Gelsolin - A Regulator of Postsynaptic Actin Assembly and AMPA Receptor Expression

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Pingwei Zhao
aus China

Basel, 2003

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

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Basel, den 16.09.2003

Dekan

Prof. Dr. Marcel Tanner

Acknowledgements

I would like to thank everybody who helped me in any way during my time as graduate student at the Friedrich Miescher-Institut. Particularly I thank Prof. Andrew Matus for give me the opportunity to work in his group, for his constant scientific support, many enthusiastic discussions, and for proofreading this thesis.

I am very grateful for the practical and intellectual support I got from working colleagues, especially Virginie Biou, Manuel Ackermann, Martijn Roelandse, Andreas Birbach, Dong Liu, Arkadiusz Welman, Ina Bruenig, Heike Brinkhaus, and Urs Mueller.

I would like to thank Prof. Heinrich Reichert and Dr. Pico Caroni for their great work in my thesis committee, give me valuable suggestions and make my study facilitated.

And last, but not least I want to thank my parents and my wife who gave me their moral support during my time as a PhD student.

Contents

1. Summary

Dendritic spines are the postsynaptic contact sites for the majority of excitatory synapses in the brain. Synaptic activity influences the number, shape and motility of dendritic spines and these effects are likely mediated by dynamic actin filaments, which are highly concentrated in spine heads. Drugs that inhibit actin dynamics block spine motility and interfere with the development of long-term potentiation (LTP), a long-lasting increase in synaptic strength considered to be closely related to learning and memory. This suggests that actin may serve as a link between activity-induced modulation of synaptic transmission and long-term changes in synaptic morphology. Despite this evidence for the importance of actin dynamics in synaptic plasticity, very little is known about its regulation at the synapse. In particular the mechanisms linking synaptic activity to the actin cytoskeleton in dendritic spines are not well understood.

The experiments described in this thesis were focused on gelsolin as a promising candidate for mediating synaptic activity to actin cytoskeleton in dendritic spines. It is shown here that exposure of cultured hippocampal neurons to glutamate results in the accumulation of gelsolin in dendritic spines. This effect is the consequence of activation of NMDA receptors and influx of Ca^{2+}. It is also shown that the F-actin binding domain of gelsolin is necessary for its enrichment at postsynaptic sites. Further experiments showed that actin filaments are more vulnerable to disruption by glutamate stimulation in gelsolin over-expressing neurons. The disruption of actin filaments in these neurons is also dependent on NMDA receptor activation and Ca^{2+} influx. LTD-related electric field stimulation likewise increased the loss of filamentous actin in gelsolin expressing cells compared with untransfected cells. The disruption of actin filaments required the severing function of gelsolin, which is associated with the specific filament-severing domain (domain 1) of the gelsolin molecule.

Severing of F-actin by active gelsolin reduces the amount of AMPA receptors (GluR1) associated with dendritic spines.

These results indicate that gelsolin plays an important role in linking synaptic activity to the postsynaptic actin cytoskeleton. Our results are also consistent with evidence that activation of NMDA receptors and influx of calcium ions play a crucial role in regulating the actin cytoskeleton in dendritic spines and hence are involved in the regulation of postsynaptic glutamate receptor plasticity at excitatory synapses via a feedback mechanism. This could occur in both the developing and mature brain under both normal and pathologic conditions. Taken together, our data support a model in which activity-dependent targeting of proteins into dendritic spines is a major mechanism for regulating synaptic plasticity at excitatory synapses.

2. Introduction

2.1. The actin cytoskeleton in dendritic spines

During development neurons grow in size, move, send out branches, transport substances and organelles within those branches, and make synapses with other cells. All of these processes rely on the neuronal cytoskeleton to provide the required structural flexibility and movement. The neuronal cytoskeleton is crucially important for normal neuronal function, since the development and maintenance of neuronal morphology is determined by the organization of the cytoskeleton in developing and adult brain and its regulation by extracellular and intracellular signals (Matus et al., 1982a; Burgoyne, 1991). The neuronal cytoskeleton also has dynamic roles in the transport of materials in both directions along axons and dendrites, in cell division in developing neurons and in mediating morphologically and functionally plastic changes in the adult brain that may underlie the long term modifications required for learning and memory (Fifkova and Delay, 1982; Smart and Halpain, 2000). The neuronal cytoskeleton is formed by three types of filamentous proteins: microtubules, neurofilaments and microfilaments (actin filaments). In dendritic spines the actin filaments are the major form of neuronal cytoskeleton (Fifkova and Delay, 1982; Matus et al., 1982a; Fischer et al., 1998; Matus, 2000; Matus and Shepherd, 2000).

2.2. Pyramidal cell in hippocampus is a good model to study synaptic plasticity

Generally synaptic plasticity has been accepted as a major mechanism for memory formation. In studying the synaptic plasticity one cell type has been

studied extensively as a good model for a long time --- the pyramidal cell of the hippocampus. The reason for this is because of the special characterization of this cell type. This highly specialized cell type owes its name to its pyramid shaped cell body, and is most abundant in the cerebral cortex and the hippocampus. Their typical dendritic organization consists of apical and basal dendrites, which give these cells their "pyramidal" shape. Pyramidal cells are glutamatergic, excitatory neurons which have most of excitatory synapses on their dendritic spines whereas inhibitory inputs are mainly mediated through GABA-receptors on the dendritic shafts. The extensive dendritic tree facilitates the integration of different kinds of inputs. In the hippocampus there are different subtypes of pyramidal neurons in regions, such as CA1 or CA3. Each pyramidal neuron can receive up to ten thousand of excitatory and inhibitory synaptic inputs onto their dendrites.

2.3. Plasticity in dendritic spines

In the adult brain dendritic spines are microspecializations of the postsynaptic membrane present on many types of neurons. These specialized structures function as integrative units in synaptic circuitry. Spines behave as individual postsynaptic compartments by preventing diffusion of protein complexes and small molecules between neighboring synapses (Svoboda et al., 1996). The emergence of dendritic spines occurs during postnatal development (Harris et al., 1992; Harris and Kater, 1994; Dailey and Smith, 1996), but the precise sequence of events leading to spine formation remains obscure.

Many reports have documented changes in the numbers and shapes of dendritic spines in both vertebrate and invertebrate organisms (Frotscher et al., 1975; Coss and Globus, 1978; Horner, 1993; Harris and Kater, 1994; Engert and Bonhoeffer, 1999; Toni et al., 1999; Fischer et al., 2000a; Lendvai et al., 2000; Korkotian and Segal, 2001). Changes in spine shape and density occur under both physiological and pathological conditions, and it seems likely that such changes alter the function of neural circuits in still undefined ways (Matus, 2000; Matus and Shepherd, 2000; Smart and Halpain, 2000).

Live cell imaging has shown that the morphology of dendritic spines is highly dynamic (Fischer et al., 1998; Dunaevsky et al., 1999). With the use of actin coupled to green fluorescent protein (GFP-actin), dynamic activity of the actin cytoskeleton in spines has been directly visualized, confirming its capacity for driving changes in synaptic morphology (Fischer et al., 1998). This motility is blocked by cytochalasin D and latrunculin, drugs that inhibit actin dynamics. It is also blocked by volatile anesthetics, suggesting a possible relationship to global brain function (Kaech et al., 1999), and by stimulation of AMPA glutamate receptors (Fischer et al., 2000a), suggesting a possible mechanism coupling synaptic transmission to the regulation of dendritic spine motility.

Brief titanic stimulation produces a long lasting form of synaptic plasticity, long-term potentiation (LTP) that can last for hours or days in the mammalian hippocampus. The involvement of the hippocampal formation in memory has been established for a long time (Scoville and Milner, 2000). Although the relation of LTP to learning is not universally accepted, LTP is a widely used paradigm for long-term synaptic plasticity in central synapses. Over the past two decades many studies have demonstrated changes in the morphology of spines after LTP, such as enlargement of the spine head and shortening of the spine neck. In addition, studies using time-lapse imaging have reported increased spinogenesis (Engert and Bonhoeffer, 1999; Toni et al., 1999). A link between this phenomenon and actin based spine motility is suggested by studies showing that drugs that inhibit actin dynamics suppress LTP (Kim and Lisman, 1999; Krucker et al., 2000).

Dendritic spines have different shapes, and their density and morphology are influenced by age, hormones, neurotrophins, learning and synaptic activity. Jacobs *et al* (1997) reported a decrease in the order of 50% in spine number on basal dendrites of layer III pyramidal cells in human cortex when comparing older (>50years) and younger age groups (≤50 years) (Jacobs et al., 1997). Similar findings have been reported in aged monkeys. In quantitative EM studies two groups reported a reduction of synaptic density with aging in

monkeys (Uemura, 1980; Peters et al., 1998). Counts of synapses in layer 1 in these monkeys revealed that compared to young monkeys, there is a 30-60% reduction in the density of synapses per unit volume in old monkeys. Such a decrease of dendritic spines of neocortical pyramidal cells in aged individual may represent loss of synapses leading to disruption of neuronal circuits during normal aging.

Morphological changes in dendritic spines have also been associated with learning and memory *in vivo*. In honeybees spines are modified during learning. Spine stem becomes shorter following the honeybees' first orientation flight (Brandon and Coss, 1982). As with the honeybee, spine changes are also associated with learning and memory in vertebrate species. After operant conditioning period the averages of spine densities of CA3 pyramidal neurons of the hippocampus showed an overall increase in the learning group over the control groups of Wistar rat pups (Mahajan and Desiraju, 1988). In the chicks, after one-trial passive avoidance training there was an increase in the density of synapses and dendritic spines in the forebrain (Stewart and Rusakov, 1995). A transient increase in spine density has also been reported to occur in rat dentate gyrus granule cells 3-6 hours after training in a passive avoidance paradigm (O'Malley et al., 1998).

In addition to physiological conditions that alter spine shape and number, some neurological and neuropsychiatric diseases are associated with changes in dendritic spines as well. In many types of mental retardation disease the density and morphology of spines is abnormal. Dendritic spines have been described as long and tortuous in fragile X syndrome (Marin-Padilla, 1972; Rudelli et al., 1985; Hinton et al., 1991; Comery et al., 1997). Spine shape and density have also been reported to be altered in Huntington's disease (Graveland et al., 1985). These observations suggest a close link between dendritic spines and normal brain function. Of course, it still remains to be investigated whether such spine changes are causal in impaired brain function, or secondary to it.

Excitotoxic neuronal damage is thought to be the main cause of dendritic spine loss in epilepsy, ischemia, and trauma to the brain (Pokorny and Trojan, 1983; Choi and Rothman, 1990; Park et al., 1996; Brown et al., 1998). Spine loss has been documented in both human epilepsy and in animal models, where there is usually a partial or complete loss of spines on surviving neurons within the hippocampus after a seizure (Scheibel et al., 1974; Paul and Scheibel, 1986; Geinisman et al., 1990; Isokawa and Levesque, 1991).

2.4. Regulation of actin assembly in dendritic spines

The mechanisms accounting for the differential stability of dendritic spines are still not clear yet. At present, filamentous actin appears to be the most important cytoskeletal component of spines to support their growth and maintain their structure. It has been proposed that there is a stable pool of actin filaments in the spine's core and a more dynamic pool of actin filaments in the surrounding periphery (Smart and Halpain, 2000). The dynamic filaments may enable the spine to change shape rapidly in response to stimuli. Stable actin filaments are possibly more long-lived and resistant to depolymerization by the presence of capping proteins.

Dynamic actin filaments can be regulated by some actin binding proteins that interact directly with actin. These proteins have specific and sometimes multiple effects on actin dynamics. Generally actin-binding proteins possess functions such as filament capping, severing, crosslinking, actin monomer sequestering and influencing the actomyosin contractile machinery. Many these proteins have multiple activities *in vitro*, and it is often unclear what their precise roles are *in vivo* (Smart and Halpain, 2000).

The major function of proteins of the ADF/cofilin family, including ADF, cofilin, actophorin, depactin and destrin, is to mediate actin filament disassembly (Maciver, 1998). The disassembly of actin filament by ADF/cofilin occurs in two ways: by severing, thereby creating more filaments ends that disassembly; and

by increasing the rate of actin monomer loss from filament ends (Theriot, 1997).

Capping protein (CapZ) was originally considered as capping barbed ends of actin filaments, where actin monomers can be added thus prolong the filaments, to stop filament growth. Whereas the discovery that capping protein is necessary for actin polymerization and motility of *Listeria* (Loisel et al., 1999) supports a hypothesis for localized actin assembly, proposed by Carlier and Pantaloni (Carlier and Pantaloni, 1997). In this model, actin polymerization is confined or 'funneled' to the free barbed ends of filaments since nearly all barbed ends are capped by capping protein.

Arp2/3 complex binds pointed ends and nucleates the formation of actin filaments with free barbed ends (Mullins et al., 1998). The presence of Arp2/3 was necessary for *Listeria* motility (May et al., 1999). The entire Arp2/3 complex has been observed in electron microscopic images of branching actin filaments (Volkmann et al., 2001). Assembly of monomers at the barbed ends of these branching filaments generates the force needed to push the ruffling membrane forward.

Profilin is a high-affinity actin monomer-binding protein, and best known for its ability to promote the exchange of nucleotide in actin monomers (Goldschmidt-Clermont et al., 1991). Profilin can promote polymerization by transporting actin monomers to the barbed ends of filaments.

Ena/VASP proteins seem to function by binding to the barbed ends of filaments and competing with capping protein, allowing for longer filament extension (Bear et al., 2002), whereas capping protein binds tightly to the barbed ends of actin filaments, causing branches to be short. Ena/VASP proteins could function to inhibit capping and allow longer filaments to form.

Gelsolin is a calcium dependent actin severing and capping protein. The detailed function and regulation of gelsolin are discussed in **chapter 2.5**

(Regulation of actin assembly by gelsolin) and **chapter 2.6 (Gelsolin function *in vivo*).**

The actin cytoskeleton of dendritic spines is influenced by a number of regulatory pathways activated by transmembrane signals. The Rho family of GTPases, of which there are three main family members (Rac, Rho, and Cdc42), has been implicated in the regulation of cytoskeletal dynamics and spine morphology and density (Luo et al., 1996; Ridley, 1996). Rho, Rac and Cdc42 all activate LIM-kinases, thereby inducing the phosphorylation and inhibition of cofilin (Arber et al., 1998; Lawler et al., 1999). Rac and Cdc42 have also been shown to activate WAVE and N-WASP, respectively, thereby inducing actin polymerization via the Arp2/3 complex (Miki et al., 1998). Rac induces polyphosphoinositide 4,5-bisphosphate {PI(4,5)P2} synthesis (Hartwig et al., 1995), and influences profilin and gelsolin function. PI(4,5)P2 synthesis leads directly to the removal of gelsolin and capping protein from actin filaments, thus increasing the probability of actin polymerization on these filaments (Schafer et al., 1996; Lin et al., 1997).

In neurons actin cytoskeleton is regulated by activation of ion-channel-linked glutamate receptors which are closely associated with it. The actin bundling protein, α-actinin-2, has the ability to bind simultaneously to actin and the NR1 subunit of NMDA-receptors (Wyszynski et al., 1997). The latter interaction is competed by calcium/calmodulin. There is evidence that Ca^{2+}-influx through the NMDA-receptor associated channel leads to depolymerization of postsynaptic actin (Shorte, 1997b; Halpain et al., 1998b) and produces a negative feedback effect on the receptor itself, causing a gradual slowdown of its associated Ca^{2+}-activity (Rosenmund and Westbrook, 1993b) which is largely a result of breaking the α-actinin-2 link between actin filaments and the NMDA-receptor. Furthermore, NMDA-receptors were shown to be influenced by two other Ca^{2+}-dependent actin binding proteins, namely by the actin-bundling protein spectrin (Wechsler and Teichberg, 1998) and the actin severing protein gelsolin (Furukawa et al., 1997b).

AMPA-receptors are attached to the actin cytoskeleton via the PDZ domain containing actin interacting proteins such as SAP97 (Sans et al., 2001) and neurabin I (Satoh et al., 1998). AMPA-receptors, like NMDA receptors, are displaced from synaptic sites when actin filaments are depolymerized (Allison et al., 1998b). However a functional link between AMPA-receptor activation and the regulation of the actin cytoskeleton has yet to be determined.

2.5. Regulation of actin assembly by gelsolin

Gelsolin is best known for its involvement in dynamic changes in the actin cytoskeleton during a variety of forms of cell motility. Gelsolin is the most potent actin filament severing protein identified to date (Figure 1.). Severing is the weakening of enough non-covalent bonds between actin molecules within a filament to break the filament in two. These interactions are regulated by Ca^{2+} ion (at micromolar levels), which activate gelsolin's binding to actin. Severing is initiated after gelsolin binds to the side of an actin filament which occurs rapidly and with high affinity (Kd 50nM). However, severing slowly (Kinosian et al., 1998) and the delay may reflect the time required for structural rearrangement within gelsolin and in the filament (McGough et al., 1998) prior to severing. This involves gelsolin changing the conformation of actin filament thereby inducing kinks the actin filament (McGough et al., 1998), suggesting a mechanical basis for severing.

After severing, gelsolin remains attached to the barbed end of the filament as a cap. As a result, short actin filaments that cannot re-anneal with each other or elongate at their barbed ends that are generated. In this way, the actin filaments are cut short and prevent from prolongation. The importance of Ca^{2+}-mediated actin severing has been clearly documented during platelet activation (Hartwig, 1992).

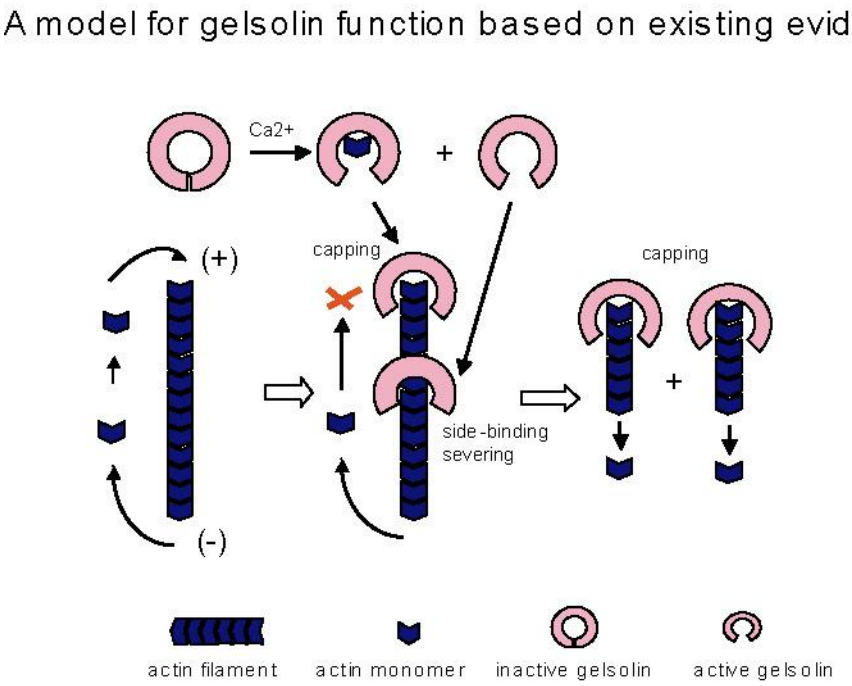

Figure 1. A model for gelsolin regulation of actin assembly. Actin monomers can be released from pointed ends (-), and then recruited to the barbed ends (+) of actin filaments. This is so called "tread-milling". Inactive gelsolin is in a tightly closed conformation where its actin binding domains are not accessible. When calcium levels reach micromolar concentration, gelsolin is activated by the opening of its functional domains. If the active gelsolin does not bind actin monomer, then this form of active gelsolin can bind to the side of an actin filament. Subsequently gelsolin severs the actin filament, it remains bound to the barbed end, functioning as a 'cap'. If the active gelsolin is already bound to an actin monomer, then this gelsolin/actin complex can only cap the barbed end of an actin filament. Capping actin filaments by gelsolin thus results in a net loss of actin monomers from actin filaments by inhibiting the adding of actin monomers to filaments' barbed ends.

The shortening of actin filaments by gelsolin occurs in the presence of micromolar calcium ion concentrations and is partially reversed following removal of calcium ions. One actin monomer remains bound to gelsolin following removal of calcium. The gelsolin-actin complexes (1:1) differ from

free gelsolin in that they have a higher affinity for actin filament barbed ends and they cannot sever filaments in the presence of calcium (Bryan and Coluccio, 1985; Janmey et al., 1985). That is why gelsolin does not dissociate readily from barbed ends when calcium is removed. Microinjection of these gelsolin-actin complexes had not effect on the cells (Cooper et al., 1987), possibly because there is a consituent of cytoplasma that can dissociate gelsolin/actin complexes (Chaponnier et al., 1987).

Gelsolin severing can also have a constructive effect because it increases the number of filaments. Membrane associated polyphosphoinositide 4,5-bisphosphate {PI(4,5)P_2} releases gelsolin from actin filament ends, providing free sites for actin assembly. PI(4,5)P_2 removes gelsolin from the barbed ends of actin filaments possibly through direct competition for gelsolin's F-actin binding site or through induction of structural changes of gelsolin (Xian and Janmey, 2002). Lysophosphatidic acid (LPA) can reduce the threshold concentration at which PI(4,5)P_2 is active (Meerschaert et al., 1998). As a result, LPA significantly promoted the release of gelsolin from barbed actin filaments in permeabilized human platelets. Uncapping of gelsolin from these filaments generates many polymerization-competent ends from which actin can grow to rebuild the cytoskeleton to new specifications. Therefore, gelsolin can promote actin polymerization by severing followed by uncapping (Yin and Stull, 1999).

2.6. Gelsolin function *in vivo*

In living fibroblasts gelsolin has a diffuse cytoplasmic distribution, suggesting that it is not tightly bound to actin filaments, most likely because gelsolin is associated with actin filaments in short-lived complexes (Cooper et al., 1988). Gelsolin incorporates preferentially into the submembranous actin arcs at the leading edge of the lamellipodia when fibroblast motility is induced by epidermal growth factor (EGF) (Chou et al., 2002). During EGF-induced

motility, the leading edge's submembranous region constitutes a distinct subcellular locale. Gelsolin preferentially concentrated near the leading edge in a punctuate fashion incorporating with actin cytoskeleton (Chou et al., 2002), suggesting gelsolin associates with dynamic actin cytoskeleton to push the ruffling membrane during EGF-induced motility. During the course of this thesis work I reexamined the distribution of gelsolin in living fibroblasts using gelsolin-GFP and confirmed this result (see **Results: 3.1. Gelsolin is enriched in the leading edges of Swiss 3T3 cells**)

Gelsolin appears to play an important role in situations where actin assembly is induced. In most cells at rest, gelsolin is not bound to actin filaments, but stimulation with increased calcium or H^+ , which activate gelsolin, can cause gelsolin to sever and cap actin filaments (Kwiatkowski, 1999). Gelsolin does not only have a destructive role on actin filaments. If gelsolin-capped filaments can be uncapped to create free barbed ends, polymerization may be induced. Activation of Rac dissociated gelsolin from barbed ends (Hartwig et al., 1995; Arcaro, 1998), thus promoted actin assembly. This gelsolin role was further proven in gelsolin knockout cells. Fibroblasts from gelsolin knockout mice were defective for formation of lamellipodia in response to Rac signaling (Azuma et al., 1998), which depends on actin polymerization.

The gelsolin null mouse, produced by genetic inactivation of the gelsolin gene, has established the importance of gelsolin for multiple cell functions. Cells from gelsolin null mice exhibit a variety of motility and actin defects. Most strikingly gelsolin null fibroblasts have abnormally pronounced actin stress fibers (Witke et al., 1995), and this phenotype is consistent with lacking the ability to sever and remodel actin filaments. Probably as a consequence, gelsolin null fibroblasts do not ruffle in response to growth factor (Azuma et al., 1998), and they exhibit defective chemotaxis and wound healing. In addition to fibroblasts, neutrophil migration is also compromised (Witke et al., 1995). The rate of clotting is reduced (Witke et al., 1995), consistent with the absence of gelsolin and its requirement of actin severing for platelet activation (Hartwig, 1992). In gelsolin null developing neurons it showed delayed retraction of filopodia along

neurites (Lu et al., 1997). Histories of individual filopodia in gelsolin knockout mice revealed that elongation rates did not differ from controls but the retraction was slowed down (Lu et al., 1997). These findings establish the importance of gelsolin in maintaining motility and actin dynamics.

Membrane ruffling is a functional readout for a coordinated series of membrane and cytoskeletal events, and it is activated by the small GTPase, Rac. Gelsolin null fibroblasts have increased Rac expression (Azuma et al., 1998), and Rac·GTP dissociates gelsolin/actin complexes (equivalent to uncapping) in cell extracts but not purified gelsolin-actin complexes (Arcaro, 1998). These results suggest that gelsolin is a downstream effector of Rac, but there are additional steps between Rac and gelsolin activation/inactivation. A number of studies suggest that linkage through the type I phosphatidylinositol 5-kinases (PIP5KIs), the major enzymes that synthesize $PI(4,5)P_2$ (Fruman et al., 1998; Anderson et al., 1999), is an attractive possibility.

$PI(4,5)P_2$ has a pivotal role in the phosphoinositide cycle that drives signaling, cytoskeletal organization, and membrane trafficking (Toker, 1998). Numerous cytoskeletal proteins are affected by $PI(4,5)P_2$ *in vitro*. They include gelsolin family proteins (Janmey and Stossel, 1987), profilin (Lassing and Lindberg, 1985), capping protein (Lassing and Lindberg, 1985), ADF/cofilin (Yonezawa et al., 1990), α-actinin (Fukami et al., 1992), vinculin (Gilmore and Burridge, 1996), ezrin/radixin/moesin (Hirao et al., 1996), and WASP family proteins (Miki et al., 1996; Higgs and Pollard, 1999). The first four are inactivated by $PI(4,5)P_2$, whereas the latter four proteins are activated by $PI(4,5)P_2$.

Gelsolin over-expression increases membrane ruffling and chemotaxis (Cunningham et al., 1991; Sun et al., 1997), consistent with the role of gelsolin in dynamic actin remodeling. However, the concentration of total intracellular gelsolin does not appear to be a central determinant of cell migration, because non-migrating fibroblasts have higher levels of gelsolin compared with migrating cells (Arora and McCulloch, 1996). Actually compared with non-migrating cells, migrating cells contain more free gelsolin and exhibit gelsolin-

dependent F-actin severing activity, which required calcium. Surprisingly, CapG, a gelsolin relative that caps but does not sever actin, and the completely unrelated capping protein also increase cell motility when overexpressed (Hug et al., 1995; Sun et al., 1995). Originally pure capping proteins are expected to be less effective in promoting actin dynamics than severing/capping proteins, because they do not increase the number of actin filaments *per se*. These results indicate that capping/uncapping may be sufficient to increase actin dynamics. More detailed study will be required to distinguish between the contributions of severing and capping.

Overexpression studies reveal that gelsolin may have other roles in addition to direct cytoskeletal regulation. Overexpressed gelsolin (Sun et al., 1997) and CapG (Sun et al., 1995) modulate phospholipase Cγ and phospholipase Cβ activity in a biphasic manner both *in vivo* and *in vitro*. These effects depend on $PI(4,5)P_2$ binding (Sun et al., 1997), suggesting that gelsolin enhances or competes with other $PI(4,5)P_2$-binding proteins for their common substrate. This potent effect may be achieved by altering the packing of $PI(4,5)P_2$ molecules within the membrane bilayer (Flanagan et al., 1997).

These results suggest that as $PI(4,5)P_2$ content and availability change during signaling, cross talk between $PI(4,5)P_2$-regulated proteins provides a selective mechanism for positive as well as negative regulation of phosphoinositide signaling. This is particularly relevant as more $PI(4,5)P_2$-regulated proteins are identified. Gelsolin coimmunoprecipitates with several PIP_2-interacting proteins, and it alters the activity of phosphatidylinositol 3-kinase and phospholipase D as well (Liu and Yin, 1998; Kwiatkowski, 1999). Gelsolin is phosphorylated by c-Src *in vitro*, and phosphorylation is enhanced by $PI(4,5)P_2$ (De Corte et al., 1997). The physiological significance of these associations and phosphorylation has not been determined.

Beyond that, gelsolin is also an activator of DNase I (Davoodian et al., 1997). Gelsolin enhances permeabilized mast cell secretion (Borovikov et al., 1995), it has been correlated with cancer and cellular transformation in various

instances (Fujita et al., 1995; Tanaka et al., 1995; Asch et al., 1996), and it participate in caspase-3-mediated apoptosis (Kothakota et al., 1997). Also, gelsolin may function as an anti-amyloidogenic protein in the plasma and cerebrospinal fluid (CSF), where it prevents Abeta from fibrillization, and helps to maintain it in the soluble form (Chauhan et al., 1999; Ray et al., 2000).

2.7. Structure of gelsolin and gelsolin/F-actin

Gelsolin is an 80-kDa protein consisting of two tandem homologous halves, each of which contains a 3-fold segmental repeat (segments S1-S3 and S4-S6, respectively) (Kwiatkowski et al., 1986; Burtnick et al., 1997) (Figure 2). The N- and C-halves are connected by a long linker, which is cleaved by caspase-3 (Kothakota et al., 1997; Kamada et al., 1998) *in vivo* and *in vitro*. Fragments of gelsolin that contain from one to five of its domains can be generated by limited proteolytic digestion (Kwiatkowski et al., 1985) or by expression in bacterial cell lines (Way et al., 1989). Investigations of the activities of these products have led to the assignment of discrete functions to individual domains. The S1 domain binds actin monomers in the absence of calcium. The C-terminal half of gelsolin, S4-S6, contains a second, calcium-dependent, actin monomer-binding fragment that is located in S4. S2 contains a calcium-independent F-actin-binding site. The tail at the C-terminus of S6 latches the second half of the protein to the first until released by calcium (Burtnick et al., 1997).

The structural basis for gelsolin regulation by Ca^{2+} is now beginning to be understood. The isolated C-half binds a single actin molecule only when Ca^{2+} is above 10^{-6} M (Kwiatkowski, 1999). The isolated N-half binds two actin molecules to sever and cap, even in the absence of Ca^{2+}. Because severing by full-length gelsolin requires 10^{-6} M Ca^{2+}, the C-half must act as a regulatory domain to inhibit severing by the N-half. In addition, the C-half potentiates severing by the N-half, possibly through cooperative binding to the filament (Kinosian et al., 1998).

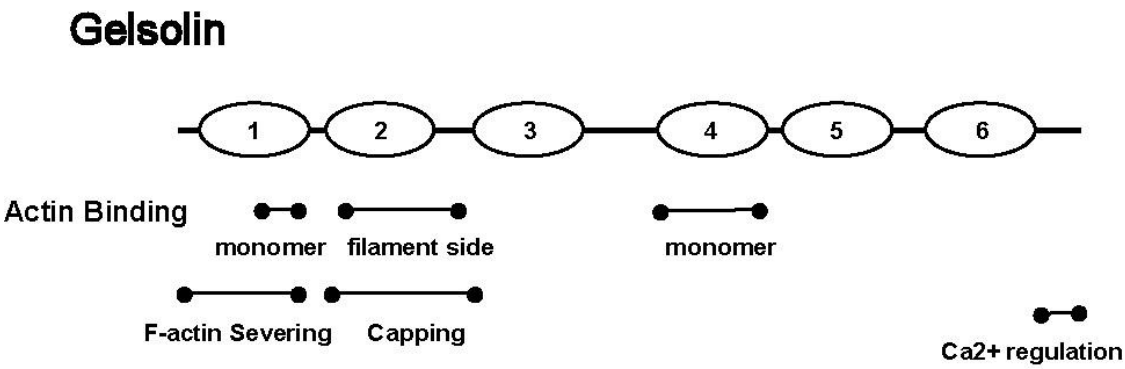

Figure 2. Gelsolin structure-function domains. Gelsolin consists of six tandem copies of a core domain. Domain 1 and 4 each have an actin monomer-binding site. An actin filament binding site resides from domain 2 to the beginning of domain 3. Domain 1 is needed for gelsolin's severing function. The capping function of gelsolin requires almost the same area as the actin filament-binding site. The key calcium regulation site for gelsolin is located at the C-terminal tail of gelsolin.

Gelsolin was first identified as a calcium-regulated protein that binds two calcium ions with a Kd of 10^{-6} M (Yin et al., 1980). Subsequent studies have shown that gelsolin interacts with calcium in a more complex way, and that isolated gelsolin domains have at least three distinct calcium binding sites, with submicromolar and micromolar Kd values (Pope et al., 1997). The conformation of gelsolin changes in response to calcium. Nanomolar concentrations of calcium initiate the unlatching of structural constraints that maintain the inaccessibility of the actin binding sites, but actin binding occurs only after additional micromolar calcium sites in both the N-terminal and C-terminal halves of the molecule are occupied. Once binding to actin, intermolecular and intramolecular calcium binding sites are created (McLaughlin et al., 1993; Weeds et al., 1995), so that gelsolin can potentially bind more calcium ions. By using synchrotron foot printing method S1 (49-72), S1 (121-135), S2 (162-166), and S6(722-748) of gelsolin showed a three-state

activation process with a calcium concentration of 1-5 µM that is competent for gelsolin's capping and severing activity (Kiselar et al., 2003).

Other studies have documented the high resolution structure of gelsolin. The S1-actin-Ca^{2+} structure shows how a single gelsolin segment binds actin (McLaughlin et al., 1993). Because of the similarities between all the segments of gelsolin (Kwiatkowski et al., 1986; Burtnick et al., 1997), S1 can be used as a template for modeling how the other segments bind to actin. The full-length gelsolin crystal formed in the absence of Ca^{2+} (gelsolin/EGTA) shows that inactive gelsolin has a compact structure in the absence of Ca^{2+} (Burtnick et al., 1997), with its two halves held together by the C-terminal S6 tail, which latches onto S2. Within each half, the first and third segments (S1 and S3, S4 and S6, respectively, for the N- and C-halves) are joined into a 10-strand β-sheet that is incompatible with actin binding. This explains why neither S1 nor S4 binds actin in the absence of Ca^{2+}. It also predicts that Ca^{2+} must induce major conformational changes in each half and in the relation between the halves to accommodate actin binding.

A cryoelectron microscopic study of a gelsolin construct missing S1 attached to an actin filament hints at the extent of the conformation change that is required for gelsolin activation (McGough et al., 1998). The reconstructed image shows that gelsolin S2-S6 binds to actin molecules in neighboring filament strands (via S2 and S4). The distances between the S2 and S4 and the S1 and S2 actin-binding sites on the filament indicate that there must be large scale conformational changes before S1, S2, and S4 can simultaneously bind actin. The convoluted linker probably unwinds, and parts of the S1 or S2 core domain may have to unravel to extend the linker between S1 and S2 (Burtnick et al., 1997; McGough et al., 1998). On the basis of crystallographic data, Robinson et al. (Robinson et al., 1999) concluded that calcium binding induces a conformational rearrangement in which segment 6 is flipped over. The structural reorganization moves apart the continuous β-sheet core of segments 4 and 6. This exposes the actin-binding site on segment 4, enabling severing and capping of actin filaments to proceed.

2.8. Gelsolin family members and expression

Three natural forms of gelsolin are known and produced by alternative transcription initiation and splicing of the mRNA (Kwiatkowski et al., 1986). Two of these are cytoplasmic, the basic form of cytoplasmic gelsolin is predominating while a low abundant form differs from it by an additional 11 amino acid residue with unknown function at the N-terminus (Vouyiouklis and Brophy, 1997). The third form, plasma gelsolin, is secreted into the blood supply where it caps and severs actin filaments released from dying cells to prevent the formation of long actin filaments that might otherwise block microcirculatory vessels (Haddad et al., 1990; Vasconcellos and Lind, 1993). Plasma gelsolin differs from the cytoplasmic forms by an N-terminal extension of 25 amino acids (Kwiatkowski et al., 1988a; Koepf et al., 1998).

Members of the gelsolin family that have three or six gelsolin repeats have been identified (Kwiatkowski, 1999). They have distinct as well as overlapping patterns of tissue expression, which may implicate for specialized function (Arai and Kwiatkowski, 1999). Except for gelsolin, calcium regulation and actin binding sites are not separated into the two halves of the molecule in other gelsolin family proteins. For example, villin, a six-domain protein, has a calcium-dependent N-half (Matsudaira et al., 1985). CapG, a three-domain protein, has a built-in calcium regulation site in its actin-binding segment (Yu et al., 1990).

Adseverin (scinderin), the closest homologue to gelsolin, was originally discovered in bovine adrenal gland. However, in human and murine adult tissues, adseverin is expressed abundant in kidney and gut, with lower levels in adrenal gland, and very low to no expression in other organs (Lueck et al., 1998). Interestingly, the expression pattern of capG, adseverin and gelsolin is highly complementary in the mouse (Lueck et al., 1998), suggesting that these proteins may have distinct functional characteristics. There is evidence that

adseverin is involved in exocytosis (Zhang et al., 1996); however this protein could take part into other processes, because it has activity and tissue distribution in other mammalian cell types (Tchakarov et al., 1990).

Villin shares the six domain structure with gelsolin and adseverin (Arpin et al., 1988). In addition to the severing and capping activities of the gelsolin-related the protein, the C-terminal domain confers actin filament bundling activity to villin. Despite there is a large amount of villin in microvilli of wild type cells, villin knockout produces very little changes in the structure of microvilli, suggesting other redundant proteins may exist (Pinson et al., 1998). On the other hand, the severing activity of villin may have a more essential role *in vivo*. Brush borders from intestinal epithelial cells of a villin knockout mouse did not disassemble their actin bundles in response to high calcium concentration (Ferrary et al., 1999). In addition to that, *in vivo*, in an experimental model for intestinal epithelial injury that includes loss of filamentous actin from the brush border, a villin-knockout mouse showed decreased loss of actin, increased severity of epithelia injury and higher probability of death (Ferrary et al., 1999).

Advillin is a gelsolin family member that is most closely related to villin (59% amino-acid identity) (Marks et al., 1998). However the precise function of this protein has not been known yet.

Supervillin (205 kDa) is another gelsolin family member that participates in interactions between actin filaments and membrane (Pestonjamasp et al., 1997). It contains a N-terminal half which has nuclear targeting signals, and a C-terminal half which has weak similarity to villin. It also contains relatively more conserved regions corresponding to F-actin binding regions.

2.9. Gelsolin function in neuronal cells

Gelsolin is expressed ubiquitously in mammalian tissues including the nervous system (Kwiatkowski et al., 1988b; Kwiatkowski et al., 1988a; Paunio et al.,

1997), Where it has been detected in oligodendrocytes, Schwann cells, and in myelin sheath (Tanaka and Sobue, 1994). In developing rat brain gelsolin level begins to increase at 8-10 day after birth and reaches a maximum 20-30 days after birth (Tanaka and Sobue, 1994). During neuronal development gelsolin is particularly concentrated in neuronal growth cones (Tanaka et al., 1993; Lu et al., 1997). PC12 cells showed a two-fold up regulation of gelsolin upon differentiation with nerve growth factor and over-expression of gelsolin enhances neurite length and increases neurite motility rate compared to controls (Furnish et al., 2001). On the other hand, neurons from gelsolin null mice show delayed retraction of filopodia along developing neurites. Histories of individual filopodia in gelsolin knockout mice revealed that elongation rates did not differ from controls but that retraction was slowed down (Lu et al., 1997). Gelsolin knockout neurons have enhanced cell death and rapid, sustained elevation of calcium levels following glucose/oxygen deprivation. Moreover, major increase in infarct size are seen in gelsolin-null mice after reversible middle cerebral artery occlusion, compared with controls (Endres et al., 1999). During this postnatal period the emergence of dendritic spines occurs and dendritic spines undergo a huge plasticity (Harris et al., 1992; Harris and Kater, 1994). In neurons from mice lacking gelsolin, activity-dependent stabilization of actin was impaired, which is depended on the activation of N-methyl-D-aspartate (NMDA) receptors and the influx of calcium (Star et al., 2002). The above evidence suggests that gelsolin may be a key regulator linking the synaptic activity to actin cytoskeleton in dendritic spines.

2.10. Live imaging by using green fluorescent protein (GFP)

Before the finding of GFP as a valuable tag to image protein locations in cells, researchers relied primarily on immunofluorescence microscopy techniques for visualizing the localization of molecules in cells. This classic immunofluorescence technique requires the fixation and hence the killing of the specimen before analysis. During that time the sole method for visualizing molecular dynamics in living cells was through the microinjection of purified

and fluorescently labeled proteins, but technical difficulties precluded its widespread use. Since its first application as an ectopically expressed reporter gene in 1994 (Chalfie et al., 1994) and its subsequent use as a fluorescent protein tag (Wang and Hazelrigg, 1994), the green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria* has enabled visualization of proteins dynamics with minimal perturbation of living cells. The tagged protein can be imaged in the living cells, offering the option of dynamic translocation studies using time lapse recording of video microscopy. Because DNA-sequence manipulations are integral to the production of cDNA for GFP fusion proteins, site-directed mutagenesis can be readily incorporated into such experiments (Ludin and Matus, 1998).

As for neuronal studies, GFP tagged proteins have been widely used for investigating synaptic plasticity during neuronal development. GFP-actin showed large actin-dependent changes in dendritic spines shape of mature neurons (Fischer et al., 1998). AMPA receptor subunit GluR1 tagged with GFP to monitor changes in AMPA receptor distribution in living neurons was used to shown that activation of NMDA receptor induced rapid delivery of tagged receptors into dendritic spines as well as clusters in dendrites (Shi et al., 1999). Okabe *et al* showed that PSD-95 tagged with GFP (PSD95-GFP) expressed in hippocampal neurons is an effective marker of the PSD structure (Okabe et al., 2001a), and PSD-95-GFP was also used to follow the events of synapse formation in developing neurons (Marrs et al., 2001). Altogether, dynamic imaging of living neurons using GFP tagged genes has revolutionized the study of anatomical plasticity at synapses, thus makes it possible to observe directly the events underlying activity-dependent changes in dendritic spines that previously could only be inferred from examining fixed tissue.

2.11. Aim of dissertation

It has been known for a long time that actin is strongly enriched in dendritic spines (Matus et al., 1982b), and GFP tagged actin now enables us to directly

record the motility of the actin cytoskeletal structure in living cells. Fischer *et al* (Fischer et al., 1998) tagged γ-cytoplasmic actin with GFP, to create GFP-γ-cyto-actin. If the cytoplasmic actins are tagged at the N-terminal site they were shown to retain their function (Brault et al., 1999). Expression of GFP-actin in dispersed cultured hippocampal neurons showed strong actin labeling of dendritic spines and live time-lapse recordings revealed rapid changes in spine shapes that occur within the time frame of seconds (Fischer et al., 1998; Dunaevsky et al., 1999). These rapid actin-driven changes in spine shape, termed spine motility, have also been documented in slice cultures (Fischer et al., 1998), acute slices (Dunaevsky et al., 1999) and in the living mouse (Lendvai et al., 2000). It has been shown that actin-driven spine motility is sensitive to blockers of actin dynamics, such as cytochalasin D (Fischer et al., 1998; Dunaevsky et al., 1999). If these events have a meaningful role in synaptic plasticity, they should be regulated by synaptic transmission and in a previous publication it was shown that activation of glutamate receptors regulates synaptic plasticity by suppressing actin dynamics in dendritic spines (Fischer et al., 2000a). Actin motility was suppressed when AMPA-type glutamate receptors were stimulated, implying that spine morphology is stabilized by signal transmission at glutamatergic synapses. It was further shown that this effect depends on depolarization of the neuronal membrane via Na^+-influx through the AMPA-receptor associated channel and a subsequent Ca^{2+}-influx through voltage-dependent Ca^{2+}-channels (VDCCs) (Fischer et al., 2000b).

The main goal of my thesis was to investigate how the activity-dependent influx of calcium into the dendritic spine cytoplasm leads to changes in actin dynamics. For this purpose I selected gelsolin, which is the most prominent calcium-dependent actin regulatory protein and whose absence in gelsolin knockout mice has been shown to lead to functional defect in the neuronal actin cytoskeleton (see above). Based on this prior knowledge I asked whether gelsolin in present in dendritic spines and how its presence there influences synaptic structure and function. GFP tagged gelsolin or gelsolin mutant genes were created to clarify the function of gelsolin in dendritic spines. First the

activity-dependent accumulation of gelsolin in dendritic spines was found using both immunostaining and living cell recording method. Then I investigate the function domains of gelsolin that are responsible for targeting to dendritic spines. Actin cytoskeleton in dendritic spines was disrupted by gelsolin under certain synaptic activities and hence influences the AMPA receptor expression at synaptic sites, which accounts for a certain form of synaptic transmission plasticity.

3. Results

3.1. Gelsolin is enriched in the leading edges of Swiss 3T3 cells.

The distribution of gelsolin in Swiss 3T3 fibroblasts was examined to check the localization of gelsolin compared to actin filament staining. 3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% Fetal Calf Serum (FCS) to promote growth of the typical motile leading edge structures and stress fibers. We used gelsolin monoclonal antibody to stain cells and co-stained with rhodamine phalloidin to show the actin filaments. There was a strong gelsolin immuno-flurescence intensity in the leading edges of 3T3 cells colocalized with F-actin staining (Figure 3). Whereas gelsolin did not show apparent colocalization with stress fibers.

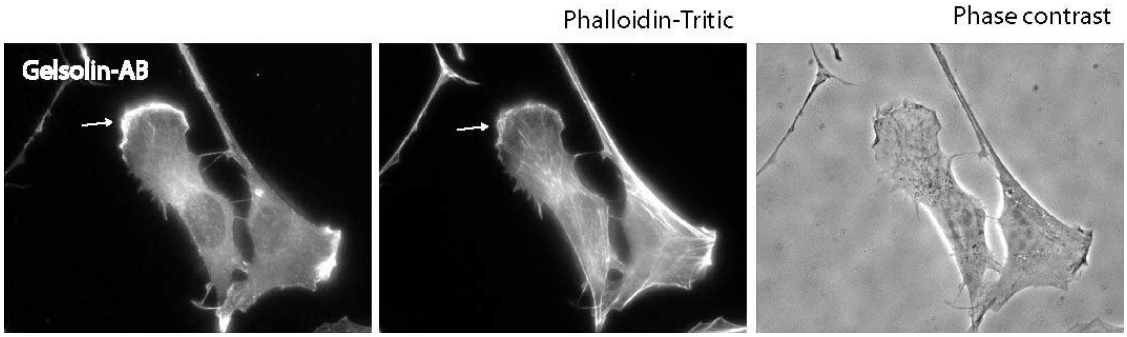

Figure 3. Distribution of gelsolin in Swiss 3T3 cells.
3T3 cells were fixed, permeabilized and stained for gelsolin and actin filaments. Gelsolin antibody showed a strong colocalization with actin filaments in lamellipodia of the leading edges (arrow). But no apparent enrichment of gelsolin in stress fibers was detected. Phase contrast image showed the ruffling membrane located in the leading edge.

From the above, the endogenous gelsolin in 3T3 cells showed that it is localized in the lamellipodia of the leading edges. But it did not show colocalization with stress fibers. This result is consistent with previous findings by other groups (Carron et al., 1986; Cooper et al., 1988), implicating a role of gelsolin in regulating the lamellipodium motility.

3.2. Gelsolin is co-localized with F-actin in dendritic spines of hippocampal neurons

At the beginning of this study it was not know whether gelsolin is localized in synapse, so we began by addressing this issue. The distribution of endogenous gelsolin was studied in hippocampal neurons (DIV21-24) by immunohistochemical staining. Primary cultures develop excitatory synapses on dendritic spines similar to those seen *in vivo* (Bartlett and Banker, 1984; Papa et al., 1995). Our result (Figure 4) showed gelsolin immunoreactivity (green) present in spiny neurons, concentrated in spine-like structures along the dendrites (Figure 4 A, B, C). These gelsolin clusters along the dendrites were co-localized with actin filament (F-actin) clusters (red) stained by phalloidin-rhodamine (Figure 4 A, B, D). Since it is well known that F-actin is highly enriched in spines compared with adjacent dendritic shafts or presynaptic terminals (Matus et al., 1982a; Cohen et al., 1985; Kaech et al., 1997; Wyszynski et al., 1997). The colocalization of gelsolin immunostaining with filamentous actin suggests that gelsolin is, like actin, concentrated within the spine cytoplasm.

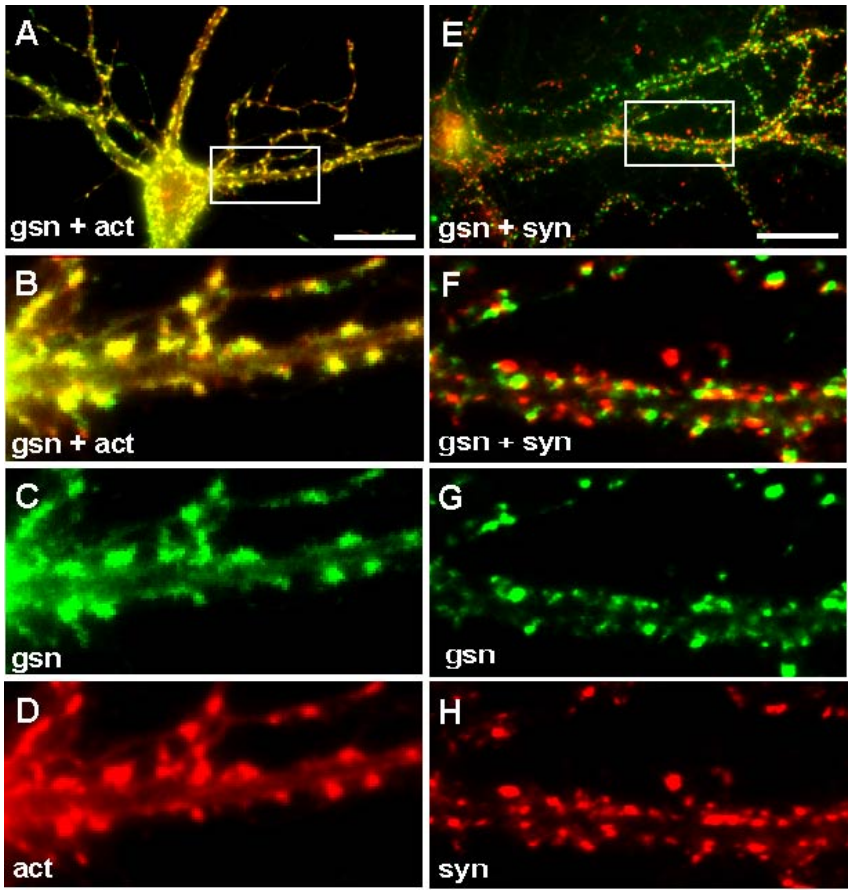

Figure 4: Gelsolin is localized in dendritic spines.
(A-D) Double stained for gelsolin (green) and actin filaments (red) in 3-week-old hippocampal neuron. In (B-D), a close-up view of the boxed region in (A) is depicted. Both gelsolin and F-actin are enriched in dendritic spines. (E-H) Double stained for gelsolin (green) and synapsin I (red) in 3-week-old hippocampal neuron. In (F-H), Higher magnification micrographs of the boxed regions are shown. Almost all the gelsolin clusters were contacted with synapsin I cluster which represent the presynaptic boutons. Abbreviations: gsn, gelsolin; act, actin filaments; syn, synapsin I. Scale bars, 10μm.

Next, neurons were stained with gelsolin and presynaptic vesicle protein synapsin I to show the presynaptic terminals. Gelsolin clusters were located opposite to, rather than precisely overlapping with, the presynaptic boutons revealed by synapsin I clusters (Figure 4 F). Taken together, the above results indicate that gelsolin is specifically concentrated postsynaptically in dendritic spines of hippocampal neurons in culture.

3.3. Glutamate induces accumulation of endogenous gelsolin in dendritic spines.

Glutamate activates postsynaptic glutamate receptors and subsequently triggers biochemical and electrical signal transduction events at excitatory postsynaptic sites. To test whether gelsolin is involved in glutamate induced postsynaptic changes, we studied putative changes in the gelsolin distribution along dendrites and in dendritic spines. Under control conditions neurons were incubated in Tyrode's solution for 5 minutes, then fixed and stained for gelsolin and F-actin (Figure 5 A). Gelsolin was located in dendritic spines, colocalized with F-actin clusters (Figure 5 A). After 5 minutes 2.5μM glutamate application, gelsolin immunostaining intensity in dendritic spines was much stronger than in control cells. Quantification data was shown in figure 5B.

Figure 5B shows fluorescence signal quantification of gelsolin levels. Based on analysis of 420 spines on 21 dendrites for each group, the ratio of gelsolin antibody signal intensity (spine/dendrite) increased 61±7.5 % upon 2.5 μM glutamate application for 5 minutes (Fig. 5 B). At the same time the ratio of F-actin signal intensity (spine/dendrite) decreased 38±4.5 %. Prolonged glutamate application time (30 minutes) gave a similar result to that of 5-minute application, so that after 30 minute exposure to glutamate gelsolin immunoreactivity increased by 59±7.1 %, while at the same time F-actin staining signal decreased by 40±5.2 %.

The above result indicates that glutamate stimulation of hippocampal neurons induces accumulation of endogenous gelsolin in dendritic spines and is associated with disruption of actin filaments in the spine cytoplasm. This is consistent with the known role of gelsolin in severing actin filaments in response to calcium influx into platelets (Hartwig, 1992).

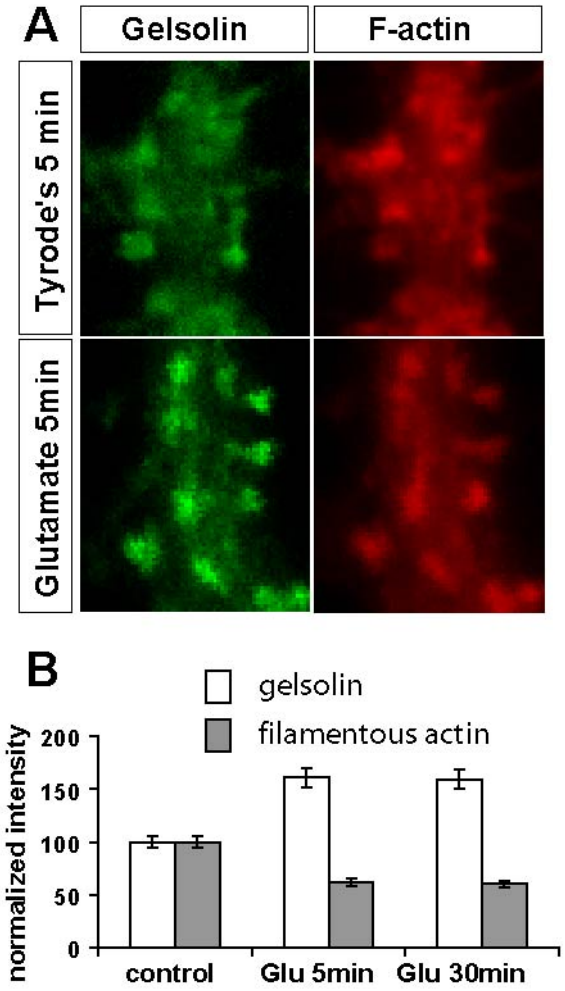

Figure 5: Glutamate induces accumulation of endogenous gelsolin in dendritic spines.

(A) Control neurons: 3-week old hippocampal neurons were incubated in tyrode's for 5 minutes then fixed and stained for gelsolin and actin filaments. Glutamate treated neurons: Cells were incubated in 2.5µM glutamate for 5 minutes then fixed and stained for gelsolin and actin filaments. (B) Glutamate induced accumulation of gelsolin in dendritic spines. Background fluorescence was subtracted using Metamorph software. A circle to encompass the spine was made by thresholding the image, then the circle was moved on its neighboring dendritic shaft. The ratio (Fs/Fd) of total fluorescent intensity in spine (Fs) and in the adjacent dendrite (Fd) was calculated. The mean values of control cells were normalized to 100%. There was a 61±7.5% increase of the ratio (Fs/Fd) for gelsolin in 2.5µM glutamate condition (± s.e.m.), while prolonged incubation time (30 minutes) did not make a difference from 5 minutes incubation. Control cells (n=21); Treated cells (glutamate for 5 minutes, n=21;

glutamate for 30 minutes n=21). 20 dendritic spines and adjacent dendrites were randomly selected for each group to be quantified for fluorescent intensity.

3.4. Gelsolin-GFP demonstrates glutamate induced accumulation of gelsolin in dendritic spines in living neurons

Immunostaining showed that endogenous gelsolin became accumulated in dendritic spines after glutamate application, but immunohistochemical method can simply answer the question "yes" or "no". To learn more about gelsolin accumulation in dendritic spines, for example the timing of gelsolin accumulation in dendritic spines or the distribution of gelsolin in the same cell before and after glutamate application, we used live cell imaging with GFP-tagged gelsolin.

In these experiments we expressed GFP tagged gelsolin in hippocampal neurons, and imaged dendrites at DIV 21 - 24 when dendritic spines with mature appearance had been formed. Under resting conditions gelsolin-GFP expressing neurons showed fluorescence signals localized in both dendritic spines as well as dendritic shafts (Figure 6). By contrast GFP-actin expressing cells showed strong accumulation of GFP-actin in dendritic spines with much lower intensity in the neighboring dendritic shafts, consistent with the previous reports (Kaech et al., 1997; Fischer et al., 1998). Contrary to GFP-actin's strong localization in dendritic spines, Cells expressing GFP alone showed strong signals in dendritic shafts with much lower signals in dendritic spines. Comparing the distribution of these three constructs in hippocampal neurons, gelsolin-GFP showed a tendency towards being concentrated in dendritic spines, but was not so enriched in spines as GFP-actin. Whereas GFP showed no tendency towards accumulating in spines.

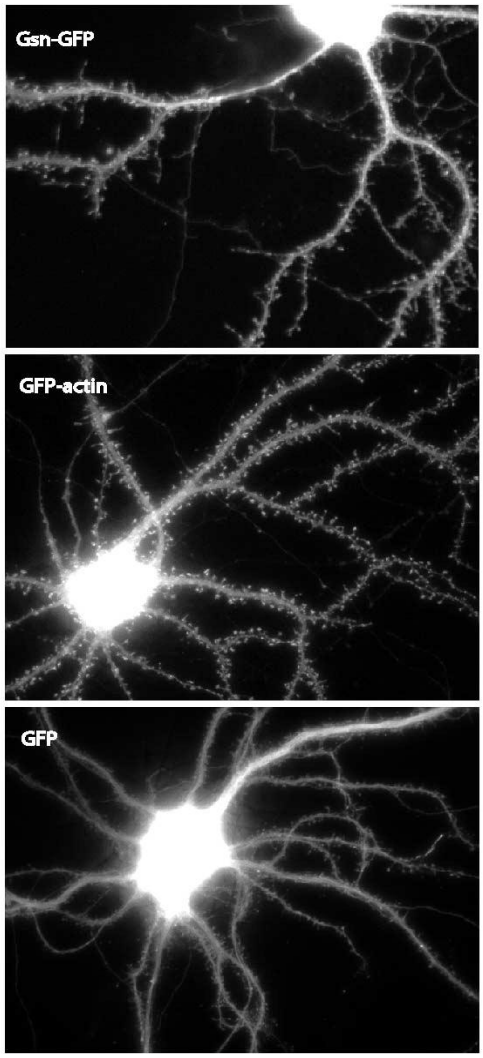

Figure 6: Distribution of gelsolin-GFP in living neurons.

Hippocampal neurons were transfected with the constructs using DOTAP method, and maintained in culture for 3 ~ 4 weeks. Gelsolin-GFP expressing neurons show fluorescence signals in both dendritic spines and dendrite shafts. GFP-actin show strong accumulations of actin-GFP in dendritic spines with much lower signal intensity in the neighboring dendrite shaft. Cells expressing soluble GFP show a strong signal in dendritic shafts with much lower intensity in dendritic spines.

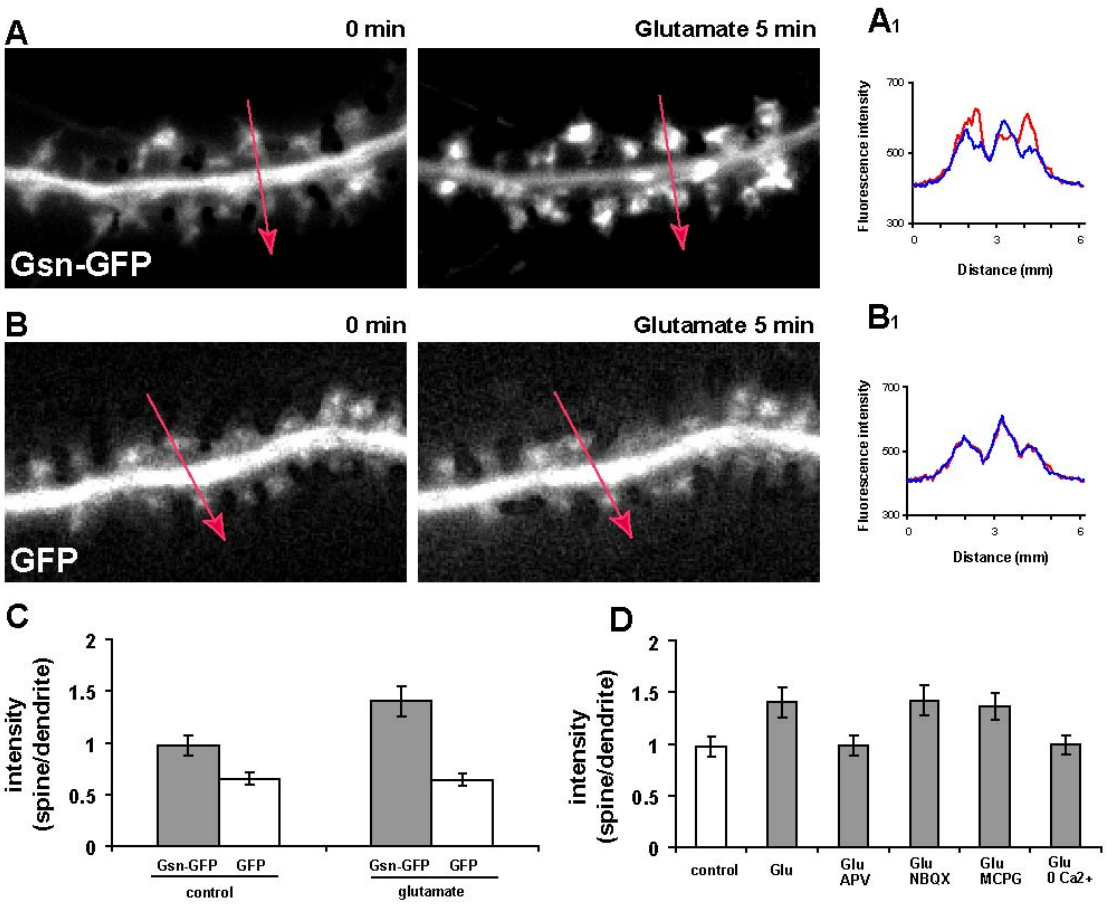

Figure 7: Activity-dependent enrichment of gelsolin-GFP in dendritic spines.

(A) Gelsolin-GFP expressing neurons treated with 2.5 µM glutamate for 30 minutes showed increased fluorescence in dendritic spines. (A1) Fluorescent intensity was plotted along the line indicated by the red arrow. The blue and red lines in A1 show the signal intensities before and after treatment respectively. After treatment the fluorescence in the peaks corresponding the dendritic spines were increased compared with the signals before treatment. (B): Control GFP transfected neurons showed no increase of GFP fluorescence in dendritic spines following treatment. (B1) Intensity plots of fluorescence intensity along the lines indicated by the red arrow in B. (C): Gelsolin-GFP accumulation in dendritic spines after 2.5 µM glutamate treatment measured as the ratio of fluorescence in the spine (Fs) compared to the dendrite shaft (Fd) (Before treatment: Fs/Fd=0.97±0.1; after treatment: Fs/Fd=1.4±0.13). Control GFP expressing cells showed no accumulation of GFP in dendritic spines after glutamate treatment (before treatment: Fs/Fd=0.65±0.06; after treatment: Fs/Fd=0.64±0.06) (± s.e.m.). (D): Both glutamate and NMDA trigger gelsolin-GFP enrichment in spines. APV and calcium free tyrode's blocked this process while the

MCPG and NBQX did not block this process. DHPG and AMPA did not trigger this process.

In the time-lapse recording method images of transfected neurons were taken at 15 second intervals to record changes of the gelsolin-GFP signal in dendritic spines and the neighboring dendritic shafts. The results of these experiments showed that application of 2.5 μM glutamate to gelsolin-GFP expressing neurons induced gelsolin-GFP accumulation in dendritic spines (Fig 7A, 7C), confirming our previous gelsolin accumulation data using fixation and antibody staining method. The increase of gelsolin-GFP in dendritic spines began after 5 minutes of 2.5 μM glutamate treatment. 30 minutes after application this effect still could be seen.

Control GFP transfected neurons showed no increasing of fluorescent intensity in spines after glutamate treatment (Fig. 7B, 7B', 7C), and the intensity of GFP signal was not changed in dendritic spines before and after application of glutamate. This control experiment excludes the possibility that accumulation of gelsolin-GFP was due to the accumulation of GFP molecules.

3.5. NMDA receptors and calcium ions are necessary for gelsolin accumulation

Glutamate receptors occur in a variety of subtypes including ionotropic receptors preferring either a-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) receptor or N-methyl-D-aspartic acid (NMDA) receptor and metabotropic glutamate receptor (mGluR). To investigate which receptor subtype is responsible for glutamate-induced gelsolin targeting to dendritic spines, the contribution of each receptor subtype to gelsolin accumulation was examined using bath application of glutamate together with selective receptor subtype antagonists. Application of glutamate (2.5 μM)

together with the NMDA receptor blocker D(-)-2-amino-5-phosphonopentanoic acid (APV) (200 μM) totally inhibited the glutamate-induced accumulation of gelsolin in spines (Figure 7 D). By contrast, blocking AMPA receptors with 20μM NBQX did not block glutamate-induced accumulation of gelsolin. Blocking mGluRs with 200 μM MCPG also did not inhibit the accumulation of gelsolin induced by glutamate (Figure 7 D). These results indicate that it is mainly NMDA receptors which mediate the activation-induced accumulation of gelsolin in dendritic spines.

Because NMDA receptor activation leads to Ca^{2+} influx into dendritic spines (Segal, 1995; Yuste et al., 1999), we tested whether Ca^{2+} influx is required for gelsolin accumulation. To exclude the Ca^{2+} influx when glutamate was applied on the cells, we repeated the experiment using calcium free solutions. We first incubated cells in calcium free Tyrode's solution for 10 minutes to equilibrate them to calcium free condition. Glutamate (2.5 μM) was then applied in calcium free Tyrode's solution exclude calcium influx. Under these conditions, activation of postsynaptic glutamate receptor neurons did not induce accumulation of gelsolin in dendritic spines, even when the incubation time was prolonged to 30 minutes (Figure 7D). Therefore, calcium influx is needed in the process of the accumulation of gelsolin in spines.

3.6. The F-actin binding domain of gelsolin is necessary for its accumulation in dendritic spines

Gelsolin has six functional distinct domains whose combined operation accounts for its calcium regulation, actin binding, severing and capping properties (Figure 8). To investigate which of these domains is responsible for its targeting to dendritic spines, we created vectors expressing various gelsolin deletion mutants tagged with GFP (Figure 8).

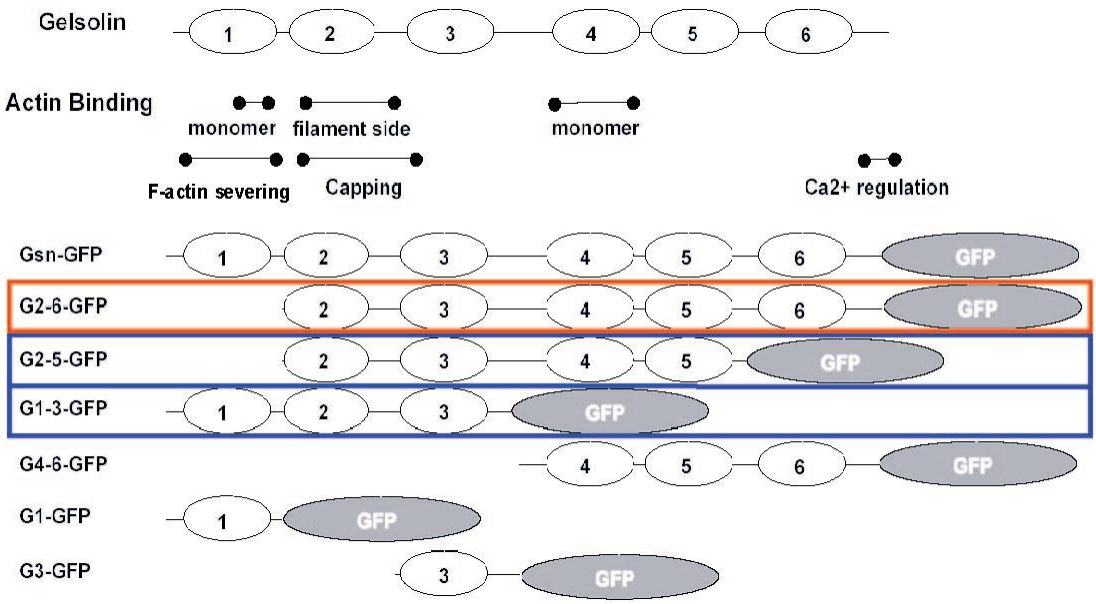

Figure 8. Domain analysis of gelsolin and gelsolin mutants.

G2-6-GFP (in red box) has an F-actin binding site, an actin monomer binding site and a calcium regulation site; G2-5-GFP (in blue box) has an F-actin binding site, an actin monomer binding site and is calcium independent; G1-3-GFP (in blue box) has an F-actin binding site, an actin monomer binding site and is calcium independent; G4-6-GFP has a monomer actin binding site; G1-GFP has an actin monomer binding site; G3-GFP does not contain any functional binding site.

G2-6-GFP contains domains 2 to 6 comprising an F-actin binding site (2), an actin monomer binding site (4) and a calcium regulation site (C-terminus). Because of the calcium regulatory site, the activity of G2-6 depends on cytoplasmic calcium concentration. When activated by calcium, G2-6 can cap or bind to the sides of actin filaments, dependent on domains 2 and 4 respectively. However, G2-6 does not have the ability to sever actin filaments, because this function is associated with domain 1, which it lacks. G2-5-GFP contains same functional domains as G2-6-GFP but lacks the calcium regulatory domain (C-terminus). So that it is constitutively active, binding to the sides of actin filaments or capping their barbed ends thus preventing further actin monomer addition. G1-3-GFP contains the F-actin severing domain (1), the actin filament binding site and is calcium independent, so that it can constitutively bind, sever and cap actin filaments. The only actin-binding site in G4-6-GFP is domain 4 so that it can only bind actin monomers. This function is

believed to be calcium independent because, although it has a fragment of the C-terminal calcium regulatory site, calcium regulation needs the co-operation of domain 2 which it lacks. G1-GFP also has an actin monomer binding site with much weak severing ability. Whereas the sequence present in G3-GFP does not contain any known functional actin binding site.

We transfected these gelsolin mutants separately into hippocampal neurons. Under control conditions without NMDA receptor activation, both G2-5-GFP and G1-3-GFP showed a strong targeting in dendritic spines (Figure 9, Figure 10). In these transfected cells GFP signals from both constructs were strong in dendritic spines, with much weaker signal intensities in the neighboring dendritic shafts. The targeting of these mutant forms to dendritic spines under control conditions is most likely because both mutants lack the calcium regulatory domain and contain F-actin binding domain which can therefore operated independently of receptor-induced calcium influx (Figure 8). G2-6-GFP, which also has a F-actin binding site, did not show enrichment in dendritic spines under resting conditions, presumably because it contains domain 6 and is therefore calcium regulated. To test this idea we exposed G2-6-GFP expressing cells with 2.5 μM glutamate for 5 minutes. Following this treatment G2-6-GFP showed strong accumulation in dendritic spines (Figure 10, Figure 11). G2-5-GFP and G1-3-GFP, which were already enriched in dendritic spines before glutamate application, showed no further increase accumulation or other variation in their relation to dendritic spines. Finally, G1-GFP, G3-GFP, and G4-6-GFP did not show targeting to dendritic spines under either control or glutamate treated conditions (Figure 10). Thus, all those constructs which lack the F-actin binding domain (G1, G3, G4-6) did not show accumulation in dendritic spines either before or after glutamate application. These results confirm that the F-actin binding domain of gelsolin is necessary for gelsolin accumulation in dendritic spines and that before gelsolin can accumulate in dendritic spines, the gelsolin needs to be activated by calcium influx.

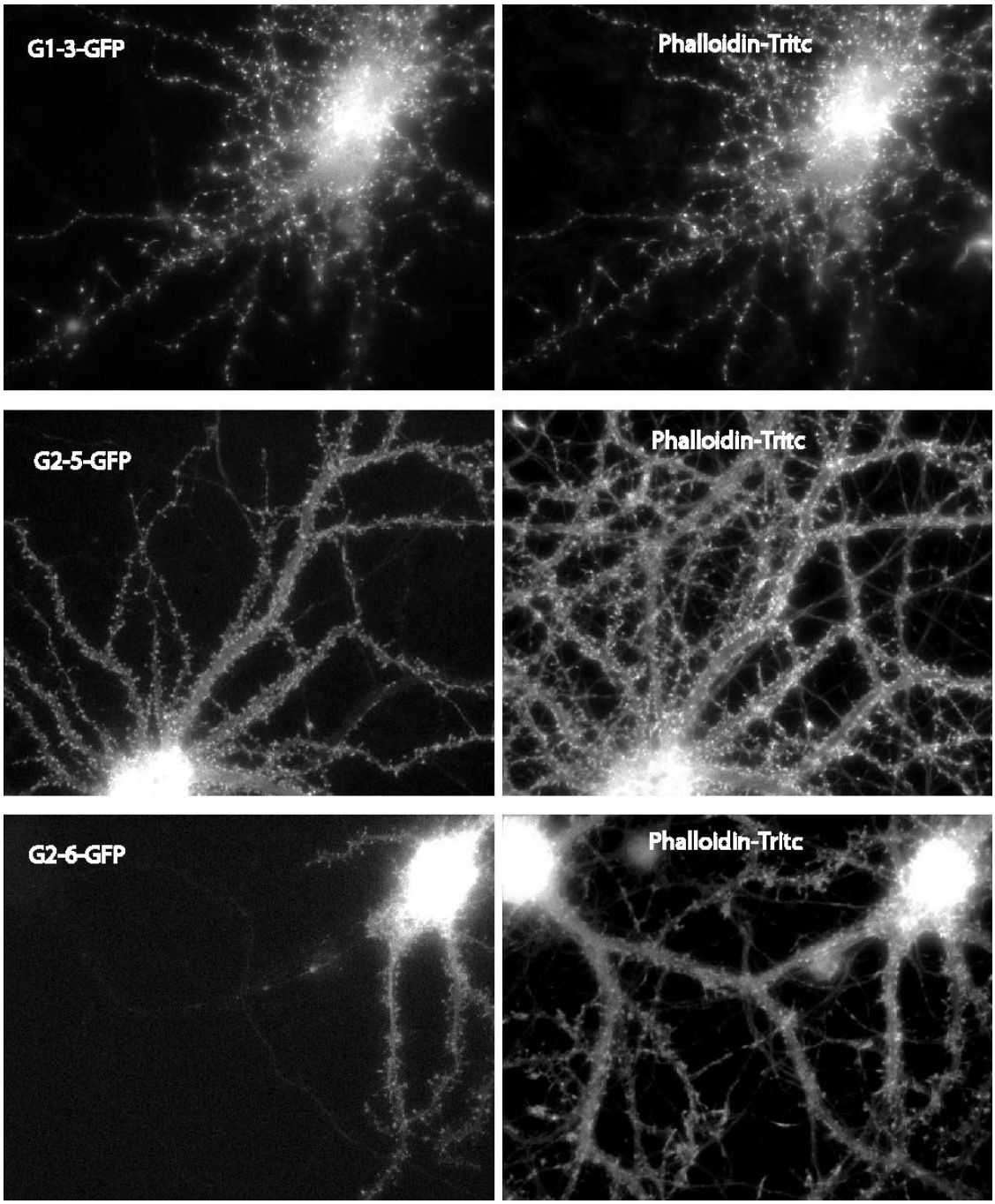

Figure 9. Expression of gelsolin mutants that has an F-actin binding domain.
Typical images of cells transfected with G1-3-GFP, G2-5-GFP and G2-6-GFP respectively. Each of these three gelsolin mutants has an F-actin binding site. Of them, G1-3-GFP and G2-5-GFP showed strong accumulation in dendritic spines in resting state. Whereas G2-6-GFP, which also has an F-actin binding site, showed a similar distribution pattern as that of gelsolin-GFP (figure 6, figure 7). G2-6-GFP was localized in dendritic spines, but not so strong as G1-3-GFP and G2-5-GFP.

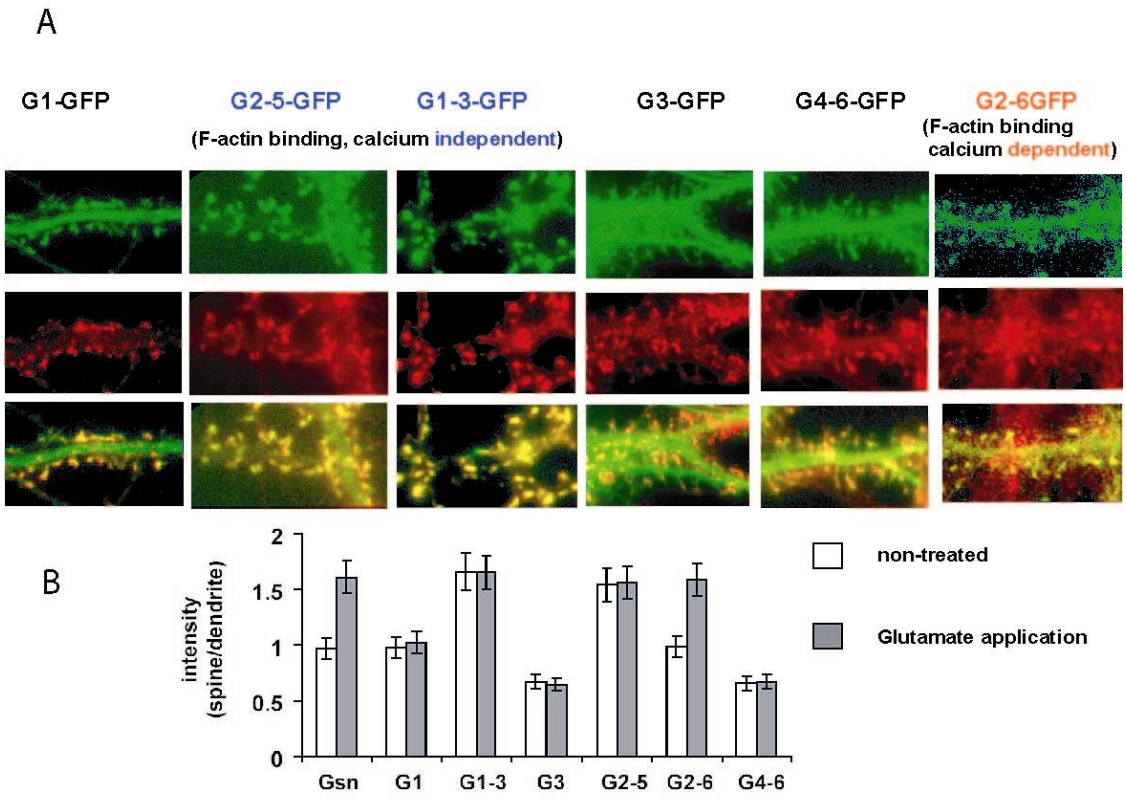

Figure 10. Targeting domain of gelsolin and calcium regulation.

A. Gelsolin mutants were transfected into hippocampal neurons. In the untreated condition, only G2-5-GFP (cell n=9) and G1-3-GFP (n=9) showed strong enrichment in dendritic spines (green color). F-actin staining (red color) shows the location of dendritic spines. The merged images (bottom row) show the enrichment of G2-5-GFP and G1-3-GFP (yellow) in dendritic spines. Whereas G1-GFP (n=11), G3-GFP (n=10), G4-6-GFP (n=8) and G2-6-GFP (n=41) did not show enrichment in dendritic spines. B. Quantification data showed the ratio of the intensity of GFP tagged gelsolin mutants in dendritic spines vs. in dendritic shafts. Open bar showed the untreated cells; shadow bar showed the cell treated with 2.5 μM glutamate for 5 minutes. After 5 minutes glutamate (2.5 μM) application G2-6-GFP was accumulated in dendritic spines. Other mutants did not show change in distribution of GFP signal in dendritic spines vs. dendritic shafts.

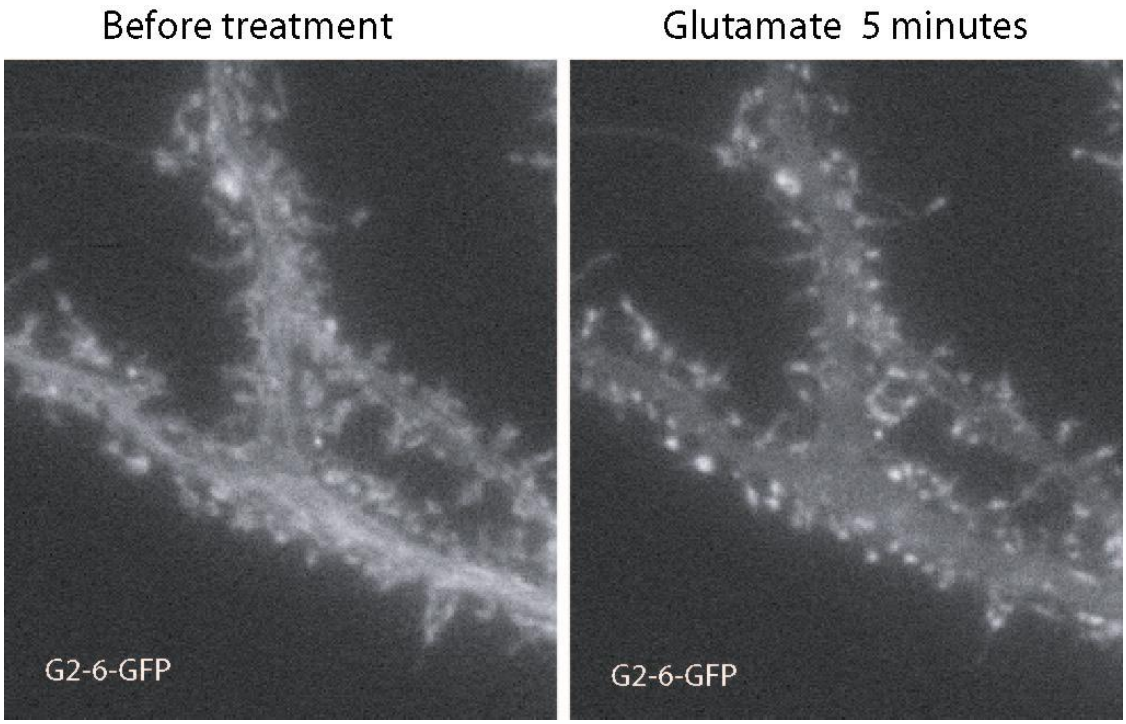

Figure 11. Glutamate induced targeting of G2-6-GFP to dendritic spines.
Before treatment G2-6-GFP did not show specific localization in dendritic spines. It showed a strong G2-6-GFP signal intensity in dendritic shafts. Application of 2.5 μM glutamate for 5 minutes clearly induced accumulation of G2-6-GFP to dendritic spines. It showed a strong G2-6-GFP signal in dendritic spines and a weak signal in the neighboring dendritic shafts. (Cells, n = 21).

3.7. Gelsolin severs actin filaments in dendritic spines upon glutamate application

Upon glutamate application, gelsolin is activated and translocated to dendritic spines. The next obvious question is what gelsolin would do in dendritic spines after it accumulates there? Post studies have shown that neurons treated with NMDA or glutamate show calcium dependent loss of F-actin in dendritic spines (Halpain et al., 1998a). Gelsolin is known to sever actin filaments in a calcium-dependent manner (Yin and Stossel, 1979; Yin et al., 1981b; Kinosian et al.,

1998). Consequently we tested whether gelsolin couples synaptic activity to the regulation of actin filaments in dendritic spines. Rhodamine-phalloidin was used to stain actin filaments in hippocampal neurons and its fluorescence intensity in dendritic spines was quantified to compare the relative ratio of F-actin (spine/dendrite) in gelsolin-GFP expressing cells and in untransfected wild type cells. First, hippocampal neurons transfected with gelsolin-GFP were allow to develop in culture for 21 days. In the experiment they were treated for 5 minutes with 7.5 μM glutamate before fixation. This resulted in a significantly weaker rhodamine-phalloidin staining of F-actin in dendritic spines of gelsolin-GFP expressing cells compared with untransfected cells (Figure 12 A), indicating glutamate-induced loss of F-actin in dendritic spines was significantly enhanced by gelsolin overexpression. In control experiments, cultures were treated with regular Tyrode's solution, which did not induce a significant difference in F-actin staining in spines between transfected and untransfected cells (Figure 12 C).

Because the loss of F-actin could be attributed to the severing or the capping function of gelsolin, we next examined whether the loss of F-actin staining in dendritic spines was due to the severing. To do this we transfected neurons with the gelsolin deletion mutant G2-6-GFP, which lacks the severing function. Application of 7.5 μM glutamate for 5 minutes on G2-6 transfected neurons did not significantly change F-actin staining in dendritic spines compared with untransfected neurons (Figure 12 C), and prolonging the incubation time to 30 min did not change the F-actin staining compared to control neurons either (data not shown). These results indicate that the actin filament loss in dendritic spines after glutamate treatment depend on the F-actin severing function of gelsolin.

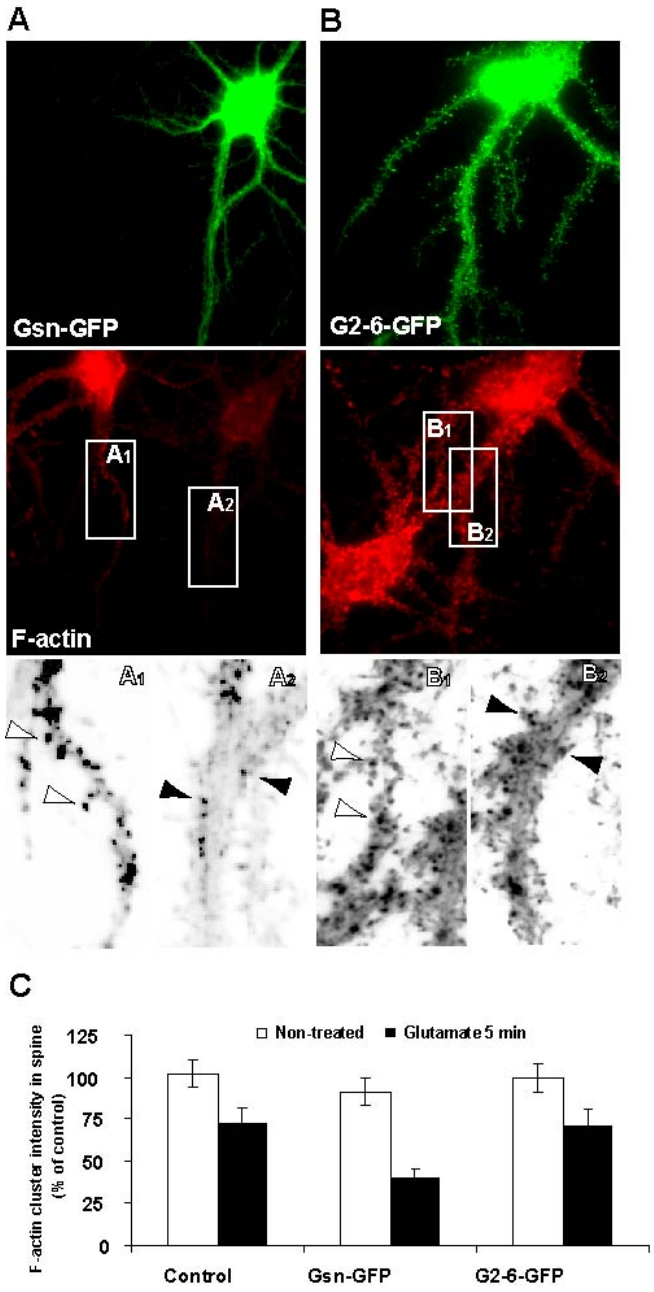

Figure 12. Gelsolin severed actin filaments in dendritic spines upon glutamate application.

(A) Neurons were treated with 7.5 µM glutamate, then fixed and stained for F-actin with rhodamine-phalloidin. The F-actin cluster intensity in Gsn-GFP expressing dendritic spines (A2) (filled arrow) was decreased compared with the control untransfected neighboring neuron (A1) (open arrow). (B) Neurons expressing G2-6-GFP (B2), a gelsolin mutant that does not sever F-actin, did not show the decreased F-actin staining in dendritic spines (filled arrow) compared with the control neuron (B1) (open arrow). (C) Quantification of F-actin cluster intensity in dendritic spines. In

untreated neurons expressing Gsn-GFP (34 cells) or G2-6-GFP (25 cells) did not change the F-actin staining significantly. Treatment with 7.5 μM glutamate for 5 minutes Gsn-GFP transfected neuron (cell n=37) showed a much lower level of F-actin in dendritic spines than control untransfected cells (n=42) and G2-6-GFP transfected cells (n=39).

3.8. Low frequency electrical stimulation activates F-actin severing by gelsolin

Electrical stimulation is often used to change the synaptic transmission efficiency of synapses and evidence indicates that long-term depression stimulation can inhibit F-actin dynamics in dendritic spines (Star et al., 2002). We tested whether gelsolin mediates LTD pattern stimulation effect on actin filaments in dendritic spines. A low frequency electrical stimulation protocol known to induce long-term depression (LTD) in dissociated hippocampal neurons was applied to cultures. This consisted of pulses delivered at 1 Hz for 15 minutes. After a further 15 minutes the cultures were fixed, and then stained for actin filaments using rhodamine-phalloidin. The relative ratio (spine/dendrite) of F-actin in gelsolin-GFP expressing neurons measured 15 min after LTD related electric field stimulation was significantly lower in gelsolin-GFP expressing cells (45±5.1%) than in untransfected cells (74±7.2%) (Figure 13B, 13C). Therefore, the loss of F-actin in dendritic spines caused by low frequency stimulation was enhanced in cells expressing gelsolin-GFP.

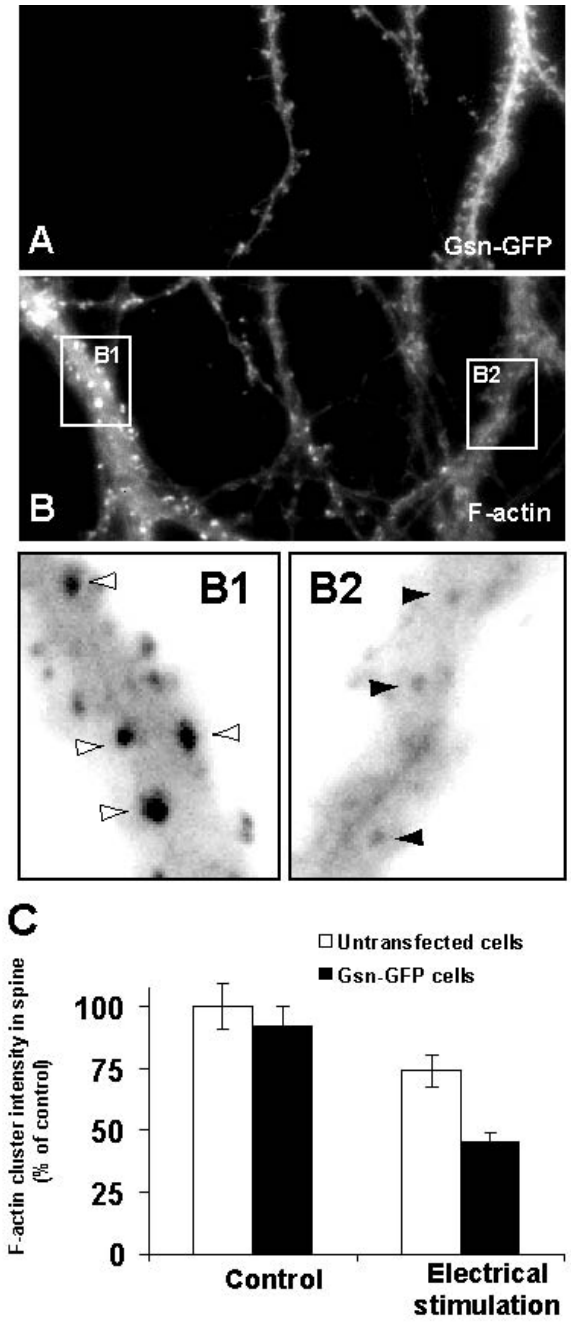

Figure 13. Gelsolin decreased F-actin staining in dendritic spines after LTD pattern stimulation.

(A): Gelsolin-GFP transfected cell. (B): F-actin staining in a gelsolin-GFP transfected cell (right) compared to an untransfected cell (left). F-actin staining in dendritic spines was weaker in the gelsolin-GFP expressing neurons (B2) than in the control neuron (B1) 15 minutes after stimulation. (C): 15 minutes after LTD pattern stimulation F-actin staining had decreased in both cells, but the decrease was more in gelsolin-GFP expressing cells (n=12). Control cells (n=19).

3.9. Activation of gelsolin requires activation of NMDA receptor and influx of calcium

Glutamate application and LTD pattern stimulation of glutamatergic neurons activates various receptor subtypes, including AMPA, NMDA and mGlu receptors. Next, we examined the contribution of each of these receptor subtypes to the glutamate induced actin loss using selective agonists and antagonists for AMPA, NMDA and mGlu receptors. Previous data have implicated calcium influx via NMDA receptor channels in the glutamate-induces disruption of actin filaments in dendritic spines. When we applied the glutamate in calcium free Tyrode's solution to exclude the calcium influx presumed to happen after NMDA receptor activation, there was no decrease in F-actin staining between gelsolin-GFP transfected and control untransfected cells (Figure 14B), confirming that the extracellular calcium is necessary for the gelsolin. Application of AMPA did not lead to a greater decrease of actin filaments in transfected cells than untransfected cells, and the mGluR agonist DHPG similarly showed no difference in actin staining between transfected and untransfected cells (Figure 14 C). By contrast application of NMDA produced same effects as that of glutamate (Figure 14C).

These indications that severing of actin filaments depends on selective activation of NMDA receptor were confirmed using glutamate receptor subtype-specific antagonist. APV, an antagonist of NMDA receptors, blocked the actin loss effects induced by glutamate (Figure 14 C), whereas NBQX, an antagonist of AMPA receptors, and MCPG, an antagonist of mGluRs, had no effect (Figure 14C).

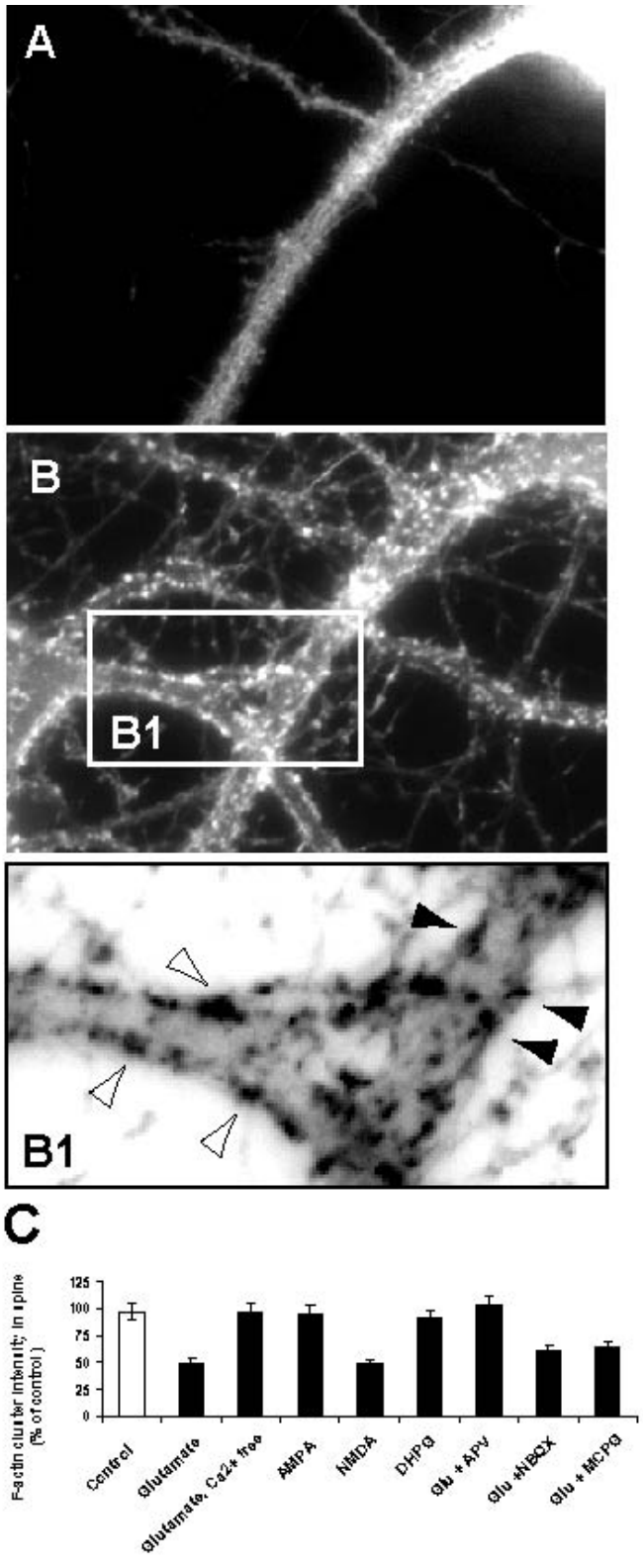

Figure 14. Signals for activating gelsolin in dendritic spines

Gelsolin-GFP expressing neuron (A) and control untransfected neuron (showed in B left) were treated with 7.5µM glutamate in calcium free tyrode's for 5 minutes, then stained for F-actin (B and B1). Under these calcium-free conditions there was no

difference between wild type and gelsolin-GFP expressing neurons concerning the F-actin staining in dendritic spines (B, B1). C) The F-actin decrease in dendritic spines of Gsn-GFP transfected neurons is treatment dependent. In normal Tyrode's solution glutamate (37 cells) and NMDA (41 cells) triggered loss of F-actin staining. The NMDA receptor antagonist APV blocked this effect (20 cells), while NBQX (27 cells) and MCPG (19 cells) were ineffective. The decrease of F-actin staining induced by glutamate was also blocked in calcium free condition (22 cells).

These experiments show that, like glutamate induced targeting of gelsolin to dendritic spines, the loss of F-actin in the spine cytoplasm is mainly mediated by calcium-influx through NMDA receptors, and that AMPA and mGlu receptors do not contribute significantly to those effect. Considering the known role of NMDA receptors in activation of gelsolin, this suggested a pathway in which influx of calcium through NMDA receptors activates the entry of gelsolin into dendritic spines, and its severing effect on actin filaments of the spine cytoskeleton.

3.10. Gelsolin activation decreases AMPA receptor expression in dendritic spines

The actin cytoskeleton is known to play an important role in anchoring AMPA receptors to synapses of pyramidal neurons. These AMPA receptors disperse from synaptic sites when F-actin is depolymerized by latrunculin A (Allison et al., 1998a; Shen et al., 2000b). Since our data show that activated gelsolin disrupts F-actin in dendritic spines we next asked whether gelsolin regulates the anchoring of AMPA receptors (GluR1) to postsynaptic sites in dendritic spines by influencing F-actin assembly.

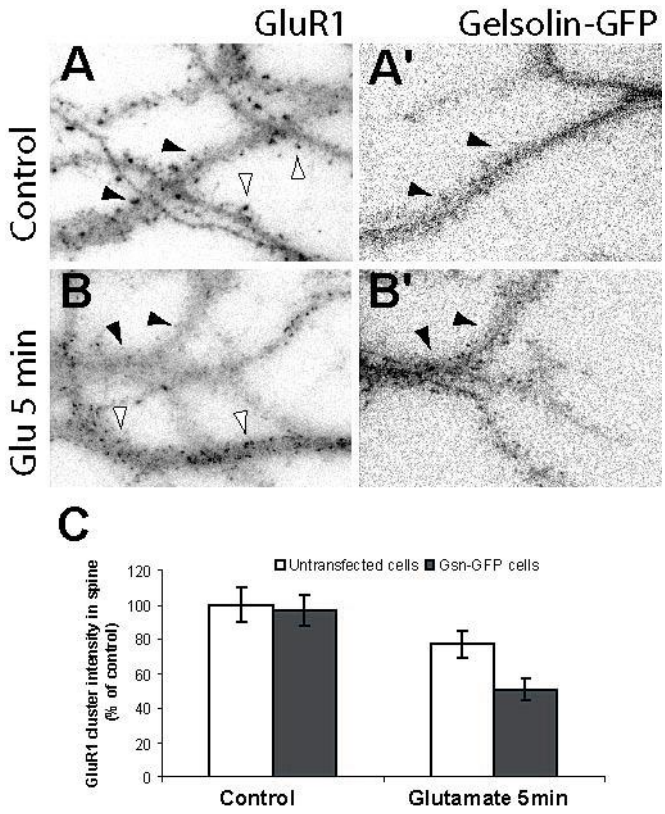

Figure 15. Gelsolin decreases GluR1 staining in dendritic spines upon glutamate treatment.

(A) Immunostaining with antibodies against GluR1 show the presence of AMPA receptor in dendritic spines in both gelsolin-GFP transfected cells (filled arrow) and untransfected control cells (open arrow) under resting conditions. (B) Incubating cells in 7.5µM glutamate for 5 minutes decreased the level of GluR1 staining in gelsolin-GFP transfected cells (filled arrow) compare to untransfected cells (open arrow). (C) GluR1 staining intensity in dendritic spines was normalized to 100% in control cells (n=28) (incubated in Tyrode's solution for 5 minutes). Gelsolin-GFP transfected cells (n=26) show similar GluR1 staining (97±9.1%) to untransfected cells in dendritic spines in control condition. However, after exposure to 7.5µM glutamate for 5 minutes both the transfected cells (n=24) and untransfected cells (n=22) show a decrease of GluR1 staining in dendritic spines which was greater in gelsolin-GFP expressing cells (51±5.9%) than in untransfected cells (77±8.1%). Error bar ± s.e.m.

Cells fixed under control conditions and stained with antibodies against GluR1 showed similar staining in both gelsolin-GFP transfected and untransfected

cells. In Figure 15A the black arrows head to the gelsolin-GFP transfected cells and the open arrows head to untransfected cells. This lack of difference reflects the fact that F-actin was not disrupted in either transfected or control cells under resting conditions (Figure 12 C). However, incubating cells in 7.5μM glutamate for 5 minutes caused a decrease in GluR1 staining in gelsolin-GFP transfected cells (Figure 15 B, black arrow heads) compared to untransfected cells (Figure 15 B, open arrow heads). Treating cells with glutamate actually induced reduction of AMPA receptor staining in both transfected cells and untransfected cells (Figure 15 C) showing that glutamate induced disruption of actin filaments in both cells (Figure 12 C), but the reduction of AMPA receptors was much greater in transfected cells. These results indicate that gelsolin, as an endogenous F-actin assembly regulator, further regulates the anchoring of AMPA receptors (GluR1) in dendritic spines.

4. Discussion

The issue addressed in this thesis is concerned with the morphological plasticity of central nervous system circuits, which is widely believed to be important for learning and memory. Recent evidence suggests the dendritic spines play a key role in this phenomenon. Dendritic spines undergo changes in shape over a matter of seconds shown by neurons expressing GFP-actin (Fischer et al., 1998; Dunaevsky et al., 1999; Korkotian and Segal, 2001). Synaptic activity can change spine numbers and shape which depend on this dynamic actin cytoskeleton (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Matus, 2000; Segal, 2001). Furthermore the morphological heterogeneity of spine shape and number may lead to the functional diversity of brains (Harris, 1999). Although it has been well established that the synaptic activity influences the dynamic actin cytoskeleton in dendritic spines, the mechanisms of mediating the synaptic activity to actin cytoskeleton remain largely unknown. In this thesis we found that gelsolin, a calcium-regulated F-actin severing and capping protein (Yin and Stossel, 1979; Janmey et al., 1985), plays an important role in mediating synaptic activity to actin cytoskeleton, and subsequently influences the AMPA receptor plasticity in dendritic spines.

4.1. Association of gelsolin with actin filament in cells

In this thesis work I first reexamined the distribution of gelsolin in fibroblasts using gelsolin immunostaining method and confirmed that gelsolin is associated with dynamic actin rich structure - lamellipodia - but not with stress fibers, which are stable actin structure (see **Chapter 2.1.**)

Compared to the well-established model for the interaction of gelsolin with actin *in vitro*, the question how gelsolin binds to actin and influences the dynamics of actin filaments in living cells has been a difficult issue for researchers because of the technical difficulties and sometimes conflicting data. The earliest studies on the localization of gelsolin in cells using immunofluorescence methods gave conflicting results. With some experiments studies showing gelsolin associated with actin filament enriched structures (Yin et al., 1981a; Rouayrenc et al., 1984; Wang et al., 1984), whereas, others reported a diffuse distribution of gelsolin in the cytoplasm and little colocalization with actin filaments (Carron et al., 1986). A significant advance in solving this question was made by Cooper et al. (1988) who showed that in living fibroblasts gelsolin has a diffuse cytoplasmic distribution, most likely because it is associated with actin filaments in short-lived complexes. When fibroblast motility is induced by epidermal growth factor (EGF), gelsolin incorporates preferentially into the ruffling membranes at the leading edge of the lamellipodia (Chou et al., 2002), suggesting that gelsolin associates with the dynamic actin cytoskeleton to push the ruffling membrane during EGF-induced motility.

Experiment on platelets provide another good example for the interaction between gelsolin and actin. In resting platelets the barbed ends of actin filaments are capped by capping proteins (Barkalow et al., 1996). Exposure of platelets to thrombin initiates a signal transduction cascade that promotes actin polymerization (Carlsson et al., 1979), which is preceded by a rapid severing of cytoplasmic actin filaments induced by free gelsolin activated by a transient increase in calcium levels (Hartwig, 1992). Platelet from gelsolin knockout mice showed a reduced rate of clotting (Witke et al., 1995), consistent with the requirement of actin severing by gelsolin for platelet activation (Hartwig, 1992).

Gelsolin knockout cells further establish the importance of gelsolin in maintaining motility and actin dynamics, since gelsolin null fibroblasts have abnormally pronounced actin stress fibers (Witke et al., 1995), and this phenotype is consistent with a lack of the ability to sever and remodel actin

filaments. Probably as a consequence, gelsolin null fibroblasts do not ruffle in response to growth factor (Azuma et al., 1998).

4.2. Accumulation of gelsolin in dendritic spines by synaptic activity

Whether gelsolin is located in dendritic spines was an obvious question when we started to investigate gelsolin function at the synapse. The immunostaining data clearly showed the localization of gelsolin at the postsynaptic sites. To exclude the possibility of artifacts that may be introduced by fixation and immunostaining, we also expressed the GFP tagged gelsolin in hippocampal neurons. Gelsolin-GFP is located in dendritic spines of resting cells, but the dendritic shafts also have relatively strong gelsolin-GFP signal. A possible explanation for this difference is that gelsolin-GFP showed the correct distribution pattern in living neurons, and that gelsolin antibody staining enhances the signal ratio between spine and shaft by permeabilizing cells during the fixation activating redistribution of gelsolin process. This kind of enhancement of gelsolin signal has been observed in fibroblasts, where gelsolin was found to be associated with stress fibers after immunostaining which is not the case in living cells (Cooper et al., 1988).

Possible changes in the distribution of gelsolin in the neuronal cytoplasm when neurons are stimulated are very interesting because gelsolin's localization is closely related to its possible function in dendritic spines. My experiments showed that glutamate treatment triggered the accumulation of gelsolin in dendritic spines (Chapter 3.3. and 3.4.), and that this effect is apparently mediate by NMDA receptors in dendritic spines (Chapter 3.5.). Glutamate treatment in calcium-free Tyrode's solution did not induce accumulation of gelsolin in spines, indicating calcium that influx through NMDA receptors is a key process in triggering the movement of gelsolin from dendritic shafts to spines. Gelsolin is a calcium-regulated actin binding protein that is activated by

increase in calcium concentration to the micromolar range, therefore calcium influx through NMDA receptors upon glutamate application is a likely trigger for the translocation of gelsolin from shafts to spines where it can bind to actin filaments. Indeed my experiment with gelsolin deletion mutants expressed in neurons confirmed that the F-actin binding domain of gelsolin is required for translocation. Appropriate deletion constructs (G2-5-GFP and G1-3-GFP), which can bind F-actin independent of calcium, strongly targeted to dendritic spines in un-stimulated cells (Chapter 3.6.) indicating that in resting cells native gelsolin is inhibited from actin binding. G2-6-GFP, a deletion construct which contains the F-actin binding domain but is calcium regulated, did not target to dendritic spines in the unstimulated state, but after glutamate application it strongly targeted to dendritic spines (Chapter 3.6.).

It is also conceivable that gelsolin binds other dendritic spine components after being activated, such as PI(4,5)P2, however, PI(4,5)P2 has been shown to be hydrolyzed in response to glutamate stimulation (Nahorski, 1988; Chuang, 1989; Fisher et al., 1992; Furuichi and Mikoshiba, 1995), thus decreasing the PI(4,5)P2 level making more likely that gelsolin mainly binds to F-actin rather than PI(4,5)P2 in dendritic spines after glutamate application.

In the recent years activity-dependent translocation has been demonstrated for a number of postsynaptic proteins (Inoue and Okabe, 2003), including for example CaMKII and PSD-Zip45. Stimulation of NMDA receptors with glutamate induced a dramatic translocation of the CaMKII-GFP to postsynaptic sites (Shen and Meyer, 1999). However after first associating with the PSD, CaMKII was subsequently phosphorylated and became dissociated from the PSD. CaMKII that had been dissociated from the PSD could re-located back more rapidly after subsequent stimulation (Shen et al., 2000a), implicating CaMKII translocation in the molecular mechanism underling neuronal plasticity (Fink and Meyer, 2002). Similarly the Homer family metabotropic glutamate receptor interacting protein PSD-Zip45 shows rapid redistribution associated with neuronal activity, but the direction of its translocation is opposite to that of CaMKII (Okabe et al., 2001b), so that the clusters of PSD-Zip45 are dispersed

by NMDA receptor activation. In contrast, a transient increase of intracellular calcium through voltage-dependent calcium channels induces PSD-Zip45 clustering. The functional consequence of PSD-Zip45 translocation may influence mGluR functions and its cell surface expression (Inoue and Okabe, 2003).

4.3. Gelsolin regulates the actin cytoskeleton in dendritic spines

Since gelsolin targets to dendritic spines after glutamate stimulation, it is quite likely gelsolin may participate into spine plasticity in some way. In this direction, first the actin cytoskeleton in dendritic spines was investigated, because gelsolin is well know for regulating actin dynamics and dynamic actin filaments have an essential role in supporting spine plasticity.

For non-neuronal cells, it is well-known that gelsolin contributes to the regulation of the dynamic actin cytoskeleton which underlies the morphology and motility of the cells (Cooper et al., 1987; Cunningham et al., 1991; Arora and McCulloch, 1996). For neuronal cells, our results show that overexpression of gelsolin rendered the actin cytoskeleton in dendritic spines more vulnerable to glutamate or LTD stimulation (Chapter 3.7. and 3.8.). Overexpression of gelsolin disrupted more actin filaments in dendritic spines in response to glutamate or LTD stimulation, suggesting gelsolin is activated by these stimuli and indicates a clear effect on actin depolymerization.

Activity-dependent stimulation of gelsolin requires the activation of NMDA receptors, since blocking NMDA receptors inhibits the loss of actin filaments caused by gelsolin (Figure 14 C). Further experiments suggest a close link between activation of NMDA receptors and gelsolin mediated by calcium ion influx through opened NMDA receptor channels. Based on previous experiments showing that calcium is required for activation of gelsolin, I

performed experiment in which glutamate receptor were stimulated in calcium-free medium and found that this inhibits the activation of gelsolin. Taken together, these results suggest the following scenario: glutamate or LTD-pattern stimulation activates NMDA receptors, then calcium ions enter through opened NMDA receptors and when calcium ion concentration reaches above the threshold (μM) to activate gelsolin, then activated gelsolin depolymerizes actin filaments in dendritic spines.

Calcium in dendritic spines has a crucial role in the induction of LTP and LTD --- the putative cellular mechanisms of learning and memory. Calcium regulates postsynaptic enzymes that trigger rapid modification of synaptic strength and also activates transcription factors that induce the expression of other genes for long-term maintenance of these modifications (Bito et al., 1997). Calcium enters spines in response to synaptic excitation and postsynaptic electrical activity. In pyramidal neurons, action potentials open voltage-sensitive calcium channels (VSCCs) that admit calcium into dendrites and spines (Yuste and Denk, 1995). NMDA receptors also clearly have a major role in spine calcium dynamics, as several groups have shown that blocking NMDA receptors abolishes the increasing of calcium levels in spines (Yuste and Denk, 1995). Although most studies suggest that calcium influx through NMDA receptors accounts for most of synaptic spine calcium, other results point towards calcium induced calcium release (CICR) (Emptage et al., 1999) or VSCCs (Schiller et al., 1998).

Phosphorylation of gelsolin may be another mechanism regulating its function following neuronal stimulation. Artificial phosphorylation of N-terminus region of gelsolin activates gelsolin (Takiguchi et al., 2000), so that it no longer requires calcium for activation; it then severs and subsequently caps actin filaments, and nucleates filament formation in calcium-free solution, suggesting phosphorylation can change the conformation of gelsolin. Gelsolin can be phosphorylated by c-Src *in vitro* and phosphorylation will be enhanced in the presence of PI(4,5)P2 (De Corte et al., 1997). The major phosphorylation site (Tyr438) was located in subdomain 4 (S4). Other phosphorylation sites were

identified as Tyr59, Tyr383, Tyr576, and Tyr624 (De Corte et al., 1999). In neuronal cells glutamate application induces c-Src kinase activation (Khanna et al., 2002). But *in vivo* phosphorylation of gelsolin by Src has not yet been proven.

To date the significance of remodeling the actin cytoskeleton in dendritic spines by glutamate receptor activation is still unclear. The partial disruption of actin cytoskeleton in dendritic spines has been suggested to be a mechanism to protect neurons from excitotoxicity, because disruption of actin filaments helps the rundown of calcium level in neurons (Furukawa et al., 1997a). NMDA receptors are linked to the actin cytoskeleton by the actin-binding protein α-actinin (Wyszynski et al., 1997), so that dynamic F-actin participates in membrane targeting of NMDA receptor clusters (Allison et al., 1998a). Compounds that inhibit actin filament assembly decrease the magnitude of LTP (Kim and Lisman, 1999), and impair the maintenance of LTP at the Schaffer-collateral-CA1 pyramidal cell synapses (Krucker et al., 2000), possibly because that depolymerization of actin filaments causes internalization of AMPA receptors (Allison et al., 1998a; Zhou et al., 2001)(also see **Chaper 4.5**).

4.4. Severing vs. capping F-actin by gelsolin in dendritic spines

The F-actin disassembly caused by gelsolin in dendritic spines may be either due to the severing or capping function of gelsolin, or both. Severing requires gelsolin binding to the side of the actin filaments through a site in segments 2 and 3 (S2-3) to position another site in segment 1 (S1) which severs the filaments. Segment S2 and S3 also caps actin filaments (Sun et al., 1994), and detailed study showed that the sequence required for actin filament side binding and capping are located close to the N terminus of S2 (residues 161-172). S3 contributes to stable capping of actin filaments, so that the gelsolin

mutant G2-6-GFP is supposed to only cap actin filaments but lack the severing ability. Our data showed that the severing function of gelsolin is the main mechanism causing disassembly of the actin cytoskeleton in dendritic spines. The G2-6 mutant which lacks the severing function, but still caps barbed end of F-actin (Sun et al., 1994; Fujita et al., 1997), accumulates in dendritic spines upon glutamate treatment, but unlike whole length gelsolin, this G2-6 mutant did not disrupt actin filament in dendritic spines upon glutamate treatment (Figure 12B; 12C). This makes it unlikely that the capping function of gelsolin is involved in the F-actin disassembly in dendritic spines.

4.5. Relationship between activation of gelsolin and synaptic localization of AMPA receptors

Neurotransmitter receptor movement in and out of synapses has been proposed as one of the main mechanisms for rapidly changing the number of functional receptors during synaptic plasticity. Our data show a decreasing of AMPA receptor amount in dendritic spines of gelsolin overexpressing neurons upon glutamate application (Chapter 3.10.). This decrease of AMPA receptors in dendritic spines could be due to the endocytosis since it has been shown that LTD or glutamate application increases the amount of AMPA receptors sequestered by endocytosis (Carroll et al., 1999; Luthi et al., 1999; Hirai et al., 2001). Using immunofluorescence and surface biotinylation assay, a rapid basal AMPA receptor endocytosis rate in cultured hippocampal neurons has been detected and the AMPA receptor endocytosis rate is further accelerated in response to synaptic activity, ligand binding, and insulin (Lin et al., 2000). AMPA-induced AMPA receptor internalization is mediated in part by depolarization and calcium influx through voltage-dependent calcium channels and in part by a novel ligand-binding mechanism that is independent of receptor activation. The endocytosis of AMPA receptors depends on dynamin and the internalized AMPA receptors can be sorted to different destinations. Thus AMPA receptors internalized in response to AMPA stimulation enter a

recycling endosome system, whereas those internalized in response to insulin stimulation turn to a possibly degradative compartment (Beattie et al., 2000; Lin et al., 2000).

AMPA receptors also are sensitive to the assembly state of actin. Disruption of F-actin in hippocampal neurons in culture by actin assembly blocking drugs decreased the number of clusters of AMPA receptors on dendritic spines, indicating the immobilization of AMPA receptors depends on the integrity of the F-actin network (Allison et al., 1998a). Electrophysiological studies (Kim and Lisman, 1999; Krucker et al., 2000) support this observation by showing that dynamic actin filaments are important for basal synaptic transmission as well as induction and maintenance of long term potentiation. Depolymerization of the actin cytoskeleton causes internalization of AMPA receptors, whereas a drug that stabilizes actin filaments blocks internalization (Zhou et al., 2001). This suggested that glutamate might induce a dissociation of AMPA receptors from the anchors that associate them with actin cytoskeleton. This might then lead to movement of AMPA receptors into the extrasynaptic domain of the membrane via lateral movement where extrasynaptic AMPA receptor are readily captured by the constitutive endocytotic pathway (Zhou et al., 2001). The decrease in AMPA receptor levels in gelsolin overexpressing neurons thus can be understood as following: upon glutamate application actin filaments are disrupted much more in gelsolin overexpressing neurons than in control untransfected cells and the subsequent increased loss of actin filaments accelerates the AMPA receptor internalization.

In addition to increasing of endocytosis by the indirect route, disruption of AMPA receptor anchoring to actin cytoskeleton, actin filaments may also directly influence endocytosis. Actin filament assembly could generate mechanical forces to induce membrane invaginations, cut off deep invaginations to create vesicles, and move newly formed vesicles away from the plasma membrane. Thus interactions of components of the endocytic machinery with actin filaments could initiate the assembly of the endocytic machinery and anchor it at nascent internalization sites. Local disassembly of

actin filaments of the cell cortex near internalization sites could promote formation of nascent pits or facilitate transportation of newly formed vesicles into the deeper cytoplasm by clearing the way for vesicle diffusion or interactions with microtubules (Schafer, 2002).

How are the glutamate receptors anchored at the postsynaptic sites? Given that AMPA receptors are localized to PSDs and do not bind F-actin directly, their expression must requires certain PSD proteins that link them to F-actin (Kasai et al., 2003). Such interactions may be mediated, for example, by complexes of AMPAR-SAP97-protein 4.1N-F-actin (Lisman and Zhabotinsky, 2001) and AMPAR-stargazin-PSD95-GKAP-shank-cortactin-F-actin (Chen et al., 2000). SAP97 is one of a large number of synaptic junction proteins with PDZ domains (Craven and Bredt, 1998), which binds to the actin cytoskeleton through its amino terminus and to the GluR1 AMPA receptor subunit through its PDZ domain (Leonard et al., 1998). Protein 4.1N, a homolog of the erythrocyte membrane cytoskeletal protein 4.1, might act as another important bridge between AMPA receptors and the actin cytoskeleton in dendritic spines. It has been shown that disruption of the interaction of GluR1 with 4.1N decreased the surface expression of GluR1 in heterologous cells (Shen et al., 2000b).

For NMDA receptors, α-actinin-2, an actin-bundling protein, is the linker between actin filaments and NMDA receptors. It binds to actin through its amino terminus and to the NR1 subunit of NMDA receptors through its central rod domain (Wyszynski et al., 1997). The influx of calcium ions through activated NMDA receptors can depolymerize postsynaptic actin (Shorte, 1997a; Halpain et al., 1998a) and produce a negative feedback effect on NMDA receptors themselves, causing a gradual rundown of their calcium conductivity (Rosenmund and Westbrook, 1993a; Furukawa et al., 1997a). Calcium ions disrupt this interaction by binding to α-actinin-2 both directly (Krupp et al., 1999) and indirectly via calmodulin, which competes for the NR1-binding site (Wyszynski et al., 1997; Zhang et al., 1998).

Generally the rapid gain and loss of receptors from synaptic sites is accounted for by endocytosis and exocytosis, as well as by lateral diffusion of receptors in the plane of the membrane. These events are dependent on each other and are regulated by neuronal activity and interactions with scaffolding proteins (Choquet and Triller, 2003)

4.6. Model of gelsolin function in dendritic spines

The relatively thin neck and large head confer on dendritic spines the properties of diffusional and electrical compartments (Nimchinsky et al., 2002). As for diffusional compartment the spine neck could serve to restrict diffusional exchange of signaling molecules between spine head and parent dendrite (Nimchinsky et al., 2002); this could be important for localizing biochemical changes to a specific synapse. The main advantages of localizing signaling proteins are increased efficiency and increased specificity (Shen et al., 1998). By targeting gelsolin to dendritic spines, its local concentration is elevated increasing the efficiency of gelsolin binding to its partners including monomeric actin, actin filaments and phosphoinositides {PI(3,4)P2, PI(4,5)P2, and PI(3,4,5)P3} (Chellaiah and Hruska, 1996).

Based on these considerations and the results of my studies I propose a model for gelsolin function in dendritic spines (Figure 16). In hippocampal neurons a proportion of gelsolin is inactive, distributed in dendritic shafts and spines, whereas the active portion of gelsolin binds to and colocalizes with F-actin in dendritic spines. When neurons are stimulated by presynaptically released glutamate, calcium ions enter the cell through NMDA receptors, so that calcium levels reach the micromolar threshold for activating gelsolin. The binding of calcium ions changes the conformation of the gelsolin molecule, opening its functional domains for actin binding. Activated gelsolin then moves into dendritic spines, binds to F-actin, subsequently severs and caps actin filaments, causing disruption of the actin cytoskeleton in the spine cytoplasm. Depolymerization of actin filaments breaks the anchoring of AMPA receptors to

synaptic sites so that they diffuse away from synaptic function by lateral diffusion. These extrasynaptic AMPA receptors then captured in the endocytotic machinery and are cleared from the membrane surface of dendritic spines.

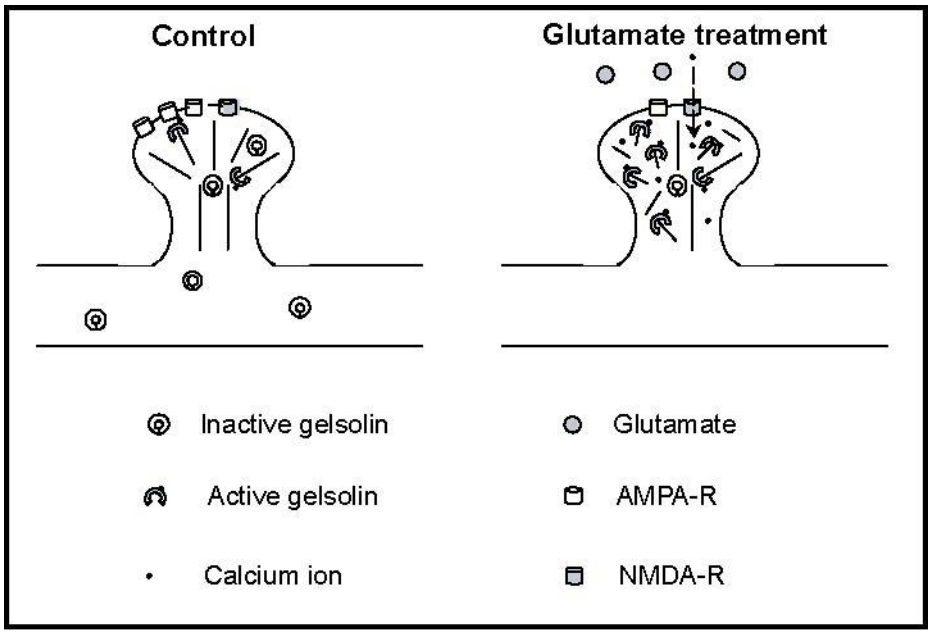

Figure 16. Diagrammatic summary of gelsolin activity regulation and gelsolin function in dendritic spines (see text).

The enrichment of gelsolin in dendritic spines may also have other physiological functions besides the regulation of actin cytoskeleton. It has been shown that gelsolin inhibits phospholipase C activity through phospholipid binding (Sun et al., 1997). The physical interaction between gelsolin and phospholipase D causes stimulation of phospholipase D (Steed et al., 1996). Gelsolin also plays a key role in recruitment of SH2 containing signaling proteins (including c-Src and PI3-kinase) to the plasma membrane through

phospholipid-protein interactions and regulation of their phosphorylation status through its association with the tyrosine phosphatase PTP-PEST (Chellaiah et al., 2001). However, whether these activities can really take place in dendritic spines and how they can influence the plasticity of synapses are still need to be solved in the future.

5. Materials and Methods

5.1. Constructs

GFP-tagged gelsolin and GFP-tagged gelsolin mutants were prepared using a eukaryotic expression plasmid carrying GFP cDNA under the control of a β-cytoplasmic actin promoter (Figure 17). Gelsolin gene was amplified from mouse cDNA by PCR with created AscI restriction site on both ends. Gelsolin deletion mutants were created by PCR also with AscI restriction site on both ends. The PCR products of gelsolin gene and gelsolin mutant gene were purified and digested by AscI, then purified again and inserted into AscI site of the plasmid (Figure 17). All constructs in this work were sequenced and checked for correctness.

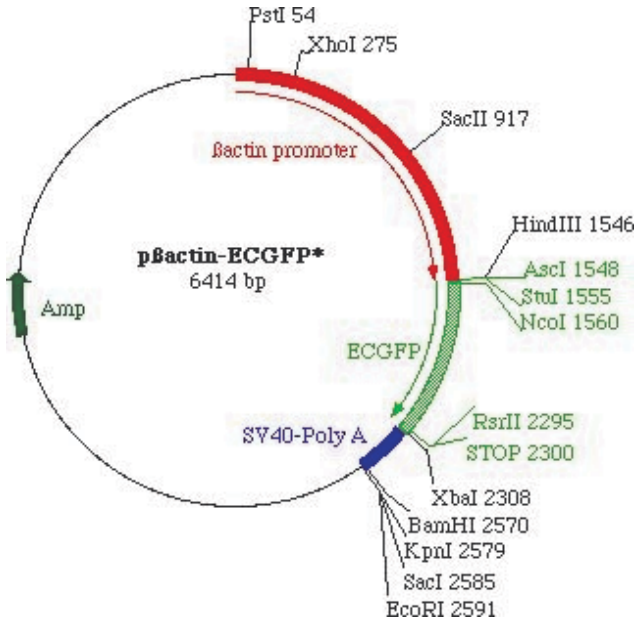

Figure 17. Diagram of the plasmid in which gelsolin wild type gene or mutants are inserted.

5.2. Dispersed cultures of hippocampal neurons

Hippocampal cultures were prepared from E19 (embryonic day 19) rat embryo and mouse newborns (P0). Hippocampal cultures were prepared and grown as described by Dr. Gary Banker, however with following changes:

1) The glia was cultured (before and after freezing of glia in aliquots) in fetal calf serum (FCS, Gibco) instead of horse serum.

2) Neurons were plated at higher density: Untransfected neurons were plated at a density of 350’000 neurons per 100 mm dish.

3) A different culture medium was used. After plating the neurons were grown in 1.5 ml neurobasal medium supplemented with B27 (Gibco, #17504-044), glutamine (0.5mM) and glutamate (25 μM). 3 days after plating 0.5 ml neurobasal medium supplemented with AraC (sigma), N2 (Gibco, #17502-048), and glutamine (0.5 mM) was added. 6 days after plating 1 ml of the medium was discarded and 1 ml of neurobasal, N2, glutamine was added. The goal is to exchange B27 medium with N2 medium. 11 days after plating, cells were fed with 0.8 ml neurosbasal/N2-medium. From now on cells were fed every 5 days with 0.7 ml neurobasal/N2-medium. Note: Neurobasal medium (Gibco) was optimized for neurons and therefore distinguishes from previously used minimal essential medium (MEM), e.g. neurobasal medium contains glycine, a co-activator of the NMDA-receptors, which is absent from MEM.

4) DOTAP transfection (see below)

5.3. Transfection of hippocampal neurons

Neurons were transfected in suspension before plating using Dotap liposomal transfection reagent (Roche) or between DIV10-16 in culture using calcium phosphate.

Dotap transfection method:

Dissect hippocampi from E19 rat embryos in Ca, Mg-free Hanks' Buffered Saline Solution (HBSS) with 10mM Hepes pH7.2. Transfer hippocampi in a 15ml polystyrene Falcon tube and add 5ml of 0.25% Trypsin in HBSS (Trypsin from 2.5% frozen stock, no EDTA). Incubate at 37 ºC for 15 minutes. Wash out trypsin by replacing the liquid with 5ml of HBSS for 3 times, 5 minutes each. Triturate hippocampi in 2 ml of HBSS with a narrow bore Pasteur pipet. Collect cells by gentle centrifugation (8min, 1000rpm), resuspend in HBSS and count in hematocytometer. Dissected, trypsinized and triturated hippocampi from E19 rat embryos were put in suspension in MEM containing 0.6% glucose (1.2 Mio cells in 3 ml). Cells were added to 3ml Ca^{2+}- and Mg^{2+}-free HBSS (Hanks balanced salt solution; Gibco), buffered with 10 mM Hepes pH 7.2, supplemented with 24 µl Dotap. Subsequently, cells were incubated at 37 degrees for 10 minutes. Then DNA (3.5 µg in 100 µl MEM) was added. After incubation at 37 degrees for 1 hour, cells were plated on a 100 mm culture dish.

Calcium phosphate transfection

2ml glia-conditioned medium for each coverslip in a 12-well plate was equilibrated at 37 ºC at 5% CO_2 for at least 30 minutes. Neurons were transferred from original wells to equilibrated glia-conditioned medium. DNA (2.5 µg per coverslip) was mixed with 60 ml $CaCl_2$ (250 mM solution) and immediately added to 60 µl 2 X BBS (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM

BES, pH 7.1) and vortexed. DNA mixture was dropped onto cells. Cells were incubated for about 90 minutes at 37 ºC at 2.5% CO_2 (check for calcium-phosphate precipitate). Cells were washed twice with pre-warmed HBS (135mM NaCl, 20mM Hepes, 4mM KCl, 1mM Na_2HPO_4, 2mM $CaCl_2$, 1mM $MgCl_2$, 10mM glucose, pH 7.35) and subsequently returned to original plate. Put back in original culture.

5.4. Fixation and staining of neurons

A 20% (w/v) stock solution of 20 g paraformaldehyde to 80 ml H_20 was prepared and heated to 65 degrees on a stirrer/hotplate. About 10 drops of 1 N NaOH were added, just enough to clear the solution. 20 % PFA stock solution was cooled to room temperature and filter through a Whatman paper. To prepare the final 4% PFA fixation solution (prepared each day freshly), 10 ml 20%-PFA stock-solution was mixed with 7.5 ml 20% (w/v) sucrose solution, 5 ml 10x PBS (Ca^{2+},Mg^{2+}-free) and 27.5 ml H_20 and prewarmed at 37 ºC. Cells were fixed in 4%-PFA for 12 minutes and then 4 times washed with PBS. Cells were permeabilized with 0.2% tritonX-100 in PBS for 10 minutes and then blocked with 3% normal goat serum, 0.5% BSA in PBS for 30 minutes. First antibodies were diluted in blocking solutions (Primary antibodies used included mouse anti-gelsolin monoclonal antibody (Becton Dickinson) 1:1000 and rabbit anti-synapsin I affinity-purified antibody (RBI) 1:200 and anti-GluR1 (from Anawa), 1:500) and subsequently 150 µl was dropped onto a coverslip that was put on parafilm. Various secondary antibodies were used, including FITC-conjugated goat anti-mouse (Jackson), TRITC-conjugated goat anti-mouse (Jackson) and Alexa488 labeled goat anti-rabbit (Molecular probes). F-actin was labeled with rhodamine phalloidin (Sigma). All antibodies were incubated between 1 and 2 hours at room temperature. Subsequently, coverslip was dipped in H_20 and mounted in Moviol.

5.5. Microscopy

Cultures of dispersed neurons were transfected with cDNA constructs and maintained in glia-conditioned, serum free medium for 19 to 28 days prior to imaging. Imaging was performed at 37°C in Tyrode's solution (119 mM NaCl, 5mM KCl, 25mM Hepes, 33mM Glucose, 2mM $MgCl_2$, 2mM $CaCl_2$ and 5µM Glycine; pH 7.3), with GFP-optimized filters (Chroma Technologies, Brattleboro, Vermont) and a MicroMax cooled CCD camera (Princeton Instruments, Trenton, New Jersey). For experiments involving glutamate receptor blockade, cells were preincubated for 15 minutes with the appropriate antagonist prior to stimulation. For calcium free experiment, cells were preincubated for 15 minutes in calcium-free tyrode's prior to application of chemicals in calcium-free tyrode's. Electric field stimulation patterns, as described, were programmed using a Master-8 pulse generator (AMPI, Jerusalem, Israel) and delivered via platinum electrodes in purpose built electrically isolated observation chamber (LIS, Olten, Switzerland). A stimulus of 900 action potentials delivered at 1 Hz to induce long-term depression (LTD), which leads to a robust and consistent induction of depression of synaptic transmission in dissociated cultures (Goda and Stevens, 1996; Carroll et al., 1999).

5.6. Image analysis and quantification

Activity-induced targeting of gelsolin-GFP to dendritic spines was assessed after the time-lapse recording. To assess the extent of stimulation-induced gelsolin-GFP accumulation in dendritic spines the fluorescence intensities of spine heads and of a circular spot of the same area in the underlying dendrite shaft was measured by integrating pixel intensities using MetaMorph, prior to and after stimulation. For each cell, 40 spines on a dendritic segment were examined. Spine outlines were generated from threshold images using an

edge-detection function of MetaMorph imaging software (Universal Imaging corporation, West Chester, Pennsylvania).

5.7. Source of active compounds

N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), 1,2,3,4-Tetrahydro-6-nitro-2, 3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), D(-)-2-amino-5-phosphonopentanoic acid (APV) from Alexis, (S)-3,5-dihydroxyphenylglycine ((S)-3,5-DHPG), (R,S)- α-methyl-4-carboxyphenylglycine (MCPG).

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7. List of Abbreviations

ADF	actin depolymerizing factor
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate
APV	D(-)-2-amino-5-phosphonopentanoic acid
ARP 2/3	actin related protein 2/3
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium
CaMKII	calcium/calmodulin-dependent kinase II
cAMP	cyclic adenosine monophosophate
CDC42	cell division cycle 42 (GTP-binding protein)
CICR	calcium induced calcium release
DHPG	(S)-3,5-dihydroxyphenylglycine
DIV	days in vitro
E19	embryonic day 19
F-actin	filamentous actin

GABA	gamma-aminobutyric acid
G-actin	globular actin
GFP	green florescent protein
GluR	glutamate receptor
G-protein	GTP-binding proteins
IP_3	inositol trisphosphate
KCl	potassium chloride
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotrophic glutamate receptors
Mg^{2+}	magnesium
MK-801	(5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10- imine hydrogen malate
Na^{+}	sodium
NMDA	N-methyl-D-aspartic acid
NR1	NMDA receptor 1
N-WASP	neuronal Wiskott-Aldrich Syndrome protein

PCR	polymerase chain reaction
PDZ domain	PSD-95/Discs large/zO-1 domain
PI3K	phosphatidyl inositol-3-kinase
PIP2	phosphatidyl inositol 4,5,bisphosphate
PIP5K	phosphatidylinositol-4-phosphate-5-kinase
PLC	phospholipase C
PP1	protein phosphotase 1
PP2	protein phosphotase 2
PSD	postsyaptic density
SAP 97	synapse-associated protein 97
SER	smooth endoplasmic reticulum
STP	short-term potentiation
VASP	vasodilator stimulated phosphoprotein
VCA	verprolin homology, cofilin homology and acidic domain
VDCC	voltage-dependent calcium channels

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Publications:

Zhao, PW., Matus, A. Gelsolin mediates activity-dependent regulation of actin assembly in dendritic spines. in preparation.

Wu-Deming, Zhao-Pingwei, Yan-Yunhua, Zheng-Chengrui and Dilana-Aishan, 1998, Analysis of Sex Pheromone Gland Extracts of the Cotton Bollworm in Xinjiang. Journal of Huazhong Agricultural University, 17(2): 111-116