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The Regulation of Dendritic Spine Plasticity by EphB and N-methyl-D-aspartate
Receptors Through Spatial Control Over Cofilin Activity in
Mature Hippocampal Neurons

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

Crystal Gayle Pontrello

December 2010

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The Dissertation of Crystal Gayle Pontrello is approved:

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Shi Y, Pontrello CG, DeFea KA, Reichardt LF, and Ethell IM (2009) Focal adhesion kinase acts downstream of EphB receptors to maintain mature dendritic spines by regulating cofilin activity. *J Neurosci* 29(25):8129-42.

The Corresponding author Dr. Iryna Ethell listed in these publications directed and supervised the research, which forms the basis for this dissertation. Dr. Yang Shi completed work on EphB receptor control of FAK and actin dynamics in the regulation of dendritic spine morphology and synapses, the results of which lead to the continuation of this project. Dr. Louis Reichardt (Professor, Department of Physiology, University of California, San Francisco) provided the fak knockout mice and contributed to the editing of the manuscript. Dr. Kathryn DeFea (Associate Professor, University of California, Riverside) provided her expertise on the regulation of cofilin activity by LIMK, beta-arrestin knockout mice, and reagents. She has also contributed to the interpretation of the results and editing of the manuscript.

Dedication

I would like to thank my advisor, Dr. Iryna Ethell, for her guidance, direction, and support in the completion of my dissertation research project. Her expertise in the field and our frequent discussions of recent relevant findings always led to exciting new directions for the project, which certainly kept things interesting! Her diligence in overseeing my project and her standard of excellence to which I always aspired provided the impetus that kept the work moving, and I always felt supported in my efforts. If I gave her a draft of a manuscript, she would revise it within hours. If I needed a motorized Z-stage for the microscope, she would buy it. Her involvement, feedback, and participation were constant, and so the potential for my project in her lab felt limitless. That is an incredible feeling to take into the lab everyday, especially when so many other factors in science are unpredictable. And in the midst of all the hard work and sleepless nights... it was a lot of fun! Thank you also to my Dissertation Committee members, Dr. Monica Carson and Dr. Todd Fiacco, for all the time spent advising me and providing feedback on my work, and my Qualifying Committee members Dr. Glenn Hatton, Dr. Peter Hickmott, and Dr. Christian Lytle. The time and effort of so many people has contributed to the completion of my research project here, and I thank all involved.

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ABSTRACT OF THE DISSERTATION

The Regulation of Dendritic Spine Plasticity by EphB and N-methyl-D-aspartate Receptors Through Spatial Control Over Cofilin Activity in Mature Hippocampal Neurons

by

Crystal Gayle Pontrello

Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, December 2010
Dr. Iryna M. Ethell, Chairperson

Dendritic spines are the postsynaptic sites of most excitatory synapses in the brain, and changes in their morphology are implicated in synaptic plasticity and long-term memory. F-actin dynamics are thought to be a basis for both the formation of dendritic spines during development and their structural plasticity (Ethell and Pasquale, 2005; Pontrello and Ethell, 2009). We have shown that the F-actin-severing protein cofilin, which is regulated by phosphorylation, can induce remodeling of mature dendritic spines in hippocampal neurons (Shi *et al.*, 2009). We also demonstrate that β -Arrestins play an important role in spatial control over cofilin activity in dendritic spines, which underlies NMDA-mediated dendritic spine remodeling. Cofilin activity in dendritic spines is regulated through CaMKII-mediated suppression of cofilin activity by phosphorylation, and calcineurin-dependent cofilin activation through its dephosphorylation. In addition, while the EphB receptor promotes spine stabilization through cofilin inactivation, the NMDA receptor prevents EphB-mediated cofilin inactivation through calcineurin.

NMDAR activation also promotes the translocation of cofilin to dendritic spines, an event that requires cofilin dephosphorylation and is also dependent on β -Arrestins, which have recently been shown to scaffold cofilin with its regulators, LIM kinase and slingshot phosphatase (Zoudilova *et al.*, 2010). Our studies demonstrate that cofilin clustering in the spines is affected in both β -Arrestin1- and β -Arrestin2- deficient neurons under normal synaptic activity, and a constitutively-active cofilin^{S3A} mutant fails to translocate to spines in response to NMDA in β -Arrestin2 KO neurons. Moreover, while wt neurons display dendritic spine remodeling in response to NMDA or with over-expression of cofilin^{S3A}, β -Arrestin2-deficient neurons are resistant to both NMDA-induced and cofilin^{S3A}-induced spine remodeling. In contrast, dominant-negative cofilin^{S3D} prevents NMDA-induced dendritic spine remodeling in wt neurons, and also rescues a mature spine phenotype that is lost in β -Arrestin1 KO neurons. In addition, over-expression of β -Arrestin in the KO neurons rescues spine abnormalities. β -Arrestin1-deficient neurons also develop immature spines *in vivo*, whereas hippocampal neurons lacking β -Arrestin2 develop normal mature spines, but fail to remodel in response to NMDA. Our studies demonstrate novel functions of β -Arrestin1 in the development of mature dendritic spines, and β -Arrestin2 in NMDAR-mediated dendritic spine plasticity through spatial control over cofilin activity.

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Abbreviations

ADF: actin-depolymerizing factor

ADP: adenosine diphosphate

AMPA: α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

A β : amyloid β

ATP: adenosine triphosphate

β -Arrestin: beta-Arrestin

cAMP: cyclic adenosine monophosphate

CIN: chronophin

DAG: diacylglycerol

DIV: days *in vitro*

E15: embryonic day 15

Eph receptor: erythropoietin-producing hepatocellular (Eph) receptor

EPSP: excitatory post-synaptic potential

ERK: extracellular signal-regulated kinase

F-actin: filamentous actin

FAK: Focal Adhesion kinase

G-actin: globular actin

γ -secretase: gamma-secretase

GPCR: G-protein-coupled receptor

GPI: glycosylphosphatidylinositol

GRK: G-protein-coupled receptor kinase

IP₆: D-myo-inositol hexakisphosphate

KO: knock-out

LIMK: Lin-11, Isl-1, Mec-3 kinase

LTD: long-term depression

LTP: long-term potentiation

MAPK: mitogen-activated protein kinase

MMP: matrix metalloproteinase

NGF: nerve growth factor

PAK: p21-activated kinase

PAR: protease-activated receptor

PI3K: phosphoinositide 3-kinase

PKA: protein kinase A

PSD: post-synaptic density

Chapter 1: Introduction

1.1 Dendritic spines and actin-binding proteins

Excitatory postsynaptic sites in the brain are usually formed on dendritic spines, small protrusions on the surface of dendrites that are highly enriched in filamentous actin (F-actin) (Rao & Craig, 2000; Sorra & Harris, 2000; Hering & Sheng, 2001; Yuste & Bonhoeffer, 2004; Ethell & Pasquale, 2005). Dendritic spines form and mature as synaptic connections develop in the brain, and their morphogenesis directly correlates with synapse formation and maturation. The most widely-held view of dendritic spine formation suggests that dendritic spines originate from dendritic filopodia-like protrusions both in vitro and in vivo (Dailey & Smith, 1996; Ziv & Smith, 1996; Maletic-Savatic *et al.*, 1999; Chen *et al.*, 2000; Marrs *et al.*, 2001; Okabe *et al.*, 2001; Trachtenberg *et al.*, 2002; Portera-Cailliau *et al.*, 2003; Ziv & Garner, 2004; Knott *et al.*, 2006). Although mature dendritic spines can also emerge from the dendritic shaft, it is widely accepted that long, thin filopodia-like morphologies are features of immature precursors to spines, while mature spines are characterized by mushroom-like or stubby shapes (Lippman & Dunaevsky, 2005; Matus, 2005; Tada & Sheng, 2006). Several studies have demonstrated a correlation between dendritic spine morphology and synaptic function (Matsuzaki *et al.*, 2001; Murthy *et al.*, 2001; Smith *et al.*, 2003; Lang *et al.*, 2004; Matsuzaki *et al.*, 2004; Nimchinsky *et al.*, 2004; Ashby *et al.*, 2006; Nicholson *et al.*, 2006). While immature thin spines with smaller heads are flexible and can rapidly enlarge or shrink in response to changes in synaptic activity (Matsuzaki *et al.*, 2001;

Murthy *et al.*, 2001; Smith *et al.*, 2003; Nicholson *et al.*, 2006), mature mushroom-shaped spines with larger heads are less likely to change, but show higher sensitivity to glutamate than immature thin spines (Matsuzaki *et al.*, 2001; Murthy *et al.*, 2001; Smith *et al.*, 2003; Nicholson *et al.*, 2006). These differences in synaptic strength are suggested to relate to the number of neurotransmitter receptors. Mature spines have a large spine head area that is proportional to postsynaptic density (PSD) and synapse size, as well as receptor complement (Fig. 1-1). Two-photon glutamate uncaging studies have shown that the mature mushroom-like spines have large PSDs with a high number of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Matsuzaki *et al.*, 2001). Indeed, numbers of AMPA receptors and N-methyl-D-aspartate (NMDA) receptors directly correlate with PSD size (Takumi *et al.*, 1999; Racca *et al.*, 2000). Spine head volume and PSD size are also proportional to the number of neurotransmitter-containing vesicles at the presynaptic bouton (Schikorski & Stevens, 1997) and the amount of neurotransmitter released at the synapse (Murthy *et al.*, 1997). Mature dendritic spines also contain elements of endoplasmic reticulum, called the spine apparatus, and provide means for compartmentalization within the neuron, which results in localized control over factors such as intracellular Ca^{2+} concentration (Llinas *et al.*, 1992) and local protein synthesis (Tanaka *et al.*, 2008).

Dendritic spines remain plastic in the adult brain and can rapidly grow, change, or collapse in response to normal physiological changes in synaptic activity that underlies learning and memory. Long-term potentiation (LTP) can result in spine head enlargement (Lang *et al.*, 2004; Matsuzaki *et al.*, 2004), whereas long-term depression (LTD) has been

reported to induce shrinkage of dendritic spine heads and spine elimination (Zhou *et al.*, 2004). Synaptic activity was also shown to shape dendritic spines in area CA1 of the hippocampus (Maletic-Savatic *et al.*, 1999), and long-lasting synaptic enhancement leads to new spine development (Engert & Bonhoeffer, 1999). Although changes in synaptic activity may not always lead to structural remodeling (Sorra & Harris, 1998), experience-dependent motility of spines and change in their morphology has been reported using various learning paradigms in developing rat barrel cortex (Lendvai *et al.*, 2000), motor cortex (Kleim *et al.*, 1998), and hippocampus (Geinisman *et al.*, 2000; Leuner *et al.*, 2003). Pathological stimuli can also lead to changes in dendritic spine shape and number in the developing and adult brain. Immature dendritic spine profiles are found in subjects with Fragile X, Down syndrome, and Rett syndrome, whose brains exhibit many long, thin spines and filopodia-like protrusions (Rudelli *et al.*, 1985; Kaufmann & Moser, 2000). Moreover, dendritic spine loss is a hallmark of several neurodegenerative diseases, and may also contribute to impaired brain function in these diseases (Halpain *et al.*, 2005). The ability of spines to rapidly turnover can lead to recovery from damage (Cotman & Nieto-Sampedro, 1984) and stressors such as sleep deprivation (Chen *et al.*, 2009).

Many molecular signals that control the changes in dendritic spine morphology act through the regulation of F-actin, some through direct interaction with actin, and others via downstream effectors (Ethell & Pasquale, 2005; Pontrello & Ethell, 2009). Actin dynamics at the leading edge in motile cells (Pollard *et al.*, 2001) and in growth cones (Lowery & Van Vactor, 2009) control cell locomotion and axon pathfinding, respectively. There is also a tight regulation of actin dynamics within dendritic spines and

filopodia-like protrusions. While the stabilization of mature spines is required for synapse maintenance, F-actin remodeling underlies synaptic plasticity and may result in spine turnover, triggering both formation of new spines and filopodia, as well as pruning of existing spines (Smart & Halpain, 2000; Kolb *et al.*, 2008). As globular actin (G-actin) monomers join together to form actin filaments (F-actin), there is a bidirectional polymerization that is biased toward what is known as the plus end or barbed end (Woodrum *et al.*, 1975). In this way, the F-actin barbed end is fast-growing, while the opposite pole of the filament, the minus end or pointed end, elongates more slowly. The constant turnover of the actin filaments in spines results from a steady-state of actin treadmilling, in which G-actin monomers are added quickly to the barbed end and are disassembled from the pointed end of F-actin filaments, while exchanging ATP for ADP (Kirschner, 1980; Stossel, 1993; Bindschadler *et al.*, 2004; Okamoto *et al.*, 2004). However, this F-actin treadmilling may or may not lead to changes in dendritic spine shape and size.

Actin assembly regulatory proteins are responsible for shifting the balance between spine assembly and disassembly that is required for normal synaptic function. Some regulate spine morphology through direct interaction with actin, while others control actin dynamics indirectly (Zhang & Benson, 2000; Sekino *et al.*, 2007; Sala *et al.*, 2008; Pontrello & Ethell, 2009). These proteins may regulate actin polymerization, branching, cross-linking and bundling, or severing and depolymerization. By affecting actin dynamics, actin-binding proteins have the ability to form, re-arrange, stabilize, or

remodel dendritic spines. These proteins also aid in clustering postsynaptic proteins and in regulating neurotransmitter receptor activities to create functional synaptic connections.

Some proteins contribute to the elongation of dendritic spines through bundling and cross-linking of actin networks. For example, α -actinin can form short branched actin filaments or elongate existing ones, depending on certain conditions and factors such as the α -actinin/actin ratio, Ca^{2+} concentration, and binding partners (Grazi *et al.*, 1991; Wachsstock *et al.*, 1993; Sjoblom *et al.*, 2008). Drebrin A bundles actin into thick winding fibers (Shirao *et al.*, 1994), and has been implicated in the development of dendritic spines and synapses (Aoki *et al.*, 2005; Mizui *et al.*, 2005; Takahashi *et al.*, 2009). The Actin-Related Protein (Arp) 2/3 complex (Fig. 1-2) is responsible for nucleating branches from the sides of existing actin filaments and capping the pointed ends, thus creating additional fast-growing barbed ends for further actin polymerization and elongation (Mullins *et al.*, 1997; Welch *et al.*, 1997; Pantaloni *et al.*, 2000; Amann & Pollard, 2001; Pollard, 2007). In maintaining the balance between assembly and disassembly of actin filaments, there are some proteins and factors that compete against the Arp2/3 complex, while others work in cooperation (Fig. 1-3). Cortactin is a protein that promotes branching and stabilization of actin filaments by binding and activating the Arp2/3 complex (Urano *et al.*, 2001; Weaver *et al.*, 2001; Ammer & Weed, 2008). Phosphorylation of the Arp2/3 complex, which is necessary for its nucleating activity and cellular localization (LeClaire *et al.*, 2008), can be achieved by such proteins as MAPK-activated protein kinase 2 (MAPKPK2) (Singh *et al.*, 2003) and the p21-activated kinase (PAK) (Vadlamudi *et al.*, 2004). Proteins of the Wiskott-Aldrich Syndrome protein

(WASP) family, Neural-WASP (N-WASP), Scar, and the WASP-family verprolin-homologous protein (WAVE), are well-known to bind to and activate the Arp2/3 complex (Takenawa & Suetsugu, 2007), each stimulating a different rate of actin nucleation (Zalevsky *et al.*, 2001). Tropomyosin inhibits the branching activity of Arp2/3 (Blanchoin *et al.*, 2001), and ATP hydrolysis also acts to antagonize its activity by de-branching older actin filaments (Le Clainche *et al.*, 2003).

In contrast to the actin-polymerizing activity of α -actinin, drebrin, and Arp2/3, some actin-regulatory proteins like ADF/cofilin and gelsolin work by severing F-actin filaments, which can result in spine elongation and remodeling by increasing actin filament turnover, or lead to spine stabilization through gelsolin capping activity. In addition, ADF/cofilin dissociates Arp2/3 from actin filaments (Chan *et al.*, 2009), but also uses its severing activity to increase the number of preferred ends for Arp2/3 nucleation (Ichetovkin *et al.*, 2002), and Arp2/3 cooperates with gelsolin in that it polymerizes actin in the presence of gelsolin-capped filaments (Ressad *et al.*, 1999).

Some proteins such as profilin (Fig. 1-4) have multiple functions in dendritic spines and promote opposing effects on actin (Theriot & Mitchison, 1993), depending on several factors and conditions, such as its subcellular localization and intracellular Ca^{2+} levels (Faivre-Sarrailh *et al.*, 1993; Ackermann & Matus, 2003; Neuhoff *et al.*, 2005; Birbach *et al.*, 2006), interactions with other proteins or phospholipids (Goldschmidt-Clermont *et al.*, 1986; Sohn *et al.*, 1995; Giesemann *et al.*, 2003; Reeve *et al.*, 2005; Ferron *et al.*, 2007; Krishnan *et al.*, 2009), and phosphorylation state (Vemuri & Singh, 2001; Sathish *et al.*, 2004; Shao *et al.*, 2008). Spinophilin (Allen *et al.*, 1997) and

neurabin I (Nakanishi *et al.*, 1997) are two proteins that are similar in structure and activity, but exert opposing effects on dendritic spines through yet unknown mechanisms (Sato *et al.*, 1998; Feng *et al.*, 2000; Oliver *et al.*, 2002; Wu *et al.*, 2008). In addition to displaying actin-bundling activity, spinophilin participates in targeting PP1 to the postsynaptic membrane (Allen *et al.*, 1997; Hsieh-Wilson *et al.*, 1999), which allows PP1 to dephosphorylate its synaptic substrates (Fig. 1-5). In this way, spinophilin can indirectly modify the activities of AMPA and NMDA receptors, two known substrates of PP1. *In vivo*, spinophilin also takes part in the anchoring of AMPA receptors to the plasma membrane and promotes their dephosphorylation through PP1 (Yan *et al.*, 1999). Similarly, the myosin motor proteins, which induce formation of short mushroom-shaped spines by enhancing actomyosin contractility (Ryu *et al.*, 2006), are also involved in modulation of NMDA and AMPA receptor membrane insertion and function (Ampanan *et al.*, 2005; Osterweil *et al.*, 2005; Correia *et al.*, 2008; Z Wang *et al.*, 2008).

The actin-binding proteins that contribute to dendritic spine dynamics are regulated by signaling cascades that can be initiated at the cell surface through trans-synaptic interactions, neuron-glia communications, and contacts with the extracellular matrix (Ethell & Pasquale, 2005). Cell surface receptors such as glutamate receptors, EphB receptors and ephrins, neuroligins and neuexins, integrins, cell adhesion molecules, growth factor receptors, and some proteoglycans, mediate these interactions linking extracellular events to the actin cytoskeleton in dendritic spines by initiating cytoplasmic signaling cascades. Cytoplasmic signaling proteins such as PIP₂ exhibit diverse control through a variety of mechanisms, from promoting α -actinin activity through direct

binding, to activating Arp2/3 through WASP, to dissociating the profilin-actin (profilactin) complex from actin. The Rho family of small GTPases, such as Rac1, Cdc42, and RhoA, are also key regulators of actin-binding proteins in dendritic spines and play an important role in dendritic spine formation, maintenance, and remodeling. In addition to the structural role of actin in dendritic spines, actin assembly was recently suggested to influence neuronal motility through a regulation of gene transcription (Stern *et al.*, 2009). This may have implications for a role of actin-binding proteins in regulating gene transcription and protein synthesis in spines.

Dendritic spine dynamics have been implicated in processes of learning and memory (Segal, 2005; Newpher & Ehlers, 2009), and abnormalities in the shape and number of spines are seen in neurodegenerative diseases, as well as some forms of mental retardation and the autistic spectrum disorders (Kaufmann & Moser, 2000; Halpain *et al.*, 2005). It is the dynamic interplay among actin-binding proteins, cell-surface receptors and their downstream effectors, and regulatory factors such as intracellular Ca^{2+} concentration, cellular localization, and protein phosphorylation state that defines the rate of growth or disassembly of actin. Ultimately, it is the actin dynamics that determine the morphology of dendritic spines and development of synapses, which underlies physiological as well as pathological conditions.

1.2 Cofilin

Cofilin is an actin-binding protein that has F-actin severing and depolymerizing activity, both actions leading to an increase in the G-actin monomer pool within the cell

(Moriyama *et al.*, 1990; Yahara *et al.*, 1996; Carlier *et al.*, 1997; Lappalainen & Drubin, 1997; Rosenblatt *et al.*, 1997; Bamburg, 1999; Bamburg *et al.*, 1999). Cofilin binds preferentially to ADP-actin (Carlier *et al.*, 1997; Maciver, 1998; Ressad *et al.*, 1999) in a 1:1 ratio and introduces a twist in F-actin filaments, thus severing them (Nishida, Maekawa Sakai, 1984; McGough *et al.*, 1997; Maciver & Hussey, 2002) (Fig. 1-6). Cofilin increases the turnover rate of F-actin by severing the filaments, which creates new barbed ends for continued F-actin assembly and growth. In this process, which is a part of actin “treadmilling,” F-actin fibers are disassembled slowly from the pointed or minus end, while G-actin monomers are added more rapidly to the barbed or plus end, leading to an overall elongation and growth of the F-actin filaments. Cofilin increases the off-rate of actin monomers from F-actin pointed ends (Carlier *et al.*, 1997), and can also bind and sequester monomeric actin (Lappalainen & Drubin, 1997).

A member of the actin-depolymerizing factor (ADF)/ cofilin family that forms “co-filamentous structures” with actin, cofilin is a 21 kD protein that was first isolated from porcine brain (Nishida, Maekawa, Muneyuki *et al.*, 1984), following several isolations of ADF from various sources (Bamburg *et al.*, 1980; Harris *et al.*, 1980; Berl *et al.*, 1983). Cofilin has an actin-binding domain and another highly conserved domain that has been suggested to be an important binding site for another protein. Several studies have identified sites that are necessary for cofilin-actin binding and actin severing (Moriyama *et al.*, 1990; Fedorov *et al.*, 1997; Lappalainen *et al.*, 1997; Moriyama & Yahara, 1999; Moriyama & Yahara, 2002). The activity of cofilin is regulated by phosphorylation on its serine-3 residue, which can be achieved by Lin-11, Isl-1, Mec-3

kinases (LIMK) and testicular kinase (TESK), leading to inactivation of cofilin, while dephosphorylation and activation can occur through slingshot (SSH) and chronophin (CIN) phosphatases (Mizuno *et al.*, 1994; Agnew *et al.*, 1995; Arber *et al.*, 1998; Yang *et al.*, 1998). Intracellular second messengers or cell surface receptors can influence the phosphorylation state of cofilin. An increase in Ca^{2+} or cyclic adenosine monophosphate (cAMP) leads to an increase in cofilin activity through dephosphorylation, as does treatment with nerve growth factor (NGF) or insulin, the latter also promoting cofilin translocation to ruffling membranes (Meberg *et al.*, 1998). We have seen in our studies that cell surface receptors such as EphB (Shi *et al.*, 2009) or NMDA receptors can induce intracellular signaling cascades that lead to the inactivation or activation of cofilin through affecting its phosphorylation. EphB receptor activation promotes cofilin phosphorylation in a RhoA-LIMK-dependent manner that leads to stabilization of mature dendritic spines (Shi *et al.*, 2009), whereas NMDA receptor activation leads to spine remodeling (Shi & Ethell, 2006), which may be mediated through calcineurin-SSH or phosphoinositide 3-kinase (PI3K)- SSH pathways.

In addition to phosphorylation state, cofilin can be regulated by pH (Yonezawa *et al.*, 1985; Hawkins *et al.*, 1993; Hayden *et al.*, 1993; Carlier *et al.*, 1999). While cofilin is still able to bind to F-actin, it displays weaker activity at pH less than 7.1. Phosphatidylinositol (4,5)-bisphosphate binds cofilin directly, inhibiting cofilin/ F-actin interaction (Yonezawa *et al.*, 1991; Kusano *et al.*, 1999), while tropomyosin can inhibit cofilin in another way, through the stabilization of actin filaments (Ono & Ono, 2002; Kuhn & Bamberg, 2008). The spatial regulation of cofilin within the cell also leads to

promotion or prevention of its activity. PIP, PIP₂, and the 14-3-3 protein can bind cofilin and sequester it to specific areas of the cell, inhibiting its activity (Fu *et al.*, 2000). β -Arrestins are scaffolding proteins that control the cellular localization of cofilin and its regulators, LIMK and SSH or CIN phosphatase (Zoudilova *et al.*, 2007; Zoudilova *et al.*, 2010). In this way, β -Arrestins control the activity state of cofilin and its distribution throughout the cell.

Cofilin is concentrated in ruffling membranes (Yonezawa *et al.*, 1987; Obinata *et al.*, 1997), at the leading edge of migrating cells (DesMarais *et al.*, 2005), and in areas of the cell where actin is dynamic (Bamburg & Bray, 1987; Nagaoka *et al.*, 1996; Obinata *et al.*, 1997; Racz & Weinberg, 2006). Cofilin controls cell polarity (Dawe *et al.*, 2003; Garvalov *et al.*, 2007), as well as growth cone and filopodial dynamics (Kuhn *et al.*, 2000; Gungabissoon & Bamburg, 2003; Fass *et al.*, 2004; Gehler *et al.*, 2004). Cofilin is localized in dendritic spines to a specific area of dynamic F-actin called the “shell” (Racz & Weinberg, 2006), suggesting its role in regulation of actin and spine dynamics. Our studies have shown that over-expression in mature hippocampal neuronal cultures of constitutively-active cofilin^{S3A}, but not dominant-negative cofilin^{S3D}, leads to elongation of spines and development of immature filopodia (Shi *et al.*, 2009), and knock-down of cofilin with siRNA was shown to promote immature spines and abnormal filopodia (Hotulainen *et al.*, 2009). LIMK knock-out (KO) or inhibition of LIMK translation using microRNA leads to immature spines with small heads (Meng *et al.*, 2002; Schrott *et al.*, 2006), suggesting that LIMK and its downstream substrate cofilin are both essential regulators of dendritic spine maintenance. NMDA receptor activation leads to transient

spine remodeling (Shi & Ethell, 2006) and cofilin activation (Carlisle *et al.*, 2008), followed by spine stabilization and cofilin inactivation. Long-term potentiation (LTP) promotes an increase in mature dendritic spines and synapses, which is dependent on LIMK-dependent cofilin inactivation (Lisman, 2003; Chen *et al.*, 2007), whereas long-term depression (LTD) leads to cofilin-dependent spine shrinkage (Zhou *et al.*, 2004). The activity state of cofilin seems to modulate the diverse effects on dendritic spines of NMDA and AMPA receptor activation, as well as promotion of LTP or LTD that are thought to underlie learning and memory processes.

Under conditions of cellular stress, cofilin translocates to the nucleus in a dephosphorylation-dependent manner (Matsuzaki *et al.*, 1988; Nebl *et al.*, 1996; Minamide *et al.*, 2000), where it forms rod-like inclusions similar to those found in Alzheimer brains (Nishida *et al.*, 1987; Bamberg, 1999; Davis *et al.*, 2009). Indeed, these cofilin-saturated actin structures can be induced by amyloid β (A β) application and are found in close proximity to the senile plaques that are hallmark structures of Alzheimer disease (Maloney & Bamberg, 2007; Bamberg & Bloom, 2009). Dominant-negative cofilin^{S3D} has been shown to have protective effects against A β -induced dendritic spine loss (Shankar *et al.*, 2007), further implicating cofilin in this pathology. Hirano bodies are another example of aberrant cofilin-rich structures that are found in the aging brain, especially in those with cognitive impairment (Hirano, 1994; Maciver & Harrington, 1995; Bamberg & Wiggan, 2002). Cofilin is a powerful modulator of many diverse cellular activities, and malfunction of its activity or localization can lead to neurological or developmental disorders.

1.3 Eph Receptor Tyrosine Kinases

Eph receptors are named for their expression in an Erythropoietin-Producing human Hepatocellular carcinoma cell line and are the largest family of cell-surface receptor tyrosine kinases that mediate cell-cell adhesion/ repulsion (Flanagan & Vanderhaeghen, 1998; Mellitzer *et al.*, 2000; Wilkinson, 2001; Pasquale, 2005), thereby spatially regulating cells and determining whether they will contact and interact with one another. The ephrin ligand is also membrane-bound, requiring cell-cell contact for Eph-ephrin interaction and signaling through the receptor and/ or ligand. There are nine mammalian EphA receptors that can promiscuously bind five different glycosylphosphatidylinositol (GPI)-linked ephrinA ligands, and there are five EphB receptors that can bind any of three transmembrane ephrinB ligands. EphA4 can also bind ephrinBs, and EphB receptors can bind to ephrinA5 (Pasquale, 2005).

Eph-ephrin interaction and signaling can be bi-directional (Kullander & Klein, 2002; Murai & Pasquale, 2003; Noren & Pasquale, 2004). Forward-signaling commences when tyrosine kinase activity is promoted in the Eph-containing cell, and reverse-signaling involves Src-dependent signaling in the ephrin-expressing cell. In the central nervous system, while canonical Eph-ephrin signaling involves pre-synaptic ephrins binding and activating post-synaptic Eph receptors, which is important in spine and synapse formation (Irie & Yamaguchi, 2002; Klein, 2009), Ephs and ephrins can be located both pre- and post-synaptically and can interact laterally. This can be inhibitory, but Ephs/ ephrins were shown in growth cones to have the ability to spatially segregate

into microdomains in order to avoid these inhibitory interactions (Marquardt *et al.*, 2005). Following Eph-ephrin signaling, termination of the signal is achieved through dephosphorylation, or internalization and degradation (Marston *et al.*, 2003; Zimmer *et al.*, 2003; Lin *et al.*, 2008). Eph receptors also regulate intracellular signaling cascades through their tyrosine kinase activity. The EphB receptor modulates FAK activity by complexing with FAK, Src, paxillin, and Grb2, which leads to activation of FAK, Src, paxillin, and the downstream effector RhoA (Moeller *et al.*, 2006). Moreover, Ephs or ephrins can be cleaved by gamma-secretase (γ -secretase) following matrix metalloproteinase (MMP) cleavage, leaving an intracellular fragment with signaling capabilities (Hattori *et al.*, 2000; Georgakopoulos *et al.*, 2006; Litterst *et al.*, 2007; Lin *et al.*, 2008; Inoue *et al.*, 2009).

Diverse cellular functions that rely on cell responses to attractive and repulsive guidance cues are mediated by Eph receptors. Processes such as cell migration and membrane ruffling (Nagashima *et al.*, 2002), platelet aggregation (Prevost *et al.*, 2002), and cell differentiation and proliferation (Batlle *et al.*, 2002; Aoki *et al.*, 2004), are promoted by Eph receptors. The role of Eph receptors in tumor angiogenesis and cancer has also been well-studied (McCarron *et al.*, 2010; Mosch *et al.*, 2010; Pasquale, 2010). During central nervous system development, Eph receptors regulate axon pathfinding, fasciculation, and pruning (Hattori *et al.*, 2000; Yates *et al.*, 2001; Coulthard *et al.*, 2002; Xu & Henkemeyer, 2009), and development of projections such as those in the olfactory system and visual system (Flanagan & Vanderhaeghen, 1998; Hindges *et al.*, 2002; Mann *et al.*, 2002; Dufour *et al.*, 2003). In addition, dendritic spine morphogenesis is regulated

by the Eph receptor, as EphB inhibition disrupts spine maturation (Ethell *et al.*, 2001), and EphB1, B2, and B3 triple knock-out mice display more immature dendritic spines and filopodia than do wt mice (Henkemeyer *et al.*, 2003).

In addition to their roles in development of dendritic spines, Eph receptors also regulate spine dynamics in mature neurons (Lai & Ip, 2009). One mechanism is through MMPs that regulate EphB-ephrinB2-mediated cell repulsion, which is achieved through EphB receptor cleavage (Lin *et al.*, 2008). MMP-7 and MMP-9 were shown to induce remodeling of dendritic spines, leading to an increase of immature spines and filopodia that is blocked by NMDA receptor antagonists (Bilousova *et al.*, 2006; Ethell & Ethell, 2007; Bilousova *et al.*, 2009). In contrast, other studies have shown that under different circumstances, MMP-9 can promote β 1 integrin-dependent dendritic spine enlargement and LTP, which depends on cofilin inactivation (XB Wang *et al.*, 2008). Indeed, MMPs have been implicated in spatial learning, memory, and LTP (Meighan *et al.*, 2006; Nagy *et al.*, 2006), which could be controlled differentially through MMP regulation of Eph receptors, NMDA receptors, and integrins. In addition to MMP-mediated dendritic spine regulation, EphB receptor activation of tyrosine kinase activity in hippocampal neurons can lead to the maintenance of mature dendritic spines through recruitment and activation of FAK, which promotes a signaling cascade that results in LIMK-mediated cofilin inactivation (Shi *et al.*, 2009) (Fig. 1-7). On the other hand, EphB receptors can potentiate NMDA receptors (Dalva *et al.*, 2000; Grunwald *et al.*, 2001; Takasu *et al.*, 2002), which promote transient dendritic spine remodeling (Shi & Ethell, 2006), followed by spine stabilization. Eph receptors and their ephrin ligands mediate cell-cell adhesion/

repulsion events, as well as intracellular signaling cascades through their receptor tyrosine activity. Acting alone or in cooperation with other receptors such as integrins or NMDA receptors, Eph receptors are able to differentially mediate cytoskeletal changes that underlie actin dynamics and dendritic spine remodeling.

1.4 N-methyl-D-aspartate (NMDA) Receptors

NMDA receptors are ionotropic glutamate receptors that allow Ca^{2+} and Na^+ ions into the cell in a voltage-dependent manner (Malenka & Bear, 2004; Traynelis *et al.*, 2010). When depolarization of the cell causes a relief of the Mg^{2+} ion that occludes the NMDAR pore at resting membrane potential, glutamate or NMDA will cause opening of the receptor in the presence of the cofactor glycine. NMDA receptors are comprised of four pore-forming subunits, which consist of two NR1 subunits and either two NR2s, or a NR2 and a NR3 subunit. In the adult rat hippocampus, NR2A and NR2B are the most common NR2 subunits (Laurie *et al.*, 1997; Wenzel *et al.*, 1997). It has been reported that the dominant subunit in the NMDA receptor switches from NR2B to NR2A in some areas of the brain during development (XB Liu *et al.*, 2004), which is important because the subunit composition determines receptor gating and pharmacological properties, to which intracellular signaling events the receptor is linked, and whether LTP or LTD will be induced upon NMDA receptor activation (Monyer *et al.*, 1994; Hrabetova *et al.*, 2000; Sheng & Pak, 2000; Kohr *et al.*, 2003; L Liu *et al.*, 2004). LTP and LTD are thought to underlie learning and memory, and there are forms of both that require NMDA

receptor activation (Bliss & Collingridge, 1993; Bear & Malenka, 1994; Malenka & Nicoll, 1999).

It is the amount of Ca^{2+} that enters the neuron following NMDA receptor activation that will determine which intracellular kinases and phosphatases are active, and whether LTP or LTD is produced (Cummings *et al.*, 1996; Nishiyama *et al.*, 2000). NMDA receptor activation that leads to high levels of Ca^{2+} influx activates calcium/calmodulin-dependent protein kinase II (CaMKII) through calmodulin activation, leading to AMPA receptor insertion into the cell membrane, where they can be activated by glutamate and allow more Na^+ to flux into the cell. Phosphorylation by CaMKII of a specific AMPA receptor serine residue is associated with LTP (Barria *et al.*, 1997; Lee *et al.*, 2000). Modulating factors such as cofilin (Gu *et al.*, 2010; Rust *et al.*, 2010), which can promote re-arrangement of the actin cytoskeleton for AMPA receptor cycling into and out of the membrane (Luscher *et al.*, 1999), and PKA (Esteban *et al.*, 2003), which can phosphorylate and target AMPA receptors for insertion, can also regulate AMPA receptor trafficking and synapse localization. In this way, NMDA and AMPA receptors can promote strengthening of synapses, stabilization of mature dendritic spines, and LTP (Bliss & Collingridge, 1993; Lisman & Zhabotinsky, 2001).

A type of LTD can be produced that is dependent on NMDA receptor-induced increase in intracellular Ca^{2+} (Dudek & Bear, 1992; Mulkey & Malenka, 1992; Kandler *et al.*, 1998; Lee *et al.*, 1998; Kamal *et al.*, 1999; Li *et al.*, 2004). This LTD is achieved when low levels of Ca^{2+} flux into the cell, leading to AMPA receptor internalization (Beattie *et al.*, 2000; Man *et al.*, 2000; Morishita *et al.*, 2005). This happens

predominantly through a pathway that involves Ca^{2+} -mediated activation of protein phosphatases rather than CaMKII (Mulkey *et al.*, 1993; Kirkwood & Bear, 1994; Mulkey *et al.*, 1994; O'Dell & Kandel, 1994). Indeed, internalized AMPA receptors have been shown to be dephosphorylated at the GluR1 serine-845 residue (Ehlers, 2000), an event that also decreases AMPA receptor open probability (Banke *et al.*, 2000). Calcineurin is a protein phosphatase that is critical for LTD (Torii *et al.*, 1995; Winder & Sweatt, 2001). Calcineurin phosphatase participates in LTD through interaction with dynamin-1, which is necessary for clathrin-mediated endocytosis (Lai *et al.*, 1999). In addition, the actin-remodeling protein cofilin that has recently been implicated in AMPA receptor trafficking (Gu *et al.*, 2010; Rust *et al.*, 2010) is a substrate for SSH phosphatase, which is activated by calcineurin. Calcineurin promotes AMPA receptor internalization and LTD through a variety of mechanisms, including the regulation of the cell cytoskeleton through cofilin.

There is evidence that Ca^{2+} -induced LTP and LTD of NMDAR-mediated EPSPs is mediated by cytoskeletal changes (Lisman, 2003; Matus, 2005; Morishita *et al.*, 2005), such as the actin dynamics underlying dendritic spine plasticity. Indeed, NMDA receptors have been implicated in regulating dendritic spines dynamics (Hosokawa *et al.*, 1995; Engert & Bonhoeffer, 1999; Maletic-Savatic *et al.*, 1999; Toni *et al.*, 1999). Most dendritic spines of excitatory synapses in the brain contain NMDA receptors, but some do not contain AMPA receptors and are known as silent synapses (Kullmann, 1994; Isaac *et al.*, 1995; Durand *et al.*, 1996; Montgomery *et al.*, 2001; Kerchner & Nicoll, 2008). When NMDA receptor activation leads to AMPA receptor insertion into the membrane

and LTP, or AMPA receptor internalization and LTD, changes in dendritic spines are also seen. LTP promotes a transient remodeling of dendritic spines (Shi & Ethell, 2006), followed by an enlargement of spines and post-synaptic densities, as well as growth of new spines (Yuste & Bonhoeffer, 2001; Abraham & Williams, 2003; Matsuzaki *et al.*, 2004), and cofilin inactivation by phosphorylation is necessary for the stabilization of mature dendritic spines that is seen following LTP induction (Chen *et al.*, 2007). On the other hand, LTD induces dendritic spine shrinkage (Zhou *et al.*, 2004), which could be achieved through a calcineurin-slingshot phosphatase pathway (Huang *et al.*, 2006) that would lead to the dephosphorylation and activation of cofilin (Fig. 1-6), as the over-expression of active cofilin has been shown to induce dendritic spine remodeling and an increase in immature spines and filopodia (Shi *et al.*, 2009). The transient remodeling and subsequent stabilization of dendritic spines with LTP and the shrinkage of spines and AMPA receptor internalization with LTD are dependent on a tight regulation of cofilin activity. In addition, EphB receptors have been shown to inactivate cofilin through a FAK-RhoA-LIMK pathway, promoting stabilization of mature dendritic spines (Shi *et al.*, 2009); however, EphB receptors are also known to associate with and potentiate NMDA receptors (Dalva *et al.*, 2000; Grunwald *et al.*, 2001; Takasu *et al.*, 2002; Salter & Kalia, 2004), and NMDA receptor activation leads to a transient dephosphorylation and activation of cofilin (Carlisle *et al.*, 2008). Activation of both receptors concurrently leads to a different effect on cofilin and dendritic spines than activation of each receptor alone (Chapter 3).

NMDA receptor activation can lead to cofilin-mediated changes in dendritic spine morphology that support LTP or LTD processes that underlie learning and memory, whereas aberrant NMDA receptor activity has been linked to several diseases. An excess of Ca^{2+} influx into the cell through NMDA receptors can lead to excitotoxicity and disorders such as epilepsy and stroke (Lee *et al.*, 1999), and glutamate excitotoxicity in dementia and Alzheimer disease has been targeted in clinical trials by memantine, a NMDA receptor inhibitor (Doraiswamy, 2003). NMDA receptor malfunction has also been linked to schizophrenia (Mohn *et al.*, 1999; Rowley *et al.*, 2001) and Huntington's disease (Zeron *et al.*, 2002). The NMDA receptor mediates many cell processes through the regulation of Ca^{2+} influx, which leads to differential activation of protein kinases and phosphatases, ultimately determining dendritic spine dynamics that underlie LTP and LTD, and learning and memory processes.

1.5 β -Arrestins

β -Arrestins are known to be involved in the desensitization and internalization of seven-transmembrane receptors, also known as G-protein-coupled receptors (GPCRs), such as adrenoceptors, muscarinic acetylcholine receptors, rhodopsin, protease-activated receptors (PARs), mu opioid receptors, and dopaminergic receptors (Lohse *et al.*, 1990; Lefkowitz *et al.*, 1992; Bunemann & Hosey, 1999; Ferguson, 2001; Luttrell & Lefkowitz, 2002; DeWire *et al.*, 2007; Nelson *et al.*, 2007; Defea, 2008; Dang *et al.*, 2009; Soh *et al.*, 2010). G-protein-coupled receptor kinases (GRKs) phosphorylate the agonist-activated GPCRs (Bunemann & Hosey, 1999), and β -Arrestins subsequently bind and physically

inhibit the receptors from interacting further with G-proteins in an event known as homologous GPCR desensitization. β -Arrestins were first discovered when it became clear that something in addition to phosphorylation was also necessary to inactivate GPCRs (Pfister *et al.*, 1985; Lohse *et al.*, 1990; Attramadal *et al.*, 1992). Following desensitization, GPCRs are endocytosed into clathrin-coated vesicles and then either re-sensitized and recycled to the cell surface or sent to lysosomes for degradation (Pippig *et al.*, 1995; Yu *et al.*, 1997; Zhang *et al.*, 1997). β -Arrestins also participate in this process by binding to elements of clathrin-coated pits and targeting the GPCRs for endocytosis (Zhang *et al.*, 1996; Goodman *et al.*, 1997; Krupnick *et al.*, 1997; Laporte *et al.*, 1999; Laporte *et al.*, 2000). In addition to interacting with and inactivating GPCRs, β -Arrestins also promote degradation of second messengers such as diacylglycerol (DAG) that are activated downstream of GPCRs (Nelson *et al.*, 2007). Through multiple mechanisms, β -Arrestins participate in the cessation of GPCR signaling.

β -Arrestins can be regulated by phosphorylation. β -Arrestin1, which can be phosphorylated on its serine-412 by extracellular signal-regulated kinases (ERKs) (Lin *et al.*, 1999), exists mostly in the phosphorylated state in the cytoplasm and is quickly dephosphorylated upon recruitment to GPCRs (Lin *et al.*, 1997). While translocation to the cell membrane does not depend on phosphorylation state (Oakley *et al.*, 2000), dephosphorylation of β -Arrestin1 is required for interaction with clathrin and receptor endocytosis (Lin *et al.*, 1997). The serine-412 phosphorylation site is not conserved on β -Arrestin2, so it must be phosphorylated on a different site, perhaps by a kinase other than ERK. In addition, β -Arrestins have a phosphoinositide binding site, and there is

evidence that they are controlled by D-myo-inositol hexakisphosphate (IP₆) binding. IP₆ binding is needed for the localization of β -Arrestins to clathrin-coated pits, but not for targeting to the cell membrane upon GPCR activation (Gaidarov *et al.*, 1999).

In addition to their classical roles in GPCR signal termination, some other functions of β -Arrestins have recently been emerging. Chemotaxis, the migration of a cell toward or away from a chemical stimulus, involves reorganization of the cell cytoskeleton in order to form a leading edge and to cause the cell to protrude in the desired direction (Ridley *et al.*, 2003). While studies of chemokine receptors have revealed that β -Arrestins may be involved in the desensitization and recycling of receptors for continued sensing of the chemical gradient, there is clearly another role for β -Arrestins in chemotaxis (Cheng *et al.*, 2000; Fan *et al.*, 2001; Fong *et al.*, 2002; Richardson *et al.*, 2003; DeFea, 2007). Indeed, the scaffolding activity of β -Arrestins that is now being elucidated is likely to be involved in the spatial regulation of molecules needed for chemotaxis (DeFea, 2007).

The scaffolding activity of β -Arrestins can be important for the down-regulation of GPCR activity through the spatial regulation of proteins that degrade downstream second messengers such as protein kinase A (PKA) (Perry *et al.*, 2002). In addition, β -Arrestins regulate diverse cellular functions by scaffolding proteins involved in many different signaling pathways (Kovacs *et al.*, 2009; Rajagopal *et al.*, 2010), such as the serine-threonine kinase mitogen-activated protein kinases (MAPKs), which are involved in a variety of cell activities ranging from cell proliferation, differentiation, and apoptosis to inflammation, migration, RNA transcription and protein translation (McDonald *et al.*,

2000; Pearson *et al.*, 2001; Morrison & Davis, 2003; Brown & Sacks, 2009). β -Arrestins recruit Src to β 2-Adrenergic receptors (Luttrell *et al.*, 1999), and Raf, MEK, and ERK to the protease-activated receptor (PAR) (DeFea *et al.*, 2000), enhancing MAPK signaling in both cases. β -Arrestins also scaffold proteins that can operate either downstream or independently of GPCRs, including those that are involved in regulating actin and cytoskeletal dynamics. The actin-binding protein cofilin was shown to complex with its regulators, LIMK and CIN phosphatase in a β -Arrestin-dependent manner upon PAR2 activation (Zoudilova *et al.*, 2007; Xiao *et al.*, 2010; Zoudilova *et al.*, 2010). The β -Arrestin1 pathway leads to scaffolding and regulation of LIMK, whereas β -Arrestin2 is involved in the activation of CIN phosphatase (or SSH phosphatase in neurons), which may lead to a decrease in cofilin phosphorylation and an increase in cofilin activity (Fig. 1-6). In this way, the β -Arrestins are able to regulate cofilin phosphorylation state through scaffolding of cofilin with its modulators, as well as the spatial localization of cofilin within cells. In addition to the classical role in GPCR signal termination, β -Arrestins are necessary for spatially regulating many factors within the cell.

1.6 References

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Figure 1-1 Dendritic spines and filopodia of hippocampal neurons

(A) A GFP-expressing hippocampal neuron at day 14 in vitro displays dendritic filopodia-like protrusions and spines with different shapes and sizes (B, C) The high magnification image of the dendrite (B) and a drawing show examples of main categories of dendritic protrusions: filopodia-like protrusions, mushroom spine, thin spine, and stubby spine. (C) Filopodia-like protrusions are precursors of dendritic spines. Mature mushroom spines display the largest heads and thin necks.

Figure 1-1

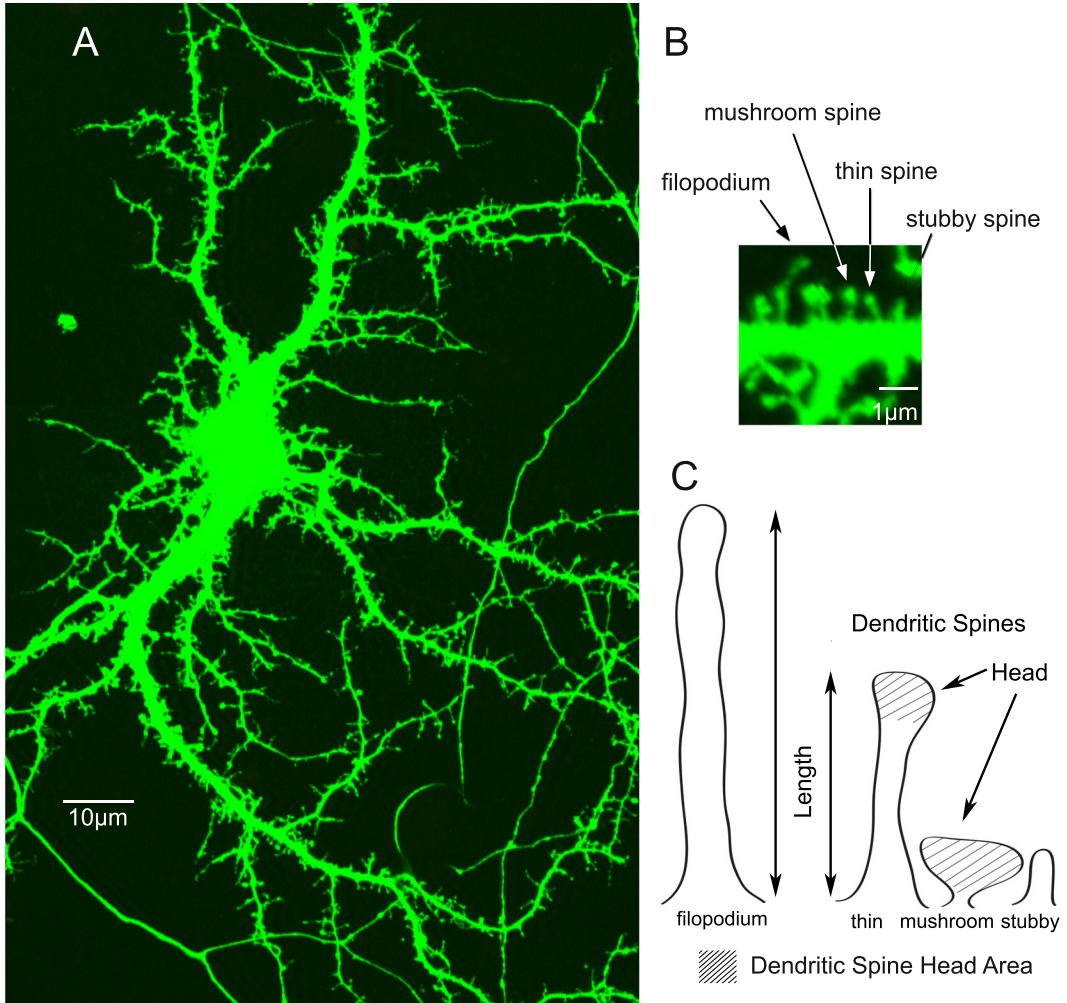


Figure 1-2 The Arp2/3 complex

Arp2/3 nucleates new branches from existing F-actin filaments and caps pointed ends, creating fast-growing barbed ends and promoting actin polymerization and outgrowth.

Figure 1-2

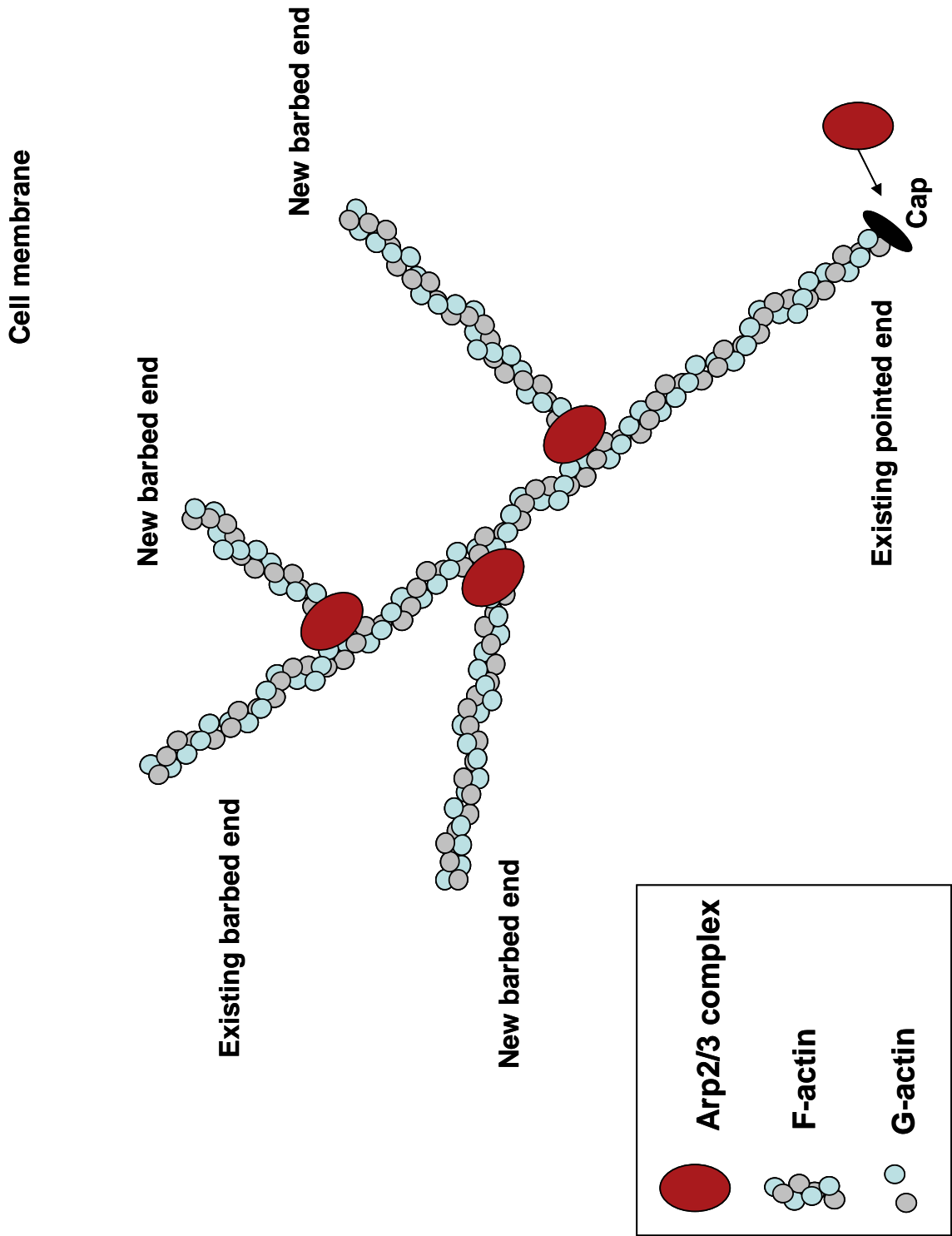
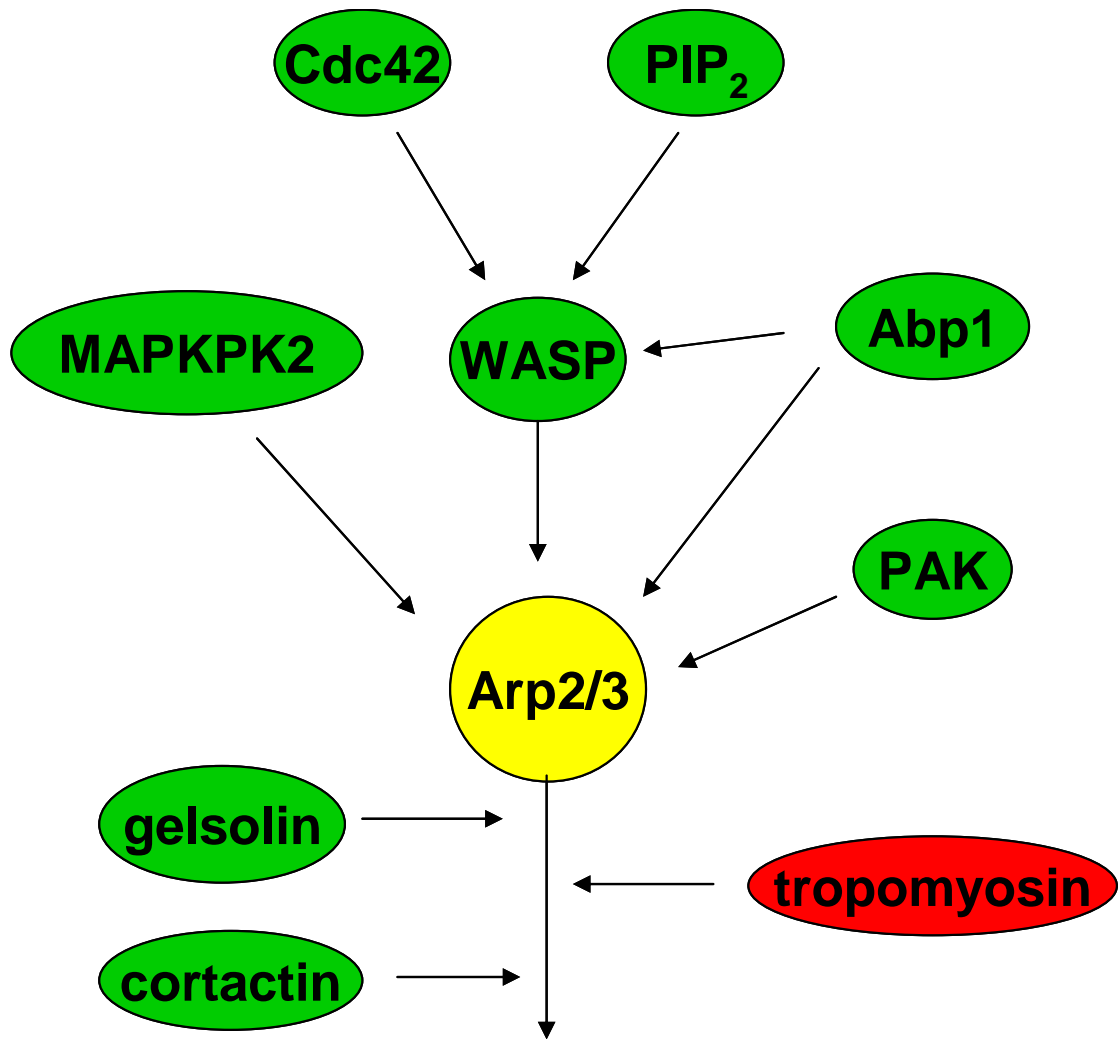


Figure 1-3 Regulatory proteins of the Arp2/3 complex

Several proteins cooperate with Arp2/3 to promote F-actin polymerization and outgrowth, while others compete with and inhibit its actin-branching activity.

Figure 1-3

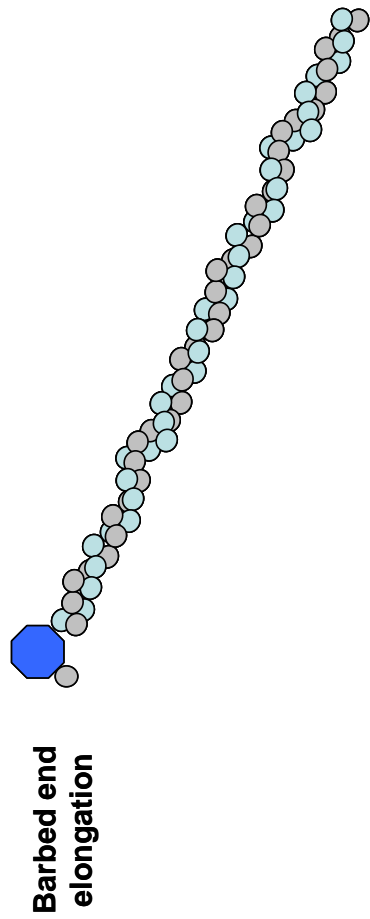
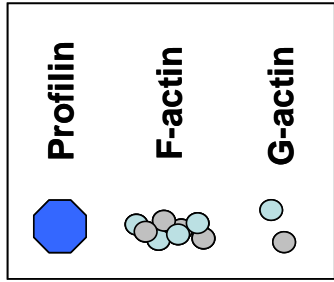


**F-actin branching
dendritic spine maturation**

Figure 1-4 Opposing actions of profilin on actin

G-actin-sequestering promotes F-actin depolymerization, while the profilin-actin complex induces polymerization by binding to F-actin barbed ends and promoting formation of ATP-actin monomers.

Figure 1-4



Pointed end

Promotion of ATP-actin monomers

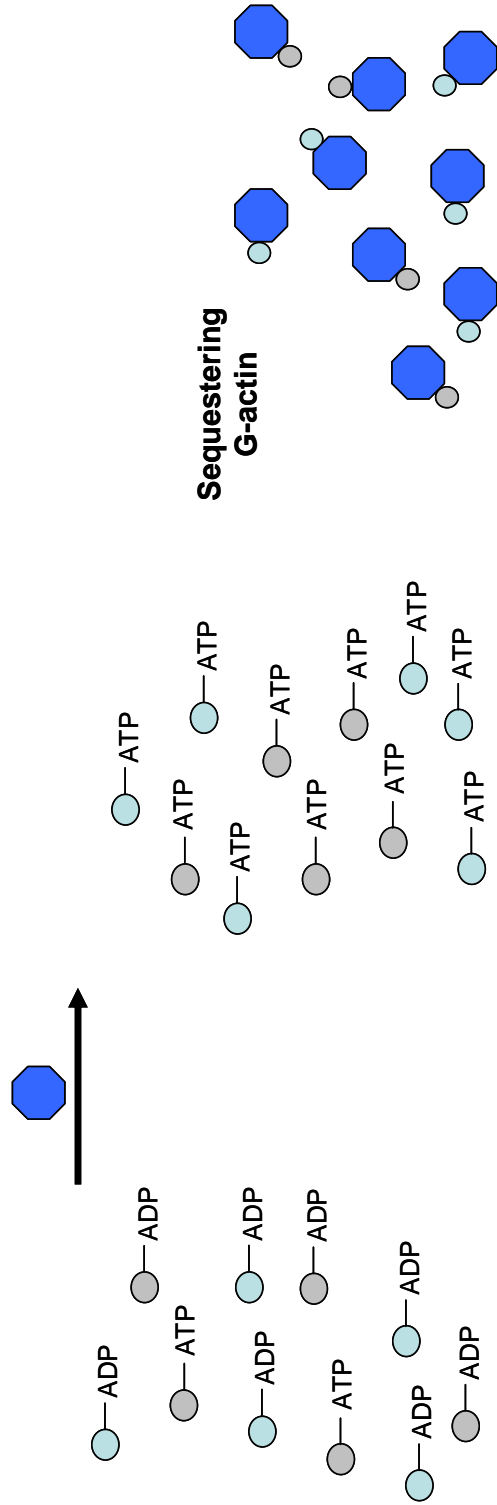


Figure 1-5 Spinophilin

Spinophilin participates in localization of PP1 to the cell membrane, where it dephosphorylates NMDA and AMPA receptors, down-regulating their activity. The actin-bundling activity of spinophilin prevents outgrowth of filopodia-like protrusions.

Figure 1-5

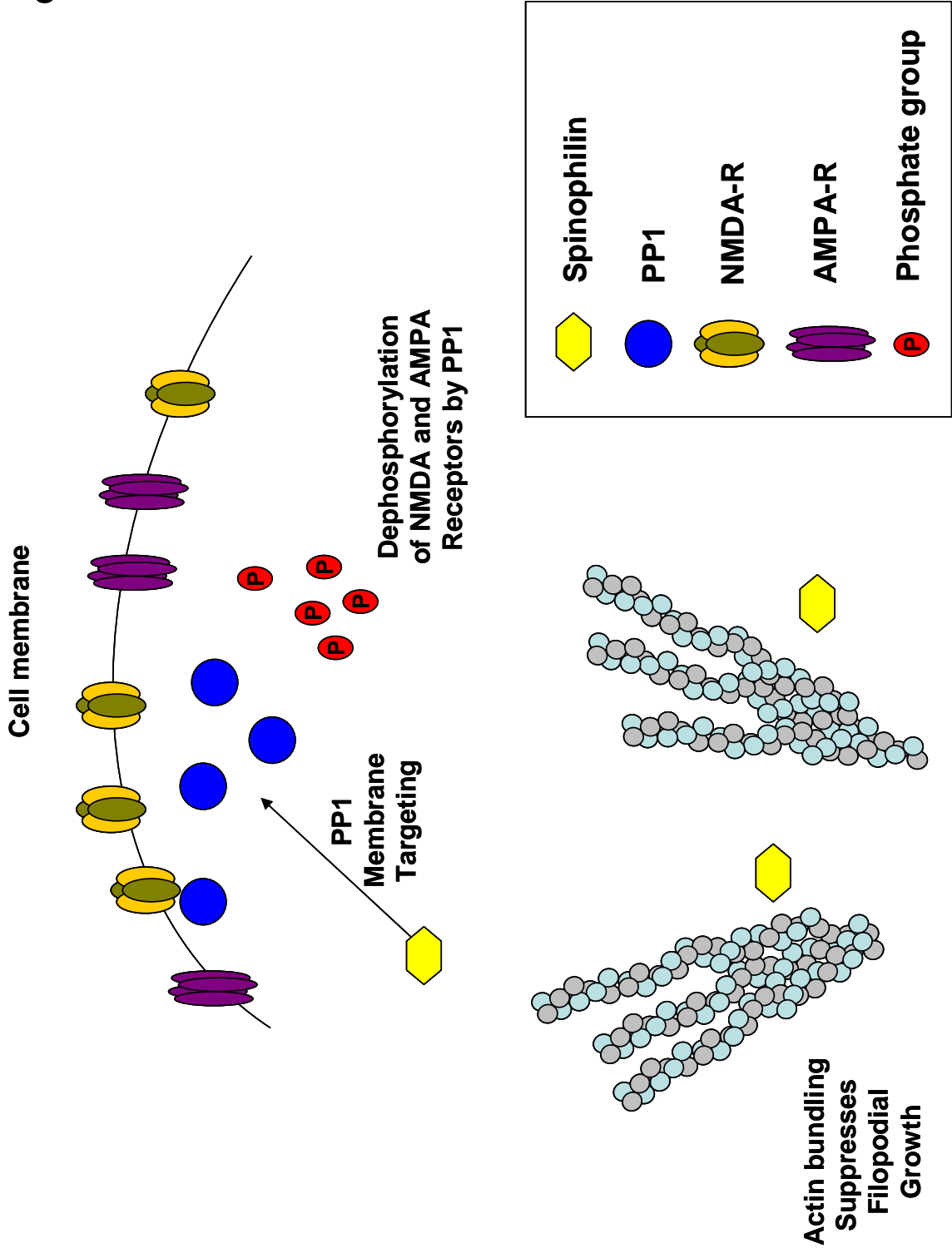


Figure 1-6 Cofilin

The actin-severing activity of cofilin promotes F-actin and dendritic spine remodeling. Meanwhile, cofilin can sequester G-actin monomers, but also creates new barbed ends for increased actin polymerization and outgrowth.

Figure 1-6

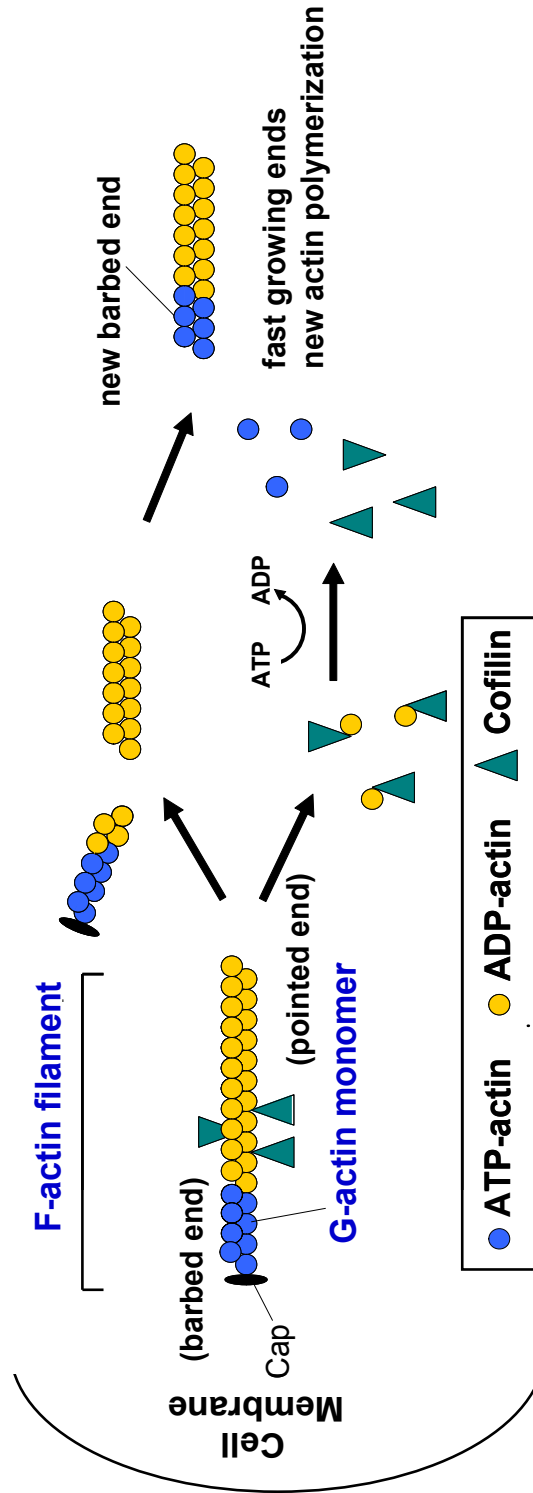
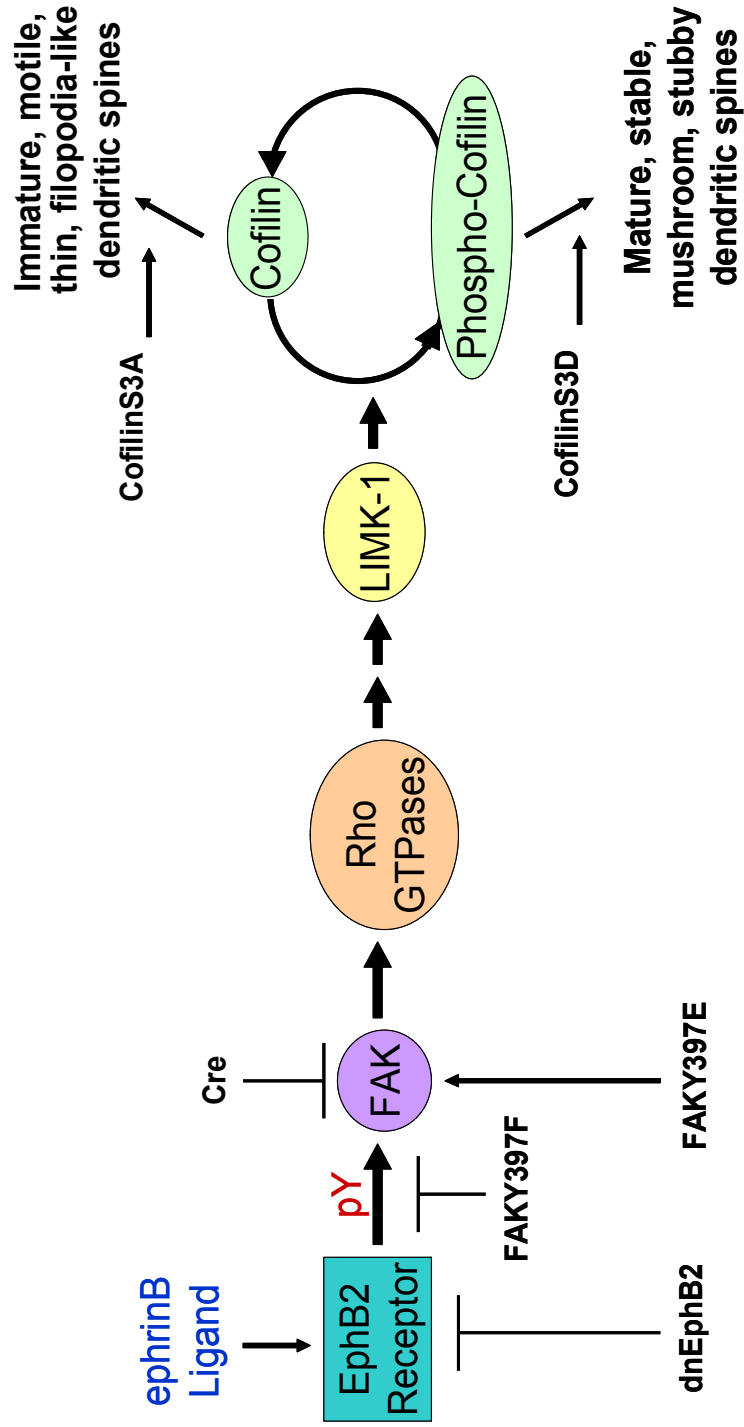


Figure 1-7 The EphB receptor pathway leads to cofilin regulation

Activation of the EphB receptor sets in motion an intracellular signaling cascade that leads to stabilization of mature dendritic spines, which is achieved through cofilin phosphorylation and inactivation.

Figure 1-7



Chapter 2: The Regulation of Cofilin-Mediated Dendritic Spine Morphology by EphB Receptors in Mature Hippocampal Neurons

2.1 Abstract

Dendritic spines are the postsynaptic sites of most excitatory synapses in the brain, and are highly enriched in polymerized F-actin, which drives the formation and maintenance of mature dendritic spines and synapses. We propose that suppressing activity of the actin-severing protein cofilin plays an important role in the stabilization of mature dendritic spines, and is accomplished through an EphB receptor-FAK pathway. Our studies demonstrate that FAK acts downstream of EphB receptors in hippocampal neurons, and that EphB2-FAK signaling controls the stability of mature dendritic spines by inhibiting cofilin activity through phosphorylation. While constitutively-active non-phosphorylatable cofilin^{S3A} induced an immature spine profile, phospho-mimetic cofilin^{S3D} restored mature spine morphology that was disrupted by either inhibition of EphB receptor activity or Cre-mediated deletion of fak. Further, we found that EphB-mediated regulation of cofilin activity at least partially depends on the activation of ROCK and LIMK-1. These findings indicate that EphB2-mediated dendritic spine stabilization relies, in part, on the ability of FAK to activate the RhoA-LIMK1 pathway that works to suppress cofilin activity and inhibit cofilin-mediated dendritic spine remodeling.

2.2 Introduction

Dendritic spines contain a number of cell surface receptors that can initiate intracellular signaling cascades, resulting in either stabilization or remodeling of the spines (Ethell & Pasquale, 2005). Spines are highly enriched in F-actin, and it is the actin dynamics that result in the plasticity of dendritic spines (Fischer *et al.*, 1998; Carlisle & Kennedy, 2005; Matus, 2005). Actin-binding proteins within the cell regulate such activities as F-actin branching, bundling, elongation, and severing (Lippman & Dunaevsky, 2005; Pontrello & Ethell, 2009), which may result in spine and filopodia growth, stabilization, or elimination. In the hippocampus, dendritic spine dynamics are thought to underlie learning and memory (Harris, 1999; Hering & Sheng, 2001; Yuste & Bonhoeffer, 2001).

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that can be activated by integrins and EphB receptors, and regulates such processes as cell migration, growth cone dynamics, and dendritic spine remodeling through its downstream effectors (Menegon *et al.*, 1999; Beggs *et al.*, 2003; Contestabile *et al.*, 2003; Rico *et al.*, 2004). FAK is enriched in several areas of the brain including the hippocampus (Burgaya *et al.*, 1995), where it may be involved in synaptic plasticity. EphB receptor activation in hippocampal neurons leads to recruitment and activation of FAK, which activates a signaling cascade that results in maintenance of mature dendritic spines (Moeller *et al.*, 2006). Cre-mediated knock-out of loxP-flanked *fak* in mature hippocampal neurons leads to an increase in immature spines and filopodia, increased spine motility, aberrant spine branches with multiple synapses, and re-arrangement of F-actin, effects that are reversed by over-expression of FAK. FAK phosphorylation and the ability of FAK to interact with

the Rho-GTPases are both necessary factors for the stabilizing effects of FAK on dendritic (Shi *et al.*, 2009).

Inhibition of the EphB receptor using dominant-negative EphB results in a reversion of spines to a more immature morphology, an effect that is reversed by a constitutively-active, but not a dominant-negative, FAK mutant (Shi *et al.*, 2009). The EphB receptor modulates FAK activity by complexing with FAK, Src, paxillin, and Grb2, which leads to activation of FAK, Src, paxillin, and the downstream effector RhoA (Moeller *et al.*, 2006). We proposed therefore that the EphB receptor might achieve spine stabilization through a FAK-RhoA-LIMK pathway, which results in the phosphorylation and inactivation of the F-actin-severing protein cofilin.

We found that EphB receptor activation induces an increase in LIMK phosphorylation at 15 min, as well as an increase in cofilin phosphorylation that is blocked by Rho-kinase (ROCK) inhibition. Over-expression of FAK or constitutively-active FAK leads to an increase in cofilin phosphorylation (a decrease in cofilin activity), but introduction of dominant-negative FAK or a FAK mutant that is unable to interact with the Rho-GTPases does not increase phospho-cofilin levels. These results show that activation of either the EphB receptor or FAK is sufficient to phosphorylate and inactivate cofilin. Furthermore, phospho-mimetic dominant-negative cofilin, but not constitutively-active cofilin, is able to restore mature dendritic spines that are disrupted by either Cre-mediated FAK deletion or inhibition of EphB receptor activity with dominant-negative EphB, suggesting that cofilin inactivation is the mechanism by which EphB receptors and FAK suppress mature dendritic spine remodeling. Our findings

indicate that EphB receptors control the stability of mature dendritic spines through a FAK-RhoA-LIMK-pathway, resulting in down-regulation of cofilin activity.

2.3 Materials and Methods

Expression vectors. The expression vectors used were: pEGFP-N1 and pDsRed-C2 (Clontech), pcDNA3-EphB2K662R (Ethell *et al.*, 2001), pPGK-Cre (from Dr. Marc Schmidt-Supprian, Harvard Medical School), pYFP-FAK (from Dr. Donna Webb, University of Virginia, Webb *et al.*, 2004), pcDNA3-GFP-cofilin, pcDNA3-GFP-cofilin^{S3A} and pcDNA3-GFP-cofilin^{S3D}. Single amino acid point mutations in FAK were generated by mutagenesis of tyrosine (Y) 397 into phenylalanine (F) or glutamate (E); proline (P) 878 into alanine (A); leucine (L) 1034 into serine (S) (Shi *et al.*, 2009), using QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Hippocampal neuronal cultures and transfection. Cultures of hippocampal neurons were prepared from embryonic day 15 (E15) or E16 mice as previously described (Shi & Ethell, 2006). Briefly, after treatment with papain (0.5mg/ml) and DNase (0.6µg/ml) for 20 min at 37°C and mechanical dissociation, cells were plated on glass coverslips or plastic dishes coated with poly-DL-ornithine (0.5mg/ml) and laminin (5µg/ml). The cells were cultured in Neurobasal medium with 25µM glutamine, 1% penicillin-streptomycin, and B-27 supplement (Invitrogen, Carlsbad, CA) under a 5% CO₂/ 10% O₂ atmosphere at 37°C. The cultures were transfected at 12 DIV using the calcium-phosphate method as previously described (Jiang & Chen, 2006; Shi & Ethell, 2006). The protocol achieves

high transfection efficiency (~30-50%), a high co-transfection rate (~95%), and low cell toxicity.

Conditional FAK KO. Hippocampal neurons were harvested from E15–16 FAK conditional (Cre-loxP) KO mice (Beggs *et al.*, 2003) and cultured as described above. The pPGK-Cre vector was obtained from Dr. Marc Schmidt-Supprian (Harvard Medical School). The cultures were transiently transfected at 12 DIV using the calcium-phosphate method. The neurons were transfected with pEGFP or pDsRed to visualize dendritic spine morphology and/or with pPGK-Cre to abolish FAK expression. For FAK overexpression and cofilin experiments, the neurons were transfected with pDsRed, pDsRed and pPGK-Cre, or pDsRed and pPGK-Cre with one of the following: pcDNA3-GFP-wt cofilin, pcDNA3-GFP-cofilin^{S3A}, or pcDNA3-GFP-cofilin^{S3D}. At 14 DIV, the cultures were fixed and processed for indirect immunofluorescence. Dendritic spines were visualized by GFP or DsRed fluorescence, counted, and measured as described in "Image Analysis." Cre expression was detected in the nuclei of the pEGFP/ pPGK-Cre transfected neurons by immunostaining.

Activation and inhibition of the EphB2 receptor. HEK293 cells in 10% FBS/ DMEM were transfected using Lipofectamine 2000 (Invitrogen) with pcDNA3-ephrinB2-Fc (Lin *et al.*, 2008). Media was collected from these cells for 5 days, filtered, and the secreted ephrinB2-Fc was bound to protein-A agarose beads (Sigma) at 4°C with rotation overnight. EphrinB2-Fc was washed with PBS, eluted from the beads with 150mM NaCl/

50mM glycine, pH 2.3, and dialyzed against PBS with 0.5mM DTT and PMSF (Sigma). To activate the EphB2 receptor in cultured hippocampal neurons, ephrinB2-Fc was mixed with goat-anti-human IgG antibody (Jackson ImmunoResearch Laboratories) at a ratio of 2:1 for 1 hour at 4°C in order to pre-cluster the ephrinB2-Fc prior to application. Human gamma globulin (Jackson ImmunoResearch Laboratories) was also pre-clustered with goat-anti-human IgG to be used as a control. Just prior to application, the Fc/ IgG mixes were diluted in cell culture media or PBS to a final concentration of 4µg/ml Fc.

To inhibit EphB receptor activity, cultured hippocampal neurons were transfected with pcDNA3-EphB2K662R (Ethell *et al.*, 2001) at 12 DIV. For cofilin experiments, the neurons were transfected with pDsRed alone, pDsRed with pcDNA3-dnEphB2K662R, or pDsRed with pcDNA3-dnEphB2K662R and one of the following: pcDNA3-GFP-wt-cofilin, pcDNA3-GFP-cofilin^{S3A}, or pcDNA3-GFP-cofilin^{S3D}. At 14 DIV, the cultures were fixed and processed for immunostaining. Dendritic spines were visualized by GFP or DsRed fluorescence, counted, and measured as described in "Image Analysis."

Biochemical Analysis. Hippocampal neurons (approximately 1.2 million cells on 10cm dishes at 14 DIV) were treated with the appropriate reagent (in PBS or cell culture media) under 5% CO₂/ 10% O₂ at 37°C. Following treatment, the dishes were placed on ice and immediately washed with ice-cold PBS, then scraped from the plate in 1ml lysis buffer (25mM Tris-Cl, pH 7.4, 150mM NaCl, 1% TritonX-100, 5mM EDTA, pH 8.0, 1x protease inhibitor cocktail (Sigma-Aldrich), and 2mM sodium vanadate. After 30 minutes rotation at 4°C, cell lysate was centrifuged for 15 min. at 16.1K r.c.f at 4°C. Supernatant

was mixed 1:1 with 2x Laemmli loading buffer (Sigma-Aldrich), boiled for 10 minutes, and loaded onto an 8-16% Tris-glycine SDS PAGE gel (Invitrogen). The contents of the gel were transferred onto a Nitrocellulose membrane (Perkin Elmer), which was blocked in 5% milk/ 0.2% Tween 20/ TBS, and specific primary antibodies (1:1000) were applied overnight at 4°C in 3% BSA/ 0.2% Tween 20/ TBS. The primary antibodies used were: rabbit anti-pLIMK1/2 (Cell Signaling; 1:1000 for WB); rabbit anti-LIMK1 (Chemicon; 0.76µg/ml for WB); rabbit anti-cofilin (ACFL02, Cytoskeleton, Denver, CO; 0.25µg/ml for WB; 1µg per 20µl Protein A-agarose beads (Sigma, St. Louis, MO)); and rabbit anti-phospho-cofilin (S3) (ab12866, abcam, Cambridge, MA; 1:1000 for WB). Secondary HRP-conjugated antibodies were applied (1:100,000) for 1 hour at room temperature in 0.2% Tween 20/ TBS. The secondary antibodies used were the following: HRP-conjugated goat anti-rabbit (0.08 µg/ml; Jackson ImmunoResearch, West Grove, PA) and HRP-conjugated donkey anti-mouse (0.08 µg/ml; Jackson ImmunoResearch). Signal was detected on film using the ECL Plus detection kit from GE Healthcare. Phospho-LIMK and phospho-cofilin levels were quantified by densitometry (Adobe Photoshop) and normalized to total LIMK and total cofilin levels, respectively. Three independent experiments were performed for each condition. Statistical differences were compared using Student's t test.

To inhibit ROCK, 14 DIV hippocampal neurons were pre-incubated with 10µM or 75µM Y-27632 for 30 minutes and then treated with 4µg/ml ephrin B2-Fc or 4µg/ml control Fc for 20 minutes in the presence of the ROCK inhibitor. Cell lysates were prepared as indicated above and subjected to immunoblotting against p-cofilin, cofilin, p-

LIMK1 and LIMK1 using specific antibodies. The levels of p-cofilin or p-LIMK1 were quantified by densitometry and normalized to total cofilin or LIMK1 levels, respectively. Three independent experiments were performed for each condition. Statistical differences between Fc-treated and ephrinB2-Fc-treated samples were compared by Student's t test.

Immunocytochemistry. 14 DIV hippocampal neurons were fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100 or 0.2mg/ml saponin, then blocked in PBS containing 5% normal goat serum and 1% BSA. Dendritic spines and filopodia were visualized by GFP fluorescence. The primary antibodies used were the affinity purified rabbit anti-phospho-cofilin (4321; 1µg/ml for ICC) and mouse anti-cofilin (MAb22; 15µg/ml for ICC) antibodies, generous gifts from Dr. James Bamberg (Colorado State University, Fort Collins, CO), and mouse anti-synaptophysin (SVP-38, Sigma, 61 µg/ml). The secondary antibodies used were the following: 4µg/ml Alexa Fluor 660-conjugated anti-mouse IgG and 4µg/ml Alexa Fluor 594-conjugated anti-rabbit IgG. Immunostaining was analyzed under a confocal laser-scanning microscope (model LSM 510; Carl Zeiss MicroImaging, Oberkochen, Germany).

Ratio imaging of total/ phospho-cofilin. Cultured hippocampal neurons were transfected with pEGFP, pYFP-FAK, pYFP-FAK^{Y397F}, pYFP-FAK^{Y397E}, or pYFP-FAK^{L1034S} at 12 DIV using the calcium-phosphate method and processed for immunocytochemistry at 14 DIV. The neurons were fixed for 30 min in 2% paraformaldehyde in PBS or cytoskeletal preservation buffer containing 10mM MES, pH 6.1, 138 mM KCl, 3mM MgCl₂, 10 mM

EGTA, and 0.32 M Sucrose. The cultures were washed in PBS and permeabilized in 0.5% Triton X-100 for 10 min, then blocked in PBS containing 5% normal goat serum and 1% BSA. The cultures were immunostained for phospho-cofilin with affinity-purified rabbit anti-phospho-cofilin antibody (4321; 1 μ g/ml) and for total cofilin with mouse anti-cofilin antibody (MAb22; 15 μ g/ml; both are generous gifts from Dr. James Bamberg). The secondary antibodies used were the following: 4 μ g/ml Alexa Fluor 660-conjugated anti-mouse IgG and 4 μ g/ml Alexa Fluor 594-conjugated anti-rabbit IgG. Immunostaining was analyzed under a confocal laser-scanning microscope (model LSM 510; Carl Zeiss MicroImaging, Oberkochen, Germany). A series of five high-resolution optical sections (1024x1024 pixel format) were taken for each neuron with a 63x water immersion objective (1.2 NA) with 1x zoom at 0.5 μ m step intervals (z-stack). All images were acquired under identical conditions. Each Z-stack was collapsed into a single image by projection (Zeiss LSM Image software), converted to tiff file and analyzed using Adobe Photoshop software. Seven to 10 neurons were randomly selected for each experimental group, and three to four proximal dendrites per each neuron were analyzed. The levels of phospho-cofilin and total cofilin were assessed by measuring the immunofluorescence signals in dendrites of neighboring transfected (GFP positive) and un-transfected (GFP negative) neurons immunostained with anti-phospho-cofilin and anti-cofilin antibodies. The background fluorescence was subtracted. The phospho-cofilin levels or ratio of total/ phospho-cofilin in transfected neurons was normalized against the phospho-cofilin levels or ratio of total/ phospho-cofilin in un-transfected neurons, respectively. Three independent experiments were performed for each condition.

Statistical differences between transfected and un-transfected neurons were compared by Student's t test. Statistical differences for multiple groups were assessed by one-way ANOVA followed by Newman–Keuls post hoc tests.

Image analysis. The dendritic morphology was examined in 14 DIV GFP-expressing hippocampal neurons as previously described (Moeller *et al.*, 2006; Shi & Ethell, 2006). Briefly, experimental and control samples were encoded for blind analysis, and GFP-expressing hippocampal neurons were randomly selected and imaged with a confocal laser-scanning microscope (model LSM 510; Carl Zeiss MicroImaging). A series of five high-resolution optical sections (1024x1024 pixel format) were taken for each neuron with a 63x water immersion objective (1.2 NA) with 1x zoom at 0.5 μm step intervals (z-stack). During the image acquisition, the detector gain and amplifier offset were adjusted for each GFP-expressing neuron to have just a few red (white saturated) and a few blue (black zero) pixels using the range-indicator option (Zeiss LSM Image software), to achieve a similar signal-to-noise ratio in all images. Note that GFP fluorescence signal-to-noise ratio was calculated for each image. There was no significant difference between the groups ($p > 0.05$). Each Z-stack was collapsed into a single image by projection (Zeiss LSM Image software), converted to tiff file, and analyzed using Adobe Photoshop software. The proximal dendrites that are at least 1 μm in diameter were selected for analysis of the length and number of dendritic protrusions. Hidden protrusions that protruded toward the back or front of the viewing plane were not counted. Ten to fifteen neurons were randomly selected for each experimental group, and three to five proximal

dendrites per each neuron were analyzed (~2000 μ m of total dendritic length per group). The length of the protrusion was determined by measuring the distance between its tip and the base using Image-J software. Dendritic spines were identified as dendritic protrusions connected with synaptophysin-positive pre-synaptic terminal. Dendritic protrusions that lack detectable synaptophysin immunoreactivity were classified as dendritic filopodia. The dendritic spine heads were defined as enlarged areas at the tips of dendritic spines. The areas were manually traced and calculated using Image-J software. The values represent mean \pm standard error of mean (SEM). Three independent experiments were performed for each condition. Statistical differences for multiple groups were assessed by one-way ANOVA followed by Newman-Keuls post hoc tests.

2.4 Results

2.4.1 EphB2 receptors induce cofilin and LIMK phosphorylation in cultured hippocampal neurons. Our results show that FAK acts downstream of EphB receptors and may promote dendritic spine stability through Rho family GTPase regulatory proteins. Moreover, our previous studies demonstrated that EphB2 receptor activation in hippocampal neurons up-regulates RhoA activity (Moeller *et al.*, 2006). Altogether, these findings suggest that EphB2-mediated dendritic spine stabilization may rely on the ability of FAK to activate the RhoA-LIMK1 pathway that works to suppress cofilin activity and discourage cofilin-mediated dendritic spine remodeling.

To investigate this hypothesis, we first assessed whether the EphB2 receptor regulates cofilin activity. Cofilin induces F-actin disassembly and reorganization through

its severing activity, which can be down-regulated by phosphorylation of serine-3 (Bamburg, 1999). To investigate whether EphB receptor activation regulates cofilin activity, we examined cofilin phosphorylation following EphB2 receptor activation in 14 DIV hippocampal neurons. Western blot revealed a higher level of cofilin phosphorylation in neurons treated with ephrin-B2-Fc as compared to neurons treated with control Fc ($p=0.00831$; Fig. 2-1A, 2-1B). Activation of EphB receptors also induced LIMK-1 activation in ephrin-B2-Fc treated hippocampal neurons as compared to Fc-treated controls, as demonstrated by an increased level of LIMK-1 phosphorylation by western blot ($p=0.04484$; Fig. 2-1C, 2-1D). Moreover, ephrin-B2-induced cofilin phosphorylation was significantly inhibited with the specific Rho-associated kinase (ROCK) inhibitor Y-27632 (Fig. 2-1A, 2-1B). These results demonstrate that cofilin activity is controlled by EphB receptors at least partially through the regulation of ROCK and LIMK activities, and suggest that downregulation of cofilin activity by LIMK-mediated phosphorylation may be responsible for maintaining mature dendritic spines.

2.4.2 FAK-mediated regulation of mature dendritic spine morphology and cofilin phosphorylation depends on its activation/ phosphorylation and its ability to

interact with regulators of Rho family GTPases. Transfection of pYFP-FAK and the FAK mutant constructs allowed for analysis of the effects of over-expressing FAK, constitutively-active FAK, and inactive FAK on dendritic spine morphology. We also analyzed whether the different forms of FAK can regulate cofilin phosphorylation and the cofilin/ phospho-cofilin ratio in dendrites of cultured mouse hippocampal neurons. Our

studies demonstrate that overexpression of FAK significantly increased cofilin phosphorylation ($p < 0.05$) and reduced the cofilin/ phospho-cofilin ratio ($p < 0.05$) in dendrites of cultured mouse hippocampal neurons as compared to control GFP-expressing neurons (Fig. 2-2F, 2-2G). Moreover, overexpression of constitutively-active FAK^{Y397E} further increased cofilin phosphorylation, and significantly decreased the cofilin/ phospho-cofilin ratio as compared to control GFP expressing neurons ($p < 0.001$). In addition, the overexpression of FAK or constitutively-active FAK^{Y397E} also increased the size of dendritic spine heads as compared to those in control GFP expressing neurons ($p < 0.05$ and $p < 0.01$, respectively), shifting the spine head to length ratio toward a more mature spine phenotype. However, the overexpression of either the FAK^{L1034S} mutant, which is unable to interact with regulators of Rho family GTPases, or the non-phosphorylatable FAK^{Y397F}, failed to induce cofilin phosphorylation or to reduce the cofilin/ phospho-cofilin ratio as compared to control GFP-expressing neurons. The levels of phospho-cofilin in the dendrites of neurons expressing FAK^{L1034S} or FAK^{Y397F} were significantly lower than in neurons overexpressing FAK or constitutively-active FAK^{Y397E} ($p < 0.05$ and $p < 0.01$, respectively). Furthermore, spine head size for neurons expressing FAK^{L1034S} or FAK^{Y397F} were significantly smaller than control neurons and those over-expressing FAK or FAK^{Y397E} ($p < 0.001$), shifting the spine head-to-length ratio toward a more immature spine phenotype. Our observations suggest that FAK activation on Y397 and its ability to interact with regulators of Rho family GTPases are involved in the regulation of cofilin phosphorylation and dendritic spine morphology in mature hippocampal neurons.

2.4.3 The constitutively active cofilin^{S3A}, but not inactive phospho-mimetic cofilin^{S3D}

or wt-cofilin, induces immature dendritic spines. As a prelude to determining the role of cofilin activity in EphB2-mediated stabilization of dendritic spines, we analyzed the overall effects of the constitutively-active cofilin^{S3A} or the inactive cofilin^{S3D} mutants on dendritic spine morphology. The overexpression of the non-phosphorylatable cofilin^{S3A}, but not the phospho-mimetic cofilin^{S3D} or wt-cofilin, induced remodeling of established mature dendritic spines and the extension of new filopodia in 14 DIV hippocampal neurons (Fig. 2-3). Neurons expressing non-phosphorylatable cofilin^{S3A} had dendritic spines that were longer and with smaller heads than neurons expressing inactive cofilin^{S3D} or wt-cofilin (Fig. 2-3E-2-3H). Interestingly, wt-cofilin accumulated in mature spines (Fig. 2-4), but did not affect the size of dendritic spine heads (Fig. 2-3H), suggesting the existence of a signaling mechanism that suppresses cofilin activity in mature spines. In addition, the overexpression of inactive cofilin^{S3D} significantly decreased the length of dendritic spines as compared to those in control neurons or neurons expressing active cofilin^{S3A}, shifting the spine head-to-length ratio toward a more mature spine phenotype. The ability of cofilin^{S3D} to promote mature dendritic spines suggests that it may act on endogenous cofilin in a dominant-negative manner, probably by competing for binding to phosphatases that dephosphorylate and activate cofilin. These results demonstrate that only non-phosphorylatable cofilin^{S3A} prompted the transformation of mature mushroom-shaped spines into thin immature spines with

smaller heads, indicating that cofilin activity is down-regulated in mature dendritic spines by phosphorylation.

2.4.4 Phospho-mimetic cofilin^{S3D} restores mature dendritic spines disrupted by inhibiting EphB receptor activity. To determine whether EphB signaling promotes dendritic spine stability through cofilin inhibition by phosphorylation, we disrupted this pathway at the level of EphB2 receptor activity. We then assessed the effects of constitutively-active cofilin^{S3A} or phospho-mimetic cofilin^{S3D} mutants on dendritic spine morphology. The inhibition of EphB receptor forward signaling by overexpression of dnEphB2 significantly changed dendritic spine length and morphology toward an immature spine profile (Fig. 2-5). These changes were reversed by the overexpression of phospho-mimetic cofilin^{S3D}, but not non-phosphorylatable cofilin^{S3A}, suggesting that the regulation of cofilin activity plays an important role in EphB2 receptor-mediated dendritic spine maturation. This study suggests that the EphB2 signaling pathway promotes mature dendritic spines through the regulation of cofilin activity.

2.4.5 Phospho-mimetic cofilin^{S3D} restores mature dendritic spines disrupted by FAK depletion. In order to confirm the EphB receptor pathway's regulation of spine morphology through cofilin, we next disrupted this pathway at the level of FAK expression. We assessed the effects of constitutively-active cofilin^{S3A} or phospho-mimetic cofilin^{S3D} mutants on dendritic spine morphology in neurons with Cre-mediated FAK deletion. The inhibition of EphB receptor forward signaling by FAK knockout

significantly changed dendritic spine length and morphology toward an immature spine profile (Fig. 2-6). The disruption of mature spine phenotype seen in FAK-deficient neurons was reversed by overexpressing phospho-mimetic cofilin^{S3D}, but not non-phosphorylatable cofilin^{S3A}. Taken together, these studies show that the EphB2-FAK signaling pathway promotes the stabilization of mature dendritic spines and synapses through the regulation of cofilin activity by phosphorylation.

2.5 Discussion

Our previous studies revealed that the non-receptor tyrosine kinase FAK plays an important role in the maintenance of mature dendritic spines in cultured hippocampal neurons (Shi *et al.*, 2009). Over-expression of FAK or constitutively-active FAK^{Y397E}, but not inactive FAK^{Y397F} or FAK^{L1034S}, shifted spine morphology toward a mature phenotype, whereas Cre-mediated knock-out of loxP-flanked fak induced remodeling of dendritic spines and synapses. The effects of FAK deletion on dendritic spine morphology and synapses were reversed by the overexpression of wild-type FAK and constitutively-active FAK^{Y397E}, but not FAK^{Y397F}, indicating the significance of FAK activation in the maintenance of mature dendritic spines (Shi *et al.*, 2009).

In addition to integrins, several other cell surface receptors and their ligands were shown to regulate FAK activity, including the DCC receptor and netrin, and Eph receptors and ephrins (Cowan & Henkemeyer, 2001; Miao *et al.*, 2001; Kruger *et al.*, 2004; Li *et al.*, 2004; Nikolopoulos & Giancotti, 2005; Moeller *et al.*, 2006). Our previous studies demonstrated that EphB2 activation in 7 DIV hippocampal neurons

induces FAK activation and assembly of a FAK/ Src complex that contributes to the formation of dendritic spines and dendritic filopodia shortening/ elimination (Moeller *et al.*, 2006). Our previous studies also showed that when EphB receptor forward signaling is inhibited by the overexpression of dnEphB2 in mature 14 DIV hippocampal neurons, there is a shift toward an immature spine morphology that is reversed by the overexpression of constitutively active FAK^{Y397E}, but not non-phosphorylatable FAK^{Y397F}. These results show that FAK acts downstream of the EphB receptors and that FAK activation plays an important role in EphB2 signaling to maintain mature dendritic spines and synapses (Shi *et al.*, 2009).

We have previously demonstrated that EphB-mediated activation of RhoA in hippocampal neurons depends on FAK activity (Moeller *et al.*, 2006), suggesting that FAK may direct activity of actin-regulating proteins in dendritic spines. FAK is known to associate with regulators of Rho family GTPases through its FAT domain and to regulate their activities in non-neuronal cells (Hildebrand *et al.*, 1996; Zhai *et al.*, 2003; Mitra *et al.*, 2005; Torsoni *et al.*, 2005; Iwanicki *et al.*, 2008). In neurons, FAK was shown to control axonal branching in part by regulating Rho family GTPases, as the overexpression of FAK^{L1034S} and FAK^{P878A} mutants, which are unable to interact with regulators of Rho family GTPases, failed to rescue the axonal arborization phenotype observed in FAK deficient neurons (Rico *et al.*, 2004). Our previous studies demonstrated that FAK^{L1034S} and FAK^{P878A} mutants were also unable to restore mature dendritic spines in FAK deficient hippocampal neurons (Shi *et al.*, 2009). Therefore, it is

possible that FAK also regulates actin organization in dendritic spines through the Rho family GTPases.

In our current studies, we investigated the mechanism by which the EphB2-FAK pathway promotes dendritic spine stabilization. Members of the Rho family of small GTPases, such as RhoA, Cdc42, and Rac, are essential regulators of actin polymerization (Luo, 2002). As multiple genetic defects in Rho-related signaling have been linked to mental retardation (van Galen & Ramakers, 2005), the Rho pathway appears to represent a major axis of control in the formation of dendritic spines (Govek *et al.*, 2004; Irie *et al.*, 2005; Zhang *et al.*, 2005; Tolia *et al.*, 2007; Xie *et al.*, 2007; Saneyoshi *et al.*, 2008; Wegner *et al.*, 2008). Rac1, Cdc42, and RhoA can also promote activation of LIMK-1 through PAK and ROCK, respectively (Yang *et al.*, 1998; Edwards *et al.*, 1999; Maekawa *et al.*, 1999). LIMK-1 is a non-receptor serine/ threonine kinase that inhibits the F-actin severing protein cofilin by serine-3 phosphorylation (Arber *et al.*, 1998; Bamberg, 1999; Sumi *et al.*, 1999). LIMK-1 has been shown to be involved in dendritic spine development, as LIMK-1 KO mice fail to form morphologically mature dendritic spines (Meng *et al.*, 2002). Rather, neurons from these knockouts form thin spines with small heads and postsynaptic densities, similar in morphology to those seen in EphB1/2/3 triple KOs (Henkemeyer *et al.*, 2003). The inhibition of LIMK translation by microRNA-134 also resulted in smaller spine heads (Schratt *et al.*, 2006). LIMK-1 may stabilize the actin cytoskeleton in mature dendritic spines by inhibiting cofilin activity. Our findings suggest that EphB2-mediated dendritic spine stabilization may rely on the ability of

EphB2 receptors to activate the RhoA-ROCK-LIMK-1 pathway that works to suppress cofilin activity and inhibits cofilin-mediated dendritic spine remodeling.

Cofilin is an actin-severing protein that preferentially binds ADP-actin subunits, twisting and breaking pre-existing actin filaments. This increases the pool of G-actin monomers used by actin polymerizing factors and also creates free barbed ends on pre-existing actin filaments, resulting in their elongation (Bamburg, 1999; Condeelis, 2001; Suetsugu *et al.*, 2002; Sarmiere & Bamburg, 2004). Low levels of cofilin activity are detected in resting cells and contribute to F-actin depolymerization at the pointed ends and F-actin polymerization at the barbed ends, resulting in steady-state F-actin turnover at a slow rate. On the other hand, enhanced cofilin activity is often detected in ruffling membranes and at the leading edge of migrating cells leading to fast F-actin dynamics. Cofilin activity is regulated by phosphorylation. LIMK-1 suppresses cofilin activity by serine-3 phosphorylation, which inhibits cofilin binding to F-actin. The cofilin-specific phosphatase slingshot (SSH) dephosphorylates and activates cofilin. Thus, the action of cofilin in cells depends on its phosphorylation state (Andrianantoandro & Pollard, 2006). In synapses, enhanced cofilin activity could induce elongation and remodeling of actin-rich stable dendritic spines and extension of new filopodia, while low cofilin activity would support F-actin maintenance in mature spines.

We have shown here that a constitutively-active non-phosphorylatable cofilin^{S3A}, but not wt-cofilin or dominant-negative cofilin^{S3D}, induced remodeling of established mature dendritic spines and extension of new filopodia, suggesting the existence of a signaling mechanism that down-regulates cofilin activity in mature spines. Our results

demonstrate that EphB2 activation in 14 DIV hippocampal neurons induced cofilin phosphorylation and LIMK-1 activation as demonstrated by an increased level of LIMK-1 phosphorylation. Moreover, ephrin-B2-induced cofilin phosphorylation was inhibited with a specific ROCK inhibitor Y-27632, suggesting that EphB-mediated regulation of cofilin activity at least partially depends on the activation of ROCK and LIMK-1. EphB receptors can also regulate Rac1 activity in dendritic spines through the Rac exchange factors kalirin-7 and Tiam1 (Penzes *et al.*, 2003; Tolia *et al.*, 2007). Moreover, the Rac-PAK-LIMK pathway was recently suggested to contribute to SynGAP's regulation of steady-state cofilin phosphorylation in dendritic spines (Carlisle *et al.*, 2008). Therefore, it is possible that the PAK-LIMK pathway may also contribute to EphB-mediated regulation of cofilin phosphorylation in dendritic spines. If the regulation of cofilin activity by phosphorylation is the mechanism behind the spine-stabilizing effect of the EphB2-FAK-LIMK pathway, then interference at any point in this pathway would lead to lower LIMK activity and/ or higher SSH activity, shifting the p-cofilin/ cofilin equilibrium toward cofilin dephosphorylation, and inducing cofilin-mediated dendritic spine remodeling. Overexpressing the phospho-mimetic cofilin^{S3D} might then restore the p-cofilin/ cofilin equilibrium by inhibiting dephosphorylation of endogenous p-cofilin, and rescue mature spine morphology. Indeed, the inhibition of EphB2 activity by overexpressing dnEphB2 or Cre-mediated fak deletion induced dendritic spine remodeling, and phospho-mimetic cofilin^{S3D} restored the mature dendritic spine phenotype. Moreover, neurons over-expressing FAK or FAK^{Y397E}, but not FAK^{Y397F} or FAK^{L1034S}, showed an increase in levels of inactive phospho-cofilin over control cells.

Our findings suggest that the EphB2-FAK pathway promotes dendritic spine stability at least partially through LIMK-mediated cofilin phosphorylation. However, the role of the cofilin phosphatase SSH in EphB2-mediated increase in cofilin phosphorylation is still unclear. Moreover, the EphA4 receptor has been recently shown to modulate the association of cofilin with the plasma membrane by regulating PLC activity (Zhou *et al.*, 2007). Future studies will determine whether the EphB receptors also regulate the activity of SSH and PLC.

Recent evidence reveals that long term potentiation (LTP) occurs within individual synapses, and that actin reorganization underlies synaptic plasticity (Carlisle & Kennedy, 2005; Lin *et al.*, 2005; Kramar *et al.*, 2006; Chen *et al.*, 2007; Fedulov *et al.*, 2007; Lynch *et al.*, 2007; Rex *et al.*, 2007; Becker *et al.*, 2008). The effects of FAK depletion on actin organization, mature spine morphology, and regulation of cofilin suggest that FAK signaling and cofilin phosphorylation may contribute to synaptic plasticity. Indeed, FAK was found to play a role in LTP induction in the dentate gyrus (Yang *et al.*, 2003), and the importance of cofilin phosphorylation in synaptic plasticity during LTP induction, as well as the protective effects of the phospho-mimetic cofilin^{S3D} mutant against A β -mediated spine loss, have been reported (Chen *et al.*, 2007; Shankar *et al.*, 2007). Moreover, two key upstream regulators of FAK activity in dendritic spines, EphB receptors and integrins, also play important roles in synaptogenesis, synaptic plasticity, and LTP (Dalva *et al.*, 2000; Chavis & Westbrook, 2001; Chun *et al.*, 2001; Ethell *et al.*, 2001; Grunwald *et al.*, 2001; Henderson *et al.*, 2001; Contractor *et al.*, 2002;

Penzes *et al.*, 2003; Gall & Lynch, 2004; Huang *et al.*, 2006; Kramar *et al.*, 2006; Webb *et al.*, 2007).

Our findings conclusively demonstrate that EphB receptors control the stability of mature dendritic spines, in part by suppressing cofilin activity through recruitment/activation of FAK and cofilin phosphorylation. Future studies will determine whether the mechanisms by which this pathway regulates maintenance of mature dendritic spines also orchestrate structural changes in dendritic spines that underlie synaptic plasticity and LTP.

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Figure 2-1 The EphB2 receptor induces cofilin and LIMK phosphorylation in cultured hippocampal neurons

(A, C) 14 DIV hippocampal neurons were treated with ephrinB2-Fc to activate EphB receptors or with control Fc for 15 min, with or without the ROCK inhibitor Y-27632 at 10 μ M or 75 μ M. Cell lysate was subjected to immunoblotting with (A) anti-phospho-cofilin or (C) anti-phospho-LIMK1/2 antibodies. The blots were stripped and re-probed against total cofilin or LIMK1.

(B, D) The levels of p-cofilin or p-LIMK1 were quantified by densitometry and normalized to total cofilin or LIMK1 levels, respectively. Experimental values represent mean \pm SD (n=3). Values significantly different in ephrinB2-Fc treated samples as compared to control Fc samples are indicated by **, p< 0.01; *, p<0.05. Cofilin phosphorylation levels were significantly lower in samples treated with ephrinB2-Fc in the presence of 10 μ M Y-27632 (a, p=0.03856) or 75 μ M Y-27632 (b, p=0.00172) as compared to samples treated with ephrinB2-Fc alone. EphB2 receptor activation with pre-clustered ephrinB2-Fc led to increased levels of phosphorylated cofilin that were inhibited with the ROCK inhibitor Y-27632.

Figure 2-1

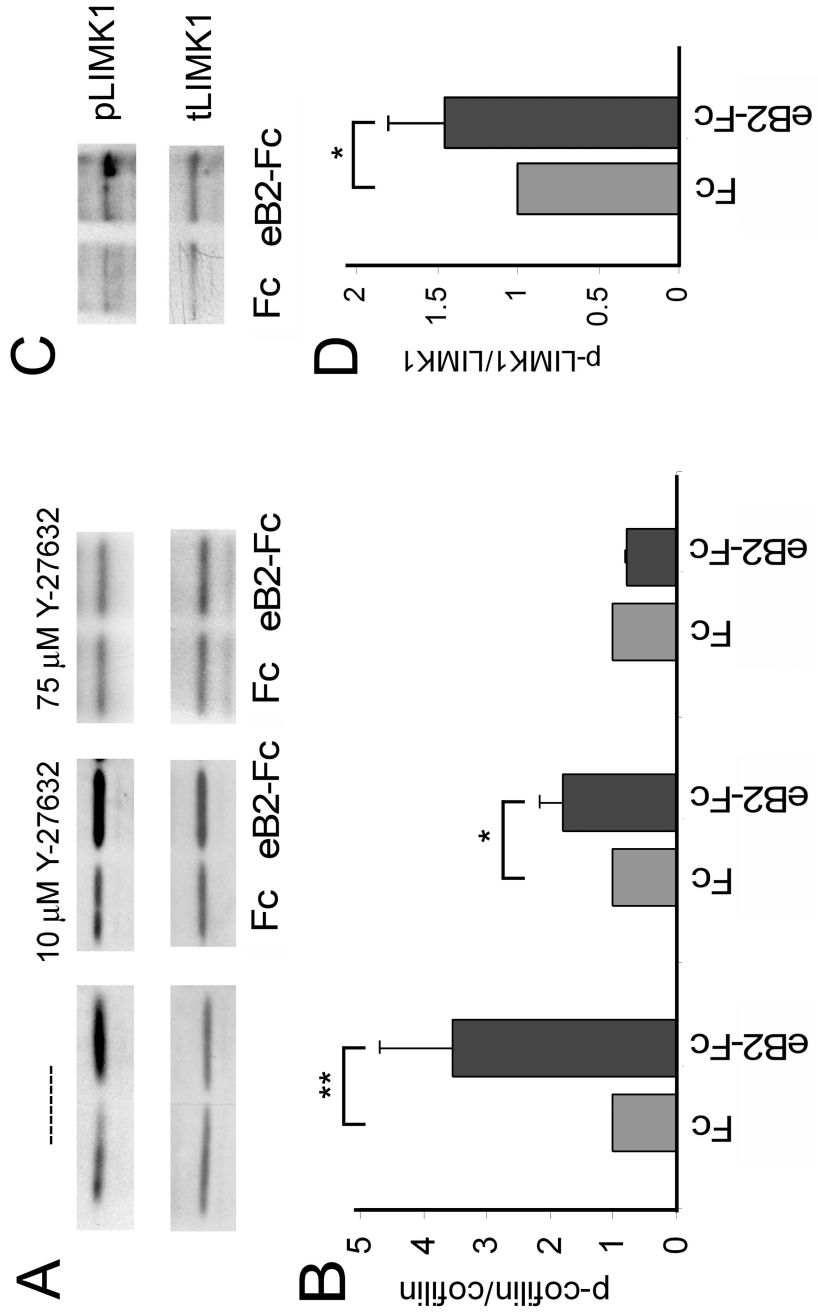


Figure 2-2 FAK-mediated regulation of mature dendritic spine morphology and cofilin phosphorylation depends on its activation/ phosphorylation and its ability to interact with regulators of Rho family GTPases

(A-E) Confocal images of 14 DIV hippocampal neurons expressing (A) GFP alone, (B) GFP and FAK (GFP + FAK), (C) GFP and FAK^{Y397E} (GFP + FAKY397E), (D) GFP and FAK^{Y397F} (GFP + FAKY397F), (E) GFP and FAK^{L1034S} (GFP + FAKL1034S). Neurons were obtained from E15 hippocampi of wt mice, transfected at 12 DIV and processed for indirect immunofluorescence at 14 DIV. Dendritic spine morphology was observed with GFP fluorescence (green), and the distribution of p-cofilin (red) and total cofilin (blue) was detected by immunostaining. Scale bar, 10 μ m.

(F) Quantification of the phospho-cofilin levels in dendrites of transfected (GFP-positive) and control (GFP-negative) neurons, and (G) the cofilin/ phospho-cofilin ratio. The levels of phospho-cofilin and cofilin were quantified by densitometry. Experimental values represent mean \pm SEM (n=20 dendrites from 5 neurons per group; ***, p<0.001; **, p<0.01; *, p<0.05).

(H-L) Quantification of (H) dendritic spine length, (I) dendritic spine density, (J) distribution of dendritic protrusion lengths: <2 μ m, 2-4 μ m, and >4 μ m, (K) dendritic spine head area, and (L) spine head area-to-length ratio. Vertical bars indicate SEM (n=500 dendritic protrusions from 5 neurons per group; ***, p<0.001; **, p<0.01; *, p<0.05).

Figure 2-2

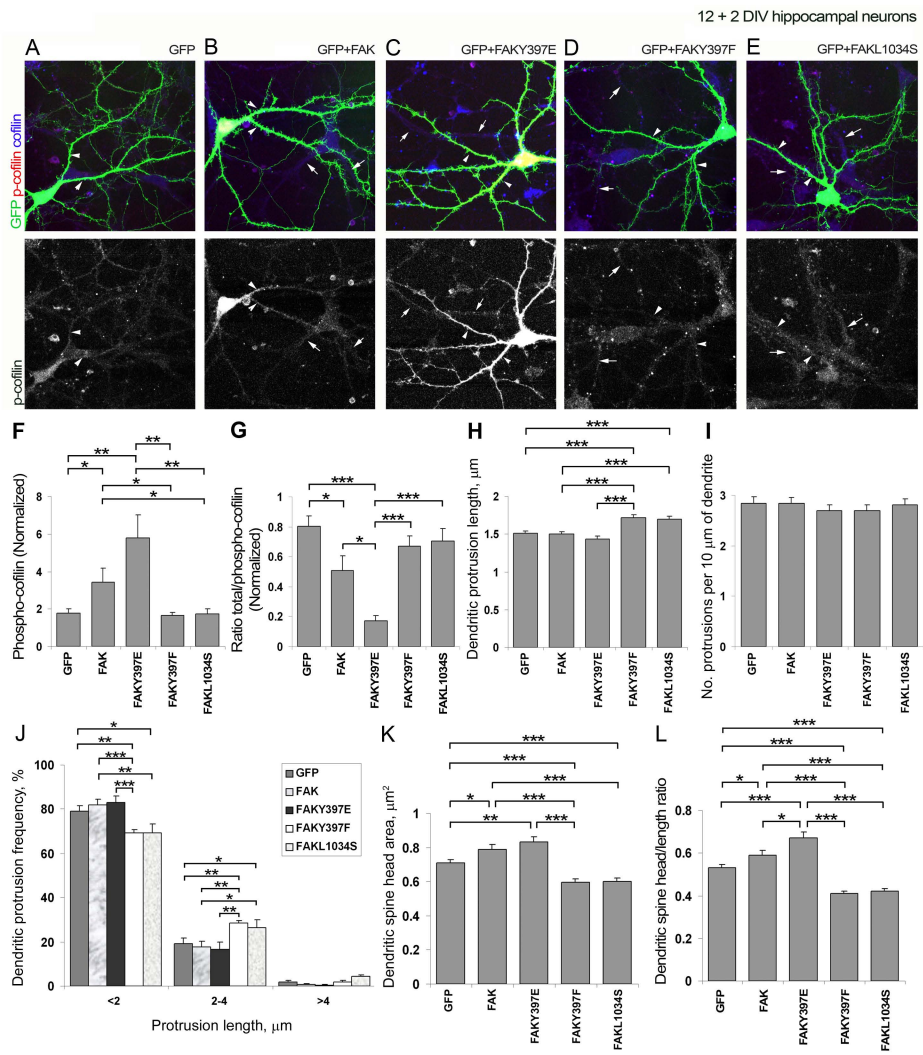


Figure 2-3 The constitutively active cofilin^{S3A} mutant, but not the inactive cofilin^{S3D} mutant or wt-cofilin, induces immature dendritic spines

(A-D) Confocal images of 14 DIV hippocampal neurons expressing (A) dsRed alone, (B) dsRed with GFP-wt-cofilin, (C) dsRed with GFP-cofilin^{S3D}, or (D) dsRed with GFP-cofilin^{S3A}. Scale bar, 10 μ m.

(E-H) Quantification of (E) dendritic protrusion length, (F) dendritic protrusion density, (G) dendritic spine head area, and (H) spine head to length ratio. Vertical bars indicate SEM (n=300 dendritic protrusions from 7-10 neurons per group); ***, p <0.001; **, p<0.01. The overexpression of the constitutively-active non-phosphorylatable cofilin^{S3A} mutant, but not the dominant-negative phospho-mimetic cofilin^{S3D} mutant or wt-cofilin, prompted the reversion of mushroom-shaped mature spines into thin spines with small heads.

Figure 2-3

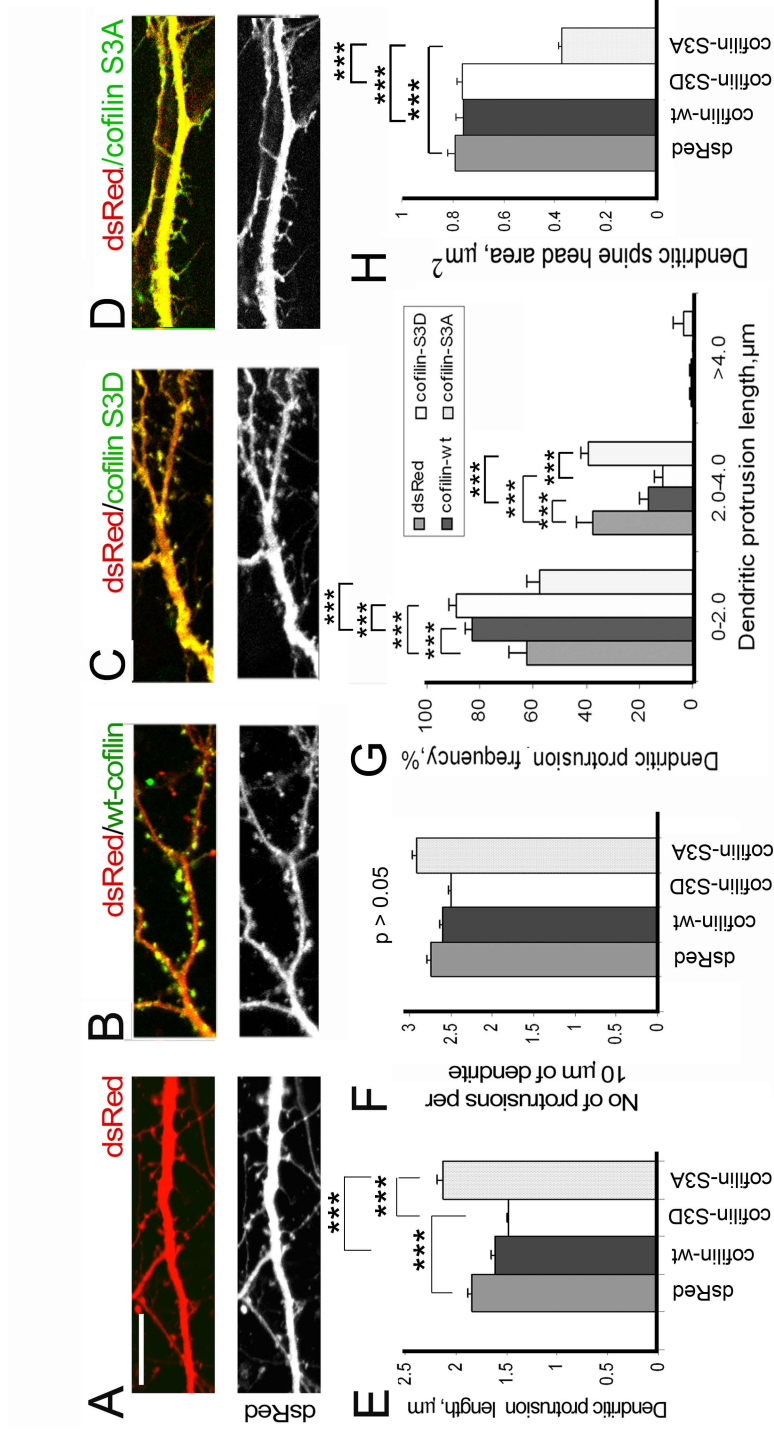


Figure 2-4 Dominant-negative phospho-mimetic cofilin^{S3D} and wt-cofilin, but not the non-phosphorylatable cofilin^{S3A}, were accumulated in the heads of mature mushroom-shaped spines

(A) Confocal images of 14 DIV hippocampal neurons expressing dsRed alone (dsRed), dsRed and GFP-wt-cofilin (dsRed/ wt-cofilin), dsRed and GFP-cofilin^{S3D} (dsRed/ CofilinS3D), or dsRed and GFP-cofilin^{S3A} (dsRed/ CofilinS3A). Squares depict areas of measurement used to quantify DsRed fluorescence (red, middle panel) or GFP fluorescence (green, lower panel).

(B and C) For each spine, DsRed fluorescence (middle panel) and GFP fluorescence (lower panel) were measured in the dendritic spine head and the adjacent area of dendritic shaft. The ratio of spine head-to-dendritic shaft DsRed fluorescence (upper graph) and GFP fluorescence (lower graph) is shown for cells expressing (B) dsRed and GFP-wt-cofilin (dsRed/ wt-cofilin) or (C) dsRed and GFP-cofilin^{S3D} (dsRed/ cofilinS3D). The ratio of spine head-to-dendritic shaft GFP fluorescence greater than 1 indicates accumulation of GFP-tagged cofilin in the dendritic spine head. Both wt-cofilin and phospho-mimetic cofilin^{S3D}, but not the non-phosphorylatable cofilin^{S3A} were accumulated in the heads of mature mushroom-shaped spines.

Figure 2-4

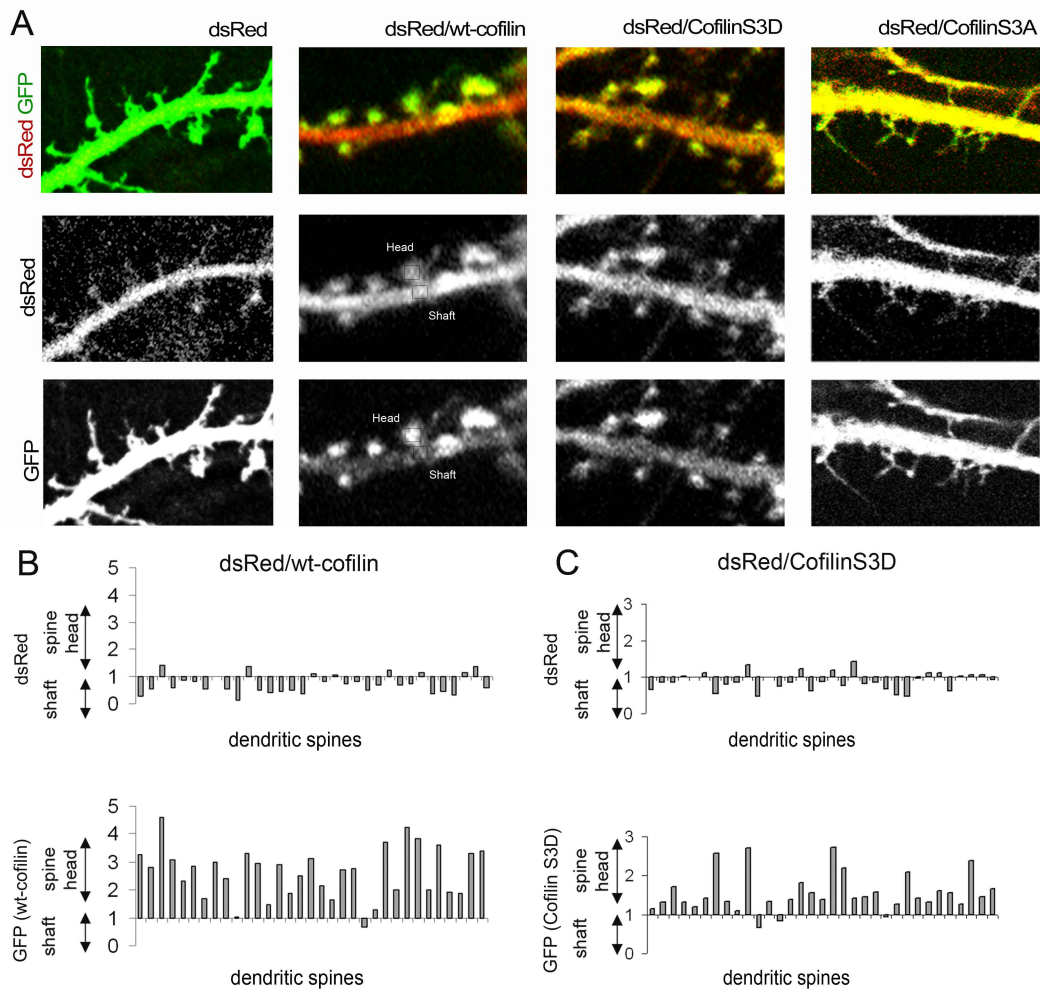


Figure 2-5 Cofilin inactivation restores mature dendritic spines in hippocampal neurons with inhibited EphB receptor activity

(A-D) Confocal images of 14 DIV hippocampal neurons expressing (A) dsRed alone (dsRed), (B) dsRed and dnEphB2K662R (dsRed/ dnEphB2), (C) dsRed, dnEphB2K662R and GFP-cofilin^{S3A} (dsRed/ dnEphB2/ cofilinS3A), or (D) dsRed, dnEphB2K662R and GFP-cofilin^{S3D} (dsRed/ dnEphB2/ cofilinS3D). Scale bar, 10 μ m.

(E-H) Quantification of (E) average dendritic protrusion length, (F) dendritic protrusion density, (G) distribution of dendritic protrusion lengths: <2 μ m, 2-4 μ m and >4 μ m, and (H) dendritic spine head area. Vertical bars indicate SEM (n=500-800 dendritic protrusions from 7-10 neurons per group); ***, p<0.001; **, p<0.05. The overexpression of the phospho-mimetic cofilin^{S3D} mutant, but not the non-phosphorylatable constitutively-active cofilin^{S3A} mutant, reversed the effects of EphB2 receptor inactivation on dendritic spine morphology.

Figure 2-5

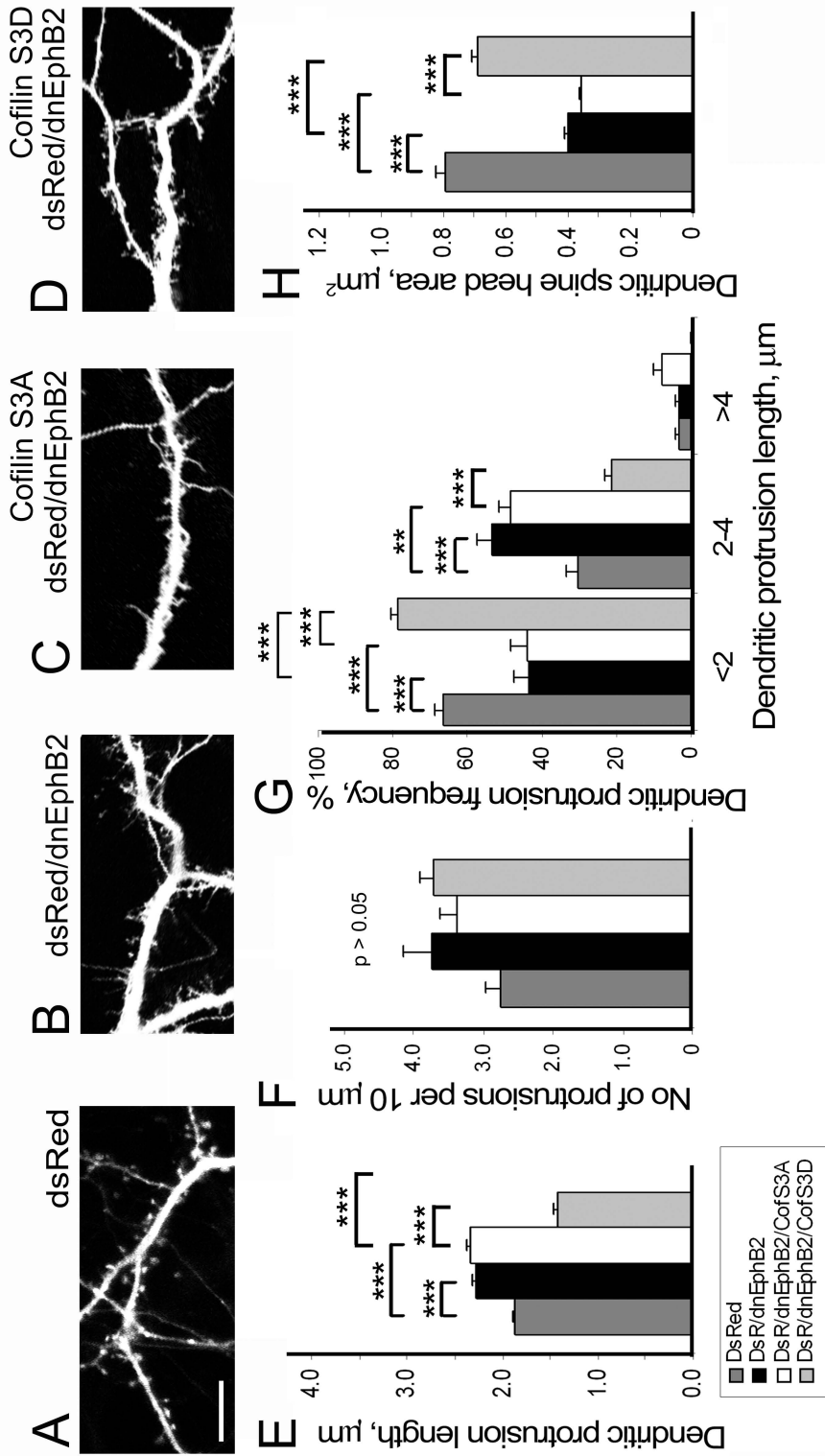


Figure 2-6 Cofilin inactivation restores mature dendritic spines in hippocampal neurons with Cre-mediated fak KO

(A-D) Confocal images of 14 DIV hippocampal neurons expressing (A) dsRed alone (dsRed), (B) dsRed and Cre (dsRed/ Cre), (C) dsRed, Cre, and GFP-cofilin^{S3A} (dsRed/ Cre/ Cofilin^{S3A}), or (D) dsRed, Cre, and GFP-cofilin^{S3D} (dsRed/ Cre/ CofilinS3D). Scale bar, 10 μ m. (E-H) Quantification of (E) average dendritic protrusion length, (F) dendritic protrusion density, (G) distribution of dendritic protrusion lengths: <2 μ m, 2-4 μ m and >4 μ m, and (H) dendritic spine head area. Vertical bars indicate SEM (n=500-800 dendritic protrusions from 7-10 neurons per group); ***, p<0.001; **, p<0.05. The overexpression of the phospho-mimetic cofilin^{S3D} mutant, but not the non-phosphorylatable constitutively-active cofilin^{S3A} mutant, reversed the effects of Cre-mediated deletion of fak on dendritic spine morphology.

Figure 2-6

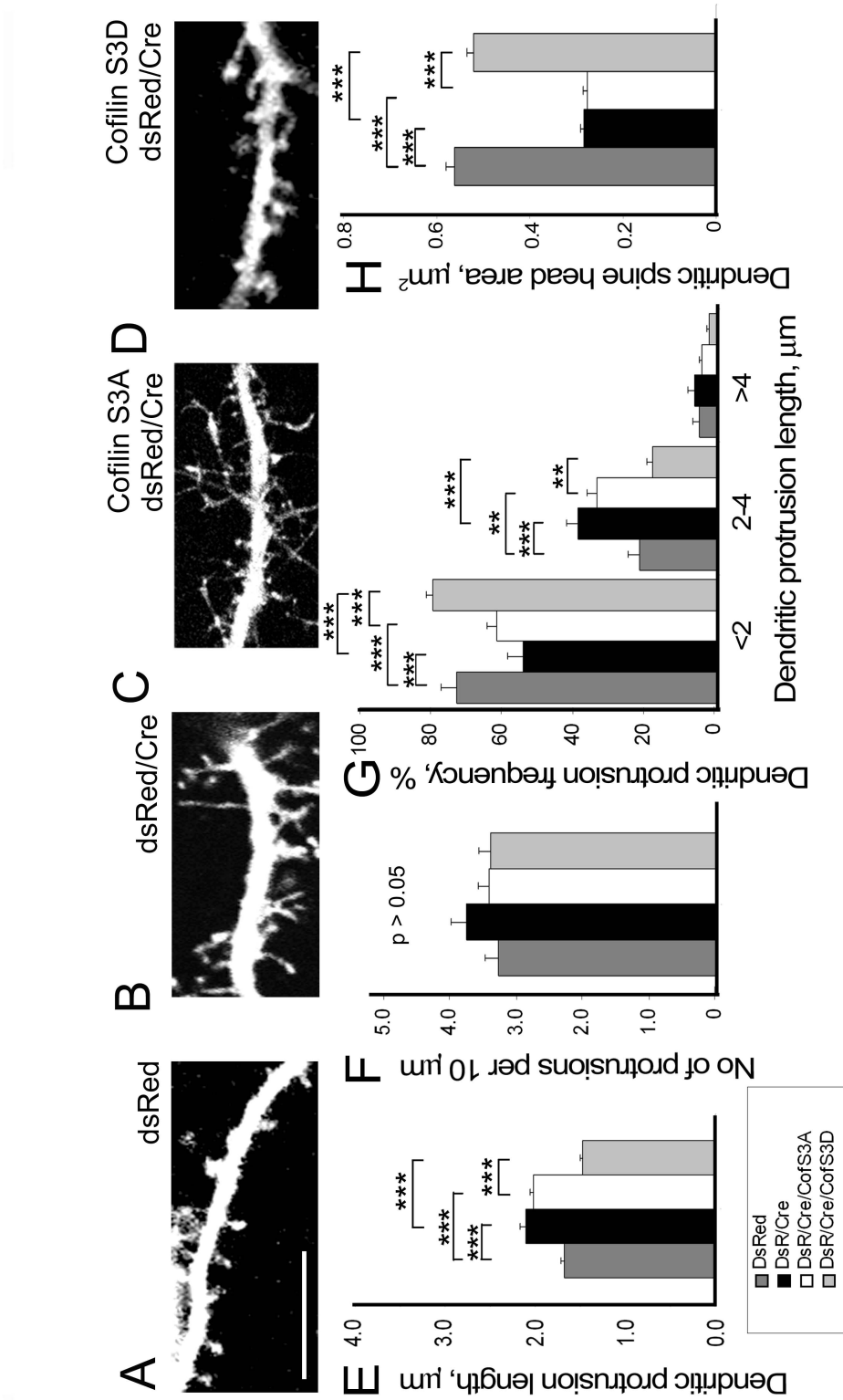
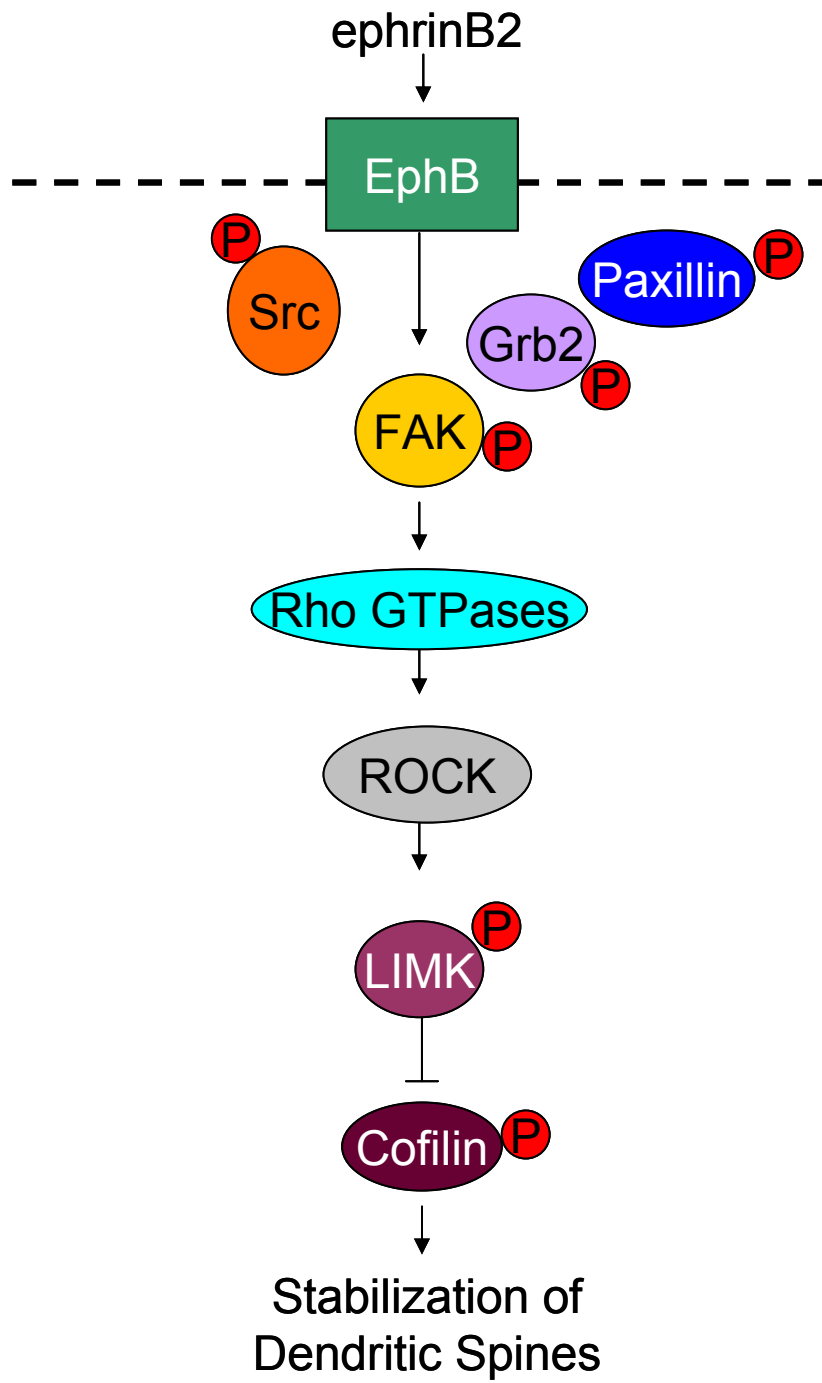


Figure 2-7 EphB receptor regulation of cofilin activity and dendritic spine morphology

Activation of the EphB receptor by the ephrinB2 ligand leads to recruitment and activation of FAK, followed by a downstream signaling cascade that results in cofilin phosphorylation and inactivation and stabilization of mature dendritic spines.

Figure 2-7



Chapter 3: The Regulation of Cofilin-Mediated Dendritic Spine Remodeling by NMDA Receptors in Mature Hippocampal Neurons

3.1 Abstract

Dendritic spines are the postsynaptic sites of most excitatory synapses in the brain, and changes in their morphology are implicated in synaptic plasticity and long-term memory. F-actin dynamics are thought to be a basis for both the formation of dendritic spines during development and their structural plasticity (Ethell & Pasquale, 2005; Pontrello & Ethell, 2009). We have previously shown that the F-actin-severing protein cofilin, which is regulated by phosphorylation, can induce remodeling of mature dendritic spines in hippocampal neurons (Shi *et al.*, 2009). Cofilin activity in dendritic spines is regulated by phosphorylation through two competing pathways, CaMKII-mediated suppression of cofilin activity by phosphorylation and calcineurin-dependent cofilin activation through its dephosphorylation. We show that NMDAR activation triggers calcineurin-mediated cofilin dephosphorylation/ activation, most likely through activation of slingshot phosphatase. NMDA receptor activation also promotes the translocation of cofilin to dendritic spines, an event that requires cofilin dephosphorylation. However, the calcineurin inhibitor alone did not block NMDA-induced cofilin dephosphorylation or cofilin translocation to spines. Our results suggest that both calcineurin and PI3K contribute to NMDA-induced cofilin dephosphorylation in hippocampal neurons. In addition, we have found that NMDAR activation also inhibits EphB-mediated cofilin phosphorylation. While EphB receptors suppress cofilin activity through LIMK-mediated

cofilin phosphorylation under normal synaptic activity, NMDAR activation results in cofilin dephosphorylation and suppression of EphB-mediated LIMK phosphorylation/activation. Here we investigate the effects of NMDAR activation on the phosphorylation state and localization of cofilin within hippocampal neurons, and its role in the remodeling of mature dendritic spines.

3.2 Introduction

NMDA receptors are ionotropic glutamate receptors that allow Ca^{2+} influx into the cell, setting in motion different intracellular signaling cascades and activating different downstream effectors, depending upon the amount of Ca^{2+} that enters the cell (Nicoll & Malenka, 1999; Lau *et al.*, 2009). Changes in dendritic spine morphology in response to NMDAR activation are implicated in synaptic plasticity and long-term memory processes. F-actin dynamics underlie the structural plasticity of dendritic spines, which allows for process such as LTP and LTD (Ethell & Pasquale, 2005; Pontrello & Ethell, 2009). NMDA application leads to dendritic spine remodeling (Shi & Ethell, 2006), an event that may be mediated by cofilin and its actin-severing activity. Cofilin activation could lead to spine head shrinkage and LTD (Beattie *et al.*, 2000; Malenka & Bear, 2004; Zhou *et al.*, 2004), or cofilin phosphorylation to spine head enlargement and facilitation of LTP (Chen *et al.*, 2007). Cofilin was recently shown to be also involved in AMPA receptor trafficking and addition of AMPA receptors to the cell membrane during LTP (Gu *et al.*, 2010). Indeed, the control of cofilin activity downstream of NMDA

receptor activation can regulate cytoskeletal dynamics and dendritic spine response to synaptic activity.

While NMDA receptors may induce spine remodeling through cofilin activation, we have previously shown that EphB receptors suppress cofilin activity through phosphorylation (Shi *et al.*, 2009), leading to spine stabilization. Interestingly, several of the EphB receptor's downstream effectors are also involved in NMDAR-mediated signaling, such as Rac, RhoA, PAK, LIMK, and cofilin. In addition, the EphB receptor is known to potentiate the NMDA receptor through activation of Src (Yu & Salter, 1999; Grunwald *et al.*, 2001; Takasu *et al.*, 2002; Salter & Kalia, 2004), leading to an increase in NMDAR gating. Therefore, we were interested in examining the cross-talk between these two pathways, and specifically looking at the regulation of cofilin and dendritic spine remodeling by these two receptors and their downstream effectors.

We show here that NMDAR activation by bath application of 50 μ M NMDA results in the phosphorylation of LIMK at 1, 5, and 15 minutes, but a decrease in p-cofilin at 5 and 15 minutes. The upregulation of pLIMK is achieved through a CaMKII-PAK pathway, while the decrease in p-cofilin is mediated by calcineurin-SSH or PI3K-SSH pathways. NMDA receptor activation promotes a rapid translocation of both wt-cofilin and constitutively-active cofilin^{S3A}, but not the inactive cofilin^{S3D} mutant, to the heads of dendritic spines, an effect that is not blocked by inhibition of CaMKII or calcineurin alone. However, the inhibition of calcineurin does prevent NMDAR-induced remodeling of dendritic spines, but this is prevailed by over-expression of wt cofilin or constitutively active cofilin^{S3A}. The accumulation of dephosphorylated (active) cofilin in dendritic

spines may underlie dendritic spine remodeling induced by NMDAR activation. Dephosphorylation is necessary for cofilin activity, and NMDA receptor activation achieves this dephosphorylation through a calcineurin-SSH or a PI3K-SSH pathway. The phosphorylation and activation of cofilin can regulate dendritic spine dynamics, which is believed to underlie LTP and LTD, as well as learning and memory.

3.3 Materials and Methods

Hippocampal neuronal cultures, transfection, and expression vectors. Cultures of hippocampal neurons were prepared from embryonic day 15 (E15) or E16 mice as previously described (Shi & Ethell, 2006). Briefly, after treatment with papain (0.5mg/ml) and DNaseI (0.6µg/ml) for 20 min. at 37°C and mechanical dissociation, cells were plated on glass coverslips or plastic dishes coated with poly-DL-ornithine (0.5mg/ml) and laminin (5µg/ml). The cells were cultured in Neurobasal medium with 25µM glutamine, 1% penicillin–streptomycin, and B-27 supplement (Invitrogen) under a 5% CO₂/ 10% O₂ atmosphere at 37°C. The cultures were transfected at 12 DIV using the calcium phosphate method as previously described (Jiang & Chen, 2006; Shi & Ethell, 2006). The expression vectors used were: pEGFP-N1 and pDsRed-C2 (Clontech); pcDNA3-EGFP-cofilin, pcDNA3-EGFP-cofilin^{S3A}, and pcDNA3-EGFP-cofilin^{S3D}.

Biochemical Analysis. Hippocampal neurons (approximately 1.2 million cells on 10cm dishes at 14 DIV) were rinsed quickly with Hank's Balanced Salt Solution (Invitrogen) containing 1.8mM CaCl₂, 1µM glycine, 0.493mM MgCl₂ and 0.407mM MgSO₄, then

treated with the appropriate reagent (in Hank's Balanced Salt Solution with 1.8mM CaCl₂ and 1μM glycine, with or without MgCl₂ and MgSO₄) under 5% CO₂/ 10% O₂ at 37°C. For NMDA receptor activation, cultures were treated with 50μM NMDA in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1μM glycine. Following treatment, the dishes were placed on ice and immediately washed with ice-cold PBS, then scraped from the plate in 1ml lysis buffer (25mM Tris-Cl, pH 7.4, 150mM NaCl, 1% TritonX-100, 5mM EDTA, pH 8.0, 1x protease inhibitor cocktail (Sigma-Aldrich), and 2mM sodium vanadate. After 30 min. rotation at 4°C, cell lysate was centrifuged for 15 min. at 16.1K r.c.f at 4°C. Supernatant was mixed 1:1 with 2x Laemmli loading buffer (Sigma-Aldrich), boiled for 10 min., and loaded onto an 8-16% Tris-glycine SDS PAGE gel (Invitrogen). The contents of the gel were transferred onto a Nitrocellulose membrane (Perkin Elmer), which was blocked in 5% milk/ TBS-Tween20 (0.2%), and specific primary antibodies (1:1000) were applied overnight at 4°C in 3% BSA/ TBS-Tween20. Secondary HRP-conjugated antibodies were applied (1:100,000) for 1 hour at room temperature in TBS-Tween20. The secondary antibodies used were the following: HRP-conjugated goat anti-rabbit (0.08μg/ml; Jackson ImmunoResearch, West Grove, PA) and HRP-conjugated donkey anti-mouse (0.08μg/ml; Jackson ImmunoResearch). Signal was detected on film using the ECL Plus detection kit from GE Healthcare. Phospho-LIMK and phospho-cofilin levels were quantified by densitometry (Adobe Photoshop) and normalized to total LIMK and total cofilin levels, respectively. Five to 10 independent experiments were performed for each condition. Statistical differences were compared using Student's t test.

Immunocytochemistry. Hippocampal neurons on glass coverslips (14 DIV) were treated with the appropriate reagent under 5% CO₂/ 10% O₂ at 37°C, then quickly rinsed with PBS and fixed in 2% paraformaldehyde, permeabilized in 0.1% TritonX-100, and blocked in PBS containing 5% normal goat serum and 1% BSA. Primary antibodies were applied in blocking solution for 2 hours at room temperature, and secondary antibodies were applied in PBS-Tween20 (0.2%) for 1 hour at room temperature. Coverslips were mounted in Vectashield anti-fade medium with DAPI (Vector Labs) and sealed with Cytoseal 60 (Fisher), then viewed under a confocal laser scanning microscope (model LSM 510; Carl Zeiss MicroImaging).

Antibodies. The primary antibodies used were as follows (all 1:1000 for Western blot): rabbit anti-phospho-LIMK1 (Thr508)/ LIMK2 (Thr505) (Cell Signaling Technology); mouse anti-LIMK1 (C-10) (Santa Cruz Biotechnology); rabbit anti-phospho-cofilin (phospho S3) (abcam); rabbit anti-cofilin (Cytoskeleton); mouse anti-synaptophysin SVP-38 (Sigma-Aldrich, 1:100 for immunocytochemistry). The secondary antibodies used were as follows: HRP-conjugated Donkey anti-Mouse IgG (Jackson ImmunoResearch, 1:100,000 for Western blot); HRP-conjugated Goat anti-Rabbit IgG (Jackson ImmunoResearch, 1:100,000 for Western blot); Alexa Fluor 660-conjugated anti-mouse IgG and anti-rabbit IgG (Invitrogen, 1:500 for immunocytochemistry).

Confocal microscopy. Fluorescence was analyzed using a confocal laser-scanning microscope (model LSM 510; Carl Zeiss Micro-Imaging). A series of five high-resolution optical sections (1024x1024 pixel format) were taken for each neuron with a 63x water-immersion objective (1.2 numerical aperture), 1x zoom at 0.5 μ m step intervals (z-stack). All images were acquired under identical conditions. Each z-stack was collapsed into a single image by projection (Zeiss LSM Image software), converted to a tiff file, and analyzed using Image J Software. Seven to 10 neurons were randomly selected for each experimental group, and three to four proximal dendrites per each neuron were analyzed.

Live imaging and fluorescence intensity. Time-lapse imaging of live cells was performed under an inverted fluorescent microscope (model TE2000; Nikon) with 40x oil Fluor objectives, and monitored by a 12-bit CCD camera (model ORCA-AG; Hamamatsu) using Image-Pro software (Media Cybernetics). During imaging, the cultures were maintained in Hank's Balanced Salt Solution (Invitrogen) supplemented with 1.8mM CaCl₂, and 1 μ M glycine, at 37°C with 5% CO₂. Images were captured at 1 min. intervals for 1 hour. For quantification of cofilin levels in spine heads versus dendrites, the EGFP fluorescence level (normalized to the DsR fluorescence level) was measured in each spine head and in an equally-sized region of the dendrite at the base of each spine (Adobe Photoshop). For each spine, the sum of EGFP fluorescence detected in the head and in the base was set equal to 100% EGFP signal for that spine. The percentage of the total EGFP present in each head and in each base was determined.

Pharmacology. Activation of the EphB receptor was achieved with 4µg/ml ephrinB2-Fc, pre-clustered before use for 2 hours with goat anti-human IgG (Jackson Labs). The NMDA receptor was activated with N-Methyl-D-aspartic acid (NMDA) (Sigma-Aldrich, 50µM), and inhibited using (+)-MK-801 Hydrogen Maleate (MK801), (Sigma-Aldrich, 10µM). CaMKII activity was inhibited with 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N methylbenzylamine (KN93) (Calbiochem, 10µM, 30 min. pre-treatment), and the inactive analog to the CaMKII inhibitor was used as a control: 2-[N-(4-Methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine Phosphate (KN92), (Calbiochem, 10µM). CyclosporinA (Sigma-Aldrich, 50µM, 2 hours pretreatment) was used for inhibition of calcineurin phosphatase, LY294002 for inhibition of PI3-kinase (Sigma-Aldrich, 50µM, 10 min. pre-treatment), and IPA-3 for inhibition of PAK (Sigma-Aldrich, 20µM, 10 min. pre-treatment).

3.4 Results

3.4.1 NMDAR activation triggers LIMK phosphorylation/ activation, as well as cofilin dephosphorylation/ activation.

We have previously shown that under normal synaptic activity, EphB receptors promote the stabilization of mature dendritic spines (Shi *et al.*, 2009), whereas NMDAR activation results in dendritic spine remodeling (Shi & Ethell, 2006). Our previous findings indicate that EphB-mediated mature spine maintenance relies on the LIMK-mediated suppression of cofilin activity through its

phosphorylation, and that a non-phosphorylatable constitutively-active cofilin^{S3A} induces rapid remodeling of established mature dendritic spines (Shi *et al.*, 2009), effects that are similar to dendritic spine remodeling triggered by acute NMDA treatment (Shi & Ethell, 2006). In order to determine whether up-regulation of cofilin activity by its dephosphorylation underlies NMDAR-mediated spine remodeling, we first examined phosphorylation levels of cofilin and its kinase LIMK in response to NMDAR activation. Surprisingly, activation of the NMDA receptor by bath application of 50 μ M NMDA with in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1 μ M glycine resulted in the phosphorylation of LIMK at 1, 5, and 15 minutes as compared to control untreated neurons (Fig. 3-1A, 3-1C), which was blocked by the NMDA receptor inhibitor MK801 (10 μ M). However, this up-regulation of LIMK phosphorylation did not lead to an increase in cofilin phosphorylation (Fig. 3-1B, 3-1D); rather, there was a significant decrease in p-cofilin at 5 and 15 minutes, suggesting activation of an additional pathway that induces cofilin dephosphorylation.

3.4.2 NMDAR activation inhibits EphB-mediated LIMK phosphorylation/activation and cofilin phosphorylation/inactivation.

As EphB receptors promote LIMK-mediated cofilin phosphorylation, we next examined whether NMDAR activation will also inhibit EphB-induced cofilin phosphorylation mediated by LIMK. While under normal synaptic activity, EphB receptor activation with 4 μ g/ml ephrinB2-Fc (eB2Fc) promoted LIMK phosphorylation at 5 minutes, followed by cofilin phosphorylation at 15 minutes, concurrent activation of EphB and NMDA receptors down-regulated both

pLIMK and p-cofilin levels (Fig. 3-2A-3-2D). Inhibition of calcineurin with cyclosporinA (CycA; 50 μ M) abolished the inhibitory effects of NMDA on ephrinB2-mediated LIMK and cofilin phosphorylation, showing that NMDA-mediated enhancement of calcineurin activity may be responsible for the decrease in pLIMK and p-cofilin levels. Our studies demonstrate that NMDAR activation shifts the p-cofilin/cofilin equilibrium toward active cofilin through a signaling pathway that triggers cofilin dephosphorylation and counteracts EphB-mediated cofilin phosphorylation. Concurrent activation of NMDA and EphB receptors enhances this signaling pathway and results in LIMK and cofilin dephosphorylation.

3.4.3 NMDAR-induced increase in LIMK phosphorylation depends on CaMKII

activation. Upon NMDA receptor activation, different pathways may be activated, depending on the amount of Ca²⁺ influx into the cell. The activation of CaMKII by the NMDAR can lead to PAK and LIMK activation, followed by cofilin inactivation, whereas the activation of calcineurin by the NMDAR could up-regulate SSH phosphatase activity and activate cofilin. Our results demonstrate that CaMKII activation is the primary mechanism by which the NMDA receptor achieves an increase in pLIMK levels, as NMDA-induced LIMK phosphorylation can be blocked by the CaMKII inhibitor KN93 (10 μ M), but not the inactive analog KN92 (Fig. 3-3A, 3-3C). Inhibition of CaMKII also reduced cofilin phosphorylation levels under normal synaptic activity, but did not enhance NMDA-mediated cofilin dephosphorylation (Fig. 3-3B, 3-3D),

suggesting that another pathway is involved in NMDA-mediated regulation of cofilin phosphorylation.

3.4.4 NMDAR-induced cofilin dephosphorylation is regulated through calcineurin

and PI3K pathways. Inhibition of PI3-kinase (PI3K) with LY294002 (LY; 50 μ M), PAK with IPA-3 (20 μ M), or Rho kinase (ROCK) with Y-27632 (Y-27; 75 μ M) partially blocked NMDA-induced LIMK phosphorylation (Fig. 3-4A, 3-4C), confirming that these pathways are involved in NMDA-induced LIMK phosphorylation. Calcineurin does not seem to participate in this pathway, as its inhibition with cyclosporinA (CycA; 50 μ M) did not affect NMDA-induced increase in pLIMK levels, and concurrent inhibition of PI3K and calcineurin had the same effect on pLIMK levels as did PI3K inhibition alone. The NMDAR-induced up-regulation of LIMK phosphorylation seems to be achieved predominantly through a CaMKII- and PI3K-mediated activation of Rac-RhoA and Rac-PAK pathways (Fig. 3-9).

While NMDAR activation alone decreases p-cofilin levels, additional PAK or ROCK inhibition leads to a further decrease in p-cofilin levels (Fig. 3-4B, 3-4D). These results indicate that although SSH may be the primary factor leading to cofilin dephosphorylation following NMDAR activation, NMDA-induced ROCK and PAK activation of LIMK, mediates cofilin phosphorylation and may play an important role in maintaining a dynamic spatial and temporal balance between cofilin phosphorylation and dephosphorylation. Surprisingly, inhibition of calcineurin or PI3K alone did not block NMDA-induced cofilin dephosphorylation; however concurrent inhibition of calcineurin

and PI3K did return p-cofilin to control levels, which suggests that in addition to calcineurin, PI3K can also contribute to NMDA-induced cofilin dephosphorylation, probably through a Ras-PI3K-SSH pathway (Fig. 3-9). These studies show that although the NMDAR activates LIMK through CaMKII-mediated PAK and ROCK activation, cofilin phosphorylation by LIMK is counteracted, most likely through calcineurin- and PI3K-mediated activation of SSH, causing an overall decrease in p-cofilin levels.

3.4.5 NMDA receptor activation promotes rapid translocation of cofilin to dendritic

spines. Cofilin dephosphorylation also appears to underlie NMDAR-induced translocation of cofilin to dendritic spines. Our recent studies have shown that cofilin is localized in dendritic spines, and its activity is suppressed in mature spines by phosphorylation. Moreover, a non-phosphorylatable constitutively-active cofilin^{S3A}, but not wt-cofilin or inactive cofilin^{S3D}, induces rapid remodeling of established mature dendritic spines and extension of new dendritic filopodia (Shi *et al.*, 2009). Here we demonstrate that NMDA receptor activation promotes the rapid translocation of both wt-cofilin and non-phosphorylatable constitutively-active cofilin^{S3A}, but not the phospho-mimetic inactive cofilin^{S3D} mutant, to the heads of dendritic spines and the tips of dendritic filopodia (Fig. 3-5A, 3-5B, Fig. 3-6), an effect that is blocked by the specific NMDAR inhibitor MK801 (Fig. 3-5C, Fig. 3-6). The accumulation of dephosphorylated (active) cofilin in dendritic spines may underlie dendritic spine remodeling induced by NMDAR activation (Shi & Ethell, 2006). Our data also show that the inhibition of CaMKII with KN93 did not affect NMDAR-induced translocation of wt-cofilin to

dendritic spines (Fig. 3-5D, 3-5E, 3-6B). In addition, calcineurin inhibition with cyclosporinA alone was not sufficient to prevent cofilin clustering in dendritic spines (Fig. 3-5F, 3-5G, 3-6B), suggesting that the PI3K pathway that regulates cofilin dephosphorylation may also be involved in cofilin translocation. However, dephosphorylation alone is not sufficient to trigger cofilin clustering in the spines, as the non-phosphorylatable cofilin^{S3A} is diffusely distributed throughout the dendrites of untreated neurons (Fig. 3-5A, 3-6C). These results show that cofilin dephosphorylation was not sufficient to trigger cofilin clustering in the dendritic spines, suggesting that in addition to phosphorylation state, cofilin translocation into dendritic spines is also regulated by other mechanisms. Potential candidates for future studies would be scaffolding proteins such as the β -Arrestins, which could spatially control cofilin and its regulators.

3.4.6 Calcineurin mediates dendritic spine remodeling following NMDA application.

Our studies indicate that acute treatment of 14 DIV hippocampal neurons with 50 μ M NMDA in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1 μ M glycine results in the transformation of mature spines with large heads into immature thin spines with small heads, showing a significant decrease in the spine head-to-length ratio 20 minutes after NMDA application (Fig. 3-7A, 3-8A). A small head area and head-to-length ratio would indicate an immature spine phenotype. NMDA-induced changes in spine morphology were blocked by the NMDAR antagonist MK801 and by the calcineurin inhibitor cyclosporinA (Fig. 3-8A), suggesting that calcineurin-mediated

cofilin dephosphorylation may underlie dendritic spine remodeling induced by NMDAR activation. CaMKII inhibition with KN93 did not have an effect on NMDA-induced spine remodeling (Fig. 3-8A), showing that NMDA application promotes spine remodeling predominantly through a calcineurin, but not CaMKII, pathway. In contrast, inhibition of calcineurin did not block NMDA-induced dendritic spine remodeling in the neurons overexpressing wt cofilin (Fig. 3-8A). Moreover, overexpression of constitutively-active cofilin^{S3A} mimicked the effects of NMDA, and the calcineurin inhibitor did not reverse cofilin^{S3A}-induced spine remodeling (Fig. 3-7C, 3-8C), suggesting that over-expression of active cofilin can overcome calcineurin inhibition and lead to spine remodeling. This supports our hypothesis that cofilin activation through calcineurin/ SSH is a mechanism behind NMDAR-induced dendritic spine remodeling.

3.5 Discussion

The tight regulation of cofilin activity and localization underlies dendritic spine morphology changes that may lead to strengthening or disassembly of spines and synapses. We have previously shown that over-expression of constitutively-active cofilin leads to more immature spines and filopodia, whereas dominant-negative cofilin promotes stable mature mushroom spines. EphB receptor activation leads to the phosphorylation and inactivation of cofilin through a RhoA-LIMK pathway, resulting in spine stabilization (Shi *et al.*, 2009). Here we focus on the role of cofilin activity in NMDA receptor-mediated dendritic spine plasticity. NMDA application leads to an increase in active CaMKII, Ras, Rac, and PAK (Carlisle *et al.*, 2008), resulting in LIMK

activation, which would be expected to enhance cofilin phosphorylation and inactivation. However, we see that cofilin phosphorylation decreases with NMDA application, implicating involvement of another pathway that promotes cofilin dephosphorylation, most likely through the cofilin phosphatase SSH. Our results demonstrate that NMDA-induced activation of calcineurin and PI3K are both involved in cofilin dephosphorylation seen in response to NMDAR activation in 14 DIV hippocampal neurons. Upon NMDA receptor activation, the amount of Ca^{2+} that enters the cell determines the intracellular pathway of effectors that will be activated. High levels of Ca^{2+} lead to CaMKII activation, whereas low Ca^{2+} activates calcineurin (Nicoll & Malenka, 1999; Lau *et al.*, 2009). This Ca^{2+} -dependent specificity leads to control of many diverse cellular functions such as growth cone guidance (Wen *et al.*, 2004), neuronal differentiation (Joo *et al.*, 2007) and migration (Komuro & Rakic, 1993), and synaptogenesis (Bressloff, 2006). In addition, NMDA-induced CaMKII activation leads to LTP, whereas calcineurin activation promotes LTD (Rusnak & Mertz, 2000; Malenka & Bear, 2004).

Changes in spine morphology and LTP/ LTD are thought to underlie learning and memory process. Mature mushroom spines are more resistant to changes induced by LTP than are thin spines (Matsuzaki *et al.*, 2004), so the more flexible thin spines are thought to be involved in learning processes (Bourne & Harris, 2008). Cofilin and its actin-severing activity are involved in spine plasticity, through transient activation and actin remodeling, followed by inactivation that allows spines to stabilize (Chen *et al.*, 2007). We see here that NMDA application leads to the up-regulation of LIMK activity, which

is achieved through a CaMKII-PAK pathway. Cofilin, on the other hand, is dephosphorylated through calcineurin-SSH and PI3K-SSH pathways. This activation of cofilin is a type of NMDAR-mediated plasticity that results in transient dendritic spine remodeling. Interestingly, we also found that the NMDAR inhibits EphB-mediated LIMK activation and cofilin phosphorylation. Concurrent EphB and NMDA receptor activation leads to a decrease in pLIMK levels, resulting in an even greater decrease in p-cofilin levels than in response to NMDA alone. This suggests that the EphB receptor predominantly activates different pathways under normal synaptic activity or in response to NMDAR activation. Indeed, there is cross-talk between EphB and NMDA receptors (Dalva *et al.*, 2000). The EphB receptor is known to potentiate the NMDA receptor through activation of Src (Yu & Salter, 1999; Takasu *et al.*, 2002; Salter & Kalia, 2004). It is clear that cross-talk between the EphB and NMDA receptors leads to an emphasis on different effectors in the downstream signaling pathways than activation of either receptor alone.

The phosphorylation state of cofilin affects not only its activity, but also its localization within cells (Nebl *et al.*, 1996). In several systems studied, different stimuli induced cofilin localization to different cellular locations, from the nucleus, to the cytoplasm, to the cell membrane (Ohta *et al.*, 1989; Abe *et al.*, 1993; Samstag *et al.*, 1994; Suzuki *et al.*, 1995). Here we examined the localization of phosphorylated and dephosphorylated cofilin in hippocampal neurons under normal synaptic activity and in response to NMDA receptor activation. Application of NMDA led to a rapid translocation of cofilin to dendritic spine heads. Using non-phosphorylatable and

phospho-mimetic mutants, we found that cofilin dephosphorylation is necessary for cofilin to translocate to dendritic spines with NMDA application, but dephosphorylation is not sufficient to cause spine localization under normal synaptic activity. It is likely that dephosphorylated active cofilin is trafficked to spine heads in order to promote the transient spine remodeling that is seen with NMDA receptor activation (Shi & Ethell, 2006), which is also needed for AMPA receptor trafficking and insertion into the cell membrane (Malinow & Malenka, 2002; Brecht & Nicoll, 2003; Gu *et al.*, 2010; Rust *et al.*, 2010). This could be followed by the inactivation of cofilin, spine stabilization, and LTP consolidation (Chen *et al.*, 2007), or destabilization of the cell membrane, AMPA receptor internalization, spine shrinkage, and LTD (Beattie *et al.*, 2000; Man *et al.*, 2000; Zhou *et al.*, 2004).

Cofilin dephosphorylation alone was not sufficient to trigger cofilin clustering in the spines, as the non-phosphorylatable cofilin^{S3A} was diffusely distributed throughout the dendrites of untreated neurons. This indicates that although phosphorylation state is important to cofilin localization within cells, there is another factor that also contributes to cofilin translocation. Likely candidates include scaffolding proteins that could control not only cofilin phosphorylation by localizing it with its regulators, but also the spatial distribution of cofilin. Some scaffolding proteins are associated with the post-synaptic density (PSD) and are known to regulate dendritic spine morphology, such as PSD-95 (Keith & El-Husseini, 2008), Homer (de Bartolomeis & Iasevoli, 2003), and Shank (Kreienkamp, 2008). The 14-3-3 scaffolding protein is known to interact with cofilin and SSH, and to increase p-cofilin levels (Gohla & Bokoch, 2002), which has an effect on

acetylcholine receptor clustering (Lee *et al.*, 2009). The 14-3-3 protein regulates potassium channel trafficking (Rajan *et al.*, 2002) and Ca_v2.2 calcium channel activity (Li *et al.*, 2006). β -Arrestins are known to be involved in the inactivation of GPCR signaling, but they are also scaffolding proteins that can effect changes on the actin cytoskeleton (Barlic *et al.*, 2000; Bhattacharya *et al.*, 2002; Wang & DeFea, 2006). Moreover, β -Arrestins have recently been shown to play an important role in the spatial localization of cofilin and its regulating proteins (Zoudilova *et al.*, 2010). β -Arrestins' functions extend beyond GPCR desensitization and into the regulation of cytoskeletal dynamics, and will therefore be important for future studies of cofilin localization with hippocampal neurons.

Dendritic spines remain plastic even in the adult brain, due to such proteins as cofilin that can re-arrange the F-actin cytoskeleton (Bamburg *et al.*, 1999), thus dictating spine morphology (Shi *et al.*, 2009). The phosphorylation state of cofilin in hippocampal neurons is tightly regulated by LIMK and SSH phosphatase, both of which are controlled by cell surface receptors such as EphB and NMDA receptors. In addition to activation state, the localization of cofilin within the cell is important for its actin remodeling activity. If cofilin is located aberrantly in the cell, it will not effect the appropriate changes on actin and dendritic spines when it is active. Abnormality in any of the many factors that control cofilin activity could lead to aberrant dendritic spines and neurological deficits such as those seen in Down syndrome and the autism spectrum disorders (Kaufmann & Moser, 2000; Sorra & Harris, 2000; Halpain *et al.*, 2005). In addition, cofilin-actin rods are stress-induced structures that are found in the brains of

patients with Alzheimer disease (Maloney & Bamberg, 2007), and inactive cofilin has been shown to prevent dendritic spine loss caused by soluble A β oligomers in rat organotypic slices (Shankar *et al.*, 2007), suggesting cofilin as potential therapeutic target to prevent A β -induced spine loss in patients with Alzheimer disease. This implicates cofilin as an important regulator of cytoskeletal and dendritic spine dynamics, whose activity and localization must be tightly controlled for proper synaptic function.

3.6 References

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Figure 3-1 NMDAR activation triggers LIMK phosphorylation/ activation, as well as cofilin dephosphorylation/ activation

(A-D) 14 DIV hippocampal neurons were treated with 50 μ M NMDA in Mg²⁺-free solution to activate NMDA receptors for 1, 5, or 15 minutes with or without the NMDAR inhibitor MK801 (10 μ M). Cell lysates were subjected to immunoblotting with anti-phospho-LIMK1/2 (A) or anti-phospho-cofilin (B) antibodies. The blots were stripped and re-probed against total LIMK1 or cofilin. The levels of phospho-LIMK1 (C) or phospho-cofilin (D) were quantified by densitometry and normalized to total LIMK1 or cofilin levels, respectively. Experimental values represent mean \pm SEM (n= 5-10). Statistical differences were compared using Student's *t* test; ***, p < 0.001; **, p, 0.01; *, p < 0.05.

Figure 3-1

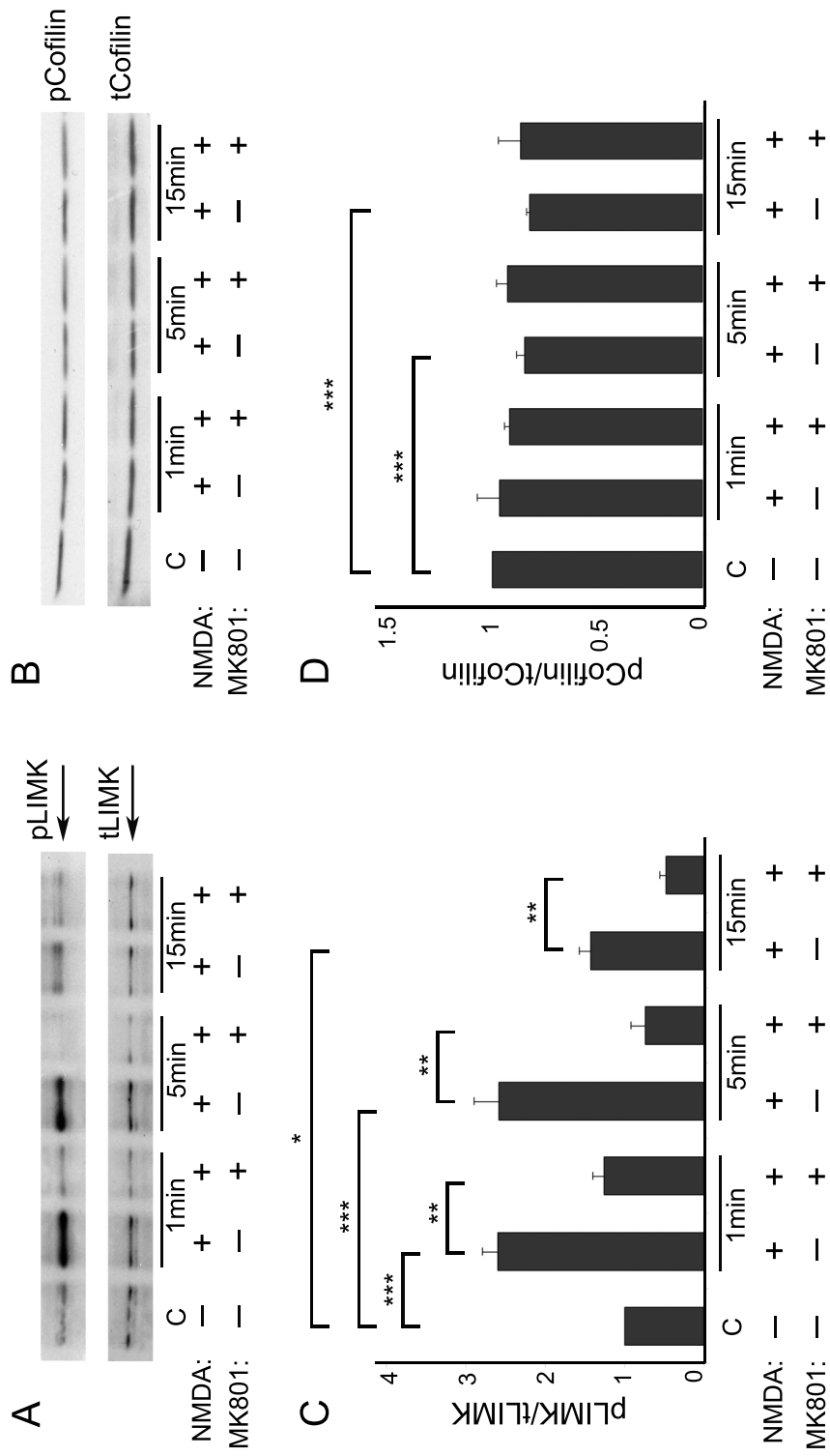


Figure 3-2 NMDAR activation inhibits EphB-mediated LIMK phosphorylation/activation and cofilin phosphorylation/inactivation

(A-D) 14 DIV hippocampal neurons were treated with 50 μ M NMDA in Mg²⁺-free solution to activate NMDA receptors, with 4 μ g/ml pre-clustered ephrinB2-Fc (eB2Fc) to activate EphB receptors, or with 4 μ g/ml pre-clustered ephrinB2-Fc (eB2Fc) plus 50 μ M NMDA in Mg²⁺-free solution for 5 min. Cell lysates were subjected to immunoblotting with anti-phospho-LIMK1/2 (A) or anti-phospho-cofilin (B) antibodies. The blots were stripped and re-probed against total LIMK1 or cofilin. The levels of phospho-LIMK1 (C) or phospho-cofilin (D) were quantified by densitometry and normalized to total LIMK1 or cofilin levels, respectively. Experimental values represent mean \pm SEM (n = 5-10). Statistical differences were compared using Student's *t* test; ***, p < 0.001; **, p, 0.01; *, p < 0.05.

Figure 3-2

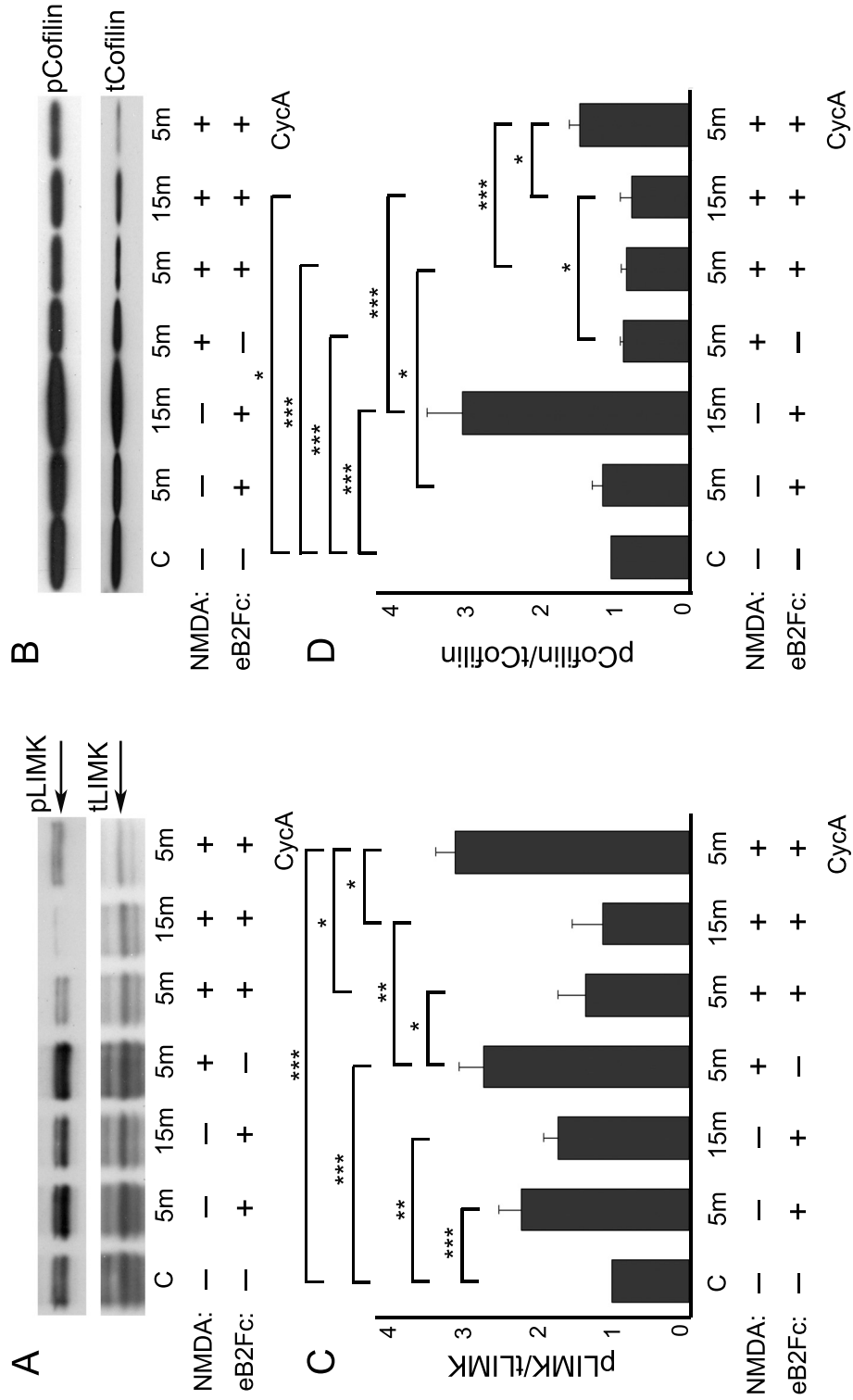


Figure 3-3 The NMDAR-induced increase in pLIMK depends on CaMKII

(A-D) 14 DIV hippocampal neurons were treated with 50 μ M NMDA in Mg²⁺-free solution to activate NMDA receptors for 5 minutes with either the CaMKII inhibitor KN93 or its inactive analog KN92 at 10 μ M. Cell lysates were subjected to immunoblotting with anti-phospho-LIMK1/2 (A) or anti-phospho-cofilin (B) antibodies. The blots were stripped and re-probed against total LIMK1 or cofilin. The levels of phospho-LIMK1 (C) or phospho-cofilin (D) were quantified by densitometry and normalized to total LIMK1 or cofilin levels, respectively. Experimental values represent mean \pm SEM (n= 5-10). Statistical differences were compared using Student's *t* test; ***, p < 0.001; **, p, 0.01; *, p < 0.05.

Figure 3-3

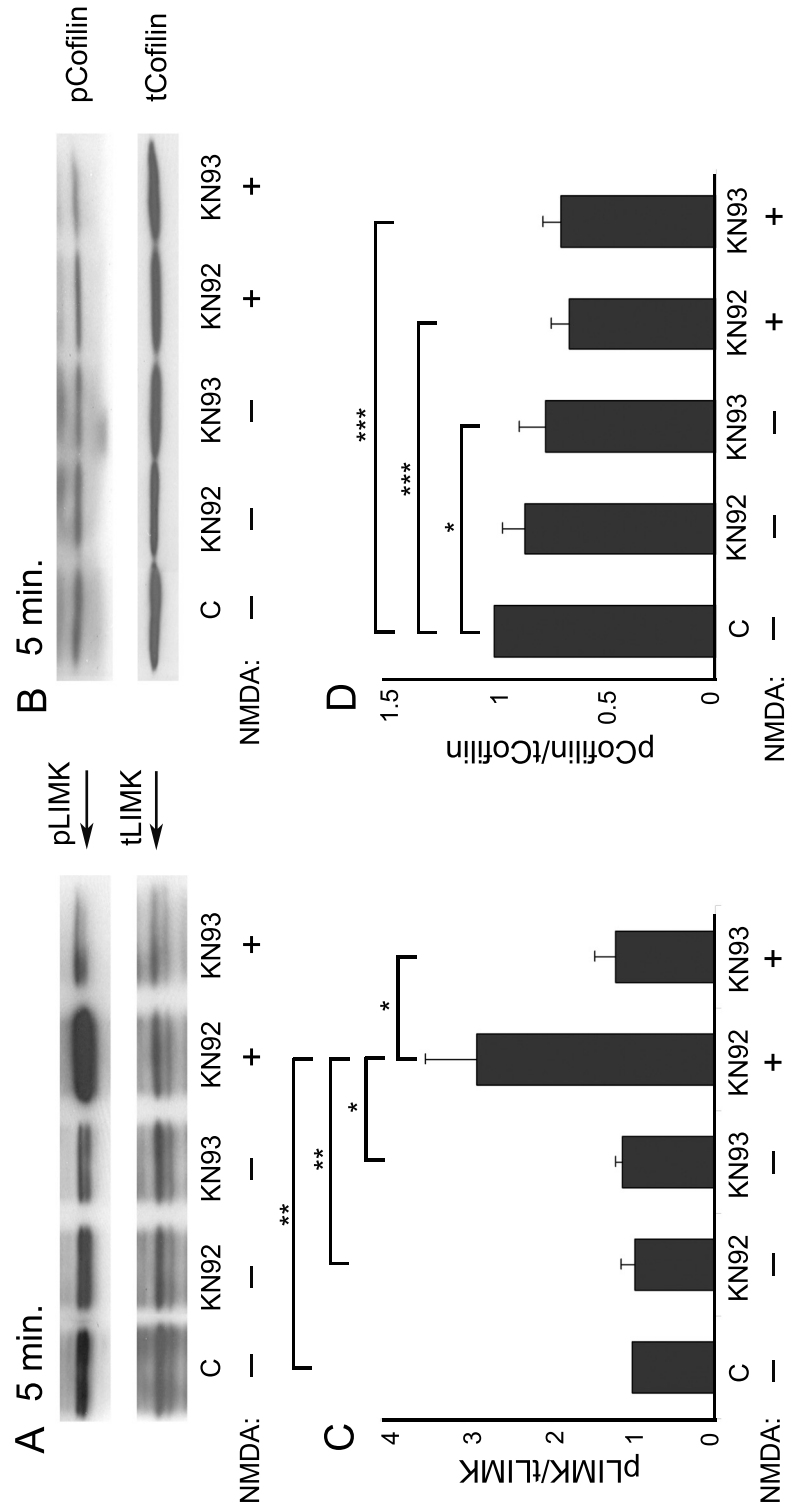


Figure 3-4 The NMDAR-induced decrease in p-cofilin is mediated by calcineurin

(A-D) 14 DIV hippocampal neurons were treated with 50 μ M NMDA in Mg²⁺-free solution to activate NMDA receptors for 5 minutes. NMDA was applied alone, or together with inhibitors of either PI3K (LY294002; 50 μ M, 10 min pretreatment), PAK (IPA-3; 20 μ M, 10 min pretreatment), or calcineurin (cyclosporinA; 50 μ M, 2 hr pretreatment). Cell lysates were subjected to immunoblotting with anti-phospho-LIMK1/2 (A) or anti-phospho-cofilin (B) antibodies. The blots were stripped and re-probed against total LIMK1 or cofilin. The levels of phospho-LIMK1 (C) or phospho-cofilin (D) were quantified by densitometry and normalized to total LIMK1 or cofilin levels, respectively. Experimental values represent mean \pm SEM (n= 5-10). Statistical differences were compared using Student's *t* test; ***, p < 0.001; **, p, 0.01; *, p < 0.05.

Figure 3-4

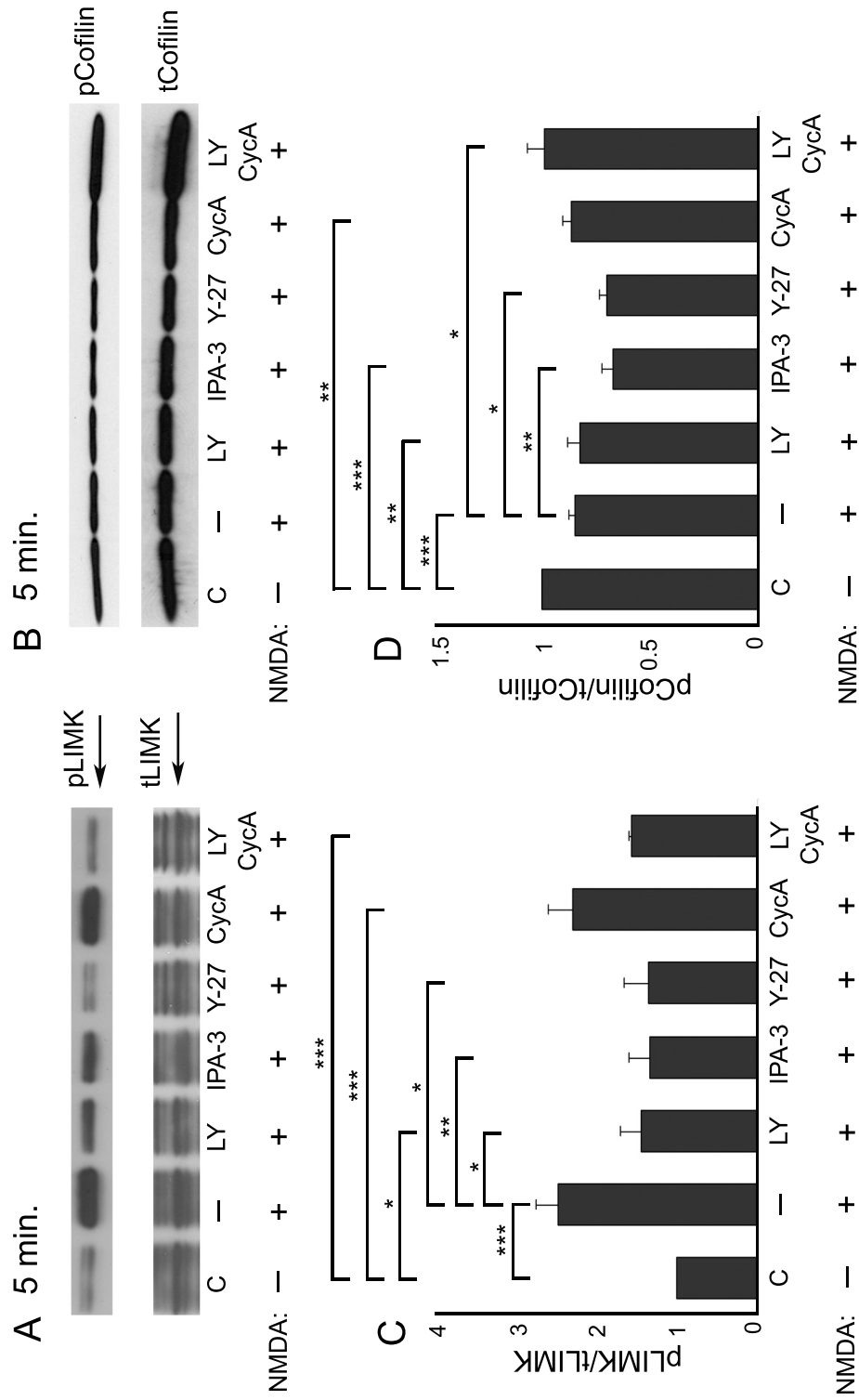


Figure 3-5 NMDAR activation promotes rapid translocation of cofilin to dendritic spines

(A-G) Time-lapse fluorescent images showing the dendrites of 14 DIV hippocampal neurons expressing DsRed (red) and GFP, wt-cofilin-GFP, cofilin^{S3A}-GFP, or cofilin^{S3D}-GFP. Neurons were (A) untreated, or treated with (B) NMDA alone or with (C) MK801, (E) KN93, or (G) cyclosporinA for 5 minutes. Scale bar= 10 μ m.

Figure 3-5

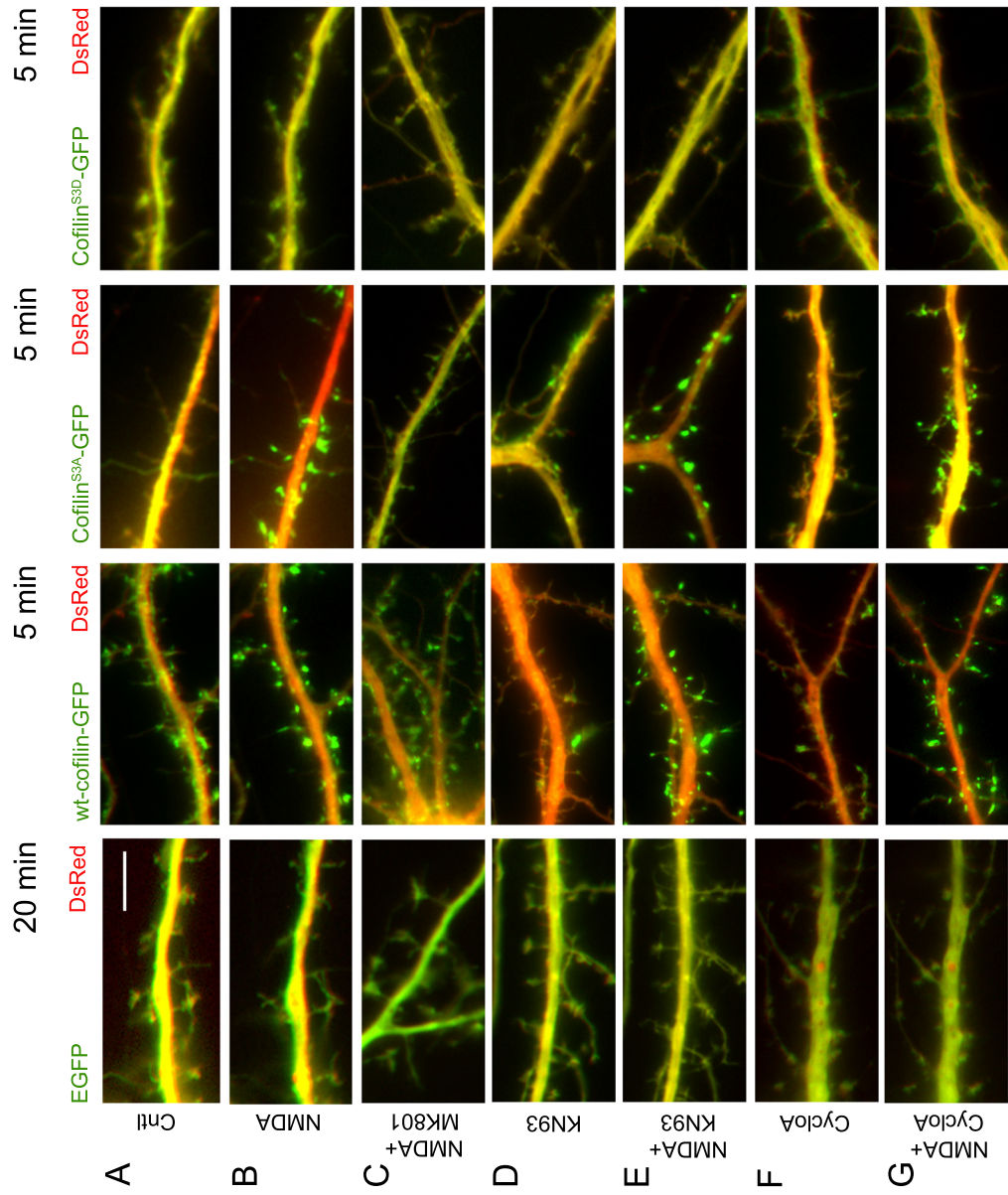


Figure 3-6 Quantification of cofilin-GFP translocation into spine heads with NMDAR application

(A-D) Graphs show dendritic spine head-to-base GFP fluorescence ratios normalized against DsRed fluorescence. A ratio of 1 would indicate uniform distribution of GFP or GFP-tagged cofilin in the spine head and dendritic shaft (base), whereas a ratio that is significantly higher than 1 would indicate specific targeting of the GFP-tagged cofilin to the spines. Experimental values represent mean \pm SEM (n= 150-300 spines from 6-10 neurons). Statistical differences were compared using one-way ANOVA followed by Tukey's Multiple Comparison post-test; ***, $p < 0.001$; **, $p, 0.01$; *, $p < 0.05$.

Figure 3-6

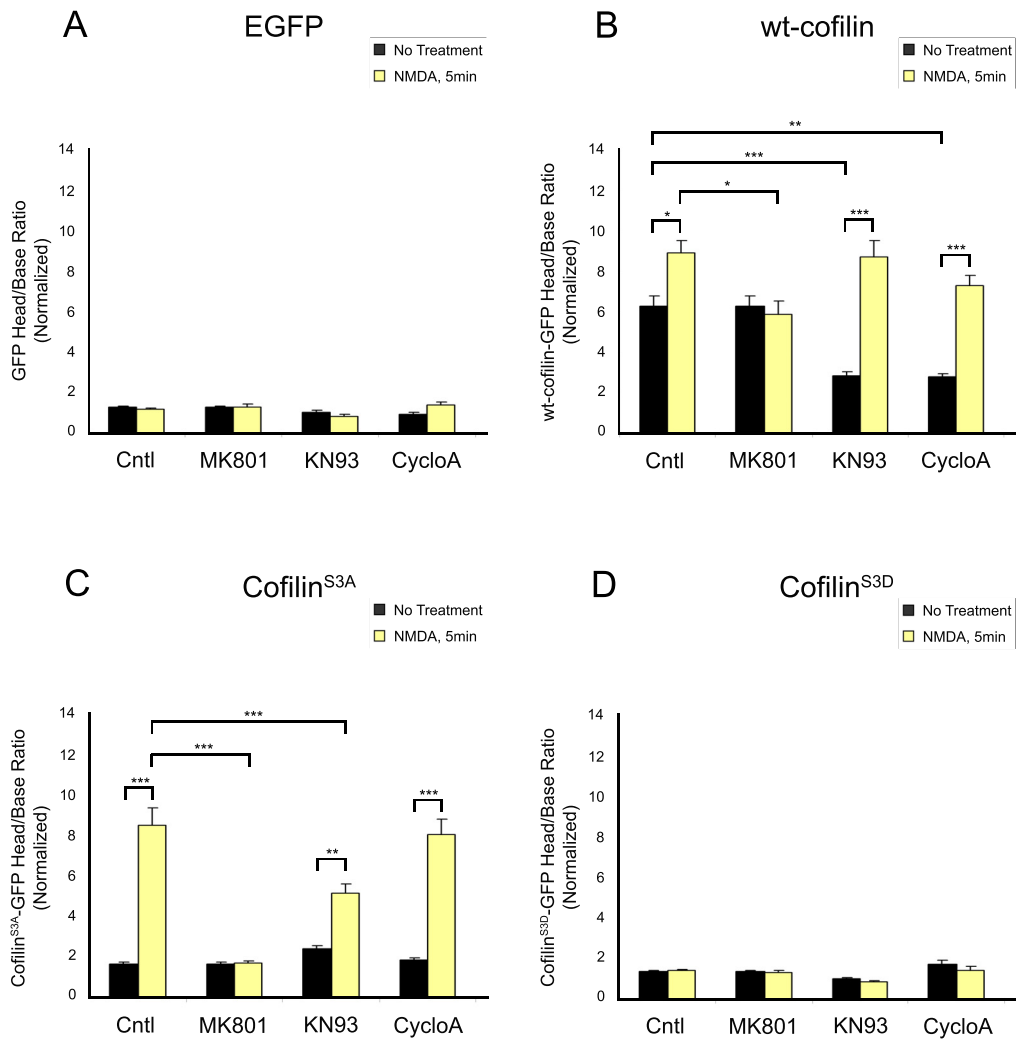


Figure 3-7 NMDAR activation promotes dendritic spine remodeling in wt neurons expressing EGFP or wt-cofilin

(A-D) Time-lapse fluorescent images showing the dendrites of 14 DIV hippocampal neurons expressing DsRed (red) and (A) GFP, (B) wt-cofilin-GFP, (C) cofilin^{S3A}-GFP, or (D) cofilin^{S3D}-GFP. Neurons were treated with 50 μ M NMDA for 20 minutes. Arrows denote (A, B) mature dendritic spines that remodel with NMDA treatment, (C) immature spines that remain immature with NMDA treatment, and (D) mature spines that do not remodel with NMDA treatment. Arrowheads denote (B) wt-cofilin and (C) cofilin^{S3A} clusters in spine heads and (D) spine heads showing no specific cofilin^{S3D} targeting. Scale bar= 10 μ m.

Figure 3-7

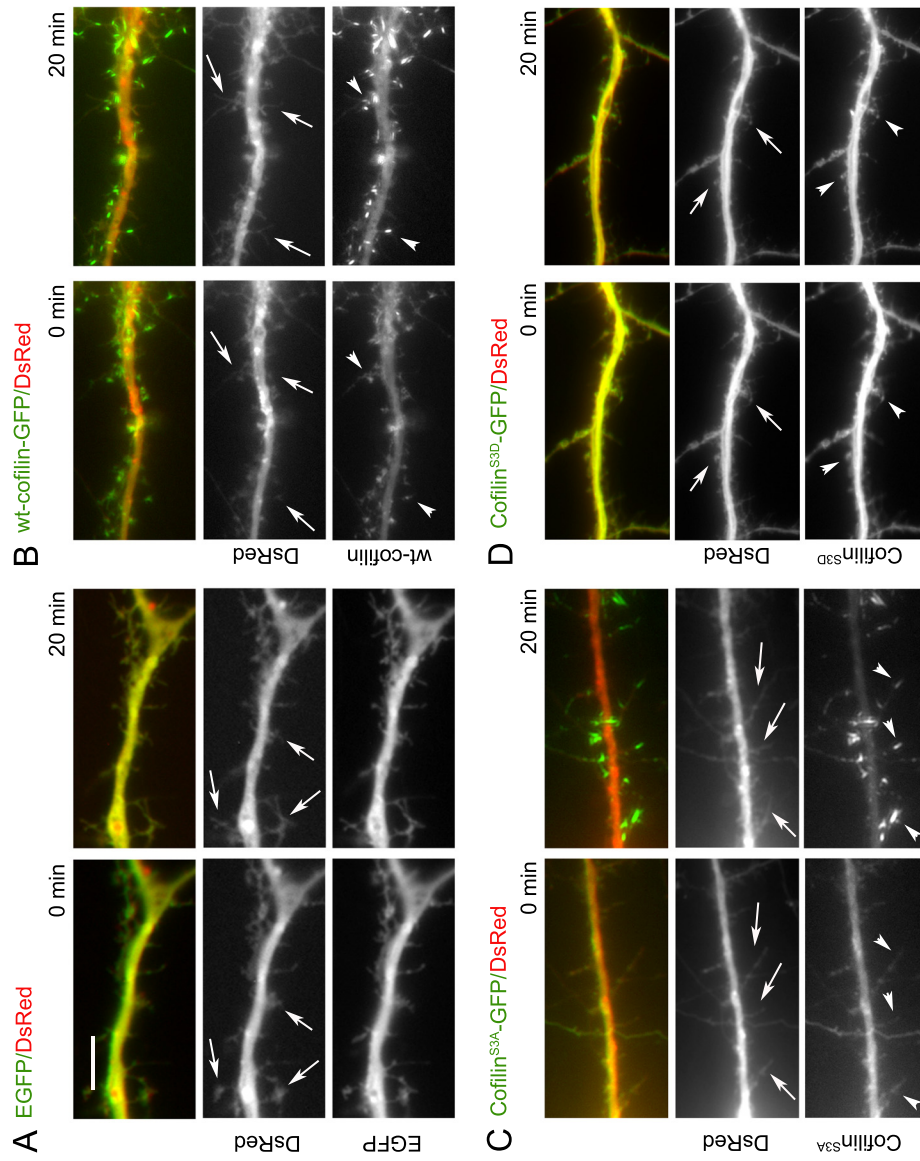


Figure 3-8 Calcineurin mediates dendritic spine remodeling following NMDA application

(A-D) Image J was used to analyze the dendritic spines from the 14 DIV hippocampal neurons that were used for live imaging studies of cofilin-GFP translocation into spines. The head-to-length ratio of spines was analyzed following NMDA bath application for 20 minutes (50 μ M). Experimental values represent mean \pm SEM (n= 150-300 spines from 6-10 neurons). Statistical differences were compared using one-way ANOVA followed by Tukey's Multiple Comparison post-test; ***, p < 0.001; **, p, 0.01; *, p < 0.05.

Figure 3-8

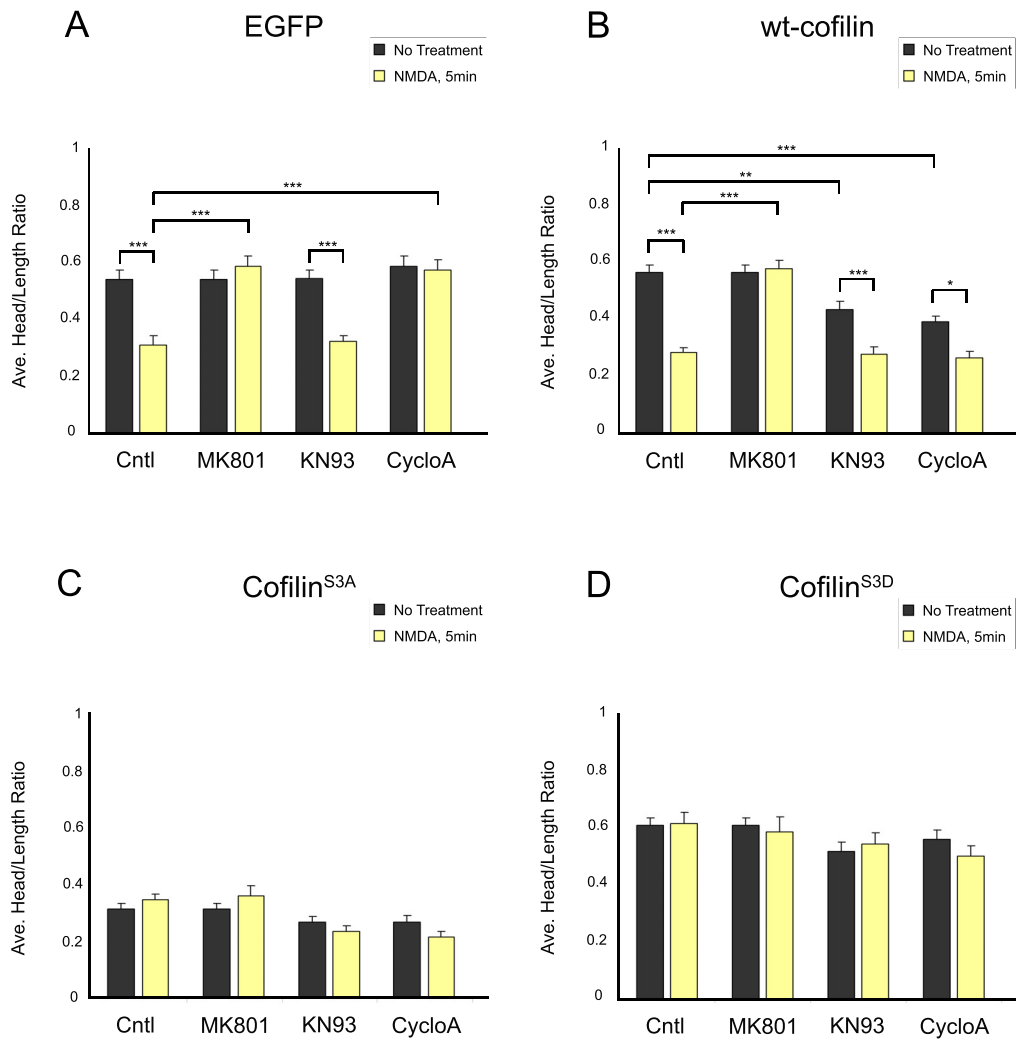
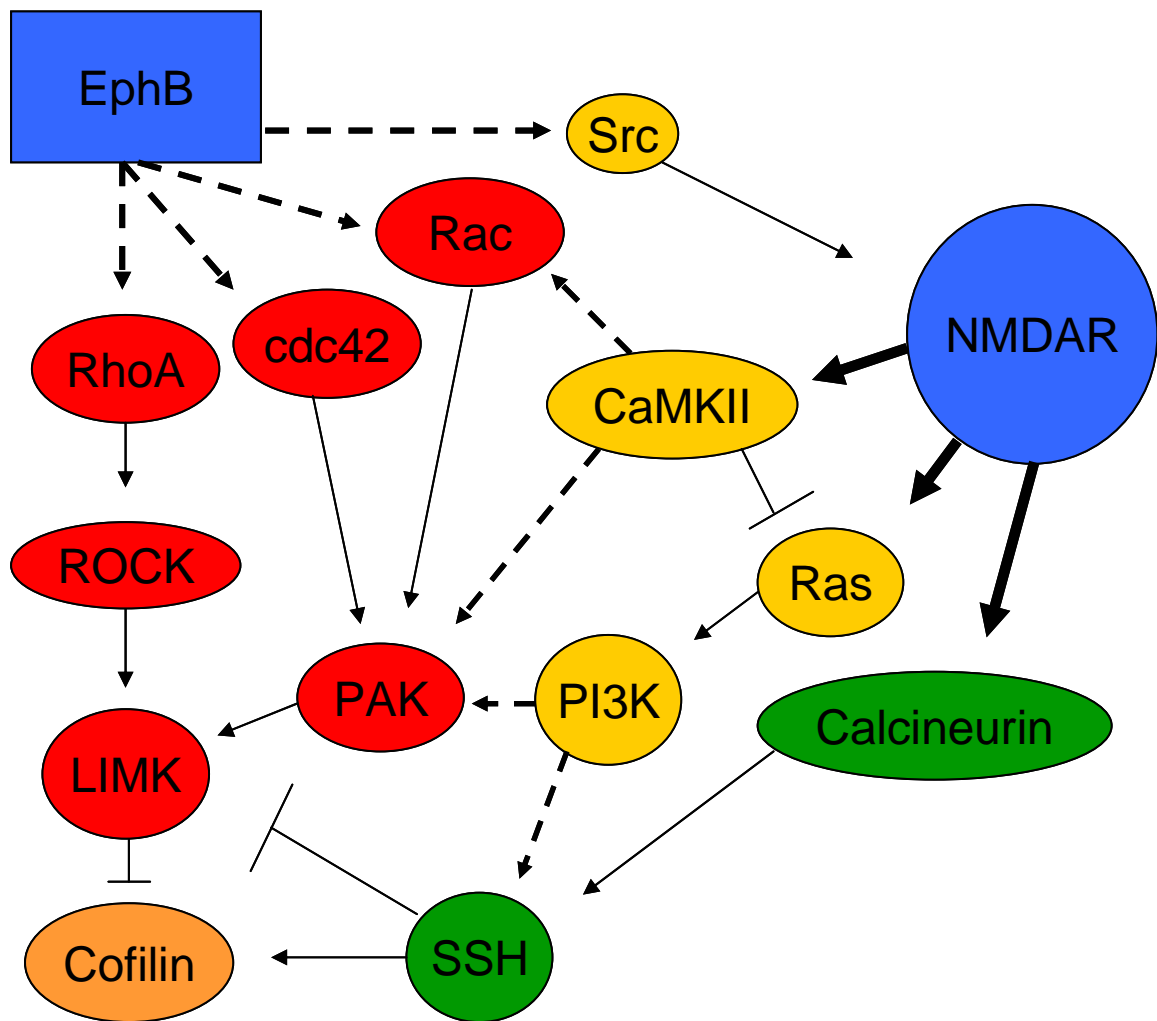


Figure 3-9 Cross-talk between the EphB and NMDA receptor pathways

EphB receptor activation promotes cofilin inactivation and spine stabilization through a RhoA-ROCK-LIMK pathway. NMDAR activation leads to LIMK activation, but also cofilin activation and spine remodeling, which is achieved through calcineurin-SSH and PI3K-SSH pathways. When EphB and NMDA receptors are co-activated, there is a decrease in the phosphorylation of both LIMK and cofilin, an effect that is eliminated by inhibition of calcineurin. This suggests that different downstream effectors may be activated depending on the activation state of EphB and NMDA receptors, leading to variable cofilin activation and dendritic spine dynamics.

Figure 3-9



Chapter 4: The Regulation of Cofilin and Dendritic Spines by β -Arrestin1 and β -Arrestin2 Scaffolding Proteins in Mature Hippocampal Neurons

4.1 Abstract

Dendritic spine plasticity is mediated by F-actin dynamics, and is thought to be a basis for both the formation and retention of memories. Actin-binding proteins shape these spine dynamics by controlling the F-actin cytoskeleton (Ethell & Pasquale, 2005; Pontrello & Ethell, 2009). We have previously shown that the F-actin-severing protein cofilin, which is regulated by phosphorylation, can induce remodeling of mature dendritic spines in hippocampal neurons (Shi *et al.*, 2009). Our current studies demonstrate that β -Arrestins play an important role in spatial control over cofilin activity in dendritic spines, which underlies NMDA-mediated dendritic spine remodeling. NMDA receptor activation promotes the dephosphorylation and activation of cofilin, as well as the translocation of cofilin to dendritic spines, an event that requires cofilin dephosphorylation. However, cofilin dephosphorylation is not sufficient to trigger cofilin clustering in dendritic spines, an effect that is also dependent on β -Arrestins. While β -Arrestins were first identified as mediators of G-protein coupled receptor signaling, they were recently suggested to regulate cofilin activity/ localization through scaffolding cofilin with enzymes that regulate its activity. Our studies demonstrate that cofilin clustering in the spines is affected in both β -Arrestin1 and β -Arrestin2 deficient neurons under the normal synaptic activity. Moreover, loss of β -Arrestin1 affects normal spine development, whereas β -Arrestin2 is involved in NMDAR-dependent re-distribution of active cofilin and dendritic

spine remodeling. Our studies demonstrate novel functions of the β -Arrestins in the regulation of cofilin activity and localization in dendritic spines and its role in NMDAR-mediated dendritic spine plasticity.

4.2 Introduction

Dendritic spine plasticity is important for proper synapse development and function, and it is the activity of actin-binding proteins such as cofilin that can modify the cytoskeleton and spine dynamics. We have shown here that cofilin is dephosphorylated and activated in response to NMDA through calcineurin-SSH and PI3K-SSH pathways, and this promotes spine remodeling activity. We see that wt-cofilin and constitutively-active cofilin^{S3A} translocates to spine heads in response to NMDA, and this is not blocked by inhibitors of CaMKII or calcineurin. Therefore, we were interested in finding other mechanisms in addition to the regulation of cofilin activity by phosphorylation that might account for the NMDA-mediated localization of cofilin to dendritic spines.

The importance of the β -Arrestins as scaffolding proteins that spatially regulate kinases and phosphatases that modify the dynamics of the cytoskeleton has recently been reported (Zoudilova *et al.*, 2010). Among the many proteins that are affected by the scaffolding activity of β -Arrestins are LIMK kinase and SSH phosphatase, two enzymes that directly affect cofilin phosphorylation state and activity. β -Arrestin1 regulates cofilin activity through the scaffolding of cofilin with LIMK, while β -Arrestin2 promotes cofilin activity through cofilin dephosphorylation by SSH and/ or CIN (Defea, 2008; Kovacs *et al.*, 2009). In this way, β -Arrestins could not only alter the localization of cofilin within

the cell, and therefore its ability to affect the actin cytoskeleton organization, but they also may regulate the activity level of cofilin by scaffolding it with LIMK or SSH. While a competing calcineurin-SSH or PI3K-SSH pathway leads to cofilin activation and transient spine remodeling (Shi & Ethell, 2006; Carlisle *et al.*, 2008), cofilin can be inactivated in response to NMDAR-mediated activation of CaMKII, presumably through phosphorylation by LIMK, leading to spine stabilization and LTP (Chen *et al.*, 2007). β -Arrestins have been shown to recruit and activate CaMKII upon β_1 -adrenergic receptor activation in cardiac cells (Mangmool *et al.*, 2010), showing that CaMKII localization and function can also be modified by β -Arrestins. Their roles in regulating several effectors downstream of the NMDA receptor make β -Arrestins good candidates for the regulation of cofilin and spine remodeling following NMDAR activation.

We show here that NMDA application leads to dendritic spine remodeling in wt neurons, but not in β -Arrestin2 KO neurons. In addition, neurons from β -Arrestin1 KO mice display more immature spines than do wt or β -Arrestin2 KO neurons. While mature dendritic spine development is affected in β -Arrestin1 KO neurons, β -Arrestin2 KO neurons develop normal mature spines as compared to wt neurons, but fail to remodel spines in response to NMDA application. While NMDA receptor activation promotes a rapid translocation of both wt-cofilin and constitutively-active cofilin^{S3A} to spines in wt neurons, NMDA-induced cofilin localization and translocation is disrupted in β -Arrestin KO neurons. NMDA treatment triggers the translocation of constitutively-active cofilin^{S3A} into the spines in wt and β -Arrestin1 KO neurons, but not in β -Arrestin2 KO neurons. In addition, spines of β -Arrestin2 KO neurons fail to remodel with over-

expression of constitutively-active cofilin^{S3A}. This agrees with the observation that β -Arrestin2 KO neurons also resist NMDA-induced remodeling, which is most likely a cofilin-mediated event. While β -Arrestin1 is required for normal development of dendritic spines, β -Arrestin2 is needed for NMDAR-mediated remodeling of mature spines.

4.3 Materials and Methods

Hippocampal neuronal cultures, transfection, and expression vectors. Cultures of hippocampal neurons were prepared from embryonic day 15 (E15) or E16 mice as previously described (Shi & Ethell, 2006). Briefly, after treatment with papain (0.5mg/ml) and DNaseI (0.6 μ g/ml) for 20 min. at 37°C and mechanical dissociation, neurons were plated on glass coverslips or plastic dishes coated with poly-DL-ornithine (0.5mg/ml) and laminin (5 μ g/ml). The neurons were cultured in Neurobasal medium with 25 μ M glutamine, 1% penicillin–streptomycin, and B-27 supplement (Invitrogen) under a 5% CO₂/ 10% O₂ atmosphere at 37°C. The cultures were transfected at 12 DIV using the calcium phosphate method as previously described (Jiang & Chen, 2006; Shi & Ethell, 2006). The expression vectors used were: pEGFP-N1 and pDsRed-C2 (Clontech); pcDNA3-EGFP-cofilin, pcDNA3-EGFP-cofilin^{S3A}, pcDNA3-EGFP-cofilin^{S3D}; pcDNA3- β -Arrestin1-FLAG, and pcDNA3- β -Arrestin2-FLAG.

Biochemical Analysis. Hippocampal neurons (approximately 1.2 million neurons on 10cm dishes at 14 DIV) were rinsed quickly with Hank's Balanced Salt Solution

(Invitrogen) containing 0.493mM MgCl₂ and 0.407mM MgSO₄, then treated with the appropriate reagent (in Hank's Balanced Salt Solution with 1.8mM CaCl₂ and 1μM glycine, with or without MgCl₂ and MgSO₄) under 5% CO₂/ 10% O₂ at 37°C. For NMDA receptor activation, cultures were treated with 50μM NMDA in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1μM glycine. Following treatment, the dishes were placed on ice and immediately washed with ice-cold PBS, then scraped from the plate in 1ml lysis buffer (25mM Tris-Cl, pH 7.4, 150mM NaCl, 1% TritonX-100, 5mM EDTA, pH 8.0, 1x protease inhibitor cocktail (Sigma-Aldrich), and 2mM sodium vanadate. After 30 min. rotation at 4°C, cell lysate was centrifuged for 15 min. at 16.1K r.c.f at 4°C. Supernatant was mixed 1:1 with 2x Laemmli loading buffer (Sigma-Aldrich), boiled for 10 min., and loaded onto an 8-16% Tris-glycine SDS PAGE gel (Invitrogen). The contents of the gel were transferred onto a Nitrocellulose membrane (Perkin Elmer), which was blocked in 5% milk/ TBS-Tween20 (0.2%), and specific primary antibodies (1:1000) were applied overnight at 4°C in 3% BSA/ TBS-Tween20. Secondary HRP-conjugated antibodies were applied (1:100,000) for 1 hour at room temperature in TBS-Tween20. The secondary antibodies used were the following: HRP-conjugated goat anti-rabbit (0.08μg/ml; Jackson ImmunoResearch, West Grove, PA) and HRP-conjugated donkey anti-mouse (0.08μg/ml; Jackson ImmunoResearch). Signal was detected on film using the ECL Plus detection kit from GE Healthcare. Phospho-LIMK and phospho-cofilin levels were quantified by densitometry (Adobe Photoshop) and normalized to total LIMK and total cofilin levels, respectively. Five to 10 independent

experiments were performed for each condition. Statistical differences were compared using Student's t test.

Immunocytochemistry. Hippocampal neurons on glass coverslips (14 DIV) were treated with the appropriate reagent under 5% CO₂/ 10% O₂ at 37°C, then quickly rinsed with PBS and fixed in 2% paraformaldehyde, permeabilized in 0.1% TritonX-100, and blocked in PBS containing 5% normal goat serum and 1% BSA. Primary antibodies were applied in blocking solution for 2 hours at room temperature, and secondary antibodies were applied in PBS-Tween20 (0.2%) for 1 hour at room temperature. Coverslips were mounted in Vectashield anti-fade medium with DAPI (Vector Labs) and sealed with Cytoseal 60 (Fisher), then viewed under a confocal laser scanning microscope (model LSM 510; Carl Zeiss MicroImaging).

Antibodies. The primary antibodies used were as follows (all 1:1000 for Western blot): rabbit anti-phospho-LIMK1 (Thr508)/ LIMK2 (Thr505) (Cell Signaling Technology); mouse anti-LIMK1 (C-10) (Santa Cruz Biotechnology); rabbit anti-phospho-cofilin (phospho S3) (abcam); rabbit anti-cofilin (Cytoskeleton); mouse anti-synaptophysin SVP-38 (Sigma-Aldrich, 1:100 for immunocytochemistry). The secondary antibodies used were as follows: HRP-conjugated Donkey anti-Mouse IgG (Jackson ImmunoResearch, 1:100,000 for Western blot); HRP-conjugated Goat anti-Rabbit IgG (Jackson ImmunoResearch, 1:100,000 for Western blot); Alexa Fluor 660-conjugated anti-mouse IgG and anti-rabbit IgG (Invitrogen, 1:500 for immunocytochemistry).

Adult brain slices. Adult mice (aged 3-12 months) were anesthetized with isoflurane and perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA). Brains were extracted, post-fixed for 3 hours in 4% PFA at 4°C, and sectioned coronally into 300µm slices on a vibratome. Sections were labeled with DiI using a gene gun, incubated in PBS at 4°C for 3 days, then mounted onto slides in PBS and sealed with Cytoseal. Pyramidal neurons from the stratum radiatum of CA1 were imaged using a confocal microscope (model LSM 510; Carl Zeiss Micro-Imaging), and dendritic protrusions were quantified using image J software.

Confocal microscopy. Fluorescence was analyzed using a confocal laser-scanning microscope (model LSM 510; Carl Zeiss Micro-Imaging). A series of five high-resolution optical sections (1024x1024 pixel format) were taken for each neuron with a 63x water-immersion objective (1.2 numerical aperture), 1x zoom at 0.5µm step intervals (z-stack). All images were acquired under identical conditions. Each z-stack was collapsed into a single image by projection (Zeiss LSM Image software), converted to a tiff file, and analyzed using Image J Software. Seven to 10 neurons were randomly selected for each experimental group, and three to four proximal dendrites per each neuron were analyzed.

Live imaging and fluorescence intensity. Time-lapse imaging of live neurons was performed under an inverted fluorescent microscope (model TE2000; Nikon) with 40x oil

Fluor objectives, and monitored by a 12-bit CCD camera (model ORCA-AG; Hamamatsu) using Image-Pro software (Media Cybernetics). During imaging, the cultures were maintained in Hank's Balanced Salt Solution (Invitrogen) supplemented with 1.8mM CaCl₂, and 1μM glycine, at 37°C with 5% CO₂. Images were captured at 1 min. intervals for 1 hour. For quantification of cofilin levels in spine heads versus dendrites, the EGFP fluorescence level (normalized to the DsR fluorescence level) was measured in each spine head and in an equally-sized region of the dendrite at the base of each spine (Adobe Photoshop). For each spine, the sum of EGFP fluorescence detected in the head and in the base was set equal to 100% EGFP signal for that spine. The percentage of the total EGFP present in each head and in each base was determined.

4.4 Results

4.4.1 β-Arrestin1 and β-Arrestin2 are involved in cofilin targeting to dendritic spines under normal synaptic activity and in response to acute NMDAR activation.

The scaffolding proteins β-Arrestins were recently suggested to regulate cofilin activity/localization through scaffolding cofilin with enzymes that regulate its activity, such as LIMK, chronophin, and slingshot (Zoudilova *et al.*, 2010). While β-Arrestins were first identified as mediators of G-protein coupled receptor signaling, increasing evidence indicates the requirement for β-arrestins in a variety of downstream signaling pathways, including activation of MAPKs, the small GTPase RhoA, and the actin filament-severing protein cofilin. Therefore, we used the β-Arrestin1 KO and β-Arrestin2 KO mice to

investigate the role of β -Arrestins in the regulation of cofilin activity and translocation in response to changes in synaptic activity.

Dendritic spine targeting of wt-cofilin was affected in β -Arrestin1 and β -Arrestin2 KO neurons under normal synaptic activity (Fig. 4-1A-4-1C, 4-2B). However, NMDA treatment triggered wt-cofilin-GFP clustering in dendritic spines in both β -Arrestin1 KO and β -Arrestin2 KO neurons similar to wt neurons (Fig. 4-2B). Interestingly, active non-phosphorylatable cofilin^{S3A} failed to cluster in dendritic spines of wt and β -Arrestin2 KO neurons under normal synaptic activity (Fig. 4-2C). Furthermore, NMDA treatment triggered the translocation of active non-phosphorylatable cofilin^{S3A} into the spines in wt and β -Arrestin1 KO neurons, but not in β -Arrestin2 KO neurons, demonstrating that β -Arrestin2 is required for the translocation of active non-phosphorylatable cofilin^{S3A} to the spines in response to NMDAR activation.

4.4.2 While β -Arrestin1 KO neurons develop immature spines, β -Arrestin2 KO neurons exhibit mature spines that are resistant to NMDA-induced remodeling.

To investigate whether the ability of β -Arrestin2 to localize active dephosphorylated cofilin into dendritic spines may underlie NMDA-induced remodeling of dendritic spines, we examined the effects of NMDA treatment on dendritic spine morphology in hippocampal neurons that lack β -Arrestin1 or β -Arrestin2. Dendritic spine morphology was analyzed in live 14 DIV hippocampal neurons before and after NMDA application through live imaging (Fig. 4-3, 4-4). Fixed 14 DIV hippocampal neurons were also analyzed using confocal microscopy under normal synaptic activity and 60 minutes after NMDA

treatment (5 min of treatment with 50 μ M NMDA, followed by washout for 60 min; Fig. 4-5). The spine lengths, spine head areas, and spine head-to-length ratios were evaluated for each condition (Fig. 4-4, 4-5D to 4-5F, 4-5I). A lower head-to-length ratio would indicate more immature spines. The number of protrusions per 10 μ m of dendrite and the percentage of protrusions that did not have heads (filopodia) were also counted (Fig. 4-5G, 4-5H). Our results indicate that deletion of the scaffolding protein β -Arrestin2 abolished the abilities of NMDA to induce dendritic spine remodeling. While NMDA treatment of wt neurons results in a more immature spine phenotype as indicated by a lower head-to-length ratio in comparison to untreated wt neurons (Fig. 4-4A, 4-5F, 4-5I), β -Arrestin2 KO neurons treated with NMDA show similar spine head size and head-to-length ratio to untreated β -Arrestin2 neurons. Interestingly, untreated β -Arrestin1 KO neurons display more immature spines with smaller heads and lower head-to-length ratios than wt or β -Arrestin2 KO neurons under normal synaptic activity (Fig. 4-4A, 4-5F, 4-5I), suggesting that β -Arrestin1 is also required for normal dendritic spine development. Moreover, both β -Arrestin1 and 2 KO neurons failed to show an increase in the number of new spines in response to NMDA application (Fig. 4-5G), suggesting that both β -Arrestin1 and β -Arrestin2 are likely to be involved in the formation of new spines triggered by NMDA. These results demonstrate that dendritic spine development is affected in β -Arrestin1 KO neurons, whereas β -Arrestin2 KO neurons develop normal mature spines, but fail to remodel spines in response to NMDA application.

4.4.3 Dominant-negative cofilin^{S3D} prevents NMDA-induced dendritic spine remodeling in wt neurons and rescues mature spine phenotype in β -Arrestin1 KO neurons.

To investigate whether NMDA-induced dephosphorylation/ activation of cofilin is responsible for spine remodeling, we examined the effects of NMDA on dendritic spine morphology in the neurons expressing dominant-negative cofilin^{S3D}. Indeed, dominant-negative cofilin^{S3D} prevented NMDA-induced spine remodeling. NMDA treatment did not induce immature spine morphology in wt neurons expressing dominant-negative cofilin^{S3D}, showing similar spine head size and head-to-length ratio before and after NMDA treatment (Fig. 4-4B). We next examined the effects of cofilin inhibition on dendritic spines in β -Arrestin1 KO neurons. Dominant-negative cofilin^{S3D} rescued a mature spine phenotype in β -Arrestin1 KO neurons (Fig. 4-4B), suggesting that the effects of β -Arrestin1 deletion on dendritic spines were also mediated through misregulation of cofilin activity in the dendritic spines. This evidence points to cofilin as the mediator underlying NMDA-induced spine remodeling and β -Arrestin1-dependent mature spine development.

4.4.4 β -Arrestin2 deletion partially prevents dendritic spine remodeling induced by constitutively-active cofilin^{S3A}.

If the ability of β -Arrestin2 to regulate localization/ scaffolding of cofilin in dendritic spines underlies NMDA-mediated spine remodeling, then deletion of β -Arrestin2 would also affect dendritic spine remodeling induced by constitutively-active cofilin^{S3A}. In order to examine this possibility, we transfected wt, β -

Arrestin1, and β -Arrestin2 KO neurons with wt-cofilin, constitutively-active cofilin^{S3A}, or inactive cofilin^{S3D} at 12 DIV and analyzed by confocal microscopy at 14 DIV (Fig. 4-6). While constitutively-active cofilin^{S3A} induced a more immature spine phenotype in wt hippocampal neurons, similar to the effects of NMDA treatment (Fig. 4-3A, 4-4A, 4-5F, 4-5I), β -Arrestin2 KO neurons expressing constitutively-active cofilin^{S3A} exhibited more mature spines with larger heads and head-to-length ratios than their wt counterparts or β -Arrestin1 KO neurons expressing constitutively-active cofilin^{S3A} (Fig. 4-6E to 4-6G, 4-6J). Our results suggest that β -Arrestin2 plays an important role in the control over cofilin activity in dendritic spines to regulate spine remodeling in response to changes in synaptic activity.

4.4.5 Overexpression of β -Arrestin1 or β -Arrestin2 reverses the effect of β -Arrestin1 or β -Arrestin2 deletion on dendritic spine morphology under normal synaptic activity or in response to NMDA.

We were able to reverse the effects of the deletion of β -Arrestin1 on dendritic spine morphology by overexpressing FLAG-tagged β -Arrestin1 in β -Arrestin1 deficient neurons (Fig. 4-7C, 4-7E). β -Arrestin1 KO neurons expressing FLAG- β -Arrestin1 showed a more mature dendritic spine morphology with a higher head-to-length ratio than did β -Arrestin1 KO neurons (Fig. 4-7A, 4-7C, 4-7E). Overexpression of FLAG- β -Arrestin2 induced immature spine morphology in both wt and β -Arrestin2 KO neurons and also rescued NMDA-induced spine remodeling in β -Arrestin2 KO neurons (Fig 4-7B, 4-7D, 4-7E), which is consistent with a model in which β -Arrestin2 promotes cofilin activity through enhancement of SSH activity. Analysis of

the distribution of transfected FLAG- β -Arrestin1 or FLAG- β -Arrestin2 revealed that transfected β -Arrestins were found to localize in both the dendritic shaft and spines (Fig. 4-7F). The results suggest that both β -Arrestins are involved in spine development and maintenance under normal synaptic activity, while β -Arrestin2 is also involved in dendritic spine remodeling in response to NMDA.

4.4.6 Deletion of β -Arrestin1, but not β -Arrestin2, disrupts the development of

mature dendritic spines in the mouse hippocampus *in vivo*. In cultures, dendritic spine development is affected in β -Arrestin1 KO neurons, whereas β -Arrestin2 KO neurons develop normal mature spines in cultured hippocampal neurons as compared to wt neurons, but fail to remodel spines in response to NMDA application. Therefore, we next examined whether development of mature dendritic spines is affected in the hippocampus of β -Arrestin1 KO and β -Arrestin2 KO mice (Fig. 4-8A-4-8C). CA1 hippocampal neurons from the stratum radiatum of β -Arrestin1 KO mice displayed longer spines with a significantly smaller head area and lower head-to-length ratio than either wt or β -Arrestin2 KO neurons (Fig. 4-8D to 4-8F, 4-8I). Overall, β -Arrestin1 KO neurons showed dendritic spines that are less mature than in wt or β -Arrestin2 KO neurons (Fig. 4-8F, 4-8I). There were no changes in the number of protrusions (Fig. 4-8G) or percentage of filopodia (Fig. 4-8H) between wt and β -Arrestin KO neurons. Abnormal development of mature dendritic spines in β -Arrestin1 KO hippocampal neurons, both *in vitro* and *in vivo*, could indicate that the scaffolding activity of β -Arrestin1 that regulates cofilin and LIMK localization/ activity are needed for dendritic spine maturation.

4.5 Discussion

The regulation of cofilin activity and localization underlies changes in dendritic spine morphology that may lead to strengthening or disassembly of spines and synapses. Our previous studies have shown that overexpression of constitutively-active cofilin^{S3A} leads to more immature spines and filopodia, but dominant-negative cofilin promotes stable mature mushroom spines (Shi *et al.*, 2009). Here we focus on the role of β -Arrestins in the regulation of cofilin activity and localization and NMDA-mediated dendritic spine plasticity. NMDA application to cultured hippocampal neurons leads to a decrease in cofilin phosphorylation, which is dependent on calcineurin-SSH and PI3K-SSH pathways. The phosphorylation state of cofilin affects not only its activity, but also its localization within cells (Nebl *et al.*, 1996). We found that application of NMDA led to a rapid translocation of cofilin to dendritic spine heads, and event that depends on cofilin dephosphorylation. However, dephosphorylation alone was not sufficient to trigger cofilin clustering in the spines, as the non-phosphorylatable cofilin^{S3A} was diffusely distributed throughout the dendrites of untreated neurons, which indicates that there is another factor in addition to phosphorylation state that also contributes to NMDA-induced cofilin translocation to dendritic spines. β -Arrestins are known to be involved in the inactivation of GPCR signaling, but recently their importance as scaffolding proteins has emerged (Barlic *et al.*, 2000; Bhattacharya *et al.*, 2002; Wang & DeFea, 2006). β -Arrestins have been shown to spatially regulate cofilin, as well as its regulating enzymes LIMK and CIN or SSH phosphatases (Zoudilova *et al.*, 2007;

Zoudilova *et al.*, 2010). In mouse embryonic fibroblasts, chemotaxis is achieved when cofilin activity allows a leading edge to form, a process that is dependent on β -Arrestin regulation of cofilin localization (Zoudilova *et al.*, 2010). Cofilin activity is also regulated by PAR-2 receptors through recruitment of β -Arrestins which inhibit LIMK and scaffold cofilin with its phosphatase CIN (Zoudilova *et al.*, 2007). β -Arrestins have important roles that extend beyond GPCR desensitization and into the regulation of cytoskeletal dynamics.

Due to the recent evidence that β -Arrestins scaffold cofilin and its regulators, and the important role of cofilin in dendritic spine development and plasticity (Shi *et al.*, 2009; Rust *et al.*, 2010), we chose to focus on these proteins. We investigated the involvement of β -Arrestins in normal dendritic spine development and NMDA receptor-induced spine remodeling in hippocampal neurons overexpressing or lacking β -Arrestin1 or β -Arrestin2. We found that β -Arrestin1 and 2 play different roles in dendritic spine regulation. We show that β -Arrestin1 is required for normal development of mature dendritic spines, as β -Arrestin1 deficient neurons demonstrate immature spine morphology. Indeed, β -Arrestin1 scaffolds cofilin with its regulator LIMK, which seems to be important for the inactivation of cofilin in neurons. Deletion of β -Arrestin1 could therefore lead to a decrease in LIMK-mediated cofilin phosphorylation and an increase in cofilin activity and dendritic spine remodeling.

In contrast to β -Arrestin1 KO neurons, dendritic spines in β -Arrestin2 KO neurons develop normally, but fail to remodel in response to NMDA application. In addition, the spatial distribution of cofilin in β -Arrestin1 and β -Arrestin2 KO neurons is

aberrant under normal synaptic activity, and constitutively-active cofilin^{S3A} fails to translocate to spine heads of β -Arrestin2 KO neurons in response to NMDA application. The results suggest that β -Arrestin2 plays an important role in the translocation of dephosphorylated active cofilin to dendritic spine heads, which is responsible for NMDA-induced dendritic spine remodeling, resulting in transformation of mature mushroom-like spines with large heads into immature thin spines with small heads. In support of this, β -Arrestin2 KO neurons are also resistant to dendritic spine remodeling induced by constitutively-active cofilin^{S3A}. We have shown here a novel function of β -Arrestin2 that does not involve GPCR signaling, but regulates the effects of cofilin on dendritic spine morphology in response to NMDA receptor activation.

Dendritic spines remain plastic even in the adult brain due to such proteins as cofilin that effect changes on the F-actin cytoskeleton (Bamburg, 1999) and dictate spine morphology (Shi *et al.*, 2009). While LIMK and SSH modify the activation state of cofilin, the localization of cofilin within the cell is equally important in order for its actin remodeling activity to modulate dendritic spine morphology (Fig. 4-9). Cofilin will not be able to effect the appropriate changes on the cytoskeleton if it not correctly spatially localized within the cell. β -Arrestins are scaffolding proteins that can control both the phosphorylation state and the spatial regulation of cofilin, and we have shown here that they play roles in regulating both normal spine development, and NMDA-induced spine plasticity. β -Arrestins have been implicated in several disorders ranging from depression (Avissar *et al.*, 2004) and dopamine receptor-related disorders (Beaulieu *et al.*, 2005; Ikeda *et al.*, 2007) to metabolic dysfunction such as insulin resistance (Rodgers &

Puigserver, 2009). Here we demonstrate a new role of β -Arrestins in the control of actin dynamics in dendritic spines through spatial control of cofilin activity. We show that β -Arrestin1 is required for normal development of dendritic spines, whereas β -Arrestin2 is needed for NMDAR-mediated remodeling of mature spines, which is achieved through a calcineurin-SSH-cofilin pathway. Our results demonstrate that abnormal β -Arrestin expression or localization in neurons can lead to cofilin mis-regulation in synapses, aberrant dendritic spine development, and impaired NMDA-dependent plasticity, which potentially may lead to neurological deficits (Sorra & Harris, 2000; Halpain *et al.*, 2005).

4.6 References

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Figure 4-1 β -Arrestin1 and β -Arrestin2 are involved in cofilin targeting to dendritic spines under normal synaptic activity and in response to acute NMDAR activation

(A-C) Time-lapse fluorescent images showing the dendrites from 14 DIV cultures of (A) wt, (B) β -Arrestin1 KO, or (C) β -Arrestin2 KO neurons expressing DsRed (red) and GFP, wt-cofilin-GFP, cofilin^{S3A}-GFP, or cofilin^{S3D}-GFP. Scale bar= 10 μ m. For NMDA receptor activation, neurons were treated with 50 μ M NMDA in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1 μ M glycine.

Figure 4-1

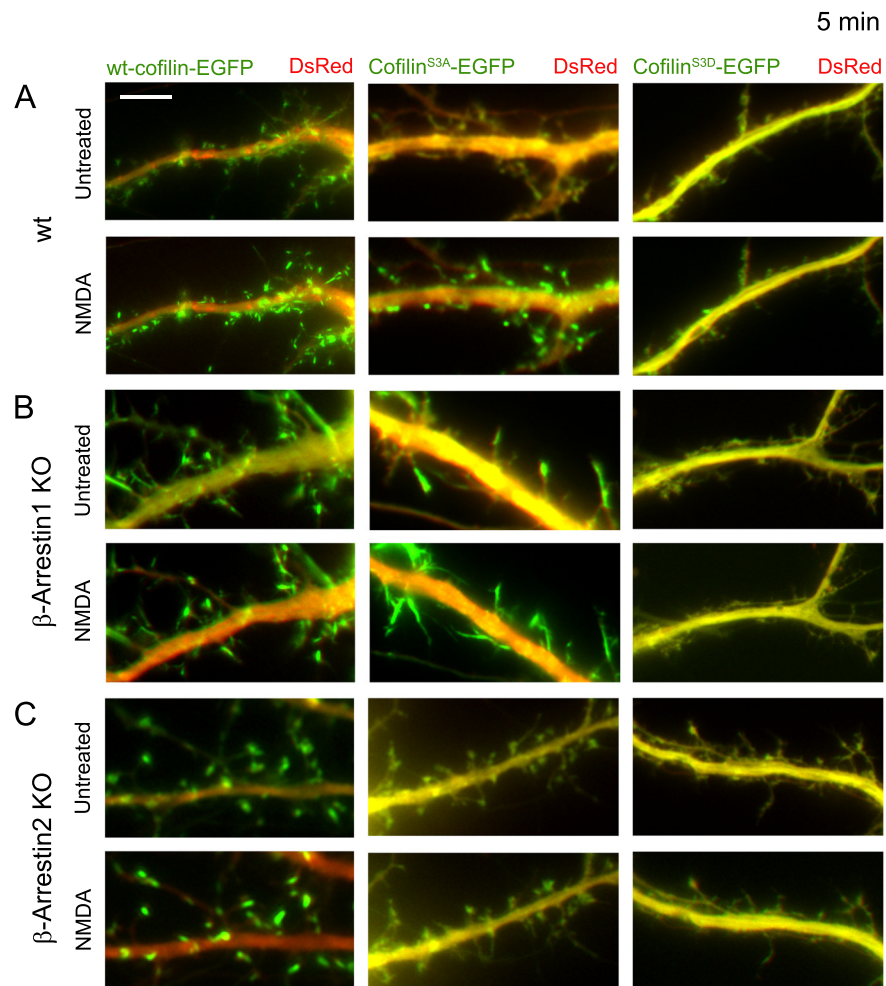


Figure 4-2 Quantification of cofilin-GFP translocation into spine heads of β -Arrestin1 and β -Arrestin2 KO neurons with NMDA application

(A-D) Image J was used to measure dendritic spine head-to-base ratio of GFP fluorescence that was normalized against DsRed fluorescence in time-lapse fluorescent images. Graphs display mean \pm SEM (n= 200-250 spines from 5-10 neurons per condition). Significant differences were determined using one-way ANOVA followed by Tukey's Multiple Comparison post-test. *, p<0.05, **, p<0.01, ***, p<0.001.

Figure 4-2

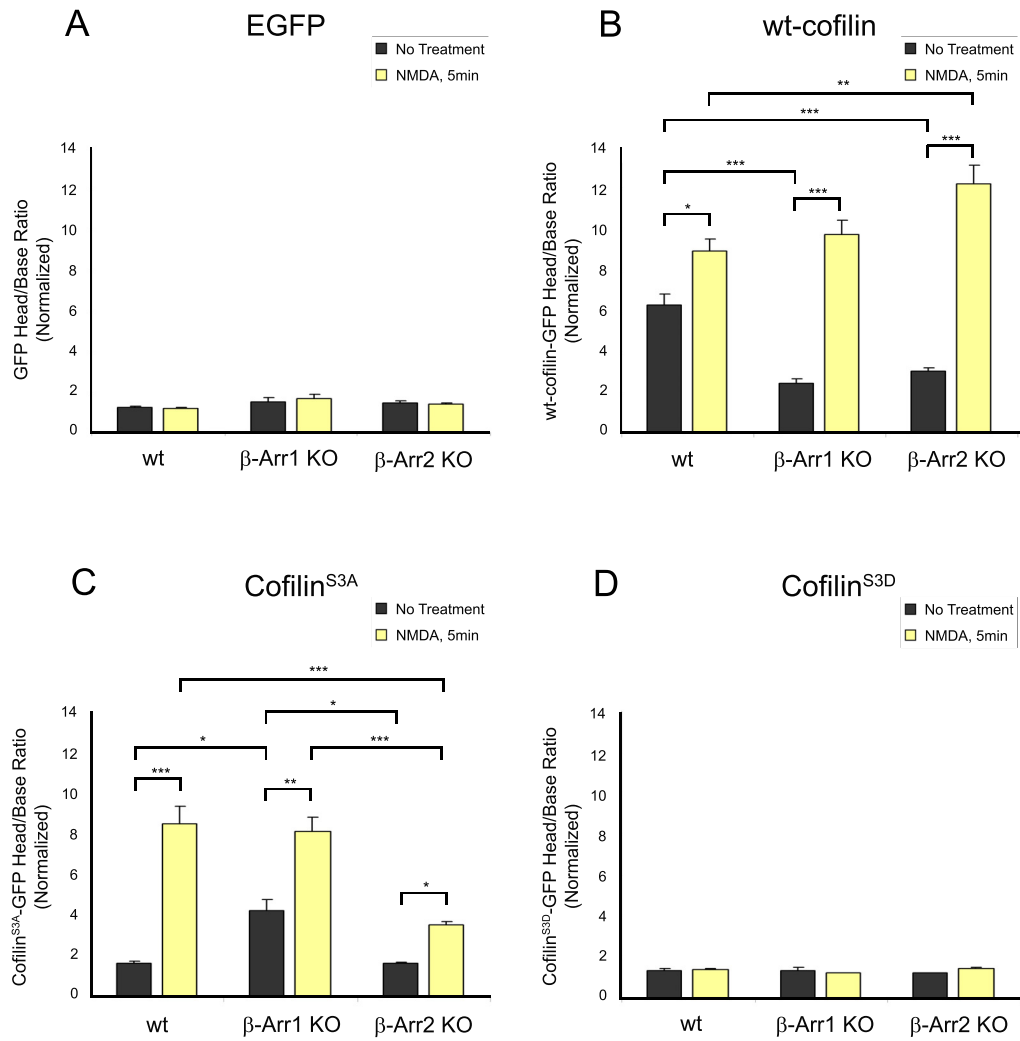


Figure 4-3 NMDA fails to trigger dendritic spine remodeling in β -Arrestin2 KO neurons

(A-C) Time-lapse fluorescent images showing the dendrites from 14 DIV cultures of (A) wt, (B) β -Arrestin1 KO, or (C) β -Arrestin2 KO neurons expressing DsRed (red) and GFP, before treatment and 20 minutes following bath application of NMDA (50 μ M) in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1 μ M glycine. Arrows denote (A) mature dendritic spines that remodel with NMDA treatment, (B) immature spines that remain immature with NMDA treatment, and (C) mature spines that do not remodel with NMDA treatment. Scale bar= 10 μ m.

Figure 4-3

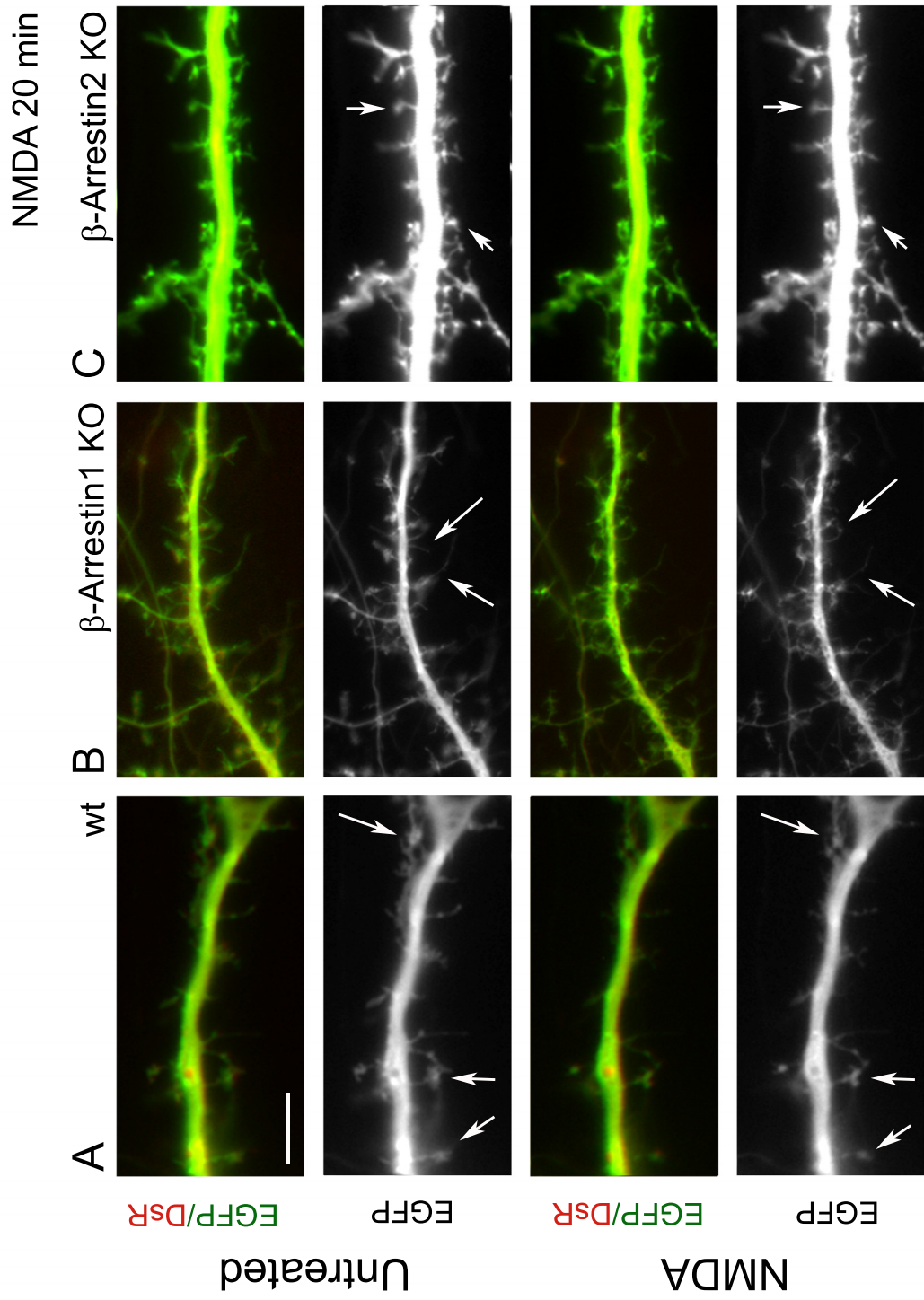


Figure 4-4 Analysis of dendritic spines in β -Arrestin1 and β -Arrestin2 KO neurons before and 20 minutes after NMDA treatment

(A-B) Image J was used to measure spine length and head area in time-lapse fluorescent images before and 20 minutes following NMDA application (50 μ M NMDA in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1 μ M glycine). Graphs display mean \pm SEM (n= 200-250 spines from 5-10 neurons per condition). Significant differences were determined using one-way ANOVA followed by Tukey's Multiple Comparison post-test. *, p<0.05, **, p<0.01, ***, p<0.001.

Figure 4-4

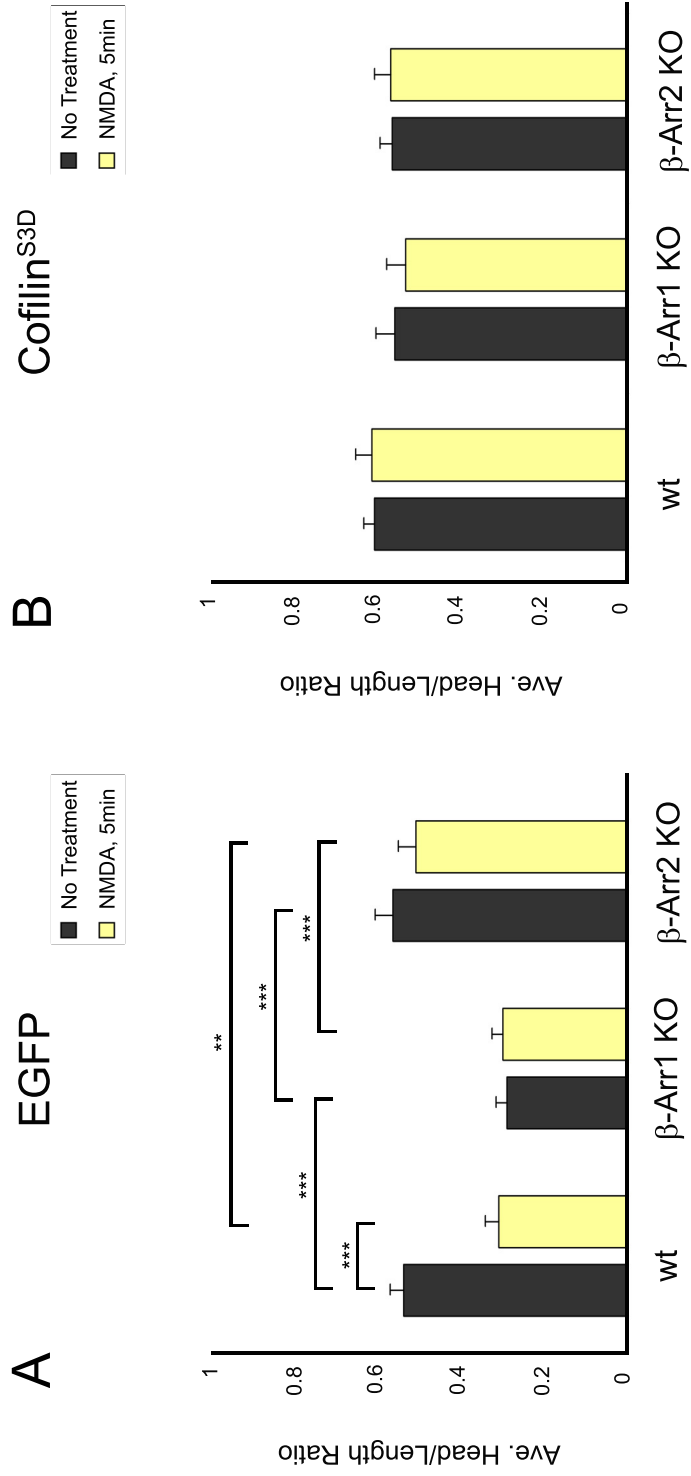


Figure 4-5 β -Arrestin1 KO spines are immature before NMDA treatment, and β -Arrestin2 KO spines are resistant to NMDA-induced remodeling

(A-C) Confocal images showing the dendrites of 14 DIV hippocampal neurons from (A) wt, (B) β -Arrestin1 KO, or (C) β -Arrestin2 KO mice. The neurons were treated with NMDA (50 μ M in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1 μ M glycine, 5 minutes), followed by washout and incubation in conditioned media for 60 minutes. Scale bar= 10 μ m.

(D-I) Image J was used to measure the length (D) and head area (E) of each dendritic spine. The head-to-length ratio was determined for each spine (F, I), as well as the number of protrusions per 10 μ m of dendrite (G) and the percentage of filopodia (H). Graphs display mean \pm SEM (n= 600-800 spines from 12-15 cells per condition). Significant differences were determined using one-way ANOVA followed by Tukey's Multiple Comparison post-test. *, p<0.05, **, p<0.01, ***, p<0.001.

Figure 4-5

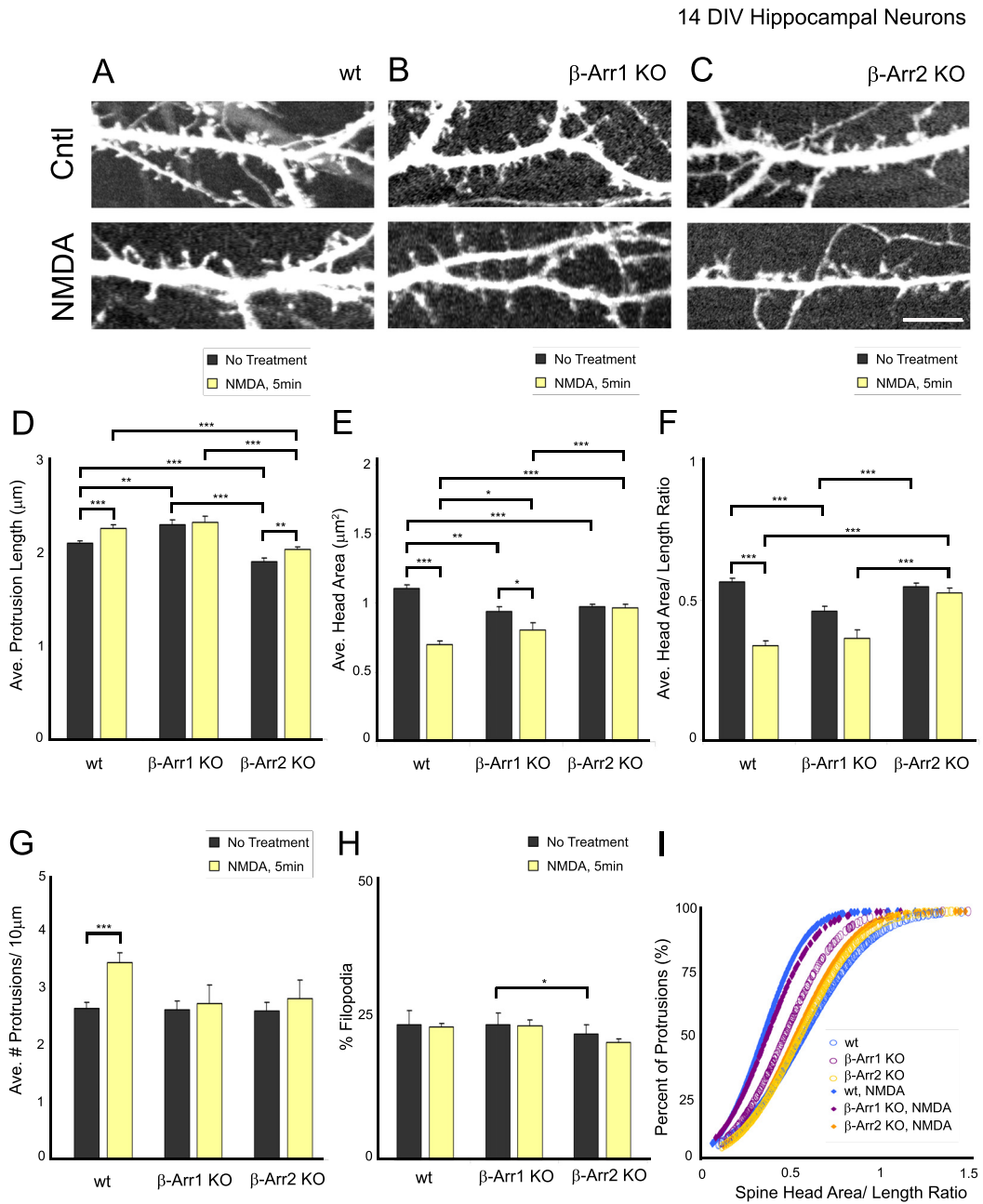


Figure 4-6 β -Arrestin2 deletion partially prevents dendritic spine remodeling induced by constitutively-active cofilin^{S3A}

(A-D) Confocal images showing the dendrites of 14 DIV hippocampal neurons from wt, β -Arrestin1 KO, or β -Arrestin2 KO mice expressing DsRed (red) and (A) GFP, (B) GFP-wt-cofilin, (C) GFP-cofilin^{S3A}, or (D) GFP-cofilin^{S3D}. Scale bar= 10 μ m.

(E-J) Image J was used to measure the length (E) and head area (F) of each dendritic spine. The head-to-length ratio was determined for each spine (G, J). Graphs display mean \pm SEM (n= 600-800 spines from 12-15 cells per condition). Significant differences were determined using one-way ANOVA followed by Tukey's Multiple Comparison post-test. *, p<0.05, **, p<0.01, ***, p<0.001.

Figure 4-6

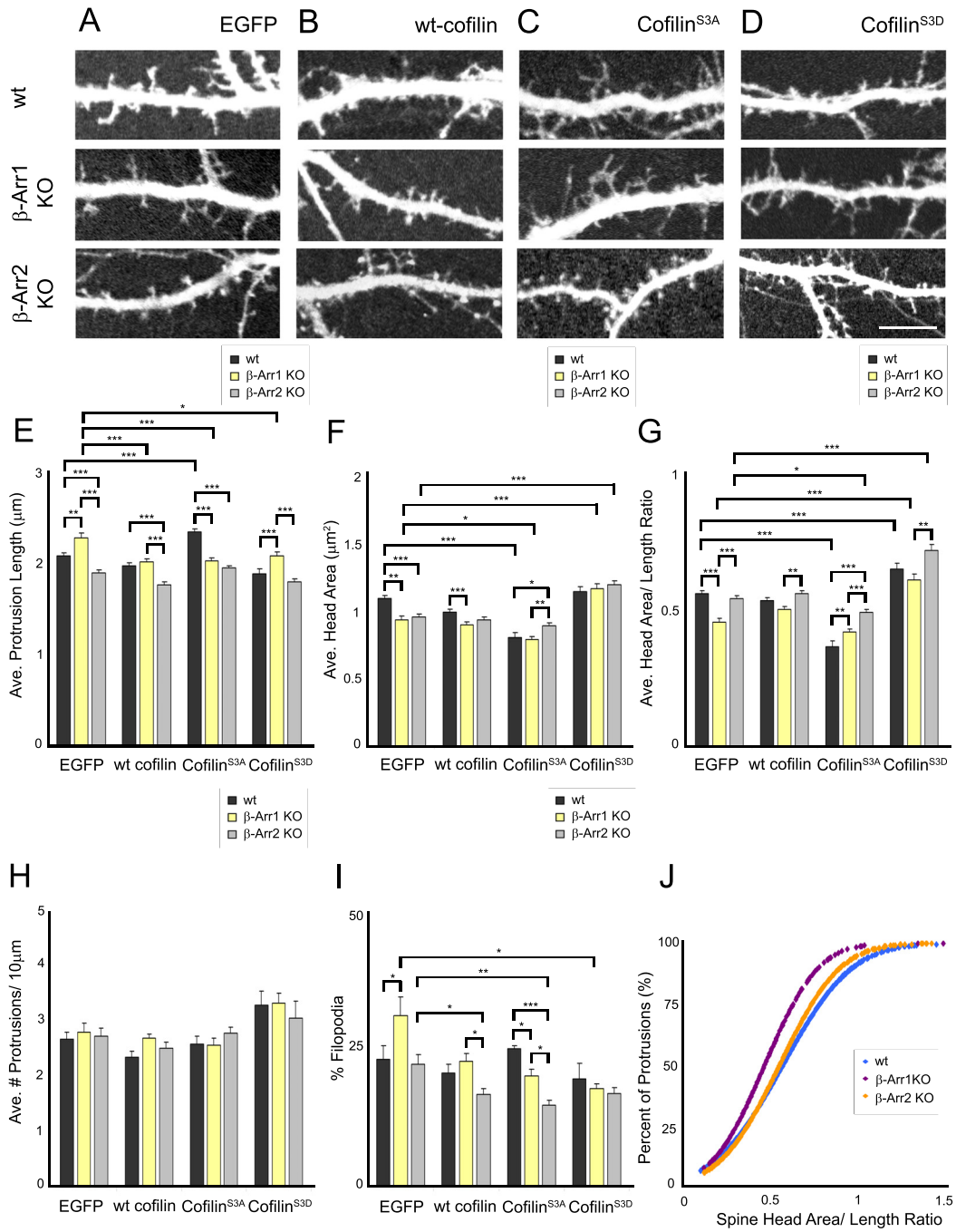


Figure 4-7 Overexpression of β -Arrestin1 or β -Arrestin2 reverses the effect of β -Arrestin1 or β -Arrestin2 deletion on dendritic spine morphology under normal synaptic activity or in response to NMDA

(A-D) Confocal images showing the dendrites of 14 DIV hippocampal neurons from β -Arrestin1 KO or β -Arrestin2 KO mice expressing DsRed (red) and GFP (green) (A, B) with (C) FLAG-tagged β -Arrestin1 or (D) FLAG-tagged β -Arrestin2. Scale bar= 10 μ m. For NMDA receptor activation, neurons were treated with 50 μ M NMDA in magnesium-free Hank's Balanced Salt Solution containing 1.8mM CaCl₂ and 1 μ M glycine.

(E, F) Image J was used to measure the head-to-length ratio (E) and the fluorescence intensity of FLAG-tagged β -Arrestin1 or 2 as detected by immunostaining, normalized to GFP fluorescence (F). Graphs display mean \pm SEM (n= 600 spines from 10 cells per condition). Significant differences were determined using one-way ANOVA followed by Tukey's Multiple Comparison post-test. *, p<0.05, **, p<0.01, ***, p<0.001.

Figure 4-7

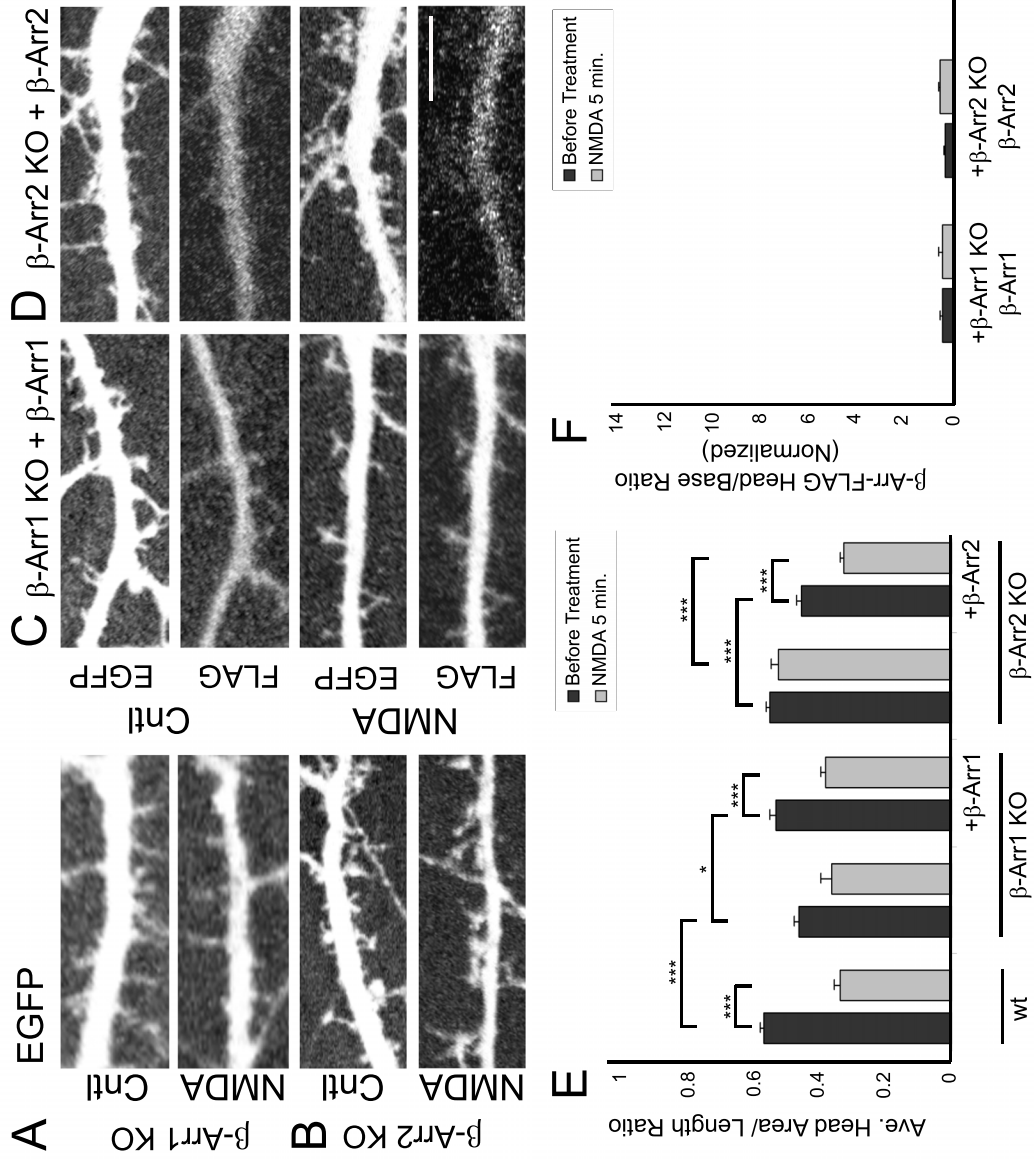


Figure 4-8 Deletion of β -Arrestin1, but not β -Arrestin2, disrupts the development of mature dendritic spines in the mouse hippocampus *in vivo*

(A-C) Confocal images of mouse hippocampal slices labeled with DiI from wt, β -Arrestin1 KO, or β -Arrestin2 KO mice. Scale bar= 10 μ m.

(D-I) Image J was used to measure the length (D) and head area (E) of each dendritic spine. The head-to-length ratio was determined for each spine (F, I), as well as the number of protrusions per 10 μ m of dendrite (G) and percentage of filopodia (H). Graphs display mean \pm SEM (n= 700-800 spines from 6-8 neurons per condition). Significant differences were determined using one-way ANOVA followed by Tukey's Multiple Comparison post-test. *, p<0.05, **, p<0.01, ***, p<0.001.

Figure 4-8

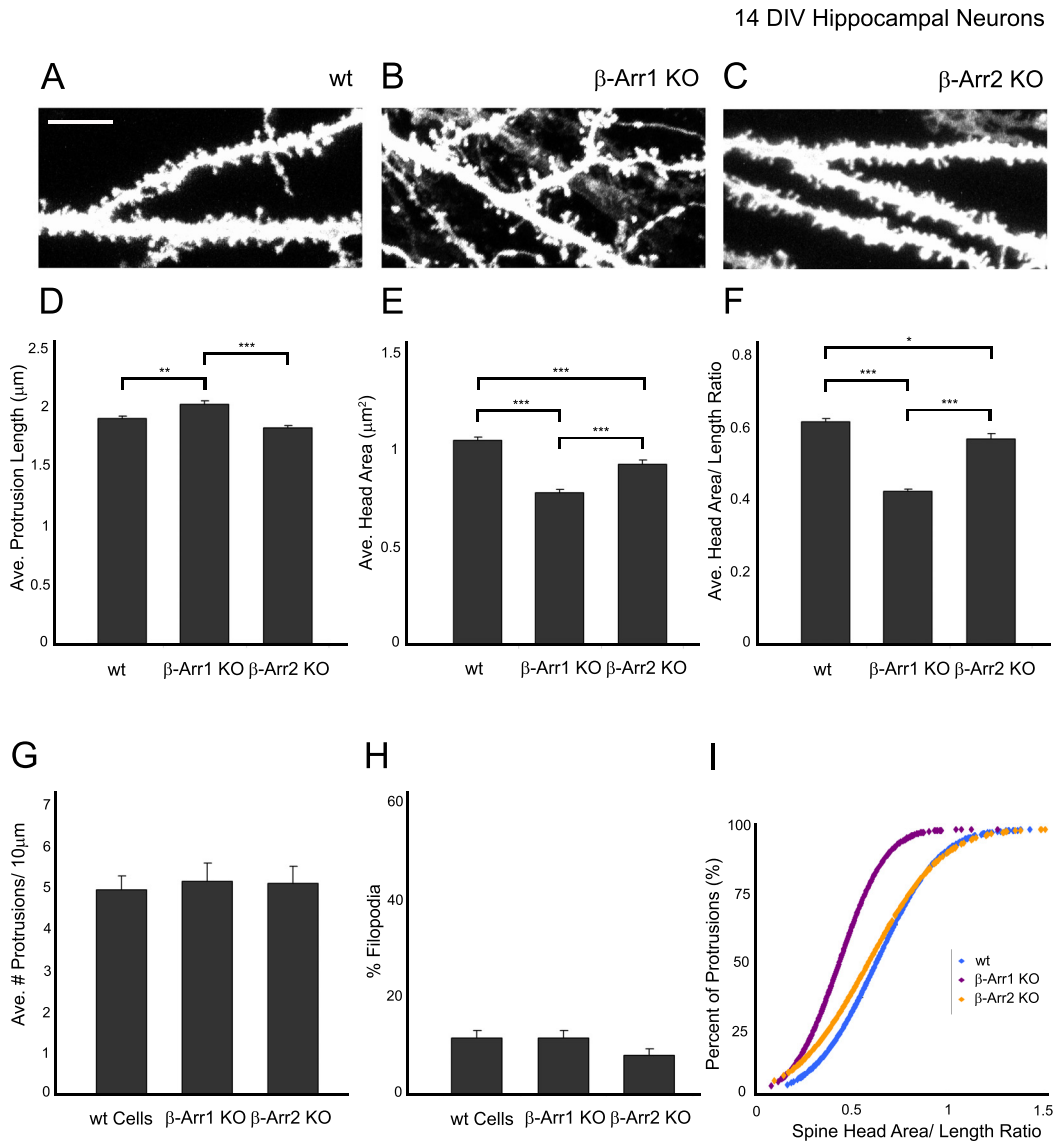
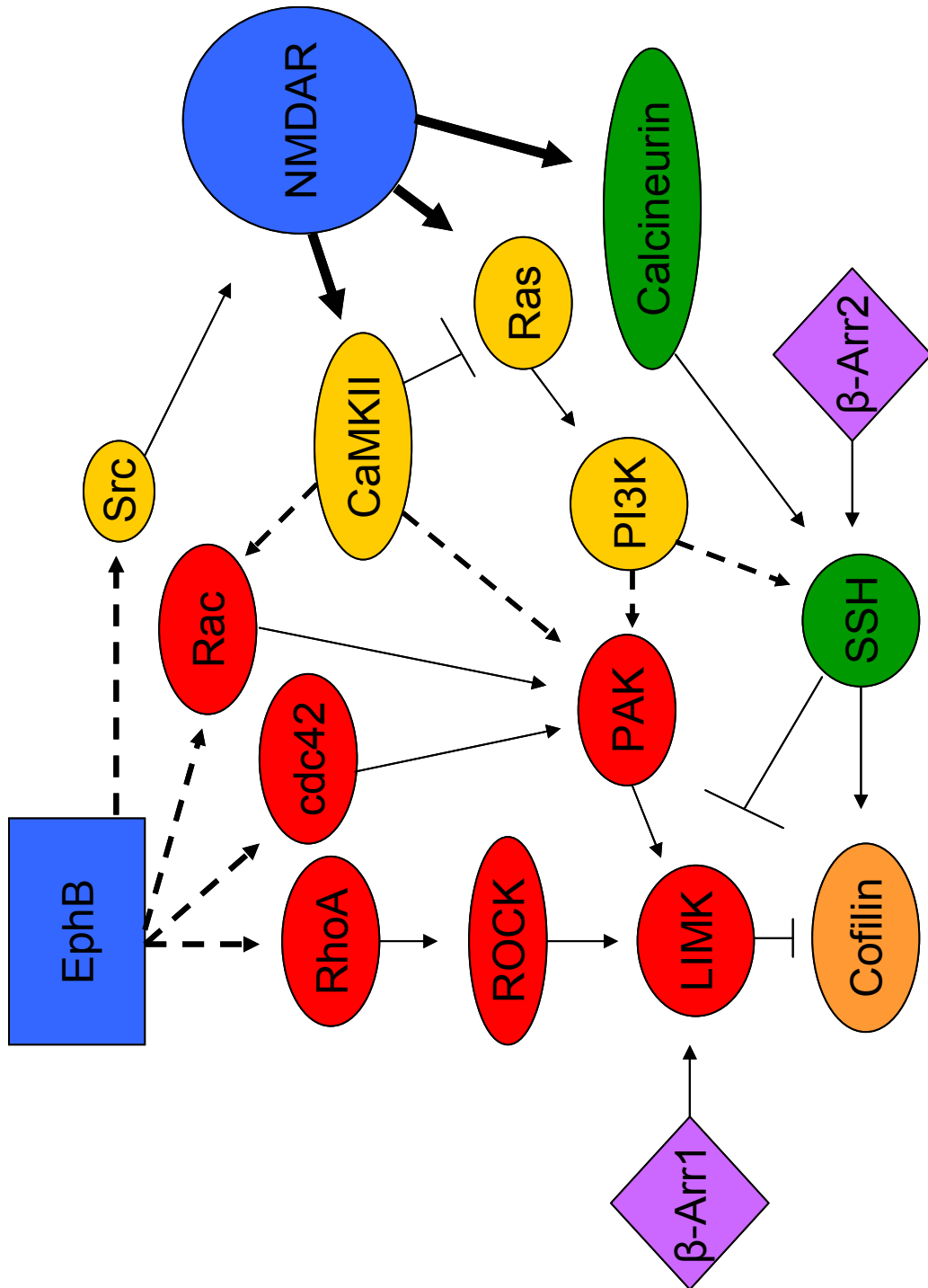


Figure 4-9 β -Arrestins participate in EphB and NMDA receptor-mediated regulation of cofilin

Cross-talk between the EphB and NMDA receptor pathways leads to tight regulation of cofilin phosphorylation and activation state, while β -Arrestins control the spatial distribution of cofilin and its regulators LIMK and SSH within hippocampal neurons.

Figure 4-9



Chapter 5: Conclusions

5.1 Results and Discussion

Dendritic spines are the post-synaptic sites of the majority of excitatory synapses in the central nervous system (Rao & Craig, 2000; Hering & Sheng, 2001; Yuste & Bonhoeffer, 2004; Matus, 2005). They are dynamic actin-rich structures that remain plastic even in the adult brain, and it is the morphology of the spine that determines the synapse maturity and stability. Mature dendritic spines tend to have short necks and large head areas, and spine head size correlates with receptor complement and PSD size (Takumi *et al.*, 1999; Racca *et al.*, 2000; Matsuzaki *et al.*, 2001). Thin spines have smaller heads and are more flexible, as well as more responsive to remodeling with synaptic input (Matsuzaki *et al.*, 2001; Murthy *et al.*, 2001; Smith *et al.*, 2003; Nicholson *et al.*, 2006). Dendritic spines remain plastic in the adult brain and can rapidly grow, change, or collapse in response to normal physiological changes in synaptic activity that underlies learning and memory. Long-term potentiation (LTP) can result in spine head enlargement (Lang *et al.*, 2004; Matsuzaki *et al.*, 2004), whereas long-term depression (LTD) has been reported to induce shrinkage of dendritic spine heads and spine elimination (Zhou *et al.*, 2004). In the hippocampus, actin-binding proteins such as cofilin are able to regulate the morphology and maturity of dendritic spines and synapses, which may underlie learning and memory, as well as neuropathology (Bamburg, 1999; Ethell & Pasquale, 2005; Pontrello & Ethell, 2009). Cofilin is inactivated by phosphorylation, which can be achieved through the activation of some cell surface

receptors and intracellular signaling cascades, while other receptors control cofilin dephosphorylation and activation. In addition, there are intracellular scaffolding proteins that spatially regulate cofilin within the neuron, and localize cofilin with its regulators. Our studies have investigated the roles of cell-surface EphB and NMDA receptors, as well as intracellular β -Arrestin scaffolding proteins, in the regulation of dendritic spine morphology through the actin-binding protein cofilin.

Cofilin is enriched at the leading edge in motile cells, in ruffling membranes and lamellipodia, and in dynamic areas of dendritic spines, where actin turnover is essential for processes such as cell migration, establishment of cell polarity, and spine remodeling (Bamburg & Bray, 1987; Yonezawa *et al.*, 1987; Obinata *et al.*, 1997; Meberg *et al.*, 1998; Fass *et al.*, 2004; DesMarais *et al.*, 2005; Racz & Weinberg, 2006; Garvalov *et al.*, 2007). While cofilin binds and severs F-actin filaments, it also creates new barbed ends for actin polymerization (Bamburg, 1999). In this way, cofilin contributes to actin treadmilling in which there is a constant turnover of F-actin filaments and net outgrowth from the fast-growing barbed ends (Kirschner, 1980; Bindschadler *et al.*, 2004; Okamoto *et al.*, 2004). Indeed, we have seen that over-expression in mature hippocampal neurons of constitutively-active cofilin^{S3A}, but not wt-cofilin or dominant-negative cofilin^{S3D}, results in spine remodeling and filopodial outgrowth (Shi *et al.*, 2009). In contrast, activation of the EphB receptor with a soluble ephrinB2-Fc ligand leads to stabilization of mature mushroom spines, which we have shown is achieved by inactivation of cofilin.

Eph receptors regulate intracellular signaling cascades through their tyrosine kinase activity by activating FAK, Src, paxillin, and the downstream effector RhoA

(Moeller *et al.*, 2006). This leads to the activation of LIMK, which directly phosphorylates and inactivates cofilin. We found that over-expression of dominant-negative EphB2 or Cre-mediated deletion of FAK downstream of the EphB receptor disrupts mature spine morphology that is induced by EphB receptor activation. Disruption of the EphB pathway at the level of the EphB receptor or FAK promotes immature spine morphology similar to that observed with over-expression of constitutively-active cofilin^{S3A}. Interestingly, mature spine morphology is not disrupted in FAK-deficient neurons or neurons with inhibited EphB receptors that also co-express dominant-negative cofilin^{S3D}, suggesting that cofilin inactivation is the mechanism behind spine stabilization seen with activation of the EphB-FAK pathway. In support of this, EphB activation leads to LIMK activation and cofilin phosphorylation and inactivation that is abolished by the ROCK inhibitor Y-27632, and neurons over-expressing FAK or constitutively-active FAK^{Y397E} show more phosphorylated inactive cofilin than non-transfected neurons or those over-expressing inactive FAK^{Y397F} or FAK^{L1034S}. These results support the role of the EphB-FAK-RhoA-LIMK pathway in suppressing cofilin activity through phosphorylation, leading to stabilization of mature dendritic spines in hippocampal neurons (Shi *et al.*, 2009).

NMDA has previously been shown to promote dendritic spine remodeling (Shi & Ethell, 2006), which could be followed by spine stabilization and long-term potentiation (Chen *et al.*, 2007) or spine shrinkage and long-term depression (Zhou *et al.*, 2004). It has recently been shown that cofilin regulates AMPA receptor trafficking and membrane insertion that is associated with synaptic plasticity (Gu *et al.*, 2010; Rust *et al.*, 2010).

Here we demonstrate that cofilin dephosphorylation and translocation underlies a type of synaptic plasticity, NMDA-mediated spine remodeling. NMDA receptor activation may lead to a variety of signaling cascades that affect dendritic spine morphology transiently or over a long period of time. NMDA receptor activation can lead to CaMKII and synGAP activation, which promotes transient Ras activation (Oh *et al.*, 2004), as the NMDA receptor promotes Ras activity but synGAP down-regulates it. In addition, a NMDA-induced Rac-PAK-LIMK signaling pathway is activated with a time delay following Ras activation. Although this would be expected to result in LIMK activation, followed by cofilin phosphorylation and inactivation, there is instead a reduction in p-cofilin levels, followed by a slow increase in phosphorylated and inactive cofilin (Carlisle *et al.*, 2008). This implicates another pathway in the predominant control over the immediate phosphorylation state of cofilin in response to NMDA, likely involving calcineurin and SSH phosphatase (Quinlan & Halpain, 1996).

In our studies, we also see a decrease in p-cofilin with NMDA treatment. Upon NMDA receptor activation, the amount of Ca^{2+} that enters the cell determines the intracellular pathway of effectors that will be activated. High levels of Ca^{2+} lead to CaMKII activation, whereas low Ca^{2+} activates calcineurin (Nicoll & Malenka, 1999; Lau *et al.*, 2009). This Ca^{2+} -dependent specificity leads to control of many diverse cellular functions such as growth cone guidance (Wen *et al.*, 2004), neuronal differentiation (Joo *et al.*, 2007), and migration (Komuro & Rakic, 1993), and synaptogenesis (Bressloff, 2006). In addition, NMDA-induced CaMKII activation leads to LTP, whereas calcineurin activation promotes LTD (Rusnak & Mertz, 2000; Malenka

& Bear, 2004). We show here that NMDA promotes LIMK phosphorylation through CaMKII, but cofilin dephosphorylation and activation through calcineurin and PI3K, resulting in overall activation of cofilin, most likely through SSH phosphatase. In addition, NMDA-induced spine remodeling can be blocked by over-expression of inactive cofilin^{S3D}, or concurrent inhibition of calcineurin and PI3K, which can activate cofilin through slingshot (SSH) phosphatase (Wang *et al.*, 2005). This suggests that SSH can act downstream of calcineurin and/ or PI3K to dephosphorylate and activate cofilin in response to NMDA. These results show that activation of cofilin underlies a type of NMDAR-mediated plasticity that results in dendritic spine remodeling.

NMDA receptor activation can also block EphB-mediated cofilin inactivation in a calcineurin-dependent manner. Concurrent EphB and NMDA receptor activation leads to a decrease in activated pLIMK levels and a decrease in p-cofilin, indicating an increase in cofilin activity. This suggests that the EphB receptor predominantly activates a different pathway when the NMDAR is concurrently activated than when it is activated alone. In this way, the EphB receptor participates in NMDAR-mediated plasticity by promoting cofilin activity. Through activation of PI3K-SSH or calcineurin-SSH pathways, the EphB and NMDA receptors could produce the LIMK and cofilin dephosphorylation that is seen here. Indeed, there is cross-talk between EphB and NMDA receptors (Dalva *et al.*, 2000). The EphB receptor is known to potentiate the NMDA receptor through activation of Src (Yu & Salter, 1999; Takasu *et al.*, 2002; Salter & Kalia, 2004). In addition, CaMKII recruitment by the EphB/ NMDA receptor complex could lead to NMDAR phosphorylation (Omkumar *et al.*, 1996), or the EphB receptor could directly

phosphorylate and potentiate the NMDA receptor through its own tyrosine kinase activity. It is clear that cross-talk between the EphB and NMDA receptors leads to an emphasis on different effectors in the downstream signaling pathways than activation of either receptor alone.

The phosphorylation state of cofilin affects not only its activity, but also its localization within cells (Nebl *et al.*, 1996). Here we examined the localization of phosphorylated and dephosphorylated cofilin in hippocampal neurons under normal synaptic activity and in response to NMDA receptor activation. NMDA induced a rapid translocation of cofilin to dendritic spine heads. Using non-phosphorylatable and phospho-mimetic mutants, we found that cofilin dephosphorylation is necessary for cofilin to translocate to dendritic spines with NMDA application, but dephosphorylation is not sufficient to cause spine localization under normal synaptic activity. It is likely that dephosphorylated active cofilin is trafficked to spine heads in order to promote the transient spine remodeling that is seen with NMDA receptor activation (Shi & Ethell, 2006), which is also needed for AMPA receptor trafficking (Malinow & Malenka, 2002; Brecht & Nicoll, 2003; Gu *et al.*, 2010; Rust *et al.*, 2010). This could be followed by the inactivation of cofilin, AMPAR insertion into the postsynaptic membrane, spine stabilization, and LTP consolidation (Chen *et al.*, 2007), or prolonged cofilin activation, AMPA receptor internalization, spine shrinkage, and LTD (Beattie *et al.*, 2000; Man *et al.*, 2000; Zhou *et al.*, 2004). The translocation of wt cofilin to dendritic spines with NMDA application was not blocked by inhibitors of calcineurin alone. This indicates that although phosphorylation state is important to cofilin localization within cells, there is

another factor that also contributes to cofilin translocation. Likely candidates include scaffolding proteins that could control not only cofilin phosphorylation by localizing it with its regulators, but also the spatial distribution of cofilin.

We found that in addition to phosphorylation state, NMDA-induced cofilin translocation into dendritic spines is also regulated by β -Arrestins. β -Arrestins are known to be involved in the desensitization and internalization of G-protein-coupled receptors (Luttrell & Lefkowitz, 2002; DeWire *et al.*, 2007; Defea, 2008), but recently their importance as scaffolding proteins that spatially regulate many other proteins and signaling molecules has emerged (Zoudilova *et al.*, 2010). Among the proteins that β -Arrestins spatially regulate are cofilin, LIMK, and SSH phosphatase. β -Arrestins can regulate the localization of cofilin within the cell, as well as its phosphorylation state through scaffolding it with LIMK or SSH. We see here that β -Arrestin1 and 2 have differential roles in dendritic spine regulation. We show that β -Arrestin1 is required for normal development of dendritic spines, as there is an increase in immature spines in the β -Arrestin1 KO hippocampal neurons under normal synaptic activity, both *in vitro* and *in vivo*. This could be due to the role of β -Arrestin1 in scaffolding LIMK with cofilin, which could lead to increased cofilin phosphorylation and inactivation. In addition, these neurons still express β -Arrestin2, which scaffolds cofilin with its activator SSH phosphatase, leading to a further increase in cofilin activation. In support of this, over-expression of constitutively-active cofilin^{S3A} leads to the formation of cofilin-actin rods in both untreated and NMDA-treated β -Arrestin1 KO neurons. Cofilin-actin rods are formed under conditions of cellular stress, when cofilin is excessively active (Minamide

et al., 2000; Bamberg *et al.*, 2010; Minamide *et al.*, 2010). In contrast, over-expression of dominant-negative cofilin^{S3D} restores mature dendritic spines in β -Arrestin1 KO neurons and prevents rod formation, most likely through competitive inhibition of SSH. Notably, over-expression of cofilin^{S3A} in the wt or β -Arrestin2 KO neurons did not induce cofilin-actin rods. In addition to the aberrant activation and localization of cofilin, abnormal GPCR signaling can also contribute to the immature spines observed in β -Arrestin1 KO neurons. Interestingly, whether it is abnormal cofilin activity/ localization and/ or GPCR signaling that disrupts spines in β -Arrestin1 KO neurons, the abnormal immature spine phenotype observed in these neurons can be rescued by over-expression of β -Arrestin1 at 12 DIV, when the majority of dendritic spines in wt neurons already display a mature morphology.

In contrast to the β -Arrestin1 KO neurons, dendritic spines in β -Arrestin2 KO neurons develop normally, but we see that β -Arrestin2 is needed for NMDAR-mediated remodeling of mature spines, as NMDA fails to promote immature spines in the β -Arrestin2 KO neurons. In addition, the spatial distribution of cofilin in β -Arrestin1 and β -Arrestin2 KO neurons is aberrant under normal synaptic activity, and active dephosphorylated cofilin fails to translocate to spine heads with NMDA treatment in β -Arrestin2 KOs. The failure of β -Arrestin2 KO neurons to respond normally to NMDAR activation could suggest that cofilin, which would promote NMDA-induced spine remodeling, may be excessively phosphorylated due to inappropriate spatial proximity to SSH, or aberrantly localized within the neurons so that it cannot effect changes on the cytoskeleton even when it is dephosphorylated and active. In support of this, β -Arrestin2

KO neurons are also resistant to dendritic spine remodeling induced by constitutively-active cofilin^{S3A}, and inactive cofilin^{S3D} blocks spine remodeling in the wt neurons. This supports the role of spatial localization of active cofilin in NMDA receptor-induced dendritic spine remodeling, which is disrupted in β -Arrestin2 KO mice. Interestingly, overexpression of β -Arrestin2 in the β -Arrestin2 KO neurons recovers the normal spine remodeling in response to NMDA. We have shown here a novel function of β -Arrestins that does not involve GPCR signaling, but regulates the effects of cofilin on dendritic spine morphology under normal synaptic activity and with NMDA receptor activation.

Dendritic spines remain plastic even in the adult brain, due to such proteins as cofilin that can re-arrange the F-actin cytoskeleton (Bamburg, 1999), thus dictating spine morphology (Shi *et al.*, 2009). The phosphorylation state of cofilin in hippocampal neurons is tightly regulated by LIMK and SSH phosphatase, both of which are controlled by cell surface receptors such as EphB and NMDA receptors. In addition to activation state, the spatial distribution of cofilin in the neurons is important for its actin remodeling activity. If cofilin is located aberrantly within the neuron, it will not effect the appropriate changes on actin and dendritic spines when it is active. β -Arrestins are scaffolding proteins that can control both the phosphorylation state and the spatial regulation of cofilin. We show here that β -Arrestins play differential roles in dendritic spines, β -Arrestin1 regulating spine development, and β -Arrestin2 mediating NMDA-induced spine plasticity. Abnormality in spatial control over cofilin activity could lead to aberrant dendritic spines and neurological deficits such as those seen in Down syndrome and the autism spectrum disorders (Kaufmann & Moser, 2000; Sorra & Harris, 2000; Halpain *et*

al., 2005). In addition, stress-induced cofilin-actin rods are found in the brain in several neurological disorders (Maloney & Bamberg, 2007), and over-expression of dominant-negative phospho-mimetic cofilin^{S3D} in hippocampal slices has been shown to prevent dendritic spine loss caused by A β oligomers, structures that are observed in the brains of patients with Alzheimer disease (Shankar *et al.*, 2007; Davis *et al.*, 2009). This implicates cofilin as an important regulator of cytoskeletal and dendritic spine dynamics, whose activity and localization must be tightly controlled under normal synaptic activity and in response to NMDA receptor activation for proper cellular function.

5.2 References

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