Non-muscle myosin-IIB and α-actinin-2 determine dendritic spine morphology and post-synaptic organization

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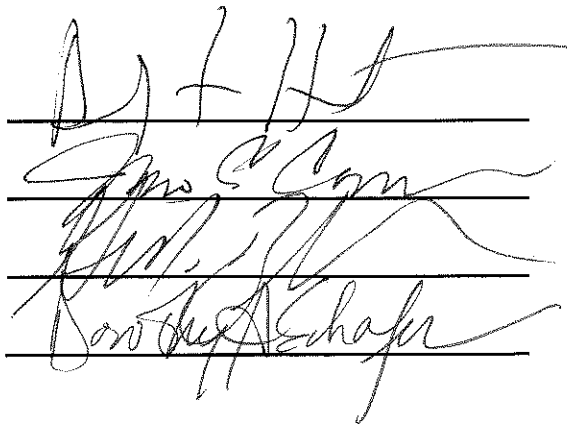

Abstract

Modulation of the actin cytoskeleton dictates the morphological changes associated with dendritic spine dynamics, which serve as the structural basis underlying learning and memory. These micron-sized protrusions mature from a filopodia-like morphology into a mushroom-shape with an enlarged post-synaptic density (PSD). The PSD contains an assembly of synaptic adhesion molecules, glutamate receptors, and signaling scaffolds; many of which respond to glutamate receptor activation and relay signals to the underlying cytoskeleton to induce structural changes in spine and PSD morphology.

Non-muscle myosin IIB (MIIB) and α-actinin-2 (ACTN2) directly effect actin organization and both proteins localize to dendritic spines. Both molecules cross-link actin filaments and MIIB also mediates contraction through its ATPase activity. Knockdown of either ACTN2 or MIIB creates an immature spine morphology that fails to mature into a mushroom-shaped spine during development and in response to chemical stimulation. Additionally, loss of ACTN2 increases spine density. Expression of an actin cross-linking, non-contractile mutant, MIIB R709C, showed that spine maturation requires contractile activity. Additionally, di-phosphorylation of the myosin regulatory light chain (RLC) by Rho kinase is required for spine maturation. Inhibition of MIIB activity via blebbistatin treatment, knockdown, or expression of a mono-phosphomimetic mutant of RLC similarly abrogated spine maturation.

MIIB and ACTN2 also determine PSD size, morphology, and placement in the spine. Loss of ACTN2 prevents the recruitment and stabilization of a PSD and NMDA-

type glutamate receptors in the spine, resulting in defective synaptic formation. Conversely, a PSD is still seen in neurons with MIIB knocked down, but its loss creates an elongated PSD morphology that is no longer restricted to the spine tip, resulting in a less-clustered distribution of NMDA receptors. In contrast, increased MIIB activity, through either over-expression of *wild type* MIIB or a RLC di-phosphomimetic mutant, enlarges the PSD area and creates an increased density of mature spines. These observations support a model whereby ACTN2 nucleates PSD formation and recruits the NMDA-type glutamate receptor to the spine, which leads to a functioning synapse. Subsequent NMDA receptor activation increases RLC di-phosphorylation to stimulate MIIB contractility, resulting in a mushroom-shaped spine with an enlarged PSD.

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Dedication

This dissertation is dedicated in loving memory of my father, McCloud Bradford Hodges III, who inspired my curiosity for science.

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List of Abbreviations

Abbreviation	Meaning
α-PIX	αPak-interacting exchange factor
Abp1	Actin-binding protein 1
ACTN2	α-actinin-2
AMP	Adenosine mono-phosphate
AMPA	α-amino-3-hydroxy-5- methyl-4-isoxazolepropionate
Arp2/3	Actin-related proteins 2 and 3
ATP	Adenosine tri-phosphate
BDNF	Brain-derived neurotrophic factor
β-PIX	βPak-interacting exchange factor
CaMKII	Calcium-calmodulin-dependent kinase II
Cdc42	Cell division control protein 42 homolog
CP	Capping protein
Daam1	Dishevelled-associated activator of morphogenesis 1
DNA	Deoxyribonucleic acid
Dock-180	Dedicator of cytokinesis - ~180kDa
EphB	Ephrin receptor B
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
FAK	Focal adhesion kinase
FGD1	Faciogenital dysplasia 1 protein

FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GAP	GTPase-Activating Proteins
GEF	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine-5'-triphosphate
GTPase	Enzymes that hydrolyze guanosine-5'-triphosphate
IgG	Immunoglobulin G
IP3	Inositol 1,4,5-triphosphate/Ca^{++}
LIMK-1	Lim kinase 1
LRRTM	Leucine-rich repeat transmembrane neuronal family
LTD	Long-term depression
LTP	Long-term potentiation
MIIB	Myosin-IIB
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan cancer foundation 7 – human breast adenocarcinoma cell line
mDia2	Diaphanous-related forming 2
mGluR	Metabotropic or heterotrimeric GTP-binding protein-linked glutamate receptors
MDCK	Madin-Darby canine kidney epithelial cells

MLCK	Myosin light chain kinase
NCAM	Neural cell adhesion molecule
NMDA	N-methyl-D-aspartate
N-WASP	Neural-Wiskott-Aldrich syndrome protein
PAK1	p21-activated kinase 1
PAK3	p21-activated kinase 3
PDZ	Protein domain – acronym comprises the first letter of 3 proteins: **P**ost synaptic density protein (PSD95), **D**rosophila disc large tumor suppressor (Dlg1), and **Z**onula occludens-1 protein (zo-1)
PI3K	Phosphatidylinositide 3-kinase
PKA	Cyclic AMP (cAMP)-activated kinase
PKC	Protein kinase C
ProSAP	Proline-rich synapse-associated protein
PSD	Post-synaptic density
PSD-95	Post-synaptic density protein 95
RhoA	Ras homolog gene family member A
RLC	Regulatory light chain of myosin II
RNA	Ribonucleic acid
RNAi	Ribonucleic acid inhibition
ROCK	Rho-associated, coiled-coil containing protein kinase 1
SH3	SRC homology 3 domain
shRNA	Small hairpin ribonucleic acid

Src	Proto-oncogene non-receptor tyrosine kinase
SynCaM	Synaptic cell adhesion molecule
TIAM1	T-cell lymphoma invasion and metastasis-inducing protein 1
TrkB	Tyrosine kinase receptor B
VASP	Vasodilator-stimulated phosphoprotein
VGlut1	Vesicular glutamate transporter 1
WAVE	WASP-family verprolin homologous protein

Chapter 1 – General Introduction

Dendritic Spines

Dendritic spines are specialized postsynaptic structures of excitatory, glutamate-releasing synapses, which serve as the main sites of excitatory neurotransmission in most principal neurons of the mammalian brain, e.g., pyramidal cells of the cortex and hippocampus, and Purkinje neurons of the cerebellum (Yuste and Denk, 1995; Sheng and Hoogenraad, 2007). These dendritic structures are micron-sized, actin-rich protrusions that undergo dynamic changes in morphology during development, as well as in response to synaptic neurotransmission. Dendritic spines develop from exploratory, filopodial-like processes that protrude from the dendrite (Sekino et al., 2007). These highly mobile protrusions undergo continual extension and retraction until physical contact is made with an axon. A combination between adhesive contact and excitatory, synaptic input from the pre-synaptic axon is thought to trigger maturation of the post-synaptic structure.

Spine maturation is characterized by a morphological change from a filopodial-like protrusion into a mushroom-shaped structure consisting of a bulbous head attached to the dendrite by a narrow stalk or neck (Sekino et al., 2007). Imaging of spines *in vitro* show that the filopodia-like protrusions transition through a lollipop-like stage, characterized by a long protrusion with a small head, before enlarging at the head and shortening at the neck into a mushroom shape. Live imaging of individual spines undergoing spine maturation, in response to pharmacological stimulation of the post-synaptic membrane, exhibit expansion of the spine head with concomitant shortening of the neck (Yuste and Bonhoeffer, 2004). While thin, filopodial-like spines are spontaneously generated and eliminated throughout development and adulthood, mature, mushroom-shaped spines are more stable (Kasai et al., 2003). The mature spine head

represents the structural entity with greatest synaptic strength, as it contains a greater number of adhesion receptors and glutamate receptors at the post-synaptic membrane mediating a strong connection to an axon.

The morphological plasticity of the spine dictates synaptic strength; neurophysiologic studies show that a thin, immature protrusion is not as effective in integrating and propagating neuronal signals as a mature spine with a larger membrane-surface area (Kasai et al., 2003; Sheng and Hoogenraad, 2007). Aberrant spine morphology and density, characterized by long tortuous spines lacking a bulbous head or dendrites lacking spines, is seen in neurons of human cadaver brains of individuals with various forms of neurodevelopmental disorders, including severe metal retardation, autism spectrum disorders, and Down's syndrome, as well as psychoses such as schizophrenia (Fiala et al., 2002; Penzes et al., 2011). Synapse loss is observed in neurodegenerative diseases such as dementia, mild cognitive impairment, Parkinson's disease, and Alzheimer's disease (Day et al., 2006; Penzes et al., 2011). Perturbations in spine morphology precede synapse loss and the neurofibrillary tangles that accompany neuronal degeneration in Alzheimer's Disease (Selkoe, 2002; Tackenberg et al., 2009). Thus, the structure of the spine directly relates to its function and a further understanding of the molecular mechanisms dictating spine dynamics and morphology is critical to unraveling the pathological causes of various neuropsychiatric disorders.

Interestingly, while the mushroom-shaped morphology represents the mature spine structure in most mammalian brains, the neck size of mature spines in the human cortex is significantly longer (Elston et al., 2001; Benavides-Piccione et al., 2002). The increased neck length found in the human brain is thought to better compartmentalize

calcium signaling from the dendrite (Elston et al., 2001). Greater spine densities are also observed in the human cortex and they are hypothesized to accommodate higher input integration and information processing (Benavides-Piccione et al., 2002). A recent study (Charrier et al., 2012) found that an ancestral gene duplication of SRGAP2, a Rho-GAP, yielded a human-specific SRGAP2 paralog that encodes a truncated domain, which mediates inhibition of the ancestral gene product. Expressing the human-specific paralog of SRGAP2 *in utero* of the mouse creates a spine anatomy *in vivo* resembling the human cortex, including an increased spine density and longer spine necks (Charrier et al., 2012). This study connects the functional outcome of human-specific gene duplication to human brain speciation and provides a potential genetic mechanism for the enhanced cognitive abilities of the human species. In addition to longer necks and greater spine densities, the spines of the human cortex harbor larger head volumes than other species, (Benavides-Piccione et al., 2002) contributing to its increased synaptic strength.

The ability of two neurons to change the strength of their connection, referred to as synaptic plasticity, is considered a key component of the molecular basis for learning and memory. The phenomena associated with the strengthening and weakening of existing synapses is respectively referred to as long-term potentiation (LTP) and long-term depression (LTD). Hallmarks of LTP include an increase in the volume of the spine head, accompanied by an increase in the size of the post-synaptic density, and the number of AMPA-type glutamate receptors inserted at the post-synaptic membrane (Matsuzaki et al., 2004). These larger spines are most adept to memory storage (Matsuzaki et al., 2004). In contrast, LTD causes spine head shrinkage and a corresponding decrease in the size of the post-synaptic density and the number of AMPA receptors docked at the post-

synaptic membrane. Structural plasticity also involves the formation and elimination of spines, which mediates changes in the brain circuitry. This can be seen in the corresponding region of the mouse neocortex *in vivo* in direct response to sensory or motor experience, or whisker trimming (Zuo et al., 2005; Holtmaat et al., 2006; Xu et al., 2009). Experience-dependent rewiring of cortical circuits provide flexibility to choose which pre-synaptic cells provide input to each post-synaptic cell, and therefore enhances the storage capacity of the brain (Chklovskii et al., 2004; Holtmaat and Svoboda, 2009). Thus structural plasticity of the spine, albeit formation, elimination, or morphology changes associated with LTP or LDP, directly corresponds to functional plasticity of the brain, as synapses along neural networks store information in response to experience.

Emerging evidence suggests a prominent role for glia cells in shaping dendritic spine morphology and synapse maturation. For example, mutant astrocytes from a Rett syndrome mouse model caused aberrant dendritic morphologies in co-cultured hippocampal neurons derived from a normal mouse (Ballas et al., 2009). Signaling between neuronal EphA receptors and Ephrin-A ligands expressed on glia cells modifies dendritic spine morphogensis (Murai et al., 2003). Specifically, glia-expressed ephrin-A3 binds to EphA4 receptor expressed on dendritic spines of hippocampal neurons and regulates the morphological plasticity of spines by modulating integrin activity (Bourgin et al., 2007). Glia cells occupy more than half the volume of the human brain and are an integral part of synapses as they release soluble factors, maintain ion homeostasis by regulating extracellular potassium concentrations and pH, and express adhesion receptors that interact with receptors on the spine surface (Eroglu and Barres, 2010).

How does a spine form? The molecular mechanisms of spinogenesis have yet to be unraveled; but the process requires both mechanical and chemical activity. Synaptic adhesion proteins expressed on both the post-synaptic and pre-synaptic side mediate a mechanical connection that initiates synaptogenesis. Non-neuronal cells expressing neurexins, a pre-synaptic adhesion protein that binds to post-synaptic neuroligins, cause the clustering of PSD-95, a canonical marker of the post-synaptic density, in contacting dendrites of hippocampal neurons (Nam and Chen, 2005). Likewise, non-neuronal cells expressing the EphB2 receptor, which binds to the pre-synaptic ephrin-B ligand, generate pre-synaptic differentiation of contacting axons in co-cultured neurons (Kayser et al., 2006). Although, post-synaptic specializations can form on either non-neuronal cells or on contacting dendrites, these specializations are without a spine structure. Since *in vivo* imaging shows that spine growth precedes synapse formation (Knott, 2006), spinogenesis likely occurs before synaptogenesis.

A dendritic filopodium, dominant in early post-natal development, serves as a precursor to spine development (Sekino et al., 2007). These filopodia-like spine precursors, seen between days *in vitro* (DIV) 6-10 in dissociated cultures, lack a post-synaptic density and do not appose a pre-synaptic terminal (Figure 1). Although their morphology is similar, filopodia-like dendritic protrusions are not the same structures as the filopodia seen on growth cones or fibroblasts. Specifically, filopodia-like spine precursors do not contain a tight bundle of parallel actin filaments cross-linked by fascin, which characterizes the actin organization constituting growth cone filopodia (Korobova and Svitkina, 2008). Growing axons release neurotransmitter before synaptic connections are formed (Scheiffele, 2003), therefore molecular mechanisms must exist

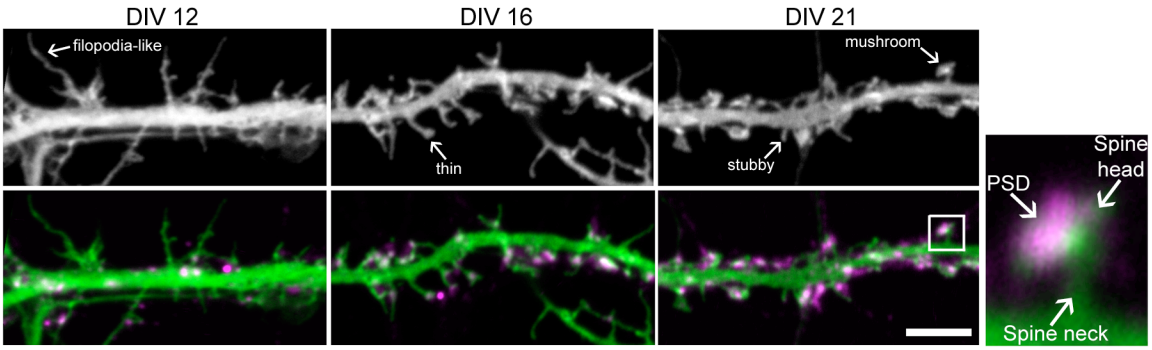
Figure 1: Spine and synaptic development in dissociated hippocampal cultures.

A) Hippocampal neurons transfected with GFP at DIV 6 were fixed and immunostained for endogenous PSD-95 (magenta) at DIV 12, 16, and 21. Top row is GFP and bottom row is the overlay image. Boxed area in DIV 21 overlay image is magnified to the right. Scale bar = 5 μm.

B) Hippocampal neurons transfected with GFP at DIV 6 were fixed and immunostained for endogenous synaptophysin (magenta) at DIV 9, 12, and 21. Top row is GFP and bottom row is the overlay image. Boxed area in DIV 21 overlay image is magnified to the right. Scale bar = 5 μm.

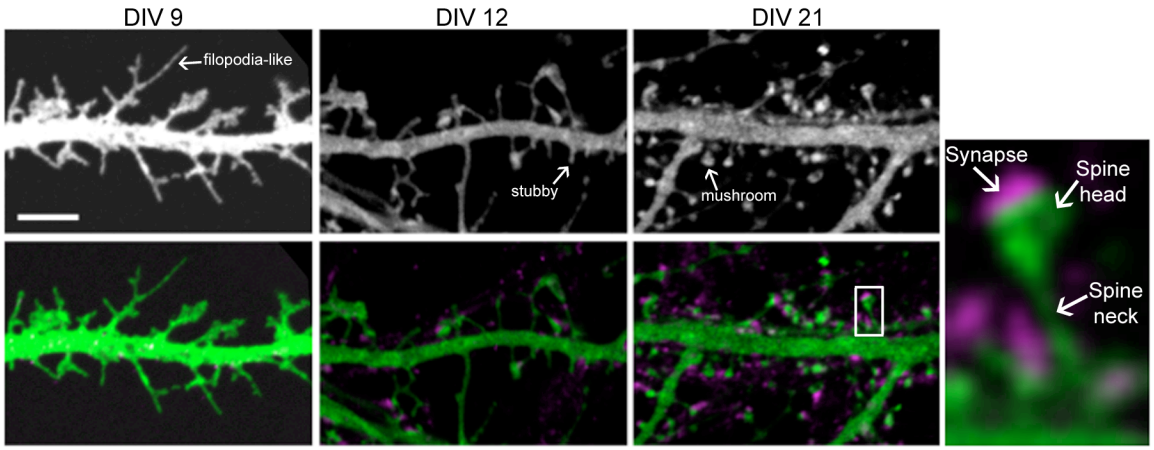
A

Localization and maturation of PSD-95 throughout spine development



B

Localization and maturation of synaptophsyin throughout spine development

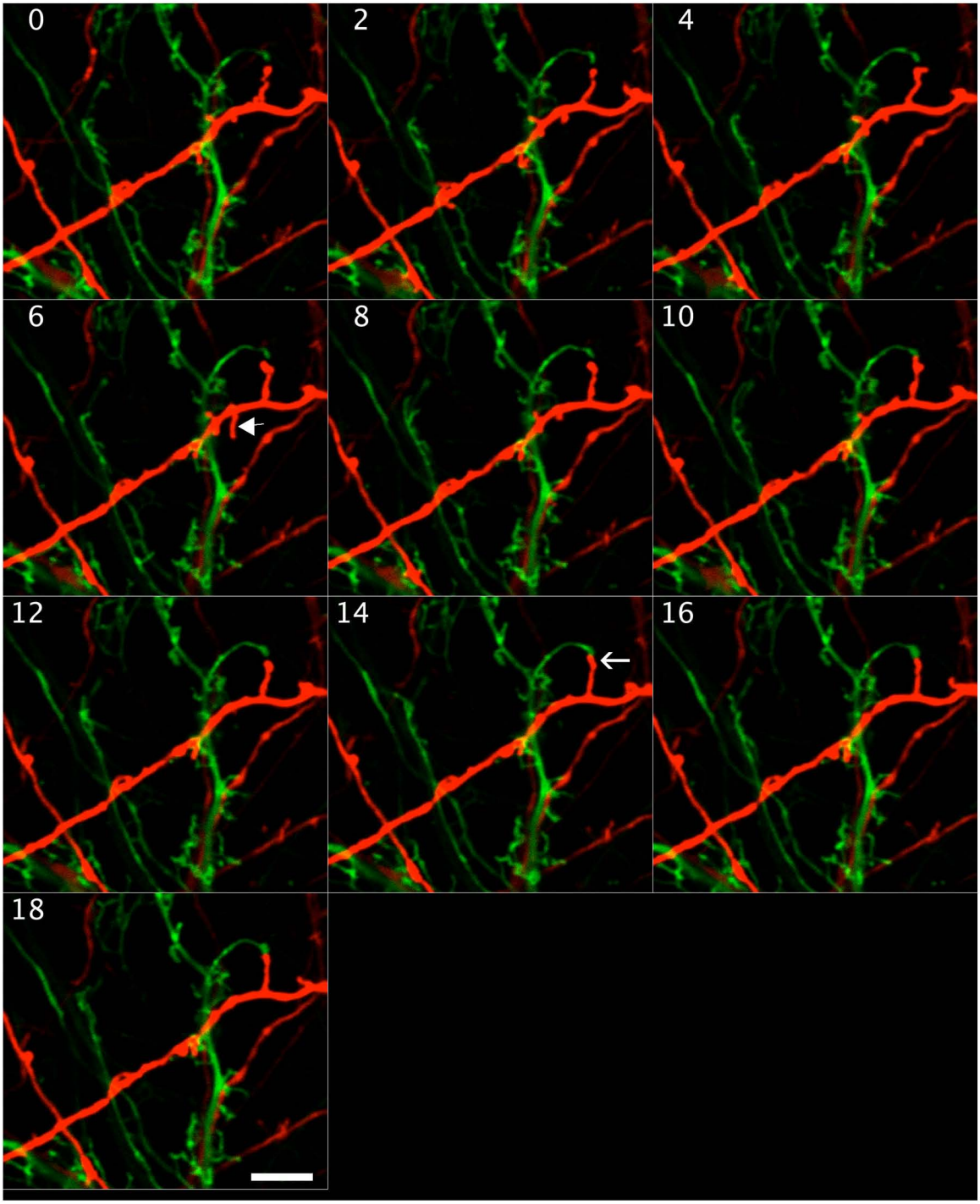


among filopodia-like dendritic protrusions to chemotactically respond to extracellular glutamate in search for a potentiated axon. To address this, one study induced spinogenesis by uncaging glutamate less than 1 μm away from the dendrites of cortical layer 2/3 pyramidal neurons in acute cortical brain slices from young mice (Kwon and Sabatini, 2011). They found that glutamate-induced *de novo* growth of spines requires the NMDA-type glutamate receptor coupled to the activation of intracellular pathways, cyclic AMP (cAMP)-activated kinase PKA and Ras-GTP-dependent mitogen-activated protein kinase (MAPK). Spinogenesis may also be facilitated by activation of G protein-coupled receptors that respond to dopamine, serotonin, or adrenalin (Kwon and Sabatini, 2011).

Interestingly, highly dynamic filopodia-like protrusions can be observed growing and retracting from pre-synaptic boutons of axons in young dissociated cultures (Figure 2), suggesting that axonal varicosities also actively participate in the search for a synaptic connection. These axonal outgrowths depend on activation of NMDA-type glutamate receptors and the retrograde release of nitric oxide from spines (Chang and De Camilli, 2001; Nikonenko et al., 2003; Tashiro et al., 2003). Once mechanical stabilization with a pre-synaptic contact occurs, many post-synaptic proteins are recruited to the spine (Figure 1). While some post-synaptic proteins form *de novo* in the spine, a pre-formed mobile complex of the postsynaptic proteins, PSD-95, GKAP, and Shank, has been observed to rapidly traffic to nascent post-synaptic synapses in an actin-dependent manner (Marrs et al., 2001; Gerrow et al., 2006). Rapid morphological changes in the spine and axonal varicosity continue to occur without losing their synaptic contact, and these overall structural changes between varicosities and spines are correlated

Figure 2: Protrusive outgrowth from dendrites and axonal varicosities. Hippocampal neurons were nucleofected with GFP or DsRed2 at DIV 0 and plated together onto poly-L-lysine-coated glass-bottomed dishes. Time-lapse confocal imaging was performed on DIV 16. Axonal varicosities on neurons expressing DsRed2 are seen protruding and retracting (arrowheads) from the axon. Some of these axonal protrusions are interacting with dendritic spines (arrows) on neurons expressing GFP. Time stamp represents minutes. Scale bar = 5 μm.

Spines on neurons expressing GFP interacting with axons on neurons expressing DsRed2



(Umeda et al., 2005), suggesting there is bi-directional control between pre- and post-synaptic components. As the synapse matures, the spine head increases in size with a coordinate increase in the post-synaptic density and pre-synaptic active zone (Figure 1). The larger spine volume is thought to better support the post-synaptic density, which contains many PDZ domain-containing proteins that cluster glutamate receptors, adhesion proteins, and signaling molecules at the post-synaptic membrane (Kasai et al., 2003). Thus, the morphology of the spine and organization of the post-synaptic density is crucial for responding to the strength of the synaptic input received and for effectively propagating excitatory synaptic transmission.

Post-synaptic density

The post-synaptic density, PSD, is an electron-dense structure on the cytoplasmic face of the post-synaptic membrane of most excitatory synapses in the brain. This structure is ~30-50 nm thick and ~200-800 nm wide, depending on the spine type, and maturation state (Sheng and Hoogenraad, 2007). It contains cell adhesion proteins like cadherins and integrins, G protein-coupled receptors, ionotropic glutamate receptors, receptor tyrosine kinases, and a plethora of cytoskeletal and signaling scaffold proteins at the post-synaptic membrane (Kennedy, 1997; Peng et al., 2004; Sheng and Hoogenraad, 2007). Core constituents of the PSD make up a well organized lattice with laminar organization of scaffolding molecules, as indicated by electron microscopy (Valtschanoff and Weinberg, 2001; Chen et al., 2008), and more recent super resolution imaging (Dani et al., 2010). This highly ordered structure supports the signaling scaffolds that are precisely arranged in space and time, effectively allowing the post-synaptic structure to

respond to synaptic input, propagate the message, and adapt. Mutations in human genes encoding PSD proteins cause approximately 133 neurological and psychiatric diseases (Bayés et al., 2011), therefore the organization of the PSD is central to its function.

A common feature to several PSD proteins is the PDZ-domain, which has a multi-domain structure enabling PDZ-containing proteins to interact with multiple binding partners simultaneously, thereby assembling into supramolecular complexes (Hung and Sheng, 2002). PSD-95 is a canonical molecule of the PSD as it contains many PDZ-domains, an SH3 domain, and directly binds to several transmembrane proteins, including the NR2 domain of the NMDA-type glutamate receptor (Kornau et al., 1995), and is thought to anchor them to the actin cytoskeleton. The number of PSD-95 molecules determines the size and strength of the synapse (Gray et al., 2006) and is required for stabilization of the synapse following synaptic activity (Ehrlich et al., 2007).

Other major scaffolding proteins at the PSD that contain PDZ and PDZ-like domains include Homer and ProSAP/Shank family members. Homer binds to group I metabotropic glutamate receptors (mGluR1 and mGluR5), which activate phospholipase C and couple to inositol 1,4,5-triphosphate/Ca^{++} (IP3) signaling (Okabe, 2007). Homer arranges the mGluRs around the outer rim of the ionotropic glutamate receptors (Kennedy, 2000). Shank proteins are layered at the cytoplasmic face of the PSD (Valtschanoff and Weinberg, 2001) and can bind to both the intracellular domains of membrane-associated molecules and several cytoskeletal proteins and regulators, including cortactin (Naisbitt et al., 1999), Abp1 (Qualmann et al., 2004), α-fodrin (Böckers et al., 2001), and the Rac1/Cdc42 GEF, βPIX (Okabe, 2007). Shank can also

bind to Homer, and multimerize with itself to create a sheet-like structure hypothesized to provide the foundation for the assembly of a higher order PSD structure (Baron et al., 2006). It is therefore no surprise that mutations in Shank genes are directly linked to synaptopathies, such as the neuropsychiatric disease Phelan-McDermid syndrome (Verpelli et al., 2012), schizophrenia (Gauthier et al., 2010), and autism (Grabrucker et al., 2011), as aberrant Shank proteins would affect the molecular framework of the PSD. Thus, organization of the PSD is paramount to post-synaptic functioning.

Adhesion proteins organized at the post-synaptic membrane mediate a mechanical connection of the PSD to the pre-synaptic bouton of an axon and induce bidirectional-signaling pathways to mediate the formation and maturation of excitatory synapses. Neuroligins, EphB receptors, cadherins, and SynCaMs (synaptic cell adhesion molecules) are major classes of post-synaptic proteins that mediate trans-synaptic adhesion. Post-synaptic neuroligins bind to neurexins expressed on the pre-synaptic surface, and PDZ domains within the intracellular portion of neuroligin connect the molecule to post-synaptic scaffolding proteins, including PSD-95 (Irie et al., 1997). Neuroligins play a key role in synaptogenesis and neuronal circuitry, as different types of neuroligin-neurexin interactions control the formation of inhibitory versus excitatory synapses (Dalva et al., 2007). Alterations in post-synaptic neuroligins are linked to autism spectrum disorders (Jamain et al., 2003).

Post-synaptic EphB receptor tyrosine kinase binds to the pre-synaptic, transmembrane ligand ephrin-B, and together orchestrate many aspects of excitatory synaptogenesis (Dalva et al., 2007). EphB2 interacts with AMPA receptors via its intracellular PDZ domain and increases their retention at the post-synaptic membrane

surface, thereby enhancing synaptic strength (Kayser et al., 2006). EphB receptor tyrosine kinase signaling leads to downstream organization of the actin cytoskeleton as it activates the Rho GTPases Rac1 and Cdc42, via activation of the GEF's kalirin (Penzes et al., 2003) and intersectin (Irie and Yamaguchi, 2002). EphB receptor tyrosine kinase signaling also results in the phosphorylation and increased association between Src, FAK, and paxillin at the post-synaptic membrane (Moeller et al., 2006). On the extracellular side, EphB directly interacts with the glutamate NMDA receptor and induces its clustering in non-neuronal cells (Dalva et al., 2000). The importance of EphB-ephrin-B interactions at excitatory synapses is highlighted by the findings that hippocampal neurons cultured from mice lacking EphB receptors 1-3 show complete loss of dendritic spines and excitatory post-synaptic specializations (Henkemeyer et al., 2003).

Classical cadherins mediate the assembly of specialized cell-cell junctions in many different cell types, including epithelia, neurons, and glia (Yamada and Nelson, 2007a). Neuronal (N)-cadherins found on both pre- and post-synaptic terminals form calcium-dependent homophilic interactions at the synapse. N-cadherin couples to the actin cytoskeleton through its intracellular interactions with α-catenin, β-catenin, and p120 catenin. Unlike neuroligins and EphB receptors, N-cadherin is dispensable for triggering synaptogenesis but does play a major role in directing spine morphogenesis and motility through its signals to Rho-GTPases (Dalva et al., 2007). Loss of function studies with any component of the N-cadherin, β-catenin, α-catenin, p120 catenin complex results in aberrant Rho-GTPase signaling and abnormal spine morphology due to erroneous organization of the actin cytoskeleton (Togashi et al., 2002; Elia et al., 2006). A common feature to trans-synaptic adhesion molecules is their ability to bind to

several components of the PSD through PDZ-domain interactions, which are critical for synapse formation, maturation, and maintenance.

Other adhesion proteins expressed on dendritic spines, but not discussed here, include integrins, the IgG superfamily of SALMs, SynCaMs and nectins, NCAMs, Netrin-G ligands, and LRRTMs (Benson and Huntley, 2012). The structural organization of these adhesion proteins in the PSD helps to align post-synaptic glutamate receptors in direct apposition of glutamate-containing pre-synaptic vesicles, mediate a mechanical connection to the pre-synaptic axon, the extracellular matrix, and glia cells, and finally induce intracellular signaling pathways to bidirectionally coordinate morphological synapse differentiation.

The ionotropic glutamate receptors α-amino-3-hydroxy-5- methyl-4-isoxazolepropionate (AMPA) and N-methyl-D-aspartate (NMDA) are found at the post-synaptic membrane of glutamatergic synapses. These transmembrane receptors respond to the excitatory neurotransmitter glutamate (Kennedy, 2000). When glutamate binds to AMPA receptors, the ion channel opens and sodium and potassium ions pass through; this depolarizes the membrane and mediates and an excitatory postsynaptic potential (EPSP). Extracellular magnesium occludes the NMDA receptor channel and requires, in addition to glutamate binding, a strong enough membrane depolarization mediated by AMPA receptors in order to relieve the blockade. Once activated, the NMDA receptors allow passage of sodium and calcium ions into the cell (Kennedy et al., 2005). Thus, the number of AMPA receptors expressed at the membrane surface directly correlates with NMDA receptor activation and the strength of the synapse. Indeed, the exocytic and endocytic machinery that traffics AMPA receptors to and from the membrane,

respectively, is an important mechanism regulating synaptic plasticity (Malinow and Malenka, 2002). The NMDA receptor is a heteromer composed of multiple NR1 subunits in combination, with at least one type of NR2 subunit (A, B, C, and/or D), and an NR3 subunit that can co-assemble with NR1/NR2 heteromers (Sugihara et al., 1992). NMDA receptor subtypes have different receptor properties and are expressed differentially throughout the brain and during development, and are implicated in normal and disease states (Cull-Candy et al., 2001). The glutamate receptors are spatially compartmentalized, such that NMDA receptors cluster in the center of the PSD with PSD-95, while AMPA receptors arrange around the periphery of the PSD (Chen et al., 2008), leading to spatially confined signal transduction pathways (Newpher and Ehlers, 2009).

A plethora of signal transduction pathways in the spine are triggered by the intracellular rise in calcium, mediated by activation of NMDA receptors (Kennedy, 2000). The large holoenzyme, calcium-calmodulin-dependent kinase II (CaMKII), is a serine/threonine kinase that is activated by calcium-bound calmodulin, and thereby amplifies the calcium signal by interacting with many downstream members of signaling scaffolds positioned at the PSD. CaMKII constitutes 1-2% of the total protein in the forebrain and is the most abundant signaling protein in the PSD (Erondu and Kennedy, 1985), priming a series of signaling cascades via phosphorylation. Many of the signaling pathways nucleated at the PSD involve the morphological maturation of the spine, such as activation of RhoGTPases that control organization of the actin cytoskeleton (Rao and Craig, 2000), and presumably spine morphology.

Signaling triggered by NMDA-type glutamate receptor activation

Signaling via the NMDA-type glutamate receptor at the post-synaptic membrane initiates spine maturation. Many signaling scaffolds are clustered at the PSD and presumably transduce the signal from the NMDA receptor to various effector targets responsible for maturation. Calcium-influx through activated NMDA receptors activates protein kinases that amplify the calcium signal, including CaMKII, protein kinase A (PKA), and phosphatidylinositide 3-kinase (PI3K) (Sheng and Kim, 2002). CaMKII primes a series of signaling cascades involved in the morphological maturation of the spine, and insertion of more glutamate receptors into the membrane via increased exocytosis of dendritic Golgi-derived vesicles (Rao and Craig, 2000). For example, the GIT1-βPIX-PAK1 signaling scaffold localizes to dendritic spines, and activation of CaMKII leads to the direct phosphorylation of the Rac-GEF, β-PIX, which enhances its GEF activity and consequently increases Rac GTPase activity (Zhang et al., 2003; Saneyoshi et al., 2008). A point mutation in β-PIX that disrupts its phosphorylation by CaMKII causes decreased spine density and reduction in the frequency of mEPSCs in cultured neurons, demonstrating the significance of this pathway and Rac activation in spine morphogenesis (Saneyoshi et al., 2008). Other examples of Rac GEFs directly phosphorylated by activated CaMKII include kalirin-7 and Tiam1 (Penzes and Jones, 2008). Many of these signaling cascades converge on the activation of the Rho-GTPases, which control the downstream activation or de-activation of different actin associated proteins. A fine-tuned balance between RhoA, Rac1, and Cdc42 signaling, mediated by upstream signaling pathways triggered by glutamate receptors, is thus important for modulating the actin cytoskeleton in response to synaptic neurotransmission.

Several diseases and neurological disorders are directly linked to genetic mutations in regulators and effectors of Rho-GTPases. Of the seven genes implicated in families with a high incidence of non-syndromic mental retardation {(Kutsche et al., 2000; Ramakers, 2000; Anon, 2002; Ramakers, 2002), three are direct regulators or effectors of Rho GTPases; including the Rho GAP, Oligophrenin1 (Billuart et al., 1998), the Rac1 and Cdc42 effector, PAK3 (Allen et al., 1998), and the Rac1/Cdc42 GEF, α-PIX (Kutsche et al., 2000). Moreover, the Cdc42 GEF, FGD1, is mutated in Aarskog-Scott syndrome, an X-linked developmental disorder associated with mental retardation and cognitive impairment (Pasteris et al., 1994). LIM domain kinase 1, LIMK1, is a downstream effector of Rho and Rac1 and stabilizes actin filaments by inhibiting the depolymerization factor, cofilin/ADF. Interestingly, a loss of function mutation in LIMK1 is implicated in the cognitive deficits associated with Williams syndrome (Frangiskakis et al., 1996). Thus, proper polymerization and organization of actin filaments is necessary for the morphological plasticity of the spine and dictates cognitive functioning.

F-actin organization underlies spine morphology

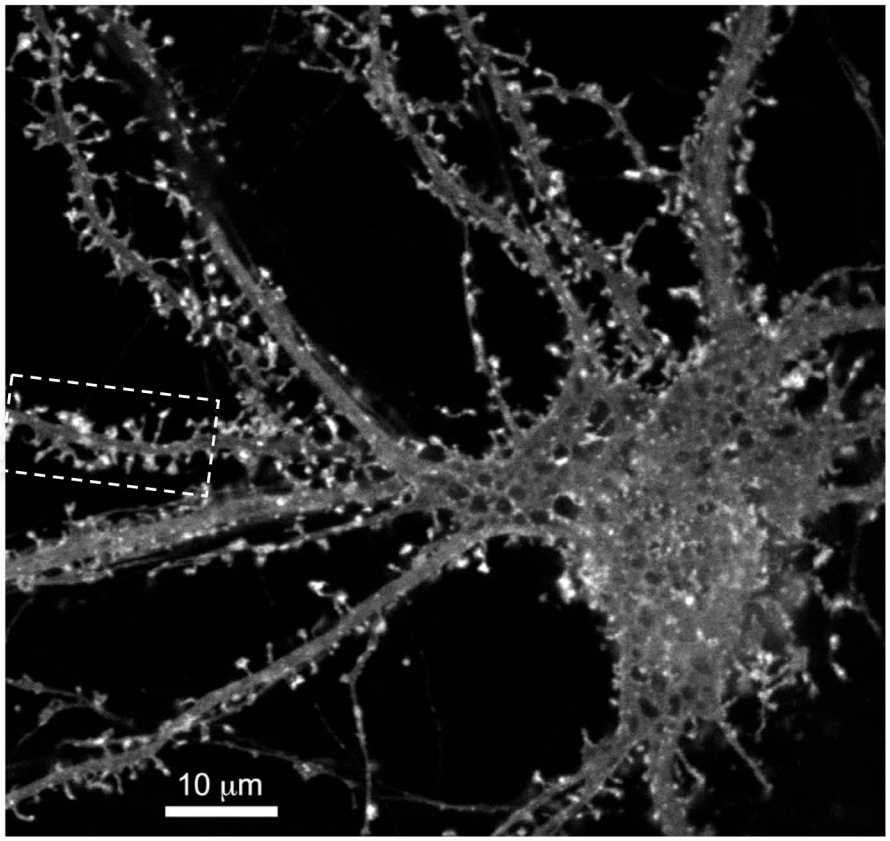
Actin is the primary structural molecule present in dendritic spines (Figure 3) (Matus, 2000). Although microtubules are observed to transiently invade the spine and modulate spine maturation by trafficking certain proteins to the spine that regulate actin (Jaworski et al., 2009; Hu et al., 2011; Merriam et al., 2011), they don't serve as the dominant structure underlying spine shape. While neurofilaments, a specialized type of intermediate filament, are also found in axons and dendrites, they do not localize

Figure 3: Actin is the primary structural determinant of dendritic spines.

A) DIV21 hippocampal neuron expressing RFP-actin. Neurons were transfected with RFP-actin on DIV6 and fixed on DIV21.

B) Outlined region of dendrite in figure 3A is magnified. After fixation on DIV21, neurons were immunostained for the endogenous microtubule marker, MAP2 (green). RFP-actin is colored magenta.

A



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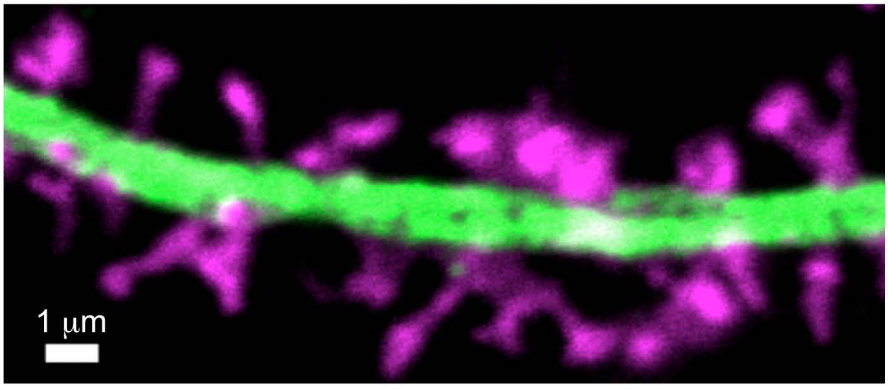


to dendritic spines (Fifková, 1985; Steward, 1989). The F-actin content of dendritic spines increases in response to LTP and actin polymerization is required for LTP maintenance (Fukazawa et al., 2003; Okamoto et al., 2004). This requirement is highlighted by the finding that treatment with the actin depolymerization agent, latrunculin B, causes a significant decrease in the number of AMPA receptors docked at the post-synaptic membrane, which is a measure of LTP (Allison et al., 1998; Kim and Lisman, 1999).

The post-synaptic density also undergoes rapid fluctuations in morphology driven by the actin cytoskeleton in response to synaptic activity (Marrs et al., 2001; Blanpied et al., 2008). Actin and actin-associated molecules such as cortactin, Arp2/3 complex, α-actinin, and others make up 12% of the PSD fraction, as estimated by mass spectrometry (Sheng and Hoogenraad, 2007). The post-synaptic reorganization underlying plasticity relies on dynamic remodeling of the actin cytoskeleton (Okamoto et al., 2004). For example, highly heterogeneous rates of filament polymerization occur within sub-domains of the spine (Frost et al., 2010b), such as the synapse region, peri-synaptic endocytic zone, and neck. This supports the proposed existence of a distinct set of actin filament networks within the micron-sized volume of the spine that serve multiple roles to support synaptic activity. Electron microscopy reveals a mixture of long actin filaments and short cross-linked filaments that exist in the spine (Korobova and Svitkina, 2010).

From studies in other systems, actin mediates a variety of activities and is under complex regulation, involving many actin-binding proteins that mediate its polymerization, localization, and organization. The Arp2/3 complex nucleates branches

off existing actin filaments and creates a branched, or dendritic actin filament network. The formins are a family of proteins that catalyze the elongation of actin filaments. In migrating cells, protrusion of the leading edge is driven by actin polymerization through formin proteins and the Arp2/3 complex: generation of a branched actin network, through the Arp2/3 complex, comprises the broad lamellipodium, and formation of parallel actin bundles, via formin proteins, constitutes a filopodium (Vicente-Manzanares et al., 2009a). Severing proteins, gelsolin and cofilin, depolymerize actin filaments and thereby control protrusion rates. Capping proteins also control protrusion rates by preventing filament elongation. α-Actinin cross-links actin filaments into various arrays and myosin II crosslinks actin filaments into antiparallel bundles, and its ATPase activity moves actin filaments past each other, which mediates contraction. Furthermore, the actin cross-linker fascin, which bundles F-actin into a tight parallel filament within filopodia of fibroblasts, is not expressed in dendritic protrusions (Sekino et al., 2007; Korobova and Svitkina, 2010). Several of these actin-associated molecules localize to spines and when mutated or impaired have deleterious effects on spine morphogenesis (Table 1). Many of these effects on spine morphology are discussed below.

The Arp2/3 complex-mediated branching of actin contributes to mature spine growth, since knock down of Arp3 or inhibition of its activator, N-WASP, dramatically decreased the number of mature spines and excitatory synapses formed, but did not perturb formation of filopodia-like spines (Wegner et al., 2008; Hotulainen et al., 2009). Similar spine pathologies were observed in the WAVE1 -/- knockout mouse (Kim et al., 2006; Soderling et al., 2007), and the consequence for knocking out an Arp2/3 activator was a decrease in hippocampal learning and memory (Soderling et al., 2003).

Table 1: Loss- and gain-of-function studies for different actin-binding proteins in spines.

Actin Regulator	Description	Approach	Spine Phenotype	Reference
Arp2/3	Nucleates branched actin filaments	-RNAi – Arp3 subunit -RNAi – p34 subunit -Scar1-WA fragment over-expression	↓ Spine density ↓ Excitatory synapses ↑ Filopodia-like protrusions ↓ Mushroom-shaped spines	(Wegner et al., 2008; Hotulainen et al., 2009)
N-WASP	Activates Arp2/3 complex	-RNAi -Wiskostatin treatment -Dominant-negative expression of N-WASP- ΔVCA	↓ Spine density ↓ Excitatory synapses	(Wegner et al., 2008)
WAVE1	Activates Arp2/3 complex	-RNAi -Knockout mouse -/-	↓ Spine density ↑ Filopodia-like protrusions ↓ Mushroom-shaped spines ↓ Learning and Memory	(Soderling et al., 2003; Kim et al., 2006; Soderling et al., 2007)
Abp1	-Actin filament side-binding molecule -Links N-WASP with actin	-RNAi -Over-expression of Abp1 SH3-domain -Over-expression of Abp1 actin-binding domain	↓ Mushroom-shaped spines ↓ Excitatory Synapses ↓ Spine density	(Haeckel et al., 2008)
		-Abp1 over-expression	↑ Length of mushroom – shaped spines ↑ Mushroom-shaped spines ↑ Synapses	(Haeckel et al., 2008)
PICK1	Inhibits Arp2/3 complex	-RNAi -DN expression of PICK1	↑ Spine area - Prevents LTD-dependent spine shrinkage	(Nakamura et al., 2011)

		PICK1 over-expression	↓ Spine area ↓ Spine length	(Lee et al., 2010; Nakamura et al., 2011)
mDia2	Formin-nucleates and elongates actin	-RNAi -DN-mDia2 expression	↓ Spine density ↓ Filopodia-like protrusions ↓ Spine length ↑ Spine head width	(Hotulainen et al., 2009)
		CA-mDia2 over-expression	↑ Filopodia-like protrusions ↓ Mushrooms	(Hotulainen et al., 2009)
Daam1	Formin-nucleates and elongates actin	Daam1 over-expression	↓ Spine density ↑ Spine length ↑ Thin protrusions	(Salomon et al., 2008)
LIMK1	Inactivates cofilin	Knockout mouse -/-	↓ Spine area ↓ PSD size ↑ Filopodia-like protrusions -abnormal LTP ↓ Spatial learning	(Meng et al., 2002)
Cofilin-1	Depolymerizes actin filaments	RNAi	↓ Mushroom-shaped spines ↑ Spine length ↑ Spine branching	(Hotulainen et al., 2009)
Gelsolin	Severs actin filaments and caps barbed ends in a calcium-dependent manner	Knockout mouse -/-	Impaired the decrease in F-actin turnover in response to NMDA receptor activation	(Star et al., 2002)
Cortactin	Facilitates Arp2/3-nucleation of actin branches	RNAi	↓ Spine density	(Hering and Sheng, 2003)
		Cortactin over-expression	↑ Spine neck length	(Hering and Sheng, 2003)

		Cortactin-ΔSH3 over-expresion	↓ Spine head width	(Hering and Sheng, 2003)
Drebrin A	Actin-binding protein	RNAi	↓ Excitatory Synapses ↓ Mature actin-enrichment spines	(Takahashi et al., 2003)
		Drebrin over-expression	↑ Spine density ↑ Spine length	(Hayashi and Shirao, 1999)
Spinophilin /Neurabin II	Bundles actin filaments	Knockout mouse -/-	↑ Spine density ↑ Filopodia-like protrusions	(Feng et al., 2000)
VASP	Bundles F-actin and protects barbed-ends from capping	RNAi	↓ Spine density ↓ Excitatory synapses	(Lin et al., 2010)
		VASP over-expression	↑ Spine density ↑ Excitatory synapses ↑ Spine head area ↑ Actin polymerization	(Lin et al., 2010)
β-adducin	Caps F-actin barbed ends and recruits spectrin to actin	β-adducin knockout -/-	↓ Synapse assembly following learning ↓ LTP	(Rabenstein et al., 2005; Bednarek and Caroni, 2011)
Eps8	Caps F-actin barbed ends	-RNAi -DN expression of Eps8TM - (abolishes actin-capping activity)	↓ Spine density ↑ Filopodia-like protrusions ↑ Spine head irregularity ↑ F-actin turnover	(Stamatakou et al., 2013)

		Eps8 over-expression	↑ Spine density ↑ Excitatory synapses ↓ Filopodia-like protrusions ↑ Stubby spines	(Stamatakou et al., 2013)
CP	Caps F-actin barbed ends -Facilitates Arp2/3-mediated nucleation	RNAi	↓ Spine density ↑ Filopodia-like protrusions ↑ Spine head branching ↓ Excitatory synapses	(Fan et al., 2011)
Spar	Actin-binding protein -Recruits PSD-95 to F-actin in heterologous cells	Spar over-expression	↑ Spine head width ↑ Spine head irregularity – less circular in shape ↓ Spine density	(Pak et al., 2001)
α-actinin-2	Cross-links actin filaments	ACTN2 over-expression	↑ Spine length	(Nakagawa et al., 2004)
		RNAi	↑ Filopodia-like protrusions ↑ Spine length ↓ Spine head width ↓ Excitatory synapses	Unpublished observations – Chapter 4
Myosin IIB	Cross-links and contracts actin filaments	-RNAi -Blebbistatin	↑ Filopodia-like protrusions ↑ Spine length ↓ Spine head width ↑ Spine head branching ↓ LTP	(Ryu et al., 2006; Rex et al., 2010; Hodges et al., 2011)
		-MIIB over-expression -RLC-DD over-expression	↑ Excitatory synapses ↑ Mushroom-shaped spines	(Hodges et al., 2011)

Key: CA = constitutive active, DN = dominant negative

Additional evidence for actin polymerization being necessary for spine development and maturation comes from the observation that profilin, the actin monomer binding partner that promotes F-actin polymerization, is redistributed to spine heads in response to synaptic activity (Ackermann and Matus, 2003). In contrast, excess F-actin elongation induced by the loss of actin-capping proteins (Mejillano et al., 2004), epidermal growth factor receptor pathway substrate 8 (Eps8) (Stamatakou et al., 2013) and capping protein (CP) (Fan et al., 2011), results in excessive filopodia-like protrusions. Therefore, actin-capping proteins in the spine provide a balance for actin filament elongation to control spine morphogenesis, and direct evidence for their importance in spine plasticity comes from the fact that decreased levels of CP are found in fetal brains of Down syndrome (Gulesserian et al., 2002).

The Diaphanous-related formin proteins, Dia1 and Dia2, which are regulated by RhoA and Cdc42, also localize to dendritic spines and overexpression of either protein led to spines with an increased protrusion length, exhibiting filopodia-like features (Salomon et al., 2008; Hotulainen et al., 2009). In spines, Dia2 promotes spine elongation, while Arp2/3 complex mediates spine head expansion (Hotulainen et al., 2009). This same study further showed that the F-actin severing molecule, cofilin, is expressed in dendritic spines and controls the proper length of actin filaments to prevent abnormal protrusions from the spine head (Hotulainen et al., 2009). Phosphorylation of cofilin by Lim kinase-1 (LIMK1), inactivates cofilin's actin depolymerization activity (Bamburg et al., 1999), and elevated levels of phosphorylated cofilin is thought to be a readout of LTP (Chen et al., 2007). LIMK1 -/- mice show diminished phosphorylated cofilin and consequently display immature spine morphologies with smaller PSD sizes

(Meng et al., 2002); therefore the temporal activity of these molecules to regulate actin filament arrangement, such as cofilin inactivation, is critical to spine morphogenesis and synaptic plasticity.

Cortactin and drebin A are two examples of actin-associated molecules that are temporally regulated. Cortactin facilitates Arp2/3-mediated nucleation of actin branches and stabilizes these newly formed branches (Weaver et al., 2001), and drebrin A is an actin side-binding protein that promotes formation of thick, curving bundles of actin (Shirao et al., 1994). Cortactin and drebin A are redistributed from spines to the dendritic shaft in response to NMDA-type glutamate receptor activation(Hering and Sheng, 2003; Sekino et al., 2007). In contrast, activation of the MAP kinase pathway via stimulation of the trkB receptor tyrosine kinase with brain-derived neurotrophic factor (BDNF), causes a dramatic redistribution of cortactin to dendritic spines, particularly during a time period when synapses are developing (Iki et al., 2005). Therefore, cortactin and drebrin A activity appear to be temporally regulated such that they promote formation of dendritic protrusions that mature into spines (Hayashi and Shirao, 1999; Hering and Sheng, 2003; Takahashi et al., 2003), but are down-regulated after the synapse engages in neuronal activity, thereby coupling activity-dependent spine morphogenesis to actin remodeling. Spatial regulation of these actin-binding proteins is also critical for spine plasticity. For example, Abp1 promotes the expansion of the spine head by spatially confining Arp2/3 nucleation at the PSD, via simultaneously binding to F-actin, ProSAP/Shank, and N-WASP (Haeckel et al., 2008).

Lastly, actin cross-linking proteins that localize to dendritic spines include spinophilin/neurabin II (Allen et al., 1997), VASP (Lin et al., 2010), α-actinin, and

myosin IIB. Loss-of-function for any of these actin cross-linkers results in the elongation of dendritic protrusions, resembling filopodia-like spine precursors (Feng et al., 2000; Ryu et al., 2006; Lin et al., 2010; Hodges et al., 2011), therefore organization of actin filaments through actin cross-linking is critical to dictating spine morphology. The roles of α-actinin and myosin IIB in spine morphogenesis are discussed in more detail below.

Rationale for the study

Regulation of spine morphology and organization of the PSD points to a key role for actin polymerization, depolymerization, and organization. The broad goal of this dissertation was to investigate some of the activities that mediate actin organization in the spine. My focus is on myosin II and α-actinin. While little is known about their functions in spine development, both of these molecules have been well studied in fibroblasts providing a starting foundation to study these proteins in cultured hippocampal neurons and interpret their neuronal functions in spine development from our findings.

Myosin II

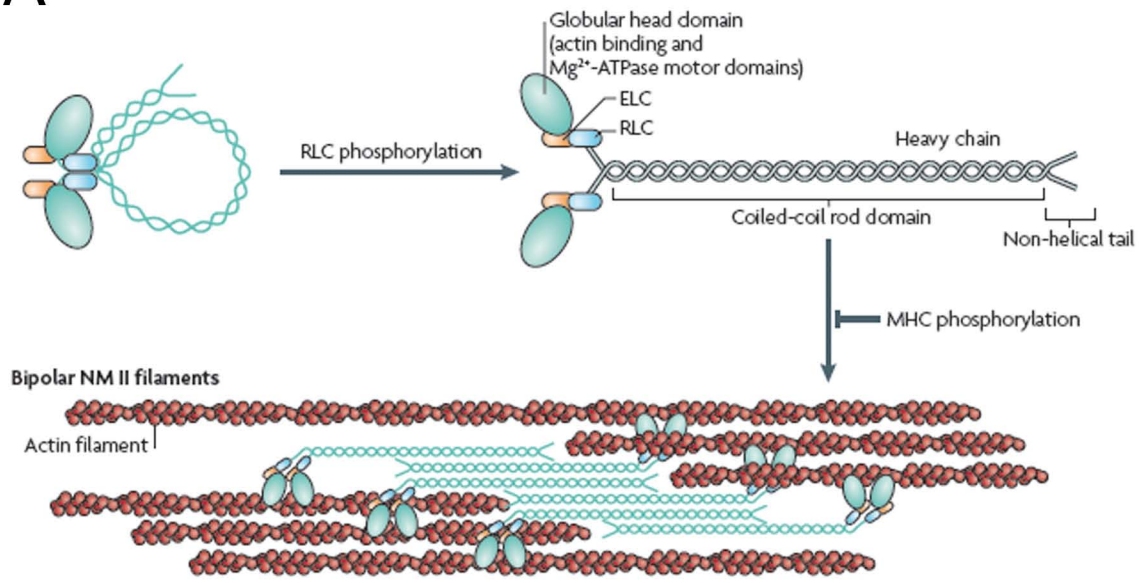
Non-muscle myosin II is a hexameric protein complex composed of two heavy chains that dimerize, two essential light chains, and two regulatory lights chains; each essential and regulatory light chain binds to a heavy chain. The long C-terminal helical coiled-coil domain of the dimerized heavy chains mediates bipolar filament bundling in an antiparallel fashion through self-association with other myosin II molecules

Figure 4: Domain structure of myosin II and myosin IIB is the predominant isoform expressed in hippocampal neurons.

A) Domain structure of non-muscle myosin II adapted from (Vicente-Manzanares et al., 2009c).

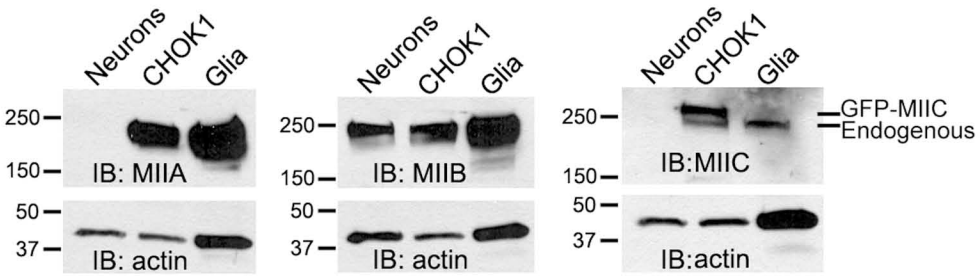
B) Myosin IIB is the only myosin II isoform expressed in DIV 13 hippocampal neurons derived from E19 rats. All three isoforms are present in glia cells and CHO-K1 cells. CHO-K1 cells were also transfected with GFP-MIIC as a positive control for the MIIC antibody. Actin immunoblot serves as a loading control.

A



Vicente-Manzanares et al., 2009

B



(Hostetter et al., 2004). The N-terminal globular head domain of the heavy chain binds to actin filaments in a specific orientation, such that the bipolar association of myosin filaments cross-links and organizes actin into an antiparallel bundle (Figure 4A). ATPase activity in the head domain induces head movement, which drives movement of actin filaments and corresponds to contraction as oppositely oriented actin filaments slide past one another (Clark et al., 2007). Therefore, bundling and contracting actin filaments constitute myosin's direct mechanical actions on the cytoskeleton. Evidence for the organization of antiparallel filaments along with the presence of branched actin networks in spines comes from EM analysis and barbed-end staining of actin, showing that actin polymerization and elongation occurs not only at the tip of dendritic protrusions, but also at the root (Hotulainen et al., 2009; Korobova and Svitkina, 2010).

Myosin II activity is regulated by phosphorylation of the regulatory light chain (RLC) and its heavy chain tail domain. Phosphorylation of RLC on Ser19, or on Thr18 and Ser19, is required for myosin's ability to assemble into bipolar filaments and activate its motor activity cycle, which binds too and contracts actin filaments (Figure 4A) (Vicente-Manzanares et al., 2009c). Rho GTPases control myosin II activity by turning on and off the kinases that directly regulate it, such as Rho-associated, coiled-coil containing protein kinase (ROCK) and myosin light chain kinase (MLCK). In contrast to RLC, phosphorylation of myosin heavy chain promotes myosin filament disassembly (Vicente-Manzanares et al., 2009c), and therefore provides another mechanism for dictating myosin II activity. It is clear that MIIB activity is present within dendritic protrusions, since the active, phosphorylated form of RLC, pRLC, localizes to spines. Intriguingly, quantification of the dendritic spine to shaft ratio shows that active pRLC is

~2-fold more concentrated in spines than total RLC, suggesting that myosin-dependent contractile events localize to dendritic spines (Zhang et al., 2005). Furthermore, ectopic expression of the constitutively active mutant, RLC-18, 19 DD, promotes spine and synapse formation by inducing an increase in dendritic spine density (Zhang et al., 2005).

There are three isoforms of non-muscle myosin II, including myosin IIA, -B, and -C. Myosin IIB (MIIB) is most highly expressed in brain and heart tissues, and its ablation results in severe cardiovascular and neuronal defects (Uren et al., 2000; Ma et al., 2007). MIIB is the only isoform expressed in hippocampal neurons derived from embryonic day 19 (E19) rats (Figure 4B). Inhibition of ATPase activity by blebbistatin inhibits contractility and induces the formation of long thin processes extending from the spine head with concomitant collapse of spine-head width (Ryu et al., 2006). Inhibition of myosin II activity also reduced the number of excitatory synapses in cultured hippocampal neurons (Zhang et al., 2005). Furthermore, *in vivo* inhibition of myosin II abrogated LTP maintenance and memory consolidation in mice (Rex et al., 2010). Taken together, these studies implicate myosin II in the maintenance of spine morphology and long-term memory.

Despite the importance of MIIB in spine morphology and long-term memory, many outstanding questions remain regarding the mechanism(s) by which MIIB dictates spine morphology and excitatory synapse formation. How do the individual functions of MIIB on organizing actin filaments, specifically cross-linking and contracting actin, determine spine morphology? How is MIIB regulated to dictate spine morphology changes? Does synaptic activity regulate MIIB activity? In addition to regulating spine

morphology, does MIIB activity dictate the organization of the post-synaptic density? Rationales for these questions are discussed below.

The organization of the post-synaptic density in response to myosin II regulation has not been studied to date. When myosin is knocked down in fibroblasts the adhesions disperse and the cells become highly protrusive (Vicente-Manzanares et al., 2009a). Just as the forces that hold together an adhesion are lost when myosin is knocked down in fibroblasts, we hypothesized that myosin activity may pull on actin filaments tethered to PSD molecules, to stabilize adhesions at the post-synaptic membrane and promote clustering of PSD molecules, thereby organizing the post-synaptic density. We further postulate that MIIB-mediated contractility enhances spine maturation through enlargement of the post-synaptic density. By pulling on actin filaments tethered to proteins within the PSD, MIIB-mediated contractility can induce conformation changes in PSD proteins thereby revealing new binding sites for the recruitment of additional proteins to the PSD. Support for this comes from the observation that exogenous expression of myosin II promotes the growth of adhesions in fibroblasts (Vicente-Manzanares et al., 2007).

Bundling and contracting actin filaments can serve different effects on cell migration; bundling is responsible for the initial events controlling adhesion maturation, whereas contractility controls protrusion rates (Vicente-Manzanares et al., 2009a). We therefore proposed that bundling and contractility via MIIB could mediate different effects on spine and PSD morphology. To separate myosin's bundling activity from contractility, we utilized a myosin mutant, MIIB-R709C, which has a mutation in its ATPase motor domain that abolishes contractility, but still allows the molecule to bind

tight too and cross-link actin (Ma et al., 2004). MIIB-R709C homozygous mice display significantly retarded migration of distinct groups of neurons in the developing mouse brain (Ma et al., 2004), demonstrating an importance for MIIB-directed contractility in the nervous system. We therefore test whether myosin's actin cross-linking activity versus contraction impose different behaviors on spine morphology and post-synaptic organization.

Phosphorylation of myosin regulatory light chain is a read-out for myosin activation and can be used as a diagnostic tool to study whether the NMDA receptor signals to MIIB. Support for NMDA receptor signaling to myosin comes from studies showing that GIT1 localizes the GIT1-βPIX-PAK signaling module to dendritic spines and functions upstream of myosin activity (Zhang et al., 2003; 2005). Where and when myosin induces contractility throughout spine development, and whether it mediates a response to synaptic stimulation, was unknown prior to this study. Furthermore, monophosphorylation versus diphosphorylation of RLC on MIIB induces different phenotypes in fibroblasts (Vicente-Manzanares and Horwitz, 2010). Specifically, a RLC-di-phosphomimetic mutant, RLC-T18D/S19D (RLC-DD) generates very large adhesions, whereas a RLC-mono-phosphomimetic mutant, RLC-T18D/S19A (RLC-DA) does not produce large adhesions (Vicente-Manzanares and Horwitz, 2010). We therefore proposed that phosphorylation of these two residues in RLC may serve different functions in spine development and growth of the PSD. Regulation of myosin activity through phosphorylation of its heavy chain has not been studied in neurons. Thus, regulation of myosin filament assembly through phosphorylation of the RLC and its

heavy chain provides an attractive mechanism to dictate myosin activity in driving spine morphogenesis.

The goal of this project was to elucidate the mechanisms by which myosin IIB organizes and regulates actin filaments within dendritic protrusions through its cross-linking function, and how contractility dictates post-synaptic organization and spine maturation in response to synaptic stimulation.

α-Actinin

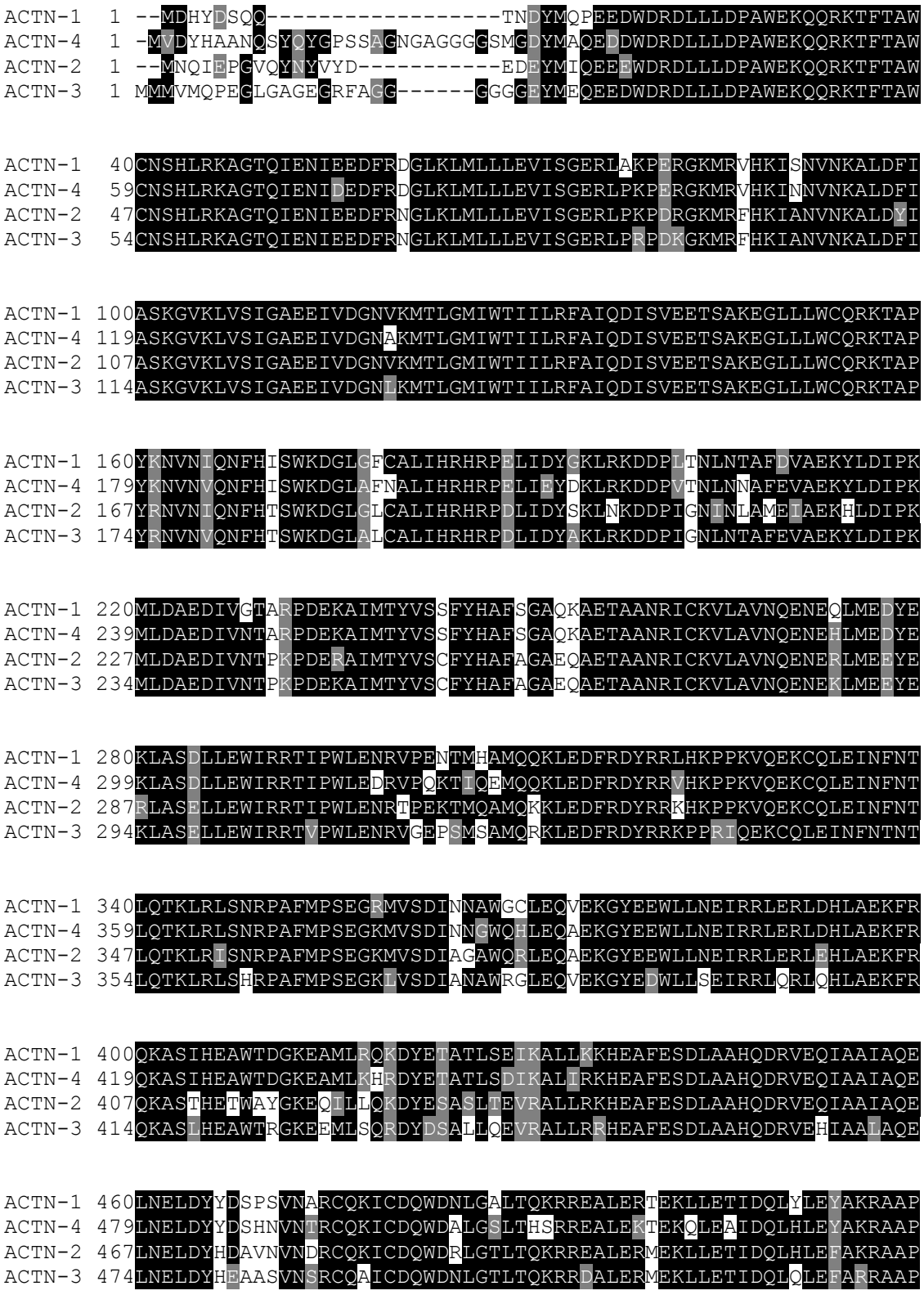
α-Actinin is another molecule that bundles anti-parallel actin filaments and localizes to many sub-cellular structures, including sarcomeres of muscle and heart cells, cell-cell and cell-matrix contact sites, lamellipodia of cellular protrusions, and stress fibers (Otey and Carpen, 2004). α-Actinin is has an actin-binding domain at its N-terminus, followed by four spectrin repeats, and calmodulin-like domain at its C-terminus. The spectrin repeats mediate bipolar dimerization such that there is an actin-binding domain on either end of the molecule, allowing α-actinin to cross-link actin filaments. In contrast to myosin II, α-actinin does not bind to F-actin in specific orientations, and can therefore adopt various cross-linking patterns. α-Actinin not only cross-links anti-parallel actin filaments like myosin II, but promiscuously cross-links actin filaments over all angles (Courson and Rock, 2010). This reveals how α-actinin is observed in both orientations of actin-filament cross-linking, including anti-parallel arrays with myosin II and parallel bundles with fascin. In this regard, ACTN2 could localize to multiple actin networks in the spine, involved in both the organization of anti- parallel actin filaments with MIIB, and the branched actin networks observed in the spine head.

There are four highly conserved α-actinin isoforms (Figure 5), two Ca^{2+}-sensitive isoforms (α-actinin-1 and -4) and two Ca^{2+}-insensitive isoforms (α-actinin-2 and -3) (Otey and Carpen, 2004). The Ca^{2+}-insensitive isoforms are highly enriched within muscle and heart tissues, while the Ca^{2+}-sensitive isoforms, whose binding to actin is regulated by calcium, are more ubiquitously expressed in other tissues (Djinović-Carugo et al., 1999). The difference in calcium sensitivity between the α-actinin isoforms resides in the EF1 hand repeat of the calmodulin-like domain; α-actinin-2 and -3 are missing several amino acids within the EF1 hand repeat, which confers Ca^{2+}-insensitivity in these isoforms (Figure 5). Of the three α-actinin isoforms (α-actinin-1, -2, and -4) found via mass spectrometry of rat brain (Walikonis et al., 2000; Peng et al., 2004), interestingly, most data points to the localization of Ca^{2+}-insensitive, α-actinin-2 (ACTN2) at post-synaptic sites of excitatory neurons of the hippocampus and cortex (Wyszynski et al., 1997; 1998; Dunah et al., 2000). ACTN2 may play a pivotal role in regulating spine morphogenesis through its cross-linking function, since over expression of ACTN2 in cultured hippocampal neurons increases the length and density of dendritic protrusions, and this phenotype was dependent on its actin-binding domain and spectrin repeats (Nakagawa et al., 2004). However, over expression studies can induce artificial phenotypes, and a more thorough study of ACTN2, via RNAi-mediated knock down, is needed to ascertain its function in dendritic spines.

In addition to cross-linking actin, α-actinin also binds to a number of other proteins, including membrane receptors, adhesion molecules, and signaling proteins (Sjöblom et al., 2008). *In vitro* binding assays reveal a direct interaction between ACTN2 and the cytoplasmic domains of the NMDA receptor subunits, NR1 and NR2B

Figure 5: α-Actinin isoforms are highly conserved.

Clustal W multi-alignment of human α-actinin isoforms. Similarities at each aligned amino acid position were scored and grouped by shade using the Clustal W2 matrix. White indicates little similarity; gray indicates high similarity; and black indicates highest similarity.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ACTN-4 1 -MVDYHAANQSYQYGPSSAGNGAGGGGSMGDYMAQEDDWDRDLLLDPAWEKQQRKTFTAW
ACTN-2 1 --MNQIEPGVQYNYVYD-----------EDEYMIQEEEWDRDLLLDPAWEKQQRKTFTAW
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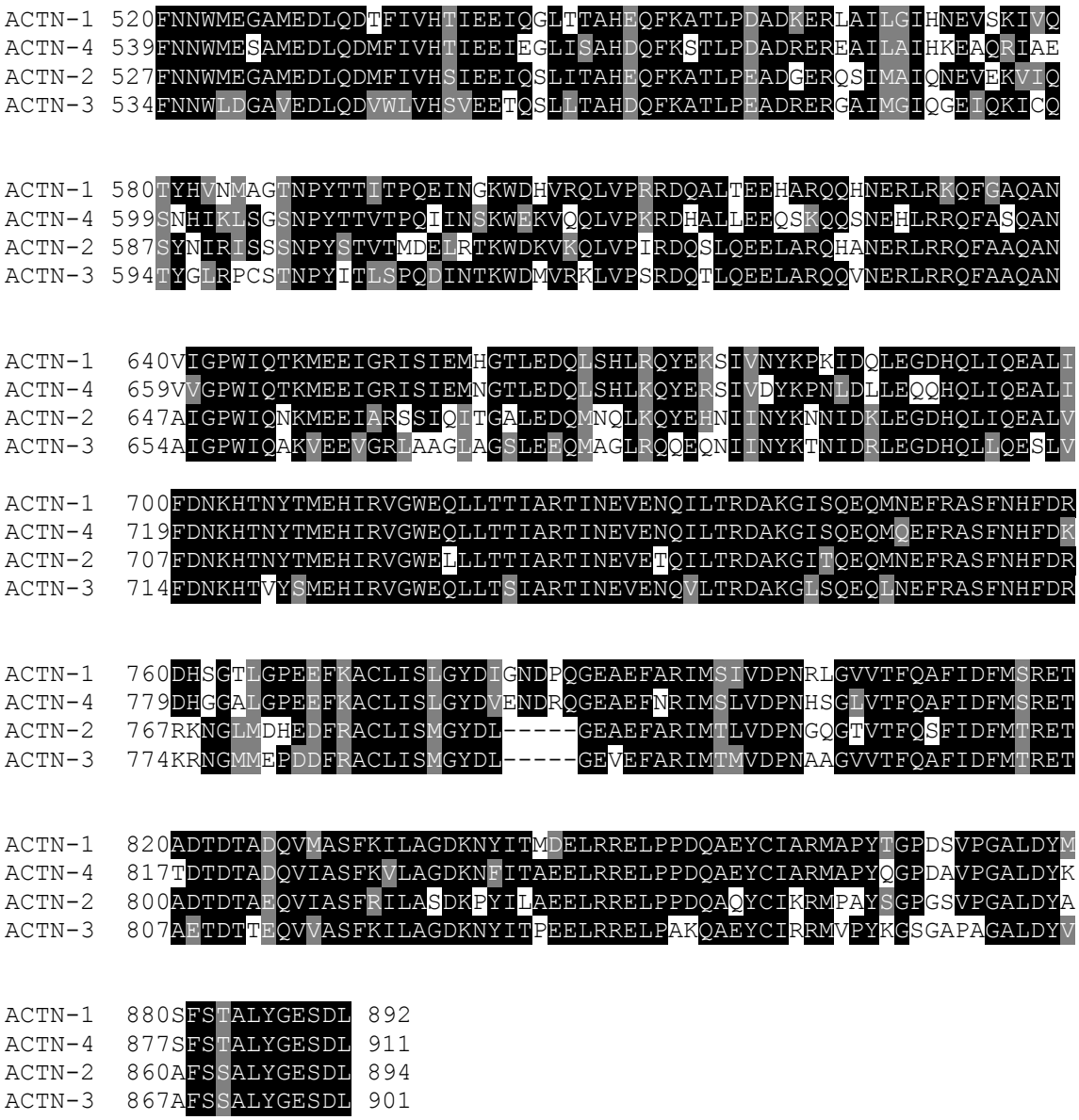
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ACTN-4 877SFSTALYGESDL 911
ACTN-2 860AFSSALYGESDL 894
ACTN-3 867AFSSALYGESDL 901

(Wyszynski et al., 1997). Although this interaction has not been shown in neurons, ACTN2 is proposed to link the NMDA receptor to the actin cytoskeleton and hold the receptor in an open state, which mediates calcium influx. The interaction between ACTN2 and the NMDA receptor is directly antagonized by Ca^{2+}/calmodulin *in vitro*, and therefore calcium influx through activated NMDA receptors triggers displacement of ACTN2 from the receptor, detaching the NDMA receptor from the actin cytoskeleton (Wyszynski et al., 1997). It is hypothesized, but not proven, that NMDA receptor release from the actin cytoskeleton destabilizes channel opening and causes its closure, thus terminating calcium influx through the channel. The significance of the Ca^{2+}-insensitivity displayed by ACTN2 comes from findings that Ca^{2+}-sensitive α-actinin isoforms could not mediate NMDA receptor inactivation in non-neuronal cells, since calcium weakens the affinity of these α-actinin isoforms for actin (Krupp et al., 1999).

The best evidence for ACTN2 regulating calcium influx through the NMDA receptor comes from electrophysiological studies of cultured hippocampal neurons expressing exogenous wild type ACTN2 or a mutant ACTN2 that is unable to bind to PtdIns(4,5)P_2**,** PIP2 (Michailidis et al., 2007). Binding to PIP2 tethers the molecule to the membrane, which is crucial for maintaining the open state of the NMDA receptor in *Xenopus* oocytes (Michailidis et al., 2007). Neurons expressing the ACTN2 mutant unable to interact with PIP2 significantly reduced peak and steady-state NMDA current compared to neurons expressing *wild type* ACTN2, suggesting that ACTN2 can't link NMDA receptors to the actin cytoskeleton and promote calcium influx without binding to PIP2 at the membrane (Michailidis et al., 2007).

Other *in vitro* binding assays indicate a direct interaction between ACTN2, densin-180, a major constituent of the PSD, and CaMKIIα (Robison et al., 2005a). Therefore, ACTN2 may serve to connect PSD molecules to the actin cytoskeleton, and thereby play a significant role in organizing the PSD. In fibroblasts, α-actinin is implicated to regulate the strength of adhesions by clustering β-integrins at focal adhesions via direct binding, as well as indirectly through interactions with vinculin (Wichert et al., 2003). Similarly, though not directly shown, α-actinin may strengthen synaptic adhesions through its reportedly direct interactions with the NMDA receptor, actin, and other components of the PSD.

It is not known how ACTN2 may be regulated in dendritic spines, and we can speculate based on regulation of α-actinin in other cells. In fibroblasts, α-actinin-1 is phosphorylated on its actin-binding domain at Tyr12 by focal adhesion kinase (FAK), which mediates its dissociation from actin and weakens the strength of the connection formed between integrins and the actin cytoskeleton (Izaguirre et al., 1999; 2001; Wichert et al., 2003). Phosphorylation of α-actinin-1 by FAK and its subsequent dissociation from actin is thought to enhance adhesion turnover, since α-actinin-1-driven crosslinking of actin nucleates adhesion formation. The amino acid sequence between all α-actinin isoforms is highly conserved, including the tyrosine residue phosphorylated by FAK, which corresponds to Tyr19 on ACTN2 (Figure 5). FAK is an important signaling molecule in dendritic spines as its conditional ablation is shown to block EphB-mediated dendritic filopodia morphogenesis into mature spines, due to failure of FAK-mediated RhoA activation (Moeller et al., 2006). Therefore, FAK may regulate ACTN2 in dendritic spines through a similar manner to weaken its association with actin. This

could have implications on the ability of ACTN2 to regulate spine morphology and possibly PSD organization. Furthermore, if the hypothesis that ACTN2 couples the NMDA receptor to actin is true, then regulating the affinity of ACTN2 for actin could have profound effects on synaptic activity.

Cross-linking is a defining factor in organizing actin filaments within focal adhesions. Knock-down of α-actinin leads to a loss of large, centripetally polarized adhesions and displays short, randomly oriented adhesions and actin filaments in fibroblasts (Choi et al., 2008). Just as actin cross-linking is important for organizing F-actin in adhesions, it may be critical to organizing adhesive molecules of the PSD within the spine. Futhermore, the significance of ACTN's function to cross-link actin in the spine and interact with the NMDA receptor, adhesion molecules, and members of the PSD is largely unknown, since most of these interaction studies were performed in non-neuronal cells or immunoprecipitations from neuronal lysates. The goals of this study, discussed in Chapter 4, were to determine a post-synaptic phenotype for ACTN2, induced by its loss of function, and deduce a functional role for ACTN2 in dendritic spine development and organization of the PSD.

From the above it should be apparent that there are many parallels between spine morphogenesis and adhesion maturation of fibroblasts (Vicente-Manzanares et al., 2009b). This dissertation applied the knowledge learned about myosin II and α-actinin in the cell migration field to study their roles in post-synaptic organization and spine morphogenesis during development and in response to chemical stimulation of the NMDA receptor.

Chapter 2 -- Dendritic Spines: Similarities with Protrusions and Adhesions in migrating cells

This chapter is based on previously published work.

Vicente-Manzanares M, Hodges J, Horwitz AR (2009) Dendritic spines: similarities with protrusions and adhesions in migrating cells. Open Neurosci J. 3: 87-96. doi:10.2174/1874082000903020087.

Abstract

Dendritic spines are specialized, micron-sized post-synaptic compartments that support synaptic function. These actin-based protrusions push the post-synaptic membrane, establish contact with the presynaptic membrane and undergo dynamic changes in morphology during development, as well as in response to synaptic neurotransmission. These processes are propelled by active remodeling of the actin cytoskeleton, which includes polymerization, filament disassembly, and organization of the actin in supramolecular arrays, such as branched networks or bundles. Dendritic spines contain a plethora of adhesion and synaptic receptors, signaling, and cytoskeletal proteins that regulate their formation, maturation and removal. Whereas many of the molecules involved in dendritic spine formation have been identified, their actual roles in spine formation, removal and maturation are not well understood. Using parallels between migrating fibroblasts and dendritic spines, we point to potential mechanisms and approaches for understanding spine development and dynamics.

Introduction

Dendritic spines are small protrusions that decorate the dendrites of Purkinje neurons in the cerebellum and pyramidal neurons in the cortex and hippocampus (Yuste and Bonhoeffer, 2004). Dendritic spines function as specialized post-synaptic structures that support excitatory neurotransmission (Zhang and Benson, 2000; Bonhoeffer and Yuste, 2002; Ethell and Pasquale, 2005). They contain ion channels and adhesive receptors, as well as a multitude of signaling intermediates and cytoskeletal components (Ethell and Pasquale, 2005; Sheng and Hoogenraad, 2007). These molecules are

essential for transmission of synaptic input and also support long-term responses to stimulation, which are central for learning and memory.

Dendritic spines adopt varied morphologies, from long, filopodia-like to short and stubby, and have a well-defined life cycle (Figure 6A). During spinogenesis, dendritic spines appear as immature precursors, which are usually long and thin (Figure 6A, *left*). A fraction of these undergo maturation, becoming shorter, thicker and wider, i.e. mushroom-shaped or stubby; and those spine precursors that are not innervated tend to turn over, undergoing cycles of growth and shrinkage (Figure 6A, *middle and right*) (Ziv and Smith, 1996; Yuste and Bonhoeffer, 2001; 2004; Schubert et al., 2006; Knott and Holtmaat, 2008).

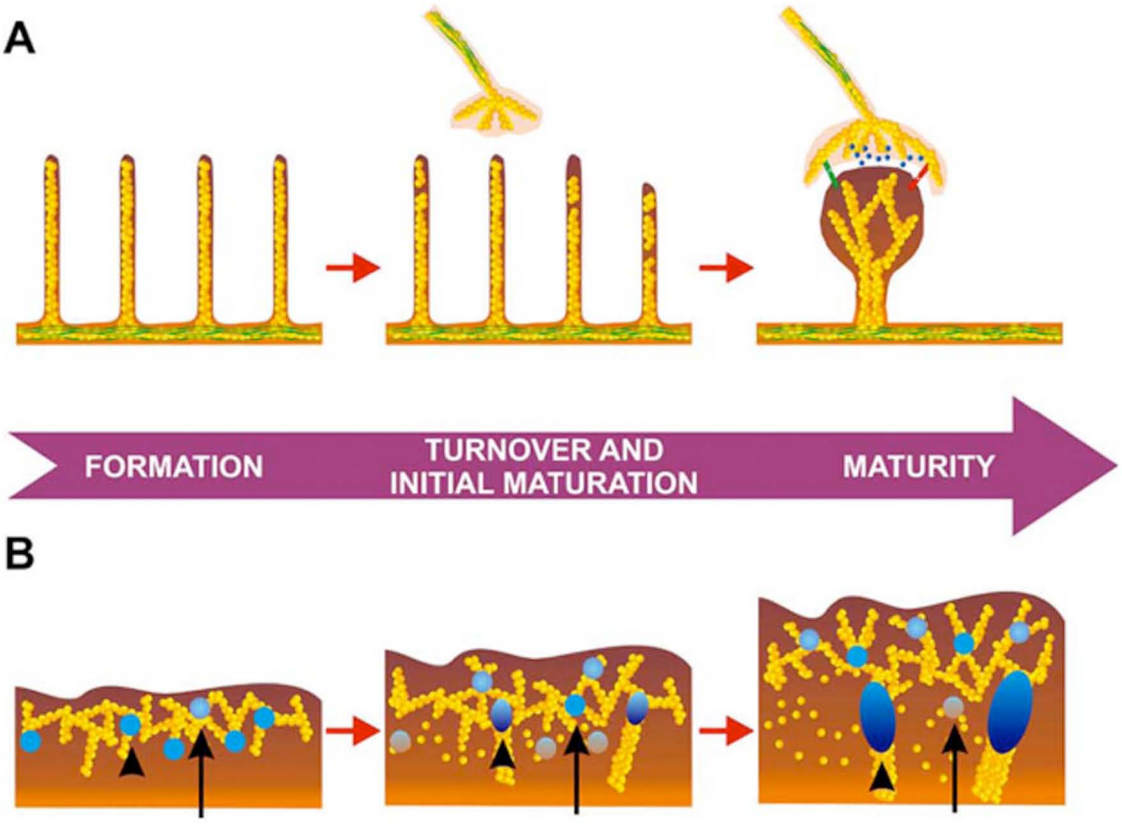
Morphological maturation of spines can be induced by physical contact with an axon and associated with synaptic stimuli. For example, mature spines of pyramidal cells are stabilized by synaptic input; but removal of afferent input, such as whisker trimming, results in the selective spines loss (Lendvai et al., 2000; Nimchinsky et al., 2002). On the other hand, dendritic spines on Purkinje cells of the cerebellum form and stabilize in the absence of afferent input (Sotelo et al., 1975).

The increase in contact area with the presynaptic terminal correlates with synaptic strength, which contributes to long-term potentiation (LTP) by increasing synaptic receptor density at the synaptic cleft. Electrophysiologic studies show that the bulbous head morphology of the mature spine is better suited to receive and propagate neuronal signals than the thin structure of the immature spine (Kasai et al., 2003). Synaptic input itself may also induce such an increase in surface contact area (Harris et al., 2003; Park et al., 2006).

Figure 6: Formation and evolution over time of dendritic spines and adhesions in migrating cells

A) Dendritic spine formation. *Left*, immature spine precursors form along the dendritic shaft, driven by actin polymerization. *Middle*, pre-synaptic contact and/or neurotransmitter secretion stabilizes an immature dendritic spine, whereas immature precursors that are not contacted by pre-synaptic portals disassemble (represented by breaking actin filaments in protrusions). *Right*, stable contact with a pre-synaptic terminal induces active remodeling of the postsynaptic terminal, which becomes shorter and wider. This process is driven by the combination of synaptic input (dark blue spheres) and adhesive signaling (green-red receptor pairs). The unselected precursors are reabsorbed in the dendritic shaft. A single actin filament in each protrusion is shown for simplicity.

B) Adhesion assembly, maturation and turnover in migrating cells. *Left*, nascent adhesions form inside the branched actin network at the leading edge (indicated by arrowhead and arrow). *Middle*, as the protrusion advances, some adhesions elongate centripetally as the actin filaments with which they associate become larger and thicker (arrowhead); newly formed adhesions are stable as long as they are associated to the branched actin network (arrow). *Right*, maturing adhesions (arrowhead) continue growing as the actin bundles become thicker and more stable. Adhesions not associated with growing actin bundles turn over and disappear as the branched actin network moves past them (arrow).


A
FORMATION
TURNOVER AND
INITIAL MATURATION
MATURITY
B

Actin is a major component of dendritic spines. Its polymerization and organization dictate the size, motility, and morphology of the spines and has a profound impact on synaptic transmission (Sekino et al., 2007). For example, inhibition of actin polymerization or depolymerization using chemical inhibitors disrupts LTP (Fukazawa et al., 2003). Furthermore, LTP induction causes an increase in F-actin, which may underlie the structural enlargement of spine heads (Okamoto et al., 2004). One mechanism is the recruitment or activation of actin regulators. For example, the actin-binding protein profilin, is targeted to spine heads in response to postsynaptic glutamate receptor activation; this increases the pool of actin monomers available for filament assembly. Profilin enrichment in spine heads also inhibits spine motility and promotes maturation (Ackermann and Matus, 2003).

The organization of actin in spines is tightly controlled by a multitude of signaling proteins. Interestingly, some diseases characterized by cognitive decline or impairment, such as nonsyndromic mental retardation, schizophrenia, Down's syndrome or Alzheimer's disease, display abnormal spine morphology and/or a decreased number of dendritic spines as a result of alterations in actin regulatory molecules. For example, long tortuous spines lacking a bulbous head and dendrites lacking spines have been described in individuals with nonsyndromic mental retardation, schizophrenia, and Down's syndrome (Fiala et al., 2002). Genomic mutation of different modulators, activators and effectors of Rho GTPases involved in actin reorganization have been linked to families with a high incidence of non-syndromic mental retardation (Allen et al., 1998; Billuart et al., 1998; Kutsche et al., 2000; Ramakers, 2002). Also, the beta-amyloid oligomers that cause inflammatory damage to the brain in Alzheimer's disease also alter the function of

key Rho GTPases that regulate actin organization, causing long-term disassembly of the synaptic actin filaments and cognitive decline (Zhao et al., 2006; Ma et al., 2008). Thus, proper regulation of the actin cytoskeleton is crucial for the morphological plasticity of the spine and provides a mechanistic link to cognitive function.

Adhesion is another critical component of dendritic spines. In general, adhesion provides anchoring, traction and communication with the cellular environment to optimize cell behavior, or to ensure a specialized response, such as immune activation, or transmission of synaptic input (Dustin and Colman, 2002). From this point of view, dendritic spines comprise the post-synaptic half of a highly specialized cell-cell adhesion structure that forms between pre-synaptic and post-synaptic terminals. Several families of adhesion receptors are found in dendritic spines, including integrins (Hynes, 2002; Geiger et al., 2009), cadherins (Takeichi, 1995; Gumbiner, 2005), neurexins/neuroligins (Lisé and El-Husseini, 2006; Craig and Kang, 2007), Eph receptors (Klein, 2009) and other families of specific neuronal receptors, such as Syn-CAMs and SALMs (Gerrow and El-Husseini, 2006; Han and Kim, 2008). These receptors are involved in both spinogenesis and synaptogenesis (Takeichi and Abe, 2005; Shi and Ethell, 2006; Webb et al., 2007).

A common property of adhesion receptors is that ligand binding induces the formation of supramolecular complexes that contain signaling adaptors and cytoskeletal molecules (Zaidel-Bar et al., 2007a). These “adhesions” are signaling centers that provide anchorage and traction for the organization of the actin cytoskeleton, which drives protrusion, adhesion modulation, and also controls gene expression (Smith et al., 2007; Geiger et al., 2009; Vicente-Manzanares et al., 2009a). Thus, actin and adhesion

are critical components not only in a variety of cell types and processes, e.g. migratory lamellipodia and filopodia in motile cells, growth cones in neurons, cell-matrix adhesions and cell-cell junctions in epithelial cells, but also in dendritic spine formation (Lin et al., 1994; Cramer et al., 1997; Svitkina and Borisy, 1999; Hartsock and Nelson, 2008; Mattila and Lappalainen, 2008). Furthermore, many regulators of both actin and adhesion are common throughout the different cellular systems. This striking resemblance is clear at a molecular level but has not been exploited explicitly and aggressively to develop insights into dendritic spine formation and structure and synaptic function.

In this mini-review, we discuss what is known about the function of actin and adhesion in nonneuronal systems and its implications and parallels for dendritic spine formation and organization. We highlight the critical role of the actin cytoskeleton and its regulators in the development, removal and maintenance of dendritic spines, pointing out the common players and their spatiotemporal regulation. Since other reviews in this volume are specifically devoted to the detailed description of some of the cytoskeletal and regulatory molecules in the synapse, we will not address their molecular characterization, but rather focus on their role in the morphological and compositional changes that take place during the lifetime of dendritic spines.

Spinogenesis, like protrusion and adhesion, is driven by actin polymerization

Two hypotheses have been postulated to explain initial spinogenesis (Yuste and Bonhoeffer, 2004). One hypothesis proposes that contact of a pre-synaptic terminal with the shaft of the post-synaptic membrane induces the formation of a protrusion.

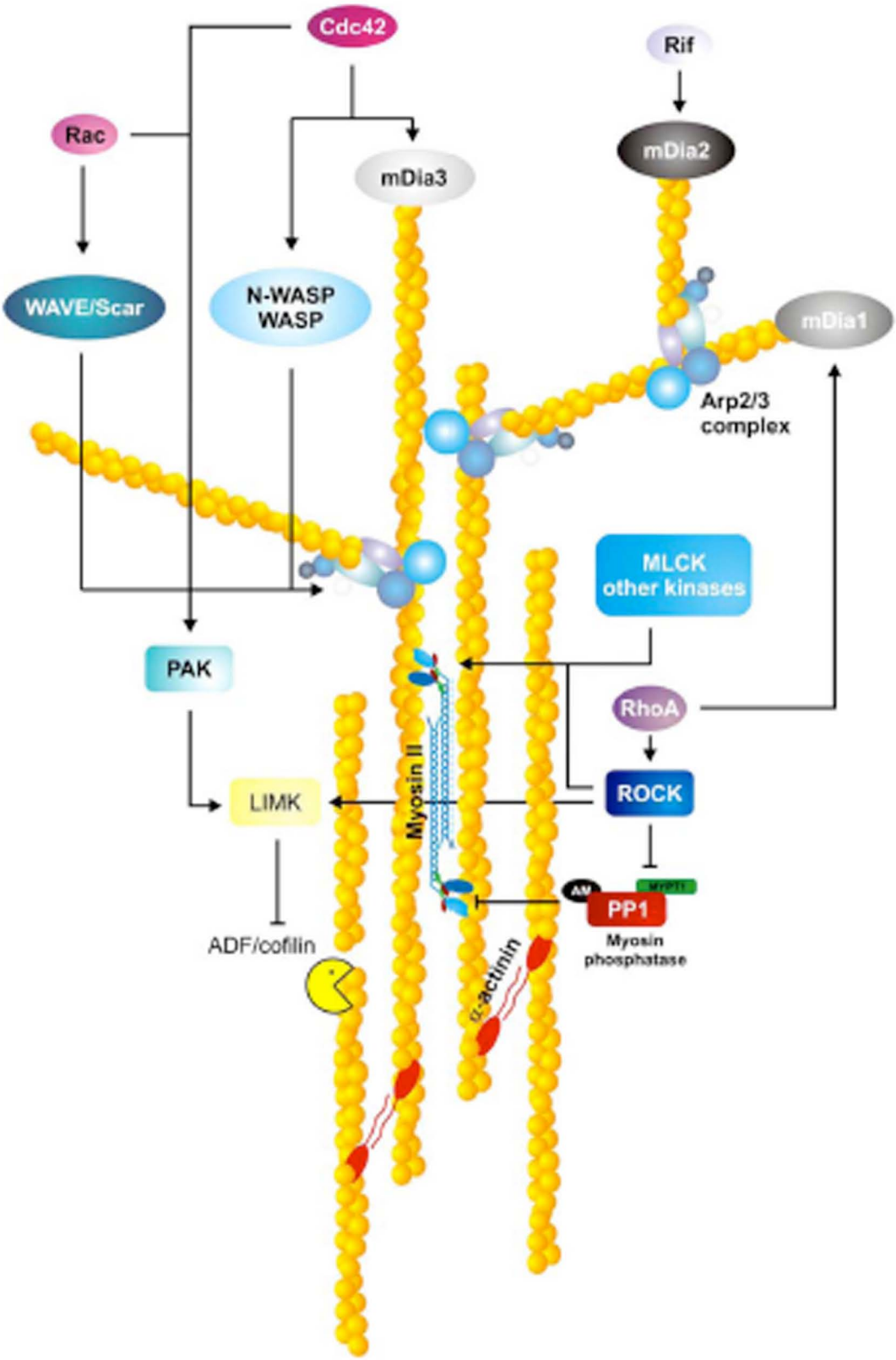
Conversely, another hypothesis proposes the spontaneous initial formation of multiple immature dendritic protrusions, followed by contact with presynaptic terminals, which induces their maturation.

Immature dendritic protrusions seem to have an active function in this process; their motion in time-lapse movies suggests they may play an exploratory role, cycling between protrusive elongation and retraction until physical contact with a pre-synaptic terminal is made (Fischer et al., 1998; Holtmaat et al., 2005). Immature spines (or dendritic spine precursors) are usually long, thin actin rich protrusions. Actin polymerization, which creates protrusions in migrating cells and growth cones, is likely to drive the initial emergence of immature dendritic precursors as well. There are two main modes of actin polymerization: a linear mode that is propelled by formins (e.g. mDia1, 2 and 3) (Figure 7); and a branched mode nucleated by the Arp2/3 complex, which binds to the side of an actin filament and promotes growth of another actin filament at a 70° angle (Figure 7) (Pollard and Borisy, 2003).

The thin, linear shape of dendritic precursors suggests the involvement of mechanisms used to generate filopodia in other cell types; however, it seems clear that these precursors are not identical to filopodia. They do not contain typical filopodial markers such as fascin, which bundles F-actin into tight parallel arrays (Sekino et al., 2007). Rather, barbed ends of F-actin are seen at the base of dendritic protrusions in addition to their tips, suggesting the existence of anti-parallel arrangements of actin filaments in immature spine precursors (Hotulainen et al., 2009). Also, Cdc42, which generates filopodia in migrating cells *via* activation of the formin mDia3, does not produce an increase in dendritic spine precursors (Hotulainen et al., 2009). Rather,

Figure 7: Mechanisms of actin regulation in dendritic spines.

The cartoon depicts the main molecules that control actin polymerization and organization in dendritic spines. Actin polymers are represented as coiled chains of yellow beads. The regulatory molecules include: 1) the Arp2/3 complex, which binds to the side of a pre-existing actin filament and promotes formation of a branched actin filament. Arp2/3 is activated by NWASP/WASP under the control of the small GTPase Cdc42, and WAVE, which is activated by the small GTPase Rac. 2) formins, including mDia1 (activated by the small GTPase RhoA), mDia2 (small GTPase Rif) and mDia3 (Cdc42), which bind to the barbed (polymerizing) end of the actin filament and promote processive incorporation of actin monomers. 3) actin crosslinkers such as α-actinin and myosin II. Myosin II activity and assembly are controlled through phosphorylation. Kinases like ROCK and MLCK can activate myosin II. ROCK is controlled by RhoA, and also inhibits the phosphatase that dephosphorylates myosin II. Finally, ADF/cofilin (yellow pac-man) severs actin filaments. It is inhibited by LIMK phosphorylation, which in turn is activated by phosphorylation *via* ROCK and PAK, which is regulated by Rac and Cdc42.


Cdc42
Rif
Rac
mDia2
mDia3
WAVE/Scar
N-WASP
WASP
mDia1
Arp2/3
complex
MLCK
other kinases
PAK
RhoA
LIMK
ROCK
Myosin II
PP1
Myosin
phosphatase
ADF/cofilin
α-actinin

expression of a constitutively active mutant of Cdc42 promotes spine head formation, causing an increase in the number of mushroomshaped and stubby spines (Hotulainen et al., 2009). RNAi inhibition or a dominant negative form of Cdc42 inhibits dendritic spine and synapse formation (Wegner et al., 2008), suggesting that Cdc42 is necessary for maturation; but its activation is not sufficient to induce the initial outgrowth of spine precursors from the shaft of dendrites. Interestingly, a similar GTPase/formin tandem, Rif/mDia2 may fulfill this role in hippocampal neurons; exogenous expression of either Rif or mDia2 promotes formation of long and thin dendritic spines (Figure 7) (Hotulainen et al., 2009). Arp2/3, which produces branched actin, also localizes to dendritic precursors and is involved in dendritic spine formation. RNAi knockdown of the Arp2/3 complex or its upstream activator N-WASP inhibited spine and synapse formation, as shown by a decrease in the total number of dendritic spines and synapses (Wegner et al., 2008). Similar results were observed in hippocampal sections from mice deficient for WAVE-1, another upstream activator of Arp2/3 (Soderling et al., 2007). This study also revealed altered neuritogenesis and field excitatory post-synaptic potential (fEPSP) in WAVE-1-deficient mice (Soderling et al., 2007). Several other studies have ascribed an important role to the small GTPase Rac and its downstream effectors in dendritic spine formation (Luo et al., 1996; Nakayama and Luo, 2000; Tashiro and Yuste, 2004).

The complementary function of the Arp2/3 complex and formins in the formation of immature spine precursors can be inferred from studies in motile cells, in which actin polymerization drives formation of filopodia and advancing protrusions. Filopodia are generated by formin-driven actin polymerization into thin parallel filaments. Close to the leading edge of the protrusion, actin is organized in a branched network nucleated by the

Arp2/3 complex (Svitkina and Borisy, 1999; Pollard and Borisy, 2003). Formins also participate in this process by inducing polymerization at the growing (barbed) ends of these branches (Yang et al., 2007). Often, advancing protrusions contain embedded filopodia that emanate from Arp2/3-dependent branching points (Korobova and Svitkina, 2008), suggesting that Arp2/3 may also participate in filopodia formation. Translating these observations to immature spine formation suggests that activation of the Arp2/3 complex in the dendritic shaft could generate a branching point, which could be subsequently extended by the action of mDia2 or Rif/mDia3, resulting in linear actin arrays typical of immature spine precursors. However, the localization of barbed ends and the Arp2/3 complex at both the tip and the base of the spine (Hotulainen et al., 2009) suggests that actin polymerization is active at both locations, where they generate antiparallel arrays of actin filaments. Also, the localized activity of ADF/cofilin, which severs actin filaments, could generate new barbed-ends within the spine.

The role of adhesion in initial spinogenesis may also parallel its role in migrating cells. As motile cells extend new protrusions, they attach to the substratum *via* small adhesions that form within the protrusion. These adhesions provide traction through their linkage to the actin cytoskeleton (Figure 6B, *left*) (Beningo et al., 2001), and they accumulate regulatory proteins that control actin polymerization, reorganization and adhesive strength. A complex network of signaling pathways originating in adhesions converge on Rac (Clark et al., 1998; Price et al., 1998; Nayal et al., 2006; Zaidel-Bar et al., 2007b), which triggers actin polymerization through binding to downstream effectors, e.g., the WAVE/Scar family, which in turn activate the Arp2/3 complex (Figure 7) (Machesky and Insall, 1998). Other adhesion-related signaling proteins, such as FAK

(Focal Adhesion Kinase) are also essential for dendritic spine formation (Rico et al., 2004). FAK also modulates the function of the actin cross-linker α-actinin (Izaguirre et al., 2001), suggesting that this pathway might be important in actin bundling during initial spinogenesis.

In addition to its role in generating signals that regulate actin, adhesion also fulfills an exploratory role. Migrating cells use filopodia and nascent adhesions as small chemo- and mechano-sensitive devices to guide cell migration (Carter, 1967; Lo et al., 2000). Similarly, immature dendritic spines seek presynaptic terminals to undergo stabilization. This process is likely to involve chemotactic, chemorepellent and/or mechanotactic signals emanating from the pre-synaptic terminal or the microenvironment of the protrusion, which is stabilized by adhesion to the presynaptic terminal. Once contacted, actin organization, contraction, and adhesion mediated signaling could drive subsequent spine maturation as these adhesions do in other cell types.

Adhesive signaling and actin depolymerization regulate adhesion and turnover, and dendritic spine removal

More than a hundred years ago, Ramon y Cajal reported that the processes of the pyramidal neurons of newborns contained more protrusions than later in development. This early observation suggested that synaptic connectivity is fine-tuned through the disassembly of unused or defective spines (Feldman, 2009). Later studies confirmed that the initial proliferation of spines is followed by a marked decrease in their number at later developmental stages (Weiss and Pysh, 1978; Rakic et al., 1986).

In one model, the removal of immature spine precursors is caused by the lack of

contact and/or pre-synaptic input; accordingly, those precursors not making contact with pre-synaptic structures would be reabsorbed into the dendritic shaft, whereas those that establish contact with pre-synaptic terminals would evolve into mature spines. A separate population of mature spines is selectively eliminated during functional rewiring of neural circuits in response to sensory experience [(Chklovskii et al., 2004).

Turnover of immature spine precursors or selective elimination of mature, innervated spines is probably linked to actin filament disassembly, or a contraction-induced retraction of actin filaments back into the dendritic shaft. Filament disassembly is more likely. Contraction requires activation of proteins like non-muscle myosin II (NM II), and the present evidence suggests that NM II activation induces maturation of precursors into dendritic spines (see below) (Zhang et al., 2005; Ryu et al., 2006). However, some synapses can survive active actin disassembly; for example, actin depolymerization induces a significant, but not complete elimination of synapses when cells are treated with the actin polymerization inhibitor latrunculin A (Allison et al., 1998). Actin filament disassembly can occur *via* two complementary mechanisms: an increase in barbed end capping, which would block actin polymerization, and actin depolymerization, mediated by filament severing proteins, such as gelsolin or ADF/cofilin (Pollard and Borisy, 2003). Gelsolin is a dual-function, calcium-sensitive actin filament-severing protein that also caps the newly formed barbed ends, impeding further polymerization (Kwiatkowski, 1999). Gelsolin-null neurons contain numerous spines that are not stabilized by synaptic stimulation, implicating gelsolin in activity-induced spine maturation and removal of unstable, immature precursors (Star et al., 2002).

The other severing protein, ADF/cofilin, is required for actin depolymerization in protrusions of migrating cells (Bamburg et al., 1999). Expression of an active mutant of cofilin, S3A, induces accumulation of branched actin, suggesting that the increased treadmilling of actin monomers and creation of new barbed ends supersede its filament-severing activity (Delorme et al., 2007). In hippocampal neurons, cofilin activity is required for the spine shrinkage observed during long-term depression (LTD), which is the activity-dependent elimination of synaptic connections (Zhou et al., 2004). Consistently, RNAi-mediated cofilin inhibition induced longer dendritic protrusions (Hotulainen et al., 2009). Expression of a constitutively active cofilin mutant significantly decreased the area of the spine head, but did not lead to its disappearance (Shi et al., 2009). These results can be explained by the dual function of cofilin. On one hand, it severs actin filaments; but it also provides the actin monomers that are recycled into *de novo* polymerization at the barbed end, *via* treadmilling (Bamburg et al., 1999; Pollard and Borisy, 2003). Therefore, the activation and inactivation of cofilin is a key regulatory step in maintaining an adequate balance of actin depolymerization and polymerization and acts in concert with capping factors. The key role of cofilin in actin function is further supported by studies of its regulation. LIMK is activated by Rho-associated kinase (ROCK) and p21-associated kinase (PAK) (Edwards et al., 1999; Maekawa et al., 1999), which are under the control of the small GTPases RhoA and Rac/Cdc42, respectively (Figure 7). LIMK phosphorylates cofilin and inhibits its binding to actin filaments, thus preventing filament severing (Arber et al., 1998; Yang et al., 1998). Consistent with this, altered cofilin phosphorylation, abnormal spine morphology and synaptic function are observed in LIMK1-deficient mice (Meng et al., 2002; Asrar

and Jia, 2009). Interestingly, a loss-of-function mutation in LIMK1 is implicated in the cognitive deficit associated with Williams' syndrome (Frangiskakis et al., 1996).

Adhesion formation in protrusions is linked to polymerized actin; adhesions disassemble or mature when and where branched actin undergoes depolymerization or reorganization, respectively (Figure 6B, *middle*) (Alexandrova et al., 2008; Choi et al., 2008). This constitutes a putative feedback loop: polymerized actin provides a physical scaffold for the formation of adhesions, which in turn generate Rac dependent signals that promote actin polymerization and inhibit filament severing. In a similar manner, filament disassembly in immature spine precursors would disrupt adhesion, also suggesting that adhesion to the pre-synaptic terminal may induce spine maturation by inhibiting filament disassembly.

In summary, the removal of immature spine precursors during development involves actin filament disassembly, presumably through a combination of actin depolymerization and inhibition of actin polymerization; the resulting actin monomers treadmill and are used to generate new dendritic precursors during the maturation of a subpopulation of dendritic spines.

Myosin II in actin organization during dendritic spine maturation

Maturing spines undergo dramatic morphological changes, including shortening, formation of a neck, widening of the head and organization of the post-synaptic density (PSD), which is an accumulation of synaptic and adhesion receptors, signaling adaptors and cytoskeletal proteins (Ethell and Pasquale, 2005; Okabe, 2007; Sheng and Hoogenraad, 2007; Feng and Zhang, 2009). The PSD itself undergoes rapid morphology

fluctuations in response to synaptic activity, and also widens concomitantly with expansion of the spine head during its maturation (Blanpied et al., 2008).

Non-muscle myosin II (NM II) is a key contractile protein that organizes and contracts actin in migrating cells. It regulates front-back polarity and modulates adhesion organization, inducing maturation (Meng et al., 2002; Vicente-Manzanares et al., 2007; 2008). It is likely that it plays an analogous role in spine and PSD organization.

NM II is a hexameric complex formed by two heavy chains (NMHC-II), two regulatory light chains (RLC), and two essential light chains (ELC). NM II binds to actin filaments and promotes their bundling; it also mediates filament contraction through ATP hydrolysis. The three isoforms of NMHC-II, NMHC II-A, II-B and II-C, are encoded by three genes, *Myh9*, *Myh10* and *Myh14*, respectively (Vicente-Manzanares et al., 2009c). Of these, NMHC II-B, is the most prominently expressed in neurons (Takahashi et al., 1992; Cheng et al., 2000). It plays a pivotal role in growth cone dynamics and in the development of the CNS. Mice ablated for NM II-B exhibit profound developmental defects, including hydrocephalus (Ma et al., 2007). NM II-B down-regulation inhibits dendritic spine maturation. RNAi targeting of NM II-B in *in vitro* cultured hippocampal neurons or acute treatment with the NM II inhibitor blebbistatin drastically reduced the number of mature spines and synapses (Zhang et al., 2005; Ryu et al., 2006).

Spine Shortening: Role of Myosin II

Spine shortening occurs concomitant with a dramatic reorganization of the actin and is likely mediated by NM II, which induces actin contraction and reorganization. NM II activation inhibits protrusion in motile cells and causes retraction of the leading

edge. It also promotes adhesion maturation and actin filament thickening (Figure 6B, *right*) (Vicente-Manzanares et al., 2007; Choi et al., 2008). In epithelial cells, NM II promotes the consolidation of the cell-cell junction, by generating contractile actin bundles parallel to the plasma membrane, increasing the contact surface between cells (contact compaction), and inducing cadherin clustering (Shewan et al., 2005; Yamada and Nelson, 2007b).

NM II-B-mediated spine shortening is likely related to its contractile activity, exerting force that would pull on the actin filaments tethered to the tip of the spine or the PSD, causing spine retraction and compaction of the material inside the spine (Figure 8). In addition, data from epithelial cell studies suggest that NM II-driven contraction may enhance adhesive strength between pre- and post-synaptic terminals by promoting clustering of adhesion receptors, e.g. cadherins (Yamada and Nelson, 2007b).

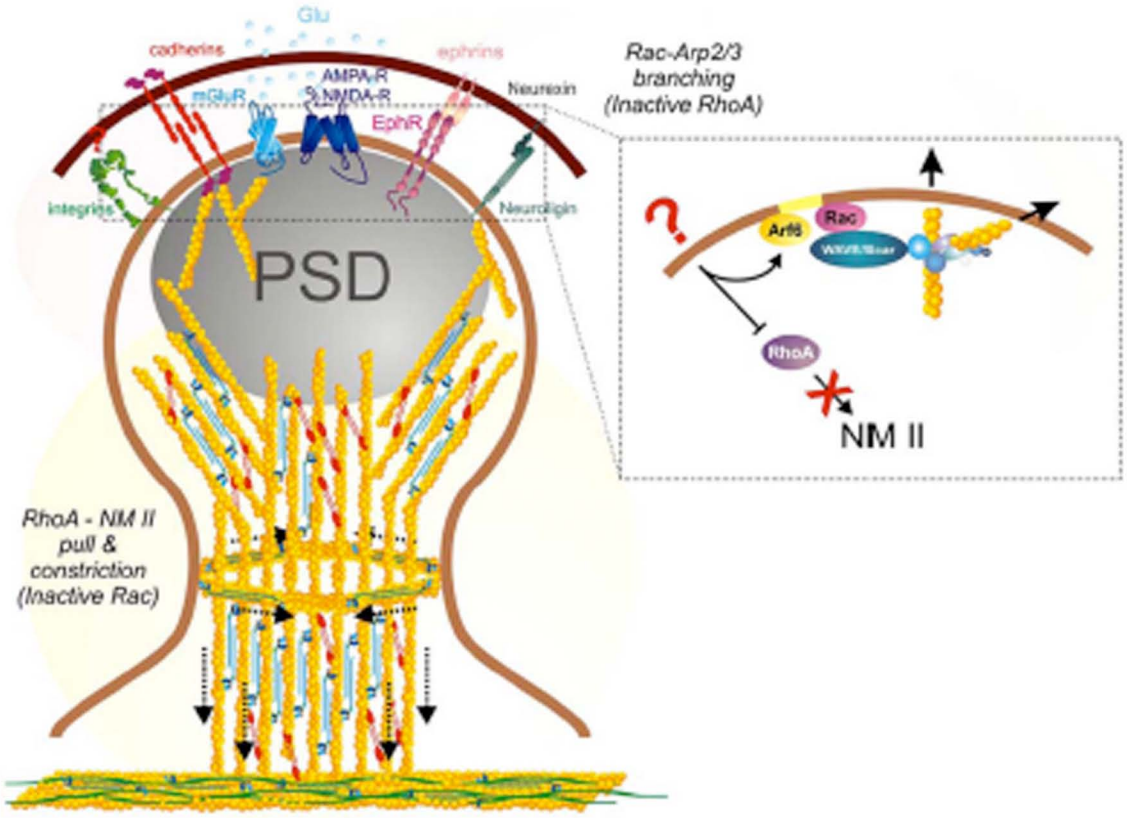
NM II function is regulated by phosphorylation of the RLC; therefore phosphorylated RLC is a marker for active NM II. Phosphorylated RLC localizes to dendritic spines, and a phosphomimetic form of RLC induces dendritic spine formation (Zhang et al., 2005). In addition, adhesion and LTP induction activate multiple signaling pathways, including RhoA/ROCK (Ren et al., 1999; Moeller et al., 2006; Rex et al., 2009), which increase the level of RLC phosphorylation in fibroblasts (Amano et al., 1996).

Formation of a Spine Neck

The spine neck is thought to be an important geometrical feature of mature spines by serving to confine neurotransmission to the spine and blocking diffusion of the signal

Figure 8: Hypothetical model of dendritic spine organization.

The cartoon represents activation of NM II (blue) at the base of the spine, which triggers retraction of the spine by pulling the actin filaments tethered to the PSD, and/or constriction of the spine neck. These movements are represented by dashed arrows. Other cross-linkers, e.g., α–actinin (shown in red), also mediate actin bundling in the spine. NM II is also found in the PSD, and controls its integrity. At the tip of the spine, activation of adhesive molecules (integrins, cadherins, neurexins/neuroligins, Eph receptors and others) or synaptic receptors (metabotropic Glu and AMPA/NMDA receptors) associated to the PSD trigger the local activation of Rac and branched actin growth by the Arp2/3 complex to support spine widening, as well as RhoA inactivation. Rac activation could be supported by the translocation of membrane domains (shown in yellow) required for spine membrane expansion under the control of the small GTPase Arf6.


Glu
cadherins
ephrins
AMPA-R
NMDA-R
mGluR
EphR
Neurexin
integrins
Neuroligin
PSD
RhoA - NM II
pull &
constriction
(Inactive Rac)
Rac-Arp2/3
branching
(Inactive RhoA)
?
Arf6
Rac
RhoA
NM II

into the shaft and adjacent spines (Ethell and Pasquale, 2005). NM II participates in actin bundles of different geometries. In migrating cells, it mainly forms thick linear actomyosin bundles (Chrzanowska-Wodnicka and Burridge, 1996); but in dividing cells it is involved in the formation of the contractile ring during cytokinesis (Matsumura, 2005). Also, NM II activation at cell-cell junctions promotes the compaction of the contact (Yamada and Nelson, 2007b). Interestingly, similar phenomena are observed at a multi-cellular level, in which coordinated cohorts of cells integrate their contractile activities: an outstanding example is the "purse-string" model of epithelial dorsal closure, which is driven by NM II activation (Young et al., 1993).

Analogously, NM II could mediate the formation of a small contractile ring-like structure that constricts the contact area of the spine with the dendritic shaft. Alternatively, the spine neck can be comprised of linear actin bundles generated during the formation of the immature spine precursor that does not undergo complete retraction. Both these possibilities are shown in Figure 8.

Spine Head Expansion

During maturation, the tip of the dendritic spine expands to provide a larger surface area of interaction with the pre-synaptic terminal; this is a hallmark of activity-induced plasticity. There are at least two coordinated mechanisms for controlling spine head expansion. One is an increase in membrane surface area, which is mediated by increased targeted delivery of vesicles under the control Rab/Arf family of GTPases like Arf6 (Park et al., 2006). The other is the reorganization of the actin cytoskeleton, in which branched actin filaments replace the linear arrays observed in immature spine

precursors. In this manner, actin branching at the tip of the spine potentially sustains the increase in volume and surface area, much like the extension of a protrusion in migrating cells.

The morphological changes that take place during spine maturation can be integrated into a model in which the local activation of Rac and Arp2/3 (and local inactivation of RhoA) at the tip of the spine supports the formation of a branched actin network that expands the head. In migrating cells, there is evidence that the activation of Rac and Rho is spatially and temporally segregated. Rac is active at the protruding edges of migrating cells, where it triggers dendritic actin formation (Nayal et al., 2006; Alexandrova et al., 2008; Choi et al., 2008). In addition, Rac signaling suppresses RhoA activation (Sander et al., 1999). On the other hand, RhoA is more active in the more posterior part of the protrusion and the center of the cell, where it induces thick actomyosin filaments, stable adhesions and inhibition of Rac activation (Chrzanowska-Wodnicka and Burridge, 1996; Sander et al., 1999).

Similarly, Rac activation closer to the synaptic cleft would promote branched actin to widen the spine head (Figure 8, insert), whereas activation of RhoA closer to the dendrite shaft would promote bundling of actin tethered to the PSD, possibly by forming an actomyosin cup, or pedestal (Figure 8). Supporting this model, it has been proposed that Arf6, which regulates vesicle trafficking and provides membrane for membrane expansion during spine widening, creates sites for targeting of Rac to the membrane (Balasubramanian et al., 2007).

CONCLUDING REMARKS

In this review, we have used insight from studies on adhesion and protrusion in migrating cells as a model for dendritic spine and PSD organization. In migrating cells, protrusions form using two actin regulators, Arp2/3 and formins, and adhesion maturation is determined by the organization of the actin cytoskeleton. Both of these are regulated by signals emanating from adhesions. Finally, the formation of epithelial adherens junctions is also mediated by actin and is accompanied by the cessation of Arp2/3 activity and stimulation of actomyosin contraction as the junctions form. Emerging evidence suggests that dendritic spine maturation is similarly mediated by actin organization and driven by contact with the pre-synaptic terminal. Thus, actin polymerization and the organization of the actin cytoskeleton remains a centerpiece of these processes, which share many common regulatory elements.

Despite the different repertoire of receptors between fibroblasts and neurons, most of the signaling pathways originate with membrane receptors and converge on the regulation of adhesion and the actin cytoskeleton through Rho GTPases. The regulators that control actin polymerization and filament disassembly downstream of the GTPases are also the same (formins and the Arp2/3 complex, and cofilin, respectively). Finally, actin cross-linkers and contractile proteins, like NM II, play similar roles in the two processes, facilitating actin reorganization and reshaping of the stable structure through actin bundling and/or contraction.

The discovery that some mental retardations are accompanied by altered Rho GTPase regulation and abnormal morphology of dendritic spines highlights the importance of understanding how the actin cytoskeleton regulates the morphological changes that dendritic spines undergo upon activation. It also points to therapeutic

targets using gene-based therapy and interventions directed at neuron specific isoforms of key adhesion and actin related molecules for the treatment of diseases with cognitive decline, such as Alzheimer's or Parkison's disease, senile dementia, or congenital and non-syndromic mental retardation.

While some clear parallels exist, many aspects of dendritic spine and PSD development remain unstudied; hopefully this discussion will provide one blueprint for a useful approach.

Chapter 3 – Myosin IIB activity and phosphorylation status determines dendritic spine and post-synaptic morphology

This chapter is based on previously published work.

Hodges JL, Newell-Litwa K, Asmussen H, Vicente-Manzanares M, Horwitz AR (2011) Myosin IIB activity and phosphorylation status determines dendritic spine and post-synaptic morphology. PLoS One 6(8): e24149. doi:10.1371/journal.pone.0024149

Abstract

Dendritic spines in hippocampal neurons mature from a filopodia-like precursor into a mushroom-shape with an enlarged post-synaptic density (PSD) and serve as the primary post-synaptic location of the excitatory neurotransmission that underlies learning and memory. Using myosin II regulatory mutants, inhibitors, and knockdowns, we show that non-muscle myosin IIB (MIIB) activity determines where spines form and whether they persist as filopodia-like spine precursors or mature into a mushroom-shape. MIIB also determines PSD size, morphology, and placement in the spine. Local inactivation of MIIB leads to the formation of filopodia-like spine protrusions from the dendritic shaft. However, di-phosphorylation of the regulatory light chain on residues Thr18 and Ser19 by Rho kinase is required for spine maturation. Inhibition of MIIB activity or a mono-phosphomimetic mutant of RLC similarly prevented maturation even in the presence of NMDA receptor activation. Expression of an actin cross-linking, non-contractile mutant, MIIB R709C, showed that maturation into a mushroom-shape requires contractile activity. Loss of MIIB also leads to an elongated PSD morphology that is no longer restricted to the spine tip; whereas increased MIIB activity, specifically through RLC-T18, S19 di-phosphorylation, increases PSD area. These observations support a model whereby myosin II inactivation forms filopodia-like protrusions that only mature once NMDA receptor activation increases RLC di-phosphorylation to stimulate MIIB contractility, resulting in mushroom-shaped spines with an enlarged PSD.

Introduction

Dendritic spines are the primary post-synaptic sites of excitatory

neurotransmission in the brain (Bourne and Harris, 2008). They are highly dynamic structures that develop from exploratory, filopodia-like processes into a compact, mushroom-shaped structure with a highly organized post-synaptic density (PSD) located at the tip (Yuste and Bonhoeffer, 2004; Sekino et al., 2007). The PSD contains cell adhesion proteins, glutamate receptors, cytoskeletal molecules, and a complex membrane-associated, cytoplasmic signaling network (Peng et al., 2004; Cheng et al., 2006; Sheng and Hoogenraad, 2007). Appropriate spine density, morphology, and PSD organization are critical for the neuronal function that underlies learning and memory (Lynch et al., 2007; Bayés et al., 2011). As such, a diverse spectrum of learning and memory disorders exhibit dendritic spine abnormalities, including neurodevelopmental disorders, such as autism, Down's syndrome, non-syndromic mental retardation, neurodegenerative diseases, like Alzheimer's, and psychoses, such as schizophrenia (Fiala et al., 2002; Newey et al., 2005).

Despite the importance of proper spine morphology and PSD organization, the structural and regulatory mechanisms that organize them are not understood. Recent evidence implicates the polymerization and organization of actin in spine organization, although how it does this is unclear (Hotulainen et al., 2009; Frost et al., 2010a). Myosin IIB (MIIB), the predominant non-muscle myosin II isoform found in brain, contributes to actin organization in most cell types through its cross-linking and contractile properties and is implicated in spine morphology (Kawamoto and Adelstein, 1991; Ryu et al., 2006; Vicente-Manzanares et al., 2009c). MIIB activity is regulated by phosphorylation on residues Thr18 and/or Ser19 in its regulatory light chain (RLC); simultaneous phosphorylation on both residues promotes maximal myosin ATPase activity and

formation of large actin bundles (Ikebe and Hartshorne, 1985; Vicente-Manzanares et al., 2009c; Vicente-Manzanares and Horwitz, 2010). We have previously identified a signaling cascade that functions through RLC phosphorylation to regulate spine density (Zhang et al., 2005). More recent evidence points to MIIB as a potentially important regulator of the spine dynamics underlying learning and memory (Zhang et al., 2005; Ryu et al., 2006; Rex et al., 2010). In particular, short-term inhibition of MIIB activity induces immature filopodia-like spines and results in a corresponding disruption of long-term potentiation (LTP) and memory acquisition (Ryu et al., 2006; Rex et al., 2010). While the importance of MIIB seems clear, the mechanism by which it shapes spine morphology is unknown.

In addition to spine morphology, proper organization of the PSD is also important for synaptic signaling, as PSD size is related to spine head area and directly correlated with synaptic strength (El-Husseini et al., 2000; Bredt and Nicoll, 2003). While many molecules that reside in the PSD have been identified, much less is known about the mechanisms that determine its morphology and organization (Peng et al., 2004; Cheng et al., 2006). The PSD is now thought to be dynamic and undergo rapid fluctuations in morphology (Blanpied et al., 2008; Frost et al., 2010b). Several proteins within the PSD scaffold reportedly interact with the actin cytoskeleton (Böckers et al., 2001; Sheng and Hoogenraad, 2007), raising the possibility that actin organization may underlie PSD morphology. The dramatic effect of MIIB on actin organization points to a likely role for it in the organization of the PSD and regulation of synaptic plasticity.

In this study, we dissect the contributions of MIIB activity to spine morphology and PSD organization during maturation and in response to stimuli. We find that MIIB

activity restricts the formation of nascent protrusions on dendrites. However, MIIB activity subsequently mediates spine maturation, with RLC T18, S19 di-phosphorylation required for mature, compact spines. This maturation is mediated by the contractile activity of MIIB since an actin-cross linking, contractile-deficient mutant of MIIB, MIIB-R709C, does not promote maturation. Stimulation induced maturation of spines also requires di-phosphorylated RLC. MIIB also plays a central role in PSD organization. When inhibited, it creates elongated PSDs localized away from the spine tip; however, when fully active, it drives PSD compaction and localization to the spine tip. Thus, MIIB activity determines spine formation and orchestrates the spine and PSD morphologies that underlie post-synaptic plasticity.

Results

Myosin IIB Regulates Spine Morphology and Dynamics

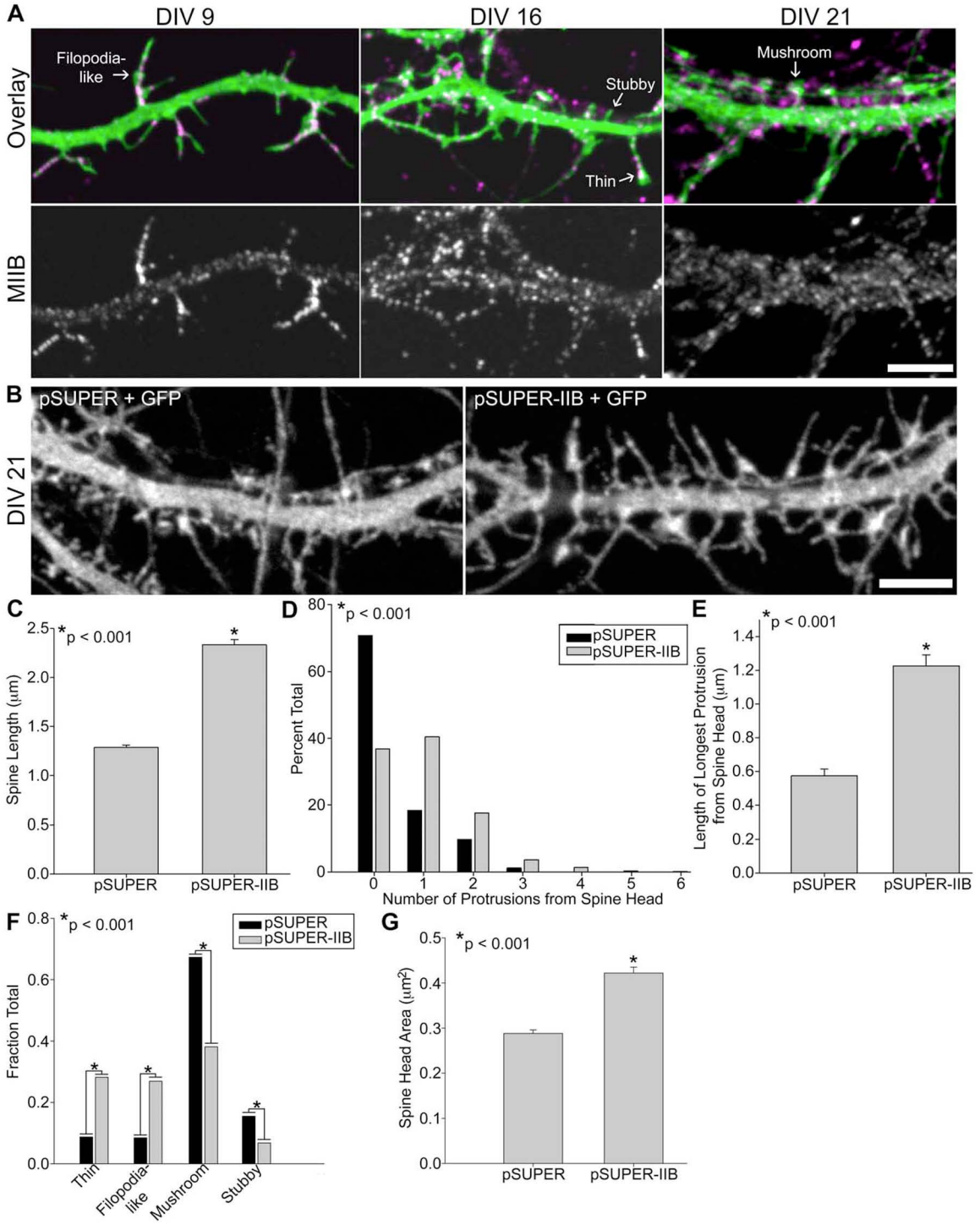
MIIB localizes to dendritic protrusions of various morphologies, including filopodia-like protrusions, as well as thin, stubby and mushroom-shaped spines (Figure 9A). Chronic inhibition of MIIB by shRNA knockdown does not change spine density detectably (~1.2 spines/μm dendrite for both day *in-vitro* (DIV) 21 control and MIIB-deficient neurons) (Ryu et al., 2006). Instead, it produces longer spines as measured from base to tip (including protrusions emanating from the spine head) (Figure 9B-C) (Ryu et al., 2006; Rubio et al., 2011). Spine heads were identified as focal expansions, which contain a PSD (see PSD-95 immunostaining in Figure 15). Noticeably, there is an increase in the number of long protrusions branching from MIIB-deficient spine heads, resulting in the spine head positioned away from the spine tip (Figure 9B, D, E).

Figure 9: Inhibition of myosin IIB activity increases the number and length of filopodia-like protrusions.

A) Hippocampal neurons transfected with GFP at DIV 6 were fixed and immunostained for endogenous MIIB at DIV 9, 16, and 21. Arrows point to different spine morphology types.

B) Hippocampal neurons were co-transfected at DIV 6 with GFP and either an shRNA vector against MIIB (pSUPER-IIB) or a control empty vector (pSUPER). Neurons were fixed at DIV 21 and scored for

(**C-G**) changes in spine length, branching number and length, morphology and head area. Knockdown of MIIB in hippocampal neurons causes a ~2-fold increase in spine length, C. Knockdown of MIIB causes a large increase in the number of protrusions branching from the spine head. Spine heads were identified by morphology and localization of PSD-95. Note the small fraction of spines that contain protrusions branching from the spine head in the controls, D. MIIB knockdown produces many long protrusions branching from the spine head, which results in spine head positioning away from the spine tip, E. MIIB knockdown creates an increase in the fraction of thin (long protrusions with small head at tip) and filopodia-like spines (long protrusions without a spine head) with a concomitant decrease in the fraction of mushroom and stubby spines, F. Spine heads present in MIIB knockdown neurons are larger in area, G. For each quantification, 512 spines from 23 control neurons and 619 spines from 36 MIIB knockdown neurons were analyzed. Error bars represent SEM. p-values were derived using the Mann-Whitney test (C, D, E, G) and Chi-square test (F). Scale bar = 5μm for all panels.


A
DIV 9
DIV 16
DIV 21
Overlay
MIIB
Filopodia-like
Stubby
Thin
Mushroom
B
DIV 21
pSUPER + GFP
pSUPER-IIB + GFP
C
*p < 0.001
Spine Length (μm)
pSUPER
pSUPER-IIB
D
*p < 0.001
Percent Total
pSUPER
pSUPER-IIB
Number of Protrusions from Spine Head
E
*p < 0.001
Length of Longest Protrusion from Spine Head (μm)
pSUPER
pSUPER-IIB
F
*p < 0.001
Fraction Total
pSUPER
pSUPER-IIB
Thin
Filopodia-like
Mushroom
Stubby
G
*p < 0.001
Spine Head Area (μm²)
pSUPER
pSUPER-IIB

At DIV 21, control neurons predominantly display mushroom-shaped spines, consisting of a large bulbous spine head on top of a short spine neck. However, MIIB knockdown neurons display significantly less mushroom-shaped spines and more filopodia-like protrusions than controls (note: mushroom-shaped spines with emanating protrusions were classified as "mushroom") (Figure 9B, F). While these MIIB-deficient spine heads exhibit a significantly larger area than controls, they are often more elongated in shape (Figure 9B, G). Thus, MIIB is required for spines to develop and maintain a mushroom-shape.

To monitor the acute effects of MIIB inhibition on spine dynamics, we used time-lapse confocal imaging of local application of blebbistatin using a micropipette. Nascent spines emerge and protrude in response to the local application of blebbistatin (Figure 10A), showing that local MII inhibition leads to formation of new protrusions (Figure 10B). However, blebbistatin micropipetting also increased spine retraction (Figure 10C), demonstrating that MIIB does not disrupt spine pruning, but promotes the dynamic assembly and disassembly of spines. Similarly, in MIIB knockdown neurons, we observed that protrusions extend and retract more frequently and were substantially longer than those in the corresponding controls (Figure 10D, E). Despite their length, these protrusions are not *de novo* dendrites, as post-imaging fixation and immunostaining reveal actin-rich structures that do not contain the dendrite marker, MAP2 (data not shown).

Myosin IIB is required for Spine Maturation in Response to NMDA Receptor Stimulation

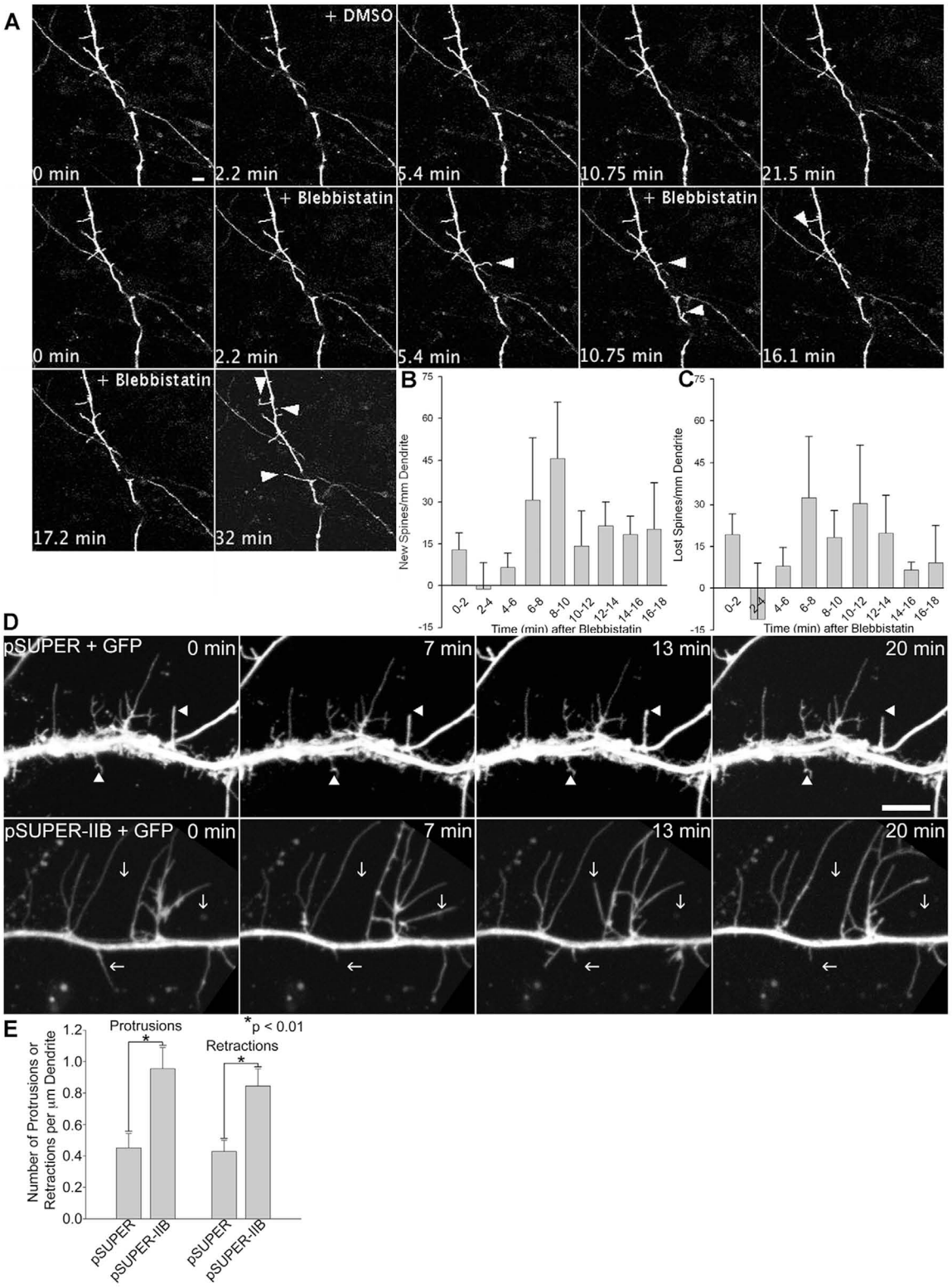
Since MIIB inhibition creates filopodia-like protrusions and inhibits spine

Figure 10: Inhibition of myosin IIB activity affects spine dynamics.

A) A DIV 7 cortical neuron expressing DsRed2 was locally micropipetted with either DMSO or 100μM blebbistatin at the indicated times. Note the increase in the fraction of spines that appear and extend in response to blebbistatin. Arrowheads indicate either nascent or elongating spines. Scale bar = 5μm.

B-C) Quantification of new spine formation (B) or loss of spines (C) following blebbistatin micropipetting (micropipetting of 5 different cortical neurons). The number of new or lost spines is corrected for the number of new or lost spines observed prior to micropipetting, i.e. the control period.

D-E) Time-lapse confocal imaging was performed on DIV 13-14 hippocampal neurons co-expressing GFP and either an shRNA vector against MIIB or a control empty vector. Scale bar = 5μm. Spines from MIIB knockdown neurons extend and retract more frequently (arrows) than spines in control neurons (arrowheads), D. MIIB knockdown increases the frequency of spine protrusion and retraction, E. Note the unusual length of the protrusions in the MIIB knockdown neurons. Quantification in (E) is based on 3 MIIB knockdown neurons and 5 control neurons each acquired for 15 minutes. Error bars represent SEM. *$p<0.01$, Mann-Whitney test.


A
+ DMSO
0 min
2.2 min
5.4 min
10.75 min
21.5 min
+ Blebbistatin
0 min
2.2 min
5.4 min
10.75 min
16.1 min
+ Blebbistatin
17.2 min
32 min
B
New Spines/mm Dendrite
Time (min) after Blebbistatin
C
Lost Spines/mm Dendrite
Time (min) after Blebbistatin
0-2
2-4
4-6
6-8
8-10
10-12
12-14
14-16
16-18
D
pSUPER + GFP
0 min
7 min
13 min
20 min
pSUPER-IIB + GFP
0 min
7 min
13 min
20 min
E
Protrusions
Retractions
*p < 0.01
Number of Protrusions or Retractions per µm Dendrite
pSUPER
pSUPER-IIB
pSUPER
pSUPER-IIB

development into compact, mushroom-shaped structures, we hypothesized that MIIB also mediates the acute, activity-induced morphology changes that underlie spine maturation. To test this, we selectively activated synaptic NMDA receptors with the co-agonist glycine and assayed for morphological changes indicative of spine maturation, including decreased spine length and increased spine tip width (i.e., mushroom-shaped) (Park et al., 2004). At DIV 14-17, neurons display many immature filopodia-like spines, allowing us to observe an accelerated, acute maturation response to stimulation. Glycine stimulation of control neurons promotes extensive maturation, including spine shortening and spine tip enlargement, resulting in the appearance of numerous mushroom-shaped spines (Figure 11). In contrast, acute inhibition of MIIB with blebbistatin prevented both spine shortening and increased spine tip width; instead, spines persisted as filopodia-like projections even when stimulated with glycine (Figure 11A-D). However, shRNA knockdown of MIIB did not prevent spine shortening in response to glycine, but did prevent an increase in spine tip width (Figure 11E-G). Thus, shRNA knockdown of MIIB also leads to the persistence of filopodia-like protrusions (Figure 11H). Together these results demonstrate that MIIB mediates the morphological transition from immature filopodia-like protrusions into mature mushroom-shaped spines.

Myosin IIB-mediated Contractility Underlies Spine Maturation

MIIB organizes actin filaments by two mechanisms: it cross-links to form actomyosin bundles, and it also moves antiparallel filaments in an ATPase-dependent manner, thereby contracting them (Vicente-Manzanares et al., 2009c). Overexpression of *wild type* (WT) MIIB accelerates spine maturation into a mushroom-shape, suggesting

Figure 11: Inhibition of myosin IIB activity prevents spine morphological changes in response to NMDA receptor activation.

A, E) When MIIB is inhibited using blebbistatin (A) or MIIB knockdown (E), spines do not shorten or assume a "mushroom" morphology in response to glycine. Hippocampal neurons either transfected on DIV 6 with GFP or co-transfected with GFP and either an shRNA vector against MIIB or a control empty vector. Neurons were treated with glycine on DIV14 (in the presence of DMSO or blebbistatin, A) or DIV16 (MIIB knockdown or empty vector control, E) to activate NMDA receptors.

B-D, F-H) Quantification of spine morphology in response to MIIB inhibition and glycine stimulation. Blebbistatin (B) or MIIB knockdown (F) prevents spine shortening in response to glycine stimulation and increases spine length compared to controls; note some decrease in spine length in the knockdown in response to glycine. Fraction of spines with a large head, spine tip width ≥ 0.4 μm, increases in response to glycine stimulation but is prevented by blebbistatin (C) or MIIB knockdown (G). In the presence of blebbistatin (D) or MIIB knockdown (H), glycine does not increase the fraction of mushroom-shaped spines in contrast to stimulated controls. For each condition, 530-895 spines from 15-21 neurons were analyzed. Error bars represent SEM. *$p<0.001$, Mann-Whitney test (B, F), t-test (C, G), Chi-square test (D, H). Scale bar = 5μm for all panels.

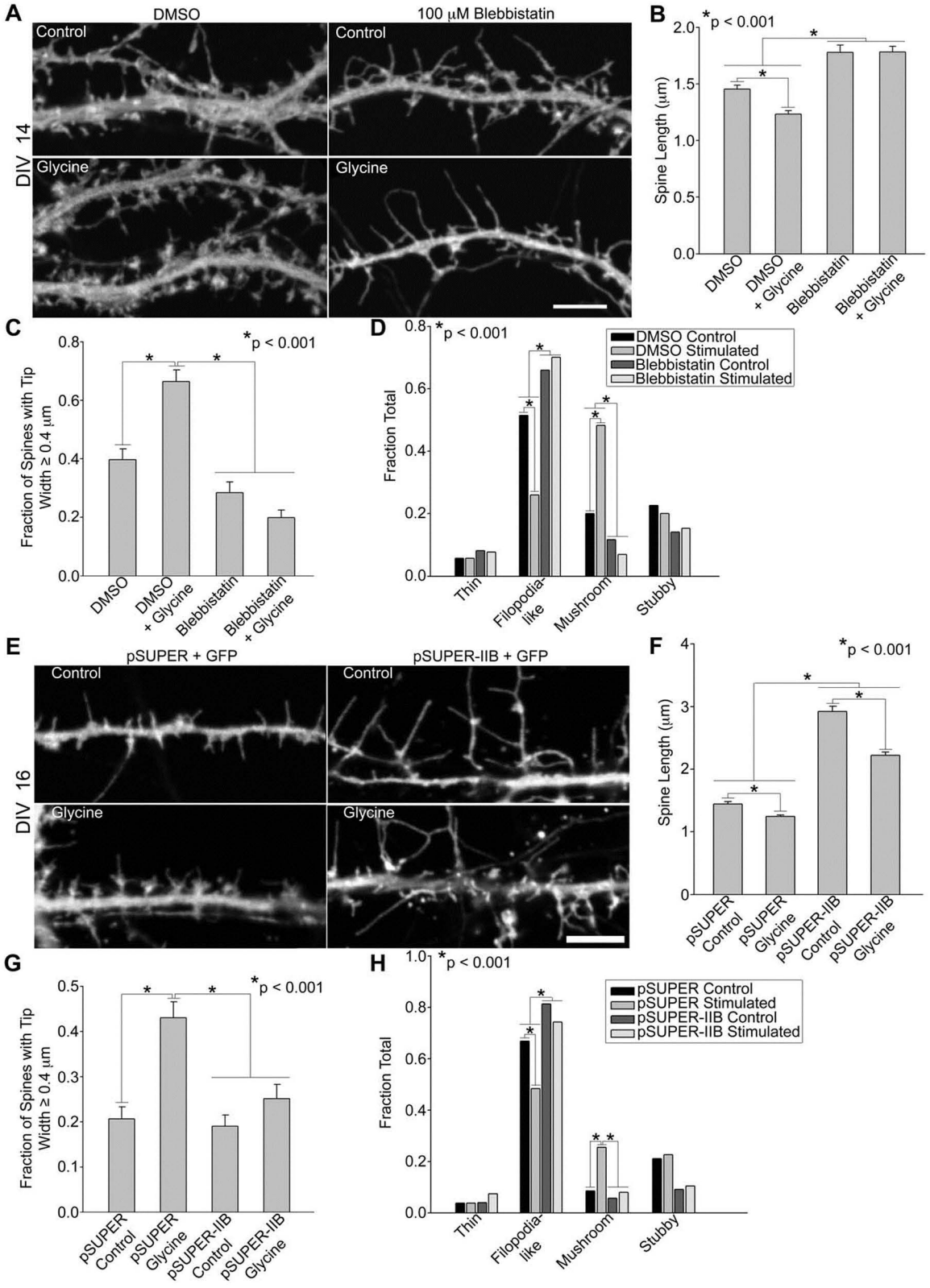
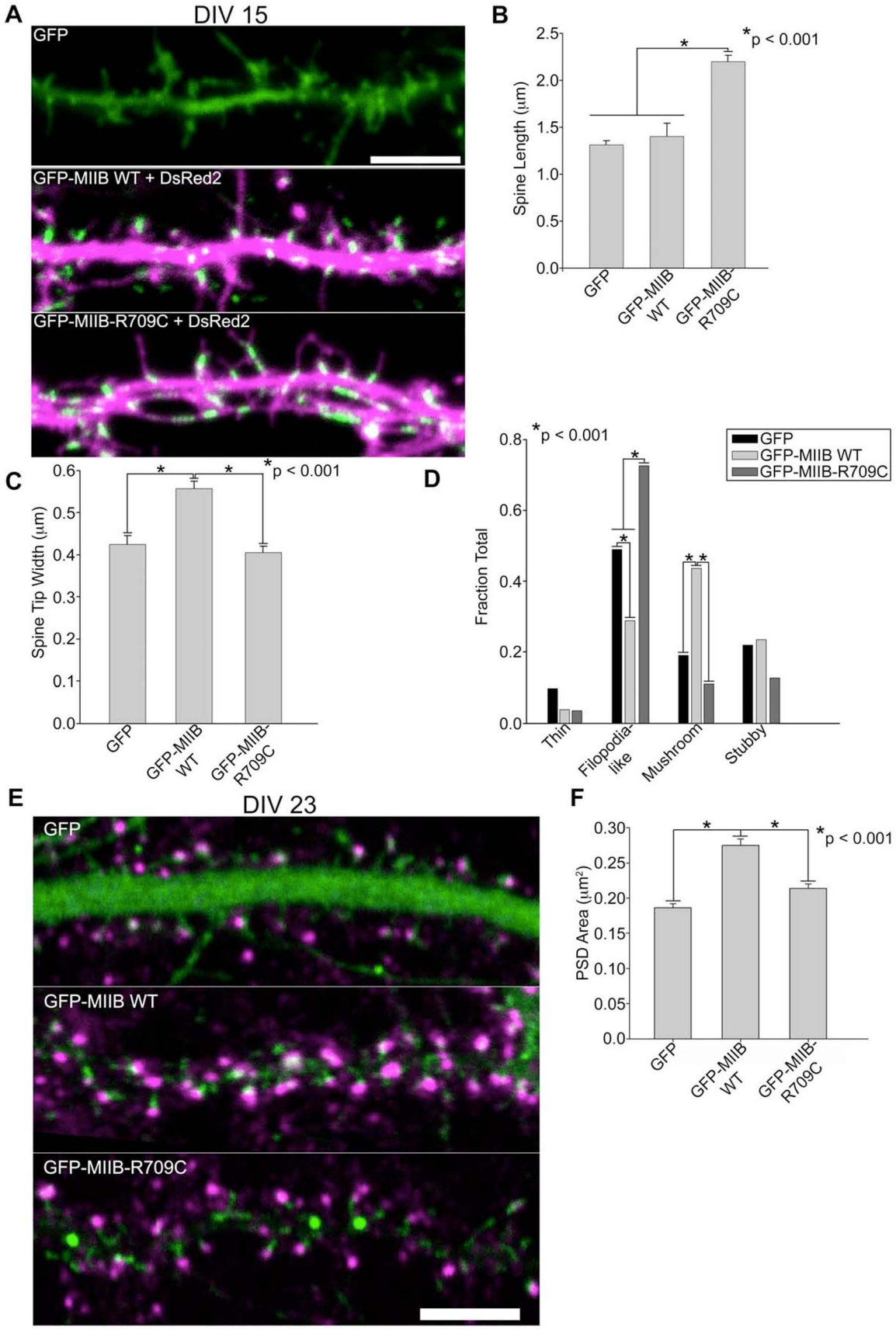

A
DMSO
100 μM Blebbistatin
Control
Control
DIV 14
Glycine
Glycine
B
*p < 0.001
Spine Length (μm)
DMSO
DMSO + Glycine
Blebbistatin
Blebbistatin + Glycine
C
*p < 0.001
Fraction of Spines with Tip Width ≥ 0.4 μm
DMSO
DMSO + Glycine
Blebbistatin
Blebbistatin + Glycine
D
*p < 0.001
DMSO Control
DMSO Stimulated
Blebbistatin Control
Blebbistatin Stimulated
Fraction Total
Thin
Filopodia-like
Mushroom
Stubby
E
pSUPER + GFP
pSUPER-IIB + GFP
Control
Control
DIV 16
Glycine
Glycine
F
*p < 0.001
Spine Length (μm)
pSUPER Control
pSUPER Glycine
pSUPER-IIB Control
pSUPER-IIB Glycine
G
*p < 0.001
Fraction of Spines with Tip Width ≥ 0.4 μm
pSUPER Control
pSUPER Glycine
pSUPER-IIB Control
pSUPER-IIB Glycine
H
*p < 0.001
pSUPER Control
pSUPER Stimulated
pSUPER-IIB Control
pSUPER-IIB Stimulated
Fraction Total
Thin
Filopodia-like
Mushroom
Stubby

Figure 12: Myosin contractility promotes spine maturation.

A) Hippocampal neurons were co-transfected at DIV 6 with DsRed2 and either GFP, GFP-MIIB WT (*wild type*), or GFP-MIIB-R709C (an actin-binding but contractile-deficient mutant) and fixed at DIV 14 or 15. Note the increased length of the non-contractile mutant and increase in mushroom-shaped spines in the cells expressing ectopic MIIB.

B-D) Spine length, measured via DsRed2, is significantly longer in neurons expressing GFP-MIIB-R709C but is not different between GFP control and neurons expressing GFP-MIIB WT, B. Spine head width, visualized using cytoplasmic DsRed2, is greater in neurons expressing GFP-MIIB WT; but there is no difference in the spine head width of neurons expressing GFP-IIB-R709C and GFP control neurons, C. The fraction of mushroom shaped spines is greater in neurons expressing GFP-MIIB WT; whereas the fraction of filopodia-like spines is greater in neurons expressing GFP-MIIB-R709C, D.

E-F) The PSD area increases in DIV 21-23 neurons expressing WT-MIIB, but not in the controls or neurons expressing R709C. For each condition, 424-582 spines from 6-15 neurons were analyzed. Error bars represent SEM. *$p<0.001$, Mann-Whitney test (B, C, F), Chi-square test (D). Scale bar = 5μm for all panels.


A
DIV 15
GFP
GFP-MIIB WT + DsRed2
GFP-MIIB-R709C + DsRed2
B
Spine Length (μm)
2.5
2.0
1.5
1.0
0.5
0.0
*
*p < 0.001
GFP
GFP-MIIB WT
GFP-MIIB-R709C
C
Spine Tip Width (μm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*p < 0.001
GFP
GFP-MIIB WT
GFP-MIIB-R709C
D
*p < 0.001
GFP
GFP-MIIB WT
GFP-MIIB-R709C
Fraction Total
0.8
0.6
0.4
0.2
0.0
Thin
Filopodia-like
Mushroom
Stubby
E
DIV 23
GFP
GFP-MIIB WT
GFP-MIIB-R709C
F
PSD Area (μm²)
0.30
0.25
0.20
0.15
0.10
0.05
0.0
*
*
*p < 0.001
GFP
GFP-MIIB WT
GFP-MIIB-R709C

that MIIB-mediated contractility enhances spine maturation (Figure 12A-D). To determine whether contractility, per se, is sufficient to create mushroom-shaped spines, we expressed a mutant, MIIB R709C, which has inhibited ATPase activity but is locked inan actin-bound state. This mutant incorporates into actomyosin bundles with high effective affinity and promotes actomyosin bundling, but not contraction (Ma et al., 2004; Vicente-Manzanares et al., 2007). When MIIB-R709C is expressed in hippocampal neurons, it leads to the persistence of filopodia-like spines, even into later stages of neuronal development (data not shown). It also induces a two-fold longer spine length when compared to WT MIIB- or GFP-expressing controls (Figure 12A, B). Furthermore, WT-MIIB, but not MIIB-R709C, increased PSD size (Figure 12E), which correlates with spine head volume and LTP (Holtmaat and Svoboda, 2009). Finally, WT-MIIB, but not MIIB-R709C, induces a significant ($p < 0.001$) increase in the number of post-synaptic sites per μm dendrite (1.29 PSDs/μm dendrite for WT-MIIB, 0.81 PSDs/μm dendrite for GFP, and 0.75 PSDs/μm dendrite for MIIB-R709C). These results suggest that MIIB contractility mediates spine and PSD maturation.

Differential Myosin Regulatory Light Chain (RLC) Phosphorylation Dictates Distinct Spine Morphologies

MIIB localizes to both immature filopodia-like protrusions as well as mature mushroom-shape spines (Figure 9A). How MIIB activity is regulated to determine spine morphology is unclear. In fibroblasts, simultaneous RLC phosphorylation on residues T18 and S19 increases MIIB activity and creates front-back polarity (Vicente-Manzanares et al., 2008; Vicente-Manzanares and Horwitz, 2010). We therefore asked

whether RLC phosphorylation regulates post-synaptic MIIB activity to create mature mushroom-shaped spines. In response to NMDA receptor activation by glycine, we stained for di-phosphorylated RLC (T18, S19), and observed a significant increase (Figure 13A-B). To determine whether RLC-T18~P, S19~P di-phosphorylation is necessary for spine maturation, we activated NMDA receptors with glycine in neurons expressing RLC-T18A, S19D (RLC-A, D), which mimics mono- but prevents di-phosphorylation (Vicente-Manzanares and Horwitz, 2010). While control neurons matured into a mushroom-shaped spine, RLC-A,D prevented spine maturation; instead they persisted as filopodia-like protrusions (Figure 13C-D). In contrast, expression of a di-phosphomimetic mutant, RLC-T18D,S19D (RLC-D,D) increased spine maturation and PSD area when compared to GFP or RLC-AD expressing neurons (Figure 13E-G). Therefore, while mono-phosphorylation inhibits spine maturation and PSD enlargement, RLC di-phosphorylation is necessary for and promotes it.

Rho Kinase (ROCK) regulates RLC T18, S19 di-phosphorylation and spine maturation.

ROCK is a kinase that increases RLC phosphorylation on T18 and S19 both directly and indirectly through inhibition of myosin light chain phosphatase (Kimura et al., 1996; Yoneda et al., 2005). We therefore determined whether ROCK regulates post-synaptic RLC di-phosphorylation and spine morphology. Neurons treated with the ROCK inhibitor, Y-27632, showed long-filopodia like spines with an increased length (Figure 14A-B) and similar to those observed when RLC di-phosphorylation is inhibited by expression of RLC-AD (Figure 13) (Tashiro and Yuste, 2004). In contrast, inhibition of myosin light chain kinase, another RLC kinase, did not increase spine length (data not

Figure 13: RLC T18, S19 di-phosphorylation mediates spine maturation.

A) Glycine-activation of NMDA receptors stimulates spine maturation and increases RLC-T18, S19 di-phosphorylation in spines (arrowheads indicate increased RLC-T18, S19 ~P in glycine-stimulated spines). DIV 21 neurons expressing GFP were chronically treated with the NMDA receptor antagonist AP-5 to inhibit spine maturation. Neurons were acutely stimulated by AP-5 withdrawal and the addition of 200μM glycine, while control neurons were continuously treated with AP-5.

B) Quantification of spine-associated RLC-T18, S19 di-phosphorylation by staining reveals a significant increase following NMDA receptor activation. 706 spines from 7 neurons were analyzed for AP5 controls and 843 spines from 8 glycine stimulated neurons.

C) RLC-AD inhibits spine maturation in response to glycine activation of NMDA receptors. DIV 21 neurons were treated as described in (A) and immunostained for the dendrite marker, MAP-2 (magenta).

D) RLC-AD prevents spine shortening in response to glycine (4C, arrows). We analyzed 2032 spines from 12 AP-5-treated GFP neurons, 1698 spines from 15 glycine stimulated GFP neurons, 1017 spines from 7 AP-5-treated RLC-AD neurons, 1116 spines from 8 glycine-stimulated RLC-AD neurons.

E) RLC-AD expression creates filopodia-like spine precursors, while RLC-DD contracts spines into a mushroom-shaped morphology with increased PSD area. Neurons between DIV 21-33 expressing GFP, RLC-AD GFP or RLC-DD GFP were fixed and immunostained for the PSD marker, PSD-95.

F) RLC-DD significantly increases PSD area in comparison to GFP or RLC-AD. PSD measurements are from neurons between DIV 21-33. We analyzed 442 PSDs from 4 GFP neurons, 2204 PSDs from 16 RLC-AD neurons, and 2167 PSDs from 15 RLC-DD neurons.

G) RLC-DD expression increases the percentage of mushroom-shape spines, while RLC-AD increases the percentage of filopodia-like spines. Spine morphology distribution of a representative culture is shown. Error bars represent SEM. *$p<0.001$, Mann Whitney test (B,D,F), t-test (G). Scale bar = 5μm for all panels.

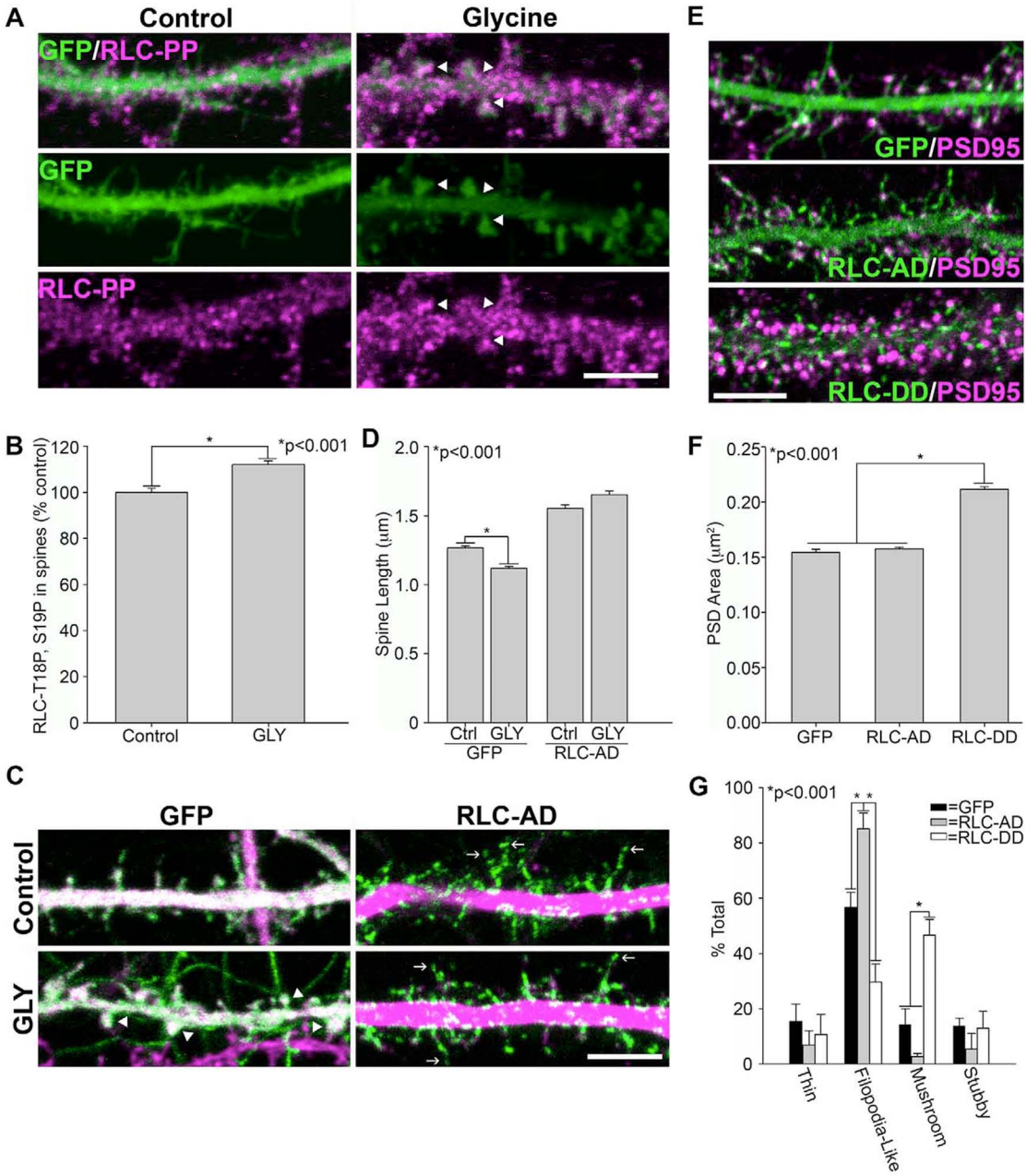

A
Control
Glycine
GFP/RLC-PP
GFP
RLC-PP
E
GFP/PSD95
RLC-AD/PSD95
RLC-DD/PSD95
B
RLC-T18P, S19P in spines (% control)
*p<0.001
Control
GLY
D
Spine Length (μm)
*p<0.001
Ctrl GLY Ctrl GLY
GFP RLC-AD
F
PSD Area (μm²)
*p<0.001
GFP
RLC-AD
RLC-DD
C
GFP
RLC-AD
Control
GLY
G
% Total
*p<0.001
=GFP
=RLC-AD
=RLC-DD
Thin
Filopodia-Like
Mushroom
Stubby

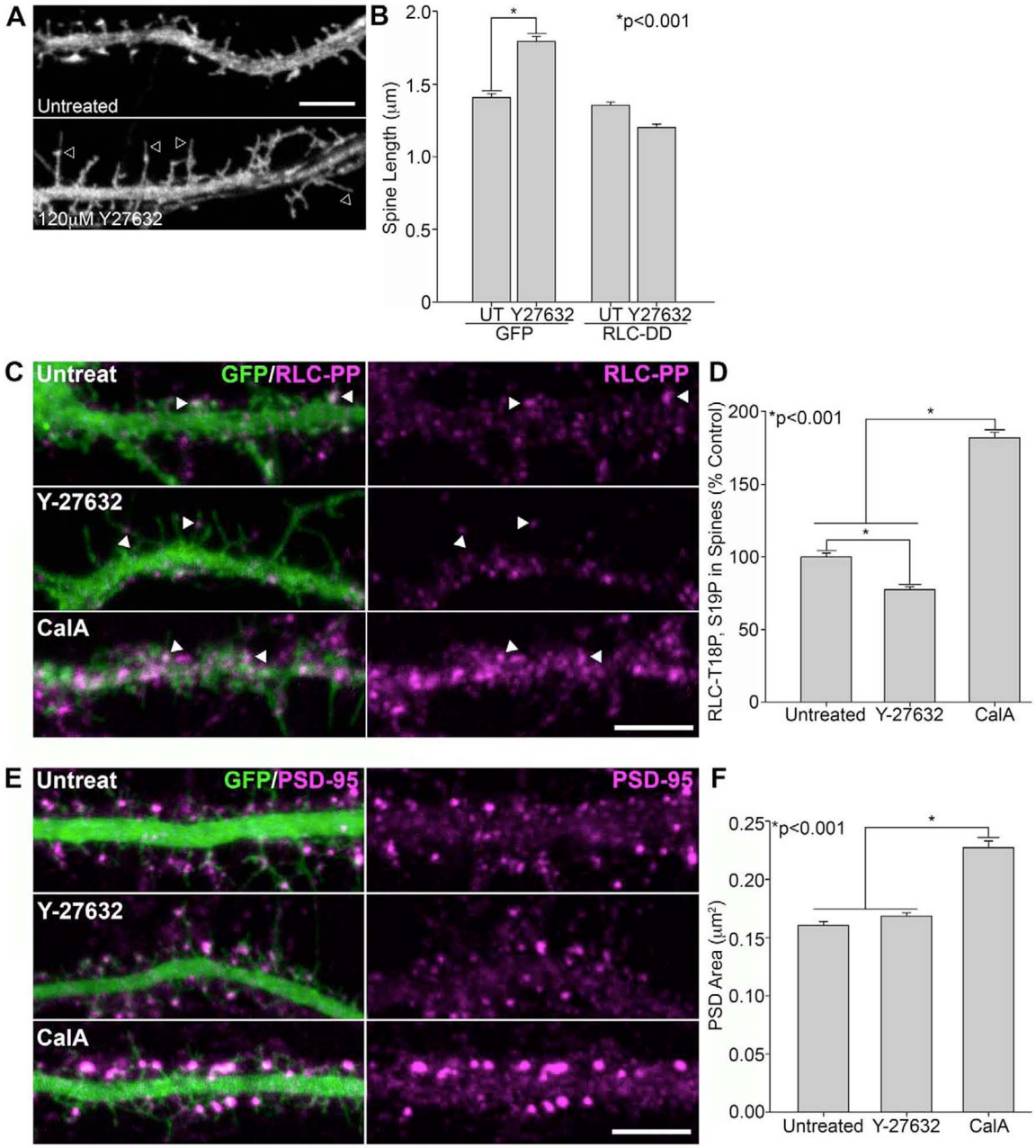
Figure 14: ROCK regulates spine morphology through RLC-T18, S19 di-phosphorylation.

A) ROCK inhibition (Y-27632) produces filopodia-like spines (arrowheads). DIV14 neurons expressing GFP were treated with 120μM Y-27632 for 2 hours or left untreated as a control.

B) RLC-DD prevents the increase in spine length with Y-27632 We analyzed 1199 spines from 13 GFP untreated neurons, 1056 spines from 9 GFP neurons treated with Y-27632, 1142 spines from 6 RLC-DD untreated neurons, and 809 spines from 8 RLC-DD neurons treated with Y-27632.

C) Y-27632 decreases endogenous RLC-T18, S19 di-phosphorylation concomitant with the formation of filopodia-like spines. In contrast, inhibition of myosin light chain phosphatase with calyculin A (CalA), increases RLC-T18, S19 di-phosphorylation. Arrowheads indicate spine-associated RLC-PP. Neurons were treated with 100μM Y-27632 for 2 hours or 20nM calyculin A for 20min or left untreated.

D) Y-27632 decreases the levels of spine-associated RLC-PP staining; whereas calyculin A increases it. We analyzed 855 spines from 10 untreated neurons, 901 spines from 9 Y-27632-treated neurons, and 989 spines from 9 calyculin A-treated neurons. E-F) Calyculin A increases PSD area in comparison with untreated or Y-27632-treated neurons. Neurons were treated as in C. We analyzed 499 PSDs from 10 untreated neurons, 519 PSDs from 9 Y-27632-treated neurons, and 452 PSDs from calyculin A-treated neurons. Error bars represent SEM. *$p<0.001$, Mann-Whitney test. Scale bar = 5μm.


A
Untreated
120μM Y27632
B
*p<0.001
Spine Length (μm)
2.0
1.5
1.0
0.5
0
UT Y27632
GFP
UT Y27632
RLC-DD
C
Untreat
GFP/RLC-PP
RLC-PP
Y-27632
CalA
D
*p<0.001
RLC-T18P, S19P in Spines (% Control)
200
150
100
50
0
Untreated
Y-27632
CalA
E
Untreat
GFP/PSD-95
PSD-95
Y-27632
CalA
F
*p<0.001
PSD Area (μm²)
0.25
0.20
0.15
0.10
0.05
0.00
Untreated
Y-27632
CalA

shown) (Potier et al., 1995). Furthermore, expression of the di-phosphomimetic RLC-D,D mutant superseded the effects of Y-27632 on spine length, suggesting that RLC is a major post-synaptic target of ROCK activity (Figure 14B). Using an antibody specific for di-phosphorylated RLC T18P, S19P, we observed an ~20% decrease in the post-synaptic levels of di-phosphorylated RLC with Y-27632, coincident with an increase in spine length (Figure 14C-D). Calyculin A, which inhibits myosin light chain phosphatase (Iizuka et al., 1999) increased RLC-P,P and induced the formation of mushroom-shaped spines with enlarged PSDs (Figure 14C-F). Thus, post-synaptic regulation of RLC di-phosphorylation underlies spine maturation.

Myosin IIB Regulates Post-Synaptic Density Organization

The PSD is a highly ordered, yet dynamic structure, undergoing continual variations in morphology (Blanpied et al., 2008). We therefore asked whether actomyosin activity regulated the size, shape, or location of the PSD in the spine. To study PSD morphology, we stained for the PDZ-containing synaptic scaffold protein PSD-95, which is a canonical PSD marker that appears early during PSD formation (Rao et al., 1998). Whereas control spines exhibit a compact, round, or slightly elliptical PSD, MIIB knockdown spines displayed an elongated PSD with larger perimeters (Figure 15A-C). Furthermore, in control cells, PSD-95 localizes mainly to the spine tip; however, in MIIB-deficient neurons, the elongated PSD localizes away from the spine tip and base, toward the center of the filopodia-like spine (Figure 15D, E). Similar results were observed using another PSD marker, shank (Figure 15F) (Böckers et al., 2001), suggesting that MIIB controls the morphology of the PSD globally, rather than through

Figure 15: Myosin IIB regulates post-synaptic density morphology.

A) Myosin IIB knockdown alters PSD morphology and positioning. Hippocampal neurons were co-transfected on DIV 6 with GFP and either an shRNA vector against MIIB or a control empty vector and fixed and immunostained for endogenous PSD-95 at DIV 21.

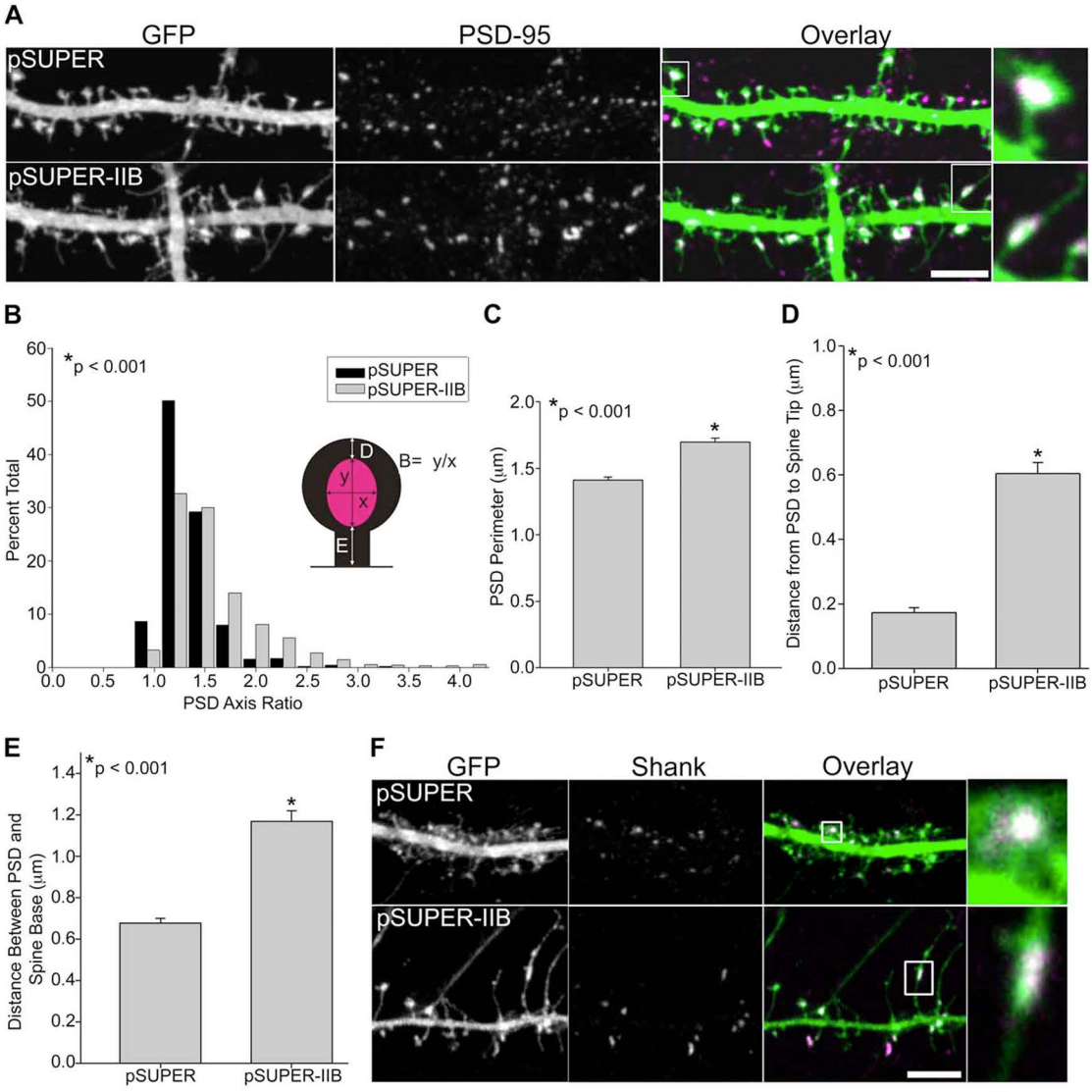
B) The PSD axis ratio (B) is expressed as the long axis (y) of each PSD divided by the short axis (x). The PSD axis ratio is significantly greater in neurons with MIIB knocked down.

C) shRNA knockdown of MIIB increases the PSD perimeter.

D) Distance from PSD-95 to the spine tip (D in diagram) is significantly greater in neurons with MIIB knocked down.

E) Distance from PSD-95 to the spine base (E in diagram) is significantly greater in neurons with MIIB knocked down. For each condition, 524-738 spines of 10-14 neurons were analyzed.

F) Immunostaining for Shank confirms the elongated PSD morphology in response to MIIB knockdown. Error bars represent SEM. *$p<0.001$, Mann-Whitney test. Scale bar = 5μm.


A
GFP
PSD-95
Overlay
pSUPER
pSUPER-IIB
B
*p < 0.001
pSUPER
pSUPER-IIB
Percent Total
PSD Axis Ratio
D
y
x
E
B= y/x
C
*p < 0.001
PSD Perimeter (μm)
pSUPER
pSUPER-IIB
D
*p < 0.001
Distance from PSD to Spine Tip (μm)
pSUPER
pSUPER-IIB
E
*p < 0.001
Distance Between PSD and Spine Base (μm)
pSUPER
pSUPER-IIB
F
GFP
Shank
Overlay
pSUPER
pSUPER-IIB

specific effects on some of its constituents.

Discussion

Non-muscle myosin II plays a major role in the organization of actin filaments and dictates the diverse morphologies and directional movement of various cell types. These include the apical constriction of epithelial cells, nuclear positioning, orientation of the microtubule-organizing center, Golgi and the contractile ring of dividing cells, and polarization of migrating fibroblasts (Vicente-Manzanares et al., 2009c). Of the MII isoforms, MIIB is the predominant one found in hippocampal neurons, and its activity and effective affinity for actomyosin filaments is regulated by RLC (Kawamoto and Adelstein, 1991; Vicente-Manzanares et al., 2009c). Previous studies have implicated MIIB as a target of a signaling pathway that is mutated in non-syndromic mental retardation and in spine development and memory formation (Zhang et al., 2005; Ryu et al., 2006; Vicente-Manzanares et al., 2009b; Rex et al., 2010). We now address the mechanisms by which MIIB acts on spines and show that differential MIIB activity determines where spines form, creates diverse post-synaptic spine morphologies, and mediates the morphology, size, and positioning of the PSD. It also mediates the changes in spine morphology in response to stimuli. Thus, MIIB emerges as a major downstream regulator of the component processes underlying post-synaptic plasticity, and implicitly, learning and memory.

Spine maturation consists of three stages: emergence of protrusions along the dendritic shaft, spine elongation, and maturation into a mushroom-shape (Yuste and Bonhoeffer, 2004). Our results demonstrate that differential MIIB activity mediates and

coordinates these diverse stages of spine development. Highly branched and dynamic spines emerge along the dendritic shaft and proceed to develop into the long dendritic protrusions that characterize immature spines, which persist in the absence of full, i.e., di-phosphorylated RLC, MIIB activation. This suggests that MIIB normally functions to restrict membrane protrusion and branching (Lin et al., 1994; Medeiros et al., 2006). It also suggests that the elongation of filopodia-like protrusions occurs in the absence of strong MIIB contractile activity. Several observations support this hypothesis. Myosin IIB inhibition or knockdown produces numerous long filopodia that do not mature (Ryu et al., 2006). In addition, the contractile-deficient myosin IIB mutant, R709C, cross-links but does not contract actin and results in persistently long spines. Similarly, inhibition of RLC T18, S19 di-phosphorylation by expressing RLC T18A, S19D or inhibiting ROCK activity using Y-27632 similarly produces filopodia-like spine precursors; however we cannot exclude contributions from other ROCK targets, like LIMK1 (Meng et al., 2002; Tashiro and Yuste, 2004; Shi et al., 2009).

Excitatory stimulation increases PSD size, which directly correlates with synaptic strength and leads to long-term potentiation (Kasai et al., 2003; Lynch et al., 2007; Holtmaat and Svoboda, 2009). MIIB determines PSD positioning as well as its morphology. When MIIB is inhibited, the PSD becomes elongated and is no longer at the spine tip. An analogous change is seen in migrating fibroblasts, where large central adhesions tend to disperse when MII activity is inhibited (Galbraith et al., 2002; Chen, 2008; Choi et al., 2008). In addition, increased myosin IIB activity via RLC T18, S19 di-phosphorylation, enlarges both the PSD and fibroblast adhesions (Vicente-Manzanares and Horwitz, 2010). In this context, the combination of crosslinking and contraction

induced by MII activity, likely serves to cluster the numerous PDZ- and SH3-domain containing actin binding proteins found within the PSD (Hung and Sheng, 2002; Kim and Sheng, 2004; Collins et al., 2006). MIIB-generated forces could also increase PSD size by inducing conformational changes in PSD components that present new binding sites for the recruitment of additional molecules, as also reported in fibroblasts (Sawada et al., 2006; Del Rio et al., 2009).

During post-synaptic development, changes in spine morphology correlate with changes in PSD organization and synaptic signaling. Specifically, maturation of spines into a mushroom-shape and PSD enlargement at the spine tip enhance the synaptic signaling that underlies learning and memory formation (Lynch et al., 2007). Our findings show that myosin IIB coordinates the spine and PSD morphological changes that occur in response to excitatory stimulation. Furthermore, differential regulation of MIIB activity through RLC phosphorylation states switches spine and PSD shape from filopodia-like spine precursors with smaller PSDs to mature mushroom-shape spines with larger PSDs. Thus, myosin IIB serves as a critical regulator of post-synaptic plasticity, consistent with the observation that myosin IIB is necessary for memory formation (Rex et al., 2010).

Our observations and previous literature lead to a model for the role of MIIB in spine formation and maturation. Spines form in regions of inactive MIIB and can extend into long filopodia-like structures in the absence of high MIIB activity. The most likely mechanism for this formation and extension is due to localized activation of Rac. The GIT1/PIX/PAK complex, which contains the Rac-activator PIX and Rac-effector PAK, is one mechanism by which Rac activation is localized to generate spines (Zhang et al.,

2003; 2005). These filopodia-like spines are highly dynamic and protrude and retract frequently; since MIIB is not required for this activity, it is likely that this arises largely from actin polymerization and depolymerization. In contrast, the maturation into a compact, mushroom-shaped structure requires MIIB contractile activity; however, Arp2/3-driven actin polymerization may contribute as well to drive spine head expansion, in analogy with the broad protrusions it mediates in migrating fibroblasts (Rácz and Weinberg, 2008; Hotulainen et al., 2009; Korobova and Svitkina, 2010). Finally, MIIB may also serve to localize signals that affect spine morphology and function, such as GEFs that mediate Rac activity, e.g., β-PIX and Kalirin-7, or other mechanoresponsive molecules that regulate signaling in other cell types (Zhang et al., 2005; Xie et al., 2007; Kuo et al., 2011). Our holistic view of the effect of myosin II on the component processes of post-synaptic development provides the framework for the identification of critical therapeutic targets, such as ROCK, for the treatment of learning and memory disorders.

Materials and Methods

Antibodies and reagents. Postsynaptic density-95 (PSD-95) monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at ratio of 1:100 for immunostaining. Non-muscle myosin heavy chain II-B polyclonal antibody was obtained from Covance (Emeryville, CA) and used at a ratio of 1:1000. A polyclonal antibody against phosphorylated RLC-T18, S19 was purchased from Cell Signaling Technologies and used at a ratio of 1:100-1:200 (Danvers, MA). Secondary anti-mouse and anti-rabbit antibodies conjugated to Alexa488, 568 and 647 were from Invitrogen.

Blebbistatin, Calyculin A, and Y-27632 were purchased from Calbiochem (La Jolla, CA) and used at the concentrations indicated in the figures. Tetrodotoxin and strychnine were purchased from Sigma and reconstituted in dH_2O.

Plasmids. The shRNA knockdown vector for MIIB has been described elsewhere (Vicente-Manzanares et al., 2007). GFP-MIIB was a gift from Robert S. Adelstein (Wei and Adelstein, 2000). RNAi-insensitive GFP-MIIB and GFP-MIIB-R709C mutants have been described previously (Vicente-Manzanares et al., 2007). The 3'-UTR encompassing 1500nt's was cut out of both GFP-MIIB and GFP-MIIB-R709C vectors using XmaI restriction enzyme. The 1.5kb DNA piece was ligated into the 9kb vector backbone and sequenced to verify correct orientation of the insert. PSD-95-GFP was a gift from David Bredt (Topinka and Bredt, 1998). RLC-GFP constructs (WT, DD) were kindly provided by Kathleen Kelly (National Cancer Institute, Bethesda, MD), and RLC-AD-GFP was generated as previously described (Vicente-Manzanares and Horwitz, 2010).

Neuronal culture and transfection. Low-density hippocampal cultures were prepared from E19 rat embryos as described previously. All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the University of Virginia Animal Care and Use Committee (Protocol Number: 2884). Neurons were plated on glass coverslips coated with 1 mg/ml poly-L-lysine at an approximate density of 70 cells/mm^2 and were transfected using a modified calcium phosphate precipitation method as described previously (Zhang et al., 2005). Cortical neurons were nucleofected with DsRed as described by (Zeitelhofer et al., 2007), and plated on poly-L-lysine coated imaging dishes. DIV 5-12 cortical neurons were micropipetted with 100uM-1mM blebbistatin for

10 msec-1 sec with 5psi pressure using an IM 300 Microinjector from Narishige International USA, Inc. (East Meadow, NY). For the chemical stimulation experiments involving knockdown or inhibition of MIIB (Figure 11), DIV14-17 neurons were removed from the glia-feeder layer and placed in 1 X Mg^{2+}-free extracellular solution containing 15mM NaCl, 0.5mM KCl, 0.2mM $CaCl_2$, 3mM glucose, 1mM Hepes, 0.5uM tetrodotoxin, and 1uM strychnine, pH7.4 (Park et al., 2004). Stimulated neurons are treated with 200uM glycine and incubated at 35°C, 5% CO_2 for 3 min. The solution is removed and replaced with 1 X Mg^{2+}-free extracellular solution with tetrodotoxin and strychnine and incubated at 35°C, 5% CO_2 for 20 minutes before fixation. For inhibition of MIIB activity with blebbistatin, neurons were pre-treated for 30 minutes and throughout the protocol with either 100μM blebbistatin or a corresponding volume of DMSO as a control. Alternatively (Figure 13), neurons were chronically treated with 100uM of the NMDA receptor antagonist, AP-5, from DIV 6-21 to inhibit NMDA receptor activation and spine maturation. Neurons were then stimulated by AP-5 withdrawal and 200uM glycine, while control neurons continued in the presence of AP-5 (200uM), as described by others (Liao et al., 2001; Lin et al., 2004).

Immunocytochemistry. Neurons were fixed in PBS containing 4% formaldehyde, methanol-free, ultra-pure EM grade (Polysciences, Inc., Warrington, PA) with 4% sucrose for 20 min at room temperature and permeabilized with 0.2% Triton X-100 for 10 min. Alternatively, for PSD-95 and RLC-T18P, S19P staining, neurons were simultaneously fixed and permeabilized in 2% formaldehyde with 4% sucrose for 10 min at room temperature and then with cold methanol for 10 min at -20°C. After blocking with 20% goat serum/PBS for one hour at room temperature, the neurons were incubated

with the appropriate antibodies in 5% goat serum/PBS for one hour at 37°C. RLC-PP staining was performed in PBS only. Coverslips were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA).

Imaging and analysis. Confocal images were collected on an Olympus Fluoview 1000 microscope (IX81 base) equipped with a 60X/1.35 NA (oil) UPLSAPO 60X objective (Olympus). Green probes (GFP and Alexa488) were excited using the 488 nm laser line of a multi Ar laser; red probes (DsRed2 and Alexa568) were excited with the 543 nm laser line of a He-Ne laser; the far-red probe Alexa647 was excited with the 635 nm line of an LD laser. Fluorescence emission was collected using the following dichroic mirror/filter combinations: SDM560/BA505-525 (GFP), SDM640/BA560-620 (DsRed2, Alexa568 and RhodamineX) and BA655-755 (Alexa647). Two-color fluorescence images of Alexa488 (GFP)/Alexa568 (RhodamineX/DsRed2) were collected in a Z-stack and in sequential mode. Images were acquired using Fluoview software (Olympus). Spine length, width, PSD-95 long and short axis, area, and perimeter were quantified using Image J software. Statistical analysis was performed using Sigma Plot 11. Spine morphologies were defined as either filopodia-like, thin, mushroom, or stubby (Yuste and Bonhoeffer, 2004). Filopodia-like spines are long and thin without a spine head, whereas thin spines contain a small head at the spine tip. Mushroom-shaped spines are shorter with a large spine head atop a neck. Stubby spines are short protrusions, either thin or wide, with no discernable neck. Statistical analysis of spine morphology in Figures 1, 3 and 4 were performed with SAS 9.2.

Addendum

These studies are unpublished research collected after submission, which supports our published findings.

Myosin IIB regulates clustering and localization of post-synaptic molecules

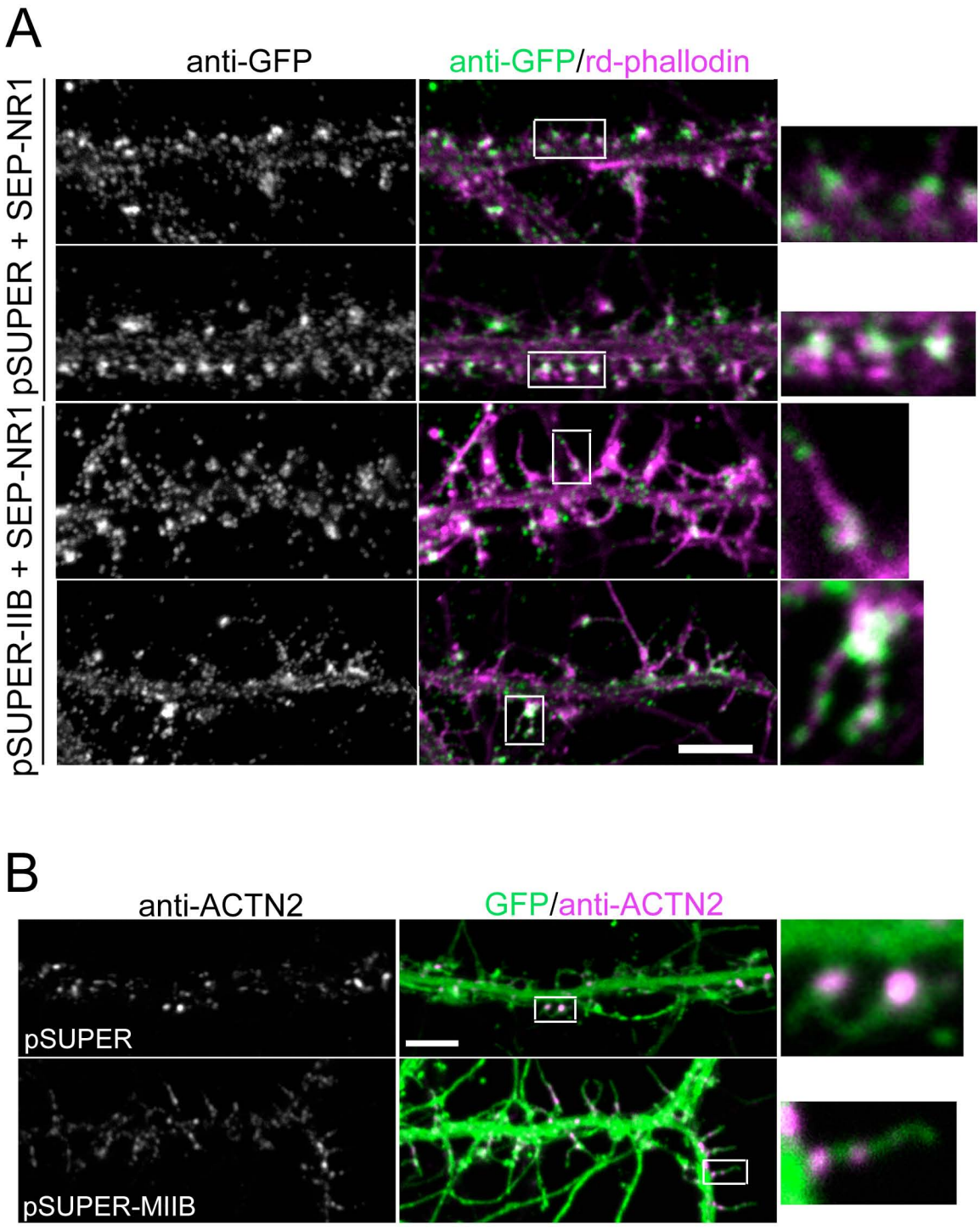
As previously shown, MIIB regulates organization of the post-synaptic density. Knockdown of MIIB in neurons causes the PSD to elongate in morphology and become mis-localized away from the spine tip (Hodges et al., 2011). NMDA-type glutamate receptors localize to the post-synaptic membrane and are clustered by PSD-95 (Lim et al., 2003). Therefore, we sought to determine whether mis-organization of PSD-95, via loss of MIIB activity, affects NMDA-type glutamate receptor localization and clustering within the spine. We co-expressed the ubiquitous NR1 subunit of the NMDA receptor fused to a super-ecliptic pHluorin (SEP-NR1), which displays GFP fluorescence at the membrane surface when SEP is exposed to a neutral environment (Kopec et al., 2006). Rhodamine-phalloidin, which clearly shows actin-rich spines, was used to visualize them. While a discrete cluster of SEP-NR1 was seen in the spine heads of control neurons, SEP-NR1 was distributed into punctate clusters throughout the filopodia-like spines of neurons lacking MIIB (Figure 16A). Larger clusters of SEP-NR1 were seen at the base of the filopodia-like spines, mis-localized from the tip of the spine, in neurons with MIIB knocked down. Therefore, as a consequence of a mis-organized PSD, due to MIIB knockdown, the NMDA-type glutamate receptor is no longer clustered appropriately at the post-synaptic membrane. This illustrates the importance of an organized actin network dictated by MIIB to properly localize and cluster glutamate receptors at the post-synaptic membrane.

The actin cross-linker α-actinin-2 (ACTN2) directly binds to NMDA-type glutamate receptors *in vitro* and this interaction is proposed to couple the glutamate receptors to the actin cytoskeleton (Wyszynski et al., 1997). Therefore, we sought to

Figure 16: Myosin IIB regulates clustering of NMDA receptors and α-actinin-2.

A) MIIB regulates clustering and localization of NMDA-type glutamate receptors. Hippocampal neurons were co-transfected with SEP-NR1 and either an shRNA vector against MIIB or a control empty vector at DIV 6 and fixed and immunostained for GFP and rhodamine-phallodin at DIV 21. Boxed region is enlarged to right. Scale bar = 5 μm.

B) MIIB regulates clustering and localization of α-actinin-2 (ACTN2). Hippocampal neurons were co-transfected with GFP and either an shRNA vector against MIIB or a control empty vector at DIV 6 and fixed and immunostained for endogenous ACTN2 at DIV 21. Boxed region is enlarged to right. Scale bar = 5 μm.

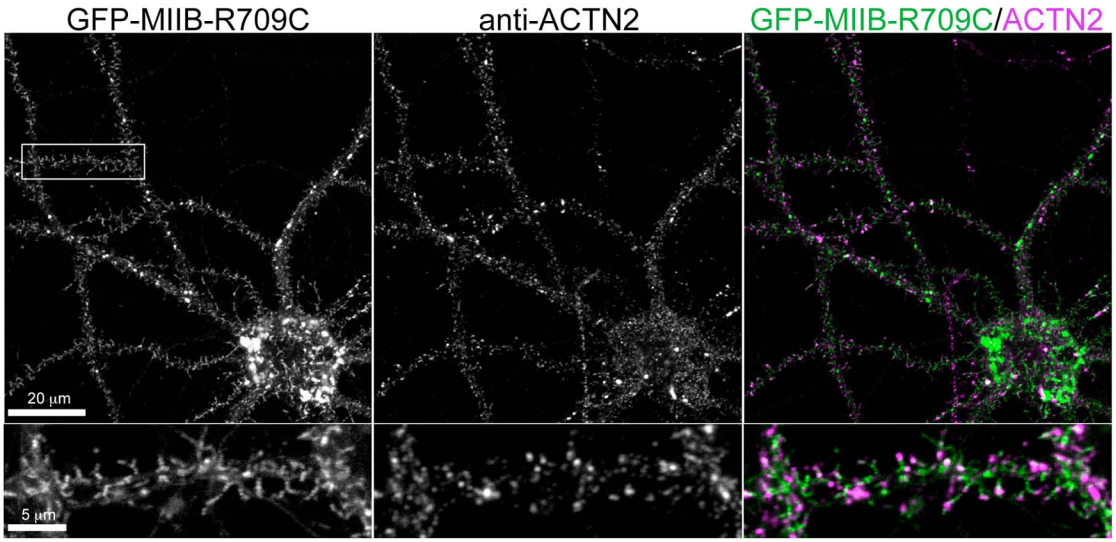

A
anti-GFP
anti-GFP/rd-phallodin
pSUPER + SEP-NR1
pSUPER-IIB + SEP-NR1
B
anti-ACTN2
GFP/anti-ACTN2
pSUPER
pSUPER-MIIB

determine whether MIIB activity had an effect on the localization of ACTN2 in the spine. In comparison to control neurons where ACTN2 localized close to the spine tip within the spine head, ACTN2 was also mis-localized away from the spine tip in the filopodia-like protrusions of neurons with MIIB knocked down (Figure 16B). Interestingly, ACTN2 was distributed into multiple, small clusters in spines of neurons lacking MIIB, whereas ACTN2 distributed as a single, large cluster in the spine heads of control neurons (Figure 16B). This indicates that the loss of MIIB has an effect on the distribution of other actin-associated molecules in the spine.

Since knockdown of MIIB affects the clustering and distribution of ACTN2, we asked whether these two actin cross-linking molecules, ACTN2 and MIIB, co-localize in dendritic spines. We specifically assayed localization of endogenous ACTN2 with respect to the non-contractile MIIB-R709C mutant. As expected, GFP-MIIB-R709C localized to the spine neck and base (Figures 17 and 12). Interestingly, we saw little overlap between ACTN2 and GFP-MIIB-R709C (Figure 17). ACTN2 localized to the tip of most dendritic protrusions (Figure 17), similar to PSD-95 (Figure 12). Some co-localization could be seen between the base of ACTN2 and the tip of GFP-MIIB-R809C. These results suggest that ACTN2 localizes predominantly to the PSD and not with actomyosin filaments.

Figure 17: MIIB and ACTN2 do not co-localize in dendritic spines.

Hippocampal neurons were transfected at DIV 6 with GFP-MIIB-R709C and fixed and immunostained for endogenous ACTN2 at DIV 21. Upper images were taken with 2x zoom and lower images (boxed region) were taken with 6x zoom.


GFP-MIIB-R709C
anti-ACTN2
GFP-MIIB-R709C/ACTN2
20 μm
5 μm

Chapter 4 -- α-Actinin-2 dictates spine morphology and nucleates assembly of the post-synaptic density

This chapter is comprised of unpublished data.

Abstract

Abundant evidence indicates that modulation of the actin cytoskeleton dictates the morphological changes associated with dendritic spine dynamics, which functions as the structural basis underlying learning and memory. Dendritic spines are micron-sized protrusions that serve as the primary post-synaptic sites of excitatory neurotransmission in the brain. Spines mature from a filopodia-like morphology into a mushroom-shape with an enlarged post-synaptic density (PSD). This electron-dense PSD contains an assembly of synaptic adhesion molecules, glutamate receptors, and signaling scaffolds; many of which respond to glutamate receptor activation and relay signals to the underlying actin cytoskeleton to induce structural changes in spine and PSD morphology. α-actinin-2 (ACTN2) cross-links actin filaments, localizes to dendritic spines, and is enriched with the post-synaptic density. We show that loss of ACTN2 creates an increased density of immature, filopodia-like protrusions that fail to mature into a mushroom-shaped spine during development and in response to chemical stimulation of the glutamate NMDA receptor. Knockdown of ACTN2 prevents the recruitment and stabilization of a PSD in the spine, resulting in the loss of NMDA receptors and synaptic formation. Furthermore, phosphorylation within its actin-binding domain can create distinct spine morphologies. These observations support a model whereby ACTN2 nucleates PSD formation in the spine to mediate synaptogenesis and promote spine maturation.

Introduction

Dendritic spines are micron-sized, actin-rich protrusions that constitute the post-synaptic sites of excitatory glutamatergic neurotransmission in the mammalian brain (Matus, 2000; Bosch and Hayashi, 2011). Spine morphology and organization of the post-synaptic density (PSD), which contains a dense cluster of adhesion molecules, glutamate receptors, and signaling scaffolds at the post-synaptic membrane, underlies the molecular basis of learning and memory (Nimchinsky et al., 2002; Sheng and Hoogenraad, 2007). Spines develop from an immature filopodia-like structure, lacking a PSD, into a mature mushroom-shaped morphology containing an enlarged PSD, that is in synaptic contact with a pre-synaptic bouton (Yuste and Bonhoeffer, 2004; Ethell and Pasquale, 2005; Sekino et al., 2007). Actin filaments are the primary structural determinant of spines, and its remodeling in response to NMDA-receptor activation is critical for spine plasticity (Allison et al., 1998; Rao and Craig, 2000; Brünig et al., 2004; Okamoto et al., 2004; Cingolani and Goda, 2008; Honkura et al., 2008; Hotulainen and Hoogenraad, 2010). Several genes encoding post-synaptic molecules that modulate the architecture of the actin cytoskeleton are mutated in non-syndromic mental retardation, autism, and schizophrenia (Allen et al., 1998; Kaufmann and Moser, 2000; Fiala et al., 2002; Carlisle and Kennedy, 2005; Penzes et al., 2011). Thus, gaining insight into the mechanisms that directly regulate actin filament dynamics in dendritic spines is crucial to understanding the cellular foundation of cognition.

Actin filament bundling by α-actinin is implicated in a variety of cellular structures such as focal adhesions, adherens junctions, and dendritic spines (Otey and Carpen, 2004). α-Actinin has an actin binding domain at its N-terminus, followed by four tandem spectrin repeats, and a calmodulin-like domain at its C-terminus that determines

each isoform's calcium sensitivity (Dixson et al., 2003; Broderick and Winder, 2005; Sjöblom et al., 2008). The functional molecule exists as an antiparallel homodimer with an actin-binding site on either end that mediates actin filament cross-linking (Djinović-Carugo et al., 1999). Although three of the four α-actinin isoforms, α-actinin-1, -2, and -4, have been identified in rat forebrain post-synaptic density fractions via mass spectrometry (Walikonis et al., 2000; Peng et al., 2004), immunofluorescence and electron microscopy studies have only shown isoform 2 (ACTN2) enriched in the post-synaptic density of glutamatergic, excitatory synapses in pyramidal neurons of the cortex and hippocampus (Wyszynski et al., 1997; 1998; Dunah et al., 2000). The sub-cellular localization of ACTN2 to dendritic spines is dependent on its interaction with actin (Allison et al., 2000; Nakagawa et al., 2004).

In addition to cross-linking actin filaments, α-actinin can interact with various membrane-associated proteins and link them to the actin cytoskeleton, including vinculin, integrins, and α-catenin (Belkin and Koteliansky, 1987; Wachsstock et al., 1987; Otey et al., 1990; Knudsen et al., 1995; Otey and Carpen, 2004). In synapses, *in vitro* binding assays suggest a direct interaction occurs between ACTN2 and the NR1 and NR2B subunits of the NMDA receptor, which is competitively inhibited by Ca^{2+}-bound calmodulin binding to NR1 (Wyszynski et al., 1997; Krupp et al., 1999). Although these interactions have not been shown to occur in intact neurons, the biochemical data proposes that ACTN2 couples the NMDA receptor to the actin cytoskeleton promoting calcium influx, until Ca^{2+}-bound calmodulin inhibits their interaction and inactivates the NMDA receptor (Krupp et al., 1999). The significance of the calcium insensitivity exhibited by ACTN2 is illustrated by the finding that Ca^{2+}-sensitive, α-actinin isoforms

cannot prevent NMDA receptor inactivation *in vitro*, as they dissociate from actin in response to calcium (Krupp et al., 1999). *In vitro* binding assays also indicate that ACTN2 can bind to the PSD-enriched molecule, densin-180, and form a ternary complex with CaMKIIα, and NR2B (Robison et al., 2005b), suggesting that ACTN2 can nucleate a CaMKII-driven signaling scaffold at the post-synaptic membrane that regulates glutamate receptors. In support for this, ACTN2 targets CaMKIIα to F-actin in HEK293 cells and enhances the interaction between CaMKII and GluN2B subunits of AMPA receptors (Jalan-Sakrikar et al., 2012). Although these interactions with ACTN2 are speculative, it points to a possible role for ACTN2 in integrating signals between PSD components and the actin cytoskeleton, and highlights the importance for determining the function of ACTN2 in spine morphogenesis and post-synaptic organization.

α-Actinin can be regulated *in vitro* to modify its actin-binding properties. PtdIns(4,5)P_2**,** PIP2, binds to the actin-binding domain of ACTN2 and tethers it to the plasma membrane, a function that is critical for maintaining the open state of the NMDA receptor in *Xenopus* oocytes (Michailidis et al., 2007). Neurons expressing an ACTN2 mutant unable to interact with PIP2 significantly reduced peak and steady-state NMDA current compared to neurons expressing *wild-type* ACTN2, suggesting that ACTN2 can't link NMDA receptors to the actin cytoskeleton and promote calcium influx without binding to PIP2 at the membrane (Michailidis et al., 2007). Focal adhesion kinase (FAK) phosphorylates α-actinin-1 in fibroblasts, which induces its dissociation from actin and promotes adhesion turnover (Izaguirre et al., 1999; 2001; Wichert et al., 2003). All α-actinin isoforms share this conserved tyrosine residue within their actin-binding domain, and FAK is an important signaling molecule in dendritic spines (Moeller et al., 2006),

and could therefore play a role in regulating ACTN2-mediated reorganization of actin to sculpt dendritic spines. Thus far, the putative interactions between components of the PSD and ACTN2, and its implication in modulating NMDA receptor activity have all been based on *in vitro* binding assays and studies in non-neuronal cells. One study found that overexpression of ACTN2 increased the length and density of dendritic protrusions in cultured hippocampal neurons, suggesting a biological role for ACTN2 in determining spine morphology (Nakagawa et al., 2004). However, over-expression studies can produce non-specific effects in cells. Despite recognition of its post-synaptic enrichment and possibly coupling membrane-associated proteins to the actin cytoskeleton, additional studies must be performed to gain a clear understanding of a function for ACTN2 in spine morphogenesis and confirm its interaction with post-synaptic proteins.

To ascertain a biological function for ACTN2 in shaping dendritic spines and organizing the post-synaptic density, we knocked down ACTN2 in hippocampal neurons via small hairpin (sh) RNA. We find that loss of ACTN2 induces an increased spine density composed of filopodia-like spine morphologies that does not mature in response to chemical stimulation. These immature spines lack a post-synaptic density and a functional synapse. Importantly, these effects could be rescued with co-expression of RNAi-resistant ACTN2. Loss of ACTN2 at later stages in spine development when synaptic contact has been established also induces an increased density of immature spines that lack a synapse, suggesting that ACTN2 is not only required for the recruitment of post-synaptic molecules, but is also required for the maintenance of the synapse. In agreement with previous findings (Nakagawa et al., 2004), over-expression of ACTN2 creates a thinner spine morphology, but expression of a non-phosphorylatable

ACTN2-Y19F mutant restores the mature, mushroom-shaped spine morphology, suggesting that ACTN2 can be regulated by phosphorylation on its actin-binding domain to create distinct spine morphologies. These studies lead us to propose a model for spine morphogenesis, whereby ACTN2 re-organizes the actin cytoskeleton in filopodia-like dendritic protrusions to promote assembly of the PSD in the spine, and mediate its transition to a mature, mushroom-shaped morphology via scaffolding PSD molecules to the actin cytoskeleton.

Results

ACTN2 Regulates Spine Morphology and Density

ACTN2 is specifically enriched in hippocampal neurons and is not in the surrounding glia cells (Figure 18A). Moreover, an antibody is specific for ACTN2 and does not cross-react with α-actinin isoforms 1 and 4, which are enriched within CHO-K1 and COS-7 cells (Figure 18A). ACTN2 localizes to dendritic spines and does not co-localize with the pre-synaptic molecule, synaptophysin, indicating that ACTN2 is solely enriched within the post-synaptic side of synapses (Figure 18B, C). To determine the role of ACTN2 in dendritic spine morphogenesis, we silenced endogenous expression of ACTN2 with shRNA expressed by the pSUPER vector (Brummelkamp et al., 2002). The shRNA sequence targeting ACTN2 mRNA is isoform-specific, and 72hrs after transfection immunofluorescence indicates the shRNA expression significantly reduced endogenous ACTN2 protein levels (Figure 18D). Co-expression of human ACTN2-SS with a silent mutation in a serine residue of the target sequence, conferring resistance to RNA inhibition (RNAi), rescued expression (Figure 18D).

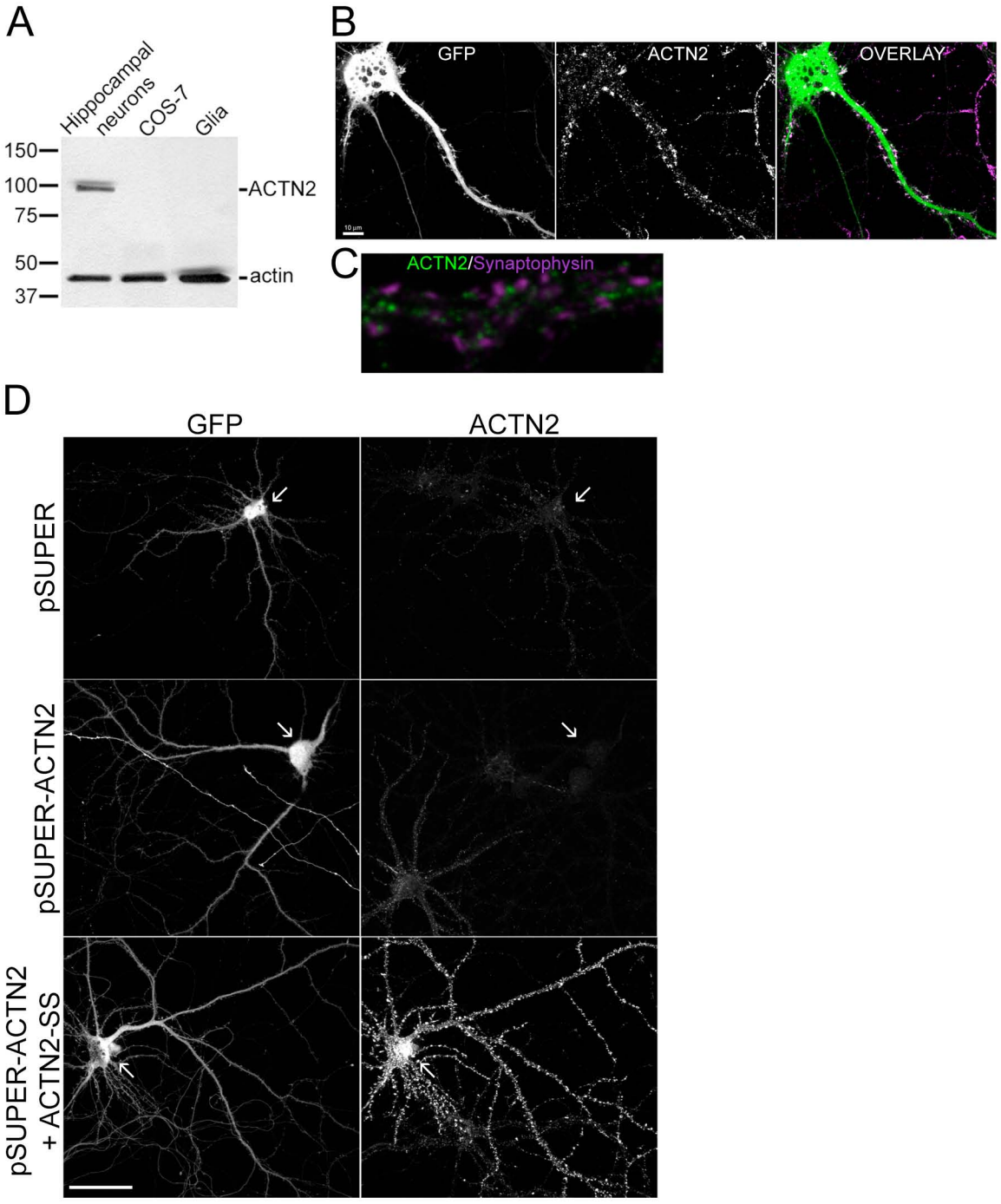
Figure 18: ACTN2 localizes to post-synaptic hippocampal neurons.

A) ACTN2 is enriched within hippocampal neurons and is not in glia cells or COS-7 cells. Cells were lysed and immunoblotted for ACTN2. Actin serves as a loading control.

B) ACTN2 localizes to dendritic spines. Hippocampal neurons were transfected at DIV 6 with GFP (green) and fixed and immunostained for endogenous ACTN2 (magenta) at DIV 16. Scale bar = 10 μm.

C) ACTN2 does not co-localize with a pre-synaptic marker. Hippocampal neurons were fixed at DIV 16 and immunostained for endogenous ACTN2 (green) and endogenous synaptophysin (magenta).

D) shRNA vector targeting ACTN2 is specific. Hippocampal neurons were co-transfected at DIV 14 with GFP and either a control empty vector or an shRNA vector against ACTN2, with or without an ACTN2 vector conferring resistance to RNAi, and fixed and immunostained for endogenous ACTN2 at DIV 17. Arrows point to the neurons expressing GFP and its immunostaining for ACTN2. Scale = 20 μm.


A
Hippocampal neurons
COS-7
Glia
150
100
75
50
37
-ACTN2
-actin
B
GFP
ACTN2
OVERLAY
10 µm
C
ACTN2/Synaptophysin
D
GFP
ACTN2
pSUPER
pSUPER-ACTN2
pSUPER-ACTN2 + ACTN2-SS

Chronic inhibition of ACTN2 by shRNA knockdown significantly increased spine density throughout development (Figure 19A, B). The spines on neurons with diminished ACTN2 expression were significantly longer and thinner (Figure 19A, C, D). While control neurons at days *in vitro* (DIV) 19-22 exhibited many spines with a "mushroom" morphology, consisting of a large bulbous spine head on top of a short spine neck, neurons with ACTN2 knocked down displayed significantly less mushroom-shaped spines and more headless, filopodia-like protrusions (Figure 19A). To determine that this phenotype was specific for knockdown of ACTN2 and did not arise from off-target effects, we co-transfected an RNAi-resistant ACTN2-SS with the shRNA at different days during development and fixed the neurons 72hrs later. At an early stage in spine development, DIV 11, spine density could be rescued to control numbers, but the spines still displayed an immature, filopodia-like morphology at this early time point (Figure 19E). At mid-development, DIV 16, spine density and the thin spine morphology, characterized by a small spine head on top of a long spine neck, could be rescued (Figure 19E). Finally, at later stages in development, DIV 20-24, spine density and the classic mushroom-shaped spine morphology could be rescued by exogenous expression of ACTN2-SS (Figure 19E). This suggests that the effects induced by shRNA-mediated knockdown of ACTN2 are specific and that ACTN2 is necessary for the proper development of spines.

ACTN2 is required for Spine Maturation in Response to NMDA Receptor Stimulation

Since neurons lacking normal levels of ACTN2 created an increased density of immature, filopodia-like protrusions that failed to develop into mushroom-shaped spines,

Figure 19: Inhibition of ACTN2 increases spine density and length and decreases width.

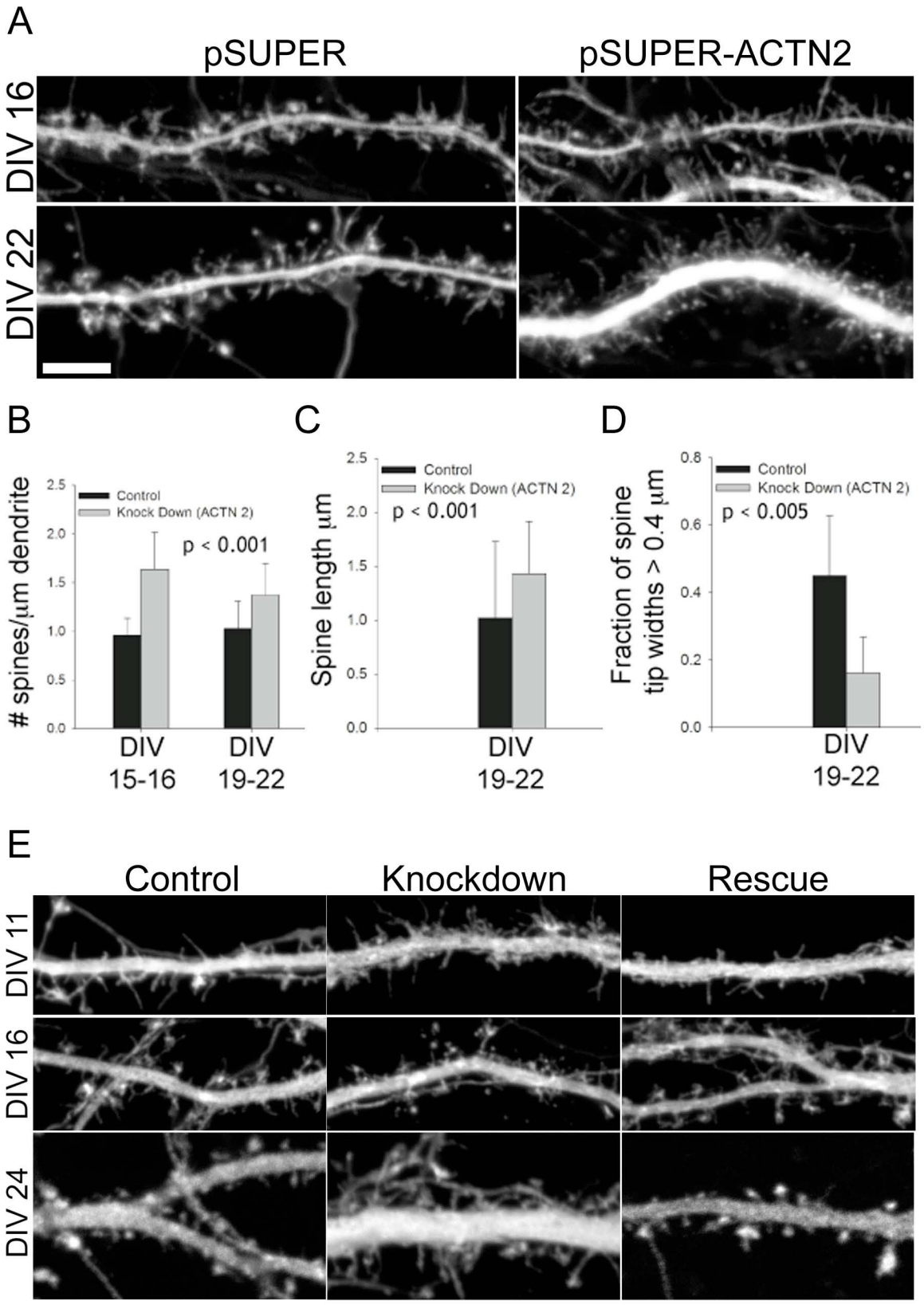
A) Chronic inhibition of ACTN2 causes an increase in spine density, spine length, and a decrease in spine head width. Hippocampal neurons were co-transfected at DIV 6 with GFP and either a control empty vector or an shRNA vector against ACTN2 and fixed on DIV 16 and DIV 22. Scale = 5 μm.

B) ACTN2 knockdown causes a significant increase in spine density. Error bars represent standard deviation.

C) ACTN2 knockdown causes a significant increase in spine length. Error bars represent standard deviation.

D) ACTN2 knockdown causes a significant decrease in the fraction of spine tip widths > 0.4 μm. Error bars represent standard deviation.

E) Spine density and morphology can be rescued. Hippocampal neurons were co-transfected at DIV 8, DIV 13, or DIV 21 with GFP and either a control empty vector or an shRNA vector against ACTN2, with or without an ACTN2 vector conferring resistance to RNAi, and fixed 72 hours later on DIV 11, DIV 16, or DIV 24.


A
pSUPER
pSUPER-ACTN2
DIV 16
DIV 22
B
spines/μm dendrite
Control
Knock Down (ACTN 2)
p < 0.001
DIV 15-16
DIV 19-22
C
Spine length μm
Control
Knock Down (ACTN 2)
p < 0.001
DIV 19-22
D
Fraction of spine tip widths > 0.4 μm
Control
Knock Down (ACTN 2)
p < 0.005
DIV 19-22
E
Control
Knockdown
Rescue
DIV 11
DIV 16
DIV 24

we hypothesized that ACTN2 was required for the acute, activity-induced spine morphology changes (Harris et al., 2003; Kopec et al., 2006). To test this, we selectively activated synaptic NMDA receptors with the co-agonist glycine (Lu et al., 2001; Park et al., 2004). As expected, 20 min following brief treatment with glycine (200 uM for 3 min), control neurons displayed a significant increase in the fraction of spines with wider heads and mushroom-shaped spines in comparison to unstimulated neurons (Figure 20A, B, C). In contrast, neurons with ACTN2 knocked down, under both conditions, continued to display an increased density of filopodia-like protrusions that failed to expand the spine head (Figure 20A, B, C). This demonstrates that ACTN2 is required for the acute morphogenesis to an enlarged, mushroom-shaped spine and corroborates our finding that ACTN2 is necessary for proper spine development.

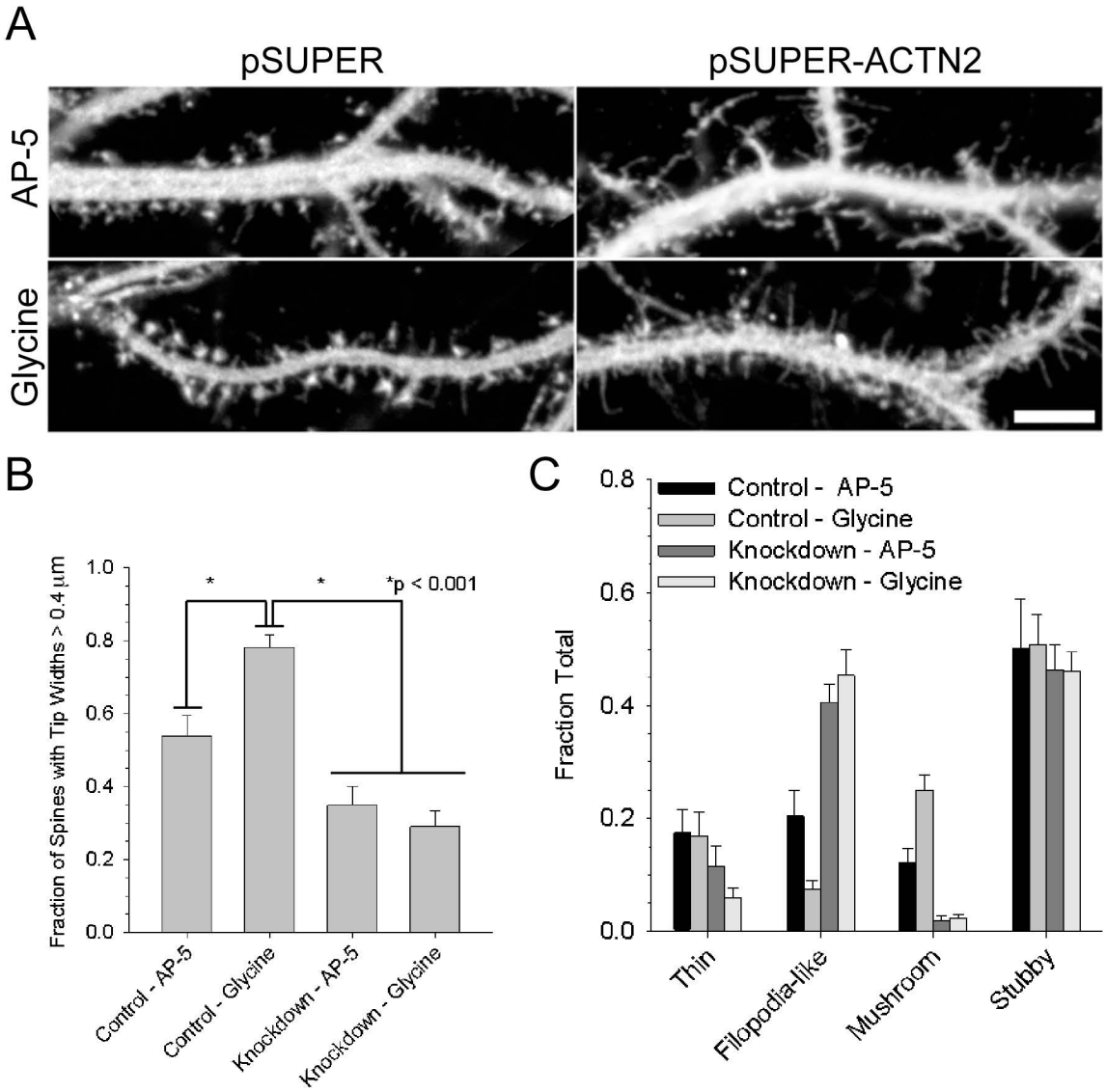
ACTN2 is required for Synapse Formation

The morphological changes associated with spine maturation require that the spine contacts a potentiated pre-synaptic bouton and for proper arrangement of post-synaptic molecules, which nucleate a signaling platform to orchestrate the structural response to NMDA receptor activation (Kennedy, 2000; Dalva et al., 2007; Chen et al., 2008). We previously found that inhibition of myosin IIB-mediated contractility in spines creates a mis-organized PSD and inhibited spine maturation in response to chemical stimulation (Hodges et al., 2011). PSD-95 is a key molecule regulating synaptic plasticity and a good marker for PDS location and organization; it is also found in synapses at early stages (Ehrlich et al., 2007). To address whether ACTN2 contributes

Figure 20: Knockdown of ACTN2 prevents spine morphological changes in response to NMDA receptor activation.

A) When ACTN2 is knocked down spines do not shorten or assume a ‘‘mushroom’’ morphology in response to glycine. DIV 21 neurons co-expressing GFP and either a control empty vector or an shRNA vector against ACTN2 were chronically treated with the NMDA receptor antagonist AP-5 to inhibit spine maturation. Neurons were acutely stimulated by AP-5 withdrawal and the addition of 200 μM glycine, while unstimulated neurons were continuously treated with AP-5. Scale = 5 μm.

B, C) Quantification of spine tip width and spine morphology in response to ACTN2 knockdown and glycine stimulation. Fraction of spines with a large head, spine tip width > 0.4 μm, increases in response to glycine stimulation but is prevented by ACTN2 knockdown (B). Glycine does not increase the fraction of mushroom-shaped spines nor decrease the fraction of filopodia-like protrusions in neurons with ACTN2 knocked down in contrast to stimulated controls (C). For each condition, 301 - 583 spines from 7 - 11 neurons were analyzed. Error bars represent SEM. *p = 0.001, Student t-test.


A
pSUPER
pSUPER-ACTN2
AP-5
Glycine
B
Fraction of Spines with Tip Widths > 0.4 µm
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*p < 0.001
Control - AP-5
Control - Glycine
Knockdown - AP-5
Knockdown - Glycine
C
Fraction Total
0.8
0.6
0.4
0.2
0.0
Control - AP-5
Control - Glycine
Knockdown - AP-5
Knockdown - Glycine
Thin
Filopodia-like
Mushroom
Stubby

to PSD organization we immunostained for PSD-95 in control and knockdowns. In contrast to control neurons, in which PSD-95 was observed in most spines, the spines of neurons with diminished levels of ACTN2 lacked PSD-95 (Figure 21A, B). In these neurons, PSD-95 only localized to a few regions on the dendrite shaft (Figure 21A, C).

Since PSD-95 interacts with the NR2 subunit of NMDA receptors (Kornau et al., 1995) and ACTN2 directly binds to the NR1 subunit *in vitro* (Wyszynski et al., 1997), we asked whether the NMDA receptor assembled at spines of neurons lacking ACTN2. We co-expressed the ubiquitous NR1 subunit of the NMDA receptor fused to a super-ecliptic pHluorin (SEP-NR1), which displays GFP fluorescence at the membrane surface when SEP is exposed to a neutral environment (Kopec et al., 2006). Rhodamine-phalloidin, which clearly shows actin-rich spines, was used to visualize them. While discrete clusters of SEP-NR1 were seen in the spines of control neurons, SEP-NR1 clusters did not localize to spines in neurons co-expressing the shRNA (Figure 21D). Instead, SEP-NR1 clustered within the dendrite shaft at the base of some filopodia-like protrusions (Figure 21D). Therefore, ACTN2 is essential for the synaptic targeting of PSD-95 and NMDA receptors.

Even though key components of the PSD were not observed in spines lacking ACTN2, these filopodia-like protrusions could potentially interact with axons of normal, untransfected neurons in the cell culture. To determine whether excitatory, pre-synaptic molecules could synapse with spines lacking a functional PSD, we immunostained for the excitatory pre-synaptic marker, VGLUT1, and did not find VGLUT1 apposed to spines in neurons with ACTN2 knocked down (Figure 22A, B). In these neurons, VGLUT1 only juxtaposed spines in a few places along the dendrite shaft, presumably in synaptic

Figure 21: ACTN2 knockdown prevents assembly of post-synaptic molecules.

A) ACTN2 knockdown prevents the recruitment and assembly of PSD-95 to spines. Hippocampal neurons were co-transfected at DIV 6 with GFP and either a control empty vector or an shRNA vector against ACTN2 and fixed and immunostained for endogenous PSD-95 (magenta) on DIV 22. Scale = 5 μm.

B) Quantification of the number of spines with co-localization of PSD-95 per total number of spines on DIV 19-24 neurons. Significantly fewer spines on neurons with ACTN2 knocked down harbor PSD-95. For each condition, 21 – 25 neurons were analyzed. Error bars represent SEM. *p = 0.001, Student t-test.

C) On DIV 19 – 24 neurons, significantly more PSD-95 co-localizes with the dendrite shaft in neurons with ACTN2 knocked down in comparison to control neurons. For each condition, 24 – 25 neurons were analyzed. Error bars represent SEM. *p = 0.002, Student t-test.

D) ACTN2 knockdown prevents the recruitment of the NMDA-type glutamate receptor to the spine. Hippocampal neurons were co-transfected at DIV 6 with SEP-NR1 (magenta) and either a control empty vector or an shRNA vector against ACTN2 and fixed and immunostained for rhodamine-phalloidin (green) on DIV 22. Scale = 5 μm.

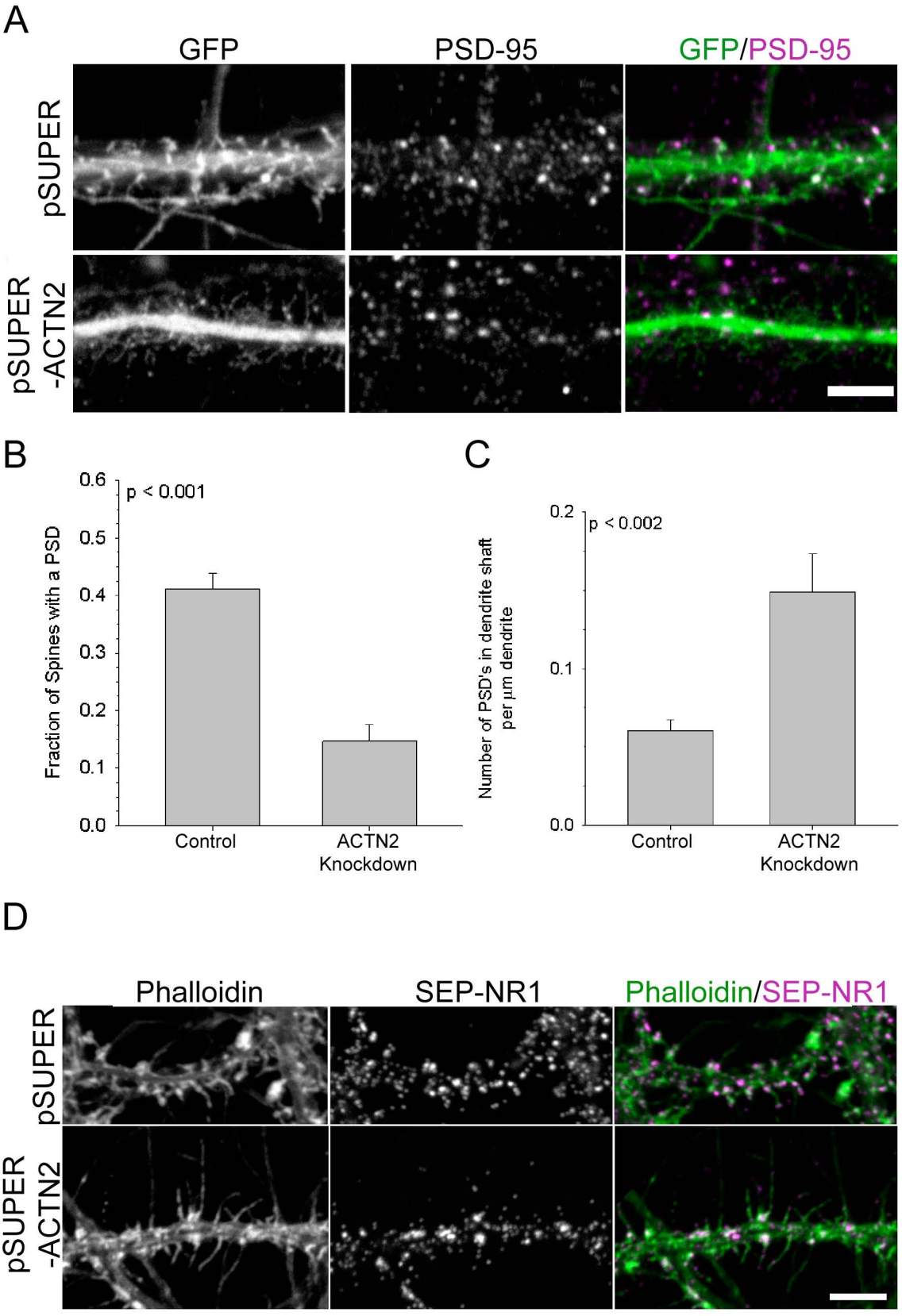
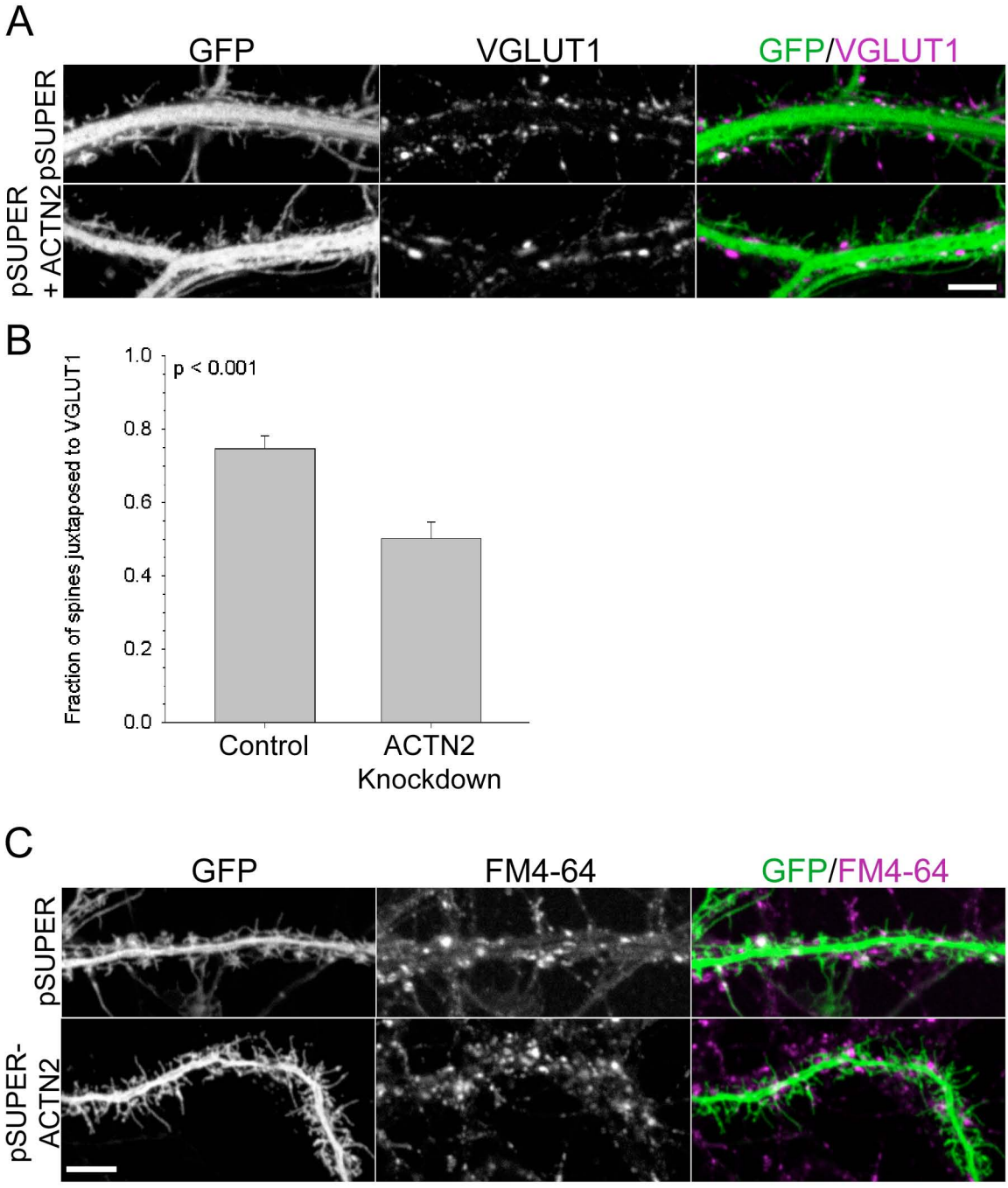

A
GFP
PSD-95
GFP/PSD-95
pSUPER
pSUPER -ACTN2
B
p < 0.001
Fraction of Spines with a PSD
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
ACTN2 Knockdown
C
p < 0.002
Number of PSD's in dendrite shaft per µm dendrite
0.2
0.1
0.0
Control
ACTN2 Knockdown
D
Phalloidin
SEP-NR1
Phalloidin/SEP-NR1
pSUPER
pSUPER -ACTN2

Figure 22: ACTN2 knockdown prevents excitatory synapse formation.

A) ACTN2 knockdown prevents synapse formation with excitatory pre-synaptic axons. Hippocampal neurons were co-transfected at DIV 6 with GFP and either a control empty vector or an shRNA vector against ACTN2 and fixed and immunostained for endogenous VGLUT1 (magenta) on DIV 15. Scale = 5 μm.

E) Quantification of the number of spines juxtaposed to VGLUT1 per total spine number on DIV 21 neurons. Significantly fewer spines are in contact with excitatory pre-synaptic boutons in neurons with ACTN2 knocked down in comparison to control neurons. For each condition, 8 neurons were analyzed. Error bars represent SEM. *p = 0.001, Student t-test.

B) Actively firing pre-synaptic boutons do not synapse with dendritic protrusions on neurons lacking ACTN2. Hippocampal neurons were co-transfected at DIV 6 with GFP and either a control empty vector or an shRNA vector against ACTN2 and treated with FM4-64 (magenta) for 5 min on DIV 19 and observed live. Scale = 5 μm.


A
GFP
VGLUT1
GFP/VGLUT1
pSUPER
pSUPER + ACTN2
B
Fraction of spines juxtaposed to VGLUT1
p < 0.001
1.0
0.8
0.6
0.4
0.2
0.0
Control
ACTN2 Knockdown
C
GFP
FM4-64
GFP/FM4-64
pSUPER
pSUPER-ACTN2

contact with non-glutamatergic synapses. In contrast, VGLUT1 apposed most spines of control neurons (Figure 22A, B). We found the same results with neurons briefly exposed to the lipophilic styryl dye, FM4-64, which marks actively firing synapses (Figure 22C). This implies that the filopodia-like protrusions of neurons deficient of ACTN2 do not form synapses with potentiated axons, as they lack functional components of the PSD, including PSD-95 and the NMDA receptor.

ACTN2 Phosphorylation Determines Unique Spine Morphologies

It is uncertain how ACTN2 is regulated to determine spine morphology and post-synaptic organization. Interestingly, both over expression and depletion of ACTN2 result in thinner spines (Figures 19A, D and 23C, D) (Nakagawa et al., 2004), therefore it is possible that ACTN2 activity is controlled to fine tune its interactions with putative binding partners in the PSD. Within its actin-binding domain, ACTN2 shares a conserved tyrosine residue with α-actinin-1 (Figure 5), which is phosphorylated by FAK in migrating cells (Izaguirre et al., 2001; Wichert et al., 2003). In fibroblasts, phosphorylation on α-actinin-1-Y12 by FAK reduces its binding affinity for actin filaments (Izaguirre et al., 2001), and thereby promotes adhesion and actin bundle disassembly (Wichert et al., 2003). To assess whether ACTN2 is similarly tyrosine phosphorylated, exogenous ACTN2 was immunoprecipitated from COS-7 cells treated with or without orthovanadate, a tyrosine phosphatase inhibitor, and immunoblotted for phospho-tyrosine (p~Tyr). The p~Tyr signal on ACTN2 was significantly higher in cells treated with orthovanadate (Figure 23A), suggesting that ACTN2 is phosphorylated on tyrosine residues in intact cells. To determine whether tyrosine phosphorylation

Figure 23: ACTN2 phosphorylation determines unique spine morphologies.

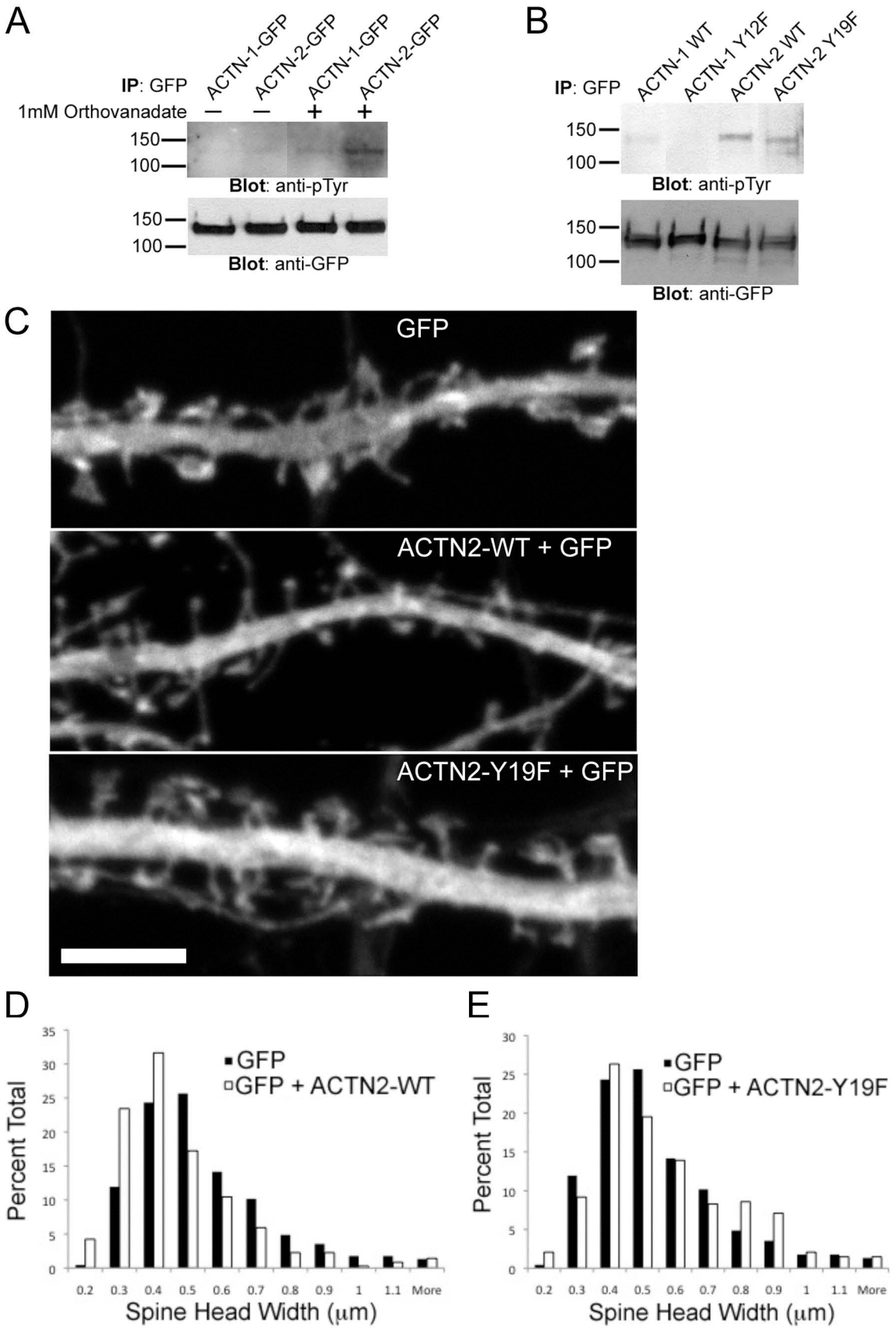
A) ACTN2 is phosphorylated on tyrosine residues *in vitro*. COS-7 cells were transfected with ACTN1-GFP or ACTN2-GFP and treated with or without orthovanadate for 24 hours before lysis and immunoprecipitation of GFP. Immunoprecipitates were immunoblotted with a p~Tyr antibody (4G10). GFP immunoblot serves as a loading control.

B) Non-phosphorylatable ACTN2-Y19F causes a slight decrease in p~Tyr signal. COS-7 cells were transfected with ACTN1-GFP-WT, -Y12F, ACTN2-GFP-WT, or –Y19F and treated with orthovanadate for 24 hours before lysis and immunoprecipitation of GFP. Immunoprecipitates were immunoblotted with a p~Tyr antibody (4G10). GFP immunoblot serves as a loading control.

C) ACTN2-WT over-expression creates thin spine morphologies and ACTN2-Y19F over-expression restores normal spine morphologies. Hippocampal neurons were transfected with GFP alone or in combination with ACTN2-WT or ACTN2-Y19F and fixed on DIV 21.

D) ACTN2-WT over-expression induces thinner spine heads.

E) ACTN2-Y19F over-expression induces normal spine head widths.


A
IP: GFP
ACTN-1-GFP
ACTN-2-GFP
ACTN-1-GFP
ACTN-2-GFP
1mM Orthovanadate
—
—
+
+
150
100
Blot: anti-pTyr
150
100
Blot: anti-GFP
B
IP: GFP
ACTN-1 WT
ACTN-1 Y12F
ACTN-2 WT
ACTN-2 Y19F
150
100
Blot: anti-pTyr
150
100
Blot: anti-GFP
C
GFP
ACTN2-WT + GFP
ACTN2-Y19F + GFP
D
Percent Total
GFP
GFP + ACTN2-WT
Spine Head Width (μm)
E
Percent Total
GFP
GFP + ACTN2-Y19F
Spine Head Width (μm)

occurs on ACTN2-Y19, the residue was mutated to a phenylalanine, rendering it non-phosphorylatable. In comparison to *wild type* ACTN2, the non-phosphorylatable mutant, ACTN2-Y19F, induced a slight decrease in p~Tyr signal (Figure 23B), indicating that Tyr19 is a potential site for phosphorylation. However, ACTN2-Y19 is not a major site of tyrosine phosphorylation, as there was still a strong p~Tyr signal on ACTN2-Y19F, indicating there are other tyrosine residues on the molecule that are possibly phosphorylated. To ascertain whether the non-phosphorylatable mutant could influence spine morphology, exogenous ACTN2-*WT* or ACTN2-Y19F were co-expressed with soluble GFP in neurons and imaged on DIV 21. In agreement with previous findings (Nakagawa et al., 2004), ACTN2-*WT* over-expression induces significantly smaller spine head widths in comparison to neurons expressing only GFP (Figure 23C, D). However, exogenous expression of the non-phosphorylatable ACTN2 mutant, ACTN2-Y19F, created mature spine morphologies with normal spine head widths in comparison to neurons expressing GFP alone (Figure 23C, E). This suggests that ACTN2 can be dynamically regulated by phosphorylation on its actin-binding domain to create distinct spine morphologies.

Discussion

The actin cross-linking protein, α-actinin, plays a central role in organizing actin filaments at various sub-cellular locations, including stress fibers (Burridge and Wittchen, 2013), the lamellipodia of migrating cells (Small et al., 2002), cell-matrix adhesions (Choi et al., 2008), cadherin-based cell-cell junctions (Knudsen et al., 1995), glomerular podoctyes (Dandapani et al., 2007), and neuronal synapses (Wyszynski et al., 1998; Otey

and Carpen, 2004). In addition to cross-linking actin, α-actinin interacts with various transmembrane proteins, linking them to the cytoskeleton, and scaffolds signaling pathways at actin-rich regions (Otey and Carpen, 2004). Thus, the versatile role of α-actinin at sub-cellular locations directly contributes to the distinct cellular functions comprising different types of tissues. Within excitatory hippocampal neurons, it is now clear from our work that ACTN2 serves to assemble key components of the PSD in dendritic spines, regulate synaptogenesis, and direct the morphogenesis of a filopodia-like protrusion into a mature spine.

Functional Mechanisms for ACTN2 in Dendritic Spines

Despite the importance of ACTN2 in shaping dendritic spines and assembling the PSD at the synapse, a clear mechanism for its function in spines remains unresolved. In fibroblasts and epithelial cells, α-actinin is involved in coupling integrins to actin filaments at focal adhesions and the cadherin complex to the actin cytoskeleton at cell-cell junctions via an α-catenin-α-actinin linkage (Otey et al., 1990; Pavalko and LaRoche, 1993; Knudsen et al., 1995). Biochemical studies *in vitro* suggest that ACTN2 binds directly to the NR1 and NR2B subunits of the NMDA receptor (Wyszynski et al., 1997)and functions to maintain the receptor in an open state (Krupp et al., 1999), coupling it to the actin cytoskeleton. Domain mapping indicates that the binding site for NR1 is localized to the fourth spectrin repeat of ACTN2 (Wyszynski et al., 1997). This is a hypothesis that we could test using an ACTN2 mutant with its fourth spectrin repeat deleted.

ACTN2 Regulation in Dendritic Spines

The differences in spine morphology induced by over expression of ACTN2-*WT* versus non-phosphorylatable mutant, ACTN2-Y19F, suggest that ACTN2 may be regulated by phosphorylation within it actin-binding domain to create distinct spine morphologies. Since exogenous ACTN2-Y19F created normal, mature spine morphologies, mechanisms may exist within dendritic spines to prevent phosphorylation on ACTN2-Y19 during spine maturation. In migrating fibroblasts, FAK phosphorylates α-actinin-1 on the conserved tyrosine residue (Y12), mediating its dissociation with actin and promoting focal adhesion turnover (Wichert et al., 2003). It remains to be determined whether FAK, which localizes to dendritic spines, phosphorylates ACTN2 in neurons or whether this tyrosine residue on ACTN2 is a substrate for tyrosine phosphorylation in dendritic spines. FAK is an important signaling molecule in dendritic spines, as it traduces EphB2 signals to activate RhoA, which regulates the actomyosin to drive spine morphogenesis (Moeller et al., 2006). Unlike fibroblasts where adhesions must disassemble for the cell to advance, post-synaptic adhesions of spines must be stabilized to remain in synaptic contact with an axon. Therefore, it seems likely that endogenous ACTN2 is protected from phosphorylation at this site in order to promote spine maturation.

Since *in vitro* studies found that phosphorylation on α-actinin-1-Y12 reduced its binding affinity to F-actin (Izaguirre et al., 2001), then the mature spine head morphology, induced by ACTN2-Y19F over expression, may be due to an increased interaction between ACTN2 and actin filaments. This could also imply a stronger association between PSD molecules and the actin cytoskeleton, bridged through ACTN2-

Y19F. FAK-mediated phosphorylation on α-actinin-1 hinders the integrin-cytoskeleton linkage, stimulating turnover of focal adhesions (Wichert et al., 2003). The PSD can be thought of as an adhesive complex (Vicente-Manzanares et al., 2009b), and increased stability of the PSD, which reinforces trans-synaptic connections, is required for spine maturation (Dalva et al., 2007; Ehrlich et al., 2007; Lucido et al., 2009; Benson and Huntley, 2012). Presumably, an increased interaction between ACTN2 and F-actin is concomitant with more F-actin bundling in the spine. To address this, FRAP experiments are currently being performed on GFP-actin in spines of neurons co-expressing the shRNA with either RNAi-resistant ACTN2-*WT* or ACTN2-Y19F. If ACTN2-Y19F enhances F-actin cross-linking activity, then GFP-actin should show significantly reduced turnover in spines expressing the non-phosphorylatable ACTN2 mutant. Increased actin cross-linking could serve to cluster the myriad of PDZ- and LIM-containing proteins in the PSD, recruiting other actin-binding proteins to the PSD, and therefore promoting its enlargement. This may account for the smaller PSD size that is observed in spines over-expressing *wild type* ACTN2 (Nakagawa et al., 2004).

It is particularly interesting that ACTN2, an isoform that is well known for it role in striated muscle, is also enriched within the PSD of dendritic spines. ACTN2 is unique from the other α-actinin isoforms (1 and 4) reportedly enriched in PSD fractions, in that it's EF-hand domain has several missing amino acids, rendering the molecule insensitive to calcium. Calcium binding to non-muscle α-actinin isoforms (1 and 4) reduces their binding affinity for actin (Burridge and Feramisco, 1981), a major mechanism in regulating the biological activity of these isoforms in migrating cells. Since the NMDA receptor mediates calcium influx and ACTN2 is proposed to couple the NMDA receptor

to the actin cytoskeleton, the calcium insensitivity displayed by ACTN2 may serve a unique function in dendritic spines, particularly in stabilizing the NMDA receptor at the PSD despite high concentrations of calcium. Support for this comes from a study showing that calcium-sensitive α-actinin isoforms do not compete with Ca^{2+}/calmodulin to prevent NMDA receptor inactivation *in-vitro* (Krupp et al., 1999). To test the hypothesis that calcium-insensitivity is critical for the function of ACTN2, the EF-hand of ACTN2 is being swapped with the calcium-sensitive EF-hand of ACTN4. We will co-express the calcium-sensitive ACTN2 mutant (ACTN2-EF4) with the shRNA and observe whether ACTN2-EF4 can rescue the spine morphology effects induced by ACTN2 knockdown, as rescued by *wild-type* ACTN2.

Conclusions

The orchestrated actions of actin binding proteins that organize the actin cytoskeleton, such as ACTN2, appear to be essential to the structural stability of the neuronal synapse (Rajfur et al., 2002). Here, we show that inhibition of ACTN2 during early spine development prevents the formation of a PSD, while inhibition during mid-development causes the disassembly of the PSD within the spine. ACTN2 serves as a platform that might affect the assembly of many protein constituents of the PSD through direct interactions with molecules like the NMDA receptor, densin-180, and CaMKII (Robison et al., 2005b). Lacking a PSD, spines without ACTN2 do not form synapses, as the pre-synaptic marker V-GLUT1 is not juxtaposed to these spines (Figure 22). Since neurons with ACTN2 knocked down do not have a functional synapse, they do not mature in response to chemical stimulation. Neurons lacking ACTN2 have an increased

density of filopodia-like protrusions that fail to develop into mature, mushroom-shaped spines. Furthermore, ACTN2 can be regulated by phosphorylation within its actin-binding domain to create distinct spine morphologies. Thus, its potential interactions with cytoskeletal, membrane-associated, and regulatory signaling molecules make ACTN2 an ideal constituent of the actin machinery to regulate synaptogenesis and spine maturation. The loss of function data for ACTN2, provided here, serves as a framework upon which future studies can build to elucidate the many functions of ACTN2 in determining spine morphology and organizing the PSD, all of which are critical cellular processes underlying synaptic plasticity.

Materials and Methods

Antibodies and reagents. α-Actinin-2 polyclonal antibody was obtained from Epitomics and used at a ratio of 1:100. Postsynaptic density-95 (PSD-95) monoclonal antibody, used at ratio of 1:100 for immunostaining, and synaptophysin monoclonal antibody, used at a ratio of 1:1000 for immunostaining, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GFP polyclonal antibody was obtained from Invitrogen and used at a ratio of 1:250. VGLUT1 monoclonal antibody was purchased from Synaptic Systems (Goettingen, Germany) and used at a ratio of 1:1000. Secondary anti-mouse, anti-rabbit, and anti-guinea pig antibodies conjugated to Alexa488, 568 and 647 were from Invitrogen. Tetrodotoxin and strychnine were purchased from Sigma (St. Louis, MO) and reconstituted in dH_2O. Rhodamine phalloidin was purchased from Cytoskeleton (Denver, CO) and used at a ratio of 1:100. FM4-64FX was purchased from Invitrogen. Sodium vanadate was purchased from Fisher Scientific.

Plasmids. Human α-actinin-2-GFP was obtained from Origene (Rockville, MD) and cloned into a GFP-N1 vector via EcoRI and BsrG1, which cuts out the GFP tag. An ON-TARGETplus set of 4 siRNA sequences of rat ACTN2 were purchased from Dharmacon-Thermo Scientific and cloned into the pSUPER cassette according to the vector manufacturer's instructions (Oligoengine). The oligonucleotide ATGAGAGGCTAGCGAGTGA, corresponding to nucleotides 938 – 956 of rat α-actinin-2 mediated knock down of endogenous ACTN2. siRNA- insensitive α-actinin was generated by site-directed mutagenesis (Quickchange kit, Stratagene) introducing one silent mutation (AGT to TCC: Ser to Ser) in the RNAi target region of human α-actinin2, which shares 100% homology with rat. pC1-SEP-NR1 was obtained from Addgene (Kopec et al., 2006).

Neuronal culture and transfection. Low-density hippocampal cultures were prepared from E19 rat embryos as described previously (Zhang et al., 2003). All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the University of Virginia Animal Care and Use Committee (Protocol Number: 2884). Neurons were plated on glass coverslips coated with 1 mg/ml poly-L-lysine at an approximate density of 70 cells/mm^2 and were transfected using a modified calcium phosphate precipitation method as described previously (Zhang et al., 2003). For the chemical stimulation experiments (Figure 20), neurons were chronically treated with 100uM of the NMDA receptor antagonist, AP-5, from DIV 6-21 to inhibit NMDA receptor activation and spine maturation. Neurons were removed from the glia-feeder layer and placed in 1 X Mg^{2+}-free extracellular solution containing 15mM NaCl, 0.5mM KCl, 0.2mM $CaCl_2$, 3mM

glucose, 1mM Hepes, 0.5uM tetrodotoxin, and 1uM strychnine, pH7.4 (Park et al., 2004). Neurons were then stimulated by AP-5 withdrawal and 200uM glycine, incubated at 35°C, 5% CO_2 for 3 min, while control neurons continued in the presence of AP-5 (200uM). The solution is removed and replaced with 1 X Mg^{2+}-free extracellular solution with tetrodotoxin and strychnine and incubated at 35°C, 5% CO_2 for 20 minutes before fixation, as described by others (Liao et al., 2001; Lin et al., 2004).

Immunocytochemistry. Neurons were fixed in PBS containing 4% formaldehyde, methanol-free, ultra-pure EM grade (Polysciences, Inc., Warrington, PA) with 4% sucrose for 20 min at room temperature and permeabilized with 0.2% Triton X-100 for 10 min. Alternatively, for PSD-95 and ACTN2 staining, neurons were simultaneously fixed and permeabilized in 2% formaldehyde with 4% sucrose for 10 min at room temperature and then with cold methanol for 10 min at -20°C. After blocking with 20% goat serum/PBS for one hour at room temperature, the neurons were incubated with the appropriate antibodies in 5% goat serum/PBS for one hour at 37°C. Coverslips were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA).

Imaging and analysis. Confocal images were collected on an Olympus Fluoview 1000 microscope (IX81 base) equipped with a 60X/1.35 NA (oil) UPLSAPO 60X objective (Olympus). Green probes (GFP and Alexa488) were excited using the 488 nm laser line of a multi Ar laser; red probes (DsRed2 and Alexa568) were excited with the 543 nm laser line of a He-Ne laser; the far-red probe Alexa647 was excited with the 635 nm line of an LD laser. Fluorescence emission was collected using the following dichroic mirror/filter combinations: SDM560/BA505-525 (GFP), SDM640/BA560-620 (DsRed2, Alexa568 and RhodamineX) and BA655-755 (Alexa647). Two-color

fluorescence images of Alexa488 (GFP)/Alexa568 (RhodamineX/DsRed2) were collected in a Z-stack and in sequential mode. Images were acquired using Fluoview software (Olympus). Spine length, width, and PSD-95 area were quantified using Image J software. Statistical analysis was performed using Sigma Plot 11. Spine morphologies were defined as either filopodia-like, thin, mushroom, or stubby (Yuste and Bonhoeffer, 2004). Filopodia-like spines are long and thin without a spine head, whereas thin spines contain a small head at the spine tip. Mushroom-shaped spines are shorter with a large spine head atop a neck. Stubby spines are short protrusions, either thin or wide, with no discernable neck.

Chapter 5 – Conclusions and Perspectives

Actin bundling and contractility are not functions typically associated with the nervous tissue; but we show here that contractility, mediated by MIIB, and actin cross-linking, driven by both MIIB and ACTN2, are integral components of excitatory post-synaptic development and plasticity. Prior to this study, the role(s) of ACTN2 and MIIB in post-synaptic plasticity, and more specifically, the organization of the post-synaptic density concomitant with spine morphology dynamics, was unclear. I have shown that ACTN2 is required for the recruitment and assembly of the PSD in dendritic spines; whereas MIIB is necessary for the localization of the PSD to the spine head and organization of the PSD (Hodges et al., 2011). Both molecules are also required for the morphological maturation of the spine in response to chemical activation of the NMDA-type glutamate receptor. Furthermore, ACTN2 and MIIB activities are regulated during spine morphogenesis: possible phosphorylation on the actin-binding domain of ACTN2 and phosphorylation of the myosin II RLC. Specifically, the di-phosphorylation of RLC by ROCK lies downstream of NMDA-type glutamate receptor activation and is required for spine and PSD maturation. Thus, the coordinate actions of ACTN2 and MIIB in organizing the actin cytoskeleton are required for proper spine morphogenesis and post-synaptic assembly.

Coordinate actions of α-actinin and myosin II on spine and PSD morphogenesis

Actin polymerization and organization of actin are the driving force behind dendritic spine morphogenesis, and ACTN2 and MIIB are integral components of the actin machinery in spines. The possible mechanisms for the coordinate actions of ACTN2 and MIIB on spinogenesis, spine maturation, and assembly and organization of

the PSD, based on our studies, are discussed below. All of this research has also drawn insight from their potential parallels in the adhesion and protrusion of migrating cells to elucidate their individual functions in driving spine morphogenesis and post-synaptic organization.

Spinogenesis

Spinogenesis involves the emergence of a filopodia-like protrusion from the dendrite. Protrusion requires the polymerization of actin filaments pushing against the membrane. The requirement for both the formin, Dia2, and the Arp2/3 complex in spine development (Wegner et al., 2008; Hotulainen et al., 2009) suggest that both branched and linear actin polymerization induce filopodia-like dendritic protrusions. Corroborating this, a mixture of branched and linear filaments are observed in dendritic spines (Korobova and Svitkina, 2010). ACTN2 and MIIB appear to be dispensable for the growth of filopodia-like protrusions, since knock down of either molecule forms filopodia-like spine precursors. However, the diminished amounts of F-actin observed in the filopodia-like protrusions of neurons lacking ACTN2 suggest that ACTN2-mediated actin-crosslinking stabilizes and enriches actin filaments in dendritic protrusions.

Actin cross-linking through ACTN2 and MIIB may better stabilize the actin filaments polymerizing at the membrane, which mediate protrusion. Support for this comes from findings that over-expression of either ACTN2 or the non-contractile myosin mutant, MIIB-R709C, which cross-links but does not contract actin, induces longer spine lengths. This also indicates a requirement for contraction of actin, through MIIB, to produce normal protrusion lengths.

Spine maturation

Spine maturation involves expansion of the spine head and shortening at the neck to generate the mature, mushroom-shaped morphology, which is a structural hallmark of activity-induced plasticity (Sekino et al., 2007). Spine head enlargement requires both exocytic trafficking of membrane components via the Rab/Arf family of GTPases to increase the post-synaptic membrane surface area (Park et al., 2006), and actin polymerization (Fukazawa et al., 2003). Actin polymerization in the spine head appears analogous to the polymerization of actin filaments in the lamellipodium of migrating cells, where similar molecules localize, including the Arp2/3 complex, cofilin, and α-actinin (Small et al., 2002; Pollard and Borisy, 2003). Cooperation between these molecules mediates the broad expansion of the spine head during maturation. For example, the severing of actin filaments by cofilin creates new barbed-ends within the spine for nucleation of branched filaments by Arp2/3 complex. Since α-actinin can cross-link actin filaments over a wide spectrum of angles (Courson and Rock, 2010), ACTN2 may help to stabilize the branched network of actin filaments nucleated by the Arp2/3 complex; this is consistent with its presence in the dendritic actin network of lamellipodia. Additionally, cofilin enhances the cross-linking of actin filaments by α-actinin, by increasing potential binding sites for α-actinin-actin cross-linking (Bonet et al., 2010). Therefore, the coordinate actions of ACTN2, Arp2/3, and cofilin may synergize to orchestrate organization and stabilization of actin polymerization within the spine head and direct its enlargement.

Spine shortening during maturation requires contraction of actin filaments by MIIB. Long protrusions extend from the spine head in neurons lacking MIIB (Hodges et al., 2011). These observations suggest that MIIB restricts protrusiveness of the spine, analogous to how myosin II controls leading edge protrusion rates of migrating cells by increasing retrograde flow of actin. Immunogold EM labeling reveals that MIIB localizes predominantly to the spine neck and the lower part of the spine head (Korobova and Svitkina, 2010). Therefore, MIIB appears to contract actin filaments within the spine neck to control spine length and spine head protrusion.

Data from myosin II in migrating fibroblasts suggests that MIIB could localize protrusive signals, i.e., Rho GTPase activation, to the spine tip where dendritic actin polymerization mediates spine head expansion during maturation. Fibroblasts expressing exogenous RLC-DD exhibit front-back polarity and localize Rac GEF's, including Dock180 and β-Pix, in the protrusion away from actomyosin bundles that reside on the sides and rear of the polarized cell (Vicente-Manzanares et al., 2011). Dock180 and β-PIX are evenly distributed around the cell periphery in cells with MIIB knocked down, indicating that myosin activity, promoted by di-phosphorylation of its regulatory light chain, restricts protrusive signaling to the front of the migrating cell (Vicente-Manzanares et al., 2011). Both β-PIX and Dock180 localize to dendritic spines and are important for spine formation, as inhibition of either GEF results in a decreased dendritic protrusion density (Zhang et al., 2003; Kim et al., 2011). Furthermore, proper localization of β-PIX to dendritic spines is required for locally regulated Rac activity in spines (Zhang et al., 2003; 2005). Increased MIIB activity through RLC di-phosphorylation may reciprocally confine Dock180 and β-PIX to the spine tip away from MIIB, thereby restricting

protrusive signaling to the spine head. Interestingly, MIIB activity functions downstream of the GIT1/PIX/Rac/PAK signaling module to promote spine and synapse formation (Zhang et al., 2005). Therefore, an intriguing model of LTP-induced spine maturation involves signaling through the GIT1/PIX/Rac/PAK cascade in response to NMDA-type glutamate receptor activation, resulting in di-phosphorylated RLC and increased MIIB activity, which could further restrict protrusive signals to the spine head, thereby promoting polarized expansion of the spine head, concomitant with spine maturation.

PSD assembly and organization

As the spine matures from a filopodia-like spine protrusion, PSD components are recruited (Gerrow et al., 2006) and assemble in spines (Figure 1A). We find that ACTN2 is indispensible for either the recruitment or assembly of a PSD in the spine (Figure 21). Nascent adhesion formation in migrating cells is coupled to polymerized actin and the adhesions then grow along an actin-α-actinin template (Alexandrova et al., 2008; Choi et al., 2008). Using this as an analogy, we find that neurons lacking ACTN2 have diminished levels of F-actin content in their spines, which provides a possible mechanism for why a PSD does not assemble in these actin-attenuated protrusions. In α-actinin-deficient fibroblasts, the lamellipodium is aborted and adhesions are short and mis-orientated and do not mature, suggesting that α-actinin is required to organize actin into an orientation appropriate for adhesion assembly (Choi et al., 2008). Similarly, actin cross-linking by ACTN2 may be required to organize actin filaments in the dendritic protrusion, and promote formation and expansion of the PSD. An additional mechanism

for recruitment of PSD molecules to the spine via ACTN2 could occur through its putative binding interactions with components of the PSD, including densin-180, CaMKIIα, and the NR1 and NR2B subunits of the NMDA-type glutamate receptor (Wyszynski et al., 1997; Robison et al., 2005a)**.** Therefore, ACTN2 may nucleate assembly and growth of the PSD through direct recruitment of PSD molecules, and scaffold these proteins to actin filaments.

This model also suggests a mechanism for spine elimination via removal of ACTN2 and disassembly of the PSD. This could occur by weakening the affinity of ACTN2 for actin, possibly through phosphorylation on its actin-binding domain. In fibroblasts, adhesion disassembly can be mediated by a reduction in the affinity of α-actinin for actin through phosphorylation of its actin-binding domain by FAK (Izaguirre et al., 2001; Wichert et al., 2003). This also suggests that there are mechanisms in place to prevent phosphorylation on the actin-binding domain of ACTN2 in dendritic spines, so that the PSD is stabilized at the post-synaptic membrane.

Ultrastructural studies reveal that ACTN2 concentrates at the post-synaptic membrane co-localizing with the PSD (Wyszynski et al., 1998). GFP-MIIB and endogenous ACTN2 are spatially segregated in the spine, with ACTN2 localizing towards the tip of the spine (Figure 17). When MIIB is knocked down, ACTN2 becomes less clustered, similar to NMDA receptor mis-distribution and concomitantly with PSD disorganization (Figure 16). These observations support the localization of ACTN2 in the spine head, where it can maintain the PSD scaffold.

MIIB is dispensable for the initial assembly of a PSD in the spine. Although knockdown of MIIB creates filopodia-like protrusions, these immature protrusions

contain PSD components, albeit they are mislocalized and not organized into a distinct cluster. Studies of myosin II contractility in fibroblasts revealed that actin cross-linking, not contraction, mediates the initial steps in adhesion maturation, since expression of a MIIA non-contractile mutant rescued adhesion elongation in MIIA knockdown cells (Choi et al., 2008). Similarly, we find a normal-sized PSD in the spines of neurons over-expressing a non-contractile MIIB mutant (Figure 12) (Hodges et al., 2011).

MIIB, however, is indispensable for the organization of the PSD, as the PSD in neurons lacking MIIB is elongated in morphology, and mislocalized away from the spine tip (Figure 15) (Hodges et al., 2011). Disorganization of the PSD via inhibition of MIIB activity leads to less clustering of the NMDA-type glutamate receptor (Figure 16), which may explain the inhibition of excitatory synaptic transmission in these neurons (Ryu et al., 2006). Furthermore, increased MIIB activity through either exogenous expression of *wild-type* MIIB or RLC-DD, but not RLC-AD, creates a larger PSD area and promotes spine maturation (Figures 12, 13) (Hodges et al., 2011). Thus, MIIB activity localizes the PSD to the tip of the spine and promotes its growth.

To dictate PSD maturation, I propose that myosin II filaments are organized within the spine neck, as supported by ultrastructural studies (Korobova and Svitkina, 2010), and pull on actin in the spine head, tethered to PSD molecules. Our immunofluorescence studies indicate a spatial segregation between GFP-MIIB and endogenous PSD-95, with only partial overlap between the molecules at the base of the PSD. This would imply that MIIB activity dictates PSD maturation at a distance within the spine, similar to its behavior in fibroblast cells.

Parallels with cell-cell junctions

Synapse formation exhibits many similarities with junction assembly between epithelial and endothelial cells. Analogous to spine growth and spine head expansion, formation of adherens junctions requires protrusion of the membrane via polymerization of both branched actin filaments, nucleated by Arp2/3 complex, and elongation of unbranched actin filaments, nucleated by formins (Collinet and Lecuit, 2013). As the junction matures, myosin II activity re-organizes radial actin bundles into bundled actin filaments aligned parallel to the contact (Collinet and Lecuit, 2013). Just as MIIB is required for the clustering of NMDA-glutamate receptors at the spine tip (Figure 16), myosin II activity regulates cell-cell adhesion by concentrating E-cadherins at the junction (Shewan et al., 2005). Inhibition of myosin II activity or upstream Rho kinase signaling caused decreased accumulation of E-cadherin at cell-cell contacts without affecting the total surface expression of E-cadherin (Shewan et al., 2005).

Unlike dendritic spines, which only contain MIIB, both myosin-IIA (MIIA) and –IIB concentrate at cell-cell junctions and these isoforms serve different functions on junction formation. Whereas MIIA is required for the clustering of E-cadherin at junctions, MIIB is necessary to support the integrity of the perijunctional actin ring to prevent fragmentation of E-cadherin clusters at the junction (Smutny et al., 2010). Furthermore, neither over-expression of MIIA nor expression of the motor-deficient MIIB mutant, MIIB-R709C, could rescue junction integrity in MCF-7 cells with MIIB knocked down, suggesting that MIIB-mediated contractility is necessary for its effects at cell-cell contacts to mechanically reinforce junctions to resist disruptive forces (Smutny

et al., 2010). The differences between the functions of the myosin II isoforms on supporting adherens junctions are hypothesized to reflect the different motor properties displayed by the myosin isoforms (Smutny et al., 2010). MIIB has a longer duty ratio than MIIA, which allows it to bind to actin longer and generate more contractile force (La Cruz and Ostap, 2004; Vicente-Manzanares et al., 2009c). An attractive hypothesis for the expression of MIIB at hippocampal synapses, and not MIIA, is that the intrinsic motor properties of MIIB make it the ideal isoform to maintain adhesive strength at the synapse and prevent turnover of the adhesive PSD in the spine.

ACTN2 is required for synapse formation and dendritic spines on neurons with ACTN2 knocked down accumulate less actin, as seen by rhodamine-phalloidin staining (Figure 21C). Similarly, knockdown of α-actinin-4 in MDCK cells inhibits actin assembly at the apical junction (Tang and Brieher, 2012). Although a mechanism for α-actinin-4-mediated nucleation of actin assembly at E-cadherin junctions is unclear, this group found that the association between α-actinin-4 and the membrane, rather than it's F-actin bundling activity, was required for Arp2/3-dependent actin assembly (Tang and Brieher, 2012). ACTN2 localizes to the post-synaptic membrane (Wyszynski et al., 1998), and therefore its direct or indirect linkage to the post-synaptic membrane may nucleate actin accumulation at post-synaptic protrusions, followed by assembly of the PSD in actin-rich protrusions. The association of ACTN2 with the post-synaptic membrane may occur indirectly through interactions with α-catenin (Knudsen et al., 1995; Catimel et al., 2005), but whether the cadherin-α-catenin complex is required for the localization of α-actinin to the synapse or cell-cell junctions is unknown.

Tension-generated signaling at synapses

Tension-generated signaling is an unexplored role for myosin II in spine development and response to stimuli. Tension can modulate adhesive signaling through phosphorylation of adhesion molecules like FAK, Src, CrkII–p130(Cas) complex, and paxillin (Tilghman and Parsons, 2008). These non-receptor tyrosine kinases localize to dendritic protrusions and may play a role in transducing tension-based signaling between trans-synaptic adhesion proteins and myosin II. EphB-mediated morphogenesis of immature spines into mature, mushroom-shaped spines leads to increased tyrosine phosphorylation of FAK, Src, and paxillin, which is transduced onto RhoA activation. Cre-mediated excision of FAK blocked EphB-mediated morphogenesis of dendritic protrusions, due to decreased RhoA signaling (Moeller et al., 2006). RhoA-GTP activates ROCK, which phosphorylates RLC and thereby activates myosin in dendritic spines (Hodges et al., 2011). Thus, increased myosin II activity mediates EphB-induced spine morphogenesis, and pTyr levels may serve as a biochemical marker for the state of tension in the dendritic protrusion.

The observation that RhoA-GTP signaling acts downstream of FAK activation to direct EphB-induced spine morphogenesis suggests that myosin II activity may positively feedback on the FAK/Src/paxillin complex to restrict their adhesive signaling to the spine head. Highly Tyr-phosphorylated dynamic adhesions, including p130(Cas) and pY(118)-paxillin, are seen at the protrusive leading-edge of migrating cells, and exogenous expression of RLC-DD in fibroblasts restricts this adhesive signaling to the front of the cell away from actomyosin bundles (Vicente-Manzanares et al., 2011). Similarly, MIIB activity, via RLC-phosphorylation, may restrict adhesive signals to the spine tip.

Integrins have been shown to mediate functional maturation of hippocampal synapses (Chavis and Westbrook, 2001; Webb et al., 2007), and adhesion molecules, such as integrin or talin, are proposed to undergo conformational changes in response to tension applied to adhesions (Del Rio et al., 2009; Friedland et al., 2009). Mechanotransduction takes place at cell-cell junctions, specifically at the cadherin-F-actin interface (Borghi et al., 2012; Huveneers and de Rooij, 2013). N-cadherin and its associated molecules, α-catenin, β-catenin, and p120-catenin, play a major role in dictating spine morphogenesis (Mysore et al., 2008). Furthermore, post-synaptic N-cadherin affects pre-synaptic neurotransmitter release in *trans* (Vitureira et al., 2011), supporting a role for N-cadherins mediating mechanotransduction at neuronal synapses. MIIB-induced reorganization of the actin cytoskeleton may exert mechanical force at the post-synaptic membrane that is sensed by N-cadherin. The finding that VE-cadherin signals to ROCK-dependent RLC phosphorylation to increase actomyosin contractility, supports the notion of actomyosin-generated tension signaling at synapses (Abraham et al., 2009).

The observation that adhesive contact triggers development of an axon bouton, containing synaptic vesicles docked at the pre-synaptic membrane, provides further support for mechanotransduction between the pre- and post-synaptic (Lucido et al., 2009; Tyler, 2012). Consistent with this, the NMDA receptor has mechanosensitive properties in the sense that procedures that increased membrane tension, such as membrane stretching, produced an increase in the NMDA response, whereas actions leading to a reduced tension had opposite effects (Paoletti and Ascher, 1994). Actomyosin may likely be involved in a feedback loop with the NMDA receptor, where NMDA receptor

activation signals to myosin and myosin-induced tension feeds back on the PSD and adhesion proteins at the membrane to adjust the membrane tension required for optimal neurotransmission. For example, the amount of contractility at the base of the spine could be fine-tuned, via RLC-phosphorylation, to adjust the protrusion rate of spine-head expansion for optimum synaptic contact with the pre-synaptic axon bouton. Tension-based signaling through actomyosin may be a provocative mechanism by which spines can adapt to changes in neurotransmission and store memory in response to learned experiences.

Future Directions

While tremendous research in understanding the molecular mechanisms underlying post-synaptic morphology and synaptic plasticity has been undertaken, the field is far from delineating the molecular signaling pathways that dictate spine morphogenesis. The molecular pathways include, but is not limited too, organization and active polymerization of the actin cytoskeleton, formation and maturation of the post-synaptic density, and trafficking of post-synaptic glutamate receptors. Furthermore, the field requires a better understanding of how dynamic behavior in the pre-synaptic release machinery is coupled to the formation of the post-synaptic assembly, and vice-versa, to mediate synaptic strengthening or weakening.

There is a plethora of actin-binding molecules present within the micron-sized volume of the spine to dynamically remodel the actin cytoskeleton during spine morphogenesis and in response to synaptic activity. The spatiotemporal regulation of actin-binding proteins and signaling molecules that exert effects on the actin cytoskeleton

is largely unknown. FRET-based probes and photoactivatable GFP-fused molecules are tools that should provide major insight into the spatiotemporal activity of these molecules within the spine. Genetically encoded calcium indicators (GCaMP reporters) in combination with photoactivatable glutamate allow monitoring of calcium signals in precise time and space at synapses. And finally, optogenetics with tools such as channelrhodopsin-2 (Nagel et al., 2003), which allows direct activation of genetically defined subpopulations of neurons with blue light, and halorhodopsin (Zhang et al., 2007), which allows inactivation of neurons with yellow light, will test hypotheses regarding how specific brain cell types are interconnected and signal in neural circuits (Peron and Svoboda, 2011). Dissecting these signaling networks is crucial to understanding the mechanisms underlying post-synaptic plasticity, so that we can deduce the causes of spine pathologies seen in numerous neurological diseases, and ultimately find treatments for these disorders.

Summary

In conclusion, the overarching goal of this thesis was to examine the roles of MIIB and ACTN2 in dendritic spine morphogenesis and organization of the post-synaptic density. We found that both of these actin cross-linking molecules were required for dendritic spine morphogenesis and the morphology changes underlying spine maturation in response to chemical activation of NMDA type glutamate receptors. ACTN2 is required for the assembly of the PSD in the spine and MIIB-mediated contractility is required for the growth and maturation of the PSD. We defined a mechanism for spine maturation whereby NMDA-receptor activation signals through Rho-GTPases to di-phosphorylate RLC, leading to increased MIIB activity and re-organization of the actin cytoskeleton, which mediates morphogenesis of spines into mature, mushroom-shaped morphologies. Our studies showing that ACTN2 is necessary for PSD assembly and spine morphogenesis, serves as a prelude for prospective examination of its mechanism to dictate these processes, possibly through interaction with the NMDA receptor or other components of the PSD. The coordination of ACTN2 and MIIB to dictate spine morphology and PSD organization position these molecules at the center of the actin machinery that is integral to post-synaptic plasticity.

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