic biology. In this dissertation, how CP contributes to this delicate machinery of actin regulation is investigated.

1.6 Figures and legends

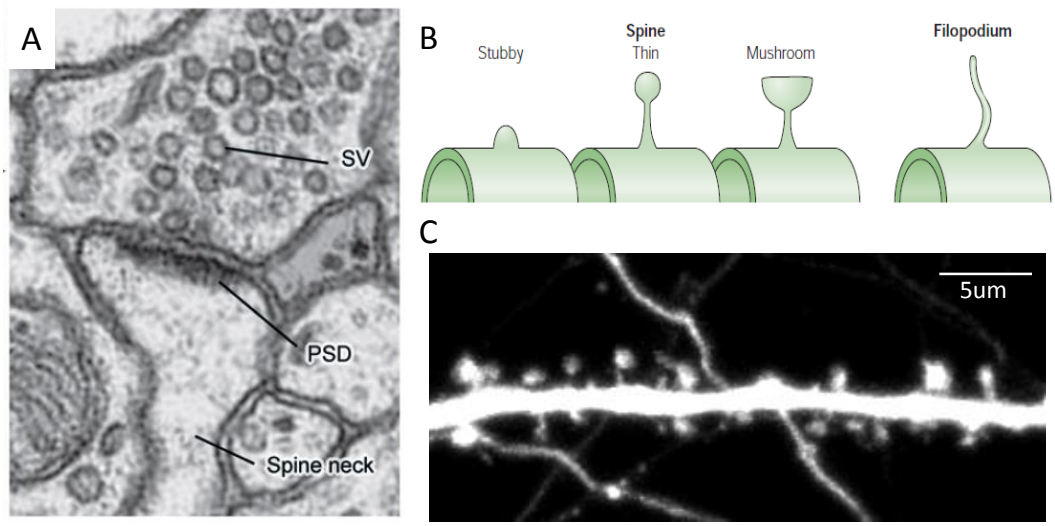

Figure 1-1. Structure of synapse and dendritic spines. A) Structure of an excitatory synapse under electron microscope. SV: synaptic vesicles; PSD: postsynaptic density; adapted from Sheng & Hoogenraad 2007 with permission; B) Categories of dendritic spines based on morphology, adapted from Yuste and Bonhoeffer 2004 with permission; C) a typical dendritic region from cultured hippocampal neurons at DIV21, with majority of spines that are mushroom shape.

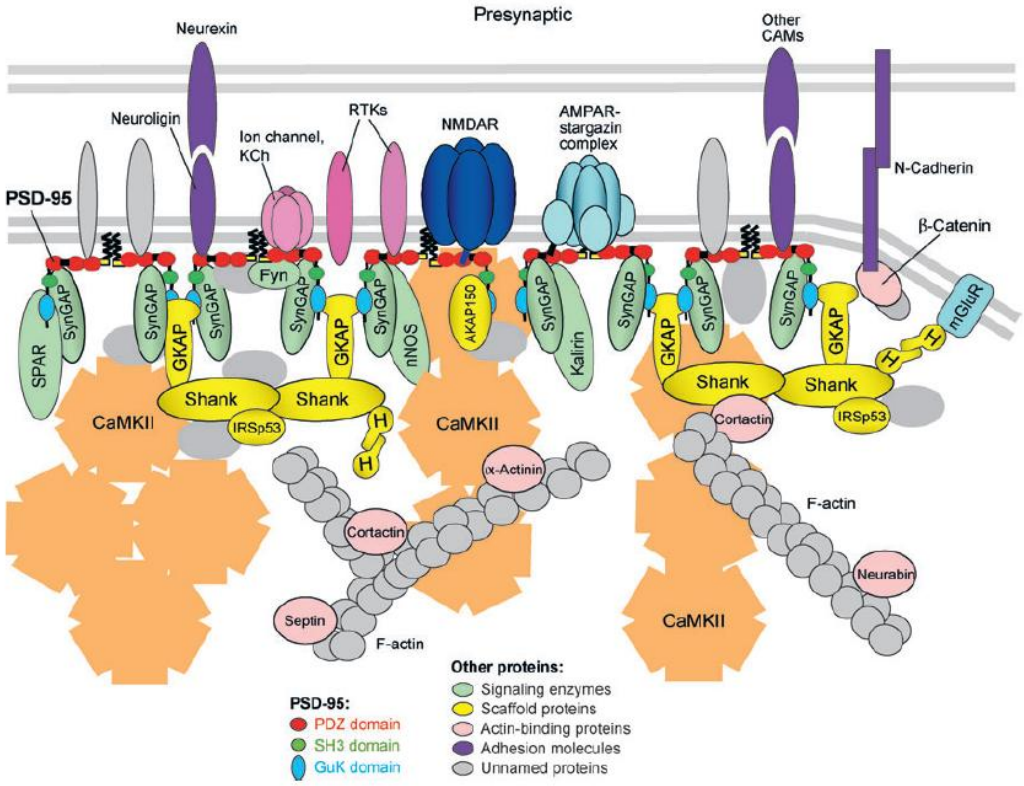

Figure 1-2. Organization of proteins and protein-protein interactions in the postsynaptic density (PSD). Major families of PSD proteins are shown in approximate stoichiometric ratio and scaled tomolecular size. Contacts between proteins indicate an established interaction between them. Domain structure is shown only for PSD-95 (PDZ domain, SH3 domain, GuK domain).Other scaffold proteins are colored yellow; signaling enzymes, green; actin binding proteins, pink. CaMKII (calcium/calmodulin-dependent protein kinase II) is depicted as dodecamer. Unnamed proteins signify the many other PSD proteins that are not illustrated in this diagram. Abbreviations: AKAP150,A-kinase anchoring protein 150 kDa; CAM, cell adhesion molecule; Fyn, a Src family tyrosine kinase;GKAP, guanylate kinase-associated protein; H, Homer; IRSp53, insulin receptor substrate 53 kDa; KCh,K+ channel; mGluR, metabotropic glutamate receptor; nNOS, neuronal nitric oxide synthase; RTK,receptor tyrosine kinases (e.g., ErbB4, TrkB); SPAR, spine-associated RapGAP. Adapted from Sheng and Hoogenraad 2007 with permission.

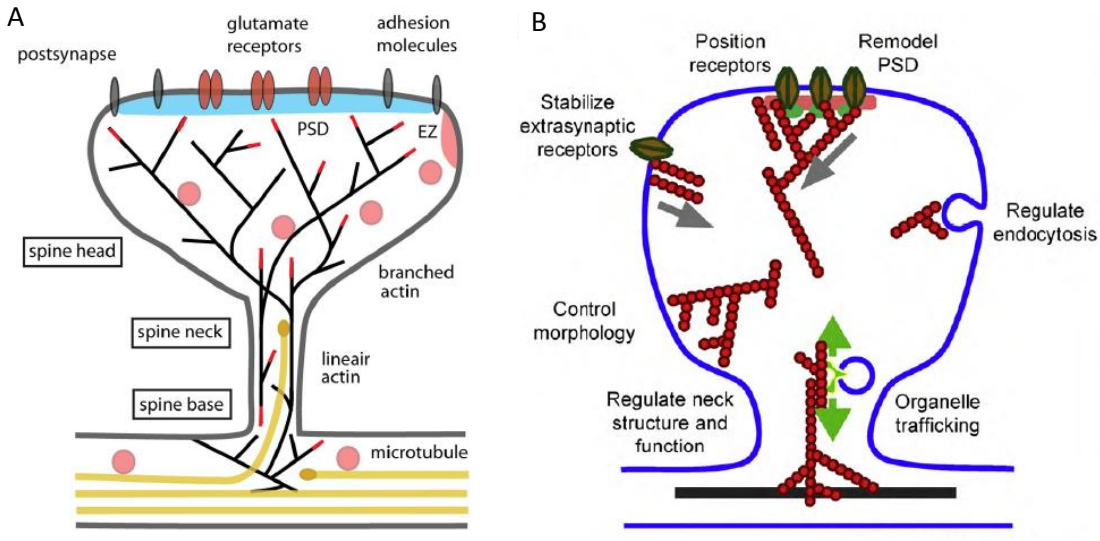

Figure 1-3. Organization of actin and microtubule cytoskeleton in dendritic spines. A) Schematic diagram of a mature mushroom-shaped spine showing the postsynaptic density (PSD; blue), adhesion molecules (gray) and glutamate receptors (reddish brown), the actin (black lines) with barbed ends (red lines) and microtubule (yellow) cytoskeleton, and organelles. Adapted from Hotulainen and Hoogenraad 2010 with permission; B) Several known subdomains of actin regulation in dendritic spines. The spine cytoskeleton is a network of networks that coordinately control synapse function via numerous distributed mechanisms. Gray arrows indicate direction of actin flow; green arrows indicate potential for cargo transport along filaments oriented in either direction. Adapted from Frost et al 2010 with permission.

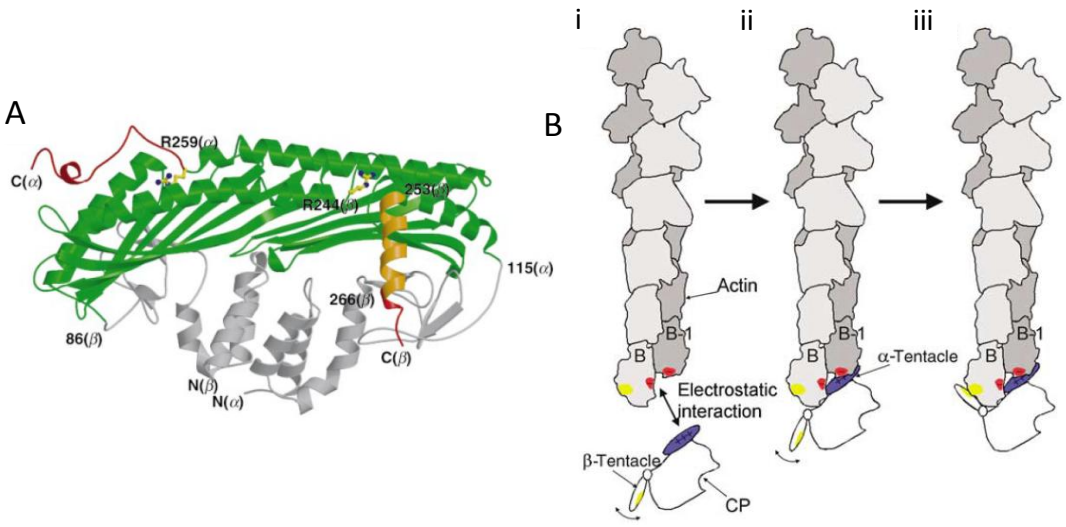

Figure 1-4. CP structure and the model of CP-actin binding. A) The regions crucial for actin binding [residues 259-C-terminus(a) and 266-C-terminus(b)], the segment which exhibits actin-binding ability [residues 253-C-terminus(b)] and the regions that are not required for high-affinity actin binding [residues N-terminus-115(a) and N-terminus-86(b)] are mapped in red, orange and gray, respectively, on the CapZ structure based on the previous deletion mutant. The two conserved arginine residues, Arg259(a) and Arg244(b), are indicated with ball-and-stick models. Adapted from Yamashita et al, 2003 with permission;

B) A model for the binding of CP to the actin filament barbed-end proposed by Narita and colleagues. First, the basic residues on the α-tentacle (blue) and the acidic residues on the bottom of the actin filament (red) attract each other. This interaction should determine the on-rate of the binding. The unbound b-tentacle (yellow) is freely mobile, due to the flexibility at the basal part (around residues 246-252). Second, the α-tentacle binds to the bottom of the actin filament. The β-tentacle remains freely mobile, and searches for its binding position on the actin filament. The β-tentacle binds to the hydrophobic cleft (yellow) on the outer surface of the end protomer B. This binding reduces the off-rate of the binding, and thereby stabilizes the binding. Adapted from Narita et al., 2006 with permission.

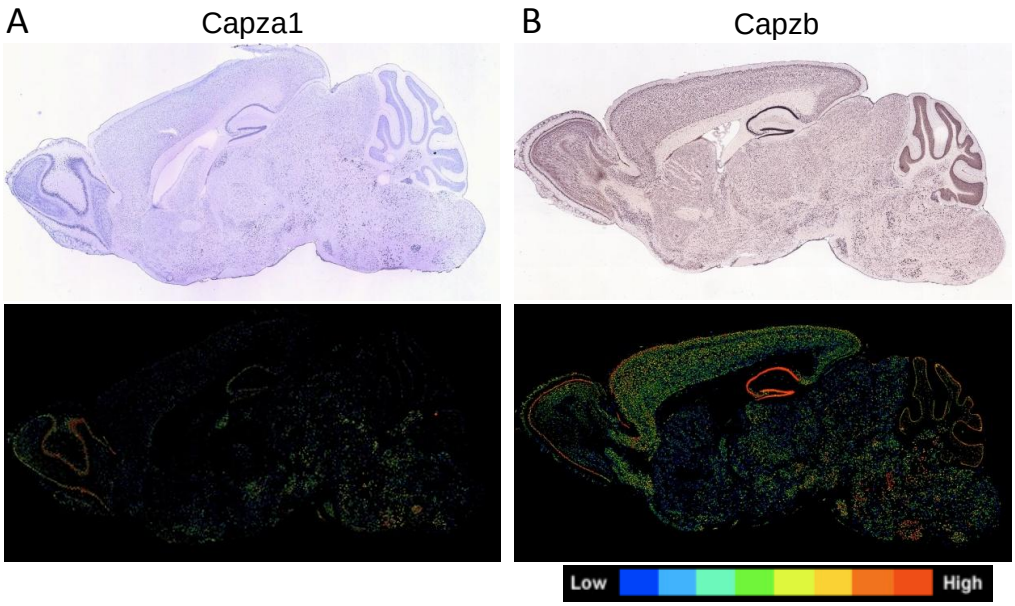

Figure 1-5. CP expression in the brain. A) In situ of Capza1 (coding for CPα1) in the brain with pseudocolored presentation of expression at the bottom, saggital view; B) In situ of Capzb (coding for CPβ2) in the brain and pseudocolored presentation of expression at the bottom. Retrieved from Allen Brain Atlas.

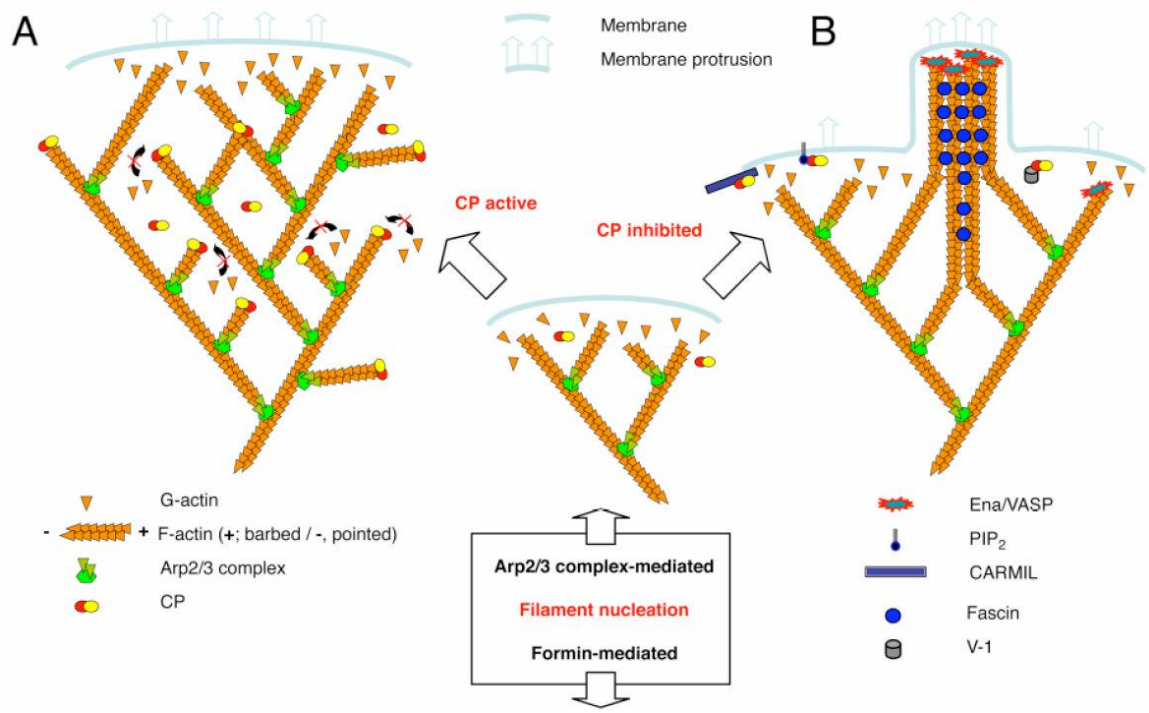

Figure 1-6. Illustration of potential modes of actin assembly in cells with respect to CP. A) When CP is active and actin nucleation is Arp2/3-mediated, lamellipodial assembly predominates. Newly created free barbed ends are near the membrane. They elongate to push the membrane forward and/or the actin filament network backward. After some time, CP caps those barbed ends, which would seem to be efficacious because the ends are no longer near the membrane and their further growth would not produce useful work. B) In this setting, when CP is inactivated in one location, by any of several potential inhibitors, then the filaments in that small region may continue to grow, producing a thin protrusion that contains a bundle of actin filaments. Adapted from Wear and Cooper, 2004 with permission.

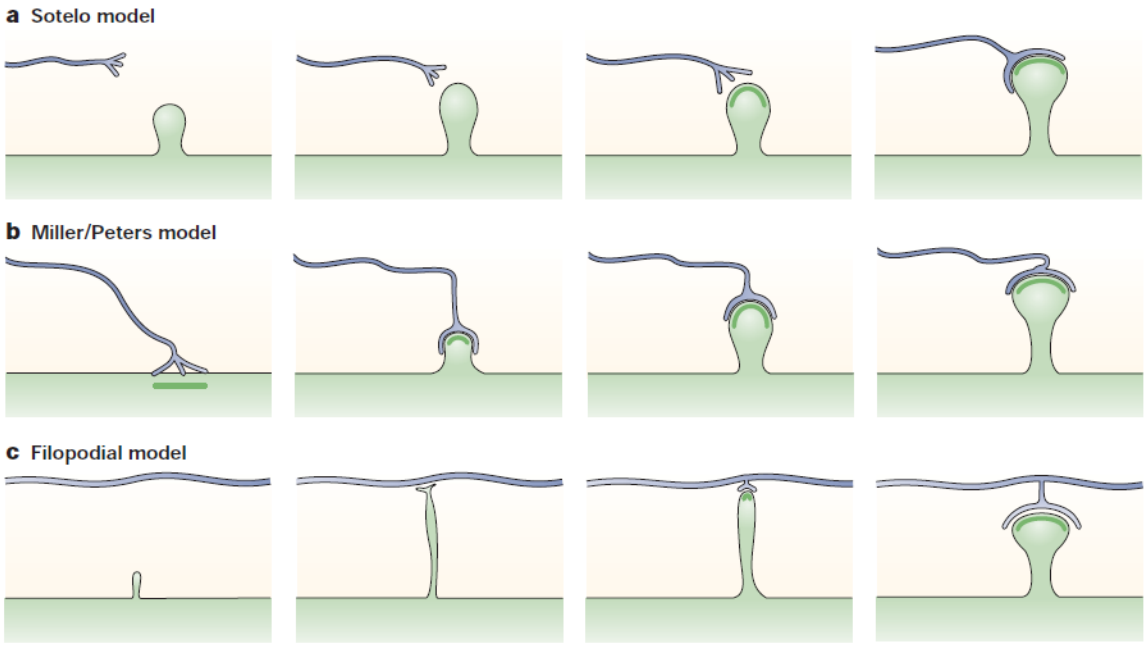

Figure 1-7. Three models for spinogenesis. This diagram illustrates the essential features of the three models of spinogenesis. In the Sotelo model (**a**), spines emerge independently of the axonal terminal. In the Miller/Peters model (**b**), the terminal actually induces the formation of the spine. Finally, in the filopodial model (**c**), a dendritic filopodium captures an axonal terminal and becomes a spine. Adapted from Yuste and Bonhoeffer, 2004 with permission.

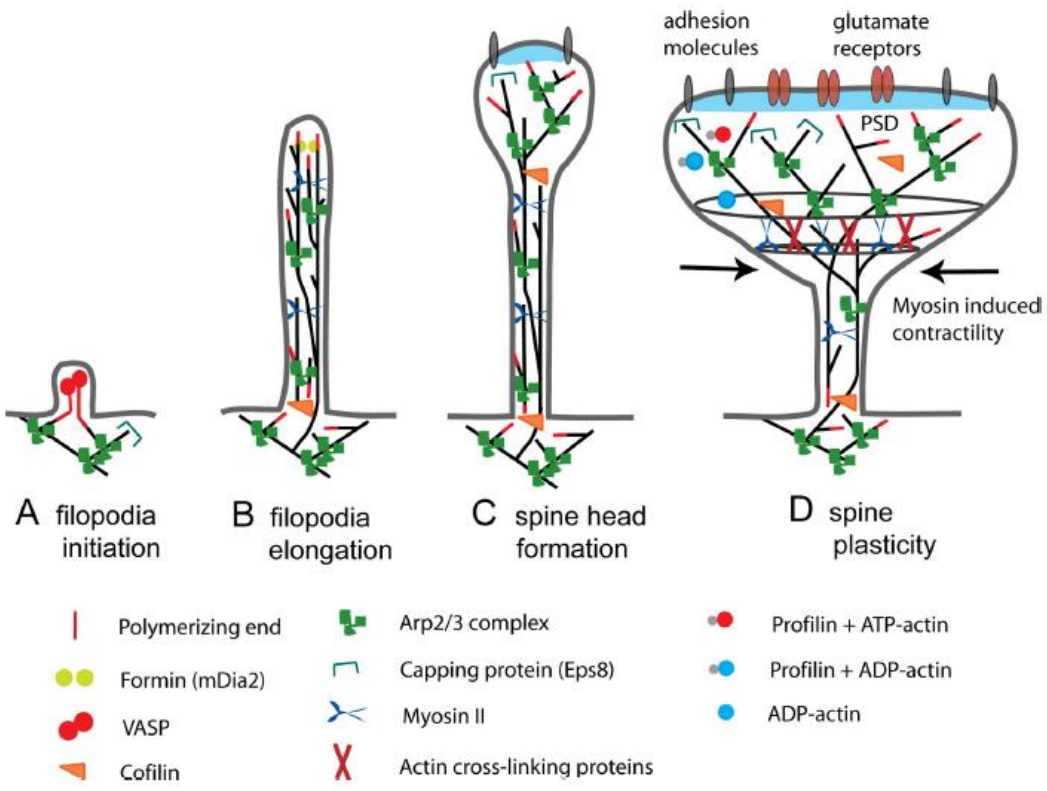

Figure 1-8. Actin regulatory mechanisms during spine development and plasticity. (A) Spine development starts with the initiation of the dendritic filopodium and its elongation. Eps8 inhibits filopodia initiation by its capping activity. Ena/VASP proteins could induce filopodia elongation from Arp2/3 complex–generated branched filaments by anti-capping the actin barbed ends. (B) mDia2 promotes actin filament polymerization in the filopodium tip. It is proposed that Ena/VASP and myosin X take part in filopodia elongation. At this stage, the elongation of dendritic filopodia protrusions is mechanistically more similar to the promotion of lamellipodia protrusions. The factors driving actin filament polymerization in the base of filopodia remain to be identified. (C) Extensive actin branching occurs at the filopodium tip and the spine head begins to form. The mechanism of actin assembly is now increased and the large Arp2/3-nucleated branched actin filament network leads to enlargement of the spine head. The function of ADF/cofilins, in addition to replenishing the cytoplasmic actin monomer pool in neurons, is to control the proper length of actin filaments and thus to prevent formation of abnormal protrusions

from spine heads. (D) Mature spines are still dynamic but maintain their overall morphology. Dynamics occur as small Arp2/3 complex–induced protrusions on the surface of the spine head (morphing). Myosin II–dependent contractility and cross-linking of actin filaments further modulate the shape of the spine head. Adapted from Hotulainen & Hoogenraad, 2010 with permission.

Chapter 2. Capping protein in the formation of dendritic spines

Parts of this chapter are adapted from:

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2.1 Summary

The formation of dendritic spines represents a key step in the functional connection of neurons. Actin cytoskeleton plays a crucial role in regulating dendritic spine formation. However, the regulatory mechanism has not been fully elucidated yet. Capping protein (CP), an actin regulatory protein that binds to barbed ends and prevents the growth of actin filaments, is highly concentrated in dendritic spines. Here we report that actin capping protein (CP), a regulator of actin filament growth, plays an essential role for spine development and synapse formation. We found that CP expression in the rat hippocampus was elevated at and after the stage of substantial synapse formation. We also observed a dynamic distribution of CP in neurons during synapse formation. While CP was initially excluded from dendritic filopodia-like protrusions, it started to accumulate in dendritic protrusions gradually along the course of spine development and eventually became highly concentrated in mature spines. This suggests CP may function in spine formation and maturation. We utilized loss-of-function approach to examine the role of CP in spine development. CP knockdown in hippocampal cultures resulted in a marked decline of the spine density accompanied by increased filopodia-like protrusions. Moreover, the spines of CP knockdown neurons exhibited an altered morphology, highlighted by multiple thin filopodia-like protrusions emerging from the spine head. The number of functional synapses was reduced by CP knockdown as evidenced by a reduction in the density of paired pre- and postsynaptic markers and in the frequency of miniature excitatory postsynaptic currents. These findings indicate that capping of actin filaments by CP represents an essential step for the remodeling of the actin architecture underlying spine morphogenesis and synaptic formation during development. We further dissected how CP functions in the spine formation process. By comparing dendritic protrusions at different stages between control and CP knockdown neurons, we found there was a decreased conversion of filopodia to spines after CP knockdown. Our subsequent time-lapse imaging showed that the extension and retraction of dendritic protrusions during the filopodia-

spine conversion stage were much more dynamic after CP knockdown. Moreover, the percentage of dendritic protrusions containing PSD95 in the conversion stage was much higher in normal developing neurons than CP knockdown neurons. Based on these results, we propose a model of dendritic spine development – CP gradually accumulates in dendritic protrusions to stabilize the structure of protrusions and facilitate the recruitment of PSD95. This process enables the subsequent morphological differentiation and clustering of synaptic receptors that are needed for the formation of mature dendritic spines.

2.2 Introduction

Most of the excitatory synapses in the vertebrate brain reside on dendritic spines that contain postsynaptic specializations including clustered synaptic receptors. During development, dendritic spines are formed upon axonal contact (Yoshihara et al., 2009), and their shape and size are tightly correlated with synaptic strength (Kasai, Matsuzaki, Noguchi, Yasumatsu, & Nakahara, 2003; R. Yuste & Bonhoeffer, 2001). Abnormality in spine number and shape has been observed in a number of neurological disorders and contributes to brain dysfunction (van Spronsen & Hoogenraad, 2010). For example, the immature spine shape with long spine neck and small head is a prominent feature of Fragile X and considered as least partially responsible for the defects of neuronal wiring (Comery et al., 1997). One important aspect of synaptic biology is to understand how dendritic spines are properly formed in normal brain development and what results in spine alteration and synaptic defects in neurological disorders.

Dendritic spine formation involves multiple steps of structural reorganization and functional specialization of dendritic protrusions. One predominant model of spine formation in hippocampal neurons is the filopodia model - dendritic filopodia initially arise from the dendritic

shaft, and explore the environment to find an axonal terminal. Once they "grab" and make contacts with axonal terminals, the filopodia are stabilized to maintain the contact, and subsequently an enlarged spine head is formed with space for postsynaptic components clustering (Hotulainen & Hoogenraad, 2010b). Two key events for spine formation are the initiation of filopodia and the transformation of filopodia to dendritic spines with mature heads. For the filopodia-to-spine conversion, the stabilization of filopodia and clustering of PSD components are observed prior to the emergence of spine heads (Marrs, Green, & Dailey, 2001; Prange & Murphy, 2001), which may ensure the stable contact with axonal terminals and provide building blocks for subsequent formation of postsynaptic specialization.

Actin is the major cytoskeletal component in dendritic spines and provides the structural basis for spine formation, modification, and elimination during synaptic development and plasticity (Cingolani & Goda, 2008; Hotulainen & Hoogenraad, 2010b; Tada & Sheng, 2006). Importantly, the actin cytoskeleton, in conjunction with other scaffolding molecules, establishes the cytoarchitecture for subcellular organization of postsynaptic components that ensures effective postsynaptic signaling (Carlisle & Kennedy, 2005). In the process of spine formation, actin cytoskeleton mediates the structural reorganization, and also regulates the trafficking of receptors and organization of other important synaptic components. Microtubule is observed in spines as well. Microtubule invasion is found in ~5% spines at steady state, but this percentage increases upon neuronal activity (Hu et al., 2008). It is considered that microtubules contribute to the activity-dependent spine formation (Gu et al., 2008). However, understanding of the microtubule function in spines is still very limited, and current cytoskeletal research largely focuses on the role of actin in spine formation.

The actin cytoskeleton and its dynamics are regulated by a broad array of binding proteins, but whether and how they function accordingly in dendritic spines remain to be

elucidated (Hotulainen & Hoogenraad, 2010a). Capping protein (CP) binds the barbed ends of actin filaments to prevent their elongation and facilitate Arp2/3-mediated nucleation (Akin & Mullins, 2008). CP exists and functions as α/β heterodimer, and single subunit is unstable (Cooper & Sept, 2008). While both α1 and α2 isoforms of α subunits are abundant in most tissues (Hart et al., 1997), β2 is the predominant β isoform in the brain (Schafer et al., 1994). A recent study showed that CP is present in dendritic spines of cultured hippocampal neurons, and the branched actin filament network containing CP appears to be a prominent feature of the spine head (Korobova & Svitkina, 2010). However, the role of CP in dendritic spines and synaptic functions has not been established.

In this study, we examined the role of CP in spine development and synapse formation. Based on loss-of-function approach in cultured hippocampal neurons, we present data supporting conclusion that CP is essential for spine development and maturation as well as the formation of functional synapses. To gain more insights into how CP is involved in the spine formation process, we analyzed the subcellular distribution of CP at different stages of spine formation and examined which key processes are affected by CP knockdown. We think CP is indispensible for the stabilization of filopodia and clustering of PSD components in dendritic spines.

2.3 Results

2.3.1 CP expression increases during hippocampal development

To gain insights into the temporal pattern of CP in brain development, we performed western blot analysis to examine CP expression in developing rat hippocampi. The peak of spine formation in rat hippocampi occurs during the first two postnatal weeks (Fiala, Feinberg, Popov, & Harris, 1998). Therefore, we chose three time points - embryonic day 18 (E18), postnatal days

9 and 18 (P9 and P18), which correspond roughly to stages before, during, and after extensive spine formation. Since CP β1 subunit was not detected in brain (Schafer et al., 1994), we examined only β2 subunit and both α subunits. We found that α and β2 subunits exhibited an increasing trend of expression over the course of hippocampal development (Figure 2-1): the CP level increased slightly at P9, but significantly at P18 over E18 (CP α1&2: $p<0.001$ for P18 vs. E18 and P18 vs. P9; CP β2: $p<0.001$ for all the pairwise comparisons, ANOVA Turkey test). These data suggest that CP functions during synapse development and refinement.

2.3.2 CP gradually accumulates in dendritic spines during development

We next examined the subcellular distribution of CP in primary cultured hippocampal neurons. Consistent with the previous study (Korobova & Svitkina, 2010), CP is highly present in dendritic spines as evidenced by immunostaining using a specific antibody against CPβ2 (Figure 2-2B) (Schafer et al., 1994). Furthermore, our live cell imaging of EGFP tagged β2 (hereafter referred to as GFP-CP) showed that CP is highly enriched in dendritic spines of DIV21 hippocampal neurons in comparison to the volume marker mOrange (Figure 2-2). The spine localization of CP is better depicted by ratiometric normalization of GFP-CP to mOrange, as the GFP-CP/mOrange ratio in spines is 2.4 ± 0.6 (mean ± 95% confidence interval, from 5 neurons over 100 spines) when normalized against that of the adjacent dendritic shaft. For neurons expressing GFP and mOrange, the ratio of GFP/mOrange in spines is about the same as that in dendritic shaft (1.0 ± 0.1, from 4 neurons over 100 spines). The localization of GFP-CP in spines is further supported by FRAP. Comparing to GFP, GFP-CP exhibited a much slower rate of recovery (halftime: ~20 s for GFP-CP vs. ~2 s for GFP) with ~12% GFP-CP fluorescence not recovered at the end of recording (Figure 2-3). These data suggest that CP is preferentially localized to spines, potentially through interactions with other spine components.

To investigate when CP starts to accumulate in dendritic protrusions and their potential function in spine formation, we analyzed the spatial distribution of CP in dendritic protrusions versus shaft at different developmental stages. We found a gradual accumulation of CP in dendritic protrusions over the course of spine development (Figure 2-4). We co-expressed GFP-tagged CP and mOrange as a volume marker in cultured hippocampal neurons at DIV13, and examined the distribution of GFP-CP versus mOrange at DIV14, 17 or 21. At DIV14, CP signal in dendritic protrusions was weak and hardly distinguished from shaft based on ratiometric analysis. At DIV21, when many mature spines were formed, CP signal was highly concentrated in spines, as shown in red (high ratio) in ratiometric analysis. At DIV17, the intensity of CP signal in protrusions fell between the DIV14 and DIV21. Due to the variation of CP/mOrange ratio in different protrusions, an accumulation curve of ratio distribution is more sensitive to detect the shift of ratio. We measured the intensity ratio of CP versus mOrange in each individual protrusion from neurons at different developmental stages, and then plotted the frequency distribution curve (Figure 2-4). From DIV14 to DIV21, the curve gradually shifted rightwards, indicating an increased ratio of CP in dendritic protrusions against shaft. These data suggest that the CP was not concentrated in the protrusions right at the beginning of filopodia initiation, but gradually emerged in some of the protrusions and eventually became highly concentrated in most protrusions when mature spines were formed. Based on this result, we hypothesized that CP functions at the critical stage of filopodia-spine transition and the maturation of dendritic spines.

2.3.3 CP knockdown impairs spine morphogenesis

To investigate the function of CP in spine development, we utilized RNAi strategy to knockdown CP in cultured hippocampal neurons. We expressed an shRNA that specifically targets CPβ2 of both mouse and rat (Mejillano et al., 2004). This shRNA construct also encodes

mRFP for identification of neurons expressing shRNA. We first verified the effectiveness of CP knockdown by this shRNA in cultured mouse CAD neuroblastoma cells (Qi, Wang, McMillian, & Chikaraishi, 1997). Consistently, the endogenous CPβ2 level was dramatically reduced three days after shRNA transfection as evidenced by western blotting (Figure 2-5). Effective knockdown of endogenous CPβ2 in cultured hippocampal neurons was verified by immunostaining (Figure 2-5). After one day of transfection, the average intensity of CP β2 immunofluorescence in shRNA-expressing neuron was not statistically different from that of non-transfected neuron; while after 4 days of transfection, the average intensity of the CPβ2 immunofluorescence in shRNA-expressing neurons (identified by the presence of mRFP fluorescence) was 53 ± 4 % of that of non-transfected neurons in the same dish (shRNA neurons: 15; non-transfected cells: >120; from different batches of experiments). This knockdown effect lasted until 7 days post transfection, when CP signal was still statistically lower in shRNA-expressing neurons compared with nearby non-transfected ones. These results confirmed the effectiveness of the CPshRNA for CPβ2 knockdown in hippocampal neurons.

To examine the effects of CPβ2 knockdown on spine development, we transfected cultured hippocampal neurons at DIV13, and performed confocal imaging on spines at DIV21. In control neurons, a large portion of dendritic spines have developed into the mushroom shape with a distinct spine head and neck (arrows) and some have acquired the stubby (arrowheads) as well as thin spine morphologies (double arrows; Figure 2-6 A). In neurons expressing CPshRNA, however, a large percentage of dendritic protrusions exhibited a thin filopodia-like morphology (red arrows), of which some were branched (red arrowheads, Figure 2-6 A). Most notably, some spine-like protrusions that did form displayed an aberrant morphology. Unlike the bulbous spine heads observed in control neurons, these spine heads appeared to be larger, irregularly shaped, and most intriguingly had thin filopodia protruding out (asterisks, Figure 2-6A). The abnormality in spine shape and size can be better appreciated by 3D reconstruction of dendritic protrusions

(Figure 2-6 C). The CPshRNA effects on spines appear to be a direct result of CPβ2 knockdown since knockdown-rescue experiments using a construct encoding both CPshRNA and a shRNA-refractory CP mutant (KDR) (Vitriol, Uetrecht, Shen, Jacobson, & Bear, 2007) produced dendritic spines similar to that of the control cells (Figure 2-6 A). Furthermore, expression of GFP-CP did not seem to perturb the spine morphology. When quantified, the number of total protrusions stemming from the dendritic shaft showed no difference among different groups (Figure 2-6B). However, the numbers of spine-like protrusions in CPβ2 knockdown cells were significantly reduced (Figure 2-6E; $p<0.05$ comparing to the control, ANOVA Dunnett's method), whereas the number of filopodia was much higher than that of control cells (Figure 2-6E). Further analysis of single spine size showed that the spine head width and neck length in CPshRNA group was larger than that of the control (Figure 2-6D; $p<0.01$and $p<0.05$, respectively, Rank sum test). These results indicate that CP is required for the formation and morphogenesis of dendritic spines.

2.3.4 Abnormal spine morphology in CP knockdown is largely actin-dependent

Similar to control cells, dendritic protrusions in CP knockdown cells are actin-based as evidenced by their concentration of F-actin (Figure 2-7A). In particular, the filopodia-like protrusions from the spine head are highlighted by F-actin (see magnified panels in Figure 2-7A). We found that most of the dendritic protrusions in CP knockdown cells do not contain microtubules except a small fraction of spines (Figure 2-7A), similar to that previously reported (Gu et al., 2008; Hu et al., 2008). Those protrusions coming out of the spine head are exclusively stained by F-actin. We didn't observe microtubule presence in the spine head protrusions (>200 spines analyzed). Base on our quantification, the baseline rate of microtubule invasion into dendritic spines is not significantly different between control and CP knockdown neurons (average 0.77/100μm vs 1.71/100μm, with $p=0.07$ student's t test).

2.3.5 CP knockdown affects pre- and postsynaptic specialization and synaptic transmission

To examine the formation of functional synapses, we first performed immunostaining to identify the pre- and post-synaptic specializations using antibodies against SV2 (presynaptic marker) and PSD-95 (postsynaptic marker). We found that almost all the dendritic spines in control cells were apposed to SV2 puncta (Figure 2-8A) and contained PSD-95 signals (Figure 2-8B), consistent with previous studies that most of the spines, if not all, represent the postsynaptic structure of functional synapses. In cells expressing CPshRNA, SV2 signals were also found to be associated with the spine-like protrusions but the density was markedly reduced (Figure 2-8A). Interestingly, the size of SV2 puncta appeared to be larger than that of the control group (Figure 2-8A). Similarly, PSD-95 signals were associated with the spine-like protrusions even though these spines had thin filopodia on the spine head (Figure 2-8B). The density of PSD-95 puncta was also reduced but their size appeared to be larger than that of the control group (Figure 2-8B).

To better determine the effects of CP knockdown on synaptic function, we performed whole-cell patch clamp recordings to examine miniature excitatory postsynaptic currents (mEPSCs). We found that the frequency of mEPSCs in cells expressing CPshRNA was markedly reduced comparing to the control non-transfected cells (Figure 2-9 A&B). Expression of the knockdown-rescue construct (KDR) restored the mEPSC frequency and expression of GFP alone did not have any effects on the mEPSCs frequency (Figure 2-9 A&B). All the groups showed similar amplitudes of mEPSCs (Figure 2-9 C). These electrophysiological data, together with the imaging findings, support a critical role for CP in spine development and synapse formation.

2.3.6 CP is involved in the conversion of filopodia to dendritic spines

The CP knockdown results are consistent with our hypothesis that CP functions in spine formation, likely at the critical stage of filopodia – spine conversion. This is supported by the increased percentage of filopodia-like protrusions after CP knockdown. To better understand why CP results in abnormal spine formation, we examined the effect of CP knockdown on dendritic protrusions at different stages before they form mature spines. Confocal imaging was performed at different days after the transfection of CPshRNA constructs to examine the spine development and compare with GFP-transfected control neurons. We transfected neurons at DIV13, and started the imaging of neurons one day after transfection. At DIV14, both control and CP knockdown neurons had similar starting point of protrusion number and morphology, possibly due to the fact that CP knockdown effect was not significant at this time (Figure 2-5). We found that filopodia were prominent in DIV14 cells with few spine-like protrusions, but a marked number of spines started to emerge from DIV17 to DIV21 in control neurons (Figure 2-10). In CPshRNA expressing cells, the thin filopodia-like protrusions were obvious by DIV17 and became predominant by DIV21, and a large portion of spines have thin filopodia-like protrusions on the spine head. We quantified the number of dendritic protrusions (Figure 2-10) and found that the increase of spine formation in control neurons was accompanied by a decrease of filopodia from DIV14 to DIV21 (Figure 2-10). However, in CPβ2 knockdown cells, the spine-like protrusions did not increase but the number of thin filopodia-like protrusions kept increasing. Together, these data suggest that CP is likely to play a role in the conversion of filopodia to dendritic spines, possibly by affecting the spine head expansion. After CP knockdown, the filopodia conversion to spines is hindered, which leads to the prominent accumulation of filopodia without the increase of spines along development.

2.3.7 Dendritic protrusions during the filopodia-spine conversion are unstable after CP knockdown

To better understand why filopodia conversion into dendritic spines was disrupted after CP knockdown, we examined the properties of filopodia after CP knockdown and compared them with filopodia in normal developing neurons. One important characteristic for a dendritic protrusion to be converted to spines is stability. Prior to the conversion, dendritic protrusion were stabilized first (Marrs et al., 2001). This stabilization step is considered to be important for maintaining the contacts with presynaptic terminals and subsequent morphological reorganization. We performed time-lapse imaging of dendritic protrusions and analyzed the dynamics of protrusions in control versus CP knockdown neurons. The difference in the rate of protrusion and retraction over a 10-min recording period was striking. In control neurons, a large number of protrusions maintained the length over the recording time, with minor morphological alterations (as shown in Figure 2-11 A). While in CPshRNA neurons, the protrusion and retraction was much faster, and the length changed over a larger range. A large number of filopodia in CPshRNA neurons underwent long protrusion to total retraction during the 10min recording time (one example in Figure 2-11A). We did notice that there were some dynamic protrusions in control group, but majority were constrained to minor length change. On the other hand, although there were some stable protrusions in CPshRNA knockdown, majority were quite unstable, similar to the example shown in Figure 2-11 A. For the purpose of quantification, we traced the length change of a single protrusion, and plotted the change against time. The ups and downs of the curve represent the dynamic range of the protrusion. In control neurons, most of the protrusions were changing in a relatively restricted region, while the change spanned a much larger area in the knockdown group (Figure 2-11B). We quantified the maximal change of length over the recording period (with or without normalization, see methods and legends of Figure 2-11B), and found the maximal length change of protrusions in knockdown group was significantly

higher than control. This reflected an increased alteration of filopodia length during a fixed amount time after CP knockdown, suggesting instability and poor maintenance of the contact with presynaptic axonal terminals.

2.3.8 Dendritic protrusions during the filopodia-spine conversion have reduced PSD clustering after CP knockdown

Prior to the conversion of filopodia to spines, clustering and transportation of synaptic components into filopodia was observed (Marrs et al., 2001; Prange & Murphy, 2001). One marker of postsynaptic specialization is PSD95. We further examined if PSD clustering in dendritic protrusions was affected after CP knockdown. We transfected neurons with GFP-tagged PSD95 to visualize the clustering of PSD in dendritic protrusions. Neurons were at DIV16, when a large percentage of protrusions were at the transitional stages between filopodia and spines. As shown in Figure2-12A, both the control and CPshRNA neurons had a large number of filopodia-like protrusions at that time, and not many mature spines were formed yet. The expression of GFP-PSD95 was at similar level as shown that the total area of PSD95 was not significantly different between control and knockdown group (Figure 2-12C). When analyzing the percentage of protrusions possessing PSD95, we defined all the protrusions with one or more PSD95 puncta in the protrusion as possession, but not those without PSD95 or with PSD95 at the base (in the shaft). Although dendritic protrusions appeared premature in both groups, majority of protrusions in control group were found to possess PSD95 puncta, while many protrusions in CPshRNA neurons were devoid of PSD95 clusters or only with PSD95 at the base of the protrusion. Quantification showed a significant difference in PSD95 possession – there were 22% of protrusions without PSD in control, while the percentage increased to 42%, almost doubled, in

the CP knockdown group (Figure 2-12B). This suggests that CP knockdown interrupts PSD95 clustering or transportation of PSD95 into dendritic protrusions during spine development.

2.4 Discussion

2.4.1 Abnormal spine morphology after CP knockdown

End capping of cytoskeletal filaments is a key mechanism to regulate filaments' elongation and disassembly, as well as the organization of the cytoskeletal architecture. It is postulated that CP is an essential component for the proper cytoarchitecture and morphology of dendritic spines. Here we show that CP knockdown promotes the formation of thin filopodia-like protrusions and reduces the formation of mature spines with proper morphology. In particular, spines with head enlargement after CP knockdown appear larger than spines in control neurons, and those spines have tiny "antenna" protruding out from the dendritic spine head. These antenna shape structures are exclusively actin-based (no single protrusion contains microtubule based on observation of over 200 spines). However, the detailed properties of those structures remain undefined. Based on PSD95 staining, those "antenna" structures are devoid of PSD95 puncta. Some of them appear protruding out on top of the PSD95 clustering. The apposing signal of presynaptic marker SV2, however, can be seen along the surface area of those spine head protrusions (like the protrusion in Figure 2-7). This presents a possibility that these structures are similar to "spinules" or "perforated" spines described in literature.

Spinules or perforated spines were first described by Westrum and Blackstad in 1962 based on the observation of tiny protrusions originating from spine heads in electron microscopy study (Westrum & Blackstad, 1962). These protrusions are typically short vesicular evaginations with double walled membranes, named "spinules". They can be engulfed by presynaptic

terminals, and thus potentially represent a retrograde signaling mechanism from the postsynaptic partner to the axonal terminal. This is considered as trans-endocytosis between spines and axonal terminals (Spacek & Harris, 2004). Harris laboratory characterized the spinule structure – the length of spinules ranges from 30 to ~700nm, and the diameter ranges from 8nm to 150nm, with maximum six spinules per spine based on their observation. The spine head protrusions in CP knockdown neurons do fit the description based on length and diameter. In our time-lapse imaging of CPshRNA cells, the spine head protrusions appeared continuously protruding and retracting, and we did not observe any truncation or break of those head protrusions that fit the description of "being engulfed" by axonal terminals. However, our recording was relatively short and may not be able to catch these events. In literature, there is limited characterization of spinules under light microscope. Based on the data we have, it is difficult to define the nature of spine head protrusions – whether they are spinules or some uncharacterized abnormal membrane protrusions, and their role in trans-endocytosis or retrograde signaling needs further investigation as well.

2.4.2 Alteration of synaptic specialization and efficacy after CP knockdown

The altered spine formation by CP knockdown appears to translate directly into a reduction in the synaptic specialization and number of functional synapses. While the spines with altered shape in CP knockdown neurons were able to form functional synapses, the number of either pre- or post-synaptic marker was largely reduced. This is consistent with the reduced spine density in neurons after CP knockdown. Staining of postsynaptic specialization marker PSD95 showed that majority of the spines with head expansions possess normal clustering of PSD95, even though there are abnormal protrusions coming out from the head, while many of the thin filopodia-like protrusions are lacking PSD95. Therefore the reduction of postsynaptic

specialization is mainly due to the insufficient formation of postsynaptic density in thin filopodia-like protrusions. Moreover, our live cell imaging of pHluorin-tagged glutamate receptor 1 (GluR1) showed that most spines (even with their aberrant shape), but not filopodia, in CP knockdown neurons exhibited a concentrated level of surface GluR1 (preliminary data not shown). Considering that our maximal knockdown effect is around 50% reduction of CP, it appears the remaining CP is still sufficient to support the spine head formation and synaptic specialization in about half of the dendritic protrusions. More careful analysis revealed that the size of both pre- and postsynaptic marker is slightly larger after CP knockdown. This corresponds to the larger size of spines after CP knockdown. One possibility is that the decreased conversion rate of filopodia to spines results in the reduction of spines that are available for successful synaptic innervation. Since the number of innervation sites is limited, the remaining contacts may conduct larger burden of synaptic transmission and are selectively enhanced to reimburse the overall reduction of connectivity.

Electrophysiology partially confirmed this. The frequency of mEPSCs is reduced after CP knockdown. The change of frequency indicates the alteration in the number of functional synapses or the release property of presynaptic terminals. The reduction of mEPSCs frequency can be explained by the reduction of both pre- and postsynaptic specializations after CP knockdown, as shown in Figure 2-8 and 2-9. However, we cannot rule out the possibility that the release frequency of presynaptic vesicles is also altered and contributes to the change of mEPSCs frequency. Even though our experiments were manufactured in a way that CP knockdown only happens in postsynaptic neurons, there is a possibility that retrograde signaling or homeostatic scaling could be affected by postsynaptic CP knockdown as well. Paired-pulse facilitation can better resolve the vesicle release property of the presynatic terminals. The median amplitude of mEPSCs reflects the sensitivity of postsynaptic receptors to single release of neurotransmitters and also the abundance of receptors. The median amplitude of mEPSCs is not statistically

different after CP knockdown, suggesting the abundance of postsynaptic receptors is probably similar to those of control neurons. This appears to contradict the observation of larger PSD size after CP knockdown. It is possible that our alteration of actin cytoskeleton directly affects the scaffolding proteins like PSD95, but not substantially alters the enrichment of postsynaptic receptors. Moreover, although our imaging data indicated that the size of spines, pre- and post-synaptic markers appeared to be larger than the control, the change was small and could only be detected by examining the cumulative size distribution. Given that the amplitude of mEPSCs was not statistically different after CP knockdown, it is possible that the postsynaptic contact area, though slightly enlarged, may contain a similar amount of neurotransmitter receptors as in control neurons. Future studies involving more rigorous electrophysiological analyses will provide definite answers to how CP affects the synaptic efficacy.

2.4.3 CP in the conversion of filopodia to dendritic spines

Dendritic spines initiate as filopodia from dendritic shafts, and then convert to mature spine structures with an expanded head (Hotulainen & Hoogenraad, 2010a; Ziv & Smith, 1996). Our data thus suggest that CP may function in the conversion of filopodia to spines. The overall density of dendritic protrusions was not significantly different between the control and CP knockdown group. When the development process was examined more carefully, there was a continuous increase of filopodia density at different days after CP knockdown, with no increase of spine density. This strongly suggests there is a failure of filopodia conversion to spines. We found those filopodia at the transitional stage were extremely unstable after CP knockdown, with many protrusions only having a life time of less than a couple of minutes. This difference is striking since majority of protrusions in control neurons could sustain during recording with minor structural changes. We also found that PSD95 clustering in filopodia was reduced as well.

Previous reports have shown that PSD95 clustering and filopodia stabilization are coupled processes. It is hard to distinguish which is the primary or preceding event in our experiments that accounts for the failure of filopodia conversion. It could be that the unstable filopodia fail to provide the necessary structural support for subsequent PSD95 clustering, or the failure of PSD95 clustering affects the signaling required for filopodia stabilization. Based on one time-lapse imaging study in literature, the authors showed a transient stabilization of filopodia preceded the clustering of PSD95 or transportation of PSD95 puncta into the filopodia, and the PSD95 entry further stabilized the filopodia structure (Prange & Murphy, 2001). However, their stabilization of filopodia was discussed in the context of hours, which is different from our short recording. In our 10min recording, there was already very prominent turnover of filopodia after CP knockdown, some of which had a life time less than 2 minutes. Most literature documented that PSD95 clustering takes half an hour to several hours, and the stabilizing effect brought in by PSD95 complex is usually in the scale of hours. In our experiments, many filopodia collapsed within a few minutes. Therefore, it is possible that those filopodia after CP knockdown could not even maintain the initial contact with axons, and could not give sufficient time for subsequent PSD95 clustering. Failure to stabilize filopodia after CP knockdown is more likely to be the primary event that resulted in the impaired filopodia-spine conversion.

It is known that the initial contact between filopodia and axon triggers signaling events to maintain the contact and stabilize dendritic protrusions. How could CP affect this process then? Besides the direct regulation of actin cytoskeleton, CP could affect the organization or transportation of other components that are necessary to stabilize the filopodia structure. Two categories of proteins known to play a key role in filopodia stabilization are adhesion molecules and scaffolding proteins.

Cadherins are known to be critical for bringing the surface of filopodia and the axonal membrane to close proximity. It is also known that cadherin is anchored to the actin cytoskeleton

through catenin and multiple actin binding proteins, like spectrin, formin and α-actinin (Takeichi & Abe, 2005). This configuration of cadherin-actin is essential for cadherins to hold the cellular junctions together, like the contact between filopodia tip and axonal surface. Cadherins are detected in filopodia at the very initial point of axon-filopodia contact, and persists until the maturation of synapses. This may serve as the initial "grasp" of contact that is required for stabilization of filopodial structure (Arikkath & Reichardt, 2008). Interestingly, cadherin knockdown produces very similar phenotypes of CP knockdown, including the instability of dendritic protrusions and abnormal spine morphology like tiny filopodia-like protrusions coming out from the spine head. Alpha-N-cadherin is required to suppress the activity of protrusions from the synaptic contacts (Abe et al., 2004). How the capping of actin cytoskeleton affects cadherin would be very interesting to explore. In Drosophila, it has been shown that E-cadherin directly binds to CD2AP/CIN85 family protein, which is further linked to actin cytoskeleton through CP, and more importantly, this interaction is crucial for the localization of E-cadherin at the epithelial junction (Johnson, Seppa, & Cagan, 2008). CD2AP/CIN85 is shown to recruit CP to the cell peripheral in response to Rac1 signaling and also enriches CP at immunological synapses (Hutchings, Clarkson, Chalkley, Barclay, & Brown, 2003; Zhao et al., 2013). While it is known that multiple actin binding proteins are required to secure the interaction of cadherin and actin cytoskeleton, it is still not clear if deregulation of actin cytoskeleton would affect cadherin localization in spines. Exploration of the potential interaction between N-cadherin and CP would bring insights into how actin cytoskeleton and cellular adhesion molecules are coordinated at the initial phase of synaptic contact, and also implicate the important question of cadherin anchoring in cell biology.

Another category of stabilization factors are scaffolding proteins, including PSD95, shank and homer. PSD95 serves as an anchor for postsynaptic receptors, while shank is the linker of postsynaptic density and actin cytoskeleton. Shank also interacts with actin binding proteins

like cortactin. It is shown that shank could stabilize filopodia structure and promotes the transformation of filopodia to spines (Arstikaitis, Gauthier-Campbell, Huang, El-Husseini, & Murphy, 2011). In our preliminary Mass Spec screening of CP binding partners, all three members of Shank family, shank 1, 2 and 3, were found. This presents an interesting possibility that shank-CP could be co-transported into filopodia. While CP controls the actin cytoskeleton, shank could provide the building blocks of postsynaptic density on the base of a stable actin cytoskeleton. This may also explain the reduced clustering of PSD95 after CP knockdown, which may be mediated by the shank. It still takes efforts to confirm this interaction and examine how this interaction affects the filopodia stability and synaptic specialization. Considering the involvement of shank in synaptic defects and pathogenesis of autism spectrum disorders, it would be interesting to explore if the interaction between shank and actin cytoskeleton plays a role in the etiology of neurological disorders.

2.4.4 CP and actin cytoskeleton in dendritic spine formation

For each step of spine formation – the initiation, elongation and stabilization of filopodia, spine head formation and synaptic specialization, there is differential requirement of capping activity at barbed ends of actin filaments.

The initiation and elongation of filopodia is dependent on anti-capping mechanisms, so lower CP activity is required at this time (Hotulainen & Hoogenraad, 2010a). This may explain why the filopodia initiation process is not much affected after CP knockdown, as shown that total dendritic protrusion number is not statistically different from control neurons. This suggests the remaining CP is enough to suffice the requirement of desired capping at this stage. The elongation of filopodia is a formin/VASP-dependent process. Since CP is antagonist of formin/VASP, CP knockdown may actually increase the rate of filopodia elongation in theory.

Based on our time-lapse imaging, the elongation rate of filopodia is indeed faster than control. The retraction of filopodia appears faster as well, which may involve actin severing proteins like cofilin (Hotulainen et al., 2009a). In all, CP requirement is low for the initial phase of spine development - the initiation and elongation of filopodia, so filopodia in CP knockdown neurons are able to arise and elongate to the desired length, even though they may not be able to maintain the length.

During the second step, the stabilization of filopodia is dependent on a stable core of actin cytoskeleton together with other scaffolding and adhesion molecules. The role of CP on actin dynamics is not definite in the cellular context. In vitro, the capping of barbed ends prevents the polymerization and depolymerization of actin filaments, thus slows the dynamics (Schafer et al., 1996). However, there is no direct evidence that CP addition could slow actin dynamics in the complicated context of filopodia. It is likely that CP affects the stability through direct regulation of actin dynamics, and at the same time affects the organization of actin network which indirectly influences other key molecules for stabilization, like scaffolding and adhesion molecules.

The spine head enlargement is a critical step for the transformation of filopodia to spines. Spine head expansion is largely dependent on the formation of an Arp2/3-mediated actin network with many short branches. Arp2/3 is highly abundant at spine head based on electron microscopy (Korobova & Svitkina, 2010). Upon the interaction of filopodia tip and axonal terminal, extensive branching replaces the previous elongation mode of actin filaments. This transition of actin machinery from elongation to branching is critical for the filopodia to stop elongating and instead switch to expansion at the tip (Hotulainen & Hoogenraad, 2010a). While the exact signal triggering the activity of Arp2/3 is not known yet, it is very clear that loss of Arp2/3 results in the reduction of synapse number and increased filopodia-like spines. The inhibition of WAVE1, which is an activator of Arp2/3, increases the number of filopodia and decreases the number of mushroom shaped mature spines (Y. Kim et al., 2006). Other regulators of Arp2/3, including

Abp1, WASP, and PICK1, are also involved in the control of spine formation especially in the morphogenesis of spine head (Haeckel et al., 2008; Nakamura et al., 2011; Wegner et al., 2008). CP is indispensible for Arp2/3-dependent branching. The synergistic activity of CP and Arp2/3 is well characterized in lamellipodia formation (Akin & Mullins, 2008). In neurons, CP may function in a similar way to facilitate Arp2/3-mediated actin nucleation and branching during spine head expansion. To keep expanding towards the plasma membrane, the actin filaments should remain short but highly branched. This way actin monomers could be added to multiple sites of the branches close to the membrane and generate protrusive force, instead of being incorporated to a few elongated filaments. In CP knockdown cells, newly formed branches cannot be efficiently capped, and actin monomers cannot be incorporated at desired sites. Instead extensive polymerization and elongation happens at the aged filament which instead hinders the branching of actin networks. Since our knockdown is not 100%, the remaining CP may provide sufficient capping for a subgroup of filopodia protrusions and ensure them to finish the Arp2/3-mediated spine head expansion. We examined the GFP-Arp3 distribution in CP knockdown neurons, and found the enrichment of Arp3 was consistent with those protrusions with the expanded head, while the signal was low in those protrusions that failed to form head expansion. From studies in other cell types, it is known that actin assembly at lamellipodia is insensitive to increase of either factor. Instead the concentration of the Arp2/3 and CP together determines the rate of actin assembly in branched network (Wakatsuki, Saffarian, & Elson, 2002). This may happen in dendritic spine formation as well – only those protrusions with sufficient capping and normal Arp2/3 activity can maintain the branching of actin cytoskeleton and make the transition to spines with enlarged heads.

Another important actin regulatory protein that is closely related to capping activity is Ena/VASP. During spine head formation, the competition between Ena/VASP and CP directly determines the final organization of actin network and the morphology of spine head. After CP

knockdown, actin dynamics tilt towards the Ena/VASP-mediated actin polymerization and filopodia elongation. Since they are direct competitors, it is sensitive to even modest decrease of CP in the spine formation process. Some protrusions that manage to make the transition from filopodia to spines, due to remaining CP after CP knockdown, may not have sufficient CP to restrict the elongation of all the actin filaments, thus there are still filopodia randomly protruding out from the spine head.

Since CP is required for the filopodia-spine conversion, we speculated that CP overexpression might favor spine formation and increase spine density, but EGFP- CPβ2 expression did not produce any effects on the morphology or density of dendritic spines. This can be explained by the fact that CP functions as a heterodimer of α and β subunits (Cooper and Sept, 2008). Therefore, a potential effect on spine formation may only be observed by overexpressing both α and β subunits. Also, the spine head formation process requires the coordination of both Arp2/3 and CP, and excess of one factor may not be sufficient to alter the whole actin assembly mechanism.

Besides CP, there is another important actin regulatory protein that caps the barbed ends of actin filaments - Eps8. Both CP and Eps8 bind to the barbed ends of actin filaments and prevent the actin polymerization, although they have different binding partners and respond to different signaling pathways. Eps8 binds to actin in response to EGFR signaling, and Eps8 also regulates actin dynamics through multiple indirect pathways. These indirect pathways include the interaction with IRSp53 to induce actin bundling, and the interaction with Abl to regulate Rac-dependent actin remodeling (Di Fiore & Scita, 2002). Eps8 overexpression promotes the formation of dendritic spines and inhibits filopodia formation. After Eps8 knockdown, the actin turnover in dendritic spines is faster, and there is a shift of synapse formation from spines to the shaft (Stamatakou, Marzo, Gibb, & Salinas, 2013). This confirms that capping activity is important in spine development and synapse formation. However, the slight difference of spine

phenotype compared with our CP knockdown may be due to the involvement of Eps8 in multiple aspects of actin regulation. The observed phenotype in Eps8 knockdown neurons may be a combined result of defects in capping, bundling and Rac-dependent signaling, since Eps8 is involved in all these pathways. Knockout mice of Eps8 exhibited increased resistance to alcohol, which may correlate to certain polymorphism of human alcoholics (Offenhauser et al., 2006). These confirm that actin capping activity is essential for normal synaptic transmission and is implicated in brain activities. However, the knowledge about how these two capping proteins overlap or differ in function is very limited. Considering that they respond to different upstream signaling and have differential regulation mechanisms on actin filaments, it is likely they work complementarily in neurons to facilitate the different requirements of actin regulation during development.

2.4.5 CP and microtubules in dendritic spines

Based on our quantification of microtubule invasion into dendritic spines, it appears the baseline rate of entry is not significantly different between control and CP knockdown neurons. Even though the statistics showed a p-value (0.07) very proximate to significance, it should be noted that the baseline rate of microtubule entry in both control and knockdown was still very low, with only one or two counts per dendritic region imaged. Therefore, the difference would be slight even if it reached a statistical significance. Also majority of the protrusions coming out of the spine head are actin-based, and microtubule is not observed in the spine head protrusions. These seemingly suggest that CP is not involved in the regulation of microtubule entry into spines. However, our quantification is a coarse estimation of the baseline entry rate. We could not rule out that the frequency of microtubule entry, the time of microtubule presence in spines, or the total number of spines explored by microtubules in accumulated time, are altered after CP

knockdown. This possibility exists as CP interaction with microtubule is found in axonal growth cones, and CP plays a key role in restricting microtubules from entering the periphery of growth cones (Davis et al., 2009). More rigorous analysis is required to examine the role of CP in microtubules of dendritic spines.

It would be an elegant model if CP could crosstalk with microtubules in the spine formation process. Hoogenraad group reported that the microtubule plus end binding protein, EB3, could connect to actin cytoskeleton through indirect bindings to cortactin, and this crosstalk between microtubule and actin cytoskeleton is important for synaptic formation (Jaworski et al., 2009). Since CP could bind to microtubules as shown in axonal growth cone, it could be an organizer of actin and microtubule arrays and coordinate these two cytoskeletal systems in spines as well.

2.4.6 Possible regulators of CP in dendritic protrusions

Subcellular distribution of CP in dendritic spines and shaft appears to be dynamic. Based on our analysis, the relative abundance of CP in dendritic protrusions gradually increases along synaptic development, from initial uniform distribution in protrusions and shaft to eventual high accumulation in mature spines. This pattern is consistent with the shifting of elongation to branching mode of actin network at different stages – less CP is required at the filopodia initiation and elongation stage, while more CP is required at the stage of spine head formation and the maintenance of spine head structure. However, the upstream signaling that regulates the distribution of CP is not known yet.

It is conceivable that CP targeting to spines is not merely based on the affinity with actin barbed ends, considering actin reorganization is such a precisely regulated process in synaptic

formation. One candidate for regulating the distribution of CP is PIP2 molecule, which is highly enriched in mature spines and could sequester CP (Brown, Morgan, Watras, & Loew, 2008; Schafer et al., 1996). PIP2 is also a signaling molecule that responds rapidly to multiple processes, which could potentially mediate the change of CP in filopodia or spines by releasing or sequestering of CP at the periphery. In platelet activation, PIP2 is the molecule that removes CP from the barbed ends of actin filaments and stores CP close to the plasma membrane (Barkalow et al., 1996). Another candidate that regulates CP localization in the spine is the CD2AP/CIN85 family protein, which responds to Rac signaling and recruits CP to the epithelial cellular junction and the surface of immunological synapses (Hutchings et al., 2003; Johnson et al., 2008). CIN85 is found in the postsynaptic compartments of dopaminergic synapses, and mice lacking CIN85 showed abnormal exploratory behaviors (Shimokawa et al., 2010). Since CD2AP/CIN85 is a major category of CP interacting proteins, it is worth further exploration if CIN85 controls the targeting of CP into synaptic contacts, in a similar way that CP is targeted to other types of cellular junctions.

2.4.7 CP and neurological disorders

Even though CP function in neurons is not well understood, several reports already point to the potential involvement of CP in neurological diseases. In fetal brains of Down syndrome, a frequent inherited disease causing mental retardation, both CP α and β levels were significantly lower than the control (Gulesserian et al., 2002). In postmortem brains with Alzheimer's disease, CP was actually found to be increased in hippocampal CA1 region. In those brains with disease progression, it is unclear if the CP increase is the cause of neuronal damage or rescuing efforts in restoring neuronal function (Kao et al., 2010). Moreover, CP level was found to decrease in a rat model of dementia, and CP accumulation in synapses was observed after LTP stimulation

(Kitanishi et al., 2010). Fragile X syndrome, which is featured by immature spine head and instability of dendritic protrusions, may also involve CP-dependent actin regulation. Although there is no direct report about altered CP expression in fragile X, pathways that could potentially alter CP activity and distribution, like the PIP2 molecule and PI3K pathways, are implicated based on transgenic mice studies (Gross et al., 2010). It is worth exploration if CP-mediated actin regulation is underlying the structural abnormality in fragile X.

In all, it is clear that proper actin organization and remodeling in the dendritic spines are crucial for the development and maturation of the postsynaptic structure and function. Alteration of actin structure and regulation is likely to result in abnormality of synapse development and function, as found in many brain disorders.

2.5 Materials and Methods

Constructs and reagents

DNA constructs of pmOrange, pEGFP-N1 and pEGFP-tubulin were from Clontech, of which mOrange was subcloned into pEGFP-N1 (Gu et al., 2008). EGFP-CPβ2, CPshRNA-mRFP, and CPshRNA-GFP-CP (Knockdown Rescue (KDR)) are described previously (Mejillano et al., 2004; Vitriol et al., 2007), of which CPshRNA targets both mouse and rat CP β2. Knockdown Rescue vector is composed of shRNA sequence targeting CP and a coding sequence of human CP that is refractory to the shRNA. GFP-PSD95 is from Dr.Bonnie Firestein at Rutgers University. Anti-CPβ2 (mAb 3F2.3), anti-CPα1&α2 (mAb 5B12.3), and anti-SV2 are from Developmental Studies Hybridoma Bank (Univ. of Iowa). Other antibodies used are: anti-tubulin antibody (PRB-435P; Convance) and anti-PSD-95 (MA1-045; Thermo Scientific). A custom-

made antibody against the C-terminus of CP β subunit (Schafer et al., 1994) was used for immunostaining (generously provided by Dr. John Hammer at NIH/NHLBI).

Neuronal culture, transfection

Rat hippocampal cultures were performed as previously described (Gu et al., 2008). Basically, E18 embryos were collected. Hippocampi were dissected, dissociated to single neurons by trypsin digestion and then plated in PDL-coated coverslips. Cells were transfected using the calcium phosphate transfection kit (Clontech) at 13 days in vitro (DIV13).

Imaging and Analysis

Live imaging was performed using a Nikon C1 confocal system or A1R system. Typically, a 3-D stack of images of a dendritic region was acquired and then projected into a 2D image (maximal intensity) for visualization and analysis. The 3-D images of spines were reconstructed using the "Surpass" function of Imaris 7.2 (Andor Technology). Fluorescence recovery after photobleaching (FRAP) was done using Nikon A1R confocal. Photobleaching was achieved by scanning only the spine of interest for 500 msec with 100% power of 488nm laser (~2mW at the 60X/1.49 objective), and subsequent imaging of fluorescence recovery was done by scanning region of interest with 2% laser power at 3 sec interval for ~3min. Time-lapse imaging of filopodia dynamics was done using Nikon A1R system, with images collected at 20sec interval for 10min.

For the analysis of spine and filopodia density, filopodia were defined as thin protrusions without a distinguishable head, and spines were defined as protrusions with a length < 4 μm and

an expanded distinguishable head (head/neck ratio>1.2) (Hotulainen et al., 2009). Spine and filopodia numbers were counted manually to calculate the density (number per unit length of the parent dendrite). Spine head width was measured as spine diameter (the longest possible axis), and neck length was measured from the proximal edge of the spine head to the edge of the dendrite. For spines with no discernible necks, a minimum value of 0.2 µm was used.

For the quantification of synaptic density, the cluster number and area of SV2 and PSD-95 per unit neurite length were counted and measured using ImageJ or Nikon elements. A threshold is set to distinguish the puncta of SV2 or PSD-95 from the diffusive signal. Particle analysis (ImageJ) or object counting (Nikon elements) was used later for automatic analysis of puncta size and number.

For the quantification of filopodia dynamics, the length of each filopodium was traced during the recording period. The maximal change of length was calculated as the absolute difference between the maximal and minimal length of the protrusion during the recording period $\Delta L_{max} = L_{max}-L_{min}$, or normalized to the maximal length $\Delta L_{max\text{-}normalized}= (L_{max}-L_{min})/L_{max}$.

Western blot

Hippocampi from E18, P9 and P18 rats of both sex were snap frozen in liquid nitrogen and then homogenized and extracted with RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Triton X-100, 0.25% (w/v) Na-deoxycholate, 1 mM EDTA, pH 7.4) supplemented with protease inhibitor cocktail (Sigma P2850). Extracts were dissolved in NuPage sample buffer (Invitrogen) with 50 mM DTT and heated at 85 °C for 5min. Equal amount of protein as determined by BCA measurement was loaded and fractioned by SDS-PAGE in a 10% acrylamide gel and subsequently transferred to nitrocellulose membrane. Membranes were treated with 5%

milk in PBS buffer with 0.05% Tween-20 and then blotted with primary antibody. Bound antibodies were detected by HRP conjugated secondary antibody (Jackson ImmunoResearch), visualized by chemiluminescence using ECL (Pierce), and quantified using the gel analysis routine of ImageJ software (NIH).

Immunostaining

Neurons were fixed for 10 min with 4% (w/v) paraformaldehyde in cacodylate buffer and permeabilized with 0.1% (w/v) Triton X-100. After blocking with 5% (w/v) BSA or goat serum in PBS for 1 hr, cells were incubated with primary antibodies overnight at 4^{o}C, followed by 1 hr labeling with fluorescent secondary antibody or fluorescent phalloidin.

Electrophysiology

Whole-cell patch clamp recordings were performed in mouse cortical cultures using Multiclamp 700A amplifier (Axon Instruments) as described before (Deng et al., 2007; Gu et al., 2010). Similarly, cells were transfected using the calcium phosphate method at DIV13-14 and fluorescent cells were identified for patch clamp recording at DIV21. The membrane potential was held at -70 mV. Data were acquired using pClamp 9 software, sampled at 5 kHz and filtered at 1 kHz. The miniature excitatory postsynaptic currents (mEPSCs) were recorded with the addition of 0.5 μm TTX and 100 μm picrotoxin to block action potentials and GABAA receptors.

2.6 Figures and Legends

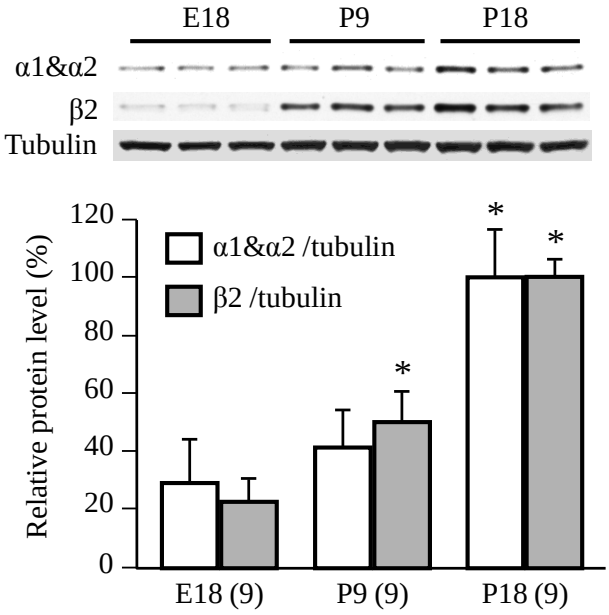

Figure 2-1. Temporal pattern of CP expression in developing hippocampi. Top: westernblot of tissue lysate from rat hippocampi at E18, P9 and P18; bottom: bar graph shows the quantification of relative protein abundance after normalization to tubulin expression. Sample number is in parentheses. *: $p<0.001$ when compared with E18 expression (CP α1&2: $p<0.001$ for P18 vs. E18 and P18 vs. P9; CP β2: $p<0.001$ for all the pairwise comparisons, ANOVA Turkey test).

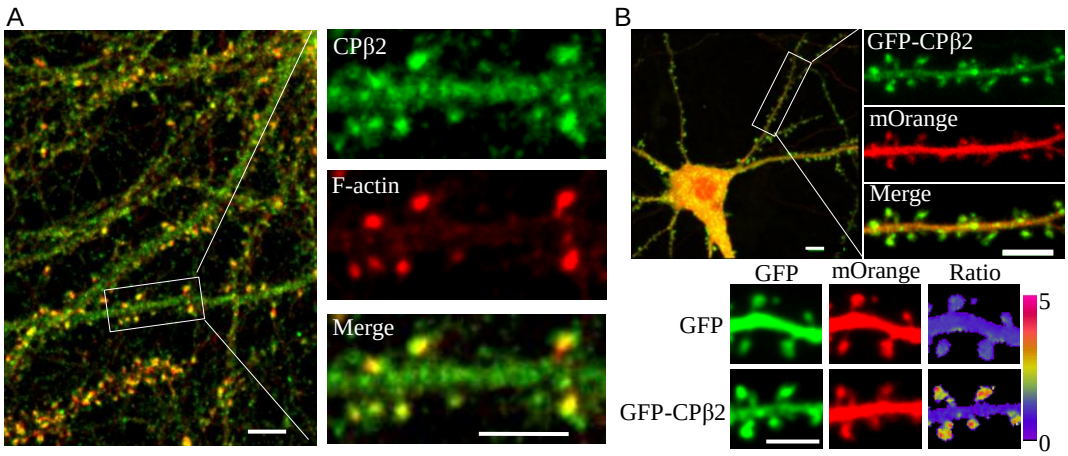

Figure 2-2. Spatial distribution of CP in primary cultured hippocampal neurons. A) Immunostaining of endogenous CPb2 (green) in hippocampal neurons together with labeling of spines by F-actin marker phalloidin (red). B) Expression of GFP-tagged CPb2 and mOrange in primary cultured hippocampal neurons, with ratiometric analysis in the bottom.

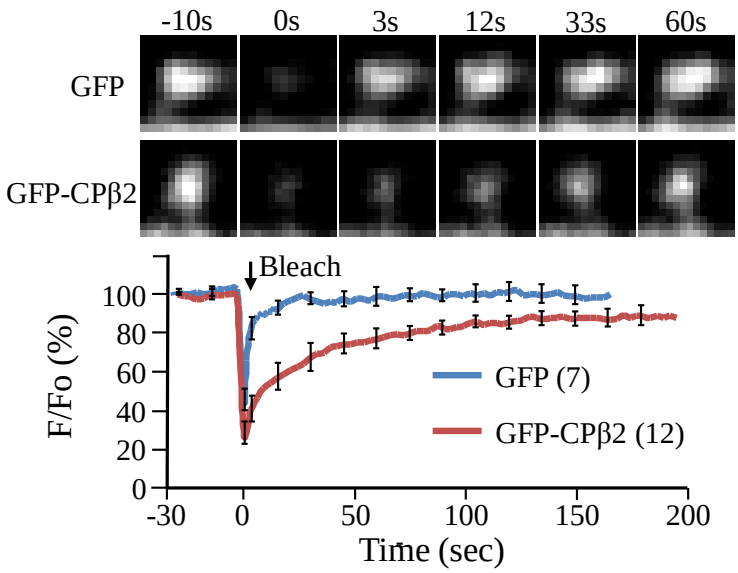

Figure 2-3. Fluorescence Recovery After Photobleaching (FRAP) of GFP-CP versus GFP in dendritic spines. Two sample time-lapse sequences are shown to demonstrate the recovery of fluorescence in spines. The curves depict the average intensity at different time points normalized to the first frame. Error bars represent the 95% confidence interval. Sample numbers are shown in parentheses.

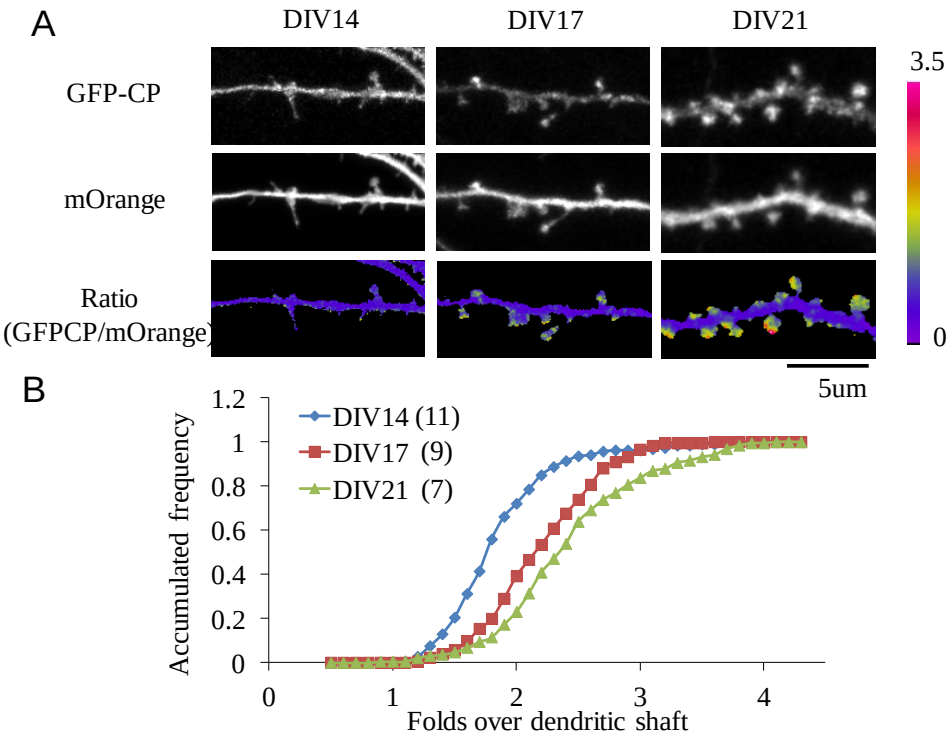

Figure 2-4. Gradual accumulation of CP in dendritic protrusions along spine development. A) Representative dendrites of neurons expressing GFP-CP and mOrange at different stages. Bottom row is the ratiometric analysis of GFP-CP versus mOrange to depict the relative accumulation of CP in dendritic protrusions versus shaft. B) Accumulated frequency of the fold of CP intensity in protrusions over dendritic shaft. For each protrusion, a ratio in the protrusion ($R_{protrusion}$) was obtained by $I_{GFP\text{-}CP}/I_{mOrange}$ (I: intensity), and a ratio in the shaft (R_{shaft}) was obtained by the average $I_{GFP\text{-}CP}/I_{mOrange}$ in the nearby shaft region. The folds of CP accumulation in the protrusion over shaft were calculated as $F=R_{protrusion}/ R_{shaft}$, and the value of F from all the protrusions were subjected to frequency analysis. An accumulation curve was plotted as shown in B. Number of neurons analyzed was included in parentheses (~30 protrusions analyzed for each neuron).

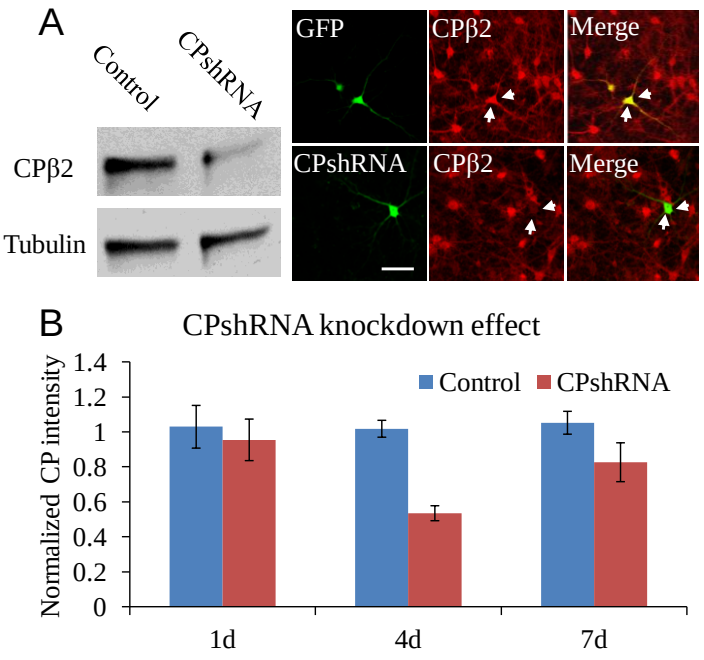

Figure 2-5. Effectiveness of CPshRNA for knockdown of CP. A) Westernblot of CAD cell lysate transfected with control or CPshRNA plasmid (left); immunostaining of CPb2 in hippocampal neurons with GFP or CPshRNA plasmids at 3 days after transfection (right); B) Quantification of CP knockdown based on immunostaining of CPb2 in cultured hippocampal neurons. For each group: ~15 transfected neurons were analyzed, the CPb2 intensity was normalized to nearby untransfected neurons. Error bars represent 95% confidence interval.

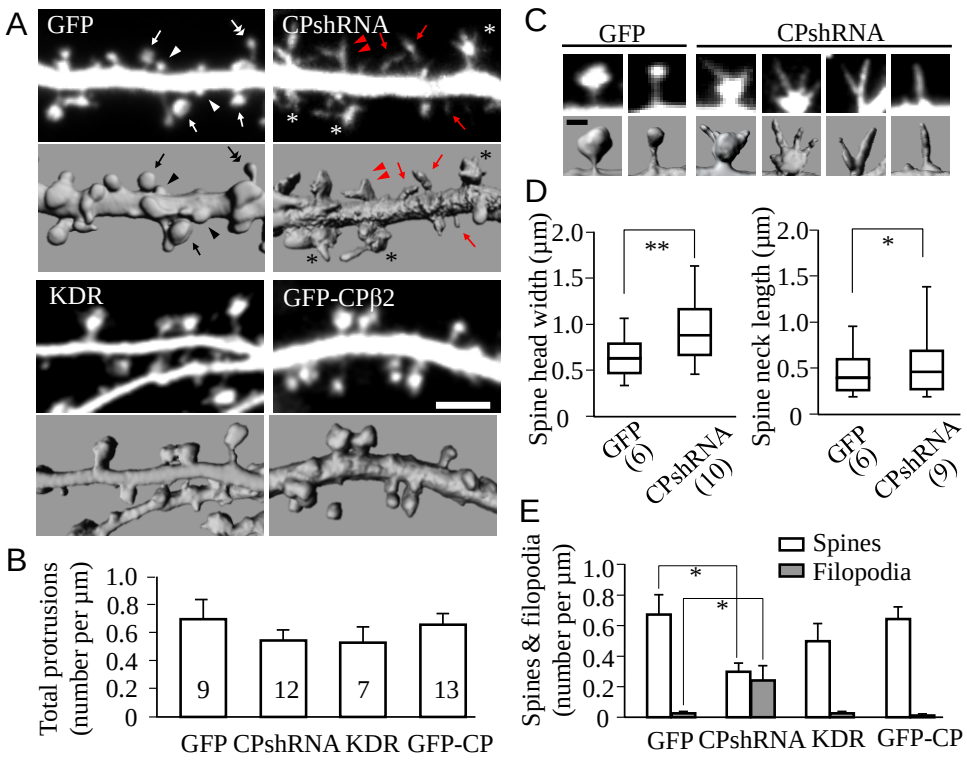

Figure 2-6. CP knockdown affects dendritic spine morphology and density. A) Representative images of dendritic protrusions in hippocampal neurons expressing GFP, CPshRNA, KDR, and GFP-CP, each accompanied by its 3D reconstruction view below. Arrows: mushroom spine; arrowheads: stubby; double arrows: thin spines; red arrows: thin filopodia; red arrow heads: branched protrusions; asterisks: spines with protrusions from the head. Scale bar: 5 µm. B & E) Quantitative analyses of total protrusions, spine and filopodia densities. Error bars represent 95% confidence interval. C) Representative images of different types of dendritic protrusions in control and CP knockdown neurons (top panel: fluorescence, bottom panel: 3D reconstructed view). Scale bar: 1 µm. D) Box-Whisker plot showing the spine head width and neck length of different groups (bottom and top of the box: the 25% and 75%; middle band: 50%; ends: 5% and 95%). *: $p<0.05$; **: $p<0.01$; ANOVA Dunnett's method for (B) and (E) and Student's *t*-test for (D).

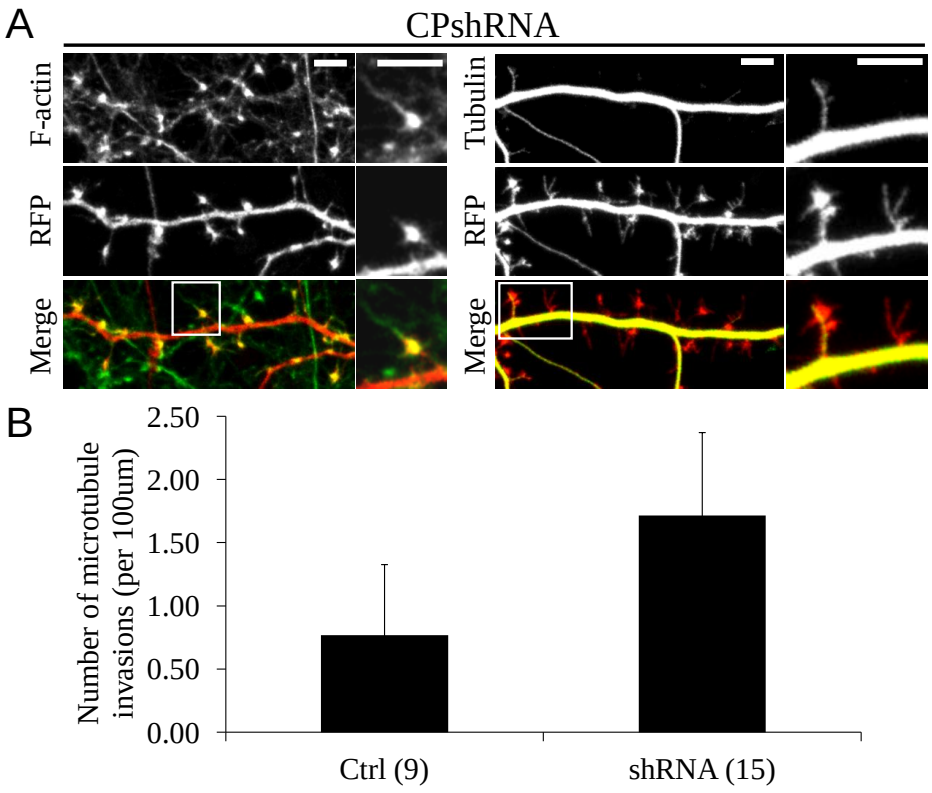

Figure 2-7. Filamentous actin and microtubule in dendritic protrusions after CP knockdown. A) labeling of F-actin by phalloidin and labeling of microtubule by expressing GFP-beta-tubulin in CPshRNA neurons; B) Bar graph shows the average density of spines with microtubule invasion at the baseline condition. Control: average 0.77/100μm; CPshRNA: average 1.71/100μm; p= 0.07, student t-test.

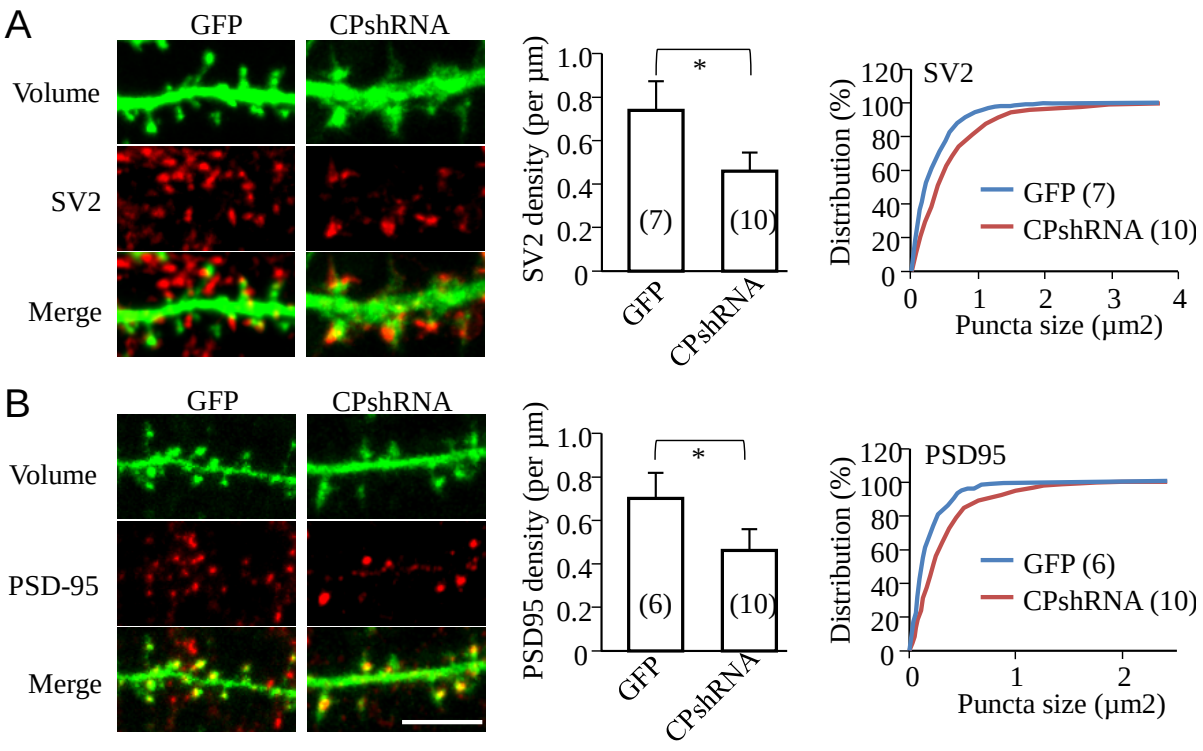

Figure 2-8. CP knockdown affected the pre- and post- synaptic specialization of dendritic spines. Effects of CP knockdown on the presynaptic marker SV2 (A) and the postsynaptic marker PSD-95 (B) as shown by the representative images of immunofluorescence (left), the density (middle bar graphs), and the size distribution (right line graphs). For CPshRNA-expressing cells, RFP is the volume marker but displayed in green for visualization purpose.

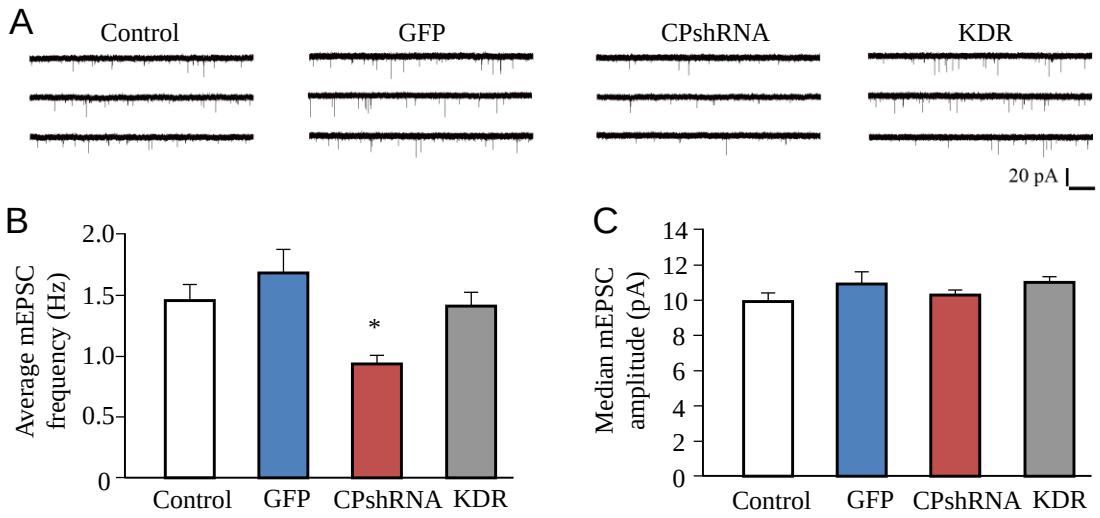

Figure 2-9. Electrophysiological recording of baseline synaptic transmission after CP knockdown. Whole-cell patch clamp recordings of mEPSCs in non-transfected neurons (control), transfected with GFP alone (GFP), with CPshRNA, and the knockdown-rescue (KDR) constructs. Sample traces of mEPSCs are shown in (A) and quantifications of the frequency and median amplitude are shown in (B) and (C), respectively. N= 24, 23, 44, 43 for control, GFP, CP shRNA and KDR, respectively. Asterisks: $p<0.01$, Student's *t*-test.

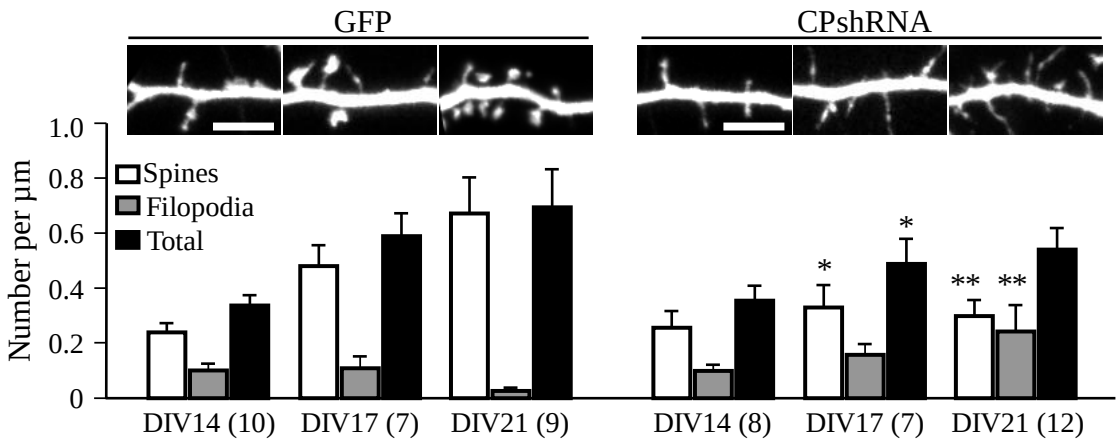

Figure 2-10. CP knockdown affects the conversion of filopodia to dendritic spines. Representative images showing the changes in filopodia and spines of cultured hippocampal neurons at DIV14, DIV17, and DIV 21 as revealed by confocal imaging. Bar graph shows quantitative analysis of the number of different dendritic protrusions at different stages in neurons transfected with GFP alone or CPshRNA. Error bars represent 95% confidence interval. *:$p<0.05$; **:$p<0.001$ (Student's *t*-test). Scale bar: 5 µm.

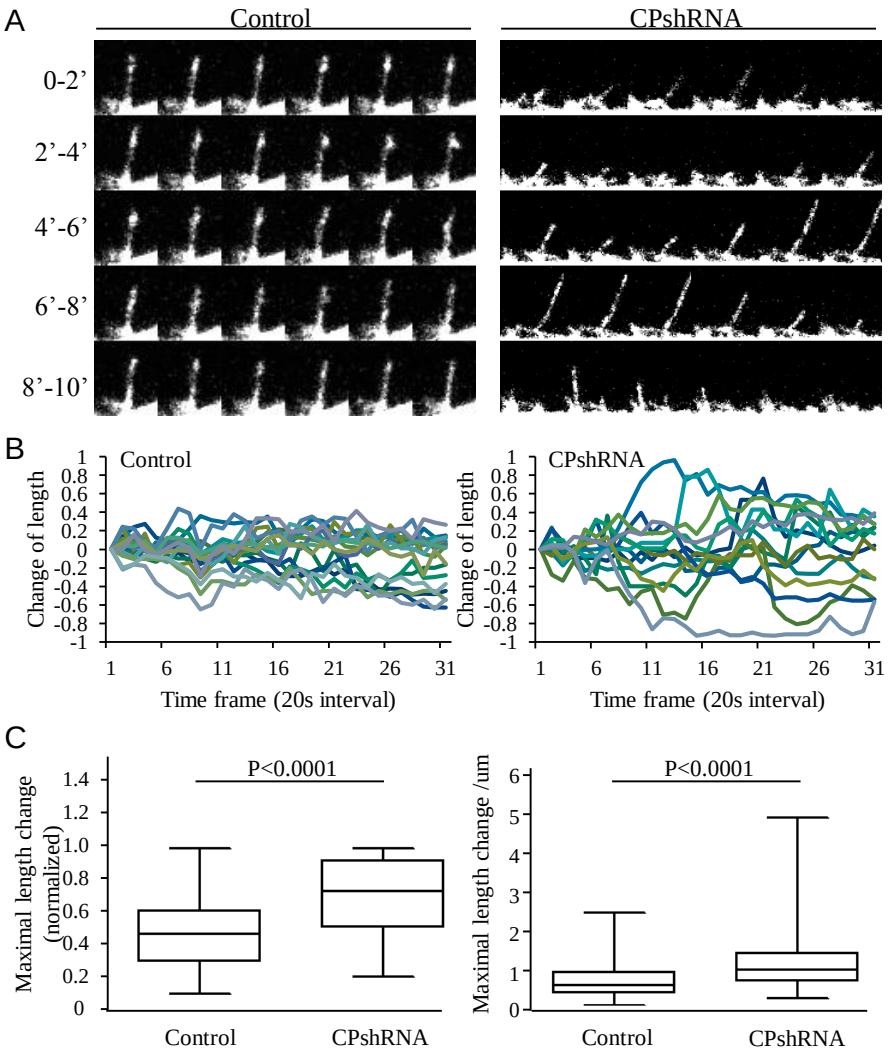

Figure 2-11. CP knockdown reduced the stability of dendritic filopodia. A: representative filopodia from control and CPshRNA neuron. Montages were made with 30 frames (20sec interval, 10min total duration); B: sample traces of filopodia length change over the recording time in one control neuron and one CPshRNA neuron. Each curve represents one dendritic filopodia; C: Box-whisker plot of maximal length change of filopodia during the recording period (Bar: max and min; box: 75% and 25%). Left: length change normalized to maximal length; right: unnormalized length change as shown in μm. P value is obtained with student t test (~120 protrusions analyzed for each group).

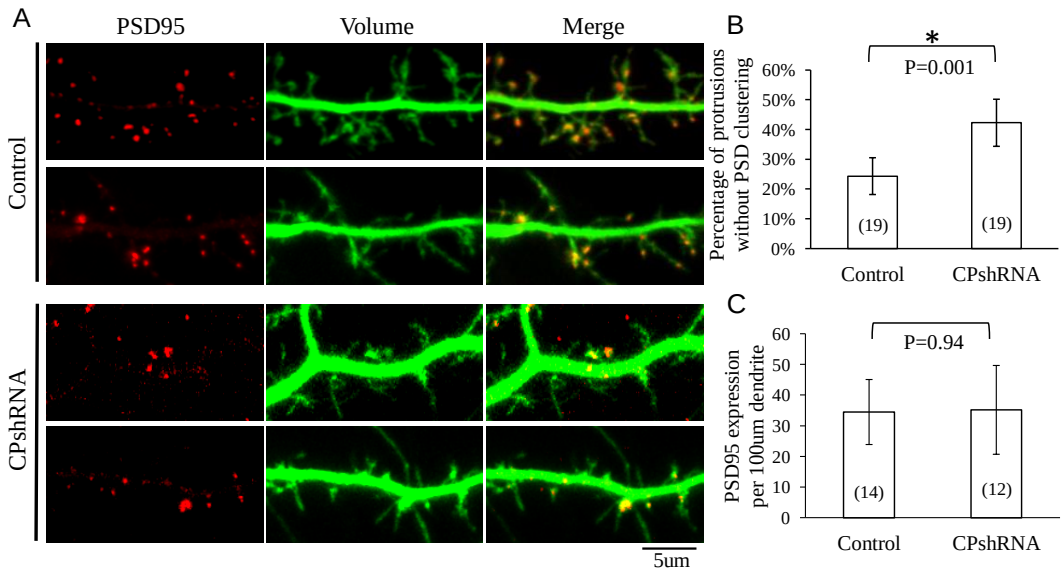

Figure 2-12. CP affects the clustering of PSD components in dendritic filopodia. A) Representative dendritic regions showing that CP knockdown results in less PSD95-containing protrusions at the conversion stage of filopodia to spines. In control, only a small percentage of protrusions are without PSD95 in the protrusion, but in CPshRNA neurons, more protrusions are without PSD95 even though some of them don't show different morphological traits when compared with control. B) Quantification of dendritic protrusions without PSD95 in control vs CPshRNA (Control: 16%±5%; CPshRNA:29%±8%; *: p=0.001, student t test). C) Quantification of total PSD95 expression in control versus CPshRNA (total area of PSD95 puncta for 100µm dendrites; p=0.001, student t test). Number of neurons analyzed was included in parentheses.

Chapter 3 Conclusion and Future Directions

3.1 Conclusion

In this dissertation, the role of CP in dendritic spine development has been investigated. We conclude that CP is an indispensible regulator of spine development and synapse formation, possibly by affecting the conversion of dendritic filopodia to spines. Our main findings include: 1. CP expression increases throughout the synaptic development process. CP dynamically localizes into dendritic protrusions during the conversion period of filopodia to spines; and eventually becomes highly accumulated in mature spines. 2. CP is indispensible for proper spine morphology and density, as evidenced by the finding that CP knockdown resulted in reduced mature spines with a concomitant increase of filopodia. Abnormal spine head protrusions were also observed after CP knockdown. 3. CP is indispensible for synapse formation, as shown by lower density of synapses and impaired baseline synaptic transmission after CP knockdown. 4. CP is possibly involved in the conversion of dendritic filopodia to spines - the dendritic filopodia after CP knockdown exhibited instability and decreased clustering of PSD components. These findings imply that CP has a regulatory role in the genesis of spines and synapses.

Based on these results, we propose a model for the role of CP during dendritic spine development. The depiction of actin regulation machinery is adapted from Hoogenraad and Hotulainen's model (Hotulainen & Hoogenraad, 2010a). We hypothesize that differential CP activity is required at various steps of spine formation in this model, as shown in Figure 3-1.

a. Filopodia initiation: low CP is required at this time. Anti-cappers like VASP and formin may play dominant roles in this process. Based on our data, the initiation of dendritic protrusions was not hindered by CP knockdown. Because no increase of filopodia initiation was observed either, the remaining CP after knockdown may be enough to antagonize effects of the anticappers at this step.

b. Filopodia elongation: low level of CP is needed for the elongation of filopodia. CP knockdown did not affect filopodia elongation, and filopodia could reach a distance that was equivalent to those in wild-type neurons.

c. Filopodia conversion to spines: high CP is required for stabilizing dendritic filopodia and clustering of postsynaptic density components, two necessary processes during the filopodia-spine conversion. Our data showed decreased stability of filopodia after CP knockdown. High dynamics and turnover rate of these filopodia may impede the formation of stable contacts with the axonal terminals. Moreover, a large percentage of filopodia after CP knockdown are devoid of the PSD clustering, which is a necessary component for forming functional synapses. Based on these data, we propose that CP is required for filopodia to proceed to the stage of dendritic spine maturation.

d. Maturation of spines: we propose that high level of CP is required for the expansion of spine head and further postsynaptic specialization. The expansion of spine head depends on the formation of highly branched actin architecture, which is mediated through the coordination of CP and Arp2/3. Without CP, filopodia could not convert to mature spines with head expansion (as shown in Figure 3-1 bottom left panel). Because our CP knockdown is only about 50%, the remaining CP may still be sufficient for about half the protrusions to make the conversion from filopodia elongation to spine maturation. However, there are tiny protrusions coming out from the head of those spines, possibly due to insufficient capping of actin filaments (as shown in Figure 3-1 bottom right panel).

This differential requirement of CP at different developmental stages is consistent with our observation of dynamic distribution of CP. CP gradually accumulates in dendritic protrusions along synaptic development (Figure 2-4). This correlation in timing supports our model. However, our imaging with an interval of several days could not capture the real-time change of

filopodia conversion. The temporal sequence of CP accumulation, filopodia stabilization and PSD clustering can only be resolved with live-cell imaging. Future live-imaging study is needed to corroborate our model that the protrusions with increased CP are indeed undergoing conversion to mature spines and will eventually form synapses.

3.2 Future directions

This dissertation explores the function of CP in dendritic spine development. Our results establish an essential role for CP in spine formation and synapse development. However, several key questions remain to be addressed. Specifically, how CP is regulated and how CP coordinates those cellular processes during spine development are not elucidated.

Central questions remain to be answered include: What regulates CP localization and expression during spine development? How does CP coordinate with other actin regulatory proteins, as well as scaffolding and adhesion molecules, in the filopodia-spine conversion and synaptic specialization? How does CP in mature spines regulate synaptic plasticity and maintenance? Finally, is CP involved in neurological disorders with spine defects? Future studies can be conducted to address the following questions.

1. What regulates CP expression and localization in dendritic spines? The regulation of CP expression at transcriptional level is poorly understood so far, and whether there is local translation of CP in dendrites is not known yet. Since the expression of CP increases dramatically in the first few postnatal weeks, one direction is to study if this increased expression is under the control of transcriptional factors, especially the factors that are active within the same time window. Future investigation of CP expression at both transcriptional and translational level

could advance our understanding of how actin cytoskeleton changes accordingly under upstream gene regulations.

Although CP is appreciated as a key regulator of actin filaments, how CP activity and localization is regulated is not fully understood yet. A few proteins that affect CP-actin binding have been identified, like CARMIL and V-1 (T. Kim, Cooper, & Sept, 2010), but these proteins are not well characterized in the nervous system. One candidate for the regulation of CP-actin binding in spines is the phospholipid molecule, PIP2, which could directly bind to CP and cause uncapping effect. PIP2 exists in spines and responds rapidly to synaptic activation (Brown et al., 2008; Horne & Dell'Acqua, 2007). Since CP and PIP2 bind directly, it is possible that CP localization in dendritic spines is under control of PIP2 level. For example, increased PIP2 in dendritic protrusions may increase the sequestration of CP, while decreased PIP2 may release CP to the cytosol. One way to examine this is to see if manipulation of PIP2 could affect the distribution of CP in spines, and also if the CP localization is associated with the PIP2 increase in spines. Another candidate that regulates CP localization is the CIN85 family, which are scaffolds known to bind CP (Hernandez-Valladares et al., 2010). One direction for future studies is to examine if the manipulation of CIN85 could affect the targeting of CP to dendritic spines. Notably, CIN85 knockout mice exhibit defects in dopaminergic transmission (Shimokawa et al., 2010), suggesting a role in synaptic function. Besides those CP regulators mentioned above, a proteomic screening of CP binding partners in neurons could provide a list of other possible regulators. This type of screening could implicate the study of CP regulation in other cell types and systems as well.

2. How does CP affect the filopodia conversion to dendritic spines and synaptic specialization? Though our data suggest that CP is required for filopodia stabilization and clustering of postsynaptic components, how CP regulates these processes is not known. We speculate that CP affects the stabilization by both directly inhibiting actin disassembly and

indirectly influencing the actin architecture. Though CP could inhibit cofilin-mediated actin disassembly based on in vitro experiment (Kueh, Charras, Mitchison, & Brieher, 2008), the effect of CP on actin disassembly in the cellular environment may be more complicated. How CP coordinates with other actin regulatory proteins, like Arp2/3 and cofilin, remains to be addressed in the future. CP could also affect filopodia stabilization by shaping the actin architecture to be a branched network. This organization of actin filaments may be important for the anchoring of certain adhesion molecules to strengthen the synaptic contact and stabilize the filopodia structure. An intriguing hypothesis is that CP regulates the localization of adhesion molecules at the filopodia tip and triggers signaling mechanisms that promote spine maturation. Several previous studies implicate an indirect interaction between CP and cadherin mediated by CIN85 (Johnson et al., 2008). How CP knockdown affects the localization and anchoring of cellular adhesion molecules is an interesting direction for future studies. For postsynaptic specialization, clustering of the PSD95 and other scaffolding proteins is necessary since they serve as the anchor for postsynaptic receptors. Whether there is potential interaction between CP and the scaffolds needs further investigation. High-resolution live imaging of CP, adhesion molecules and postsynaptic scaffolds could provide a temporal sequence of how these proteins target to the dendritic protrusions. Future analysis can be conducted regarding how the enrichment of these molecules correlates with morphological changes of filopodia. This type of analysis may address which is the triggering event of filopodia conversion to dendritic spines.

3. How does CP function in synaptic plasticity? CP is substantially high in mature spines (Figure 2-1, 2-2), implying a requirement of capping in mature spine activity. Future studies can be conducted to address how CP coordinates the morphological reorganization and functional alteration of spines during synaptic plasticity. This type of study would benefit from advanced imaging technique. By dissecting the subspine localization of CP during synaptic

modification, how CP fine tunes actin dynamics through differential distribution at subspine regions can be addressed.

4. Is CP involved in neurological disorders with spine defects, like Fragile X?

Filopodia instability and immature spine morphology are featured in Fragile X (Pan, Aldridge, Greenough, & Gan, 2010). CP knockdown neurons appear to partially resemble these phenotypes. Moreover, excess phosphoinositide 3-kinase (PI3K) is found in Fmr1-/- mice and considered to be a potential therapeutic target in Fragile X (Gross et al., 2010). The level of PIP2, one regulator of CP, is directly affected by PI3K activity. Whether CP expression or localization is altered in fragile X is worth further investigation.

With these questions answered, we can better understand how CP is involved in synaptic activity and how CP deficits influence the formation and function of neuronal network. Whether the alteration of CP affects brain activity and behaviors could be further examined in model organisms. These explorations may eventually provide novel therapeutic targets and contribute to the efforts of restoring the morphology and function of synapses in the diseased brain.

3.3 Figures and Legends

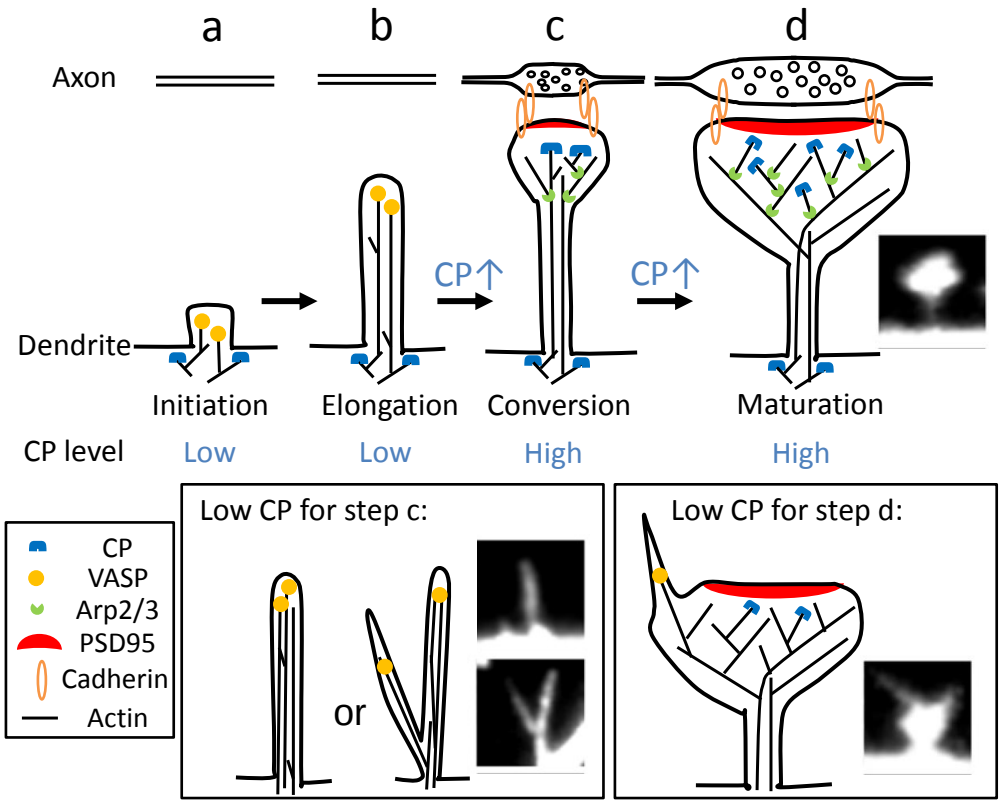

Figure 3-1. Hypothetical model of CP involvement in dendritic spine development. Low CP is required for the initiation (a) and elongation (b) of dendritic filopodia, and high CP is required for the conversion of filopodia to spines (c) and spine maturation (d). Bottom left: icon list; bottom center: defects of spine morphology when CP is low for filopodia conversion to spines (step c), with insets showing sample protrusions in CP knockdown neurons; bottom right: defects of spine morphology when CP is low for spine maturation (step d), with insets showing sample protrusions in CP knockdown neurons.

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