ARP2/3 COMPLEX HAS A NEUROPROTECTIVE ROLE AND IS REQUIRED FOR MATURE DENDRITIC SPINE HEAD MORPHOLOGY

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Dedication

I dedicate this dissertation to my family, especially…

to my mother and father, Alicia and Jesus for instilling in me the value of hard work and a higher education;

to my Godmother Melva for her constant encouragement and advise;

to my husband, Rolando for his love, encouragement, support, and patience during my long hours in lab and writing;

to my son, Emilio for bringing joy and balance to my life.

Abstract

Several lines of evidence suggest that Arp2/3 may play a role in regulating dendritic spine morphology. First, inhibition of the Arp2/3 activators N-WASP, Cortactin, and Wave alters spine morphology and density (Racz and Weinberg 2004, Pipel and Segal 2005, Soderling et al. 2007b, Wegner et al. 2008). Second, electron microscopy of dendritic spines revealed that the actin filaments within the spine head appear to be organized in "Y" shaped branches (Fifkova and Delay 1982). Since, Arp2/3 is the only complex known to make "Y" branched actin filaments, the presence of such branches in spine heads strongly suggests that Arp2/3 is involved in actin polymerization in spines. Consequently, the general hypothesis for my doctoral work is that *if Arp2/3 is a major regulator of dendritic spine morphology, then Arp2/3 will be enriched in dendritic spines heads and inhibition of Arp2/3 activity will alter the number and/or morphology of dendritic spines*. Our results show Arp2/3 localization within the dendritic spine heads of cultured hippocampal neurons. However, we observed Arp2/3 redistribution within dendritic shafts in response to induced synaptic activity. Temporal inhibition of Arp2/3 function during dendritic spine development showed severe morphological consequence in mature cultures. Finally, in collaboration with Dr. Robert Meller of Dow Neurobiology Laboratory at Legacy Research, Legacy Health in Portland Oregon, we show that Arp2/3 has a neuroprotective role of in ischemia tolerance.

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Abbreviations

ADP	adenosine diphosphate
AAV	adeno-associated virus
Abi	Abelson-interacting protein
ABP	actin binding protein
Akt	activate serine/threonine activated
AMPAR	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Arp2/3	Actin related protein
ARPC1	Actin related protein core subunit 1
ATP	adenosine triphosphate
AV	adenovirus
BAR	Bin-Amphiphysin–Rvs
BDNF	brain-derived neurothrophic factor
Bim	Bcl-2-interacting mediator
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CaM Kinase II	calcium/calmodulin dependent kinase II
Cdc42	Cell division control protein 42 homolog
CMV	Cytomegalovirus
CNS	central nervous system
CREB	Cyclic adenosine monophosphate response element-binding
CsCl	Cesium Chloride
CYFIP	Cytoplasmic fragile X mental retardation interacting protein
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
DIV	days *in vitro*
EGFP	enhanced green fluorescent protein
EGFP-CA	enhanced green fluorescent protein fused to cofillin-acidic
p21-EGFP	enhanced green fluorescent protein fused to p21-Arp2/3 subunit
EM	electron microscope
EMEM	Eagle's minimal essential medium
Eph	Ephrin
EPSC	excitatory post-synaptic current
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
EVH1	Ena-WASP homology-1
F-actin	filamentous actin
F-BAR	Extended FCH Homology Bin-Amphiphysin–Rvs

FBS	Fetal bovine serum
FRET	Förster resonance energy transfer
5-FUDR	5-Fluoro-2'-deoxyuridine
G-actin	globular actin
GABA	γ-Aminobutyric acid
GBD	GTPase binding GEF
GFP	green fluorescent protein
GKAP	guanylate kinase-associated protein
GLT1	glial glutamate transporter
GluR2	Glutamate Receptor 2
GTPase	Guanosine-5'-triphosphate hydrolase enzyme
H_2	Hydrogen
HBSS	Hanks balance salt solution
HCC	Hippocampal cell culture
HEK	Human Embryonic Kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ins(1,4,5)IP3	inositol 1,4,5-triposphate
IRSp53	insulin receptor tyrosine kinase substrate p53
IT	Ischemic tolerance
ITR	inverted terminal repeats
K^+	potassium
KCl	potassium chloride
KH_2PO_4	monopotassium phosphate
LTD	long term depression
LTP	long-term potentiation
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein (MAP) kinases
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
mGluR	metabotropic glutamate receptors
mRNA	messenger ribonucleic acid
Na^+	sodium
NaCl	sodium chloride
Na_2HPO_4	monosodium phosphate
NAPI	new API
Nck1	non-catalytic region of tyrosine kinase adaptor protein 1
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NMDAR	N-methyl-D-aspartic acid receptor
NPF	nucleation-promoting factors
N-WASP	Wiskott-Aldrich syndrome protein
NTA	amino-terminal
OGD	oxygen glucose deprivation

p34	Arp2/3 subunit
PAK	p21 activated kinases
PBS	phosphate buffered saline
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), Zonula occludens-1 protein (zo-1)
PI	propidium iodide
PICK1	protein that interacts with C kinase 1
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PLC	phospholipase C
PPS	paraformaldehyde
PSD	post synaptic density
Rac1	Ras-related C3 botulinum toxin substrate 1
RCB	Rac binding domain
RNAi	RNA interference
SAP97	Synapse-associated protein-97
SH3	SRC Homology 3 Domain
shRNA	short hairpin RNA
Sra1	Steroid receptor RNA activator 1
SV2	synaptic vesicle protein 2
Tiam1	T-cell lymphoma invasion and metastasis 1
TOCA-1	transducer of Cdc42-dependent actin assembly 1
VCA	verprolin-cofillin-acidic
WAVE	WASP family verprolin-homologous protein
WH1	WASP-homology-1
WH2	WASP homology-2 domain
WHD/SHD	WAVE/SCAR homology domain
WIP	WASP interacting protein

CHAPTER 1 Introduction

Pre- and post-synaptic targeting in the CNS

Efficient excitatory synaptic transmission in the central nervous system is important for learning and memory and requires proper synapse development. Development of excitatory synapses in the CNS often occurs in several steps: 1) axons respond to guidance cues, 2) proper axon-target contact, 3) recruitment of pre- and post-synaptic proteins to sites of synaptic contact, 4) spinogenesis and 5) maturation of dendritic spines.

During the development of the nervous system neurons extend two types of process, axons and dendrites. At the end of both axons and dendrites there are specialized tips known as growth cones, which are highly motile structures that can explore, navigate and advance through the extracellular environment. Growth cone motility is mediated by polymerization and depolymerization of its cytoskeletal structures and guidance signals the growth cone encounters (Lowery and Van Vactor 2009). Actin and microtubules are the major cytoskeletal components within growth cones that provide structural support. Arrangement of these cytoskeletal structures defines different regions within the growth cone.

The peripheral region is the most distal and dynamic part of migrating growth cones. It contains finger-like projections called filopodia that explore the surrounding extracellular environment, and veil-like lamellipodium that separate the filopodia projections (Bouquet and Nothias 2007). Both filopodia and lamellipodium are actin rich

structures, where F-actin bundles form the filopodia and branched F-actin networks give lamellipodium their veil-like structure (Cohan 2003, Strasser et al. 2004). F-actin bundles have closely packed arrays of f-actin that run in parallel, whereas, in networks the branches of f-actin crisscross and are loosely packed.

The central domain of growth cones is composed mostly of dynamic microtubules that extend from the axon or dendrite shaft. The transition zone of the growth cone is located between the peripheral region and the central domain. The transition zone is enriched with myosin motor proteins that contract F-actin network and induce formation of F-actin arcs, which regulate microtubule advancement (Lowery and Van Vactor 2009). The F-actin structures are critical for neuronal growth cones guidance and CNS development. F-actin structures can sense guidance cues, interact with microtubules and assist in growth cone steering towards intended targets (Cohan 2003). Axons are guided along the way by their response to short-range and long-range guidance cues (O'Donnell et al. 2009), which can be attractive or repellent to the growth cone. While following guidance cues, growth cones can establish transient contacts with many potential targets. However, these transient contacts can develop into stable synapses depending on the arrangement of proteins recruited to the pre- and post-synaptic sites of contact.

Several families of adhesion proteins are involved in many of the processes important in establishing excitatory synaptic transmission (Gerrow and El-Husseini 2006). For example, families of cell adhesion molecules (CAMs) are diffusely expressed on both pre- and post-synaptic membranes, but undergo specific localization to the sites

of axon-target contact and mediate recruitment of other pre- and post-synaptic molecules (Lardi-Studler and Fritschy 2007).

One of the best-studied families of CAMs is cadherins. They are calcium dependent transmembrane glycoproteins that generally play important roles in the tissue formation of developing embryos. Cadherins can form homophilic interactions via their variable N-terminus cadherin extracellular domains. The intracellular C-terminus domain is conserved among cadherins and contains a β-catenin binding domain. β-catenin interacts with intercellular α-catenin, which in turn links the cadherin/catenin complex with the actin cytoskeleton. N-cadherin, a neuronal cadherin that is localized to both the presynaptic and postsynaptic membrane of excitatory synapses, is important for neurulation and neuronal migration (Huntley G.W. 2002). Blocking of N-cadherin function by antibody addition to the chick retino-tectal system leads to retino-ganglion cells missing intended target and formation of aberrant synapses (Inoue and Sanes 1997). Thus, N-cadherins seem to be necessary for target recognition.

In dendritic filopodia, N-cadherin has widely diffused localization, but upon initial contact with axons, the cadherin-catenin complex accumulates to sites of contact providing stability by enlarging contact area (Ranscht 2000, Takeichi and Abe 2005). The N-cadherin/β-catenin complex can bind PDZ domain proteins and recruit PDZ domain proteins to the synapse (Lardi-Studler and Fritschy 2007). In addition, N-cadherin/β-catenin complex interacts with GTPase activating protein RICS, which activates Cdc42 and Rac 1 to induce rearrangement of the postsynaptic actin cytoskeleton (Okabe et al. 2002) perhaps by mediating actin binding proteins function. For example,

the Arp2/3 actin-binding protein complex interacts directly with the cadherin-catenin complex and participates in formation of cadherin mediated adherens junctions (Kovacs et al. 2002, Bershadsky 2004). Adherens junctions are structures that are formed by homophilic interaction between transmembrane adhesion proteins that allow neighboring cells to adhere and facilitate communication between cells and their environments. Formation of cadherin-mediated adherens junctions resembles the process of synapse assembly in that both events involve cell-cell interactions that are accompanied by remodeling of the actin cytoskeleton (Bershadsky 2004). Thus, the Arp2/3 complex may play a similar role in the remodeling of the actin cytoskeleton that occurs as a result of the excitatory synapse assembly, maturation and plasticity.

Another widely studied family of CAMs that are important in axon guidance, target recognition and recruitment of proteins to synaptic sites is the ephrins and their Eph receptors. The Eph receptor-ephrin ligand interaction mediates many cell-cell interactions in the nervous system. Ephrin B ligands are transmembrane bound proteins that are found on the surface of presynaptic membranes. EphB receptors are transmembrane bound proteins that are localized to the postsynaptic membrane. The extracellular N-terminus of the EphB receptor is highly conserved and interacts with the ephrin ligand. A cysteine-rich domain and two fibronectin repeats follow the ligand-binding region. The fibronectin repeats are involved in EphB receptor dimerization and interaction with other proteins, such as integrins. The intercellular C-terminus of EphB receptor contains a PDZ binding domain that helps tether EphB-ephrin ligand signaling to PDZ proteins. EphrinB/ephrin interaction promotes spine synapse formation by

recruitment of PDZ domain containing proteins to the synapse (Irie and Yamaguchi 2004). Interestingly, the EphB2 receptor regulates dendritic spine development via interactions with an adaptor protein involved in clathrin mediated endocytosis, intersectin, cdc42 and the Arp2/3 actin-binding protein complex activator, N-WASP (Irie F. 2002), suggesting Arp2/3 involvement in dendritic spine development.

Molecular composition of the excitatory post-synaptic compartment

Excitatory glutamatergic synapses are made onto the surface of dendritic spines. The postsynaptic membranes of glutamatergic synapses contain an underlying dense matrix, called the postsynaptic density (PSD, figure 2). Biochemical purification of the PSD has identified an enrichment of ionotropic glutamate receptors (NMDAR and AMPAR), receptor tyrosine kinases, G-protein coupled receptors, ion channels, cytoskeleton proteins and cell adhesion molecules that are all held in place by association with scaffolding proteins. Thus, the PSD contains the materials necessary for postsynaptic signaling and plasticity.

Na^+ and K^+ ion channels in the PSD mediate depolarization of the postsynaptic membrane and backpropagation of action potentials to the soma (Araya R. 2007, Sheng M. 2007). K^+ ion channels at cell membrane resting potential are leaky, causing slight increase in K^+ conductance across the membrane leading to minor depolarization, opening Na^+_V channels and increase electrochemical drive for Na^+ conductance into the postsynaptic membrane, which leads to increase depolarization of the membrane.

Increase in depolarization contributes to release voltage dependent Mg^{2+} block from NMDAR.

NMDAR and AMPAR are permeable to Ca^{2+}, Na^{+}, and K^{+}. Both NMDAR and AMPAR require glutamate neurotransmitter release from presynaptic vesicles and binding to glutamate binding sites. Once glutamate is bound, NMDAR and AMPAR become permeable to Na^{+} influx and K^{+} efflux, which leads to depolarization of the postsynaptic membrane. However, NMDAR become permeable to the influx of Ca^{2+} when concurrent glutamate binding to NMDAR and sufficient depolarization post-synaptic membrane relieve Mg^{2+} blockade from NMDAR. AMPAR do not exhibit voltage dependent Mg^{2+} blockade and are less permeable to Ca^{2+} than NMDAR. Influx of Ca^{2+} is supplemented by the activation of voltage gated Ca^{2+} channels that open as a result of a strong depolarization across the postsynaptic membrane.

As a result of Ca^{2+} influx, intracellular Ca^{2+} increases and activates Ca^{2+} - dependent kinases, such as calmodulin-dependent kinase II (CaM Kinase II), which has an important role in induction of long-term potentiation (Lee et al. 2009). CaM Kinase II can bind directly to NMDAR and activate a cascade of kinases. These kinases eventually lead to phosphorylation, activation and nuclear translocation of transcription factors, such as CREB, that transcribes genetic material for the production of proteins required for enhanced synaptic activity.

Increase in intercellular Ca^{2+} can also activate several guanine-nucleotide exchange factors (Block et al.), which activate several small GTPase signaling molecules, such as Ras and Rac1. These small GTPAses signaling molecule are responsible for the

activation signaling pathways that activate multiple transcription factors or modulate actin dynamics downstream of post-synaptic activity. For example, the MAPK/ERK pathway is activated by Ras activation of multiple transcription factors (Cullen P.J. 2002, Sheng and Kim 2007). In addition, Kalirin7, a Rho GEF, is activated by interaction with PDZ domain containing proteins, such as PSD-95, and activates Rac1(Penzes P. 2001). Activated Rac1 leads to activation of ADF/cofilin, an actin depolymerizing proteins, and WAVE (WASP family verprolin-homologous protein, an Arp2/3actin-binding protein complex activator) (Ethell and Pasquale 2005), suggesting that Arp2/3 complex dynamics may be modulated by post-synaptic activity.

Metabotropic Glutamate receptors indirectly contribute to the increase in intercellular Ca^{2+} concentration. mGlutamate receptors are G-protein coupled receptors that activate phosphoinositide-specific phospholipase C (PLC), which leads to formation of membrane diacylglycerol (Huntley G.W.) and soluble inositol 1,4,5-triposphate (Ins(1,4,5)IP3), which stimulates the release of Ca^{2+} from the endoplasmic reticulum (Topolnik L. 2005). mGlutamate receptors, NMDAR and AMPAR are held at the PSD by interaction with a complex of scaffold proteins, PSD-95, GKAP, Homer, and ShanK (Naisbitt S. 1999, Sheng M. 2007). Shank proteins are linked to the actin cytoskeleton through interaction with cortactin (another Arp2/3 complex activator), suggesting that the Arp2/3 complex responds to changes of the glutamate receptors activity and may be important in mediating actin dynamics that underlie the changes in dendritic spine morphology.

What are dendritic spines?

Dendritic spines are micron-sized protrusions that extend from the surface of dendrites of most excitatory neurons in the central nervous system (figure 1). Dendritic spines compose the individual postsynaptic components of excitatory synapses but information from these individual postsynaptic compartments can integrate into neuronal circuits. Actin is the major cytoskeletal component within dendritic spines and is believed to provide the structure and support that is necessary to maintain dendritic spine head morphology. Although, the prototypical mature spine is characterized by having a short skinny neck that extend from the surface of dendrites and large bulbous head which contains an electron dense structure called the post-synaptic density (PSD, figure 2) located juxtaposed to the active zone of the pre-synaptic terminal, dendritic spines can have a wide range of shapes and sizes depending on the developmental state and synaptic activity of the spines (Hayashi and Majewska 2005).

A variety of membrane bound proteins (ligand-gated, second messenger-gated ion receptors and cell adhesion molecules) are concentrated in the PSD (figure 2). Large arrays of scaffolding proteins tether the membrane-bound receptors to the underlying actin cytoskeleton and signaling proteins within the spine heads (figure 2) suggesting that changes in synaptic activity not only modulate the conformation of membrane bound receptors but may also induce changes in the activity of signaling cascades that can modulate actin-binding proteins and actin cytoskeleton dynamics within dendritic spines (Alvarez and Sabatini 2007).

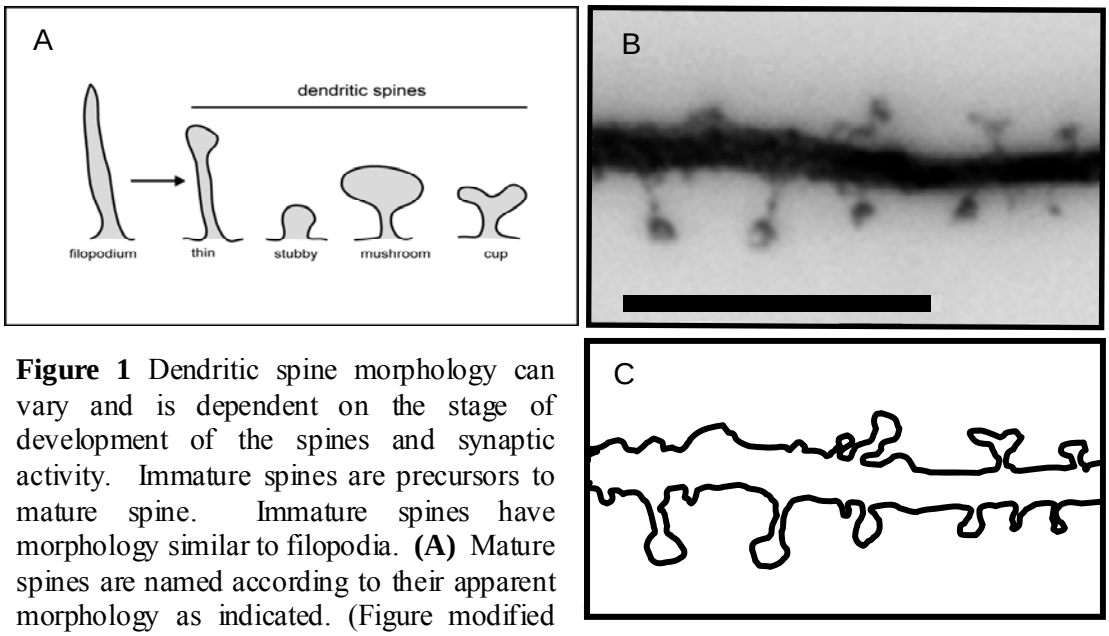

Figure 1 Dendritic spine morphology can vary and is dependent on the stage of development of the spines and synaptic activity. Immature spines are precursors to mature spine. Immature spines have morphology similar to filopodia. **(A)** Mature spines are named according to their apparent morphology as indicated. (Figure modified from Progress in Neurobiology (2005) 75:161-205)
(B) Expression of EGFP reveals that by 14DIV primary hippocampal cultures can show a variety of dendritic spine morphology within a single segment of dendrite shaft. Scale bar 10µm. **(C)** Tracing of (B) highlighting the different dendritic spine morphologies. (Maldonado M., unpublished)

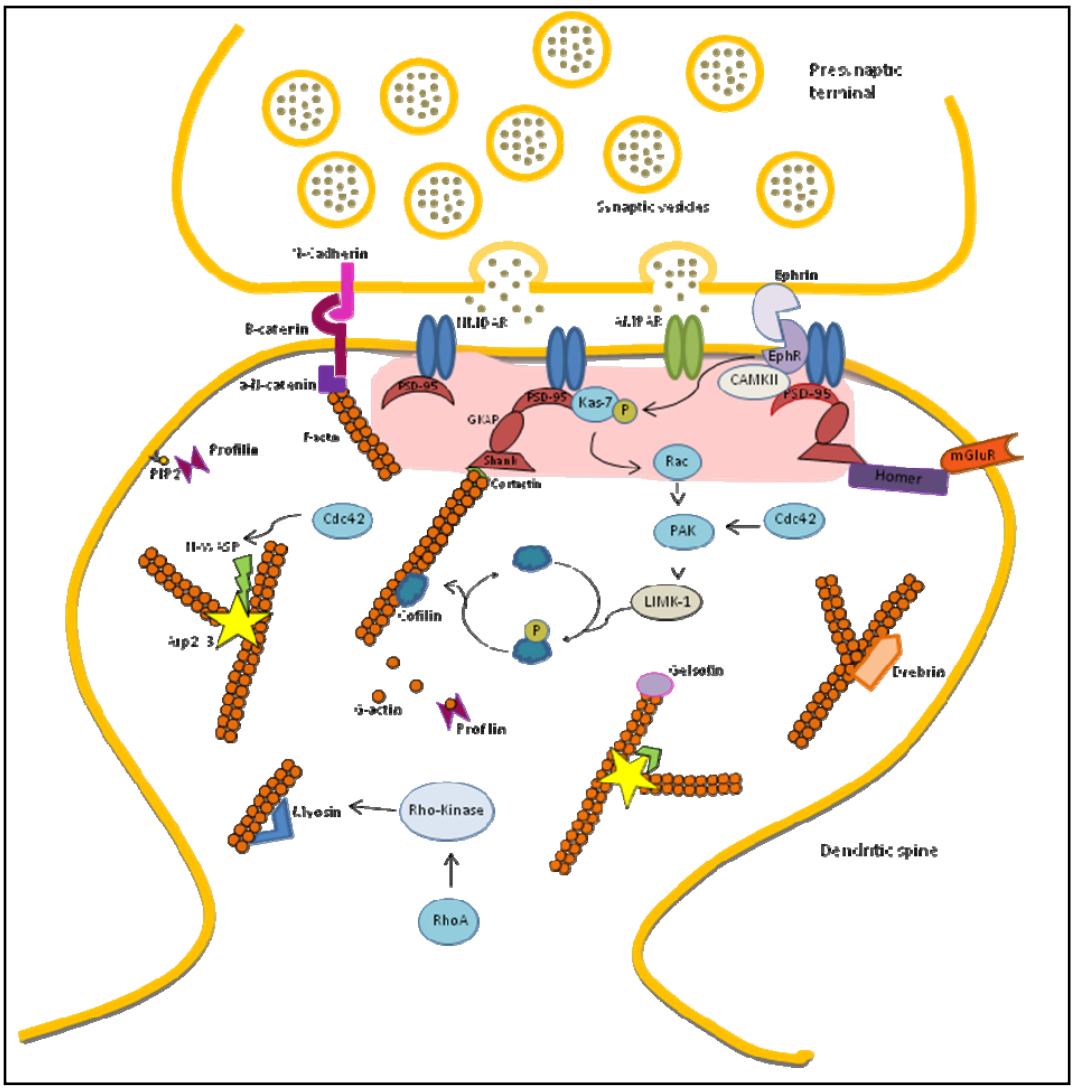

Figure 2 Dendritic spines are the postsynaptic terminals of excitatory synapses. The surface of dendritic spine heads contains an assortment of membrane bound proteins, including NMDA and AMPA receptors, that are anchored to a dense matrix called the post-synaptic density (PSD, shown in pink). Below the PSD lie a myriad of proteins and signaling molecules that relay the activity of membrane bound proteins and induce changes on the underlying actin cytoskeleton. Arp2/3 is an actin-binding protein that is able to make y-shaped branched actin filaments. The general hypothesis of my doctoral work is that these y-shaped branches of f-actin may be important in providing the structural support necessary for maintaining an enlarged spine head. (Figure modified from Physiology (2006) 21:38-47).

Why are dendritic spines important?

Dendritic spines increase the surface area of synaptic contact. The size of dendritic spines correlates with the size of the synapse and with the presence of a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Matsuzaki et al. 2001). In most regions of the developing brain dendritic spine formation occurs within the first few weeks after birth (Yuste and Bonhoeffer 2004). However, the development of dendritic spines is impaired in neurodevelopmental disorders characterized by mental retardation (Johnston et al. 2009). People with Fragile X, the most common form of inheritable mental retardation, have abnormally long and thin spines with an increase in density (Wisniewski et al. 1991) where as people with trisonomy 21 (Down's syndrome) show a decreases in spine density in both the neocortex and hippocampus (Ferrer and Gullotta 1990, Takashima et al. 1994).

Aberrant spine morphology and density have also been associated with other neurological conditions such as aging, Alzheimer's, schizophrenia, bipolar disorder, depression, and epilepsy (Fiala et al. 2002, Blanpied and Ehlers 2004, McKinney et al. 2005, Wong 2005), suggesting that dendritic spine morphology and number are important for proper cognitive function.

Dendritic spines and synapses remain motile throughout adulthood and can undergo spinogenesis, pruning, and remodeling in response to synaptic activity, learning and memory formation, hormonal changes, and aging (McKinney et al. 2005, Dickstein et al. 2007). During normal aging, the reduction in dendrite arborization and length, is combined with a loss of spines, synapses, and decrease levels of AMPAR and NMDAR,

which lead to reduced frequency and amplitude of spontaneous excitatory post synaptic currents (EPSCs) (Dickstein et al. 2007).

Since dendritic spines are important post-synaptic sites for excitatory synapses in the CNS, it is not surprising that abnormalities in spine morphology and density have a negative consequence on the formation of excitatory synapse formation and cognitive function. Any information that could increase our knowledge of the dynamics that govern the architecture of dendritic spines would be of great benefit to our current understanding of cognitive function and excitatory synaptic transmission.

Dendritic spine development

Filopodia-like protruding structures on the surface of dendrites are thought to be the precursors for mature dendritic spines. However, current literature has proposed three models to describe spinogenesis (figure 3): 1) synaptic contact from the pre-synaptic component induces changes within the filopodium precursor, which leads to development of mature spine head; 2) filopodium precursor is slowly undergoing maturation, but synaptic contact from the pre-synaptic component induces acceleration in maturation of spines; and 3) pre-synaptic component of the synapse comes into direct contact with the dendritic shaft and induce development of mature spine (Sorra and Harris 2000, Ethell and Pasquale 2005). Although, each of these three models describes a distinct origin for dendritic spines, the processes may occur concurrently.

The major hallmark for synapse maturation is the transformation of the immature glutamatergic postsynaptic compartment into a mature dendritic spine. Various mature spine morphologies have been observed in fixed brain tissue, but are generally classified into four types according to morphological appearance (figure 1): thin (slender neck and small head), stubby (no neck and large head), cup (short neck and cup head), mushroom (short thin neck and large bulbous head), (Ethell and Pasquale 2005, Sekino et al. 2007). In many instances, all of the mature morphologies of dendritic spines can be seen on a single segment of dendritic shafts (figure 1B and C).

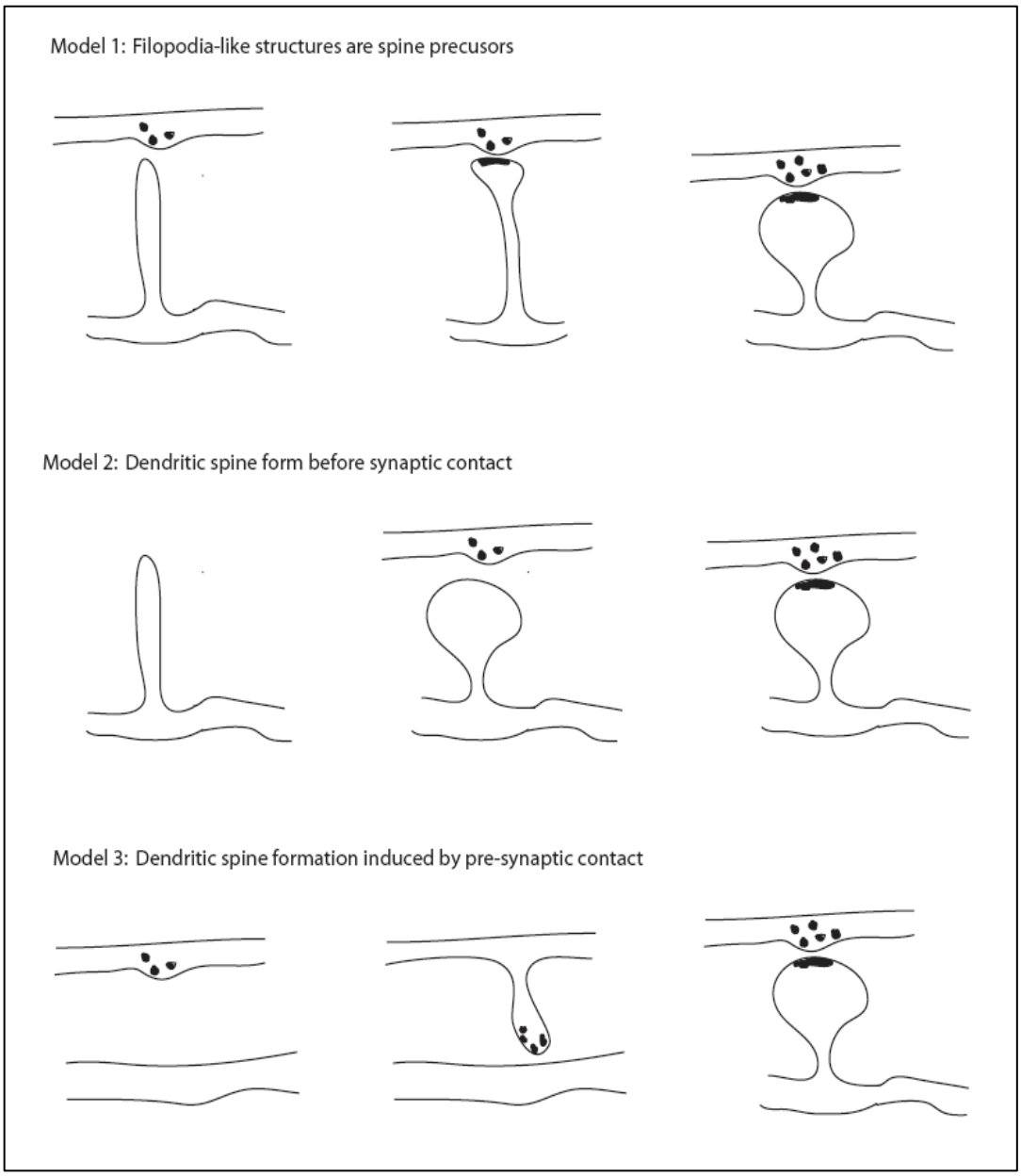

Figure 3 Three models of spinogenesis.
Model 1 shows that pre-synaptic contact induces changes within filopodium precursor and leads to development of an enlarged mature spine head. Model 2 shows that filopodium precursor is undergoing maturation slowly, but pre-synaptic contact accelerates the maturation of spines. Model 3 shows that synaptic contact on the dendritic shaft induces development of mature spine at the site of contact. (Figure modified from Progress in Neurobiology 75(2005) 161-205)

The most stable mature dendritic spines tend to have "mushroom" shape, with bulbous heads that are connected to the dendritic shaft by thin spine necks (Calabrese et al. 2006). Spine heads are thought to form solitary compartments that can isolate excitatory synaptic input and Ca^{2+} from the rest of the neuron (Sheng and Kim 2007). However, neuronal activity can regulate diffusion of molecules between spine heads and dendritic shafts and spine morphology (Bloodgood and Sabatini 2005, Araya et al. 2006). In addition, dendritic spines undergo alteration in density and morphology in response to synaptic activity. For instance, growth of new spines, or strengthening of synapse by changes in spine head morphology occurs to facilitate greater excitatory synaptic activity inducing long-term potentiation (LTP) (Sorra and Harris 2000, Grutzendler et al. 2002, Yun et al. 2007), suggesting a correlation between spine morphology and synaptic function (Hering and Sheng 2001).

What determines dendritic spine morphology?

Actin is the main cytoskeletal component of dendritic spines and filamentous actin is highly enriched within spine heads. Actin is a highly conserved globular protein with a molecular weight of 43kDa. In its monomer state it is referred to as globular or G-actin. Under physiological conditions, actin can self assemble and undergo polymerization to form helical filaments of actin (F-actin). The initial step for actin assembly requires nucleation (assembly of dimeric or trimeric complex) of monomeric G-actin. This nucleation phase is unfavorable because the short oligomers of G-actin are

unstable. Actin assembly factors, such as Arp2/3 complex, formins, spire, and cordon blue can facilitate the dimeric or trimeric actin complex assembly (dos Remedios et al. 2003). Subsequent addition of monomeric G-actin is more favorable and leads to active actin polymerization and formation of polarized actin filaments. Actin filaments have a fast-growing end (barbed end), where ATP-bound G-actin is polymerized onto the growing filaments. Actin filaments also have pointed ends (slow-growing end), where active actin depolymerization occurs. Slow hydrolysis converts ATP-actin into ADP-actin as actin monomers are shifted along the filament towards the pointed end.

Actin filaments can elongate by polymerizing actin monomers on the fast-growing end of the filament. Actin filaments can also extend by making side branches and nucleating new filaments. FRET experiments using GFP-actin show that LTP-induction can increase actin polymerization within dendritic spines (Okamoto et al. 2004). A malleable actin cytoskeleton thus appears to be the underlying component responsible for development and plasticity in morphology of spines. Local organization of F-actin depends on the composition and activity of actin binding proteins available (dos Remedios et al. 2003). Several studies have shown localization of various ABPs that may regulate actin dynamics within spine heads (figure 2). The activity of membrane bound receptors (i.e. NMDAR, mGluR and EphR) on the surface of spine heads regulates multiple downstream biochemical signaling pathways that ultimately converge to modify the function and localization of ABPs and induce changes on F-actin organization.

For example, activation of postsynaptic NMDA receptors and increase in postsynaptic Ca^{2+} levels induces increase localization of the sequestering protein, profilin II into the spine heads (Ackermann and Matus 2003). Profilin can bind G-actin, position actin monomers to barbed ends of growing filaments, and increase the exchange of ADP for ATP that facilitates actin polymerization and f-actin treadmilling (dos Remedios et al. 2003). In addition, profilin can enhance Arp2/3-dependent actin nucleation and f-actin polymerization downstream of Cdc42 (Yang et al. 2000), which may help in the stabilization of spine head morphology.

The capping protein, gelsolin function is Ca^{2+} dependent and mediates f-actin rearrangement by NMDA receptor activation (Ethell and Pasquale 2005). Gelsolin binds to the barbed ends of actin filaments and prevents further actin polymerization at exposed barbed ends (dos Remedios et al. 2003). Gelsolin also has a severing function and breaks actin filaments, which increases the number of pointed ends and promotes f-actin shortening (Cooper and Schafer 2000). Primary hippocampal neurons cultured from gelsolin knockout mice show a decrease in actin depolymerization in response to glutamate induced activation of NMDA receptor and increase Ca^{2+} influx, suggesting that gelsolin plays a major role in Ca^{2+} induced actin depolymerization in neurons (Furukawa et al. 1997).

ADF/cofilin also severs f-actin into shorter filaments, thus increasing the number of filament ends and facilitating depolymerization (Pollard 1986, dos Remedios et al. 2003, Sekino et al. 2007). ADF/cofilin function in spines is regulated by EphBR and NMDAR activation, which leads to activation of several guanine nucleotide exchange

factors (GEFs) such as kalirin (Calabrese et al. 2006). Kalirin-7 activates the Rho GTPase, Rac1 and its downstream effector p21-associated kinase (Richter et al.) (Richter et al.). PAK can directly phosphorylate and activate its downstream effector LIM kinase (Calabrese et al. 2006). Phosphorylation of ADF/cofilin by LIM kinase inhibits ADF/cofilin severing and actin depolymerization function (Sumi et al. 1999, Gungabissoon and Bamburg 2003). LIM kinase I knock–out mice show a reduced amount of phosphorylated ADF/cofilin in brain slices, reduced accumulation of f-actin in spines and abnormal dendritic spine morphology characterized by wide spine necks with small spine heads (Meng et al. 2002), suggesting that decrease in ADF/cofilin phosphorylation leads to an increase in severing and actin depolymerization activity which may increase f-actin turn over in LIM kinase I knock-out mutant mice affecting actin rich structures.

Drebrin, a side binding protein, alters the stability and mechanical properties of F-actin by altering the configuration of F-actin from kinky to straight, sometimes preventing other ABPs, such as myosin from interacting with F-actin (Hayashi et al. 1996, Sekino et al. 2007). A decrease in drebrin levels is observed in disorders accompanied by cognitive impairment, such as Alzheimer's and Down syndrome (Harigaya et al. 1996, Shim and Lubec 2002), suggesting drebrin is needed for proper cognitive function. Drebrin A is a neuron- specific isoform of drebrin that is highly enriched within dendritic spine heads (Aoki et al. 2005). Glutamate dependent activation of NMDAR induces a subcellular redistribution of drebrin from spine heads into dendritic shafts (Sekino et al. 2006) in a similar redistribution pattern as observed for f-actin in

response to enhanced synaptic activity (Ouyang et al. 2005), which may be necessary for translocation of proteins (i.e. NMDA receptors) into spine heads. Overexpression of drebrin A induces elongation of spines in mature neurons (Hayashi and Shirao 1999) and enlargement of immature filopodial spines in immature neurons (Mizui et al. 2005), suggesting drebrin has an important role in regulating spine morphology. In addition, drebrin enrichment within spines is necessary for the postsynaptic accumulation of the scaffold protein, PSD-95 and NMDAR targeting (Takahashi et al. 2003, Takahashi et al. 2006).

Although many actin-binding proteins localize to spine heads, it is not clear which actin-binding proteins are responsible for F-actin arrangement within dendritic spines. Fifkova and Delay (1982) used S-1 myosin subfragment to decorate the actin ultrastructure within dendritic spines and were able to illustrate that many of the actin filaments within the spine head had a short branched arrangement, whereas actin filaments observed within the spine necks showed longitudinal bundle arrangement similar to that observed in filopodia of growth cones (Ethell and Pasquale 2005, Korobova and Svitkina 2010). The f-actin branched arrangement observed within spine heads has similar appearance to Y-shaped branches that are made by the Arp2/3 actin binding complex. In fact, Arp2/3 has recently been shown to localize to spine heads (Racz and Weinberg 2008, Korobova and Svitkina 2010), suggesting that Arp2/3 may be involved in the production of this network of branched f-actin within spine heads. Arp2/3 complex can nucleate monomeric actin, elongate f-actin by actin polymerization, and is the only protein known to form Y-shaped branch networks of f-actin that are

important in driving membrane protrusion in motile cells (Svitkina and Borisy 1999, Sekino et al. 2007, Korobova and Svitkina 2010).

In addition, signaling molecules that are upstream of Arp2/3 are localized to dendritic spines and further suggest Arp2/3 function within spines. For example, activated EphBR forms a complex with intersectin, a GEF that specifically activate Cdc42 (Calabrese et al. 2006). Active Cdc42 promotes actin nucleation and branching by binding N-WASP, a member of the Wiskott-Aldrich syndrome family of proteins that is ubiquitously expressed in neurons (Irie and Yamaguchi 2002). Active N-WASP recruits G-actin and forms a complex with the Arp2/3 complex. Active Arp2/3 nucleates actin polymerization and promotes formation of Y-shaped branched actin filaments.

NMDAR dependent activation of the Rac-GEF, Tiam1 also leads to activation of Arp2/3 via WAVE (another Arp2/3 activator) and IRSp53 (Ten Klooster et al. 2006). Cortactin, an additional Arp2/3 activator, forms part of a group of scaffolding proteins that are associated with the cytoplasmic tail of NMDAR subunits (Sheng and Kim 2002). Thus, several signaling pathways that activate Arp2/3 appear to be available within dendritic spines suggesting Arp2/3 complex functions within spine heads in producing the meshwork of Y-shaped branches observed through EM.

Role of the Arp2/3 complex and branched actin filaments

Actin filaments not only provide structural support for cell membranes, but active polymerization of actin filaments also supplies the driving force for cellular rearrangement required in embryonic morphogenesis, cell attachment, endocytosis and many forms of motility. For example, intercellular bacterial pathogens such as Listeria, Rickettsia, Shigella, Mycobacterial, and Burkholderia, exploit the actin polymerization machinery in host cells to induce actin-based motility. The comet tails of Listeria have barbed ends (fast growing ends) of f-actin oriented to the surface of the bacteria. Thus, pathogens use the force generated from actin polymerization to propel through the host cytoplasm and infect neighboring cells (Brown 2006). Actin polymerization at the leading edge of migrating fibroblast cells and neuronal growth cones is also the driving force for the forward extension of the cell membrane (Borisy 1999, Pollard 2007).

Fibroblast motility initiates by formation and extension of a broad membranous protrusions (lamellipodium) at the leading edge. Actin at the leading edge of lamellipodia in fibroblast is very dynamic and organized into a network of short branches formed at 70º angles from the side of existing filaments with barbed ends oriented towards the cell membranes (Svitkina and Borisy 1999). This rigid meshwork of actin referred to as the "dendritic array", provides structural support for the lamellipodia. Capping proteins bind to the tips of some barbed ends and block actin polymerization, but other barbed ends of f-actin in lamellipodia are protected by anti-capping proteins and continue to elongate (Borisy and Svitkina 2000, Amann and Pollard 2001). Thus, active

polymerizations of the uncapped actin filaments in the dendritic array push the lamellipodia cell membrane forward driving membrane protrusion.

EM studies of branched filaments in lamellipodia have shown that the Arp2/3 actin-binding complex is localized to branched junctions of actin filaments (Svitkina and Borisy 1999). Arp2/3 is a ubiquitous eukaryotic actin binding protein complex composed of seven evolutionarily conserved subunits, two actin-related proteins (Arp2, and Arp3) and five polypeptides (Welch et al. 1997). All seven subunits are present together in equal stoichiometry for the Arp2/3 complex to form. Thus, presence of one subunit is indicative of the presence of the whole Arp2/3 complex. Arp2/3 functions as an actin assembly factor, where Arp2 and Arp3 form a stable dimer that facilitates favorable conditions for subsequent addition of actin monomers leading to active actin polymerization (Higgs and Pollard 2001). The Arp2/3 complex is the only actin binding protein able to form characteristic Y-shaped f-actin branches by nucleating Arp2 and Arp3 subunits to pre-existing actin filaments, facilitating actin polymerization and formation of daughter actin filament branches at 70° angles from pre-existing mother filaments (Dayel and Mullins 2004, Goley and Welch 2006).

Historically two models have been proposed to describe the formation of branched actin filaments by activated Arp2/3. The first model is known as the "side-branching model" and describes the formation of an actin branch that initiates from the side of pre-existing filaments. This model suggests that the Arp2/3 complex has two binding sites for actin, one which allows Arp2/3 to bind to the side of the existing (mother) filament and the second which allows Arp2/3 to nucleate actin monomers for

actin polymerization and formation of a new branch (daughter) filament (Mullins et al. 1997, Volkmann et al. 2001). The second model known as the "barbed-end branching model" describes the formation of a G-actin monomer-VCA-Arp2/3 complex that competes with capping proteins to bind the barbed end of an existing (mother) filament. In this model both the new daughter branch and the original mother filament elongate by actin polymerization (Suetsugu et al. 2002).

It is now generally accepted that Arp2/3 is responsible for nucleating monomeric actin to sides of existing f-actin, facilitating actin polymerization, creating a dendritic array of Y-shaped f-actin branches, which generate the protrusive force observed in fibroblast lamellipodium during cell migration (figure 4) (Borisy 1999, Higgs and Pollard 2001, Pollard 2007). My doctoral work proposes an alternate model in which the dendritic array formed by Arp2/3-actin polymerization, provides the structural support to maintain enlarged spine heads. The f-actin dendritic array may also serve as a scaffold for transmembrane proteins and provide a barrier that limits translocation of through the spine heads.

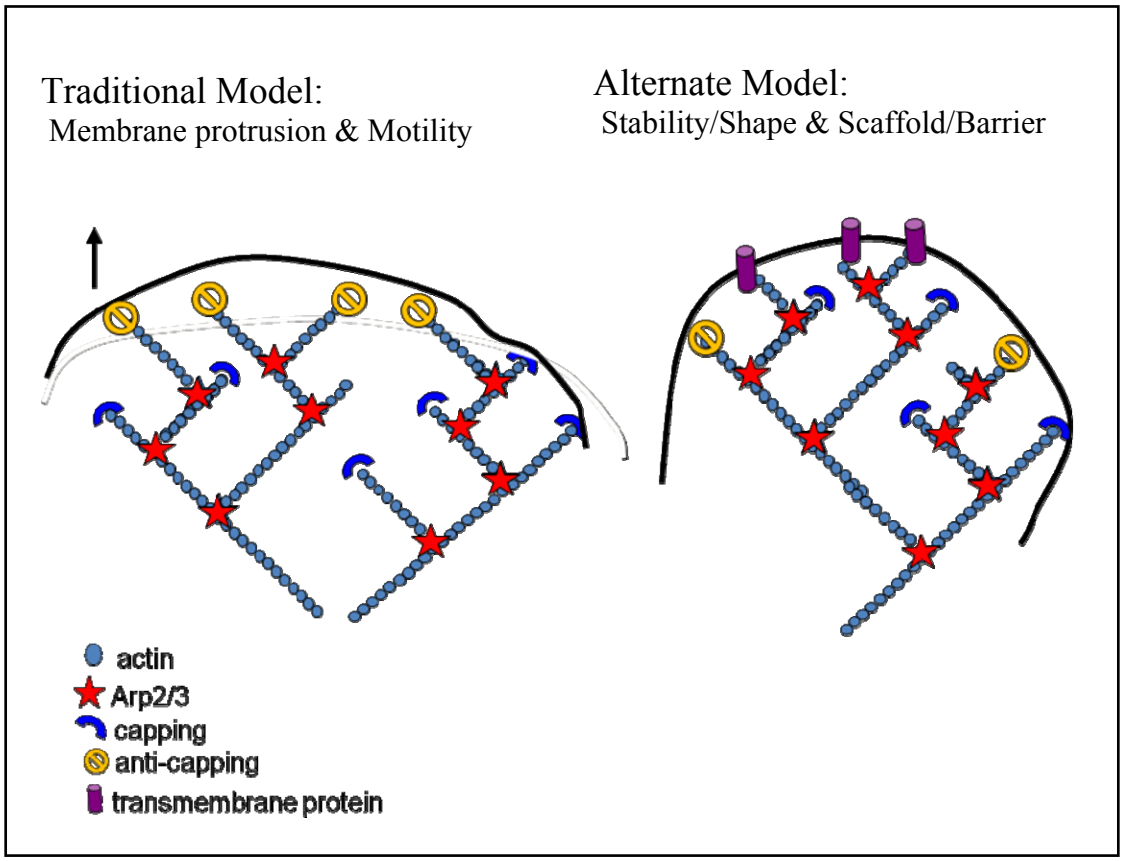

Figure 4 The traditional model is widely accepted to explain Arp2/3 dependent actin polymerization and formation a rigid meshwork of actin referred to as the "dendritic array". Capping proteins bind to the tips of some barbed ends and block actin polymerization, but other barbed ends of f-actin in lamellipodia are protected by anti-capping proteins and continue to elongate (Borisy and Svitkina 2000, Amann and Pollard 2001). Active polymerizations of the uncapped actin filaments in the dendritic array push the lamellipodial membrane forward driving membrane protrusion and motility. My doctoral work proposes an alternate model in which the dendritic array formed by Arp2/3-actin polymerization, provides the structural support to maintain enlarged spine heads. The f-actin dendritic array may also serve as a scaffold for transmembrane proteins and provide a barrier that limits translocation of proteins through the spine heads.

Regulators of the Arp2/3 complex

On its own the Arp2/3 complex has very low nucleating activity and must undergo a conformational change to be fully functional. Several nucleation-promoting factors (NPF), such as N-WASP, Cortactin and WAVE (Higgs 2001) can stimulate the nucleating activity of Arp2/3, but only PICK1 has been identified as an Arp2/3 inhibitor.

The NPFs are multidomain proteins. Two of the NPFs, N-WASP and WAVE have a common verprolin-cofillin-acidic (VCA) domain (also known as WCA domain) (figure 5), which binds and activates Arp2/3 to induce actin polymerization (Kelly et al. 2006). The verpolin-homology (V) domain, also known as the WASP homology-2 (WH2) domain, is a binding site for actin. The cofilin homology domain, also known as the central (C) domain facilitates actin and Arp2/3 binding to their respective domains. The acidic (A) domain contains acidic residues flanking a tryptophan and is the biding site for Arp2/3. Each of the NPFs also have their own unique domains, which allow them to interact with various other proteins that regulate their function and link them to different signaling pathways (Soderling 2009).

The neural Wiskott-Aldrich syndrome protein (N-WASP), which is highly enriched in the brain and localized to dendritic spines and functional synapses (Wegner et al. 2008), is the best characterized Arp2/3 activator (Takenawa and Miki 2001). A decrease in the density of dendritic spines and excitatory synapses is observed after RNA interference knockdown of endogenous N-WASP or inhibition of its activity by wiskostatin, a specific N-WASP inhibitor (Wegner et al. 2008); suggesting N-WASP activity is necessary for dendrite and synapse formation.

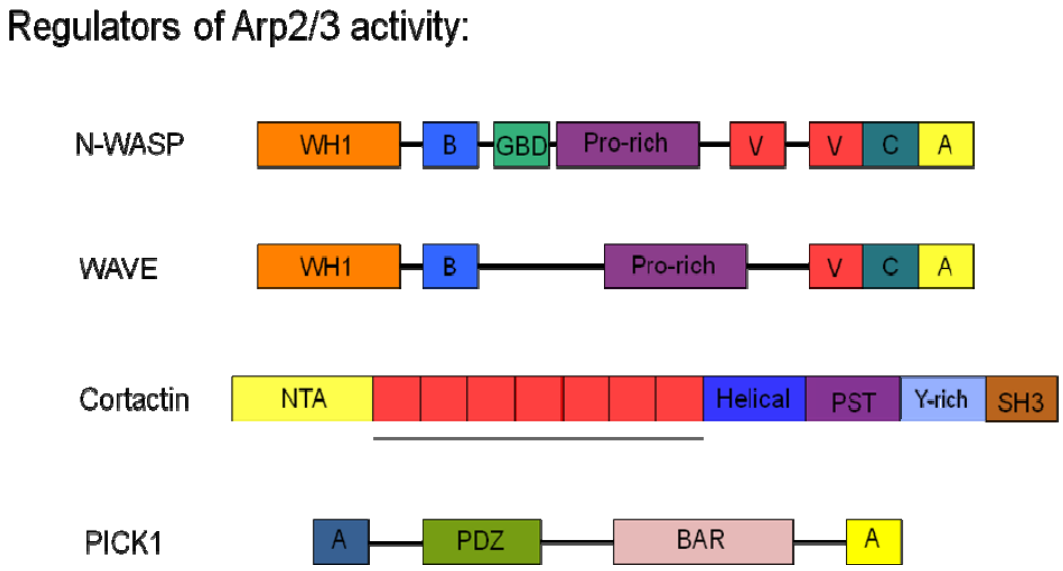

Figure 5 The Arp2/3 complex is regulated by several multi-domain proteins that have a common acidic (A) domain (shown in yellow). The acidic (A) domain contains acidic residues flanking a tryptophan and is the biding site for Arp2/3. Regulating proteins that activate Arp2/3 are referred to as nuclear promoting factors (NPFs) and include N-WASP, WAVE, and Cortactin. Two of the NPFs have a common verprolin-cofillin-acidic (VCA) domain (also known as WCA domain) (Kelly et al. 2006). The verpolin-homology (V) domain, also known as the WASP homology-2 (WH2) domain, is a binding site for actin. The cofilin homology domain, also known as the central (C) domain facilitates actin and Arp2/3 binding to their respective domains. Cortactin lacks both V and C domains that are common to other NPFs. Absence of a V domain results in the inability for cortactin to bind monomeric G-actin resulting in a weaker ability to activate Arp2/3 in comparison to the VCA domain of other NPFs (Weaver et al. 2003). However, the presence of six and a half tandem repeats (shown in red) facilitate cortactin binding to f-actin (Weed and Parsons 2001) which is required for Arp2/3 complex activation. PICK1 (protein that interacts with C kinase 1) is a recently identified inhibitor of Arp2/3 function. PICK1 is a dimeric scaffold protein that contains a C-terminal acidic (A) region (shown in yellow) and tryptophan residue that is similar to Arp2/3 activators (Rocca et al. 2008). PICK1 inhibits Arp2/3 by competing with N-WASP for VCA binding site on Arp2/3. Each of the Arp2/3 regulators also have their own unique domains, which allow them to interact with various other proteins that regulate their function and link them to different signaling pathways (Soderling 2009).

N-WASP is a multidomain protein (figure 5) that has an N-terminal WASP-homology-1 (WH1, also known as Ena-WASP homology-1 (EVH1)) domain that interacts with the WASP interacting protein (WIP) family to help maintain N-WASP stability (Anton and Jones 2006). The WH1 domain is followed by a basic (B) region that interacts with the membrane bound phosphatidylinositol 4,5-bisphosphate, PIP2 and is important for N-WASP localization in close proximity to the cell membrane (Takenawa and Suetsugu 2007). The GBD (GTPase binding) domain follows the B domain, which are both crucial for N-WASP activation. Endogenously, N-WASP is kept in an autoinhibited state through an intramolecular interaction between its GBD domain and the central domain of VCA (Derivery Emmanuel and Gautreau 2010). However, synergistic binding of Cdc42 to the GBD domain, PIP2 binding to the B domain and phosphorylation by Src tyrosine kinase induces a conformational change that releases N-WASP from autoinhibition, exposing the VCA domain for Arp2/3 binding and activation (Ma et al. 1998). N-WASP also contains a proline-rich domain that serves as a binding site for many SH3 containing proteins and profilin, which recruits GTP-actin monomers to the N-WASP V-domain (Takenawa and Suetsugu 2007). The proline-rich domain of N-WASP also binds F-BAR family proteins, such as TOCA-1 (transducer of Cdc42-dependent actin assembly 1) that are involved in endocytic vesicle formation by cell membrane deformation (Takenawa and Suetsugu 2007, Derivery Emmanuel and Gautreau 2010). F-BAR containing proteins are also able to induce N-WASP activation independent of Cdc42 by dimerization of N-WASP through F-BAR and SH3 domain interaction (Derivery Emmanuel and Gautreau 2010). Dimerization of N-WASP leads to

enhanced Arp2/3 activity by permitting two VCA domains to interact with Arp2/3 (Soderling 2009). However, deletion of the C-terminal VCA domain of N-WASP leads to a decrease in dendritic spine and synapse density, suggesting that activation of Arp2/3 by N-WASP is necessary for dendritic spine and synapse formation (Wegner et al. 2008).

The Wiskott-Aldrich verprolin homologous protein (WAVE) family is another group of Arp2/3 activators that is comprised of three proteins, WAVE1, WAVE2, and WAVE3. WAVE1 and WAVE2 are ubiquitously expressed in all tissues except skeletal muscle, while WAVE3 is highly enriched in brain tissue (Suetsugu et al. 1999). Interestingly, WAVE1 and WAVE3 localize to dendritic spine heads where they act directly downstream of Rac GTPase and the adaptor protein Nck1 (non-catalytic region of tyrosine kinase adaptor protein 1 (Pilpel and Segal 2005). In addition to the C-terminus VCA domain, each of the WAVE proteins have an N-terminal WAVE/SCAR homology domain (WHD/SHD) that contributes to WAVE heterocomplex formation with Abi (Abelson-interacting protein), NAPI (also known as p125NAP1), CYFIP (Cytoplasmic fragile X mental retardation interacting protein), and HSP300 (Takenawa and Miki 2001). Knocking down of any one member of the WAVE complex has been shown to cause degradation of the rest of the complex members (Schenck et al. 2004), suggesting that all members of the WAVE complex are essential for WAVE function. The WAVE basic (B) domain immediately follows the WHD/SHD domain and plays an important role in the localization of WAVE protein (Takenawa and Miki 2001). WAVE2 basic (B) region binds the phophoinositide PIP3 (PtdIns(3,4,5)P3) which is necessary for WAVE2 recruitment to the plasma membrane (Derivery Emmanuel and Gautreau 2010).

All WAVE proteins also have a proline–rich domain that precedes the C-terminus VCA domain. Similar to the N-WASP proline-rich domain, the WAVE proline-rich domain binds SH3 containing proteins, specifically IRSp53. IRSp53 also contains an N-terminal Rac binding domain (RCB) that facilitates binding to active Rac (Suetsugu et al. 2006). The WAVE complex exists in an inactive state in the cytosol (Derivery et al. 2009). However, activate Rac regulates the activity of WAVE proteins by associating with WAVE complex members PIR121/Sra1-NAP1 and IRPp53, thus exposing the VCA domain and connecting the Rac signal to Arp2/3 complex-mediated actin polymerization (Miki et al. 2000, Derivery et al. 2009). IRSp53 and PIP3 function synergistically to translocate the WAVE protein to the plasma membrane where IRSp53 associates with active Rac. WAVE dimerizes via it's BAR domain and binds PIP2 inducing formation of outward membrane protrusion by Arp2/3-dependent actin polymerization (Derivery et al. 2009, Derivery Emmanuel and Gautreau 2010).

Similar to N-WASP and WAVE, cortactin also has an acidic (A) domain that binds and activates the Arp2/3 complex. However, cortactin lacks both V and C domains that are common to N-WASP and WAVE. Absence of a V domain results in the inability for cortactin to bind monomeric G-actin resulting in a weaker ability to activate Arp2/3 in comparison to the VCA domain of other NPFs (Weaver et al. 2003). However, the presence of six and a half tandem repeats facilitate cortactin binding to f-actin (Weed and Parsons 2001) which is required for Arp2/3 complex activation. The A domain of cortactin is located on the amino-terminal (NTA) region (figure 5), which also contains a conserved tryptophan that is characteristic of all NPFs (Higgs and Pollard 2001, Weed

and Parsons 2001). The NTA domain of cortactin binds to Arp3 subunit while the N-WASP VCA domain binds Arp2 and ARPC1 subunits, forming a transitional Arp2/3-N-WASP-cortactin complex until N-WASP is displaced by cortactin when it gains higher affinity for activated Arp2/3 and f-actin (Daly 2004, Lua and Low 2005). The c-terminus of cortactin contains an α-helix domain, a proline-rich domain, and a SH3 domain. A stable Arp2/3-N-WASP-cortactin complex can form when the SH3 domain of cortactin binds to the proline-rich domain of N-WASP leaving two A domains available to synergistically activate the Arp2/3 (Lua and Low 2005). Additionally the SH3 domain of cortactin binds to a variety of proteins that recruit cortactin and Arp2/3 to various actin polymerization mediated cellular functions (Lua and Low 2005). In the process of endocytosis, cortactin SH3 domain interacts with dynamin2s' proline-rich domain, which recruits cortactin and Arp2/3 function to clathrin-mediated vesicle formation (McNiven et al. 2000). In dendritic spine heads, the SH3 domain of cortactin associates with the proline-rich domain of the scaffold protein, Shank; suggesting that Arp2/3-dependent actin polymerization is involved in the formation of scaffolds at the PSD of excitatory synapses (Naisbitt S. 1999). In addition to activating Arp2/3 function, cortactin can also stabilize branched filaments by forming an Arp2/3-N-WASP-cortactin complex that prevents debranching and disassembly of actin (Weed and Parsons 2001).

PICK1 (protein that interacts with C kinase 1) is a recently identified Arp2/3 regulator that is highly expressed in the brain where it is localized to dendritic spine heads and it is believed to be involved in AMPAR endocytosis (Hanley 2006, 2008). PICK1 is a dimeric scaffold protein that contains a C-terminal acidic region (figure 5)

and tryptophan residue that are similar to Arp2/3 activators (Rocca et al. 2008). However, unlike NPFs PICK1 acts as a local inhibitor of Arp2/3 function. In *in-vitro* pyrene-labeled actin polymerization assays PICK1 reduces the rate of VCA-stimulated Arp2/3 dependent actin polymerization (Rocca et al. 2008), suggesting that PICK1 inhibits Arp2/3 dependent actin polymerization. PICK1 induced inhibition of Arp2/3 is believed to occur by either of two ways: 1) PICK1 displacement of the VCA domains of Arp2/3 activators from binding sites on the Arp2/3 or 2) direct inhibition of Arp2/3 by PICK1 binding (Rocca et al. 2008). Co-immunoprecipitation experiments show that PICK1 and N-WASP VCA do not interact, suggesting that PICK1 does not inhibit VCA directly, but instead it may compete with N-WASP for VCA binding site on Arp2/3. Furthermore, GST-PICK1 pull-down assay show that PICK1 interacts directly with Arp2/3 and f-actin (Rocca et al. 2008).

In addition to the Arp2/3 binding C-terminus acidic region, PICK1 also contains an N-terminus acidic domain that acts as a calcium sensor. However, PICK1 is best known for its PDZ and BAR domains. The PDZ domain of PICK1 binds to a variety of membrane proteins including receptors, transporters and ionic channels (Xu and Xia 2006). The PDZ domain also forms an intramolecular interaction with its own BAR domain, inducing the closed inhibited conformation of PICK1. In endocytosis, the PDZ domain binding to the GluR2 subunit of AMPAR exposes the BAR domain which is free to bind to a variety of binding partners such as f-actin, phospholipids, and other BAR domain containing proteins(Rocca et al. 2008).

Co-immunoprecipitation experiments from neuronal tissue extracts show that Arp2/3, PICK1, actin and GluR2 (AMPA receptor subunit) interact to from a complex that mediates AMPA receptor endocytosis (Rocca et al. 2008). Knockdown of PICK1 by shRNA blocked GluR2 (AMPAR subunit) endocytosis (Iwakura et al. 2001, Rocca et al. 2008). Cotransfection of PICK1 shRNA and shRNA resistant wild-type PICK1 can rescue AMPAR endocytosis, but cotransfection of PICK1 shRNA and a PICK1mutant (lacking Arp2/3 binding domain) is not able to rescue AMPAR endocytosis (Rocca et al. 2008), suggesting that PICK1-Arp2/3 interaction maybe required for AMPAR internalization.

Research Aims

Electron micrograph studies of dendritic spines have illustrated the presence of a meshwork of short Y-shaped branches actin filaments in spine heads (Fifkova and Delay 1982, Korobova and Svitkina 2010, Svitkina 2010). In addition, recent studies have highlighted the presence of Arp2/3 (Racz and Weinberg 2008, Hotulainen et al. 2009, Korobova and Svitkina 2010, Svitkina 2010), Arp2/3 activators (N-WASP (Wegner et al. 2008), Cortactin (Racz and Weinberg 2006, Hotulainen et al. 2009) and WAVE1(Kim et al. 2006, Hotulainen et al. 2009)), and other proteins (profilin (Ackermann and Matus 2003) and ADF/Cofilin (Racz and Weinberg 2006, Hotulainen et al. 2009)) necessary for generating the protrusive meshwork of Y-shaped f-actin branches. Results from these studies suggest the following: 1) Arp2/3 regulates the formation of the Y-shaped branched networks within dendritic spine heads and 2) these Y-shaped networks of actin are dynamic. However, we are left to speculate on the role of branched actin meshwork within dendritic spines. Do the branched actin filaments provide membrane structural support that adapts to changes in spine maturity or synapse activity resulting in subsequent changes in morphology, or are the branched actin filaments a scaffold barrier that helps anchor transmembrane proteins to the spine head membrane?

Since Arp2/3 is the only actin binding protein known to facilitate production of branched actin filaments, I was interested in determining if the actin related protein (Arp) 2/3 complex played a role in regulating dendritic spine development and morphology. The general hypothesis for my doctoral work is that *if Arp2/3 is a major regulator of dendritic spine morphology, then Arp2/3 will be enriched in dendritic spines heads and*

inhibition of Arp2/3 activity will alter the number and/or morphology of dendritic spines. To investigate if Arp2/3 complex was necessary for dendritic spine development and maintenance of mature morphology I first needed to determine the localization of Arp2/3 in dendritic spines. I used cell fractionation assays, immunofluorescence of egfp tagged Arp2/3 subunit, and antibody staining to show Arp2/3 localization within the dendritic spine heads of cultured hippocampal neurons. Once having determined that Arp2/3 is localized to spine heads I explored the morphological consequence of inhibiting Arp2/3 function during various developmental time points of dendritic spine development in hippocampal cultures. Since there is evidence to suggest that f-actin and several actin binding proteins undergo activity dependent redistribution, I used immunofluorescence to survey the localization of Arp2/3 within dendritic spines in response to induced synaptic activity. I also explored the morphological consequence of Arp2/3 inhibition in response to synaptic activity. Finally, since f-actin redistribution is also observed in hippocampal cultures after ischemia preconditioning, the neuroprotective role of Arp2/3 in ischemia tolerance was explored in collaboration with Dr. Robert Meller of Dow Neurobiology Laboratory at Legacy Research, Legacy Health in Portland Oregon, who performed and analyzed the ischemia tolerance experiments.

CHAPTER 2 Localization of the Arp2/3 complex within dendritic spines

Introduction

Examination of dendritic spines ultrastructure suggests that a meshwork of branched f-actin is the main cytoskeletal structural component within dendritic spine heads, whereas spine necks contain an array of short parallel filaments of actin (Fifkova and Delay 1982). During development and in response to synaptic activity, spines undergo changes in morphology that are believed to be driven by rearrangement in the underlying actin cytoskeleton (Matus et al. 2000). Although many actin-binding proteins are enriched within dendritic spine heads (figure 2), the f-actin arrangement within the spine heads appears similar to Y-shaped f-actin branches that are characteristically formed by the actin-binding protein complex, Arp2/3 (Goley and Welch 2006). This similarity suggests that Arp2/3 may be involved in forming the branched f-actin within spine heads. In addition, several Arp2/3 activators (N-WASP, Cortactin, and WAVE) are enriched within spine heads and are believed to be important for regulating spinogenesis, spine morphology and spine density by regulating Arp2/3 dynamics (Hering and Sheng 2003a, Pipel and Segal 2005, Soderling et al. 2007b, Wegner et al. 2008).

In this study we used cell fractionation assays, immunofluorescence and antibody staining to explore the localization of Arp2/3 and its activators within dendritic spines of hippocampal cultures. The results from my localization experiments corroborate with other studies (Soderling et al. 2002, Racz and Weinberg 2004, Racz and Weinberg 2008,

Wegner et al. 2008, Korobova and Svitkina 2010) that show Arp2/3 and its activators are localized within dendritic spine heads where Arp2/3 dependent actin polymerization forms Y-shaped branches of f-actin that may provide the structural support needed for maintenance of dendritic spine head morphology.

Materials and methods

Primary Hippocampal Cultures

Hippocampal tissue was dissected from embryonic day (E16) Swiss Webster mice in sterile 1XHBSS (Hanks balance salt solution) with 10mM HEPES solution. Hippocampal tissue was collected and incubated in 0.25% trypsin-EDTA at 37ºC for 15minute. After rinsing 3 times for 5 min. in HBSS, tissue was suspended in hippocampal plating media (EMEM/10mM HEPES/ 1mM sodium pyruvate / 0.5mM glutamine/12.5µM glutamate/10% FBS/ 0.6% glucose and dissociated into single cells by triturating with a fire polished Pasteur pipette. Dissociated hippocampal cells were used to prepare in vitro primary hippocampal cultures (HCC), which were plated in hippocampal plating media at density of 2x10^5 cells/35mm dish. Each 35mm dish contained five Poly-D-Lysine/Laminin (100µg/ml / 4µg/ml) coated 12mm-coverslips. Poly-D-lysine hydrobromide was purchased from sigma, dissolved in borate buffer (pH= 8.5), and filtered sterilized (0.2µ filter) before use. Three hours after platting hippocampal dissociated cells, the hippocampal platting media was replaced with growth media (Neurobasal /B27/0.5mMGlutamine). After 4DIV (days in vitro), primary HCC were fed glia-conditioned media by replacing 50% of growth media with glia-conditioned growth media (growth media that was conditioned by glia cultures for 48hrs). On DIV3, 5-FUDR (5-Fluoro-2'-deoxyuridine) was added to inhibit mitosis. Primary hippocampal cultures were allowed to continue *in- vitro* development with incubation at 37ºC/5%CO_2 until 29DIV. Culture health was observed and maintained by weekly feedings of glia-conditioned media at 7DIV, 14DIV and 21DIV.

Glia Cultures and glia-conditioned growth media

Sterile 1XHBSS (Hanks balance salt solution) with 10mM HEPES solution was used for cortical tissue dissections from postnatal (P1or P2) Swiss Webster mice. Striatal tissue was removed and the remaining brain tissue was collected in 1XHBSS with 10mM HEPES solution and minced. Minced brain tissues were incubated in 1XHBSS/10mM HEPES plus .25% trypsin and DNAse 1 at 37ºC for 30 minutes. After trypsinization, equal volume of glia platting media (EMEM/10mM Hepes/1mM Sodium Pyruvate/2mM glutamine/10% FBS/0.6% Glucose/ Penicillin-Streptomyocin was added and centrifuge for 5 minutes at 1000rpms. The supernatant was removed and minced tissue was resuspended in the glia plating media and mechanically dissociated with fire polished Pasteur pipette. Dissociated cells were filtered through 0.7µm cell strainer and plated in 10 cm tissue culture dishes. Glia cultures were split 1:3 after reaching a density 90% confluent and glia-plating media was replaced every subsequent week for two months. *In vitro* glia cultures were incubated in 37ºC/ 5% CO_2 and maintained for use in preparation of glia- conditioned growth media used to feed primary hippocampal cultures. Glia-conditioned growth media was prepared by removal of glia plating media from *in vitro* glia cultures incubated in 37ºC/ 5% CO_2 and replacing with growth media (Neurobasal /B27.0.5mM glutamine) for 48hrs. Once glia conditioning of growth media is complete, the glia-conditioned growth media is removed and replaced with fresh glia plating media.

Adenovirus production

Recombinant adenoviruses were produced using the AdEasy system in which the E1 gene (necessary for virus particles assembly) is provided by adenovirus packaging cell line, 293 HEK cells (He et al. 1998). EGFP, EGFP-CA and p21-EGFP were cloned by PCR and inserted into pShuttle CMV. The resulting plasmids were linearized with Pme 1 and electroporated into BJ5183-Ad1 (electrocompetent E-coli bacteria with AdEasy-1, supercoiled viral DNA). Recombinants were selected for kanamycin resistance. Once recombinants were identified by Pac I digest, recombinant deficient DH5α bacteria were used to expand recombinant DNA. Purified recombinant DNA was digested with Pac I to expose its inverted terminal repeats (ITR), and then used for calcium-phosphate transfection into 293HEK. The 293 HEK cells were used for large-scale virus production and after four passages; the viruses were purified on CsCl gradient and tittered on 293HEK cells.

Immunostaining for Arp2/3 and post-synaptic proteins

Primary hippocampal cultures (HCC) were fixed for 20min at 4ºC with PPS (4% paraformaldehyde in PHEM buffer (60mM PIPES (pH 7.0), 25mM HEPES (pH 7.0), 10mM EGDT, 2mM $MgCl_2$, 200mM sucrose). Cultures were prepared for immunofluorescence staining: rinsed in PBS for 5 minutes at room temperature, incubated for 30min in 3% BSA (bovine serum albumin in PBS) at room temperature, permealized for 10min in 0.2% triton (in PBS), rinsed in PBS for 5min at room temperature, and blocked in 3% BSA (bovine serum albumin in PBS) for 30 min at room

temperature. HCC were incubated in primary antibodies overnight at 4ºC. Primary antibodies used: anti-psd95 (Upstate), anti-p34 (Upstate), anti-SV2 (Developmental Studies Hybridoma Bank), anti-MAP2 (Covance). Coverslips were rinsed in PBS for 5 min to remove primary antibodies; then incubated in secondary antibodies for 1hr at room temperature. Secondary antibodies used: Cy5 anti-mouse (1:100), Coumarin anti-rabbit (1:100). Coverslips were also stained for f-actin with ALEXA 594 phalloidin (1:100, purchased from Molecular Probes). Cultures were rinsed with PBS for 5 min to remove secondary antibodies and excess phalloidin stain; then mounted using a DABCO based glycerol media to reduce photobleaching.

Imaging

Primary hippocampal cultures were fixed with PPS and stained the following antibodies and stain: anti-SV2, anti-MAP2, anti-p34, anti-PSD-95 and Alexa 594 phalloidin. Infected cells were identified by EGFP expression. Images of dendritic spines were taken using Zeiss Axiovert 200M 40x, 63x and 100x-oil objective and Openlab software (Improvision). Images were processed using Adobe Photoshop.

Synaptosomal and PSD Fractionation

Synaptosomal and PSD fractions were prepared as previously described (Carlin et al. 1980, Huttner et al. 1983b) from postnatal day 14 (P14) mouse pups. Neuronal organelles were isolated by synaptosomal fractionation using mechanical and osmotic

lysis at several centrifugation speeds in non-detergent hypotonic solution. Post-synaptic material was isolated and concentrated by PSD fractionation using mechanical lysis and centrifugation in non-ionic and denaturing detergent solutions. For all fractions, samples were resuspended in 0.1%SDS/40mM Tris pH 8.0 and the protein concentration determined by BCA assay (Pierce). For western blots, 10μg of each fraction were run on 10% SDS-PAGE gels, transferred onto nitrocellulose membrane and probed with anti-p34 (Upstate Biotechnology), anti-cortactin (Upstate Biotechnology), anti-WAVE (H-180, Santa Cruz Biotechnology), anti-NWASP (4335-NW3B, produced by the Lanier Lab), anti-PSD-95 (Upstate Biotechnology), and anti-SV2 (Developmental Studies Hybridoma Bank). Signal was developed using chemiluminescence (Amersham, ECL Western Blotting Analysis System).

Results

Localization of Arp2/3 and NPFs in neuronal synapses

We used cell fractionation assays in order to determine the endogenous enrichment of Arp2/3 and its activators within synaptic compartments. Synaptosomal and PSD fractions were taken from mouse brain tissue at postnatal day 14 (P14), a time in brain development when synapses are undergoing maturation (Harris Kristen M. 1992, Pan Feng. 2008). For the synaptosomal fractionation, brain tissue was minced, homogenized, and put through various centrifugation settings under non-detergent hypotonic solutions conditions to isolate neuronal cell fractions by mechanical and osmotic lysis (figure 6A)(Huttner et al. 1983a). Proper synaptosomal fractionation was confirmed by western blots with antiserum to detect SV2 (a synaptic vesicle protein) and PSD-95 (a post-synaptic density protein). Western blot analysis using antiserum against p34 (an Arp2/3 subunit) revealed that p34 was enriched in the LP1 fraction (which contains the pre- & post-synaptic membrane and proteins tightly associated with these membranes) and in the LP2 fraction (which contains crude synaptic vesicles and parts of the actin cytoskeleton, Figure 6B). These results suggest that Arp2/3 enriched at neuronal synapses.

The Arp2/3 activators WAVE, N-WASP and Cortactin showed different enrichment patterns. WAVE was more enriched in the LP1 fraction than in the LP2 fraction, suggesting that WAVE is associated with pre- and post- synaptic membranes. N-WASP was also present in the LP1 fraction, but was not highly enriched, whereas Cortactin was not detected in the LP1 fraction, but was highly enriched in the LS2

fractions (soluble synaptic proteins), suggesting that Cortactin is not tightly associated with neuronal synaptic membranes or the actin cytoskeleton in synapses.

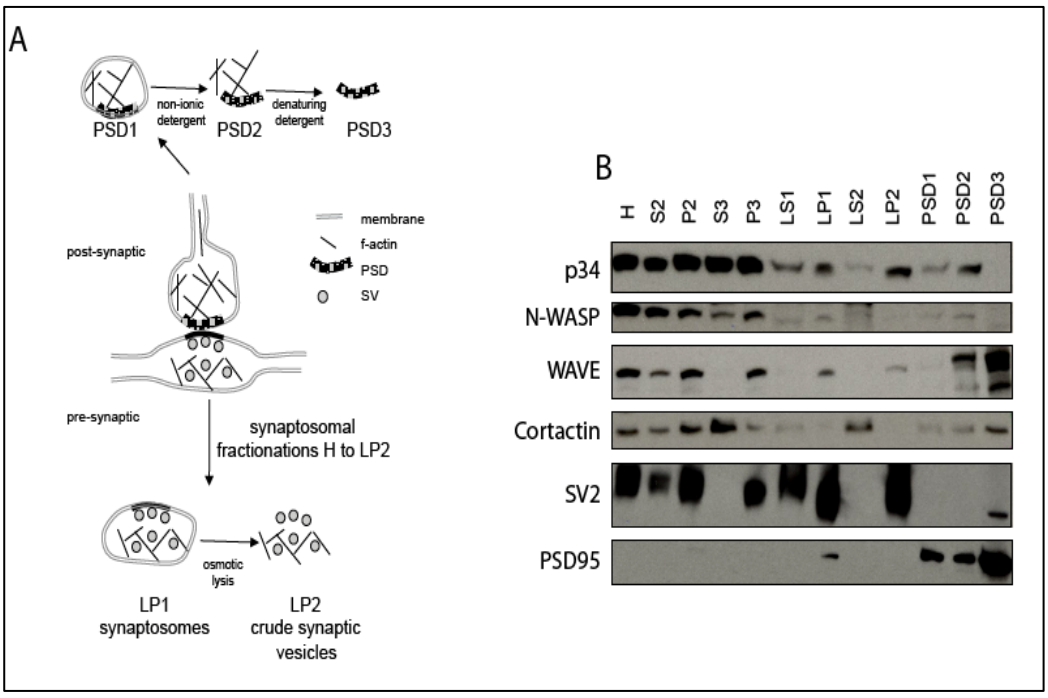

Figure 6 Endogenous enrichment of Arp2/3 and its activators in synaptosomal and post-synaptic density brain fractions. (A) Synaptosomal and PSD fractions were isolated from postnatal day 14 mouse brains. Synaptosomal fractionation assay isolates neuronal organelles (by mechanical and osmotic lysis using several centrifugation speeds in non-detergent hypotonic solution: H (crude homogenate), S2 (crude cytosol), P2 (membranes & vesicles), S3 (cytosol), P3 (golgi, ER, light membranes), LS1 (soluble synaptic proteins & small vesicles), LP1 (pre- & post-synaptic membranes), LS2 (soluble synaptic proteins), and LP2 (crude synaptic vesicles)) by mechanical and osmotic lysis using several centrifugation speeds in non-detergent hypotonic solution. PSD fractionation assay isolates and concentrates post-synaptic material by mechanical lysis using several centrifugation speeds in non-ionic and denaturing detergent solutions: (PSD1 (crude post-synaptic synaptosome), PSD2 (f-actin cytoskeleton & psd-core proteins), PSD3 (psd- core proteins)) by mechanical lysis using several centrifugation speeds in non-ionic and denaturing detergent solutions. (B) Immunoblots were probed for SV2 (a pre-synaptic marker), PSD-95 (a post-synaptic marker), p34 (a subunit of Arp2/3), and the Arp2/3 activators (N-WASP, Cortactin, and WAVE). The LP1 (lysed synaptosome membrane) fraction contained Arp2/3, N-WASP and WAVE; whereas Cortactin was highly enriched in the LS2 (soluble synaptic proteins) fraction. Arp2/3 and its activators (N-WASP, Cortactin and WAVE) were enriched in the PSD2 (f-actin cytoskeleton & psd-core proteins) fraction, only Cortactin and WAVE were enriched in the PSD3 (psd-core proteins) fraction. Together these results suggest that Arp2/3 is localized to the pre- & post-synaptic compartments and the Arp2/3 activators, Cortactin and WAVE may have other functions besides regulating Arp2/3 functions, since they associate with psd-core proteins.

A PSD fractionation was done to discern between pre- or post-synaptic localization of Arp2/3 and its activators. For the PSD fractionation, brain tissue was minced, homogenized, and put through various centrifugation settings to obtain synaptosomes that were then put through a sucrose density gradient to isolate the crude post-synaptic densities (PSD1 fraction), which contains the post-synaptic membrane, f-actin, and PSD core proteins (figure 6A). A non-ionic detergent was used on the crude post-synaptic synaptosomes to isolate the PSD2 fraction, which contains the f-actin cytoskeleton, PSD core proteins, and an enrichment of other tightly associated proteins. A denaturing detergent was then used to isolate the PSD3 fractions, which contained an enrichment of PSD core proteins (figure 6A) (Carlin Richard K. 1980).

As with the PSD fractionation, immunobloting to detect SV2 and PSD-95 confirmed that proper PSD fractionation was achieved. Western blot analysis on PSD fractions showed that p34 (Arp2/3 subunit) and N-WASP are enriched in the PSD2 fraction but not in the PSD3 fractions (figure 6B), suggesting that Arp2/3 and N-WASP are tightly associated with the f-actin cytoskeleton and may be important for regulating the actin dynamics within the dendritic spine heads. In contrast, Cortactin and WAVE are present in PSD2 fractions and are highly enriched in the PSD3 fractions (figure 6B), suggesting that both Cortactin and WAVE not only serve to modulate Arp2/3 activity and regulate actin dynamics, but may also be directly associated with post-synaptic membrane receptors (Naisbitt S. 1999, Soderling et al. 2007b) and have other functions. Together, these results suggest that Arp2/3 may be present in both the pre- and postsynaptic compartments where it could regulate actin dynamics. The differential

expression of the Arp2/3 activators in the different synaptosomal and PSD fractions suggests that N-WASP, Cortactin and WAVE may also serve other, Arp2/3-independent functions and may respond to different signaling pathways.

Localization of Arp2/3 in mature dendritic spines

Mature primary hippocampal cultures 21div (days in vitro) that have established mature synapses and have visible mature dendritic spine heads were used to investigate the localization of Arp2/3 in mature neuronal synapses. We detected endogenous Arp2/3 localization by staining with antiserum against p34 (an Arp2/3 subunit). Localization of Arp2/3 was also determined by using recombinant adenovirus to drive expression of p21-EGFP (an Arp2/3 subunit fused to EGFP). Antisera against SV2 and PSD-95 were used to visualize pre- and post-synaptic compartments. Antiserum against Map2 (a microtubule associated protein) was used to visualize dendrites and assess the health of the cells. Fluorescently labeled phalloidin was used to visualize f-actin.

Antiserum staining against p34 and PSD-95 showed co-localization of p34 (Arp2/3 subunit) and PSD-95 within dendritic spine heads (figure 7D). Similarly, expression of p21-EGFP showed an enrichment of Arp2/3 within spine heads, which appeared to co-localize with f-actin, as shown by phalloidin staining (figure 7B). Antiserum staining against SV2 showed SV2 localization juxtaposed to Arp2/3 enrichment within the spine heads, indicating the presence of mature synapses (Figure 7C). Together, these results suggest that Arp2/3 is enriched within mature dendritic spine heads, where it co-localizes with f-actin.

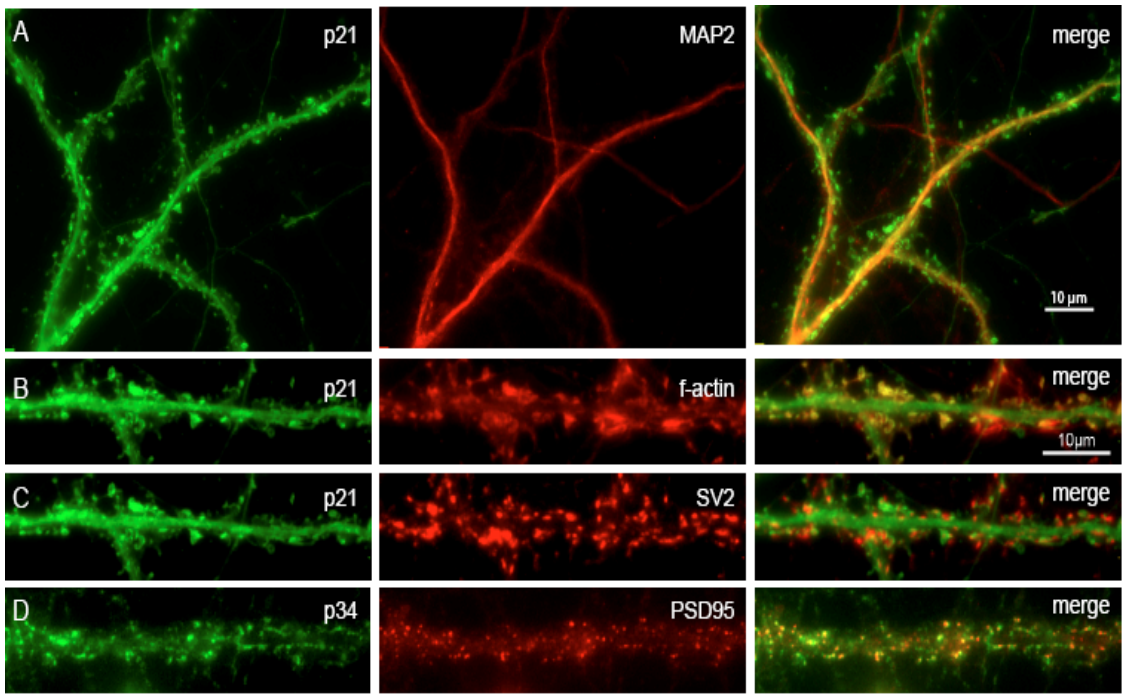

Figure 7 Arp2/3 localization in mature dendritic spine heads.
(A) p21-EGFP (Arp2/3 subunit fused to EGFP) is highly enriched within the spine heads of mature 21div (days in vitro) hippocampal cultures and co-localizes with f-actin (B). Cells were stained with phalloidin (f-actin), and antiserum against SV2 (synaptic vesicle protein, pre-synaptic marker). (C) SV2 was localized juxtaposed to p21-EGFP, suggesting the presence of mature morphological synapses. (D) p34 (Arp2/3 subunit) antiserum staining co-localized with PSD-95 (post-synaptic density, post-synaptic marker) antiserum staining, suggesting Arp2/3 is enriched post-synaptically.

Discussion

Cell fractionation assay and immunofluorescence results show that Arp2/3 is properly position within dendritic spines to be involved in regulating actin polymerization during neuronal synapse maturation. Synaptosomal and PSD fractionation assays show endogenous enrichment of p34 (Arp2/3 subunit) within the LP1 fraction (contains the pre- & post-synaptic membrane and proteins tightly associated with these membranes, figure 6B), the LP2 fraction (contains crude synaptic vesicles and actin cytoskeleton, figure 6B), and the PSD2 fraction (contains the f-actin cytoskeleton, PSD core proteins, figure 6B), suggesting that Arp2/3 is localized to neuronal synapses where it is tightly associated with the f-actin cytoskeleton within spine heads.

Adenovirus driven expression of p21-egfp (Arp2/3 subunit fused to egfp) and p34 (Arp2/3 subunit) immunolocalization in mature hippocampal cultures corroborate with other studies that show Arp2/3 complex co-localization with f-actin and PSD-95 in mature dendritic spine heads (figure 7 B and D) (Racz and Weinberg 2008, Hotulainen et al. 2009), suggesting that Arp2/3, within the torroidal domain of the spine head, (the widest part of the spine head, (Racz and Weinberg 2008)) forms Y-shaped branches of f-actin that may provide the structural support needed for spine head enlargement.

Since the Arp2/3 complex is intrinsically inactive, it needs to be activated by any one of its activators (N-WASP, Cortactin, or WAVE) to induce the nucleation of monomeric actin and drive the formation of Y-branched f-actin by actin polymerization. Our results and current literature suggest that it is possible that more than one Arp2/3 activator may modulate Arp2/3 function within dendritic spine heads. Our synaptosomal

fractionation assay revealed that several of the Arp2/3 activators (N-WASP, Cortactin, and WAVE) are present in the LP1 fraction (figure 6B), suggesting that multiple Arp2/3 activators are present at the synapses. Immunostaining of WAVE-1 in mature hippocampal cultures shows WAVE-1 localization to spine heads, where it co-localizes with f-actin and PSD-95 (Soderling et al. 2002). Electron microscopy studies on hippocampal brain slices also show Cortactin localization within dendritic spine heads (Racz and Weinberg 2004). Our PSD fractionation assay revealed the presence of N-WASP in PSD2 fractions but not in PSD3 (figure 6), suggesting that N-WASP associates with F-actin. N-WASP immunolocalization studies in hippocampal cultures corroborate our results and suggest that N-WASP plays a critical role in dendritic spine and synapse development (Wegner et al. 2008). It would be interesting to determine if the different Arp2/3 activators are involved in regulating Arp2/3 function in a temporal dependent manner.

Unlike N-WASP and Arp2/3, Cortactin and WAVE were highly enriched in the PSD3 fraction (contains enrichment of PSD core proteins, figure 6B), suggesting that both Cortactin and WAVE have Arp2/3 independent functions. Consistent with this idea, knockdown of WAVE1 in neurons leads to a decrease in neurite outgrowth (Soderling et al. 2007b), whereas inhibition of Arp2/3 activity leads to an increase in neurite outgrowth (Strasser et al. 2004). Besides its' Arp2/3 activating function, Cortactin can serve as a scaffold protein by binding to the proline domain of Shank, a scaffold protein found in complex with PSD-95 and in direct association to NMDA receptors (Naisbitt S. 1999, Sheng M. 2007). The diverse synaptosomal and PSD fractionation enrichment patterns

for the Arp2/3 activators observed, may reflect their involvement in varying signaling pathways and suggests that N-WASP, Cortactin and WAVE may be positioned at distinctive spatial locations within the spine heads to activate Arp2/3. Immunogold electron microscopy studies have shown that most of the Cortactin within the spines concentrates in the spine core, 100-150nm away from the PSD; but a small fraction of Cortactin localizes adjacent to the PSD (Racz and Weinberg 2004). Thus, the activity of Arp2/3 may be under spatial control and depend on Arp2/3 activator availability.

CHAPTER 3 Temporal acute inhibition of the Arp2/3 complex in hippocampal dendritic spines

Introduction

Filopodia-like protrusions that extend from dendritic shafts are considered to be the early precursors of mature spines. Rearrangement in the underlying actin cytoskeleton (Matus et al. 2000) is believe to drive these immature filopodia-like spines to undergo morphological changes during development and in response to synaptic activity, such that their mean length decreases and head size increases (Sala Carlo. 2008). Thus, as a result of spine development or increased synaptic activity, a marked decrease in filopodia-like spine density is observed in conjunction with an increase density of spines with mature head morphologies.

The results from Arp2/3 and Arp2/3 activators localization experiments corroborate with other studies that show Arp2/3 and its activators (N-WASP, Cortactin, and WAVE) are enriched within the dendritic spine heads (Racz and Weinberg 2004, Kim et al. 2006, Racz and Weinberg 2008, Wegner et al. 2008, Korobova and Svitkina 2010), suggesting that Arp2/3-dependent actin polymerization may be important for regulating dendritic spine development, morphology and density. In order to characterize the role of Arp2/3 during the development of dendritic spines, we used a recombinant adenovirus to drive acute expression of a competitive inhibitor of Arp2/3 function (made by EGFP fusion to the CA domain of N-WASP, figure 8) in primary hippocampal cultures. This competitive inhibitor (EGFP-CA) binds to Arp2/3, preventing the Arp2/3-

EGFP-CA complex from binding to f-actin (Strasser et al. 2004) thus inhibiting Arp2/3 induced actin polymerization. Following acute inhibition of Arp2/3 function, we quantified parameters such as spine length, spine head width and spine density, that were indicative of spine morphology during a temporal period for spine initiation, spine maturation and spine maintenance.

Materials and methods

Preparation and analysis of hippocampal cultures were performed as described in Chapter 2.

Results

Arp2/3 is not necessary for spine initiation

Our localization results showed that Arp2/3 is enriched within dendritic spine heads (figure 7), which suggests that Arp2/3 could play a role in regulating dendritic spine development and morphology. Filopodia-like protrusions that extend from dendritic shafts are considered to be the early precursors of mature spines. These immature filopodia-like spines undergo morphological changes during development and in response to enhanced synaptic activity, such that their mean length decreases and head size increases with maturation (Sala Carlo. 2008). Thus, as a result of spine development or increased synaptic activity, a marked decrease in filopodia-like spine density is observed in conjunction with an increase density of spines with mature head morphologies.

We first explored the role of Arp2/3 in dendritic spine initiation by acutely inhibiting Arp2/3 function during the formation of spines in 7 div primary hippocampal cultures. Recombinant adenoviruses were used to drive the expression of EGFP-CA or EGFP (control). Infected cultures were analyzed after 48hrs (i.e. at 9 div) to allow for complete expression of our protein of interest (detected by EGFP expression). In normal (control) hippocampal cultures at 9 div, we observed mostly immature filopodia-like protrusions on the surface of dendritic shafts (figure 9A-D). If Arp2/3 were necessary for spine initiation, then we would expect to see a decrease in spine density after Arp2/3 inhibition. However, inhibition of Arp2/3 function in 9 div hippocampal cultures had no significant effect on spine density when compared to control cultures. Both the Arp2/3

inhibited and control cultures at 9 div had about ~3.2 spines for every 10μm segment of dendritic shaft (figure 9L), which suggests that Arp2/3 is not required for dendritic spine initiation.

Inhibition of Arp2/3 in the immature cultures does seem to have an effect on the morphology of the filopodia-like protrusions as shown by the average length/width ratio. In normal spine development, a smaller length/width ratio represents a more mature spine, since these would have shorter necks (reduced length) and wider heads (enlarged width) than the immature filopodia precursors. Arp2/3 inhibition in 9div cultures resulted in spines that were longer and wider when compared to spines of control cultures (figure 9I & 9J). The average length/width ratio for immature spines in the Arp2/3 inhibited cultures was significantly larger than the length/width ratio of immature spines in control cultures (figure 9K), suggesting that the filopodia-like spines of Arp2/3 inhibited cultures were not undergoing proper spine development. Together, these results suggest that although Arp2/3 may not be required for dendritic spine initiation, it may be necessary for dendritic spine maturation.

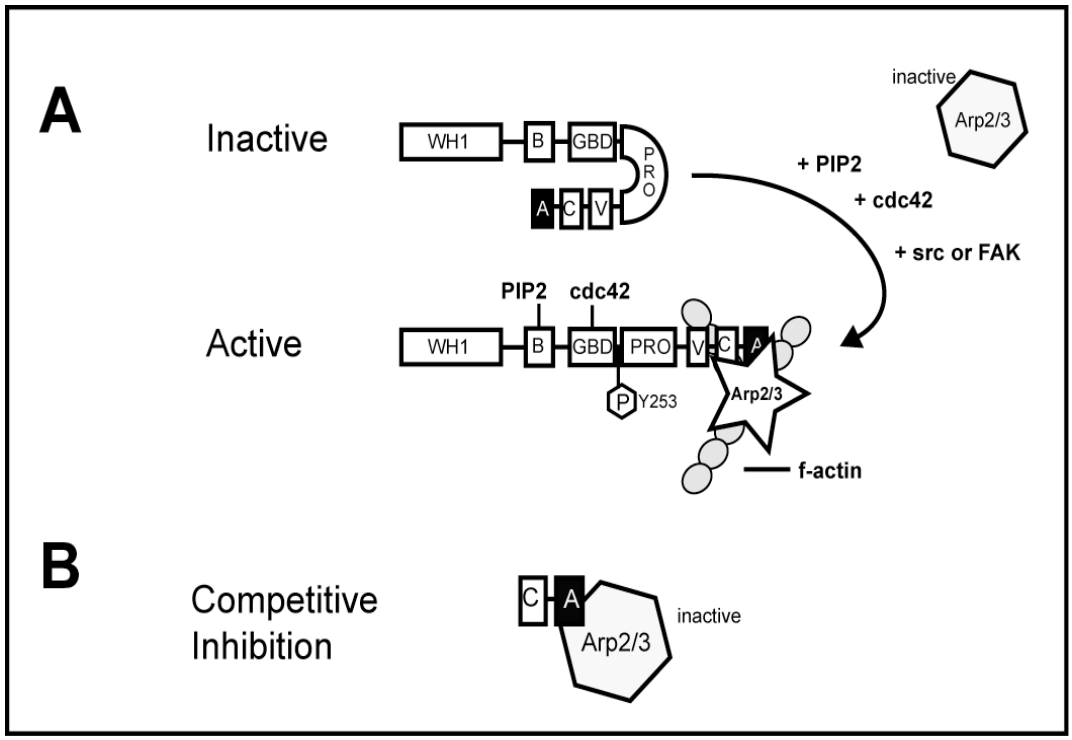

Figure 8 N-WASP regulation of Arp2/3 complex
(A) Endogenous inactive N-WASP undergoes a conformational change when activate. This conformational change exposes the binding site for Arp2/3 (A domain of N-WASP). (B) A competitive inhibitor for Arp2/3 was made by fusion of CA domain of N-WASP and EGFP. This inhibitor competes with endogenous N-WASP by binding Arp2/3 but not activating it. Inactive Arp2/3 is unable to induce actin dependent polymerization. A control for the competitive inhibitor was made by fusion of C domain of N-WASP and EGFP. The control does not bind Arp2/3 and should not affect its function.

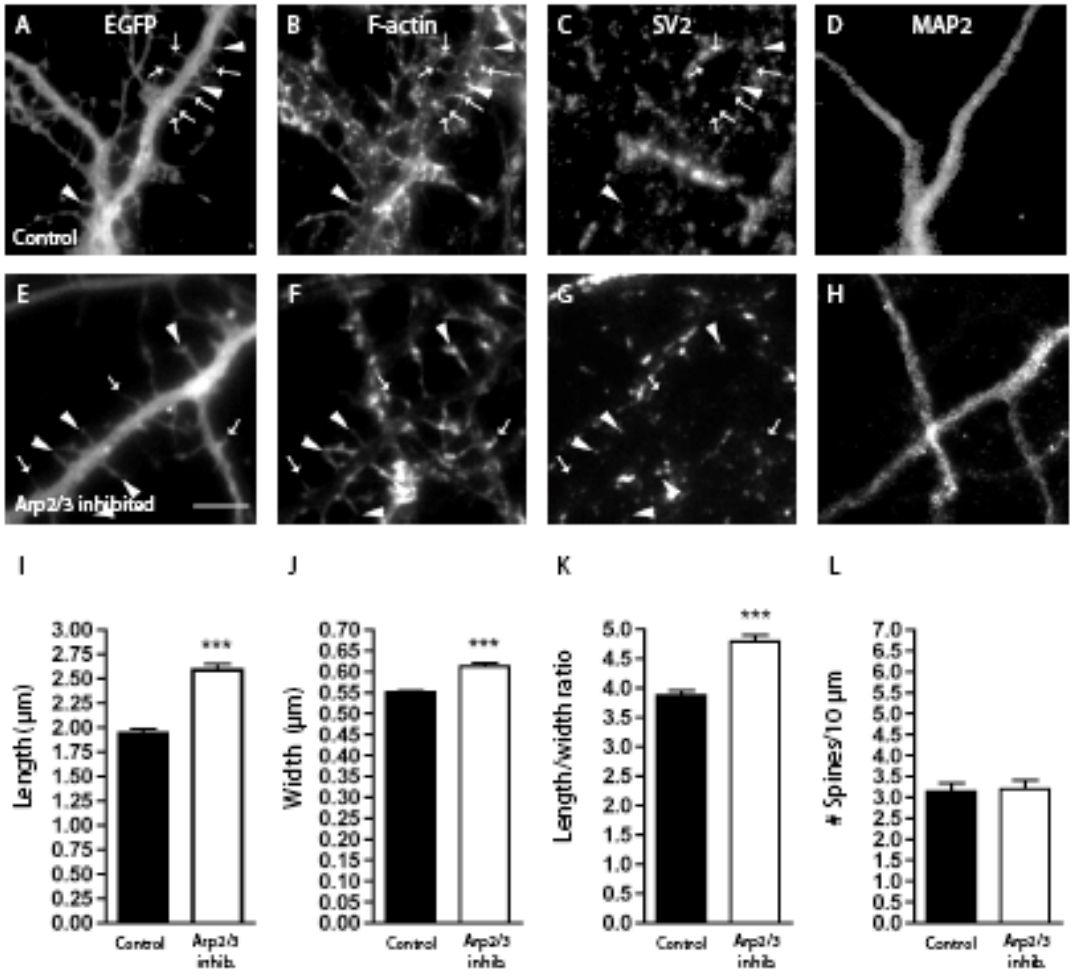

Figure 9 Effect of Arp2/3 inhibition on dendritic spine initiation.
9div primary hippocampal cultures were infected with adenovirus for 48hrs to express EGFP vs. EGFP-CA. Cells were stained with phalloidin (f-actin) (B & F), and antiserum against SV2 (synaptic vesicle protein, pre-synaptic marker) (C & G), and Map2 (Map associated protein) (D & H), which was used to identify dendrites. (A) Cultures expressing EGFP (control) and (E) cultures expressing EGFP-CA (competitive Arp2/3 inhibitor) were used to quantify Length (L, µm) of spines by measuring from the base of dendrite shafts to tips of spine heads. Width (W, µm) of dendritic spine heads was quantified by measuring across widest part of spine head. Arrows indicate protrusions without adjacent SV2 puncta (i.e. no synapse). Arrowheads indicate protrusions with adjacent SV2 puncta (i.e. asynapse). (I) Cultures expressing EGFP-CA had significantly longer spines than those of cultures expressing EGFP, $p<0.0001$ (J) Cultures expressing EGFP-CA had significantly wider spines heads than those of cultures expressing EGFP, $p<0.0001$. (K) The average dendritic spine length and width were measured, and the average length to width ratio was determined. Cultures expressing EGFP-CA had significantly greater L/W ratio than cultures expressing EGFP, $p<0.0002$ (L) The number of dendritic spines per 10µm segment of dendritic shaft was quantified and averaged. No significant difference was observed in the average number of spines per 10µm segment of dendritic shaft in cultures infected with EGFP vs. EGFP-CA.

Arp2/3 plays a role in spine maturation

To explore the role of Arp2/3 in dendritic spine maturation, we acutely inhibited Arp2/3 function in 14 div primary hippocampal cultures, a time point when dendritic spines would normally begin the process of maturation and both immature and mature spine head morphologies are observed (figure 10). Compared to 9div control cultures, control cultures at 14div had a smaller length/width ratio, with shorter necks and wider spine heads, as expected in normal maturing spines (figure 9K & 10K). In addition, 14div control cultures showed an increase in spine density when compared to 9div control cultures, also suggesting normal spine development. Dendritic spines in Arp2/3 inhibited 14 div cultures had a smaller length/width ratio than spines in 9div Arp2/3 inhibited cultures (compare figures 9K & 10K). Arp2/3 inhibited 14 div cultures showed an increase in spine density and a decrease in length compared to 9div Arp2/3 inhibited cultures (compare figures 9L & 10L). Together, these results suggest that spines in 14div cultures were undergoing maturation despite Arp2/3 inhibition. However, inhibition of Arp2/3 in 14 div cultures led to significantly fewer and longer spines when compared to control cultures at 14div (figure 10I, 10J & 10L). Together, these results suggest that Arp2/3 may play an important role during the maturation of spines, perhaps by providing structure and stability to maintain mature maturing spine heads.

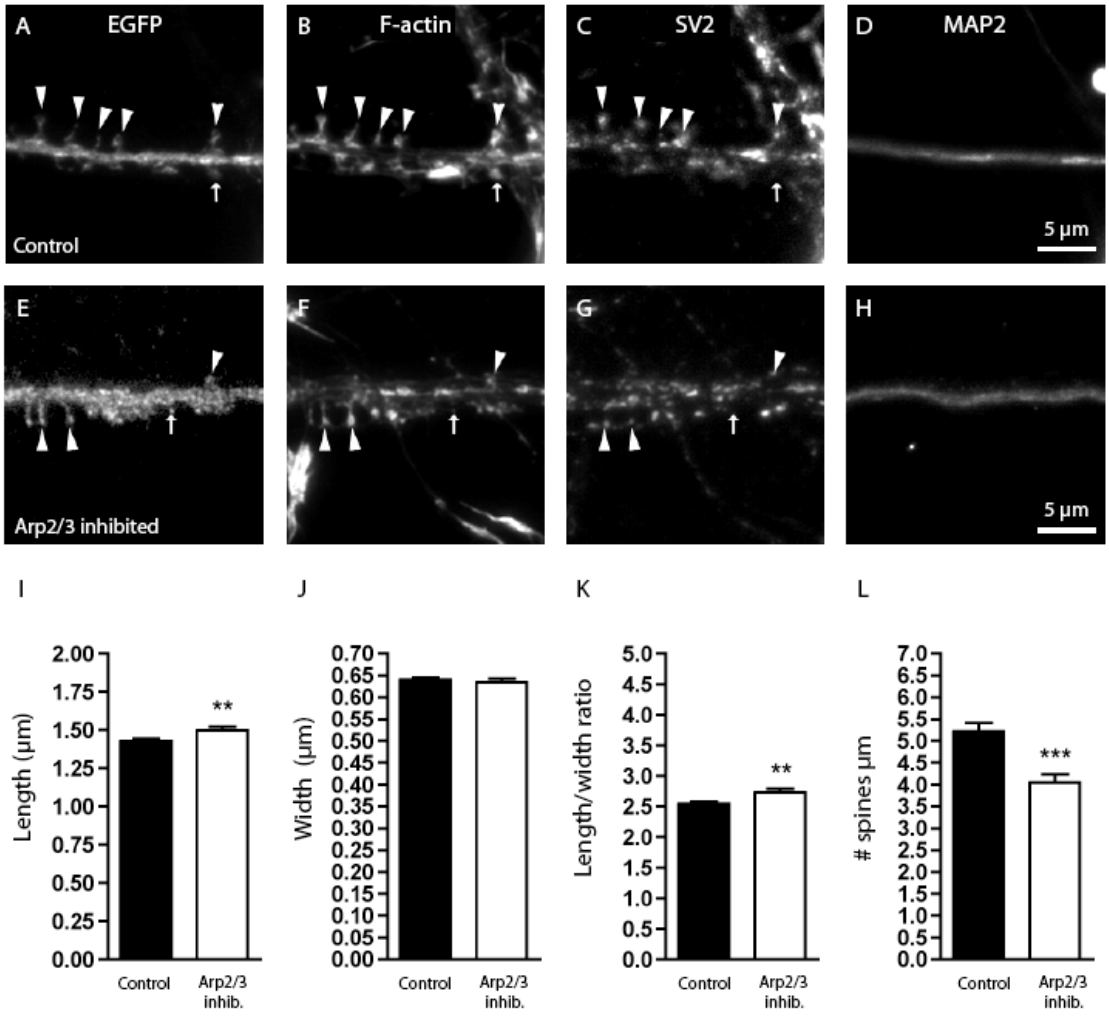

Figure 10 Effect of Arp2/3 inhibition on dendritic spine maturation.
14DIV primary hippocampal cultures were infected with adenovirus for 48hrs, to express EGFP vs. EGFP-CA. Cells were stained with phalloidin (f-actin) (B & F), and antiserum against SV2 (synaptic vesicle protein, pre-synaptic marker) (C & G), and Map2 (Map associated protein) (D & H), which was used to monitor neuronal health. (A) Cultures expressing EGFP (control) and (E) cultures expressing EGFP-CA (competitive Arp2/3 inhibitor) were used to quantify Length (L, µm) of spines by measuring from the base of dendrite shafts to tips of spine heads. Width (W, µm) of dendritic spine heads was quantified by measuring across widest part of spine head. Arrows indicate protrusions without adjacent SV2 puncta (i.e. no synapse). Arrowheads indicate protrusions with adjacent SV2 puncta (i.e. asynapse). (I) No significant difference was observed in spine length, but spines of treatment had significantly smaller spine heads than controls (p-value=0.0013) (J). (K) The average dendritic spine length and width were measured, and the average length to width ratio was determined. No significant difference was observed in average spine length to width ratio between cultures expressing EGFP or EGFP-CA. (L) The number of dendritic spines per 10µm segment of dendritic shaft was quantified and averaged. No significant difference was observed in the average number of spines per 10µm segment of dendritic shaft in cultures infected with EGFP vs. EGFP-CA.

Arp2/3 is required for the maintenance of mature dendritic spines

To explore the role of Arp2/3 in maintenance of mature dendritic spine heads, Arp2/3 function was acutely inhibited in 21 div primary hippocampal cultures, a time period when dendritic spines have established stable synapses and mature spine head morphologies are commonly observed (figure 11). Control cultures at 21div showed an increase in spine density and reduced length/width ratio when compared to 14div control cultures, as expected in normal mature spines (figures 10L & 11L). In contrast, Arp2/3 inhibition in 21div hippocampal cultures led to an increase in length/width ratio when compared to 21div control cultures and 14div Arp2/3 inhibited cultures (figures 10K & 11K), suggesting that Arp2/3 inhibition affects the maturation and enlargement of spines heads. We observed a significant increase in spine length and decrease in spine head width in 21div Arp2/3 inhibited cultures when compared to spines from either 21div control or 14div Arp2/3 inhibited cultures (figures 11I & 11J), suggesting that Arp2/3 is required for the maintenance of enlarged spine heads.

There is a strong correlation between spine head morphology and the magnitude of synaptic strength, such that a large spine head can hold larger number of glutamate receptors and can lead to stronger synaptic activity (Carlisle and Kennedy 2005). In contrast, a reduction in spine head size or spine loss will lead to reduce synaptic activity (Carroll et al. 1999). Thus, our data suggest that the absence of functional Arp2/3, mature dendritic spines may not be able to sustain an enlarged head morphology, which may eventually lead to loss of dendritic spines, as observed by the decrease in spine density (figure 11L). Interestingly, most of the remaining spines had synaptic puncta

(figure 12), suggesting that the spines maintained synaptic contact up until the time of spine loss.

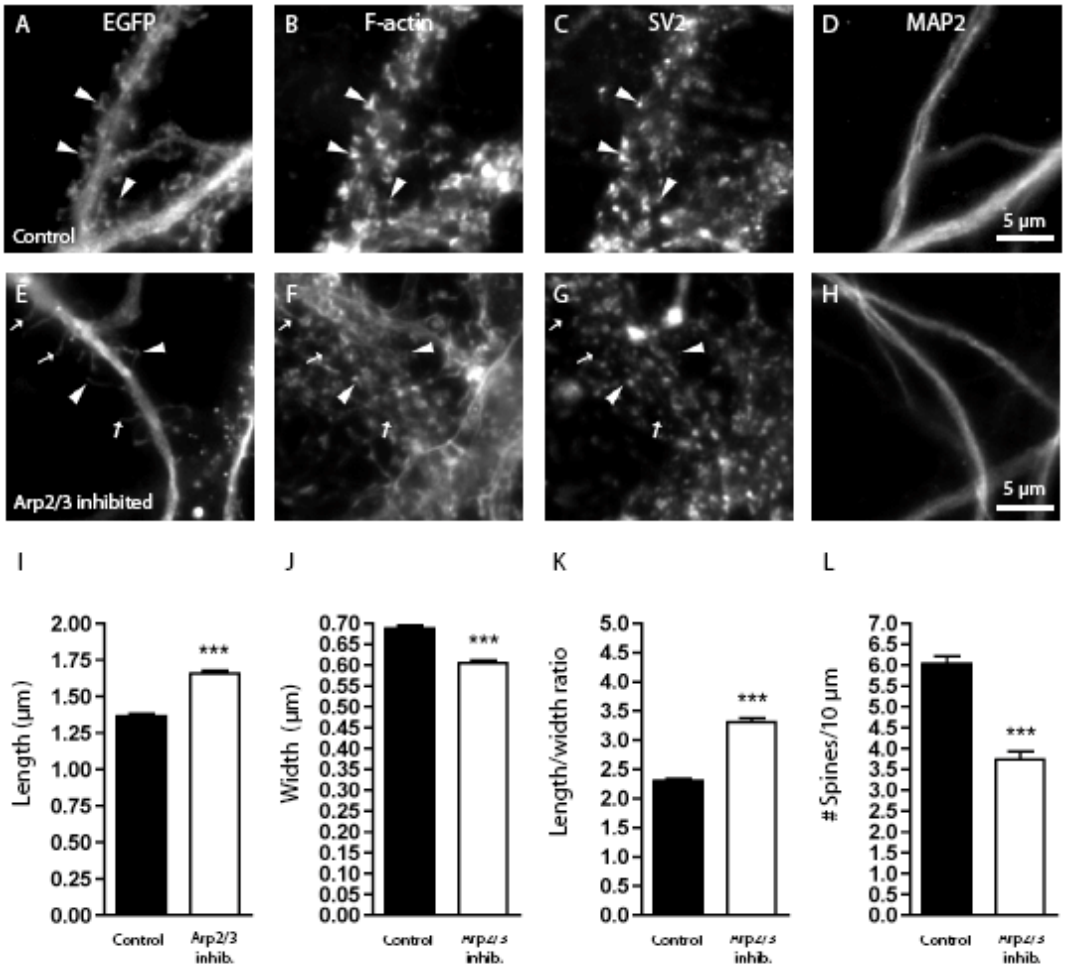

Figure 11 Effect of Arp2/3 inhibition on mature spine maintenance.
21div primary hippocampal cultures were infected with adenovirus for 48hrs, to express EGFP vs. EGFP-CA. Cells were stained with phalloidin (f-actin) (B & F), and antiserum against SV2 (synaptic vesicle protein, pre-synaptic marker) (C & G), and Map2 (Map associated protein) (D & H), which was used to monitor neuronal health. (A) Cultures expressing EGFP (control) and (E) cultures expressing EGFP-CA (competitive Arp2/3 inhibitor) were used to quantify Length (L, µm) of spines by measuring from the base of dendrite shafts to tips of spine heads. Width (W, µm) of dendritic spine heads was quantified by measuring across widest part of spine head. (I) Cultures expressing EGFP-CA had significantly longer spines than those of cultures expressing EGFP. (J) Cultures expressing EGFP had significantly wider dendritic spine heads than those of cultures expressing EGFP-CA, $p < 0.0001$. (K) The average dendritic spine length and width were measured, and the average length to width ratio was determined. Cultures expressing EGFP-CA had significantly greater L / W ratio than cultures expressing EGFP, $p < 0.0001$. (L) The number of dendritic spines per 10µm segment of dendritic shaft was quantified and averaged. Cultures expressing EGFP had significantly greater number of spines / 10µm segment than cultures expressing EGFP-CA, $p < 0.0001$.

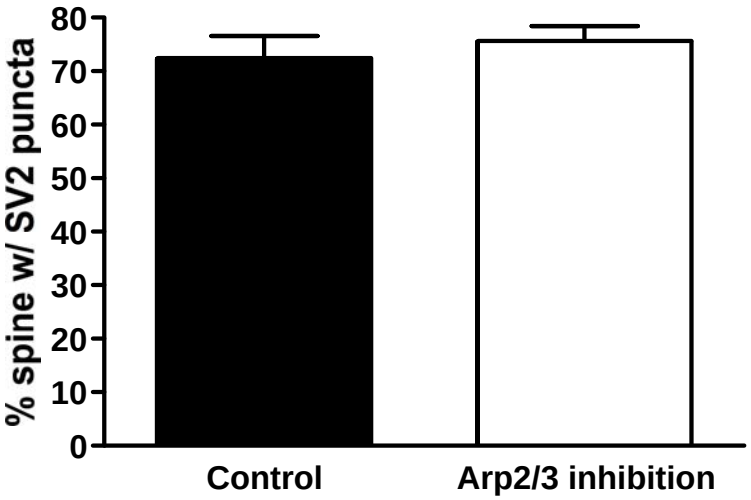

Figure 12 Effect of Arp2/3 inhibition on number of synapses. 21div primary hippocampal cultures were infected with adenovirus for 48hrs, to express EGFP vs. EGFP-CA. Cells were stained with phalloidin (f-actin) and antiserum against SV2 (synaptic vesicle protein, pre-synaptic marker). EGFP and SV2 localization was used to determine the percent of total spines with SV2 puncta localized juxtapose. There was no significant difference in the percentage of spines with synapses between controls and Arp2/3 inhibited spines.

Discussion

Electron microscopy studies suggest that branched f-actin arrangement, which resemble y-shaped f-actin branches typically made by Arp2/3, provide structural support within dendritic spine heads (Fifkova and Delay 1982, Landis and Reese 1983, Hirokawa 1989). Our localization results corroborate with other studies that show Arp2/3 is in the proper location to make y-shaped f-actin branches within the torroidal domain, the widest part of the spine head (Racz and Weinberg 2008).

Since immature spines contain unbranched f-actin arrangement (Fifkova and Delay 1982, Landis and Reese 1983, Hirokawa 1989) and Arp2/3 is responsible for forming y-shaped branched f-actin, it was not surprising that acute inhibition of Arp2/3 during the initiation of immature filopodia-like spines had no significant effect on spine density when compared to controls, suggesting that Arp2/3 is not necessary for dendritic spine initiation. mDia2 formin is an actin-binding protein that promotes formation of unbranched actin filaments (Goode and Eck 2007) and seems to be better candidate for regulating actin dynamics in immature filopodia-like spines (Hotulainen et al. 2009). However, inhibition of Arp2/3 early in spine development does seem to have an effect on spine morphology. During normal spine development, immature spines become shorter and develop wider spine heads; decreasing length/width ratios represents such changes in morphology. When Arp2/3 was inhibited early in spine development the length/width ratio remained large while the length/width ratio of dendritic spines in control cultures decreased. These results suggest that immature filopodia-like spines do not mature properly when Arp2/3 activity is inhibited early in spine development.

Increase in spine density, shortening of spine necks and enlargement of spine heads typically characterizes the maturation of dendritic spines. During the process of dendritic spine maturation, rapid changes from filopodia-like to mushroom-shaped morphologies are observed (Hotulainen et al. 2009). Acute inhibition of Arp2/3 function in 14 div primary hippocampal cultures, when dendritic spines would normally begin the process of maturation, showed an increase in spine density and decrease in length when compared to spines of 9div Arp2/3 inhibited cultures (figures 8I,L & 9I,L), suggesting that spines were undergoing maturation despite Arp2/3 inhibition. However, inhibition of Arp2/3 does hinder the normal maturation of spines since 14div Arp2/3 inhibited cultures had significantly less spine density and longer spines than those of 14div controls cultures (figures 9I & 9L)

Acute inhibition of Arp2/3 in mature hippocampal cultures at 21div, when mature spine head morphologies typically predominate (figure 10A), caused significant increase in spine length, decrease in spine head width and decrease in spine density when compared to either 21div mature control cultures or 14div Arp2/3 inhibited cultures (figures 9I, J & 10I,J), suggesting that Arp2/3 is required for the maintenance of enlarged spine heads and may be important for maintaining functional synapses.

The atypical morphology observed in 21div Arp2/3 inhibited mature cultures is marked by an increase in the length/width ratio, suggestive of immature filopodia-like spine morphology. Strengthening of synapses by enlargement of spine head morphology correlate with greater excitatory synaptic activity that can induce long term potentiation (LTP)(Sorra and Harris 2000, Grutzendler et al. 2002). The immature filopodia-like

morphology observed in the Arp2/3 inhibited cultures suggests that the small surface area of the spine heads allows for limited availability of postsynaptic proteins that would be necessary to carry synaptic activity and increase of Ca^{2+} levels after LTP induction. AMPA receptors are reportedly more abundant in mushroom spines and sparsely distributed in thin and filopodia-like spines (Matsuzaki et al. 2001). Shrinkage or absence of spines have been shown to correlate with low frequency of synaptic activity inducing long term depression (LTD)(Zhou et al. 2004, Massey and Bashir 2007). Collapse of dendritic spines may occur as a result of sparse synaptic activity. Indeed, reduced expression of Arp2/3 or inactivity of Arp2/3 has been shown to reduce the frequency of mEPSCs, suggesting that the numbers of functional synapses are reduced (Hotulainen et al. 2009). We speculate that inhibition of Arp2/3 leads to destabilization of f-actin, AMPA receptor endocytic internalization, synapse weakening, and dendritic spine head collapse leading to decreased spine density.

CHAPTER 4 Glutamate induced redistribution of the Arp2/3 complex

Introduction

Our Arp2/3 inhibition experiments showed that inhibition of Arp2/3 in mature cultures resulted in immature filopodia-like dendritic spine morphology. This result suggests that the f-actin branches made by Arp2/3-dependent actin polymerization may provide the structural support required for enlarging spine heads during the maturation of dendritic spines. In addition, the strength of synaptic activity is believed to influence dendritic spine shape, size and density (Harms and Dunaevsky 2006). Since dendritic spines are highly enriched with f-actin, any changes in dendritic spine morphology or density depend on f-actin reorganization (Schubert and Dotti 2007), suggesting that actin binding proteins that regulate actin dynamics within the spines are modulated by synaptic activity. Actin-binding protein (ABP) seems to dynamically redistribute in response to activity. For example, NMDA receptor activation induces rapid redistribution of profilin II from dendritic shafts into dendritic spine heads (Ackermann and Matus 2003, Ouyang 2005) where it can facilitate f-actin polymerization by increasing the exchange of ADP for ATP bound actin (Pollard et al. 2000). On the other hand, activation of NMDA receptors can activate cofilin, which severs f-actin filaments and facilitates the redistribution of f -actin into dendritic shafts to allow protein translocation into the spine heads (Meng et al. 2003, Ouyang 2005).

Cortactin is an f-actin binding protein that serves as an Arp2/3 activator and scaffolding protein for NMDA receptors (Naisbitt S. 1999). At basal levels of synaptic

activity, the majority of the cortactin protein within the spines concentrates in the spine core, but a small fraction of cortactin localizes adjacent to the PSD (Racz and Weinberg 2004). In response to synaptic stimulation and NMDA receptor activation, cortactin and actin redistribute from the spines heads into the dendritic shafts of neurons, suggesting that cortactin modulates Arp2/3 function and actin dynamics in an activity dependent manner inducing changes on spine morphology (Hering and Sheng 2003a). The results from the Hering and Sheng 2003 study on cortactin redistribution and results from our Arp2/3 inhibition study suggest that Arp2/3 function is crucial for providing the structural support needed to maintain enlarged heads in mature spine. These results also suggest that Arp2/3 localization and function may be mediated by synaptic activity. We used bath application of glutamate in order to induce global depolarization of pyramidal cells in culture and asses the localization of Arp2/3 (p21-egfp) in dendritic spines of pyramidal neurons immediately following glutamate treatment and six hours post-glutamate wash-out.

Materials and methods

Preparation of cultures and immunofluorescence were performed as detailed in chapter 2.

Glutamate depolarization and recovery

Primary hippocampal cultures (HCC) were prepared and maintained as stated above. At 21div HCC were infected with adenovirus for 36hrs to drive expression of p21-EGFP. Depolarization was induced by bath application of 10μM glutamate for 15min at 37ºC. After 15min of treatment, cultures were fixed and prepared for immunostained. For recovery experiments, cultures received either the mock treatment or the depolarization treatment for 15min at 37ºC, then the media replaced with conditioned growth media and cultures incubated for 6hrs at 37ºC. After 6hrs of recovery, HCC were fixed and prepared for immunostaining as mentioned above.

Results

Activity dependent redistribution of Arp2/3 to dendritic shafts

Synaptic activity drives actin rearrangement, which is thought to be the underlying mechanism for changes in spine morphology (Matus et al. 2000). Since, Arp2/3 binds to f-actin and affects actin polymerization; we wanted to determine if Arp2/3 undergoes a change in distribution in response to synaptic activity. We used recombinant adenoviruses to drive the expression of p21-EGFP (an Arp2/3 subunit fused to EGFP) in 21div primary hippocampal cultures and observed the distribution of Arp2/3 in response to depolarization induced by bath application of 10μM glutamate. Phalloidin staining was used to detect f-actin distribution and staining with antiserum against Map2 was used to identify dendrites and monitor neuronal health. Bath application of glutamate has been used to induce depolarization, stimulate redistribution f-actin from spine heads into dendritic shafts, and induce changes in spine morphology that appear to prevent GFP filling of spines (Halpain et al. 1998). Interestingly, despite the loss of GFP filling, punctate staining for pre- and post-synaptic markers are preserved (Halpain Shelley. 1998, Ackermann and Matus 2003, Hering and Sheng 2003a), suggesting that synapses are maintained despite actin redistribution. Supporting this conclusion, spines reform at the same location within a few hours of removing the stimulus (Hasbani 2001).

Bath application of 10μM glutamate also induces focal swelling along the length of neuronal dendrites (figure 13H). These focal swellings are not signs of toxicity or death of cell since they are reversible (Halpain et al. 1998) and occur via independent mechanisms: cell death is Ca^{2+} mediated whereas focal swelling formation is mediated

by Na^+ (Ikegaya et al. 2001). In addition, this global depolarization of in vitro hippocampal cultures led to a redistribution of both F-actin and Arp2/3 into the dendritic shafts (figure 13), and punctate staining for SV2 (pre-synaptic marker) was preserved (figure 13G & H), suggesting that synapses are maintained. These focal swellings coincide with an enrichment of both f-actin (figure 13E) and p21-EGFP (figure 13F), suggesting that in response to synaptic activity Arp2/3 redistributes with f-actin from spine heads into dendritic shafts. In control cultures that had basal level of activity, we found Arp2/3 co-localizes with f-actin in dendritic spine heads (figures 13A & 13B).

We also surveyed the localization of Arp2/3 and f-actin 6hrs after glutamate washout, in an attempt to recover basal level of activity. Interestingly, focal swellings disappeared and we observed f-actin and p21-EGFP co-localization in spine heads 6hrs after glutamate washout (figure 14), suggesting that f-actin and Arp2/3 redistribution is activity dependent. Together, these results suggest that addition of 10μM glutamate in 21div mature culture, with mature synapses already in place and releasing glutamate, may result in an environment that is slightly excitotoxic, but not lethal. Thus, in a protective response neurons develop focal swellings and retract their spines by inducing redistribution of f-actin and proteins important in regulating actin dynamics and spine morphology, such as Arp2/3. Washing out the excess glutamate brings synaptic activity back to normal excitability, leading to reemergence of spines with an enrichment of f-actin and Arp2/3 within the spine heads (figure 14).

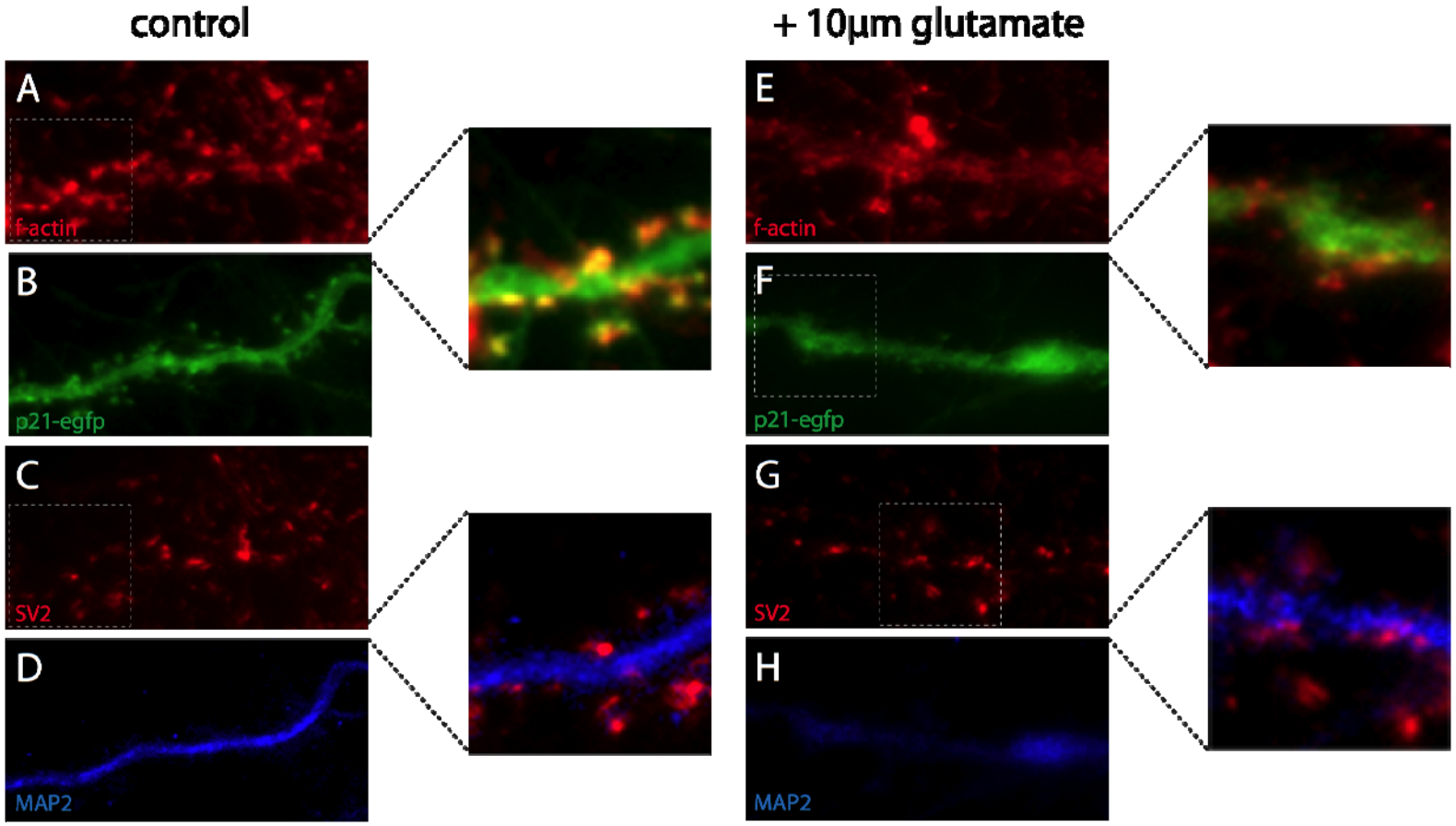

Figure 13 Activity dependent redistribution of Arp2/3 from spine heads into dendritic shafts

Figure 13 Activity dependent redistribution of Arp2/3 from spine heads into dendritic shafts.
p21-EGFP (Arp2/3 subunit fused to EGFP) is highly enriched within the spine heads of mature 21div (days in vitro) hippocampal cultures (B) and co-localizes with f-actin (A). However, 15min after depolarization with10µm glutamate both p21-EGFP and f-actin redistribute from the spines heads to the dendrite shaft. Cells were stained with phalloidin (f-actin), and antiserum against MAP2 (dendrite marker). (G) and (G) Although, no GFP filled spines are visible SV2 puncta remained visible along the dendrites after depolarization and redistribution of Arp2/3 and f-actin, suggesting the presence of mature morphological synapses.

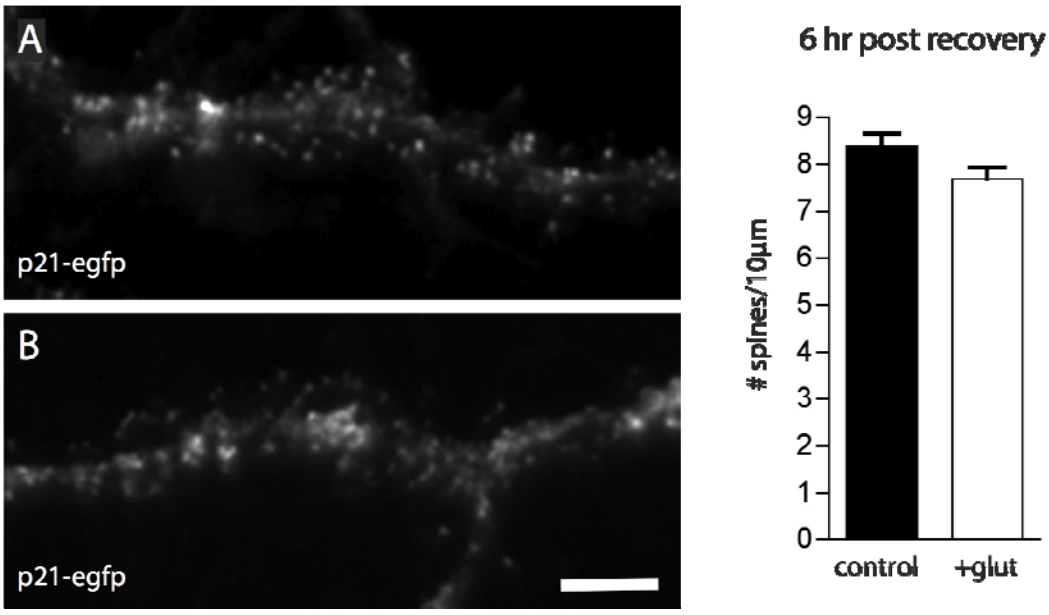

Figure 14 Removal of depolarizing stimulus restores Arp2/3 to spine heads.
6hrs after removal of glutamate, p21-EGFP returns to the spine heads and GFP-filled spines are visible. 6 hrs after recovery there is no significant difference in spine density between the control cultures and cultures that were depolarized. These results suggest that spines may not be visible during depolarization because f-actin within the spines destabilizes and is redistributed to the shafts of dendrites, remnants of the spine head material may still be available when f-actin reemerges into the spine head and structures within the spine can rearrange allowing spines to reemerge.

Discussion

Our results show that Arp2/3 co-localizes with f-actin in dendritic spine heads (figure 13A & B) at basal level of synaptic activity. Increase in synaptic activity by glutamate induced NMDA receptor activation caused focal swellings along the length of neuronal dendrites with loss of dendritic spines (figure 13G & H). Both f-actin (figure 13E) and Arp2/3 (p21-EGFP, figure 13F) colocalized to these focal swellings suggesting that in response to synaptic activity, Arp2/3 redistributes with f-actin from spine heads into dendritic shafts. Upon initial observation, focal swellings seemed to be early signs of excitotoxicity. However, we observe f-actin and Arp2/3 co-localization return to spine heads 6hrs after glutamate washout (figure 14), suggesting that f-actin and Arp2/3 redistribution is activity dependent. The redistribution of f-actin from spine heads into dendritic shafts during focal swelling and in response to LTP or ischemia preconditioning maybe temporary but necessary to allow unhindered translocation of proteins, such as AMPA receptors into the spine heads (Hasbani et al. 2001, Malinow and Malenka 2002, Ouyang 2005, Meller 2008). Polyribosomes, endosomes and lysosomes are often present within or at the base of dendritic spines, suggesting local protein synthesis and degradation can occur within spines and maybe be important for the structural changes that occur during synaptic plasticity (Steward and Schuman 2001).

Clathrin-mediated endocytosis is an important cellular process required for the internalization and trafficking of AMPA receptors from the dendritic spine head surface following long term depression (LTD) (Blanpied et al. 2002, Lu et al. 2007, Hirling 2009, Peng et al. 2009). However, the mechanism for regulating actin filament dynamics

during endocytosis in neurons is not clear. The process of clathrin-mediated endocytosis in yeast and mammalian cells begins with the assembly of clathrin-coated pits at the inner surface of plasma membrane, which deforms the membrane and causes curvature of the membrane into the cytosol. Arp2/3 and several of the Arp2/3 activators are recruited to clathrin-coated pits through interaction with coat components (Merrifield et al. 2004, Girao et al. 2008). Cortactin is recruited to coated pits during the invagination of the vesicle by binding to dynamin and participate with dynamin in the scission of the coated vesicle (Schafer 2002, Zhu et al. 2005, Chen et al. 2006). N-WASP is also believed to be recruited to coated pits through its association with SH3-domain containing proteins, such as intersectin and syndapin, which are associated directly with dynamin, (Qualmann and Kelly 2000, Schafer 2002, Kessels and Qualmann 2004). Thus several studies suggest a possible role for Arp2/3 in spines.

Our findings suggest that Arp2/3 seems to play an important role during the maturation phase of dendritic spine development. The Y-shaped f-actin branches made by Arp2/3 may provide the structural support that is necessary for maintaining an enlarged mature spine head. When Arp2/3 is activated by NPFs it can form a dense meshwork of f-actin made by the y-shaped branches. This meshwork of f-actin may also help maintain membrane bound receptors, such as AMPAR, at the surface of the spine head for a longer period by decreasing the rate of endocytosis. However, when Arp2/3 is regulated by PICK1, Arp2/3 becomes locally inactive and branched f-actin becomes destabilized allowing for bundled f-actin arrangement that may allow for faster translocation of proteins.

CHAPTER 5 ARP2/3 is necessary for rapid ischemic tolerance

Introduction

Our results suggest that high levels of glutamate induced NMDA receptor activation leads to formation of focal swellings, loss of dendritic spines (figure 13G & H), and co-localized of Arp2/3 and f-actin (figure 13E) to these focal swellings. Although focal swelling can sometimes be interpreted as a sign of excitotoxicity, we observed f-actin and Arp2/3 co-localization return to spine heads 6hrs after glutamate washout (figure 14). In addition, a 10 minute exposure to NMDA receptor agonists can induce the redistribution of f-actin and focal swelling formation which are fully reversible and do not lead to cell death (Hasbani et al. 2001). A similar reversible redistribution of f-actin and formation of focal swelling are observed during rapid ischemic tolerance (Meller 2008). Ischemic tolerance (IT), is an endogenous neuroprotective mechanism of the brain and other organs that is used to protect against future injury by adapting to sublethal levels of noxious insult (Alkan 2009).

According to the National Stroke Association, stroke is the third leading cause of death on America and a leading cause of adult disabilities. The diagnosis of stroke is given to patients that have experience considerable neuronal cell death as a result of global or focal decreases in blood flow leading to poor oxygen and glucose supply also know as cerebral hypoxia (Hertz 2008, Dirnagl et al. 2009). However, ischemic tolerance is observed in the clinical setting following prototypical preconditioning stimuli, such as cerebral hypoxia, sublethal transient global and focal ischemia (Lehotsky et al. 2009). A myriad of preconditioning stimuli such as chronic hypoxia, chronic

hypoperfusion, oxidative stress, hypothermia, hyperthermia, pharmalogical metabolic inhibitors, and inflammatory cytokines, are used to induce IT in animals, cell culture and tissue slices (Alkan 2009, Lehotsky et al. 2009). Depending on the type of preconditioning stimulus, the state of tolerance can be triggered within minutes of the sublethal insult resulting in rapid or acute tolerance or it can occur after several hours or days resulting in delayed tolerance. From a molecular perspective, the acute tolerance is most likely due to rapid posttranslational protein modifications whereas the delayed tolerance may be dependent on de novo protein synthesis (Dirnagl et al. 2009, Terasaki et al. 2010).

The concrete signaling pathways for ischemic tolerance remain unknown but ischemic preconditioning may induce tolerance by activating endogenous protective mechanism that preserve mitochondrial function, upregulate genes involved in energy metabolism, inhibit caspases and proapoptotic genes, activate serine/threonine activated (Akt), and extracellular signal regulated (ERK) kinases, activate nerve growth factor (NGF) and brain-derived neurothrophic factor (BDNF), reduce inflammatory response initiated by Toll-like receptors, suppress activation of the NFkB transcription factor and production of proinflammatory cytokines (Gidday 2006, Lehotsky et al. 2009). In the case of cerebral ischemic tolerance, preconditioning also reduces the potential for excitotoxicity by suppressing glutamate release, increasing glutamate uptake by upregulating the expression of the glial glutamate transporter (GLT1-EAAT2), downregulating expression of AMPA and NMDA receptors, enhancing release of GABA

and inhibitory synaptic transmission thus inducing a shift from excitatory to inhibitory neurotransmission (Dave et al. 2005, Dirnagl et al. 2009, Lehotsky et al. 2009).

Cerebral ischemia is modeled in vitro by oxygen glucose deprivation (OGD) in primary cell cultures or hippocampal slice cultures (Bonde et al. 2005). Ischemia preconditioning stimulus in primary hippocampal cultures results in transient formation of focal swellings, loss of f-actin from dendritic spines and activation of the proteasome pathways (Hasbani et al. 2001, Meller 2008). Thus, blocking actin reorganization and proteasome pathways blocks focal swelling formation, actin reorganization, and ischemic tolerance. Ischemic tolerance can occur by ubiquitination and degradation of a cell death associated protein Bcl-2-interacting mediator (Bim) of cell death (Meller 2008). Ubiquitin-binding pulldown assay are used to monitor changes in protein ubiqutination after ischemic preconditioning. A reduction in WAVE-1 pulldown with a P62 ubiquitin binding domain was observed following 30 min OGD, suggesting a decrease in WAVE-1 ubiquitination following ischemic preconditioning stimulus (Meller et al. 2008). This finding suggests that less WAVE-1 is targeted for degradation and may be made more available to regulated Arp2/3 and actin dynamics following OGD. To determine if inhibition of Arp2/3 function has an effect on cortical neurons response to ischemic preconditioning stimulus, Arp2/3 function was inhibited and pyramidal neuronal cell death was measured following 30min of OGD and a subsequent harmful ischemic stimulus.

Materials and methods

Ischemic tolerance

Neuronal cultures were prepared as previously described (Meller et al. 2008). Briefly cortices were removed from one-day-old rat pups (mixed sex) incubated in papain (Worthington Biochemicals) and then triturated with a fire polished glass pipette. Neurons were grown for 14 days in Neurobasal A medium supplemented with B27 (Invitrogen). Oxygen and glucose deprivation (OGD) was used to model ischemia, as previously described (Meller et al. 2005, Meller et al. 2006). Briefly, cells were washed twice with phosphate buffered saline solution supplemented with magnesium and calcium (NaCl 1.37 mM, KCl 2.7mM, Na_2HPO_4 10 mM, KH_2PO_4 1.7 mM, $CaCl_2$, 0.5 mM, $MgCl_2$ 1 mM, pH 7.4), then placed in 1.5 ml PBS solution and transferred to an anoxic chamber (85 % N_2, 5% H_2, 10 % CO_2; 35 ºC) (Bactron) for indicated periods of time. To terminate ischemia, cells were replenished with Neurobasal A media and returned to a normoxic chamber. Four sets of experimental conditions were investigated in the ischemic tolerance paradigm. Cells received either control (wash only), 30 min OGD, 120 minute OGD, or 30 min OGD followed by 1 hour recovery in normoxic conditions with Neurobasal A media followed by 120 min OGD. Cells are left to recover for 24 hours following the final wash or ischemic event and cell death is assessed by propidium iodide (PI) exclusion assay (Meller et al. 2008). Cells were fixed; counter stained with DAPI and the number of PI positive neurons counted. Coverslips were imaged at 3 random areas, and the number of DAPI positive and PI positive cells determined using Image J.

Results

Inhibition of Arp2/3 blocks rapid ischemic tolerance

Focal ischemic stroke induces WAVE-1 to form a complex with the mitochondria transmembrane protein, Bcl-XL, resulting in increase association of Bax with mitochondria, cytochrome c release and neuronal cell death (Cheng et al. 2007), suggesting a role for WAVE-1 in apoptosis. However, P62 ubiquitin binding domain-pull down assay indicates reduced ubiquitination of WAVE-1 following ischemic preconditioning stimulus (Meller et al. 2008), suggesting less WAVE-1 is targeted for degradation and may be made more available to regulated Arp2/3 and actin dynamics following OGD. To investigate whether Arp2/3 availability is altered in preconditioning, the N-WASP/ WAVE-1 VCA-domain conjugated to agarose was used to pulldown Arp2/3 from neuronal cell lysates. There was a significant increase in the pulldown of both Arp2 and Arp3 following ischemia preconditioning (30 min OGD), compared to controls without OGD (Fig. 15A, B). This suggests that OGD preconditioning alters Arp2/3 association with the actin cytoskeleton, making it more accessible to pulldown by VCA either by weakening Arp2/3 association to the actin cytoskeleton or completely releasing inactivated Arp2/3.

To determine if Arp2/3 plays a role in ischemic conditioning in cortical cultures, Arp2/3 was inhibited for 48 hrs and cells were then subject to an oxygen, glucose depravation (OGD) ischemic tolerance paradigm (Fig. 15C, (Meller 2008) Cells received either no ischemia, 30 min OGD, 120 min OGD or 30 min OGD followed by 120 min OGD one hour later and cell death was quantified 24 hrs after treatment. In

untreated cells, harmful ischemia induced approximately 40% cell death in the cultures, where as preconditioning the cells with 30 min OGD prior to the harmful ischemia significantly reduced cell death (Fig. 15D). Propidium iodide staining in cells expressing EGFP alone showed a slight increase in cell death following both 30 and 120 min OGD when compared to uninfected controls, indicating that adenoviral infection may sensitize cells to ischemic damage; however infection with control virus did not block the protective effects or preconditioning. Inhibition of Arp2/3 did not significantly alter cell death rates following PC or harmful ischemia, but completely blocked the protective effects of preconditioning compared to untreated cells ($P<0.01$). Together, these findings indicate that Arp2/3-dependent actin reorganization may play a critical role in determining the protective effects of ischemic preconditioning.

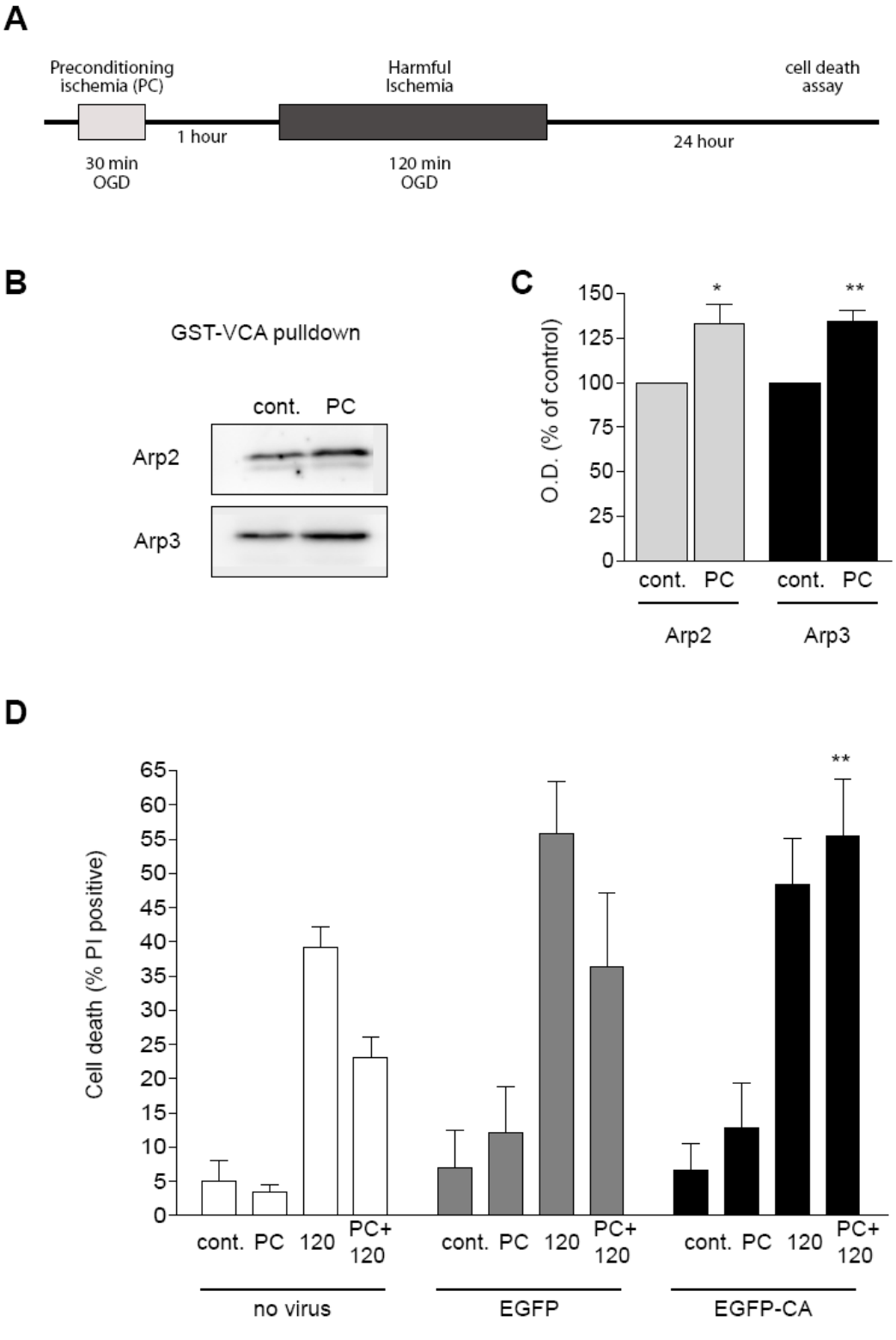
A
Preconditioning ischemia (PC)
Harmful Ischemia
cell death assay
1 hour
24 hour
30 min OGD
120 min OGD
B
GST-VCA pulldown
cont.
PC
Arp2
Arp3
C
O.D. (% of control)
150
125
100
75
50
25
0
*
**
cont.
PC
cont.
PC
Arp2
Arp3
D
Cell death (% PI positive)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
**
cont.
PC
120
PC+ 120
cont.
PC
120
PC+ 120
cont.
PC
120
PC+ 120
no virus
EGFP
EGFP-CA

Figure 15 Inhibition of Arp2/3 blocks the protective effect of Preconditioning Ischemia (A) Schematic of the rapid ischemia tolerance paradigm. PC (30min of oxygen& glucose deprivation, OGD) is administered 1hr before harmful ischemia (120min OGD). Cell death is assessed 24hrs post harmful ischemia by propidium iodine post harmful ischemia (Meller 2008). (B) N-WASP/ WAVE-1 VCA-domain conjugated to agarose was used to pulldown Arp2/3 from neuronal cell lysates. (C) There was a significant increase in the pulldown of both Arp2 and Arp3 following preconditioning ischemia (30 min OGD), compared to controls without OGD. (D) Arp2/3 was inhibited for 48 hrs and cells were then subject to (OGD) ischemic tolerance paradigm (A). Cells received either no ischemia; 30 min OGD, 120 min OGD or 30 min OGD followed by 120 min OGD one hour later and cell death was quantified by propidium iodide 24 hrs after treatment. In untreated cells, harmful ischemia induced approximately 40% cell death in the cultures, where as cell death was not increased by preconditioning with 30 min OGD. EGFP (virus control) increased the percent of cell death, when compared to no virus condition, but preconditioning decreased cell death induced by harmful ischemia treatment. However, inhibition of Arp2/3 removed the protective effect of preconditioning and showed significant increase in cell death when compared to untreated cells.

Discussion

Neurodegeneration that results from cerebral ischemia develops from glutamate induced neurotoxicity, inflammation and cell death. Within minutes of OGD resulting from cerebral ischemia, astrocytes stop or reverse the uptake of extracellular glutamate, and the ATP-dependent astroglial glutamate synthase which normally converts glutamate to inactive glutamine becomes impaired leading to extracellular increase in glutamate (Bonde et al. 2005). Excessive extracellular glutamate can increase influx of Ca^{2+} through voltage dependent calcium channels leading to Ca^{2+} dependent glutamate release. In addition, the increase in extracellular glutamate leads to activation of NMDA and AMPA receptors resulting in influx of Na^{+}, Cl^{-}, and Ca^{2+} inducing osmotic swelling and free radical production and eventual cell death (Bonde et al. 2005).

NMDA receptors are usually anchored to the actin cytoskeleton via scaffolding proteins (Shank and cortactin, an Arp2/3 regulator) (Naisbitt S. 1999), but in a protective response to ischemia and excitotoxicity dendritic spines undergo transient retraction, f-actin redistribution (Hasbani et al. 2001, Meller 2008) and NMDA receptor anchoring to the cytoskeleton is reduced after ischemia tolerance is induced by OGD preconditioning (Meller 2008).

Most AMPA receptors of the hippocampus contain a GluR2 subunit, which makes the AMPA receptors impermeable to Ca^{2+} ((Hollmann et al. 1991). However, ischemia induces down regulation of GluR2 mRNA and protein expression allowing for alternative composition of AMPA receptors that are permeable to an influx of Ca^{2+} which facilitates cell death (Pellegrini-Giampietro et al. 1992, Liu et al. 2006). Interestingly, OGD can

delay cell death in hippocampal neurons by delaying the down regulation of GluR2 mRNA expression (Liu et al. 2006). In addition, an increase in excitatory post-synaptic current (EPSC) rectification is observed following OGD, suggesting a switch in AMPA receptor composition by rapid internalization of AMPA receptors containing GluR2 subunit and delivery of GluR2-lacking AMPARs to synaptic sites (Dixon et al. 2009). This rapid internalization of GluR2 containing AMPA receptors likely occurs by PICK1 mediated-endocytosis, since GluR2-PICK1 binding has been shown to increase in hippocampal neurons following OGD and is a process also observed in response to synaptic activity (Malinow and Malenka 2002, Liu et al. 2006). Furthermore, disrupting GluR2-PICK1 interactions by peptides that interfere with the PICK1-PDZ domain blocks internalization of GluR2-AMPA receptors (Dixon et al. 2009). PICK1 is a protein that contains PDZ and BAR domains and binds to several membrane proteins including GluR2 subunits of AMPA receptors (Hanley 2006, Rocca et al. 2008, Dixon et al. 2009). PICK1-GluR2 interaction is necessary for AMPA receptor internalization from the synaptic membrane in response to Ca^{2+} influx via NMDA receptor activation (Kim et al. 2001, Hanley and Henley 2005).

PICK1 is also an Arp2/3 regulator that locally inhibits Arp2/3 function (Rocca et al. 2008). Coimmunoprecipitation experiments from neuronal tissue extracts show that Arp2/3, PICK1, actin and GluR2 interact to from a complex that is involved in NMDA-induced AMPA receptor endocytosis (Iwakura et al. 2001, Rocca et al. 2008). Locally inactive Arp2/3 permits local destabilization of branched f-actin normally formed by active Arp2/3 dependent actin polymerization allowing for bundled f-actin arrangement

that may allow for faster translocation of proteins in response to synaptic activity or OGD. In addition, inhibition of Arp2/3 by the N-WASP CA domain results in increase AMPAR internalization, suggesting that inhibition of Arp2/3 is required for AMPAR internalization (Rocca et al. 2008). Our results demonstrated that inhibition of Arp2/3 in cultured cortical neurons completely blocked the protective effects of OGD preconditioning compared to untreated cells, suggesting that Arp2/3-dependent actin polymerization is necessary for the protective effect of ischemia preconditioning (figure 15). Thus, acute inhibition of Arp2/3 by NWASP CA domain (EGFP-CA) may destabilizes branched f-actin which will no longer be able to sustain an enlarged spine head. A smaller spine head reduces the density of AMPA receptors available to respond increases in extracellular glutamate induced by OGD.

CHAPTER 6 Conclusion and Future perspectives

Localization and Inhibition of Arp2/3 function

Data presented here show that Arp2/3 is localized to dendritic spine heads (figure 6 & 7) and corroborates with recent reports of Arp2/3 localization within the widest part of the spine head, (Racz and Weinberg 2008, Korobova and Svitkina 2010) leading to the hypothesis that Arp2/3-dependent actin polymerization forms characteristic Y-shaped branches of f-actin that provide the structural support needed for spine head enlargement. Additionally, our synaptosomal and PSD fractionation assay revealed the presence of the Arp2/3 activators, N-WASP, WAVE and Cortactin (figure 6) suggesting that Arp2/3 activity can be modulated by more than one regulator. Knockdown of N-WASP by RNAi or inhibition of N-WASP activity by wiskostatin leads to decrease in spine density (Wegner et al. 2008). Similarly, knockdown of Cortactin in hippocampal neurons and hippocampal cultures from WAVE-1 knockout mice also show a decrease in spine density (Hering and Sheng 2003a, Soderling et al. 2007a) Thus, absence or inactivity of any of the Arp2/3 activators has detrimental effects on spine density, which may be reflective of limited Arp2/3 activity.

We explored the hypothesis that inhibition of Arp2/3 function would have detrimental effects on dendritic spine morphology and density during the development of spines. Our results on inhibition of Arp2/3 function are in good agreement with studies that chronically knockdown expression of Arp2/3 subunits (Arp3 or p34) by RNAi or that inactivated Arp2/3 function and show a decrease density of dendritic protrusions,

increase spine neck length and increase density of filopodia-like spines (Wegner et al. 2008, Hotulainen et al. 2009). However, no other study has explored the temporal requirement for Arp2/3 in dendritic spine initiation, maturation and maintenance of mature spine head morphology. Our results show that immature filopodia-like spines do not mature properly when Arp2/3 function is inhibited early in spine development; these spines appear wider and taller than their control counterparts (figure 9 A, E, I & J). Furthermore, during the maturation stage of spine development Arp2/3 inhibited cultures showed dendritic spines were longer and less dense than those of control cultures (figure 10 A, E, I & L). Inhibition of Arp2/3 in mature cultures had the most dramatic effect on spine morphology and density, featuring atypical filopodia-like morphology and reduced spine density when compared to control cultures (figure 11) suggesting that Arp2/3 has an important role in maintaining mature spine head morphologies. These findings are uncommon for the mature stage in dendritic spine development, where dense number spines with mature morphology are expected. Thus, inhibition of Arp2/3 may cause destabilization of the branched f-actin network and appearance of immature filopodia-like spines that contain a limited number of AMPA receptors.

Arp2/3 forms physical barrier for AMPA receptor trafficking

In non-neuronal cells multiple Arp2/3 activators play a role in the invagination and movement of vesicles during clathrin mediated endocytosis, but mutation or reduced expression of Cortactin seems to have the most deleterious effect on endocytosis (Galletta

et al. 2008). RNAi knockdown of Cortactin or mutation to Arp2/3 binding domain completely blocks transferin uptake and reduces cortactin ability to bind to dynamin (Zhu et al. 2005), whereas RNAi knockdown of N-Wasp reduces the rate of EGF (epidermal growth factor) uptake, but has no effect on the accumulation of actin or Arp2/3 at clathrin coated pits (Benesch et al. 2005), suggesting that Arp2/3 is necessary for the scission of clathrin coated vesicles from the plasma membrane. Sequestration of Arp2/3 by WA-domain of Scar1 abolished any recruitment of actin or actin polymerization to clathrin-coated pits but did not affect clathrin coat assembly (Benesch et al. 2005), suggesting that proper spatial localization of Arp2/3 is necessary for actin polymerization around the clathrin-coated pits. However, myosin VI (an actin based motor protein) can associate with a protein complex containing clathrin and adaptor protein AP-2, deform, invaginate the plasma membrane during the early stages of coated-pit formation and transport the endocytic vesicle through dense actin filaments in the cytoplasm (Schafer 2002).

In neurons, myosin VI localizes to the PSD in dendritic spine heads where it exists in a complex with the AMPAR, AP-2, and SAP97, suggesting that it is involved in clathrin mediated endocytosis of AMPAR (Osterweil et al. 2005). If actin filaments dynamics within dendritic spine heads were similarly regulated by N-WASP and Cortactin activation of Arp2/3 during AMPA receptor endocytosis, then inhibition of Arp2/3 function by EGFP-CA would reduce Arp2/3 dependent actin polymerization around clathrin-coated pits and prevent Arp2/3 dependent actin polymerization induced propulsion of endocytic vesicles through the cytosol, suggesting a reduction in AMPAR internalization. However, we observe that inhibition of Arp2/3 leads to filopodia-like

spines morphology, suggesting unbranched actin filament arrangement resembling actin cables which may facilitate myosin V1 transport of vesicles containing AMPA receptor resulting in increase rate of AMPA receptor internalization leading to reduction in synaptic strength in LTD.

Considerable evidence suggests that activation of NMDAR induces AMPAR internalization via the Ca^{2+} dependent protein PICK1, which binds to membrane bound GluR2/3 (AMPA receptor subunits) (Hanley and Henley 2005, Rocca et al. 2008). PICK1 also interacts directly with Arp2/3 and locally inhibits Arp2/3 dependent actin polymerization (Rocca et al. 2008). Coimmunoprecipitation experiments from neuronal tissue extracts show that Arp2/3, PICK1, actin and GluR2 (AMPA receptor subunit) interact to from a complex that mediates APMA receptor endocytosis (Rocca et al. 2008). Knockdown of PICK1 by shRNA blocked GluR2 (AMPAR subunit) endocytosis, suggesting PICK1 is necessary for AMPAR endocytosis (Iwakura et al. 2001, Rocca et al. 2008). Furthermore, cotransfection of PICK1 shRNA and shRNA resistant wild-type PICK1 rescued AMPAR endocytosis, but cotransfection of PICK1 shRNA and PICK1mutant (lacking Arp2/3 binding domain) was not able to rescue AMPAR endocytosis (Rocca et al. 2008), suggesting that PICK1-Arp2/3 interaction is required for AMPAR internalization. Inhibition of Arp2/3 by the N-WASP CA domain results in increase AMPAR internalization, suggesting that inhibition of Arp2/3 is required for AMPAR internalization (Rocca et al. 2008).

Thus, Arp2/3 dependent actin polymerization may provide the mechanical force that maintains the dendritic spine head membrane. Arp2/3 dependent branched f-actin

may restrict AMPA receptor endocytosis by opposing membrane invagination that occurs by clathrin and associated coat proteins assembly at the endocytic sites. Binding of PICK to GluR2 enhances PICK1-actin and PICK-Arp2/3 interactions, inhibits actin polymerization locally and destabilizing f-actin network around the coated pit (Rocca et al. 2008). The absence of f-actin network around the coated-pits reduces membrane tension and allows the coated membrane to invaginate facilitating AMPA receptor endocytosis.

Inhibition of Arp2/3 by the N-WASP CA domain results in increase AMPAR internalization, suggesting that inhibition of Arp2/3 is required for AMPAR internalization (Rocca et al. 2008) and supports our hypothesis that Arp2/3 plays an important role in maintaining mature spine head morphologies, such that inhibition of Arp2/3 leads to destabilization of the branched f-actin network and appearance of immature filopodia-like spines that contain a limited number of AMPA receptors. Since branched f-actin network are observed within the mature spine heads (Fifkova and Delay 1982), inhibition of Arp2/3 in our cultures by EGFP-CA may induce f-actin destabilization within the entire spine head leading to collapse of the mature spine head resulting in immature filopodia-like spine morphology, and increase AMPA receptor internalization resulting in weaken synaptic activity that leads to decrease in spine density (figure 16).

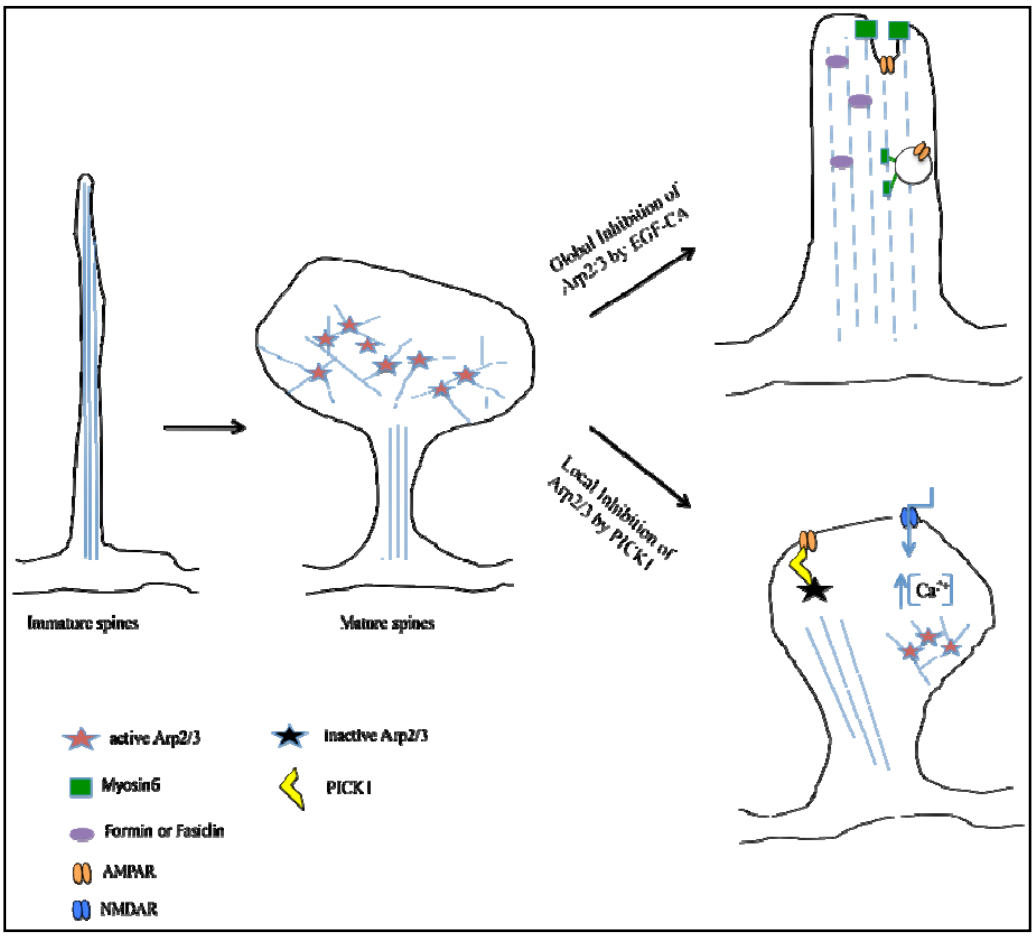

Figure 16 Proposed role of Arp2/3 within spines.
During normal development of dendritic spines Arp2/3 seems to play an important role during the maturation of spines. The Y-shaped f-actin branches made by Arp2/3 may provide the structural support that is necessary for maintaining an enlarged mature spine head. When Arp2/3 is activated by NPF it can form a dense meshwork of f-actin made by the y-shaped branches. This meshwork of f-actin may also help maintain membrane bound receptors, such as AMPAR, at the surface of the spine head for a longer period by decreasing the rate of endocytosis. However, when Arp2/3 is regulated by PICK1, Arp2/3 becomes locally inactive, and branched f-actin becomes destabilized, allowing for bundled f-actin arrangement. Endocytosis of AMPAR is facilitated by PICK1 inhibition of Arp2/3. Thus, the bundled f-actin arrangement may allow for faster translocation of proteins. The competitive inhibitor, EGFP-CA causes acute and global inhibition of Arp2/3. Global inhibition of Arp2/3, results in no formation of Y-shaped branched f-actin. Fascin is another nucleating protein that crosslinks actin filaments into unbranched bundles. These bundles facilitate myosin VI transport of AMPAR resulting in an increase of AMPAR recycling, which lead to decrease in synaptic activity, and eventual loss of synapses.

Neuroprotective role of Arp2/3 redistribution

Collectively, results from our localization and inhibition studies suggest that Arp2/3 localization and function are critical for the maintenance of mature spine head morphology that provide greater surface area for enhanced synaptic activity (Malinow and Malenka 2002, Carlisle and Kennedy 2005). However, changes in localization and regulation of actin-binding protein (ABP) activity are likely to occur as a result of changes in synaptic activity. Brief application of NMDA induces shortening of spine necks within 1-2 hrs of application (Segal 1995b), suggesting a link between glutamate receptor activation and actin mediated shape of dendritic spines. Our results show that bath application of glutamate in our cultures induces redistribution of Arp2/3 from dendritic spines to focal swellings observed in dendritic shafts (figure 13) and supports the idea for spine actin cytoskeleton disassembly occurs in response to glutamate receptor stimulation. In addition, similar redistribution has been observed for Cortactin in response to glutamate receptor activation (Hering and Sheng 2003b) and further suggests activity dependent dynamic changes of the actin network within dendritic spines.

It has been proposed that dendritic spines serve a neuroprotective role isolating the cell from the harmful consequences of synaptic activity dependent increases of intracellular Ca^{2+} (Segal 1995a). Stabilized F-actin within spine heads blocks the translocation of Ca^{2+} /calmodulin-dependent kinase and other proteins into dendritic spines (Ouyang et al. 2005), suggesting that a stabilized f-actin network within spines serves as a gate that prevents non-specific translocation of proteins into unpotentiated spines. However, a reduction of spine length is observed shortly after repeated

application of NMDA to primary hippocampal cultures, suggesting that dendritic spines shrink in response to excessive synaptic stimulation (Segal 1995b). Disruption of f-actin in cultured neurons serves a neuroprotective role and attenuates excitotoxicity by allowing the transport of newly synthesized proteins to target sites to perform the necessary remodeling within spine heads in response to synaptic stimulation (Furukawa et al. 1995, Steward and Schuman 2001, Malinow and Malenka 2002). We observe formation of focal swellings with reduced spine density in response to glutamate treatment (figure 13). Focal swellings have also been observed in neurons that have undergone ischemia and have shown recovery of dendritic spines to sites of focal swelling when noxious stimulus is removed (Hasbani 2001), suggesting that in a protective response neurons develop focal swellings and retract their spines by inducing redistribution of f-actin and proteins important in regulate actin dynamics and spine morphology.

This neuroprotection effect is also occurs in cortical neurons pretreated with 120μM glutamate for 40min which produced tolerance to subsequent severe ischemia challenge (Lin et al. 2008). The glutamate protective effect is blocked by NMDA and AMPA receptor antagonist and Ca^{2+} chelator treatment but not by voltage dependent calcium channel blockers, suggesting that AMPA and NMDA receptor activation is required for glutamate induced ischemia tolerance in cultured cortical neurons (Lin et al. 2008). Our data showed that inhibition of Arp2/3 in cultured cortical neurons completely blocked the protective effects of OGD preconditioning compared to untreated cells, suggesting that Arp2/3-dependent actin polymerization is necessary for the protective

effect of ischemia preconditioning (figure 15). Acute inhibition of Arp2/3 by NWASP CA domain (EGFP-CA) may destabilize branched f-actin globally and facilitates internalization AMPA receptors resulting in smaller dendritic spine head size (figure 16) making the cells vulnerable to subsequent excitotoxic stimulus.

Despite our interesting findings several questions remain: 1) Is Arp2/3 required to initiate the pre-conditioning ischemic response? 2) What is the role of Arp2/3 redistribution to the dendritic shaft? 3) Is Arp2/3 need to reform dendritic spines after harmful ischemia or sub-excitotoxic level of glutamate? Future experiments will need to address the temporal requirement, subcellular localization and activity of Arp2/3 in the protective effect of ischemia preconditioning and in response to sub-excitotoxic level of glutamate.

Small molecule Arp2/3 complex inhibitors

A 52% knockdown expression of the Arp2/3 subunit p34 by siRNA shows a decrease in spine density, decrease in density of thin and stubby mature spine morphologies, increase in spine length, and increase in density of filopodia-like spines (Hotulainen et al. 2009). Although the knockdown of p34 corroborates with our results, the siRNA oligonucleotide target sequence for p34 needs to be introduced into cell cultures by transfection. Cell transfection can be accomplished chemically, mechanically or biologically. Chemical methods of transfection make use of carrier molecules to overcome the cell-membrane barrier. Calcium-phosphate is a commonly used chemical

method of transfection because the components required are inexpensive and readily available. Nevertheless, calcium phosphate transfection can be toxic to primary cells and its' efficiency is variable and dependent on cell culture variables. Lipofection is another commonly used chemical method of transfection that makes use of micelle formation that packages RNA or DNA of any size and introduces it to the cell via endocytosis. Since lipofection is highly dependent on endocytosis, inhibition of Arp2/3 may make this method inefficient in primary hippocampal cultures. Electroporation is a commonly used mechanical method of transfection that makes use of high-voltage pulse of electricity which creates temporary pores in the cell membrane allowing for unobstructive entry of desired DNA. Despite the great efficiency of this method in most cell types, electroporation parameters need to be optimized for primary cells and often times the high voltage causes high mortality rates. In the current study we employed a biological method of transfection by the use of recombinant adenovirus to drive the expression of the competitive inhibitor for Arp2/3, EGFP-CA, our control vector, EGFP and Arp2/3 subunit (p21-EGFP). Although, cultures infected with these adenoviruses appeared to be healthy when observed by Map2 staining, we did observe a virus induce cell death susceptibility in our rapid ischemia tolerance experiments (figure 15D) suggesting that adenovirus have a negative consequence on cell health making them vulnerable to subsequent challenges. Thus, an alternative method to inhibit the Arp2/3 complex would be highly advantageous.

Small molecule inhibitors (CK-636 and CK-548) for Arp2/3 have been produced and shown to inhibit formation of actin filaments comet tails by Listeria (Nolen et al.

2009). Both compounds are cell permeable but neither compound interacts directly with actin, thus the lack of f-actin formation is specific to Arp2/3 inhibition. CK-636 restricts movement of Arp2 and Arp3 subunits into their active conformation by binding between Arp2 and Arp3, whereas CK-548 inserts into the hydrophobic core of Arp3 subunit and alters its' conformation (Nolen et al. 2009). The greatest advantage in use of these small molecule inhibitors is that Arp2/3 inhibition can be reversible in live cultures by simple wash-out of culture media containing the compound. This reversibility offered by presence or absence of small molecule inhibitors is an attractive and useful way to manipulate Arp2/3 function in the study of many Arp2/3-dependent actin polymerization processes during live-cell imaging.

Mental retardation and the Arp2/3 complex

Abnormalities in morphology or density of spines observed in cognitive disorders/diseases imply that dendritic spines are essential for normal cognition (Fiala et al. 2002, Halpin et al. 2005, Harms and Dunaevsky 2006). Reduced length and branching of dendrites and reduced spine density are observed in patients with Down's syndrome (Fiala et al. 2002, Halpin et al. 2005, vanGalen and Ramakers 2005). Whereas patients with fragile X syndrome often show an increase density of abnormally long thin spines, which are filopodia-like in morphology and represent an immature state (Vanderklish and Edelman 2005, Grossman et al. 2006). Thus, a common characteristic of many cases of mental retardation is the presence of neurons with lasting abnormalities

in dendrite structure, dendritic spine morphology and density (Fiala et al. 2002, Grossman et al. 2006).

In many cases of mental retardation, genes encoding for upstream regulators of Arp2/3 were shown to be mutated or absent (figure 17) (Blanpied and Ehlers 2004, Halpin et al. 2005, vanGalen and Ramakers 2005). Inactivation of Arp2/3 can persist if upstream activators are not physically or functionally available to induce activation and have a negative consequence on Arp2/3 dependent actin-polymerization.

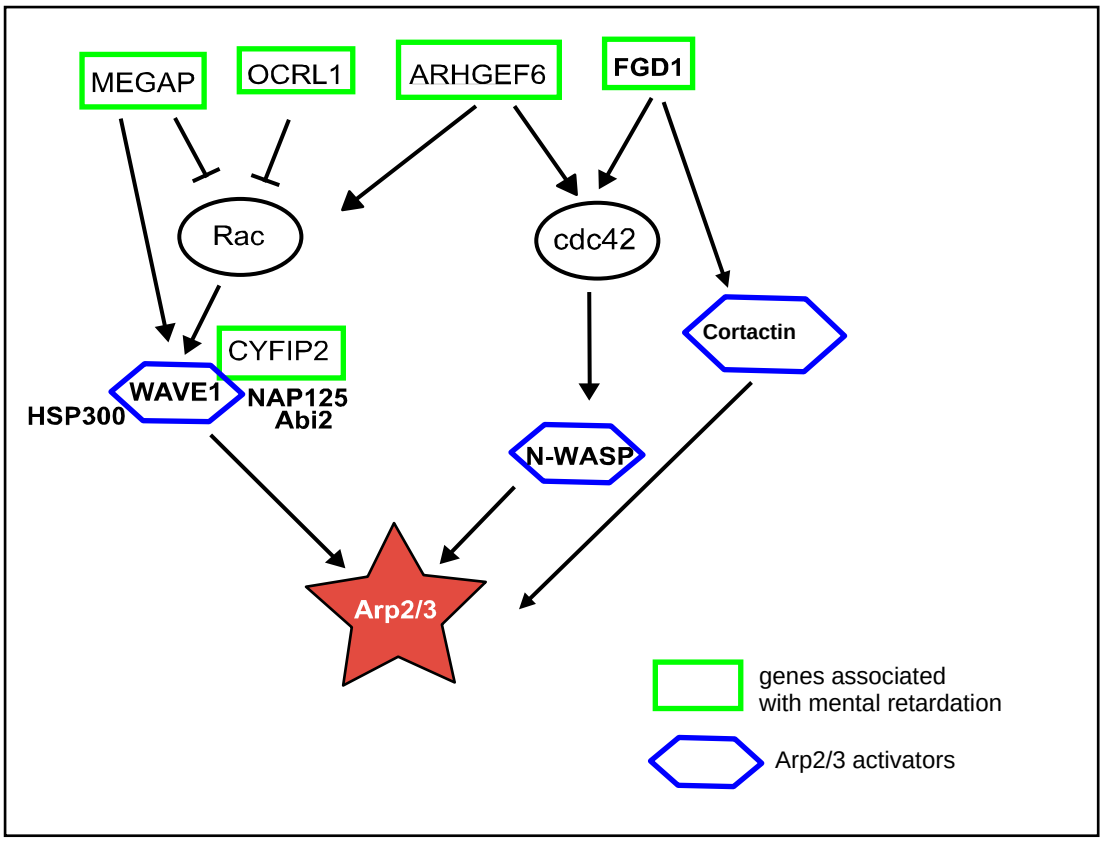

Figure 17. Genes associated with mental retardation encode for upstream regulators of Arp2/3. MAGAP (mental disorder-associated GAP protein) encodes a Rho GAP. OCRL1 (oculocerebroreneal syndrome of Lowe) encodes OCRL1 protein that contains an inositol phosphate/phosphoinositol 5-phosphate domain and Rho Gap homology domain. ARHGEF (Rho guanine-nucleotide exchange factor 6) encodes for a Cdc42/Rac1 GEF. FGD1 (Faciogenital Dysplasia) encodes Cdc42-specific exchange factor. CYFIP2 (cytoplasmic FMR1 interacting protein) encode CYFIP2 protein that is part of inactive WAVE protein complex. CYFIP2 protein is a direct target for Rac1 signaling.

Our results show that inhibition of Arp2/3 function in mature cultures leads to atypical filopodia-like morphology and reduced spine density when compared to control cultures (figure 11). These filopodia-like dendritic spines are reminiscent of those observed in individuals with Down-syndrome and Fragile X syndrome(Irwin et al. 2000, Ramakers 2000); suggesting that the Arp2/3 actin-binding protein complex is involved in maintaining mature spine head morphologies. Furthermore, proteomic analysis of actin binding proteins expression levels in fetal Down Syndrome cortex shows reduced expression of the p20 subunit of the Arp2/3 complex (Weitzdoerfer et al. 2002), further suggesting that the reduced expression of Arp2/3 complex may be involved in the aberrant development of Down Syndrome brain. In addition, knockdown of the p34 subunit of Arp2/3 by siRNA shows a correlation between a decrease in density of mature spines and decrease in frequency of mEPSPs (miniature excitatory post-synaptic potentials) (Hotulainen et al. 2009), suggesting that mature spine head morphology of spines is important for proper and efficient synaptic activity. Indeed, the size of spines is correlated to the size of excitatory synapse, such that increases in F-actin content correlates with larger spine heads that have larger synapses and support stronger synaptic transmission (Sorra and Harris 2000, Hering and Sheng 2001, Yuste R. and T. 2001, Fukazawa et al. 2003, Lisman 2003).

Although our current study provides exciting data that suggests a role of Arp2/3 in the maintenance of mature dendritic spines, it is limited and applies to primary hippocampal cell cultures. Inhibition of Arp2/3 complex function in hippocampal slice cultures would provide a useful *in vitro* model to study the physiological properties of

individual neurons as well as neuronal circuits. Since the hippocampus develops later when compared to the neocortex (Noraberg et al. 2005) and dendritic spine formation occurs within the first few weeks after birth (Yuste and Bonhoeffer 2004) we can introduce our competitive inhibitor, EGFP-CA, into the early postnatal hippocampus. Given that actin polymerization is vital to several cell processes, complete and unrestrained inhibition of Arp2/3 would perhaps be detrimental to further development of the animal. Therefore, stereotactic microinjection of recombinant AAV (adeno-associated virus) to drive expression of EGFP-CA will provide the best approach in introducing the competitive inhibitor into the mouse hippocampus. Stereotactic microinjection is a highly efficient physical method of introducing genes of interest into a single or subpopulation of cells. The use recombinant AAV to drive the expression of EGFP-CA *in vivo* offers the benefit of reduced immunogenicity over the recombinant adenovirus that was used in our in vitro culture studies (Duffy et al. 2005). In addition, recombinant AAV have been extensively used as a gene delivery vector for the nervous system with different serotypes of the AAV targeting diverse cell types (Burger et al. 2005). rAAV1 and rAAV5 have shown to transduce the entire hippocampus (Burger et al. 2004) and would most likely afford the best expression of EGFP-CA in the hippocampus.

Inhibition of Arp2/3 function in the developing hippocampus will allow the use of hippocampal slice cultures to examine their physiological properties, but our interpretations would be limited since slice cultures lack the inputs and outputs present in an intact brain. Consequently, physiological results from hippocampal slice cultures may

not be fully reflective of synaptic behavior associated with learning and memory. Thus, in order to obtain an in vivo model lacking functional Arp2/3 in hippocampal tissue we could allow the AAV microinjected mice to reach maturity while permitting the expression of EGFP-CA and Arp2/3 inhibition in the developing hippocampus. Inhibition of Arp2/3 *in vivo* will allow for a better understanding of the role the Arp2/3 complex plays in learning and spatial memory tasks, such as radial arm maze and Morris water maze.

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