VASODILATOR-STIMULATED PHOSPHOPROTEIN (VASP) PROMOTES ACTIN ASSEMBLY IN DENDRITIC SPINES TO REGULATE SYNAPTIC STRENGTH

By

Wan-Hsin Lin

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements for

the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

Aug, 2011

Nashville, Tennessee

Approved:

Professor Charles K. Singleton

Professor Donna J. Webb

Professor Terry L. Page

Professor Albert B. Reynolds

Professor Alissa M. Weaver

ACKNOWLEDGEMENTS

After countless long hours working on experiments and writing, I am now one step away from getting the doctorate degree. I look back and realize that all the excitement, happiness, struggle, and frustration that occurred in these six years really shaped the way I am today. I deeply appreciate all of the experience that I gained and all of the people who supported, encouraged, and challenged me along the way. With all of your positive influence, I see more confidence, intelligence, meticulousness, and bravery in myself. I owe my sincere gratitude to all of you, especially those who have always been there for me.

This work could not have been accomplished without many people. I am grateful for having great assistance from Caroline Nebhan and Bridget Lindsay, who both helped with tedious analysis and quantification. Also, I would like to thank Lan Hu and Devi Majumdar for the preparation of neuronal cultures, Greg Mathews for the help with electrophysiological experiment, and Andrew Benesh and Rajalakshimi Nambiar for their help with data analysis. I also want to give special thanks to Corey Evans for spending time reading through all of my papers and giving me very constructive comments in writing.

This work was supported by grants MH071674 from NIH and S10RR025524 from the National Center for Research Resources at NIH to D.J.W.

I am very grateful to have Donna Webb as my advisor; she has great patience and strong trust in me and always gives me her full support. With this, I am able to gain confidence in doing experiments and enhance my ability to thinking critically. I also appreciate the great contributions of my dissertation committee—Terry Page, Al

Reynolds, Charles Singleton, and Alissa Weaver—for providing exceptional discussion and suggestions. I also want to thank Tony Weil who always has a strong believe in me to be a good scientist, and Chang Y. Chung who initially introduced me the fascinating aspects of the actin cytoskeleton.

Things in life are not always simple and easy; this includes going abroad for school. However, I am extremely blessed to have many good friends that help me make lemonade when life throws me lemon. I would like to thank Yee Mon Thu who walked toward me the first day of the IGP class to offer to study together and who also became a very close friend. I also owe a great gratitude to Trenis Palmer, Maria Abreu, and Joshua Broussard who have been very supportive, especially when in difficult times. In addition, I am very thrilled to have a very talented undergraduate student, Monica Da Silva, who has helped out a lot in another project, and also Jonas King, Lydia King, Lane Coffee, Becky Michaud, and Jeanne Bristow who have made graduate school more fun. Finally, I would like to thank a very special friend, Joseph Roland, who has always been around—in good times or bad—to provide his expertise in experiments and all the support in life to help me move forward.

Finally, I owe deep thanks to my loving parents and to my sister, who are always there for me and support me. I am very blessed to have the sweetest and most supportive parents who respect my decisions and always care deeply for me. With all of their love, everything is possible.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABP	Actin-binding protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
Arp2/3	Actin-related protein 2/3
AZ	Active zone
$[Ca^{2+}]_i$	Intracellular concentration of calcium
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent protein kinase II
Cc	Critical concentration
Coco	Coiled-coil
CP	Capping protein
Dlg	Discs large
E	Embryonic
EM	Electron microscopy
ER	Endoplasmic reticulum
EVH	Ena/VASP homology
EVL	Ena/VASP like

FAB	F-actin binding
F-actin	Actin filament
FRAP	Fluorescence recovery after photobleaching
GAB	G-actin binding
G-actin	Globular actin
GK	Guanylate kinase
GluR	Glutamate receptor
HBSS	Hank's buffered salt solution
HEK	Human embryonic kidney
IP_3R	Trisphosphate 3 receptor
LTD	Long-term depression
LTP	Long-term potentiation
MAGUK	Membrane-associated guanylate kinase
Mena	Mammalian enabled
mEPSCs	Miniature excitatory postsynaptic currents
mGluR	G-protein-coupled metabotropic glutamate receptor
mmvvee	Mena/VASP/EVL triple knockout mice

NMDA	N-methyl-D-asparate
P	Postnatal
PALM	Photoactivated localization microscopy
PFA	Paraformadehyde
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PP	Protein phosphatase
PRD	Proline-rich domain
PSD	Postsynaptic density
R2F	Rat 2 fibroblast
RIAM	Rap1-GTP-interacting adaptor molecule
SH3	Src homology 3
SV	Synaptic vesicle
SynCAM	Synaptic cell-adhesion molecule
TIRF	Total interference reflection fluorescence
VASP	Vasodilator-stimulated phosphoprotein

ZO1	Zona occludens-1

CHAPTER I

INTRODUCTION

Synapses and Dendritic Spines

Synapses are specialized cell-cell junctions that allow neurons to communicate with each other. In the central nervous system, trillions of synapses establish neuronal circuits and convey signaling and computation from neighboring neurons. Additionally, these sophisticated structures undergo experience-mediated long-term modifications in their number, morphology, and function; therefore, synapses play a central role in cognitive functions such as information storage, learning, and memory.

Structurally, synapses are comprised of presynaptic axon terminals and postsynaptic regions [Figure 1]. For most excitatory synapses, synaptic inputs are received on tiny structures called dendritic spines, which protrude from postsynaptic dendrites [Figure 1] {Gray 1959}. To deliver synaptic information, electric signals—in the form of action potentials—first race down axons and reach presynaptic terminals, causing the fusion of synaptic vesicles to the plasma membrane. These vesicles then release stored neurotransmitter molecules that cross the synaptic cleft, an approximately 20 nm space between pre- and post-synaptic cells, and bind to their receptors on the plasma membrane of dendritic spines [Figure 1] {Schikorski et al. 1997}. Subsequently, these receptors open their channels to allow an influx of ions, which activates a variety of signal transduction pathways, including those that lead to the reorganization of the cytoskeleton. This series of events collectively forms the basis of information transfer in the brain.

Interestingly, dendritic spines, which generally consist of a bulbous head and a thin neck [Figure 1], experience altered density and changes in morphology from filopodia-like protrusions to more mature thin, stubby, or mushroom-shaped structures during development [Figure 1] {Harris et al. 1994; Sorra et al. 2000} {Peters et al. 1970}. These changes have drawn tremendous attention because abnormal spine density and morphology are usually found in neuronal disorders, such as mental retardation, Fragile-X syndrome, Down's syndrome, Alzheimer's disease, and epilepsy {Suetsugu et al. 1980; Ferrer et al. 1990; Swann et al. 2000; Chechlacz et al. 2003; Grossman et al. 2006}. However, the underlying molecular mechanisms that regulate spine formation and morphological changes are still largely unknown.

Development of Dendritic Spines

Currently, three models for the formation of dendritic spines have been proposed [Figure 2] {Ethell et al. 2005}. In the most accepted model, dendritic spines are believed to originate from thin, immature protrusions called dendritic filopodia. This assumption is based on the observation that dendritic shafts of neurons in culture at an early stage of development are decorated by dendritic filopodia. A week later, as synapses form, dendritic filopodia are replaced by dendritic spines. This filopodia-to-spine transition on dendrites is also found in the developing brain {Morest 1969; Dailey et al. 1996; Ziv et al. 1996; Fiala et al. 1998; Dunaevsky et al. 1999}. Using time-lapse microscopy, it is shown that highly motile dendritic filopodia extend and retract within minutes to probe their surrounding environment {Dailey and Smith 1996; Ziv and Smith 1996; Dunaevsky, Tashiro et al. 1999}. Once the appropriate presynaptic partner is found, the synaptic

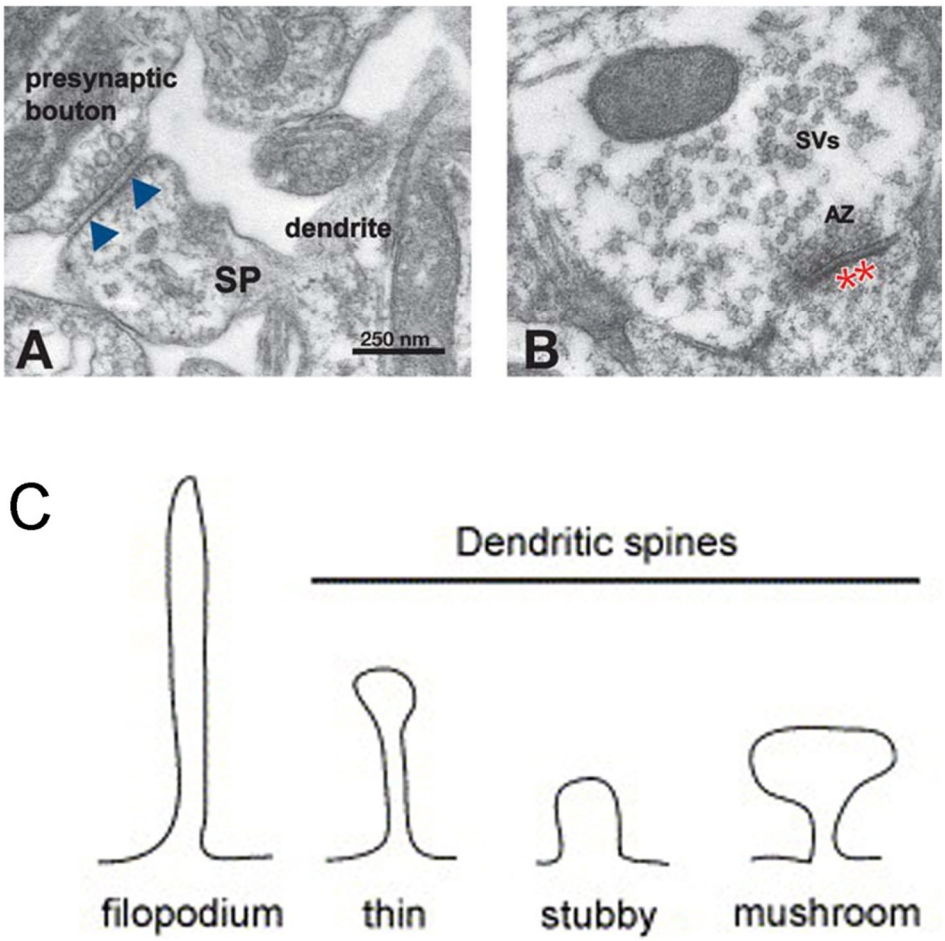


Figure 1. Ultrastructure of synapses and morphology of dendritic spines. *A*, *B* Electron microscopy images from synapses formed between hippocampal neurons grown for 15 days in culture are shown. A dendritic spine (SP) extends from a dendrite and apposes a presynaptic terminal (synaptic bouton). More than one hundred synaptic vesicles (SVs) are stored in each bouton. These vesicles can be docked in a 50-100 nm area called the active zone (AZ), which perfectly aligns with the postsynaptic density (PSD) (arrowheads in A, asterisks in B) across the synaptic cleft. *C*, Schematic diagram of a spine precursor (dendritic filopodium) in comparison with the common morphological classes of more mature dendritic spines. Reprinted from {Ethell et al. 2005; Waites et al. 2005}.

contact stabilizes and elicits the substantial shortening and the distal expansion of filopodia to yield a bulbous spine {Dailey et al. 1996; Maletic-Savatic et al. 1999; Marrs et al. 2001; Okabe et al. 2001; Trachtenberg et al. 2002}. These findings point to dendritic filopodia as the precursors of dendritic spines.

Conversely, dendritic spines have also been proposed to arise from the dendritic shaft in response to presynaptic signals {Miller 1981}. This idea is supported by the observation that the majority of synapses are located on dendritic shafts rather than on dendritic filopodia in young pyramidal neurons {Harris et al. 1992}. As development proceeds, the number of shaft synapses decreases while the number of spine synapses increases. Live-cell imaging is also used to reveal the emergence of spines directly from dendritic shafts {Dailey et al. 1996; Marrs et al. 2001}. Accordingly, the search for a synaptic partner may be initiated by axonal filopodia (not dendritic filopodia, as suggested by the previous model) due to the observation that axonal filopodia form contacts with the dendritic shaft {Fiala et al. 1998}. These shaft synapses later give rise to dendritic spines.

A third model suggests that the formation of dendritic spines is determined by intrinsic mechanisms that do not require presynaptic contact {Sotelo 1990; Takacs et al. 1997}. Indeed, dendritic spines in Purkinje cells are able to form before coming in contact with presynaptic parallel fibers. Consistently, the formation of Purkinje spines develops normally in mice lacking presynaptic parallel fibers {Sotelo 1990}.

Taken together, the first two models are generated based on studies of cultured pyramidal neurons or slices from the neocortex and the hippocampus, and they point to an involvement of synaptic contacts during spinogenesis. In contrast, spine development in the cerebellum is determined by a contact-independent mechanism.

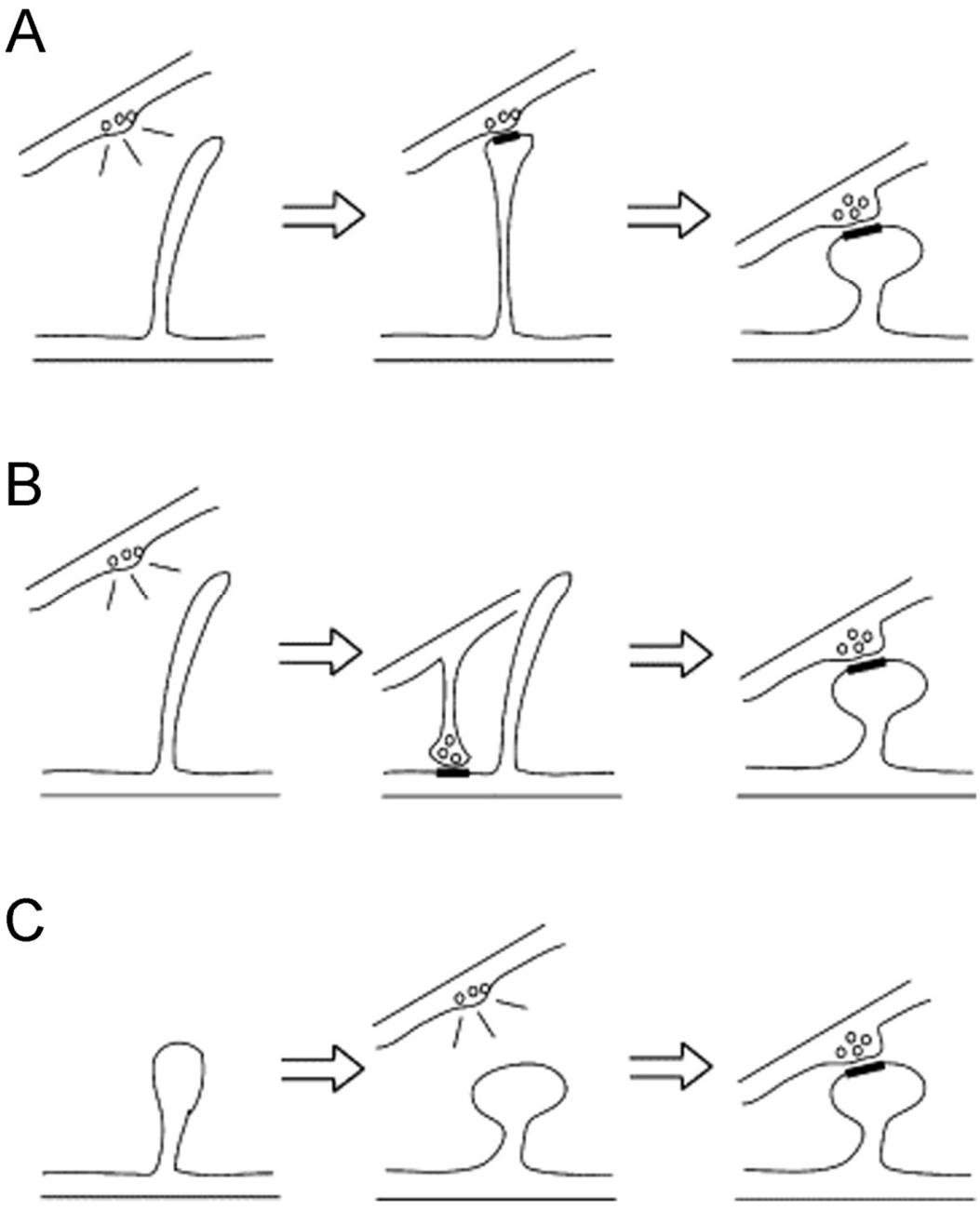


Figure 2. Models of the formation of dendritic spine. *A,* Dendritic filopodia as spine precursors. *B,* Spine formation from the dendritic shaft. *C,* Spine formation without synaptic contact. Reprinted from {Ethell et al. 2005}.

Although we still lack a clear understanding of how spines are actually formed *in vivo*, it is possible that these mechanisms can occur in different subsets of neurons in response to different presynaptic signals.

Ultrastructure of dendritic spines: the postsynaptic density

To efficiently receive presynaptic inputs, a complex of proteins is packed and clustered in a 30-50 nm-thick area at the distal tips of the spine head {Carlin et al. 1980}. This region is referred to as the postsynaptic density (PSD), which directly apposes the presynaptic active zone where neurotransmitters are released [Figure 1] {Palay 1956; Carneiro et al. 2005; Zuber et al. 2005}. The PSD is an electron-dense structure that consists of adhesion molecules, receptors, ion channels, scaffold proteins, and signaling molecules, and cytoskeletal elements [Figure 3] {Li et al. 2003; Sheng et al. 2007}. Due to their effects on the PSD's composition, structure, and size during development and in response to synaptic activity, these proteins have been well studied and show a profound effect on synaptic function.

Adhesion molecules — In order to stabilize the connections between presynaptic terminals and postsynaptic spines, a variety of adhesion molecules are present in the PSD {Li et al. 2003}. For example, cadherins—Ca^{2+}-dependent homophilic adhesion molecules—are localized to both pre- and post-synaptic sites of synapses {Takeichi 1991}. Neuronal (N)-cadherin regulates pre- and post-synaptic organization, and its expression is correlated with the stability of dendritic spines {Yagi et al. 2000; Togashi et al. 2002; Mendez et al. 2010}. Later studies have shown that catenins, downstream effector proteins that link cadherins to the actin cytoskeleton, also control synaptic functions and maturation {Murase et al. 2002; Abe et al. 2004}. Similar functions are

also reported for other cell-adhesion molecules, including integrins, neuroligin/neurexin, synaptic cell-adhesion molecules (SynCAMs), and Ephirin/Eph receptors {Li et al. 2003}.

Neurotransmitter receptors and ion channels — Glutamate is the major type of neurotransmitter used by excitatory synapses in the central nervous system. It can activate ionotropic glutamate receptors, such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type and N-methyl-D-asparate (NMDA)-type receptors, as well as a family of G-protein-coupled metabotropic glutamate receptors (mGluRs). During development and in response to stimuli, the composition and number of these receptors are altered in order to properly respond to the released glutamate from presynaptic terminals. The activation and opening of NMDA receptors allow the influx of Ca^{2+} and Na^{+} into dendritic spines. However, under basal conditions while the membrane potential is at the resting state, the channel of the NMDA receptor is blocked by extracellular Mg^{2+}; so, an initial membrane depolarization is required to dissociate Mg^{2+} from the channel. The NMDA receptor is therefore a voltage-dependent ion channel and has little contribution (if any) to synaptic transmission at the resting potential {Lu et al. 2001}. In contrast, the AMPA receptor is permeable to the movement of ions when a neuron is at its resting membrane potential. Two monovalent cations—Na^{+} and K^{+}—cross the channels of the AMPA receptors and determine synaptic responses in the form of electrical signals.

Scaffold proteins — One of the best-studied PSD scaffold proteins is postsynaptic density 95 (PSD95/SAP90), which belongs to the membrane-associated guanylate kinase (MAGUK) family {Cho et al. 1992; Kistner et al. 1993}. Like all MAGUK family members, PSD95 contains three PSD-95/Discs large (Dlg)/Zona occludens-1 (ZO1) (PDZ) domains, a Src homology (SH3) domain, and a catalytically inactive guanylate kinase (GK) region. These multiple domains allow PSD-95 to interact directly or

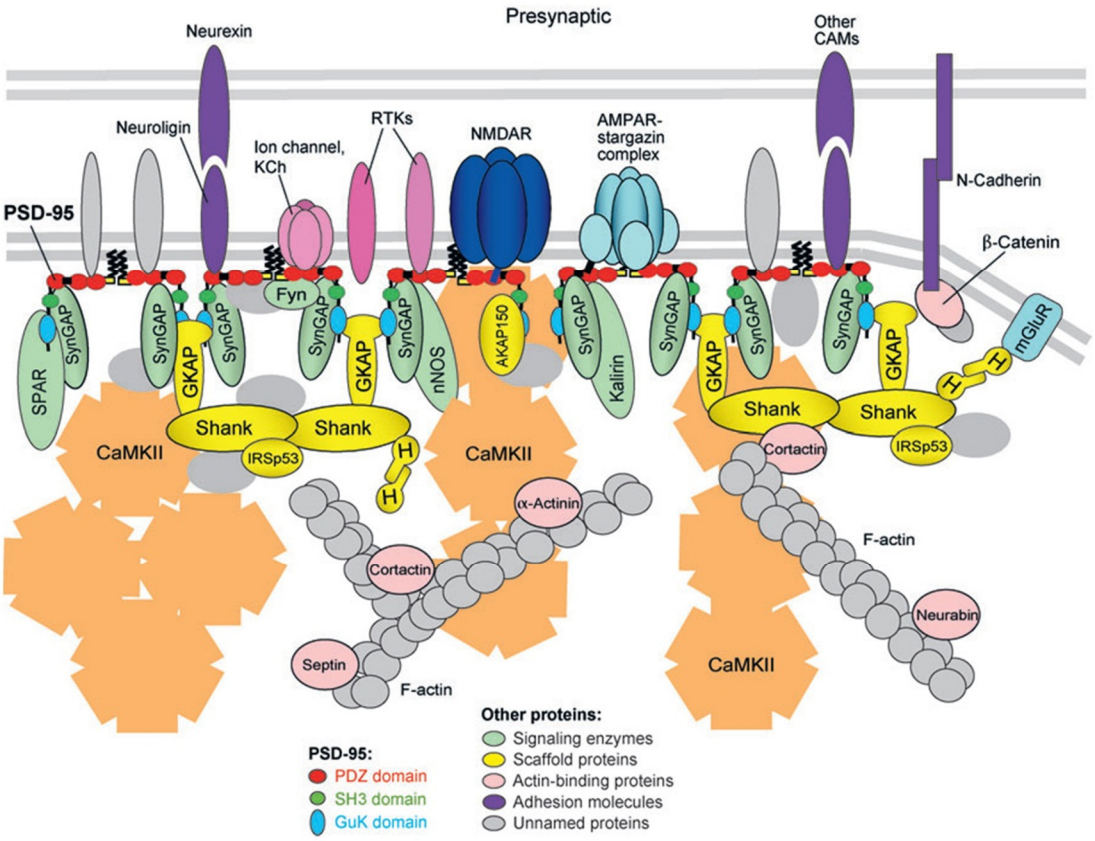


Figure 3. Organization of proteins in the postsynaptic density (PSD). Schematic diagram of the network of proteins in the PSD. Only major PSD proteins are shown. These proteins include adhesion molecules, neurotransmitter receptors, ion channels, scaffold proteins, signaling molecules (kinases and phosphatases), and cytoskeletal proteins. Reprinted from {Sheng et al. 2007}.

indirectly with different glutamate receptors, providing regions for these receptors to localize at the PSD {Niethammer et al. 1996; O'Brien et al. 1998; Chen et al. 2000; Nicoll et al. 2006}. Because PSD95 can form multimers, the receptors cluster at the PSD and therefore promote synaptic transmission {El-Husseini et al. 2000; Christopherson et al. 2003}. This critical effect prompted investigators to search for other PSD95 binding partners. This led to the discovery of the protein GKAP, which associates with the GK domain of PSD95 {Kim et al. 1997; Naisbitt et al. 1997; Satoh et al. 1997; Takeuchi et al. 1997}. The association of PSD95 with GKAP was further shown to regulate the assembly of other scaffold proteins in the PSD and to promote the stability of synapses {Romorini et al. 2004}. Later, a novel GKAP binding partner called Shank, named for the presence of an SH3 domain and multiple ankyrin repeats, was characterized to gain insight into GKAP function {Naisbitt et al. 1999}. The GKAP-Shank interaction recruits Shank to PSD-95 clusters, and the multimerization of Shank potentially leads to coupling of PSD95/NMDA receptor complexes. Interestingly, Shank proteins were also shown to interact with the actin-binding protein cortactin, and this interaction is enhanced in response to glutamate {Du et al. 1998; Naisbitt et al. 1999}. This finding suggests that a close interplay exists between synaptic activity, PSD scaffold proteins, and the actin cytoskeleton. In addition, Shank forms a polymeric complex with another PSD scaffold protein called Homer, which forms multimers in the brain and interacts with metabotropic glutamate receptors (mGluRs) in the postsynaptic membrane {Tu et al. 1998; Xiao et al. 1998; Tu et al. 1999}. As a member of the Shank/PSD95/NMDA receptor complex mentioned earlier, Homer provides a link to allow the crosstalk between signal transductions elicited by ionotropic and metabotropic receptors. Indeed, disruption of Homer-Shank interactions allows mGluR agonists to inhibit ionic currents across the NMDA receptors {Bertaso F, 2011 PLoS-One}. Homer has also been demonstrated to interact with inositol trisphosphate 3 receptors (IP_3Rs) at the endoplasmic reticulum

{Naisbitt, Kim et al. 1999}. IP_3Rs are Ca^{2+} channels activated by IP_3. The opening of these channels allows the release of calcium from the endoplasmic reticulum (ER), increasing the intracellular concentration of calcium ($[Ca^{2+}]_i$) and triggering Ca^{2+}-dependent signaling pathways. The Homer-Shank complex couples the activation of mGluRs to the stimulation and opening of IP_3Rs and thus modulates the homeostasis of Ca^{2+} and signaling {Sala et al. 2005}. This interaction turns out to be crucial for the induction of spine head enlargement and the potentiation of synaptic strength {Sala et al. 2001}.

Signaling molecules — In response to synaptic activity, many kinases and phosphatases in dendritic spines are mobilized to modulate synaptic functions and neuronal behaviors {Sheng et al. 2002}. These enzymes are activated by the elevated concentration of secondary messengers. One of the most critical secondary messengers in neurons is calcium, which can be delivered into spines through opened NMDA receptors. The increased $[Ca^{2+}]_i$ level activates a kinase called calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII), which is the most abundant protein in the PSD {Erondu et al. 1985} {Sheng et al. 2007}. Owing to its influence on the phosphorylation and subsequent activation of glutamate receptors, CaMKII plays a central role in modulating synaptic responses. Interestingly, activation of CaMKII also increases its association with NMDA receptors, and this interaction locks CaMKII in an active state that cannot be inactivated by phosphatases {Leonard et al. 1999; Bayer et al. 2001}. Such an effect prolongs the activation of CaMKII in synapses. Since activated CaMKII can also phosphorylate and thus increase the conductivity of AMPA receptors, synaptic transmission is then potentiated {Barria et al. 1997; Pratt et al. 2003}. Besides its enzymatic function, one subset of CaMKII (CaMKIIβ) can serve as a scaffold protein to cross-link actin filaments and to stabilize spine head structure

{Okamoto et al. 2007}. Therefore, CaMKII not only has enzymatic effects but also acts as a structural component in spines.

In addition to the activation of CaMKII, Ca^{2+} influx causes the activation of protein kinase C (PKC) and stimulates the production of cAMP, which further leads to the activation of protein kinase A (PKA). Both of these kinases are capable of phosphorylating the GluR1 subunit of AMPA receptors, increasing receptor exocytosis and expression at postsynaptic membrane {Kameyama et al. 1998; Lee et al. 1998}. To tightly control the phosphorylation state of GluR1, the protein phosphatases PP1 and PP2 (also termed calcineurin) counteract the phosphorylation and activation of AMPA receptors {Beattie et al. 2000; Ehlers 2000; Morishita et al. 2001}. Once GluR1 is dephosphorylated by these phosphatases, it then undergoes endocytosis, which removes the receptor from the postsynaptic membrane and decreases synaptic transmission. All these phosphorylation-dependent processes, furthermore, are shown to be critical for long-lasting modification of synaptic function.

Synaptic strength and synaptic plasticity

The main function of a synapse is to allow pre-synaptic and post-synaptic neurons to communicate with each other via electrical signals. The term *synaptic strength* is commonly used in the field to describe the efficacy of this type of communication. Synaptic strength is defined by the changes in membrane potential or in the amount of ion flow through the postsynaptic neurotransmitter receptors (i.e. synaptic transmission). A common way to detect synaptic transmission in cultured neurons is to perform electrophysiological experiments to measure spontaneous activity-mediated miniature excitatory postsynaptic currents (mEPSCs), which exclude the

responses caused by action potentials. Synaptic transmission is triggered by neurotransmitters that bind to and permit the opening of postsynaptic receptors. Therefore, the number (frequency) and size (amplitude) of mEPSCs are decided by the amount of released neurotransmitters, the probability of the opening of ion channels, and the amount of ion channels at the postsynaptic membrane. If one assumes that the amount of neurotransmitters in each synaptic vesicle is relatively fixed, the amplitude of mEPSCs would reflect the function and/or number of neurotransmitter receptors {Malenka et al. 1999}. In excitatory synapses, glutamate is the key neurotransmitter, and glutamate receptors are the main ion channels responsible for synaptic transmission. Since PSD scaffold proteins stabilize and recruit glutamate receptors to the PSD, one can imagine that the number and function of PSD scaffold proteins can indirectly contribute to synaptic strength {Kim et al. 2004}.

Interestingly, synaptic strength undergoes persistent changes in an activity- and experience-dependent manner. This phenomenon—synaptic plasticity—serves as the cellular basis of information acquisition (learning) and storage (memory) in the brain. {Malinow et al. 2002}. Synaptic plasticity is exemplified by two well-characterized models, long-term potentiation (LTP) and long-term depression (LTD), which enhances and decreases synaptic transmission, respectively {Bliss et al. 1973}. Several approaches have been developed to induce these two forms of plasticity, and the most common protocol is to apply high-frequency stimulation to induce LTP and low-frequency stimulation to produce LTD {Malenka et al. 2004}. In most cases, the expression and maintenance of LTP and LTD require the activation of NMDA receptors {Malenka et al. 2004}. The degree of NMDA receptor activation, as well as $[Ca^{2+}]_i$ due to receptor activation, determines the expression of LTP or LTD {Bear et al. 1987; Lisman 1989; Artola et al. 1993}. Specifically, during LTP strong depolarization of the

membrane potential allows a heightered activation of NMDA receptors, leading to a greater increase in $[Ca^{2+}]_i$ and thus the activation of protein kinases (e.g. CaMKII, PKA, PKC). These kinases phosphorylate AMPA receptors and increases their synaptic localization and ionic conductivity {Malinow et al. 2002; Sheng et al. 2002; Shepherd et al. 2007}. In contrast, LTD expression produces a modest activation of NMDA receptors and, therefore, only a slight increase in $[Ca^{2+}]_i$. This low amount of $[Ca^{2+}]_i$ is enough to activate phosphatases (e.g. PP1 and PP2B) that, in turn, result in dephosphorylation and endocytosis of AMPA receptors. In addition, these kinases and phosphatases modulate the phosphorylation of signaling molecules, scaffold proteins, and cytoskeletal molecules to mediate synaptic plasticity. Intriguingly, LTP and LTD expression are synonymous with spine expansion and shrinkage, respectively, linking synaptic plasticity to the regulation of underlying cytoskeletal components.

Ultrastructure of dendritic spines: the actin cytoskeleton

The cytoskeletal components play a prominent role in regulating almost all biological processes, ranging from cell division to organ development, and can be classified into three different categories—actin filaments (F-actin), microtubules, and intermediate filaments. In neurons, the structure and dynamics of microtubules and of actin filaments are more well-studied than those of intermediate filaments. Microtubules have been shown to be enriched in the shafts of axons and dendrites, while actin is enriched in the distal protrusions of these processes, such as axon terminals and dendritic spines. As revealed by electron microscopy (EM), the ultrastructure of the cytoskeleton in dendritic spines shows a combination of branched lattice networks and straight crosslinked filaments {Fifkova et al. 1982; Landis et al. 1983}. A more modern EM technique named platinum replica EM further revealed that actin molecules are

organized into a short, cross-linked branched network in spine heads and a network of branched and linear actin filaments in spine necks [Figure 4] {Skwarek-Maruszewska et al. 2010}. Time-lapse microscopy using fluorescently-labeled actin unexpectedly showed that actin is highly dynamic in spines and in presynaptic terminals {Colicos et al. 2001}. Fluorescence recovery after photobleaching (FRAP) further demonstrated that 85% of actin in spines is highly dynamic, and its turnover is modulated by neuronal activity {Moss et al. 2002}. The use of photoactivatable actin combined with time-lapse microscopy indicated that three distinct actin populations are present in spines; the subspine region as well as spine head size determine the kinetics of actin turnover {Honkura et al. 2008}. Under basal conditions, a dynamic F-actin pool is restricted to the tips of spines, while a stable pool resides at the base of spine heads. In response to glutamate, a more kinetically stable F-actin pool is formed and mediates the expansion of spines, indicating that neuronal activity can regulate actin dynamics and modulate spine function {Honkura et al. 2008}. The movement of actin filaments was also investigated using a super-resolution microscopy technique named photoactivated localization microscopy (PALM) and showed that the velocity and turnover of F-actin are highly heterogeneous within individual spines {Tatavarty et al. 2009; Frost et al. 2010; Frost et al. 2010}. These studies highlight the complex nature of actin and point to its crucial role in regulating spine development and the plasticity of synaptic strength.

Role of actin in spine development and synaptic function

To investigate a functional role for actin in spines, pharmacological approaches are used to perturb actin dynamics. The application of latrunculin A, which causes actin depolymerization, alters the number and localization of glutamate receptors {Allison et al. 1998}. This treatment also disrupts the localization of the signaling molecule CaMKII, as

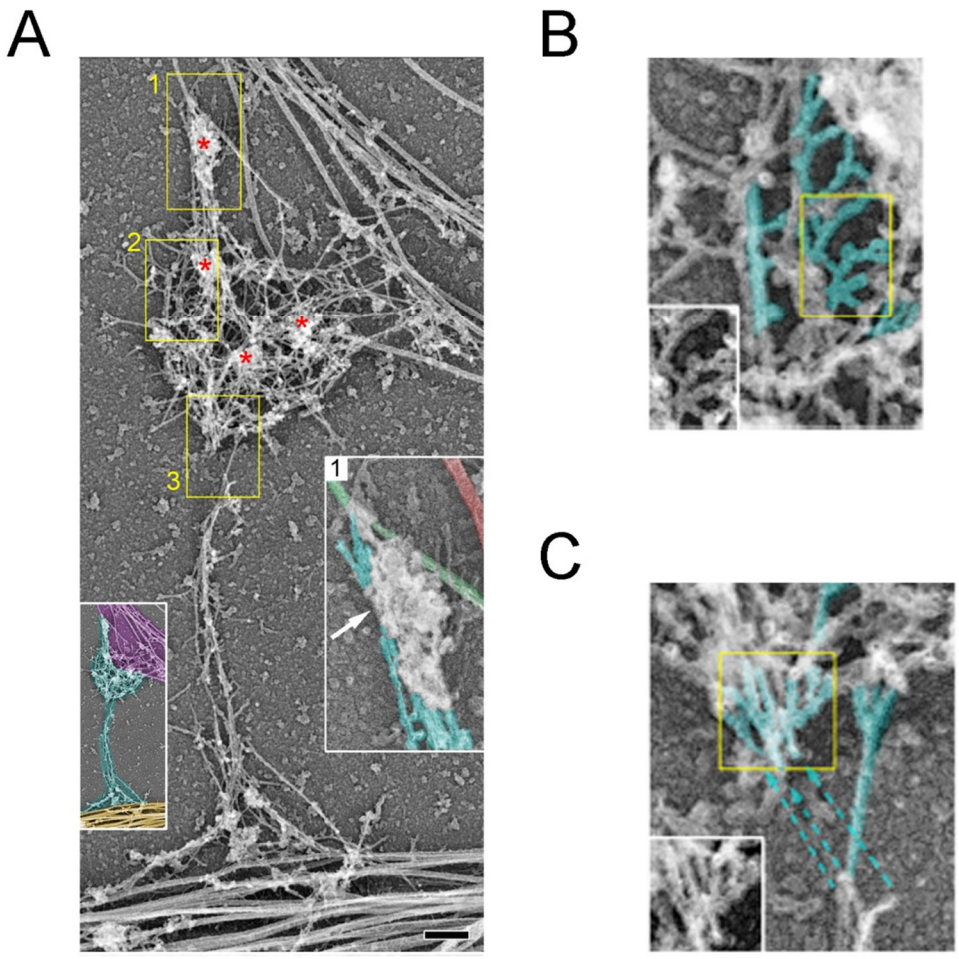


Figure 4. Ultrastructure of cytoskeletal organization in dendritic spines. Images from platinum replica electron microscopy of a mushroom-shaped spine from a hippocampal neuron grown in culture for 14 days are shown. *A,* A spine extends from a dendrite (bottom) and contacts with an axon (top). An unlabeled inset shows a smaller version of the image in which axons, dendrites, spines are color-coded in purple, yellow, and cyan, respectively. Asterisks, represent multiple PSDs. Bar, 0.2 µm. Box 1, Interaction of a putative PSD with axonal intermediate filaments (green). Actin filaments (cyan) and a microtubule (red) are also shown. *B-C,* Zoom-in images from box 2 (*B*) and 3 (*C*) show branched actin networks in a spine head (*B*) and at the spine neck junction (*C*). Dashed lines show a potential breakage of actin filaments. Reprinted from {Korobova et al. } .

well as certain actin-binding proteins and PSD scaffold proteins, to spines {Allison et al. 2000; Kuriu et al. 2006}. These studies suggest a potential role for actin in tethering neurotransmitter receptors, signaling molecules, and scaffold proteins at the PSD to modulate spine function. Indeed, latrunculin A treatment shows an inhibition in spine motility and a delay in synaptic development, indicating that actin dynamics endow spines with a high degree of plasticity {Fischer et al. 1998; Zhang et al. 2001}.

The amount of actin filaments in spines experiencing synaptic plasticity has been examined, and studies point to an association of synaptic activity, spine size, and the concentration of actin filaments in spines. When LTP is induced in the hippocampus, the amount of F-actin increases and is accompanied by the enlargement of spine heads {Fukazawa et al. 2003; Okamoto et al. 2004; Lin et al. 2005}. Conversely, induction of LTD promotes the depolymerization of actin, resulting in spine head shrinkage {Okamoto et al. 2004}. These intriguing data suggest a linkage between actin dynamics and changes in synaptic strength and spine morphology. In fact, bath application of the actin depolymerization agent latrunculin B to hippocampal slices results in the reduction of AMPA receptor-mediated basal synaptic transmission and LTP {Kim et al. 1999}. Subsequent studies also found that actin depolymerizing agents affect the maintenance of the early and late stages of LTP {Krucker et al. 2000; Fukazawa et al. 2003}. Two possible mechanisms as to how actin regulates synaptic plasticity have been proposed: (i) the actin cytoskeleton may serve as a platform for the retention of glutamate receptors in the postsynaptic density; and (ii) the actin cytoskeleton provides a path for short-distance protein trafficking in spines, a process that is required for synaptic transmission. AMPA receptors, which move between synapses and extrasynaptic sites, are the primary mediators of synaptic transmission {Sheng et al. 2002; Shepherd et al. 2007}. It is generally believed that surface expression of AMPA receptors is regulated by passive

diffusion and by active endocytosis/exocytosis {Man et al. 2000; Park et al. 2004; Gerges et al. 2006; Park et al. 2006; Yudowski et al. 2007}. Importantly, trafficking of AMPA receptors is conducted by actin motors—myosins—which carry AMPA receptors-containing endosomes along actin tracks {Osterweil et al. 2005; Lise et al. 2006; Ryu et al. 2006; Correia et al. 2008; Wang et al. 2008}. In this way, the modulation of the actin cytoskeleton may dictate the incorporation and internalization of AMPA receptors, thereby altering synaptic strength and plasticity {Zhou et al. 2001}.

Modulation of actin by actin-binding proteins (ABPs) and their roles in spines

Actin exists in two states in the cell: globular or monomeric actin (G-actin) and filamentous actin, which results from the polymerization of G-actin. Monomeric G-actin can be associated with either ATP or ADP along with a Mg^{2+} ion; however, ATP-bound actin has a higher efficiency than ADP-actin in F-actin assembly. The assembly of actin filaments occurs through three sequential steps: nucleation, which is the rate-limiting step, elongation, and ultimately steady state where there is no net change in the amount of F-actin [Figure 5]. The rate of actin assembly is determined by the available amount of unpolymerized G-actin and by the G-actin critical concentration (Cc) [Figure 5] {Oosawa et al. 1975; Carlier 1990}. The difference in Cc values at each end of actin filaments results in different elongation rates. Therefore, F-actin exhibits net polymerization at the fast growing end (the barbed or plus end) and depolymerization at the slow growing end (the pointed or minus end). Actin polymerization is initiated by the formation of nucleation seeds, composed of G-actin trimers. However, these structures are unstable under physiological conditions unless they are stabilized by the binding of certain ABPs, such as the actin-related protein 2/3 (Arp2/3) complex {Chesarone et al. 2009}. Once these stable actin seeds are formed, actin polymerization occurs with the

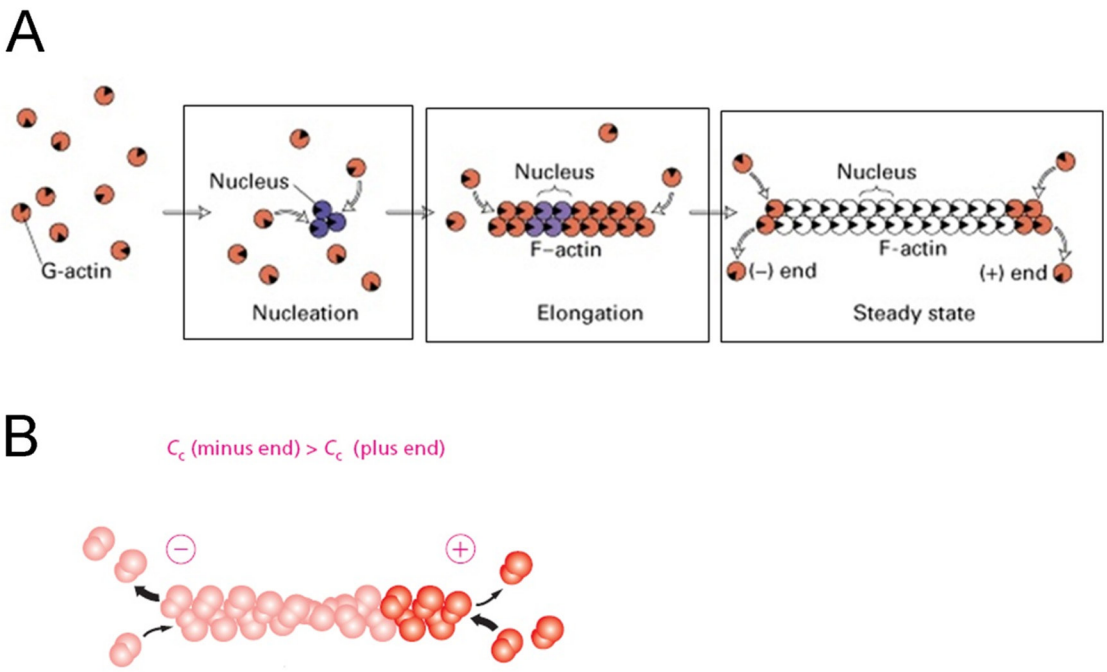


Figure 5. Actin polymerization in vitro. *A,* Actin polymerization occurs in three sequential steps: nucleation, elongation, and ultimately steady state. First, ATP-bound G-actin monomers slowly form trimeric G-actin seeds. Actin assembly occurs at both plus and minus ends of actin filaments (F-actin) and then reaches a steady state in which no net change in the amount of actin within the filament occurs. *B*, The critical concentration (Cc) of G-actin is an important factor in determining the rate of actin elongation. Reprinted from {Lodish et al. 2000} {Alberts et al. 2008}.

assistance of other ABPs. For example, actin assembly can be promoted by a G-actin-binding protein called profilin, which catalyzes the exchange of ADP for ATP on G-actin and increases the addition of actin monomers to the growing ends of filaments {Tilney et al. 1983; Pollard et al. 1984; Pring et al. 1992}. To balance the amount of actin filaments in cells, F-actin can also undergo depolymerization. This process is carried out by ABPs that possess a severing activity that breaks down actin filaments into smaller pieces and thus provides G-actin reservoirs for further actin assembly and reorganization. Other groups of ABPs, like capping proteins, do not affect the exchange of G-actin and F-actin directly, but instead stabilize F-actin by binding to the barbed or pointed ends of actin filaments and preventing the addition or loss of G-actin from these sites. Finally, some ABPs, such as α-actinin, are able to modulate the structure of the actin cytoskeleton by bundling actin filaments. Taken together, a variety of ABPs cooperate with each other through different mechanisms to regulate actin-based cellular events and shape actin-rich structures, such as dendritic spines. Indeed, emerging studies are beginning to demonstrate the importance of ABPs in spine formation and function [Table 1] {Lin et al. 2009; Pontrello et al. 2009}.

The Ena/VASP family

Some of the most important actin-binding proteins are those belonging to the Ena/VASP family. In vertebrates, this family includes mammalian enabled (Mena), vasodilator-stimulated phosphoprotein (VASP), and Ena/VASP-like (EVL) molecules; the functions of these family members are interchangeable in many cases {Kwiatkowski et al. 2003}. Previous studies have shown that they play a crucial role in a variety of cellular processes, including platelet aggregation {Aszodi et al. 1999; Hauser et al. 1999}, membrane protrusion {Bear et al. 2002; Lebrand et al. 2004; Dent et al. 2007}, cell

Table 1. Role of actin-binding proteins (ABPs) in actin regulation and spine/synaptic function. Reprinted from {Lin et al. 2009}.

Actin-binding proteins	Actin regulation	Spine density	Spine morphology	Synaptic strength and plasticity
α-actinin 2	Bundling	Overexpression: reduced spine density and more filopodia-like protrusions	Overexpression: longer spines	Undetermined
Arp2/3 complex	Branching, nucleation	Knockdown (Arp3, p34): reduced spine density Knockdown (p34): fewer mature spines	Knockdown (p34): longer protrusions	Undetermined
CaMKIIβ	Bundling	Knockdown: no effect on spine density	Knockdown: longer spines and smaller spine heads	Undetermined
α-N-catenin	Bundling	Overexpression: increased spine density Knockdown: fewer synaptic spines, but no effect on the total number of spines	Knockdown: longer, immature spines with uncontrolled motility	Undetermined
Cofilin	Depolymerization, severing	Knockdown: fewer protrusions	Knockdown: longer protrusions, but no effect on spine head size Dominant negative: shorter protrusions Constitutively active: longer protrusions and smaller spine heads Dominant negative peptide: less spine shrinkage during LTD Constitutively active peptide: more spine shrinkage during LTD	Dominant negative peptide: blockage of NMDAR-mediated, but not AMPAR-mediated LTD Constitutively active peptide: retain LTD expression
Cortactin	Nucleation-promoting factors	Knockdown: reduced spine density	Overexpression: longer spines	Undetermined
Formin (mDia2)	Nucleation	Knockdown: fewer protrusions	Knockdown: longer and larger spines Constitutively active: headless spines	Undetermined
Gelsolin	Anti-bundling, capping, severing	Undetermined	Undetermined	Knockdown: no effect on LTD
N-WASP	Nucleation-promoting factors	Overexpression: increased spine density Knockdown: reduced spine density	Dominant negative: longer, thinner protrusions	Undetermined
Neurabin I	Bundling	Overexpression (F-actin binding domain): increased spine density and fewer mushroom-shape spines	Overexpression (F-actin binding domain): longer spines and filopodia-like protrusions	Overexpression (FAB): less basal synaptic transmission Knockdown: reduced LTP, but no effect on LTD Overexpression: blockage of LTP, and enhanced LTD Overexpression (with F-actin binding deletion): increased LTP and reduced LTD
Neurabin II/Spinophilin	Bundling	Overexpression: fewer filopodia-like protrusions Knockout: increased spine density	Overexpression: longer filopodia-like protrusions	Knockout: reduced LTD, but no effect on LTP
Profilin II	Polymerization	Undetermined	Undetermined	Knockout:no effect on both LTP and LTD
WAVE 1	Nucleation-promoting factors	Knockout: reduced spine density and fewer mature spines	Undetermined	Knockout: increased LTP and reduced LTD

migration {Chakraborty et al. 1995; Bear et al. 2000}, endothelial barrier formation {Collard et al. 2002; Furman et al. 2007}, axon guidance {Menzies et al. 2004}, and neuritogenesis {Kwiatkowski et al. 2007}. These varied functions are caused by modulating actin dynamics and organization.

Regulation of the actin cytoskeleton: actin elongation, anti-branching, and bundling — Ena/VASP proteins are effective actin-regulatory molecules, and their expression promotes the assembly of actin. Two mechanisms are involved in Ena/VASP -mediated actin polymerization. In one scenario, Ena/VASP proteins recruit G-actin/profilin complexes, facilitating the addition of G-actin monomers to the growing actin filaments {Reinhard et al. 1995; Chereau et al. 2006; Ferron et al. 2007}. In the other scenario, Ena/VASP proteins act as actin anti-capping molecules that allow persistent actin assembly. Actin polymerization in cells is highly controlled in order to properly modulate actin-based cellular processes. While actin elongation molecules, such as Ena/VASP, promote actin assembly, the other groups of actin-binding proteins, such as capping protein (CP), obstruct the growing ends of actin filaments and terminate actin polymerization. The anti-capping activity of Ena/VASP proteins was first observed in an *in vitro* experiment showing that the presence of purified VASP proteins inhibits actin capping mediated by CP {Bear et al. 2002}. Further studies using total interference reflection fluorescence (TIRF) microscopy demonstrated the direct interaction between Ena/VASP proteins and actin barbed ends {Pasic et al. 2008}. Interestingly, barbed ends can only interact with Ena/VASP when they are not pre-capped by CP, indicating that Ena/VASP proteins possess an anti-capping but not an un-capping activity {Pasic et al. 2008}. In addition, several studies indicate that interaction with profilin effectively increases the anti-capping activity of Ena/VASP and thus heightens actin polymerization {Hansen et al. ; Barzik et al. 2005}. However, this argument has been challenged by a

study showing no effects of profilin on Ena/VASP-mediated barbed-end elongation {Breitsprecher et al. 2008}. This discrepancy may be due to the differences in reagent preparation and storage {Bear et al. 2009}.

Besides modulating actin dynamics, Ena/VASP is capable of regulating the architecture of the actin cytoskeleton. Actin networks can be arranged as a combination of branched and parallel actin filaments. In general, the actin-binding proteins formin and the Arp2/3 complex are responsible for the initiation of linear and branched actin filaments, respectively; other actin-binding proteins, such as Ena/VASP, are able to modify the organization of these resulting actin filaments {Campellone et al. 2010}. In an *in vitro* system, purified Ena/VASP reduced Arp2/3-mediated actin branch formation {Skoble et al. 2001}. Furthermore, platinum replica EM showed that disruption of Ena/VASP targeting to lamellipodia resulted in shorter and more branched actin filaments compared to control cells {Bear et al. 2002}. In contrast, relocalization of Ena/VASP to lamellipodia contributes to longer and less branched F-actin than controls, indicating that Ena/VASP possesses anti-branching activity {Bear et al. 2002}. One may wonder how Ena/VASP proteins regulate not only actin polymerization but also F-actin organization. One possibility is that Ena/VASP competes with the Arp2/3 complex for G-actin monomers, which are essential components for both Ena/VASP-mediated actin polymerization and Arp2/3-directed actin nucleation and branching {Bear et al. 2009}. Another possible explanation for Ena/VASP's anti-branching activity is through its anti-capping effect. This speculation is supported by data showing that increased actin capping results in higher levels of G-actin monomers and, therefore, provides more available G-actin for Arp2/3 to initiate F-actin branching {Akin et al. 2008}. Since Ena/VASP protects actin from capping, the majority of the G-actin supply would be utilized for actin elongation and would leave fewer G-actin monomers for actin branching.

In addition, Ena/VASP proteins have been demonstrated to bundle actin filaments, a role that requires direct binding to F-actin as well as Ena/VASP tetramerization {Bachmann et al. 1999}. Through their anti-branching and F-actin bundling activities, Ena/VASP proteins therefore contribute to the organization of the actin cytoskeleton.

Domain structures and interacting partners — Ena/VASP proteins are composed of three conserved domains: the N-terminal Ena/VASP homology 1 (EVH1) domain, the central proline-rich domain (PRD), and the C-terminal EVH2 region [Figure 6]. Previous studies have shown that Ena/VASP uses these distinctive regions to interact with a variety of proteins. For example, the EVH1 domain provides binding sites for proteins containing proline-rich motifs (D/E)-FPPPP-X(D/E)(D/E) (short for FP4), including the lamellipodia localization protein lamellipodin {Krause et al. 2004}, the focal adhesion molecules zyxin and vinculin {Reinhard et al. 1995; Brindle et al. 1996; Reinhard et al. 1996}, and Rap1-GTP-interacting adaptor molecule (RIAM) {Lafuente et al. 2004}. The PRD region possesses binding sites for SH3- and WW- domain-containing proteins and also allows Ena/VASP to bind to profilin {Reinhard et al. 1995; Gertler et al. 1996; Ermekova et al. 1997; Kang et al. 1997}. Through the interaction with profilin/G-actin complexes, Ena/VASP protects barbed ends from capping proteins more effectively and thus further promotes actin elongation {Barzik et al. 2005}. The EVH2 domain is the major region responsible for actin assembly, and it is comprised of a G-actin-binding (GAB) motif, an F-actin-binding (FAB) motif, and a coiled-coil (Coco) region {Bachmann et al. 1999; Walders-Harbeck et al. 2002; Zimmermann et al. 2002} [Figure 6]. The GAB motif shares close homology with the core sequences of the actin-sequestering protein β4-thymosin and exhibits a higher binding affinity to actin when actin is coupled to profilin {Walders-Harbeck et al. 2002; Chereau et al. 2006; Applewhite et al. 2007}. Interestingly, this motif is also important for targeting Ena/VASP to the tips of filopodia

where barbed ends are localized; however, it is not required for capturing barbed ends of actin filaments as demonstrated in a TIRF-based experiment {Applewhite et al. 2007{Pasic et al. 2008}. The FAB motif is crucial for actin polymerization and F-actin bundling, and it has been shown to be necessary for filopodia formation {Applewhite et al. 2007}{Bachmann et al. 1999}. The extreme C-terminal Coco domain is responsible for the tetramerization of Ena/VASP proteins {Bachmann et al. 1999; Zimmermann et al. 2002}. This region targets Ena/VASP to barbed ends and enhances actin assembly and crosslinking {Pasic et al. 2008}.

It is generally believed that both the EVH1 and EVH2 domains are important for the targeting of Ena/VASP proteins to actin-rich structures. Through direct interaction with actin molecules, the EVH2 region allows Ena/VASP proteins to modulate actin dynamics and rearrangement {Chereau et al. 2006}. The association with profilin-actin complexes via the PRD region promotes actin polymerization {Kang et al. 1997}. Since the GAB motif binds to actin more efficiently in the presence of profilin, it is likely that ATP-bound G-actin released by profilin is delivered to the GAB motif, which then cooperates with the FAB and Coco domains to boost actin assembly {Chereau et al. ; Ferron et al. 2007}.

Phosphorylation — As implied by its name, Ena/VASP can be phosphorylated, specifically by cyclic-nucleotide-dependent kinases PKA, PKG, and PKC [Figure 6] {Halbrugge et al. 1990; Nolte et al. 1991; Chitaley et al. 2004}. Notably, phosphorylation of specific sites within the EVH2 domain negatively modulates the binding affinity of Ena/VASP for actin molecules; this reduces Ena/VASP-mediated actin polymerization and F-actin bundling {Harbeck et al. 2000; Barzik et al. 2005}.

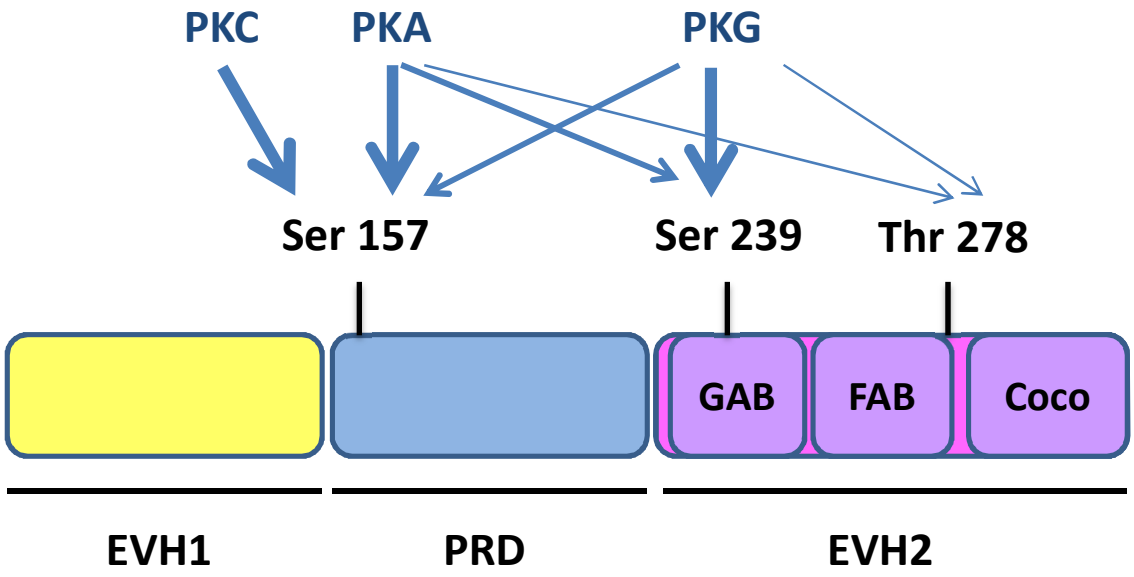


Figure 6. Domain organization of human VASP. VASP contains three conserved domains: the N-terminal Ena/VASP homology 1 (EVH1) domain, the central proline-rich domain (PRD) and the C-terminal EVH2 domain. Within the EVH2 domain, G-actin-binding (GAB), F-actin-binding (FAB) and coiled-coil (Coco) regions are crucial in regulating actin assembly and organization. Phosphorylation sites are indicated. The preferences of PKA, PKG, and PKC for different VASP phosphorylation sites *in vitro* are indicated by the thickness of the arrows.

Role of Ena/VASP proteins in neuronal cells

Proper neuron functions depend on the tight regulation of the dynamics and organization of the actin cytoskeleton {Dent et al. 2003}. At the beginning of development, neuronal cells are born with a spherical shape, and their cell bodies are surrounded by lamellipodia-like protrusions {Barnes et al. 2009; Tahirovic et al. 2009}. Later, initial neurites are sent out from the cell body and develop into axons and dendrites, thereby establishing the structural polarity of neurons. These neurites terminate in growth cones, which are specialized fan-shaped structures that navigate the local environment to steer neurites to their appropriate targets. This path-finding process, coupled with neuronal migration, is driven by guidance cues and allows neurons to migrate from the germinal layer to their final destination {Huber et al. 2003}. This migratory process, in turn, establishes the sophisticated framework of the brain.

Neurite initiation — All Ena/VASP family members are expressed in different regions of the brain during development {Lanier et al. 1999; Goh et al. 2002}. To circumvent their possible overlapping functions, Mena/VASP/EVL triple knockout mice (*mmvvee*) were generated {Kwiatkowski et al. 2007}. The deletion of all Ena/VASP proteins results in embryonic lethality, which occurs between embryonic (E) 16.5 and postnatal (P) 0 and is possibly caused by intra-amniotic hemorrhage, hydrop fetalis, and/or exencephaly {Kwiatkowski et al. 2007}. Moreover, *mmvvee* mice exhibit cobblestone cortex, a condition associated with loss of pial membrane integrity and subsequent neuronal ectopias {Olson et al. 2002}. Similar to Ena/VASP's role in endothelial barriers, the pial membrane defect further underscores Ena/VASP's function in the maintenace of tissue integrity {Furman et al. 2007}. In addition, axon tracts are found largely lost in the developing cortex of *mmvvee* mice {Sur et al. 2005}. Detailed analysis indicates that this defect is caused by a lack of F-actin bundles, which are an essential cytoskeletal

component for the formation of filopodia {Dent et al. 2007; Kwiatkowski et al. 2007}. Because the filopodia that are extended from neuronal lamellipodia later give rise to neurites, it was claimed that Ena/VASP is integral for F-actin bundling and filopodia formation to initiate neuritogenesis.

Neuronal migration — Interestingly, neurons without Ena/VASP expression or proper functions show aberrant positioning in the developing cortex {Goh et al. 2002; Lebrand et al. 2004; Kwiatkowski et al. 2007}. This defect is possibly due to altered migratory properties in neurons. In fact, the Ena orthlog UNC-34 was found in a screen designed to discover mutants with migration defects in canal-associated neurons in *C. elegans* {Forrester et al. 1997}. In this screening analysis, the recessive mutation, which reduces or eliminates UNC-34 gene function, causes modest defects in neuronal migration {Forrester et al. 1997}. However, the formation and morphology of neuronal processes, which are important for migration, seem normal in *mmvvee* mice {Kwiatkowski et al. 2007}. Further examination of the effect of Ena/VASP on neuronal migration in vertebrates is required.

Axon guidance and outgrowth — *In vivo*, neuronal migration and axon formation are concurrent processes {Noctor et al. 2004}. In response to guidance cues, axons extend and move toward their synaptic counterparts; this process is called axonal outgrowth and is led by axonal growth cones. Axonal growth cones are highly motile structures that extend numerous filopodia to explore the surrounding environment. Several different classes of guidance receptors are enriched at the filopodial tips. These receptors possess binding sites for guidance molecules and are able to transduce signals within filopodia to regulate the dynamics and organization of the actin cytoskeleton {Song et al. 1999}. As in neurites, these filopodia are composed of parallel F-actin bundles, and the modulation of actin dynamics controls the extension and

retraction of these protrusions. Ena/VASP has been shown to modulate axon guidance in several different ways {Colavita et al. 1998; Bashaw et al. 2000; Lanier et al. 2000; Yu et al. 2002; Gitai et al. 2003; Lebrand et al. 2004}. In response to the guidance cue Netrin-1, Ena/VASP exhibits increased phosphorylation in conjunction with an increased number and length of filopodia on growth cones, which are important for attractive guidance {Lebrand et al. 2004}. The Netrin-1-induced phosphorylation of Ena/VASP is PKA-dependent and is crucial for growth cones filopodia dynamics {Lebrand et al. 2004}. Intringuingly, the *C. elegans* Ena ortholog UNC-34 has been reported to mediate repulsive guidance in a Netrin-1-independent manner {Yu et al. 2002}. Furthermore, genetic approaches applied in *Drosophila* provide a more direct evidence for the role of Ena/VASP in axon guidance. In these studies, motor axons with Ena mutations fail to move toward their target muscles {Bashaw et al. 2000; Lanier et al. 2000}. However, neutralization of Ena/VASP's function in *Xenopus* does not alter axon guidance {Dwivedy et al. 2007}. Collectively, these results indicate that the effect of Ena/VASP on axon guidance may be species-dependent and could be regulated by different guidance cues and diverse signaling in the growth cones {Drees et al. 2008}.

Hypothesis

Previous studies showed that spine development and synaptic function are modulated by actin dynamics and rearrangement; however, the molecular mechanisms underlying the modulation of the actin cytoskeleton during these processes are still largely unknown. In many studies, VASP has been demonstrated to localize to dynamic actin structures and regulates actin-based cellular processes by promoting actin elongation and rearrangement. We therefore hypothesized that VASP regulates spine

formation and morphology as well as synaptic function via reorganization of the actin cytoskeleton.

CHAPTER II

MATERIALS AND METHODS

Low-density neuronal culture

Low-density hippocampal neurons were prepared and cultured as previously described [Figure 7] {Goslin et al. 1998}. Briefly, hippocampi were isolated from E19 rat brains and incubated with 0.25 % trypsin in Hank's buffered salt solution (HBSS) at 37^0C for 15 min. To dissociate neurons, hippocampal tissues were subjected to continuous pipetting until no chunks of tissue remain. Neurons were then plated onto poly-L-lysine pre-coated glass coverslips at a density of 75,000 cells/mm^2. After 3-4 hours at 37^0C, neurons were attached to the coverslips, which were then transferred neuron-side down to dishes containing a bed of astroglial cells in neuronal medium. To support this "sandwich-based" method during co-culturing, four dots of sterilized paraffin wax were placed at the edge of each coverslips. Neuronal medium is neurobasal media supplemented with B27 and 2mM L-glutamine (GIBCO, Grand Island, NY). Ara-C (5 μM) was added after 3 days cultured *in vitro* to reduce the proliferation of glial cells in neuronal culture.

Primary astroglial cells used to support the growth and survival of low-density neurons were prepared as previous described {Goslin et al. 1998}. Briefly, cerebral hemispheres of one-day-old P1 rat pups were isolated, and the meninges were carefully removed. The tissue was further minced into fine pieces with scissors and dissociated with 2.5% trypsin and 1% DNAse in HBSS. Then, cells were filtered through a 70 μm cell strainer (BD Falcon, Bedford, MA) to remove any undissociated chunks, and

trypsinization was neutralized by the addition of MEM and 10% horse serum (glial medium). Cells were then centrifuged, resuspended, and maintained in medium.

Neuron transfection

Neurons were transfected by a modified calcium phosphate method at day 5-6 in culture, unless otherwise specified {Zhang et al. 2003}. Briefly, 6 µg of plasmid DNA were mixed with 120 µl of 250 mM $CaCl_2$, and then 120 µl of 2X HBS (274 mM NaCl, 9.5 mM KCl, 15 mM glucose, 42 mM HEPES, 1.4 mM Na_2HPO_4, pH 7.05) were added dropwise while the mixture was aerated. The mixture was then immediately added drop wise to neurons in transfection media (50% neuronal medium, 50% glia-conditioned MEM supplemented with 0.1 % albumin and N2). To prevent any stress-elicited action potential during transfection, 0.5 mM kynurenic acids were added into the transfection media. After 45-90 min, a sufficient density of DNA-calcium phosphate precipitates formed, and neurons were washed with 1X HBS and returned to the home dish and maintained in neuronal media containing 0.5 mM kynurenic acid.

Immunocytochemistry

Neurons were fixed at day 11-12 in culture, permeabilized, and stained as previously described {Wegner et al. 2008}. Briefly, neurons were fixed with 4% paraformadehyde (PFA)/4% sucrose in PBS and then permeabilized with 0.2 % Triton X-100 and blocked with 20% goat serum. Primary antibodies were then diluted in 5% goat serum and incubated with neurons for an hour at room temperature or overnight at 4 ^{0}C. After several washes with PBS, secondary antibodies were added and incubated at room temperature for 45 min. After washing, coverslips were then mounted with Aqua

Poly/Mount (Polysciences, Inc., Warrington, PA) or ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). When F-actin staining was required, fluorescently-labeled phalloidin was diluted in 5% goat serum and incubated with neurons at room temperature for 45 min.

Microscopy and image analysis

Neurons were imaged using a Retiga EXi CCD camera (QImaging) on an Olympus IX71 inverted microscope (Olympus, Melville, NY) with a PlanApo 60X OTIRFM objective (NA 1.45). Image acquisition was controlled with MetaMorph software (Molecular Devices, Sunnyvale, CA), which was interfaced with a Lambda 10-2 automated controller (Sutter Instruments). Alexa Fluor 488 and EGFP were imaged with an Endow GFP Bandpass filter cube (excitation HQ470/40, emission HQ525/50, Q495LP dichroic mirror) (Chroma, Brattleboro, VT). For Alexa Fluor 555 or 546, a TRITC/Cy3 cube (excitation HQ545/30, emission HQ610/75, Q570LP dichroic mirror) was used. Alexa Fluor 647 was imaged with a Cy5^{TM} cube (excitation HQ620/60, emission HQ700/75, Q660LP dichroic mirror).

The density of spines and synapses was quantified, beginning within 5 μm of the soma, along primary and secondary dendrites as previously described {Zhang et al. 2005}. The average length of the dendrites analyzed was 60 μm. We define spines as dendritic protrusions that have a bulbous head with an average size of 0.5 μm^2 and that are in contact with presynaptic terminals. In our analyses, dendritic spines ranged in length from 1 to 4 μm.

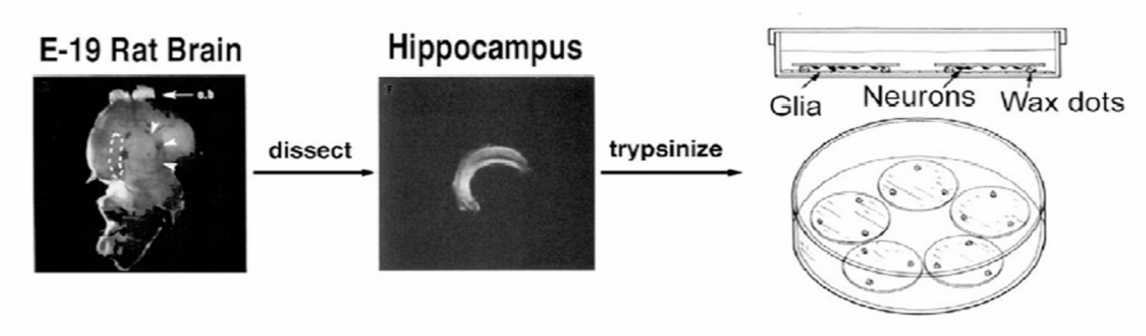


Figure 7. Protocol for culturing rat hippocampal neurons. A hippocampus (dotted line) from the E19 brain is isolated and further trypsinized to dissociate neurons. Dissociated neurons are then plated on coverslips and co-cultured with a bed of glial cells using a “sandwich-based” method. Reprinted from {Goslin et al. 1998}.

CHAPTER III

VASP PROMOTES SPINE FORMATION AND SPINE HEAD ENLARGEMENT THROUGH ITS EVH1 AND EVH2 DOMAINS

Abstract

Dendritic spines are small actin-rich structures that receive the majority of excitatory synaptic input in the brain. The actin-based dynamics of spines are thought to mediate synaptic plasticity, which underlies cognitive processes such as learning and memory. However, little is known about the molecular mechanisms that regulate actin dynamics in spines and synapses. In this study, we show that the multifunctional actin-binding protein VASP regulates the density, size, and morphology of dendritic spines. Knockdown of endogenous VASP by siRNA led to a significant decrease in the density of spines and synapses, while expression of siRNA-resistant VASP rescued this defect. The ability of VASP to modulate spine and synapse formation, maturation, and spine head enlargement is dependent on its actin-binding Ena/VASP homology 2 (EVH2) domain and its EVH1 domain, which contributes to VASP localization to actin-rich structures. Collectively, our results suggest that VASP is critical for spine formation and expansion.

Introduction

Neurons communicate via specialized structures called synapses that are composed of presynaptic and postsynaptic terminals. In excitatory synapses, the

majority of synaptic input takes place on dendritic spines, which are actin-rich structures comprised of a bulbous head and a thin neck connected to dendritic shafts {Gray 1959; Harris et al. 1994}. Dendritic spines play a central role in cognitive processes, and changes in their size, number, and morphology are associated with numerous neurological disorders {Fiala et al. 2002}. Emerging evidence indicates these processes are regulated by polymerization and reorganization of the actin cytoskeleton, pointing to the importance of actin dynamics in modulating synaptic function {Matus et al. 1982; Fischer et al. 1998; Zito et al. 2004}. Alterations in actin remodeling, in turn, are mediated by actin-binding proteins, but the role these proteins play in modulating the development, morphology, and function of spines and synapses is not well understood.

VASP is an actin-binding protein that regulates actin polymerization and bundling via direct interaction with both globular (G) and filamentous (F) actin {Reinhard et al. 1992; Bachmann et al. 1999; Walders-Harbeck et al. 2002}. In non-neuronal cells, VASP localizes to dynamic actin structures, such as focal adhesions, filopodia, and the leading edge of lamellipodia, where it regulates actin-based cellular processes {Rottner et al. 1999; Bear et al. 2000; Bear et al. 2002}. In the nervous system, VASP family proteins are required for proper positioning of neurons and neuritogenesis in the neocortex as well as filopodia formation in cortical and hippocampal neurons {Goh et al. 2002; Lebrand et al. 2004; Dent et al. 2007; Kwiatkowski et al. 2007; Lin et al. 2007}. In addition, loss of VASP family proteins significantly impairs normal brain development, indicating an important role for VASP proteins in the central nervous system {Kwiatkowski et al. 2007}.

VASP contains three conserved domains, including an EVH1 domain, a proline-rich domain (PRD), and an EVH2 domain, that have different roles in VASP function {Gertler et al. 1996}. The EVH1 domain mediates VASP localization to actin-rich structures,

possibly via association with proline-rich proteins {Gertler et al. 1996; Niebuhr et al. 1997; Carl et al. 1999; Bear et al. 2000; Applewhite et al. 2007}. The central PRD contains binding sites for WW- and SH3-domain containing proteins, as well as the G-actin binding protein profilin {Gertler et al. 1995; Reinhard et al. 1995; Gertler et al. 1996; Ermekova et al. 1997; Kang et al. 1997}. The C-terminal EVH2 domain consists of a G-actin binding motif, an F-actin binding domain, and a coiled-coil domain for VASP tetramerization {Bachmann et al. 1999; Walders-Harbeck et al. 2002; Zimmermann et al. 2002}. Like the EVH1 domain, the EVH2 domain contributes to VASP targeting to lamellipodia {Bear et al. 2002; Loureiro et al. 2002}. Moreover, it is involved in bundling F-actin, protecting barbed ends from capping, and mediating filopodia formation {Loureiro et al. 2002; Walders-Harbeck et al. 2002; Barzik et al. 2005; Applewhite et al. 2007}.

Experimental Procedures

Reagents — SV2 monoclonal antibody was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). PSD95 antibodies were purchased from Chemicon (Temecula, CA) and NeuroMAB (Davis, CA). VASP antibody was kindly provided by Frank Gertler (MIT, Boston, MA). Mena antibody (clone 21) was purchased from BD Biosciences (Franklin Lakes, NJ). Alexa Fluor® 546 phalloidin, and β-actin AC-15 monoclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO). GFP antibody, Alexa Fluor® 647 phalloidin, ProLong Gold antifade reagent, Alexa Fluor® 488, 555, and 647 anti-mouse, Alexa Fluor® 488 and 555 anti-rabbit and Alexa Fluor® 680 anti-mouse were from Invitrogen (Carlsbad, CA). IRDye 800 anti-mouse was obtained from Rockland Immunochemicals (Gilbertsville, PA). Aqua Ply/Mount was from Polysciences (Warrington, PA).

Plasmids — VASP cDNA was a generous gift from Jüergen Wehland (Technical University of Braunschweig, Braunschweig, Germany). Full-length human VASP tagged with EGFP was cloned into a neuronal expression vector, generously provided by Freda Miller, that contains a neuronal-specific α1-tubulin promoter {Gloster et al. 1999}. GluR1-GFP was kindly provided by Julius Zhu (University of Virginia, Charlottesville, VA). mCherry cDNA was generously provided by Roger Tsien (University of California, San Diego, CA) Deletion constructs were generated using PCR with the following primers: VASP ΔEVH1 (Δ1-117), forward, 5'-GGTTCCAGATCTCCCCCTCCACCCCCAGCACTTCC-3' and reverse, 5'-GGCCTTCTCGGTCAGGGAGAACCCCGCTTCC-3'; VASP ΔEVH2 (Δ226-380), forward, 5'-GGTTCCGGATCCATGAGCGAGACGGTCATCTGTTCC-3' and reverse, 5'-GGCCTTCTCGAGGCCTGGGGCCCCAGCTCCCC-3'; VASP ΔCoco (Δ336-380), forward, 5'-G-GTTCCGGATCCATGAGCGAGACGGTCATCTGTTCC-3' and reverse 5'-GGCCTTCTCGAGCGTGCAGGGTTGGGTCTCG-3'. Nested PCR methods were used to generate the ΔPRD (Δ118-225) and ΔFAB (Δ260-277) constructs, and the external primers were as follows: forward, 5'-GGTT CCGGATCCATGAGCGAGACGGTCATCTGTTCC-3' and reverse, 5'-GGCCTTCTCGAGTCAGGGAGAACCCCGCTTCC-3'. Internal primers used for ΔPRD were: forward, 5'-GCGTTGGAAGGAGGTGGGCTGGCCGCA-GCTATTGCTGG-3' and reverse, 5'-CCAGCAATAGCTGCGGCCAGCCCACCTCCTTCCAACGC-3'; for ΔFAB were: forward, 5'- GCTGAGAGTGGTCGAAGCGGAACGCAAGTTGGGGAGAAAACC-3' and reverse, 5'-GGTTTTCTCCCCAACTTGCGTTCCGCTTCGACCACTCTCAGC-3'. VASP phosphomimetic and unphosphorylatable mutants were generated using the site-directed mutagenesis kit (Stratagene, La Jolla, LA) with the following primers: S157A, forward, 5'-GCACATAGAGCGCCGGGTCGCCAATGCAGGAGGC-3' and reverse, 5'-GCCTCCTGCATTGGCGACCCGGCGCTCTATGTGC-3'; S157D, forward, 5'-CATAGAGCGCCGGGTCGACAATGCAGGAGGCC-3' and reverse, 5'-GGCCTCCTGCATTGTCGACC

CGGCGCTCTATG-3'; S-239A, forward, 5'-CTCAGGAAAGTCGCCAAGCAGGAGGA GGCC-3' and reverse, 5'-GGCCTCCTCCTGCTTGGCGACTTTCCTGAG-3'; S239D, forward, 5'-CTCAGGAAAGTCGACAAGCAGGAGGAGGCC-3' and reverse, 5'-GGCCTC CTCCTGCTTGTCGACTTTCCTGAG-3'; T-278A, forward, 5'-GGAGAAGGAAAGCCG CGCAAGTTGGGGAGAAAAC-3' and reverse, 5'-GTTTTCTCCCCAACTTGGCGGCTT TCCTTCTCC-3'; T-278E, forward, 5'-CCGGAGAAGGAAAGCCGAGCAAGTTGGGGAG AAAACC-3' and reverse, 5'-GGTTTTCTCCCCAACTTGCTCGGCTTTCCTTCTCCGG-3'. To destroy the G-actin-binding motif, two consecutive point mutations were generated at R236E-K237E with the following primers: forward, 5'-GCTGGAGCCAAACTCGAA GAAGTCAGCAAGCAGG-3' and reverse, 5'-CCTGCTTGCTGACTTCTTCGAGTTTGG CTCCAGC-3'. VASP siRNA constructs were generated by ligating 64-mer sense and antisense oligonucleotides into pSUPER vector as previously described {Zhang et al. 2008}. The VASP siRNA oligos contained the following 19-nucleotide target sequences: VASP #1, 5'-TGAAAGAGGAAATAATCGA-3' and VASP #2, 5'-TTGTGGAAGAGGTGC GGAA-3'. Scrambled sequence, 5'-CAGTCGCGTTTGCGACTGG-3'.

Cell culture and transfection — Low-density hippocampal neurons were prepared and cultured as previously described {Goslin et al. 1998}. Briefly, neurons were plated at a density of 75,000 cells/mm^2 and transfected by a modified calcium phosphate method at day 5-6 in culture, unless otherwise specified {Zhang et al. 2003}. Human embryonic kidney 293T (HEK-293T) cells and rat 2 fibroblasts (R2Fs) (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. HEK-293T and R2Fs were transfected with Lipofectamine 2000 (Invitrogen) and Amaxa kits (Lonza Cologne, Germany), respectively, according to the manufacturer's instructions.

Cell lysis and western blot analysis — Cells were lysed in lysis buffer (25 mM Tris-Cl, pH 7.5, 137 mM NaCl, 10% Glycerol, 2 mM EDTA, 1% NP40) with the addition of protease inhibitor (Sigma, St. Louis, MO) and spun for 5 min at 13,000 xg to remove undissolved debris. Samples were then mixed with Laemmli sample buffer (312.5 mM tris-base, 50% glycerol, 10% SDS, 0.035% bromophenol blue) and 10 mM DTT and boiled for 10 min. 30 μg of protein samples were loaded into 8% SDS-PAGE gel, transferred onto a nitrocellulose membrane and subjected to a Western blot analysis using the Odyssey system (Li-COR, Lincoln, NE). Briefly, membranes were blocked for 1 hour with 1% BSA/TBST and probed for primary antibodies overnight at 4 ^{0}C. After several washed with TBST, secondary antibodies are applied and incubated for 45 min at room temperature. After washed with TBST, signals were detected and quantified using Odyssey 3.0 software.

Immunocytochemistry and image analysis — Neurons were fixed at day 11-12 in culture, permeabilized, and stained as previously described {Wegner et al. 2008}. To simultaneously stain for endogenous VASP and PSD95, neurons were fixed with cold 10% formalin for 15 min at room temperature, permeabilized and stained as previously described {Wegner et al. 2008}. The spine/shaft ratio was calculated by measuring the background subtracted fluorescent intensity in individual spines and an equivalent area in the neighboring shaft. To analyze the number of spines, the images were zoomed in 200%, and the edges of the dendrites were outlined using Metamorph^{TM} software (Molecular Devices, Sunnyvale, CA). Spines were defined as a bulbous protrusion in contact with the presynaptic marker SV2 and were manually counted. The number of spines was then divided by the length of chosen dendrites to show spine density. Quantification of synapse density is obtained using SV2 staining using a similar approach. Statistical analyses were performed using a Student's *t*-test.

Results

VASP is concentrated in dendritic spines and excitatory synapses. Ena/VASP proteins are highly expressed in the brain and in hippocampal pyramidal neurons, which mediate excitatory synaptic connections via dendritic spines {Aszodi et al. 1999; Lanier et al. 1999; Kwiatkowski et al. 2007}. This led us to hypothesize that VASP plays a role in regulating spine and synapse development. To begin to test this hypothesis, we examined the localization of endogenous VASP with synaptic markers in low density cultures of hippocampal neurons. Endogenous VASP co-localized with the synaptic vesicle protein SV2 as well as the excitatory postsynaptic density protein PSD95 [Fig. 8*A*]. Quantification showed that approximately 85% of the VASP puncta co-localized with SV2 and PSD95 clusters [Fig. 8*C*], indicating that VASP is enriched in spines and excitatory synapses. Like endogenous VASP, GFP-VASP is concentrated in spines and synapses with about 85% of GFP-VASP puncta co-localizing with SV2 and PSD95 clusters [Fig. 8*B*,*D*]. To confirm the enrichment of GFP-VASP to spines, we measured the ratio of the fluorescent intensity in spines to neighboring shafts from GFP-VASP expressing neurons and normalized it to that observed in neurons expressing GFP. Indeed, the ratio of the fluorescent intensity was significantly enhanced in GFP-VASP expressing neurons [Fig. 8*E*], indicating that GFP-VASP is enriched in spines. Additionally, these results show that GFP-VASP localizes similarly to the endogenous protein and is a valid marker for examining VASP function in spines and synapses.

VASP regulates the formation of spines and synapses. To study the function of VASP in spines and synapses, GFP-VASP was expressed at relatively low levels, about 4-fold over endogenous [Fig. 9], in neurons. Expression of GFP-VASP resulted in a 35% increase in the number of spines compared with control neurons, as determined by GFP fluorescence [Fig. 10*A*,*B*]. Similar results were obtained when spine number was

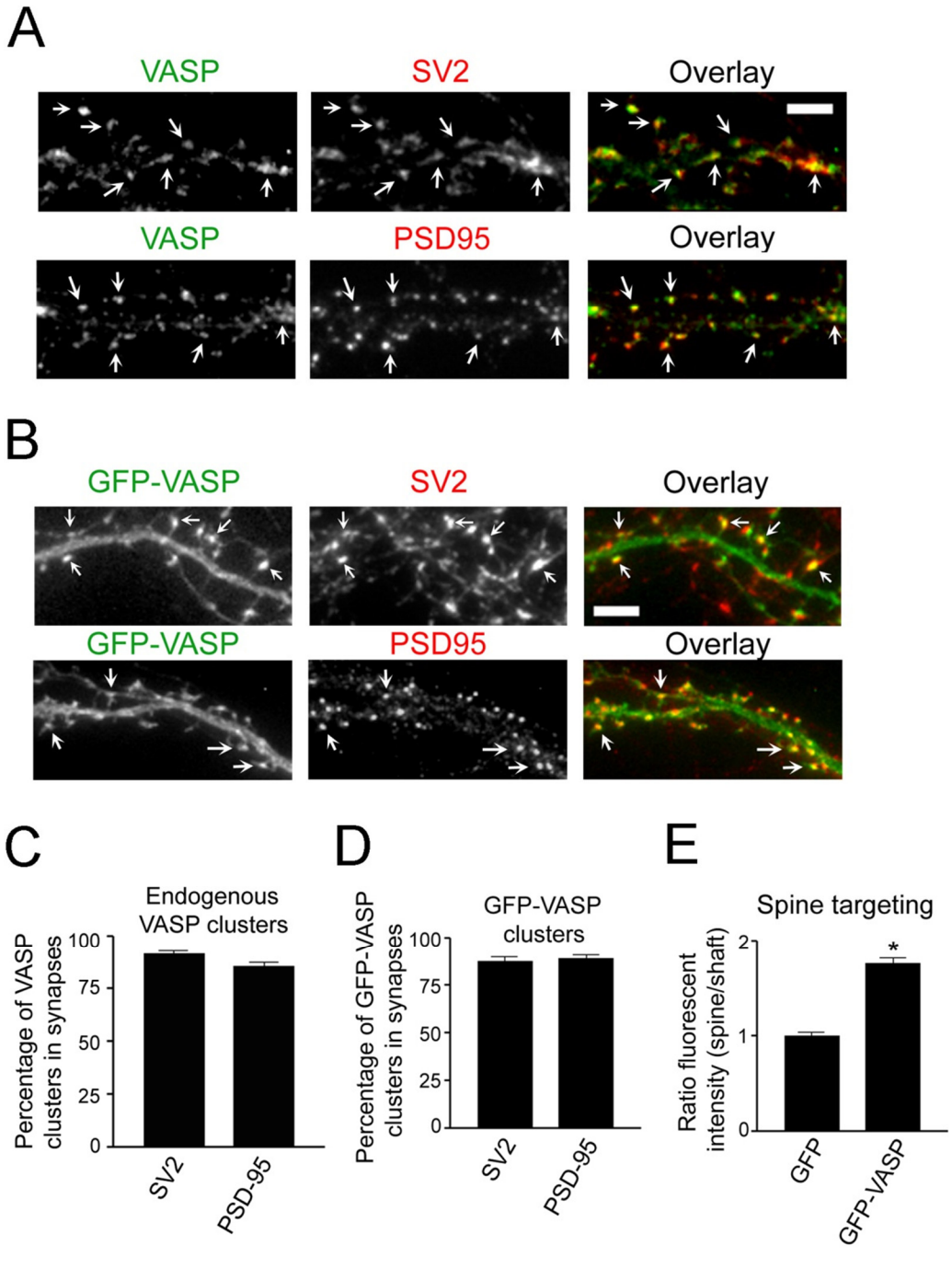

A
VASP
SV2
Overlay
VASP
PSD95
Overlay
B
GFP-VASP
SV2
Overlay
GFP-VASP
PSD95
Overlay
C
Endogenous VASP clusters
Percentage of VASP clusters in synapses
100
75
50
25
0
SV2
PSD-95
D
GFP-VASP clusters
Percentage of GFP-VASP clusters in synapses
100
75
50
25
0
SV2
PSD-95
E
Spine targeting
Ratio fluorescent intensity (spine/shaft)
2
1
0
*
GFP
GFP-VASP

Figure 8. VASP localizes to dendritic spines and synapses. *A,* Hippocampal neurons were co-immunostained at day 12 in culture for endogenous VASP and the synaptic markers SV2 (*upper panels*) and PSD95 (*lower panels*). Endogenous VASP accumulated in puncta that co-localized with the synaptic markers (*Overlays, right panels, arrows*). Bar, 5 μm. *B,* Neurons were transfected with GFP-VASP at day 5 in culture, fixed and immunostained for SV2 (*upper panels*) and PSD95 (*lower panels*) at day 12. GFP-VASP localized in puncta with SV2 and PSD95 (*Overlays, right panels, arrows*). *C,D,* Quantification of the percentage of endogenous VASP *(C)* and GFP-VASP *(D)* co-localizing with SV2 and PSD95. Error bars represent S.E.M. for at least 20 dendrites. *E,* The ratio of the fluorescent intensity in spine heads to neighboring shafts for GFP-VASP expressing neurons was normalized to that observed in control neurons expressing GFP. Asterisks indicate $p < 0.0001$.

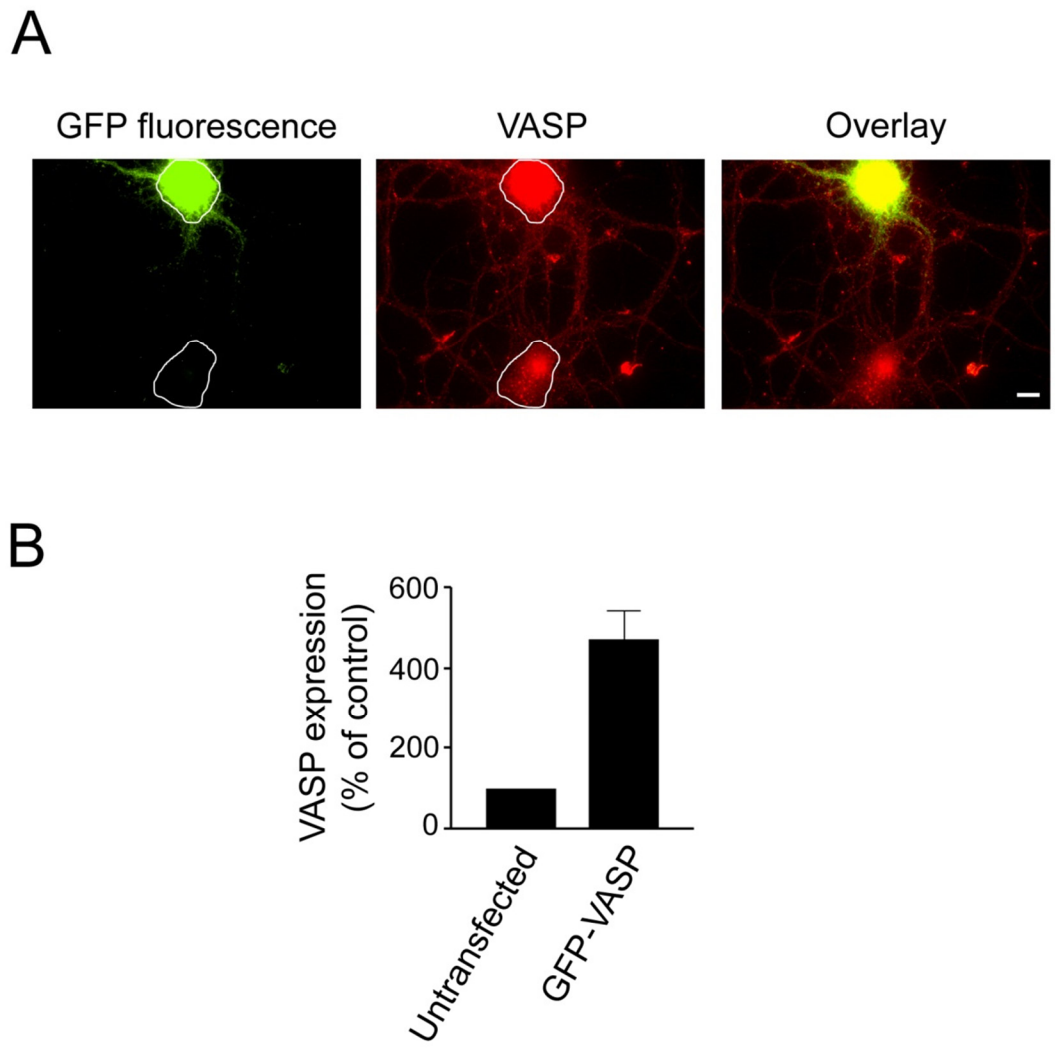


Figure 9. GFP-VASP expression level in cultured hippocampal neurons. *A,* Hippocampal neurons were transfected with GFP-VASP at day 5 in culture, fixed and immunostained for VASP at day 11. The cell body of a transfected and neighboring untransfected neurons are outlined. Bar, 10 μm. *B,* The amount of VASP was quantified by measuring the average fluorescent intensity in untransfected neurons and transfected neurons expressing GFP-VASP. Error bars represent S.E.M. for 24-26 neurons from three independent experiments. Asterisks indicate $p < 0.0001$.

assessed by staining with phalloidin [Fig. 10*A,B*], which binds to F-actin and can be used to visualize dendritic spines {Allison et al. 1998; Fischer et al. 1998}. A 40% increase in synaptic density, as determined by staining for SV2 and PSD95, was observed in neurons expressing GFP-VASP compared with GFP expressing controls [Fig. 10*A,B*]. Even though VASP family proteins were reported to influence neurite outgrowth {Dent et al. 2007; Kwiatkowski et al. 2007}, it is unlikely that the VASP effects we observe on spine and synapse formation are due to neuritogenesis. This process occurs predominately during days 1-4 in culture {Goslin et al. 1998} and we did not transfect neurons with GFP-VASP until day 5 in culture. However, to address this concern, we transfected neurons with GFP-VASP at day 10 in culture, which is a time when neurites have already reached sufficient length to form synapses, and examined the effect of VASP on spines and synapses. Consistent with our previous results, expression of VASP led to 34.8 ± 3.8% (n=20) increase in spine density. Collectively, these results suggest that VASP is critical for the regulation of spine and synapse formation. Since spines appeared to be larger in GFP-VASP expressing neurons compared with controls [Fig. 10*C*], we quantified the spine head area and length of spines. Expression of GFP-VASP resulted in a 1.5-fold increase in spine head area, but did not significantly affect spine length, when compared with GFP expressing neurons [Fig. 10*D*], suggesting an important role for VASP in regulating spine head enlargement.

To further show that VASP regulates spine development, we generated two small interfering RNA (siRNA) constructs to knock down endogenous expression of VASP. The VASP siRNAs knocked down VASP expression by approximately 70% in rat 2 fibroblasts (R2Fs) compared with pSUPER empty vector or a scrambled siRNA [Fig. 11*A*]. The siRNAs did not affect expression of Mena, another VASP family member, or other actin-binding proteins, such as N-WASP [Fig. 12], indicating their specificity for

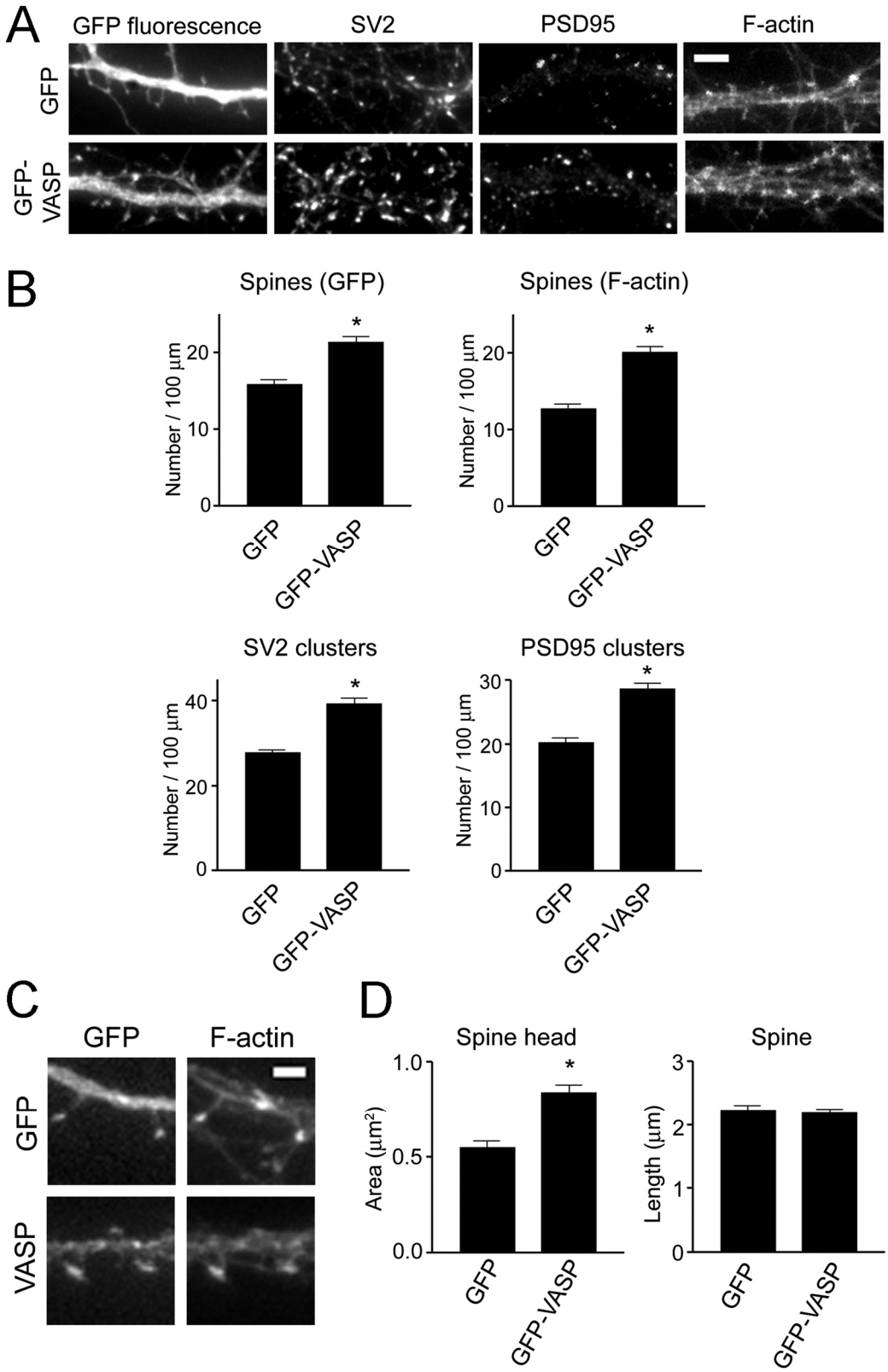

A
GFP fluorescence
SV2
PSD95
F-actin
GFP
GFP-VASP
B
Spines (GFP)
Spines (F-actin)
SV2 clusters
PSD95 clusters
Number / 100 µm
GFP
GFP-VASP
C
GFP
F-actin
VASP
D
Spine head
Spine
Area (µm²)
Length (µm)

Figure 10. VASP regulates spine and synapse formation and promotes spine head enlargement. *A,* Neurons were transfected with GFP or GFP-VASP and stained for SV2, PSD95, and F-actin (phalloidin). Bar, 5 μm. *B,* Quantification of SV2 and PSD95 clusters and spine density as determined by GFP fluorescence and F-actin staining (phalloidin) is shown for GFP and GFP-VASP expressing neurons. *C,* Higher magnification images of dendritic spines, visualized by GFP fluorescence and F-actin staining (phalloidin), from GFP and GFP-VASP expressing neurons. Bar, 2 μm. *D,* Quantification of spine length and area in GFP and GFP-VASP expressing neurons is shown. Error bars represent S.E.M. for 40-50 dendrites *(B)* or 100 spines *(D)* from three separate experiments. Asterisks indicate $p < 0.0001$.

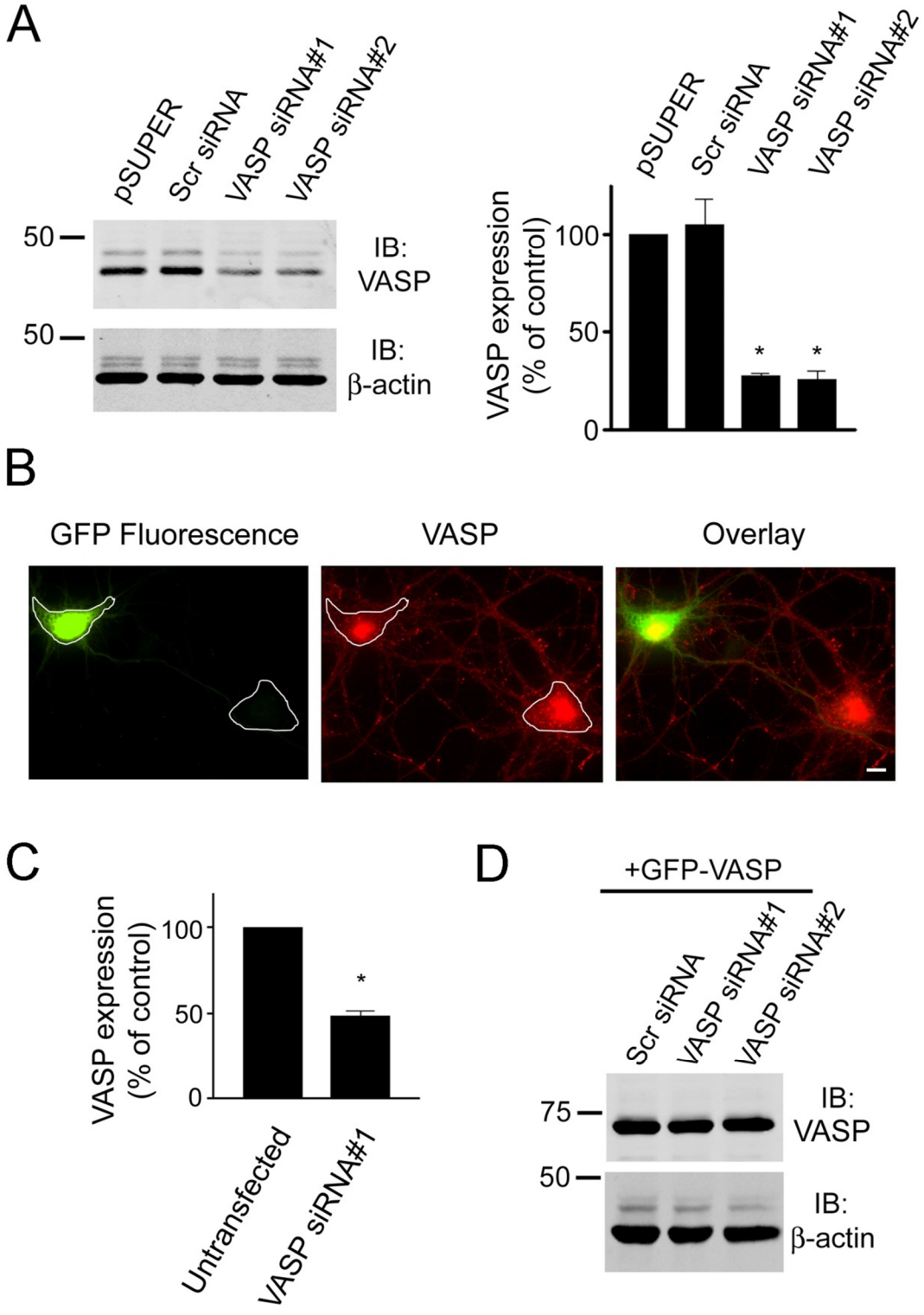

A
pSUPER
Scr siRNA
VASP siRNA#1
VASP siRNA#2
50
IB:
VASP
50
IB:
β-actin
VASP expression
(% of control)
100
50
0
*
*
B
GFP Fluorescence
VASP
Overlay
C
VASP expression
(% of control)
100
50
0
*
Untransfected
VASP siRNA#1
D
+GFP-VASP
Scr siRNA
VASP siRNA#1
VASP siRNA#2
75
IB:
VASP
50
IB:
β-actin

Figure 11. VASP siRNA knocks down endogenous expression of the protein. *A,* Cell lysates from R2Fs transfected with VASP siRNAs, pSUPER empty vector, or a non-silencing scrambled siRNA (*Scr siRNA*) were immunoblotted for VASP and β-actin (loading control). Quantification of blots from four separate experiments is shown (*right panel*). *B,* Hippocampal neurons were transfected with GFP and VASP siRNA at day 5 in culture, fixed and immunostained for VASP at day 11. The cell body of a transfected and neighboring untransfected neurons are outlined. Bar, 10 μm. *C,* The amount of VASP was quantified by measuring the fluorescent intensity in untransfected neurons and transfected neurons expressing VASP siRNA. Error bars represent S.E.M. for 24-26 neurons from three independent experiments. *D*, Cell lysates from R2Fs co-transfected with human GFP-VASP and scrambled siRNA (*Scr siRNA*) or VASP siRNAs were blotted for VASP and β-actin (loading control). For panels A,C, asterisks denote a statistically significant difference compared with pSUPER transfected cells. Error bars represent S.E.M. (*, $p < 0.0001$).

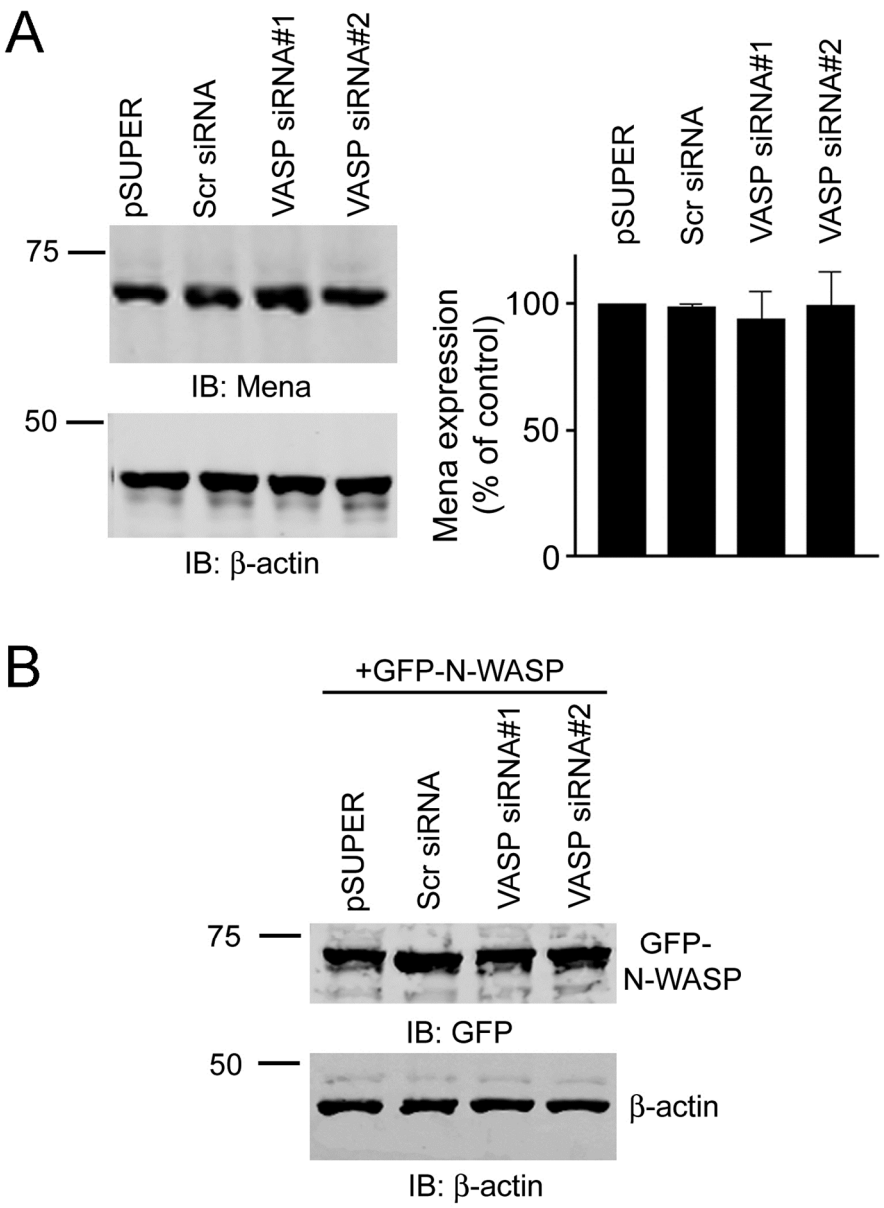


Figure 12. Specificity of VASP siRNAs. *A,* Cell lysates from R2Fs transfected with VASP siRNAs, pSUPER empty vector, or scrambled siRNA (*Scr siRNA*) were immunoblotted for Mena and β-actin (loading control). Quantification of blots from three separate experiments is shown (*right panel*). Error bars represent S.E.M. *B,* HEK-293T cells were co-transfected with GFP-N-WASP and either VASP siRNAs, pSUPER empty vector, or scrambled siRNA. Cell lysates were immunoblotted for GFP and β-actin (loading control).

VASP. The VASP siRNAs were similarly effective in neurons, decreasing endogenous VASP expression by about 55% [Fig. 11*B*,*C*]. Transfection of neurons with VASP siRNA resulted in a significant decrease in the number of spines and synapses compared with control cells expressing pSUPER empty vector or scrambled siRNA [Fig. 13]. Since our VASP siRNAs were specifically designed against the rat VASP sequence, they should not affect expression of human VASP due to several nucleotide mismatches. To confirm this, we expressed human GFP-VASP with VASP siRNA. As shown in Fig. 11*D*, VASP siRNA did not affect expression of GFP-VASP, which allowed us to perform "rescue" experiments in neurons. Expression of GFP-VASP in siRNA knockdown neurons rescued the siRNA-mediated defect on spines and synapses [Fig. 13]. Thus, these results show that the defect is due to loss of endogenous VASP and indicate an important role for VASP in regulating spine and synapse formation.

The EVH domains are necessary for VASP recruitment and function in spines and synapses. To identify the region of VASP that mediates its localization and function in spines and synapses, we generated various deletion constructs and expressed them as GFP fusion proteins [Fig. 14*A*]. The relative expression level of all of the deletion constructs was similar to that observed with full-length GFP-VASP as determined by immunoblot analysis [Fig. 14*B*]. When either the EVH1 or EVH2 domain was deleted (ΔEVH1 or ΔEVH2), VASP failed to localize to spines [Fig. 14*A*] while deletion of the PRD (ΔPRD) domain did not significantly affect VASP localization to spines [Fig. 14*A*]. The central role of the EVH1 and EVH2 domains in targeting VASP to spines suggested that these domains may also be important for VASP function in the development of spines and synapses. Indeed, deletion of either the EVH1 or EVH2 domain of VASP significantly impaired spine and synapse formation [Fig. 15]. In neurons expressing ΔEVH1- or ΔEVH2-VASP, the number of spines was decreased by 54% and 68%,

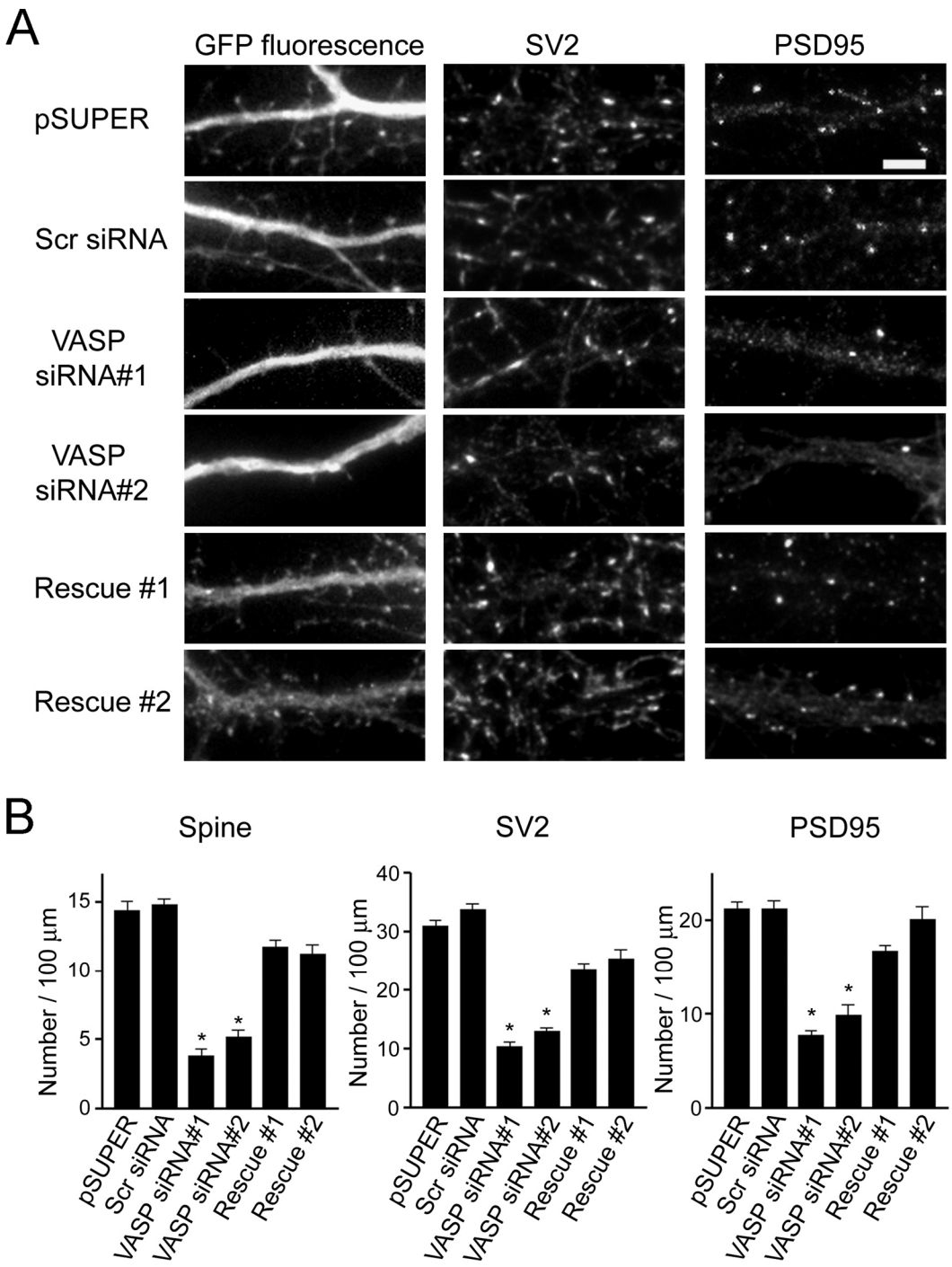

A
GFP fluorescence
SV2
PSD95
pSUPER
Scr siRNA
VASP siRNA#1
VASP siRNA#2
Rescue #1
Rescue #2
B
Spine
SV2
PSD95
Number / 100 µm
0
5
10
15
20
30
40
*
pSUPER
Scr siRNA
VASP siRNA#1
VASP siRNA#2
Rescue #1
Rescue #2

Figure 13. Knockdown of endogenous VASP inhibits the formation of spines and synapses. *A,* Neurons were co-transfected with GFP and either pSUPER empty vector, scrambled siRNA (*Scr siRNA*), or VASP siRNAs at day 6 in culture, fixed and stained with synaptic markers at day 12. To show that the siRNA-induced defect on spines and synapses was due to endogenous loss of VASP, neurons were co-transfected with human GFP-VASP and VASP siRNA#1 or VASP siRNA#2 (*lower panels, labeled "Rescue#1" and "Rescue#2"*). Bar, 5 μm. *B,* Quantification of spine and synaptic density (*SV2 and PSD95 clusters*) in neurons transfected with the indicated constructs is shown. Error bars represent S.E.M. for 40-50 dendrites from at least three separate experiments (*, p <0.0001). Asterisks denote a statistically significant difference compared with pSUPER transfected cells.

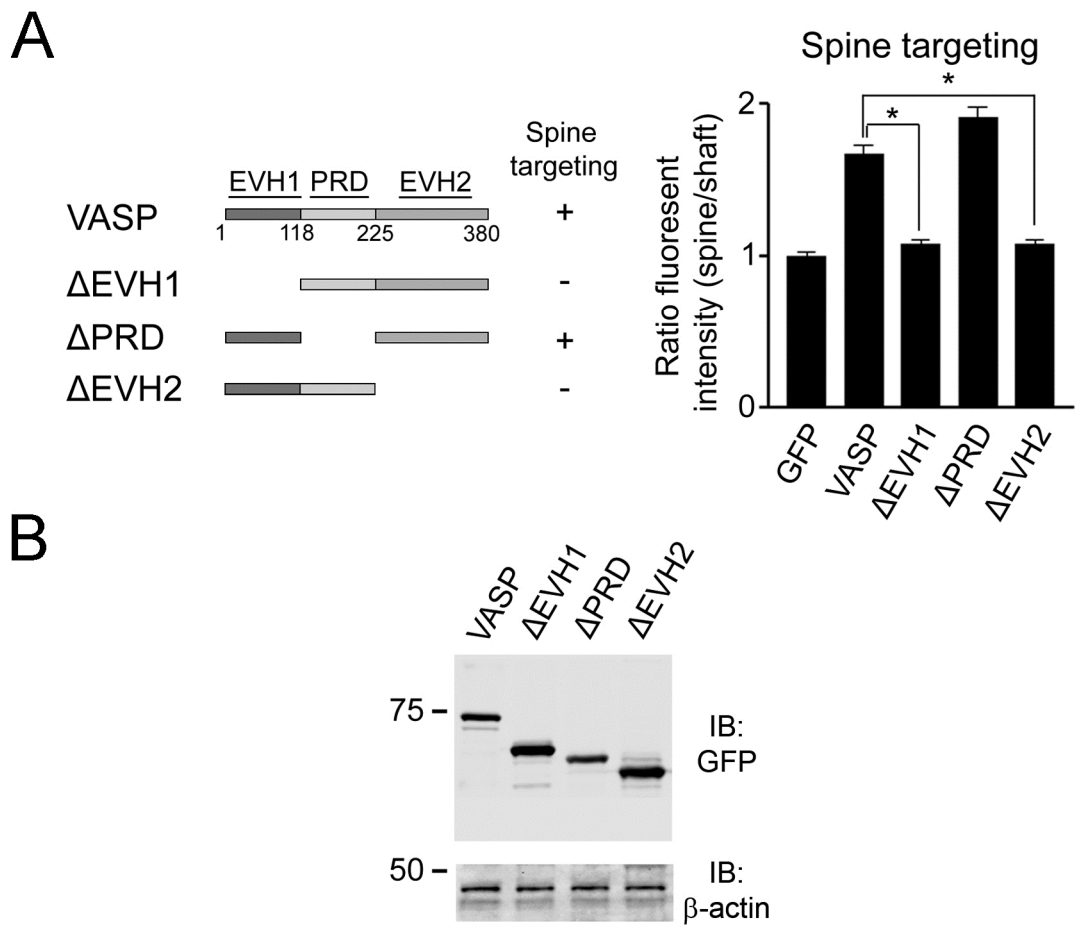


Figure 14. Localization and expression level of VASP deletion mutants. *A,* Domain structure of full-length VASP is shown. EVH1, PRD, and EVH2 domains are indicated. A schematic diagram of VASP deletion constructs is shown. The deletion mutants that localize to spines are indicated with a "+". The ratio of the fluorescent intensity in spine heads to neighboring shafts was quantified in neurons expressing the indicated constructs and normalized to control neurons expressing GFP (*right panel*). *B*, Lysates from HEK-293T cells transfected with the indicated constructs were blotted for GFP and β-actin (loading control). For panel *A*, error bars represent S.E.M. for 100 spines from three separate experiments (*, p <0.0001).

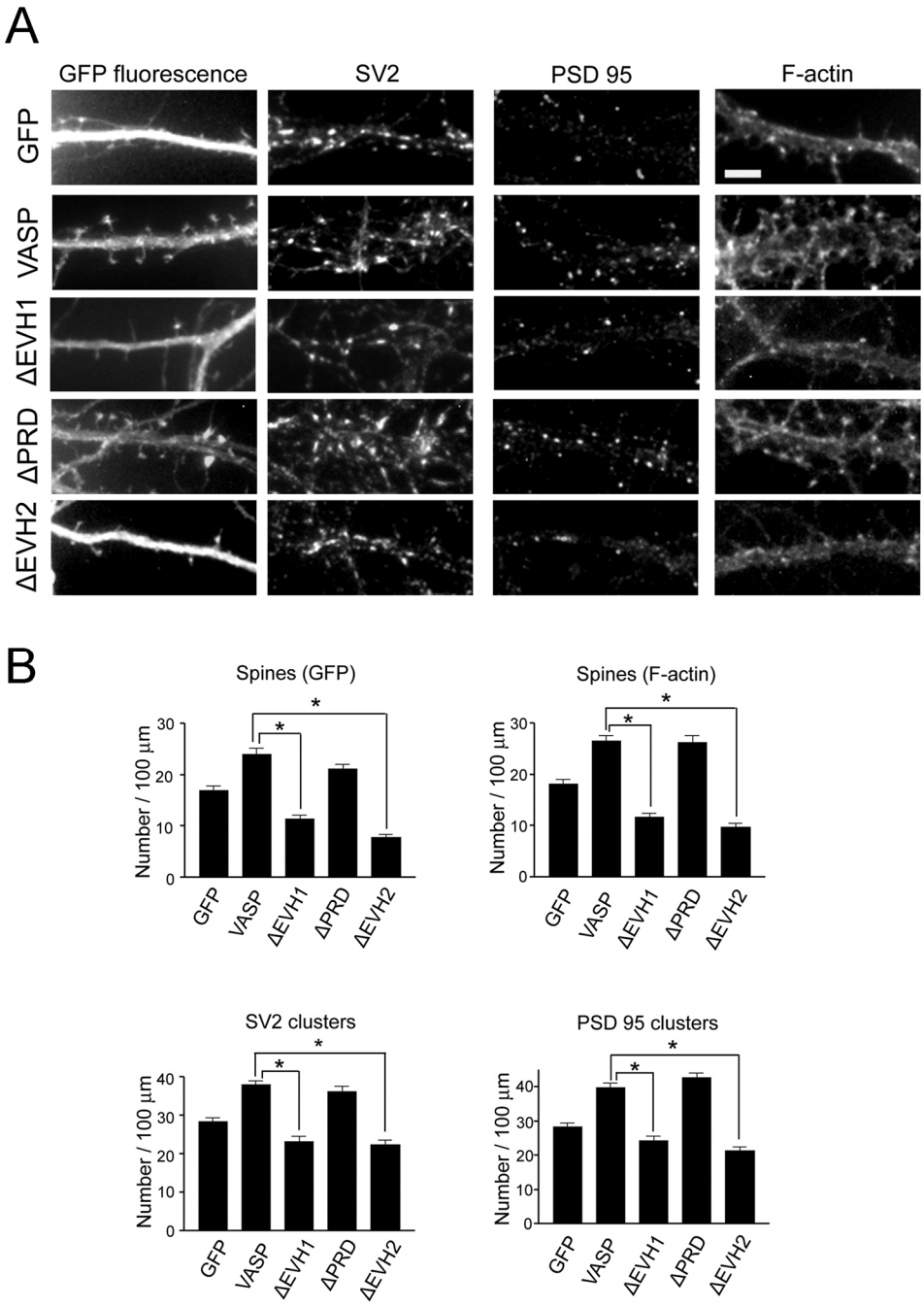


Figure 15. The EVH1 and EVH2 domains of VASP promote spine and synapse formation. *A,* Neurons were transfected with the indicated constructs and stained for SV2, PSD95, and F-actin (phalloidin). Bar, 5 µm. *B,* Quantification of synaptic density (*SV2 and PSD95 clusters*) and spine number by GFP fluorescence and F-actin staining from transfected neurons. For panel *B*, error bars represent S.E.M. for 40 dendrites from three separate experiments (*, p <0.0001).

respectively, compared with full-length VASP [Fig. 15]. Moreover, expression of ΔEVH1- or ΔEVH2-VASP resulted in a significant decrease in the number of SV2 and PSD95 clusters compared with full-length VASP [Fig. 15]. In contrast, the number of spines and synapses in ΔPRD-VASP expressing neurons was comparable to that observed in neurons expressing full-length VASP [Fig. 15].

Since VASP also promotes spine head enlargement, we determined which domains of VASP are critical for this process. The spine head area in neurons expressing VASP mutants lacking either the EVH1 or EVH2 domain was reduced compared to full-length VASP [Fig. 16], indicating the importance of these domains in promoting spine head enlargement. In contrast, the PRD domain was not necessary for VASP-induced enlargement of spines heads [Fig. 16]. Collectively, these results indicate the EVH1 and EVH2 domains of VASP regulate spine/synapse formation, targeting, and spine head enlargement.

In light of the importance of the EVH2 domain in these processes, we further dissected the role of EVH2 domain in more detail using VASP constructs with the deletions in the FAB (ΔFAB) and Coco (ΔCoco) domains and the mutation in the GAB motif (GABmt). Two basic amino acids in the extreme C-terminus of GAB motif is mutated to abolish its binding to G-actin monomers {Walders-Harbeck et al. 2002}. Interestingly, we found that deletion of either the FAB or the Coco domain of VASP impaired the formation of spines and synapses [Fig. 17]. In neurons expressing ΔFAB- or ΔCoco-VASP, the number of spines was decreased by 56.4% and 55.6%, respectively, compared with full-length VASP [Fig. 17]. Moreover, expression of ΔFAB- or ΔCoco-VASP also resulted in a significant decrease in the number of SV2 and PSD95 clusters compared with full-length VASP [Fig. 17]. In contrast, the mutation of GAB motif

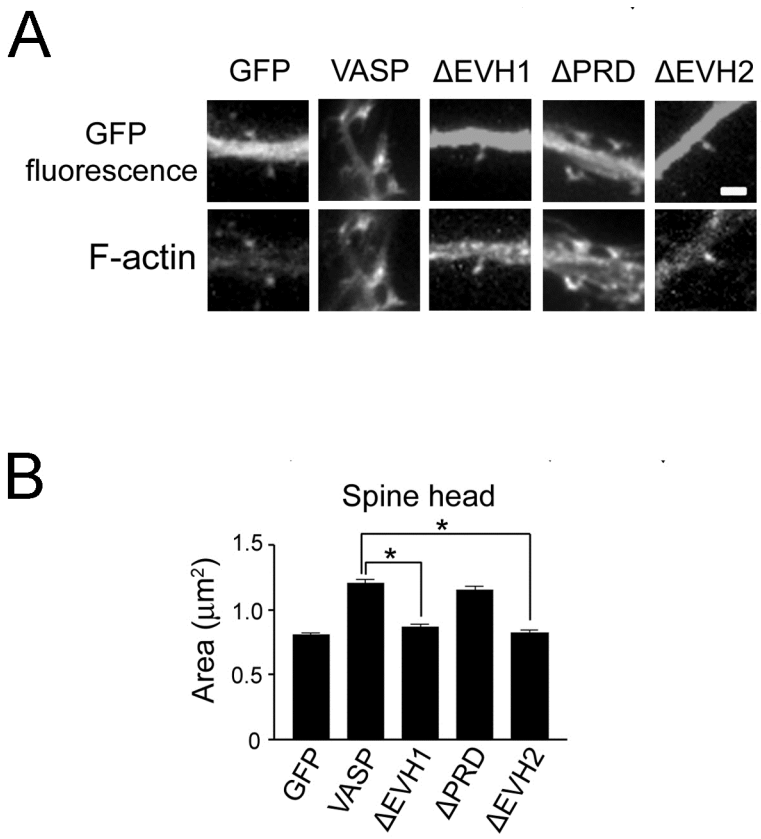


Figure 16. The EVH1 and EVH2 domains of VASP promote spine head enlargement. *A,* Higher magnification images of dendritic spines from neurons expressing GFP, GFP-VASP, or the indicated VASP deletion mutants. Bar, 2 μm. *B,* Quantification of spine head area in neurons expressing the constructs. For panel B, error bars represent S.E.M. for 100 spines from three separate experiments (*, $p < 0.0001$).

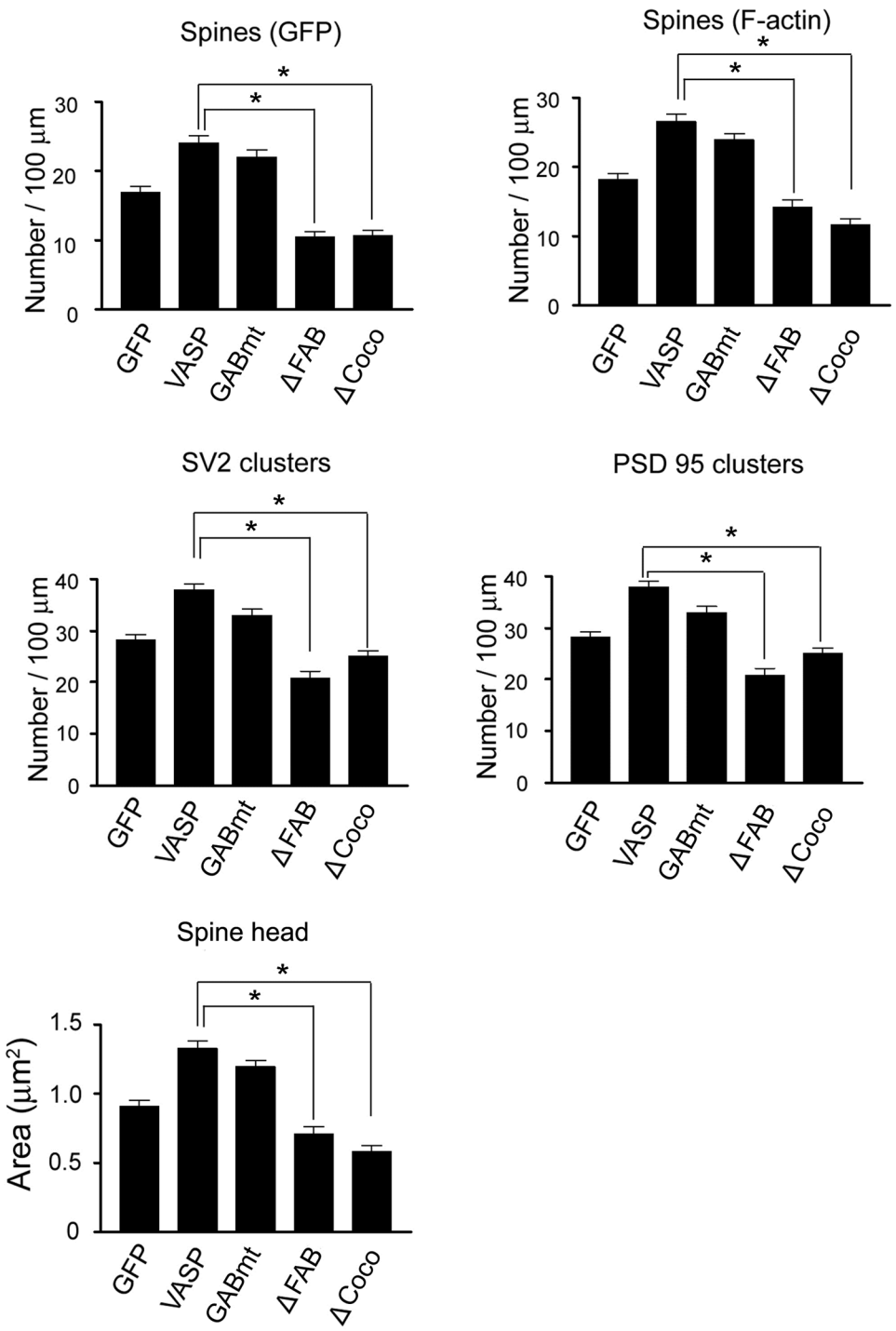


Figure 17. F-actin-binding (FAB) and coiled-coil (Coco) domains, but not G-actin binding (GAB) region are crucial for spine and synapse formation as well as spine head enlargement. Quantification of spine density and synapse density (SV2 clusters, PSD 95 clusters) as well as spine head size in neurons expressing indicated constructs is shown. Error bars represent S.E.M. for 100 spines from three separate experiments (*, p <0.0001).

did not result in a significant difference in the density of spines and synapses [Fig. 17]. These results indicate that both FAB and Coco domains, which are critical for actin assembly and F-actin bundling, play an important role in the development of spines and synapses.

VASP phosphorylation levels do not influence spine development. Previous studies have demonstrated that VASP can be phosphorylated by PKA, PKG, and PKC [Fig. 6] {Halbrugge et al. 1990; Nolte et al. 1991; Chitaley et al. 2004}. Phosphorylation reduces the effects of VASP on actin polymerization and F-actin bundling, modulating the role of VASP in actin-based cellular events {Harbeck et al. 2000; Barzik et al. 2005}. Interestingly, all of these kinases are important regulators for spine development and synaptic plasticity {Arancio et al. 1995; Arancio et al. 2001; Sheng et al. 2002; Serulle et al. 2007}. To examine the possible role for VASP phosphorylation in spine development, we generated a series of single unphosphorylatable and phosphomimetic mutations for individual serine or threonine residues (Ser-157, Ser-239, Thr-278) of VASP. Surprisingly, we did not observe any significant effect of these mutations on the formation of spines and synapses or on the sizes of spine heads. However, we cannot rule out the possibility that each VASP phosphorylation site partially contributes to spine development; therefore, we are not able to observe a defect if only a single mutation is introduced.

Discussion

While emerging evidence points to the importance of the actin cytoskeleton in spine development and plasticity, the underlying molecular mechanisms that regulate actin dynamics in spines and synapses still remain largely unknown. To address this

issue, we chose to study the role of a multi-functional ABP, VASP, in spine formation and morphological changes. Our results show that VASP promotes the formation of dendritic spines and synapses and induces enlargement of spine heads [Fig. 10]. The EVH2 domain, which binds G- and F-actin, is necessary for synaptic targeting of VASP as for VASP's regulation of spine head size and density [Figs. 14-16]. The EVH1 domain also contributes to VASP localization and function in spines and synapses, but the PRD region does not appear to be necessary in this regard [Figs. 14-16].

Knockdown of endogenous VASP using two distinct siRNA constructs led to a significant reduction in the spine and synaptic density [Fig. 13]. The VASP siRNAs did not target another VASP family member, Mena, or other related actin-binding proteins, such as EVH1-containing N-WASP [Fig. 12]. These data strongly suggest that the siRNAs are specific for VASP and validate the use of this approach to alter expression of the endogenous protein. Moreover, the siRNAs specifically target the rat VASP sequence and do not affect expression of human VASP [Fig. 11]. Indeed, expression of human VASP in siRNA knockdown neurons rescued the spine and synapse defects, which further suggests that the phenotypic changes in these structures are specifically due to the loss of endogenous VASP [Fig. 13].

Our results raise the question as to how the EVH1 and EVH2 domains contribute to VASP targeting and function in spines. EVH1 domains are found in other postsynaptic proteins belonging to the Homer family where they are thought to serve as localization modules {Tu et al. 1998; Prehoda et al. 1999}. This domain is structurally similar to the pleckstrin homology (PH) domain and could potentially mimic the membrane-targeting function of the PH domain to recruit VASP to spines {Fedorov et al. 1999; Prehoda et al. 1999}. Alternatively, VASP may be recruited to spines by yet to be identified EVH1-binding proteins. The EVH2 domain mediates F-actin bundling through its ability to bind

actin via FAB region and promotes tetramerization of VASP via the Coco domain {Ahern-Djamali et al. 1998 103; Bachmann et al. 1999}. Deletion of the FAB and Coco regions, which are within the EVH2 domain, impairs spine development and spine head expansion, suggesting that VASP may regulate these processes through its F-actin bundling activity [Fig. 17].

The PRD region of VASP binds to the actin polymerizing protein profilin II, which targets to spine heads upon neuronal activity where it is thought to stabilize spine morphology {Ackermann et al. 2003}. It has been proposed that proline-rich motifs in proteins such as VASP serve to recruit profilin to the plasma membranes of spine heads {Ackermann et al. 2003}. This points to profilin as a potential effector of VASP in regulating the activities of spines and synapses. Surprisingly, we did not observe a requirement for profilin binding, via the PRD region, in VASP function in spines and synapses. It is possible that the interaction between VASP and profilin only contributes to spine function after stimulation, when spines and synapses are actively remodeling, and not during their development. However, it is more likely that VASP exhibits activities in spines and synapses that are mediated through other effectors and are not dependent on profilin.

Previous studies have been shown that phosphorylation modulates VASP's effects on actin polymerization and organization as well as on actin-based cellular processes {Horstrup et al. 1994; Comerford et al. 2002; Krause et al. 2003; Lindsay et al. 2007; Benz et al. 2009; Lee et al. 2009}. These phosphorylation sites are regulated by PKA, PKG, and PKC, which are crucial kinases for synaptic function and plasticity [Fig. 6] {Butt et al. 1994; Smolenski et al. 2000; Howe et al. 2002; Sheng et al. 2002; Chitaley et al. 2004}. We therefore examined whether or not alteration of VASP phosphorylation levels affects VASP-mediated synaptic functions. We generated unphosphorylatable

and phosphomimetic mutations for each phosphorylation sites of VASP and expressed these mutants in hippocampal neurons. Unexpectedlly, we did not observe any significant defects in these mutants (data not shown), suggesting that the level of phosphorylation may not significantly modulate VASP-mediated effects in spine formation. However, since all of these phosphorylation sites of VASP can be regulated by the same sets of kinases, though with different preferences [Fig. 6], it is possible that we will only observe an effect when all the three sites are mutated. Therefore, a construct containing mutations of all the phosphorylation sites may be required in the future to conclude if VASP phosphorylation is involved in spine development. Alternatively, VASP phosphorylation may play a more important role in synaptic plasticity in which many kinases—including PKA, PKG, and PKC—and phosphatases are actually highly active.

CHAPTER IV

VASP MODULATES ACTIN ASSEMBLY TO POTENTIATE SYNAPTIC STRENGTH

Abstract

Dendritic spines are small actin-rich structures that receive the majority of excitatory synaptic input in the brain. The actin-based dynamics of spines are thought to mediate synaptic plasticity, which underlies cognitive processes such as learning and memory. However, little is known about the molecular mechanisms that regulate actin dynamics in spines and synapses. In this study, we show that the multifunctional actin-binding protein VASP regulates the density, size, and morphology of dendritic spines by inducing actin assembly in these structures. Moreover, VASP increases the amount of PSD scaffolding proteins and the number of surface GluR1-containing α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors in spines. VASP knockdown results in a reduction in surface AMPA receptor density, suggesting a role for this protein in regulating synaptic strength. Consistent with this, VASP significantly enhances the retention of GluR1 in spines as determined by fluorescence recovery after photobleaching (FRAP) and increases AMPAR-mediated synaptic transmission. Collectively, our results suggest that actin polymerization and bundling by VASP are critical for spine formation, spine expansion, and the modulation of synaptic strength.

Introduction

VASP has been shown to regulate a variety of cellular processes through its modulation of actin assembly. The expression of VASP recruits G-actin/profilin

complexes, facilitating the addition of G-actin monomers to the growing actin filaments {Reinhard et al. 1995; Chereau et al. 2006; Ferron et al. 2007}. This effect turns out to be important for VASP's promotion of intracellular movement in *Listeria monocytogenes* {Chakraborty et al. 1995; Laurent et al. 1999}. In addition, VASP proteins possess an anti-capping activity that prevents capping proteins from blocking the fast growing ends or barbed ends of actin filaments, thereby allowing persistent actin assembly. This function has been implicated in the underlying mechanism by which VASP proteins regulate the organization of actin networks within lamellipodia during fibroblast movement {Bear et al. 2002}. The anti-capping activity of VASP proteins was first observed in an *in vitro* experiment showing that the presence of purified VASP proteins inhibits actin capping {Bear et al. 2002}. Further studies demonstrated that VASP directly interacts with the barbed ends of actin filaments {Pasic et al. 2008}. Interestingly, interacting with profiln has been suggested to effectively increase the anti-capping activity of VASP and thus heightens actin polymerization; however, some contradictory results have been reported {Hansen et al. ; Barzik et al. 2005; Breitsprecher et al. 2008}.

The modulation of actin polymerization has been implicated in spine formation, motility, and morphology {Fischer et al. 1998; Dunaevsky et al. 1999; Matus 2000}. More recently, it has been shown to play a critical role in the organization of the PSD components, as well as the localization and expression of glutamate receptors at synapses, which determine synaptic strength. For example, depolymerization of F-actin results in reduced number of glutamate receptors at the PSD and alters the synaptic localization and/or retention of several scaffold proteins {Allison et al. 1998; Kuriu et al. 2006}. Moreover, inhibition of actin polymerization has been reported to prevent AMPA receptor-mediated synaptic transmission {Matsuzaki et al. 2004}. However, the effect of ABPs during these processes remains unclear.

Experimental Procedures

Reagents — SV2 monoclonal antibody was from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). PSD95 antibodies were purchased from Chemicon (Temecula, CA) and NeuroMAB (Davis, CA). Homer 1b/c antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Shank1 antibody was purchased from Abcam (Cambridge, MA). Unlabelled phalloidin, strychnine, and bicuculline methiodide were obtained from Sigma-Aldrich (St. Louis, MO). GluR1 antibody was from Calbiochem. GFP antibody, Alexa Fluor® 568 G-actin, Alexa Fluor® 647 phalloidin, ProLong Gold antifade reagent, Alexa Fluor® 488, 555, and 647 anti-mouse, Alexa Fluor® 488 and 555 anti-rabbit and Alexa Fluor® 680 anti-mouse were from Invitrogen (Carlsbad, CA). IRDye 800 anti-mouse was obtained from Rockland Immunochemicals (Gilbertsville, PA). Aqua Ply/Mount was from Polysciences (Warrington, PA). Tetrodotoxin was purchased from Tocris Bioscience (Ellisville, MO). ATP was from Fisher Scientific (Pittsburg, PA).

Cell culture and transfection — Low density hippocampal neurons were prepared and cultured as previously described {Goslin et al. 1998}. Briefly, neurons were plated at a density of 75,000 cells/mm^2 and transfected by a modified calcium phosphate method at day 5-6 in culture {Zhang et al. 2003}.

Immunocytochemistry — Neurons were fixed at day 11-12 in culture, permeabilized, and stained as previously described {Wegner et al. 2008}. Neurons were stained for surface GluR1 as previously described {Lu et al. 2001; Lise et al. 2006}. Briefly, cells were incubated for 30-50 min at room temperature in an extracellular solution containing 150 mM NaCl, 5 mM KCl, 2mM $CaCl_2$, 10 mM HEPES, 30 mM glucose, 0.5 μM tetrodotoxin, 1 μM strychnine, and 20 μM bicucullin methiodide, pH 7.4. For live-cell GluR1 staining, neurons were incubated with GluR1 antibody for 20 min at 37°C. These

cells were subsequently fixed in 2% paraformaldehyde/0.12 M sucrose and stained with secondary antibodies. To visualize presynaptic terminals in these neurons, cells were subsequently permeabilized with 0.2% Triton X-100 and immunostained for SV2.

Actin barbed end staining — Barbed end staining was performed as previously described with minor modifications {Chan et al. 1998; Bryce et al. 2005}. Briefly, neurons were permeabilized with 0.02% saponin in 20 mM HEPES, 138 mM NaCl, 4 mM $MgCl_2$, 3 mM EGTA, 1% BSA, 1 mM ATP, and 3 μM unlabelled phalloidin, pH 7.4. After a brief wash, free barbed ends were stained with Alexa 568 G-actin in saponin-free solution. Cells were then fixed in 4% paraformadehyde/0.12 M sucrose and visualized in fluorescence.

Fluorescence recovery after photobleaching — FRAP was performed on a Quorum WaveFX spinning disk confocal system with a Nikon Eclipse Ti microscope using a PlanApo 60X TIRF objective (NA 1.49). Four to six circular regions of interest (20 x 20 pixels) in spine heads were photobleached with a 405 nm diode laser for 700 msec with 100% laser power. EGFP images were acquired at 20 sec intervals using a Hamamatsu ImageEM-CCD camera and MetaMorph software. EGFP and mCherry were excited with 491 nm and 561 nm laser lines, respectively. For FRAP analysis, the background subtracted fluorescent intensity at each time point *t* was corrected for the loss of fluorescence due to image acquisition. The corrected data was further normalized to the baseline fluorescence (I_{pre}), which is defined as 100% and graphed according to the equation: $FI\ (t) = (I_t * I_{nf,\ pre}) / (I_{pre} * I_{nf,\ t})$ where nf represents a region that was not subjected to FRAP. A graph of the recovery traces were generated using the following equation: *Fluorescence recovery* (*FR*)= [*FI* (t)- *FI* (0)] / [*FI* (pre)-*FI* (0)]. The time constant was calculated according to the equation: $FR=\alpha-A_1*\exp(-k_1t)-A_2*\exp(-k_2t)$ where

α is the mobile fraction and A_x is the amplitude of the exponential process with rate constant k_x {Tyska et al. 2002}.

Electrophysiology — Neurons were transfected with GFP-VASP at day 6 in culture, and whole-cell patch clamp recordings were obtained at day 14-16 in culture. Cells were placed in a recording chamber in an extracellular solution, containing 140 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose, 25 mM HEPES, 0.5 μM TTX, 20 μM bicuculline methiodide and 1 μM strychnine, pH 7.4. Patch pipettes were filled with an intracellular solution, composed of 115 mM cesium gluconate, 17.5 mM CsCl, 10 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, 4 mM K_2-ATP, 0.4 mM Na-GTP, pH 7.4, and cells were recorded at room temperature at a holding potential of -60 mV using a Multiclamp 700A amplifier (Molecular Devices). Recordings were pass-filtered at 2 kHz and sampled at 10 kHz. Membrane and access resistances were monitored continuously, and recording data were rejected if series access resistance varied more than 20%. The statistical significance was calculated using a paired *t*-test.

Results

VASP regulates actin dynamics in dendritic spines. To further explore the effect of VASP on actin dynamics in spines, we stained GFP and GFP-VASP expressing neurons with phalloidin to visualize F-actin. Quantification of the fluorescent intensity of phalloidin showed a 2.5-fold increase in F-actin staining in spines of GFP-VASP expressing neurons compared with GFP controls [Fig. 18]. In addition, when we normalized the fluorescent intensity to the unit area, a significant increase in the normalized fluorescent intensity in spines was still observed in GFP-VASP expressing neurons compared with controls [Fig. 18], indicating that VASP significantly enhances

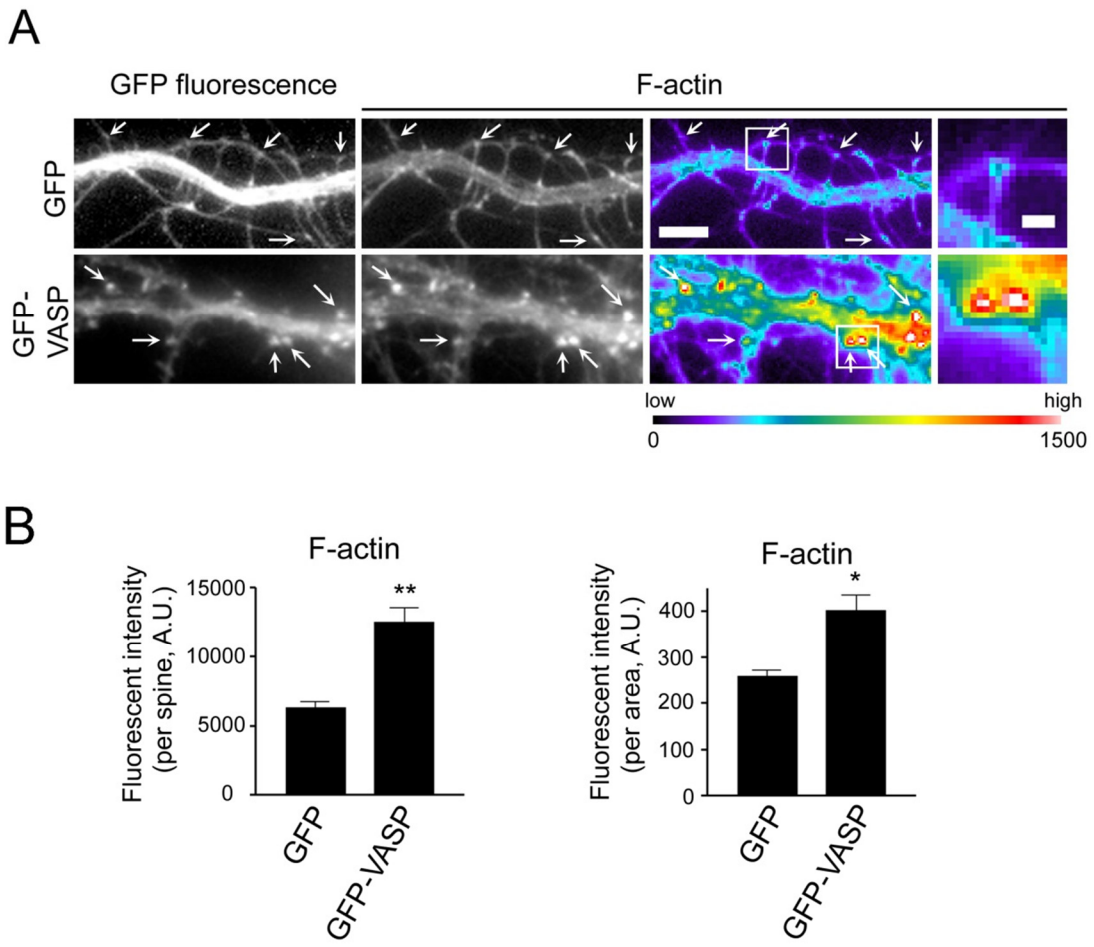


Figure 18. VASP promotes actin polymerization in spines. *A,* Images of GFP and GFP-VASP expressing neurons stained for F-actin are shown. Pseudocolor coded images (right panel) and higher magnification images of the boxed regions (far right panel) are shown. Arrows indicate dendritic spines. Bar, 5 µm; 1 µm. *B,* Quantification of the fluorescent intensity of F-actin staining in individual spines from GFP and GFP-VASP expressing neurons is shown (*left panel*). The fluorescent intensity was normalized to the spine area (*right panel*). For panel *B*, error bars represent S.E.M. for 100 spines from three independent experiments (**, p <0.0001; *, p <0.01).

the amount of F-actin in spines.

Does VASP promote actin polymerization in dendritic spines? *In vitro*, VASP induces actin polymerization in the presence of barbed end capping proteins, suggesting that VASP protects F-actin from capping proteins to promote filament elongation {Bear et al. 2002; Barzik et al. 2005; Breitsprecher et al. 2008}. This led us to hypothesize that VASP stimulates actin polymerization in dendritic spines by protecting the barbed ends of actin filaments. To test this, we stained GFP- and GFP-VASP-expressing neurons with fluorescent monomeric actin to label available barbed ends. Some barbed end staining of actin filaments was observed in spines of GFP expressing neurons [Fig. 19], which is consistent with a previous study demonstrating free barbed ends in dendritic spines {Hotulainen et al. 2009}. Importantly, a 2-fold increase in the fluorescent intensity of barbed end staining in spines was observed in GFP-VASP-expressing neurons compared with controls [Fig. 19]. Similar results were obtained when the fluorescent intensity in spines was normalized to the unit area, indicating that the enhanced staining was due to an increased number of available barbed ends and not to larger spine heads. When the actin-binding EVH2 domain was deleted, VASP no longer protected barbed ends [Fig. 20]. Interestingly, the number of barbed ends was still increased in ΔEVH1-VASP-expressing neurons compared with GFP controls, suggesting that the EVH1 domain is not essential for this function of VASP. These results suggest that VASP, via its EVH2 domain, promotes actin polymerization in spines by increasing the availability of barbed ends for further actin assembly.

VASP modulates the amount of GluR1-containing AMPARs and PSD scaffolding proteins in spines. The spine head size, the PSD area, and the intact actin cytoskeleton control the anchoring of postsynaptic receptors, which can determine the

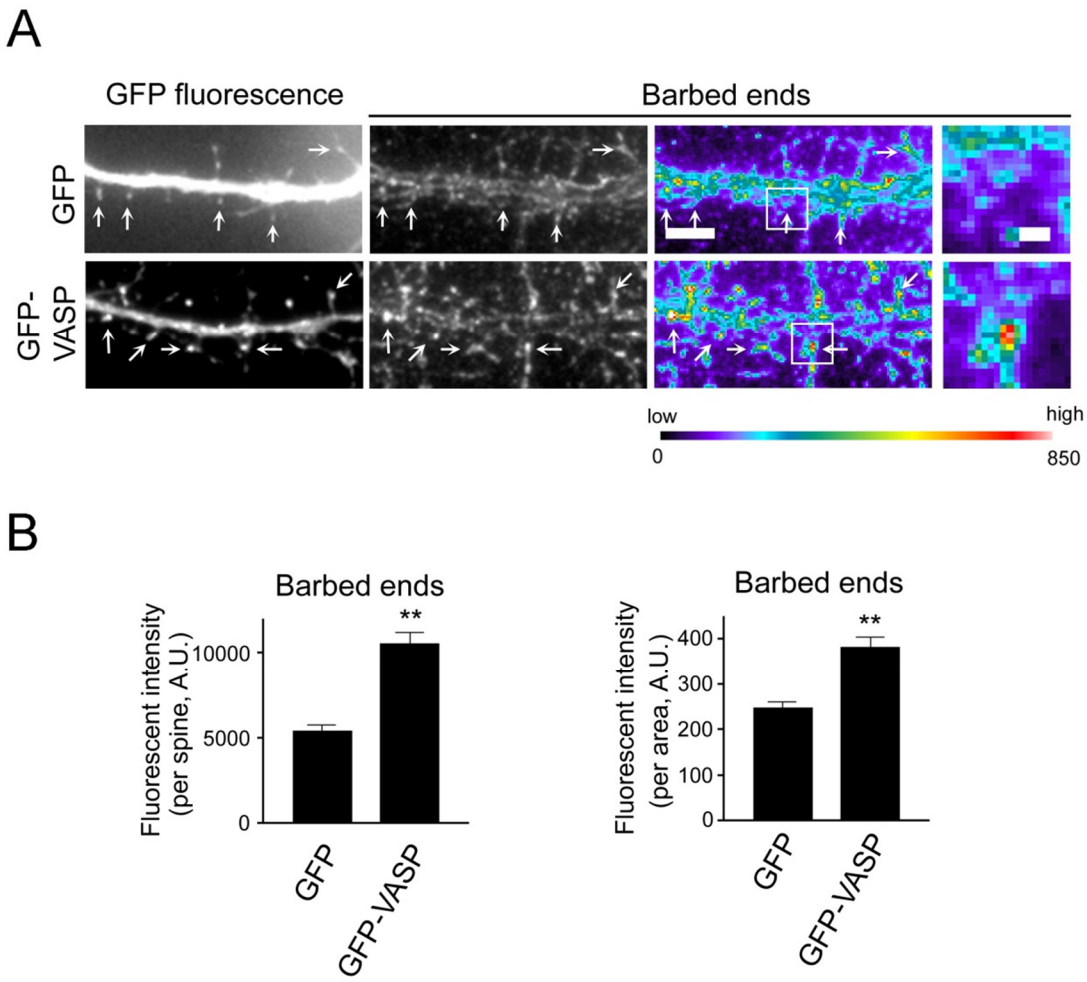


Figure 19. VASP protects barbed ends from capping. *A*, Images of GFP and GFP-VASP expressing neurons stained for free actin barbed ends are shown. Arrows indicate spines. Bar, 5 μm. *B,* Quantification of the fluorescent intensity in individual spines from barbed end staining is shown (*left panel*). The fluorescent intensity was normalized to spine area (*right panel*). Error bars represent S.E.M. for 100 spines from three independent experiments (**, p <0.0001).

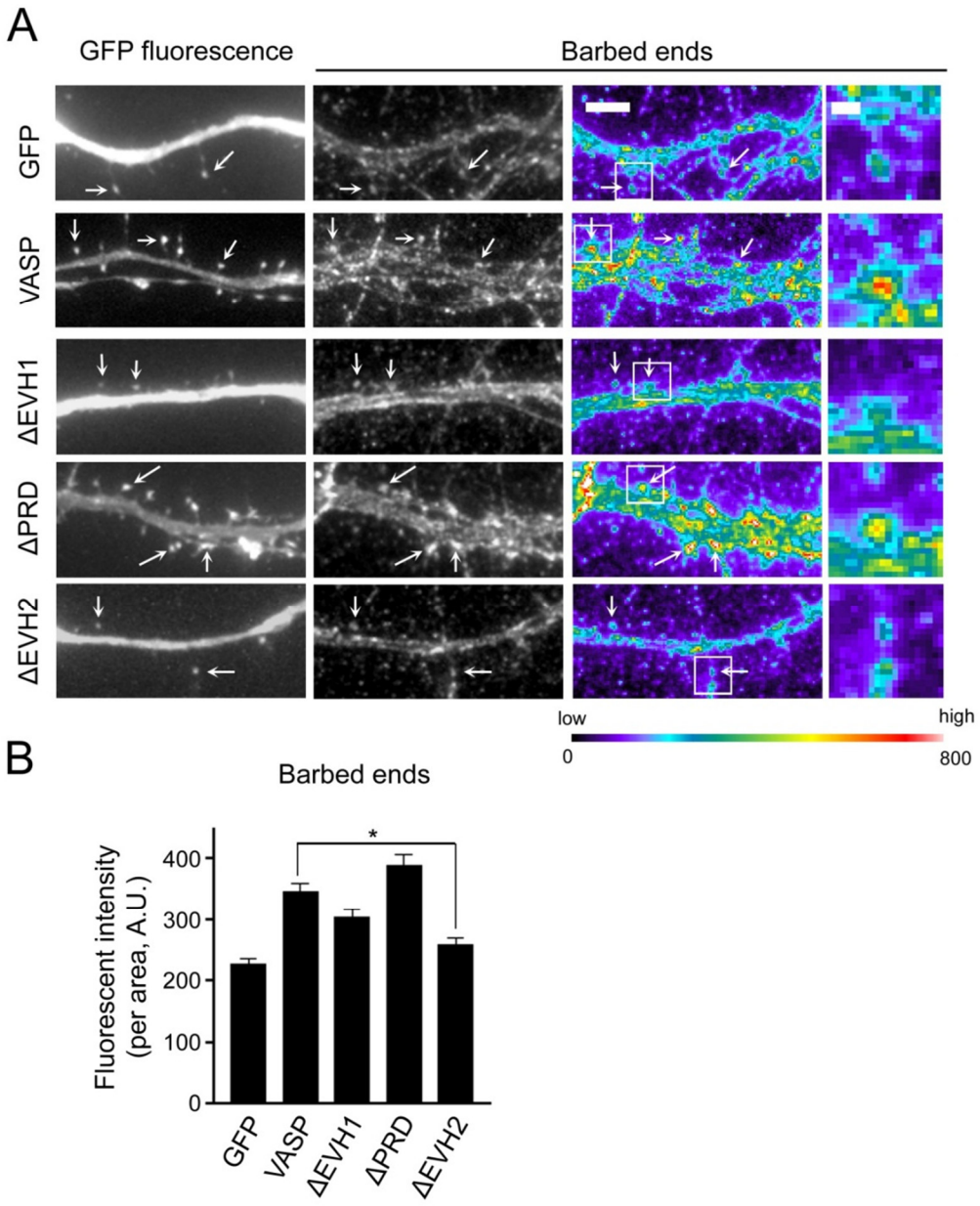


Figure 20. The EVH2 domain is essential for VASP to protect barbed ends. *A,* Images from barbed end staining of neurons expressing the indicated constructs are shown in grayscale (*middle panels*) and pseudocolor coding (*right panels*). Pseudo-color coding indicates the range of fluorescent intensities to the assigned color. Bar, 5 μm. Arrows indicate spines. Higher magnification images of the boxed regions are shown in the far right panels. Bar, 1 μm. *B,* Quantification of the fluorescent intensity in individual spines from neurons expressing GFP, GFP-VASP, or VASP deletion mutants and stained for free barbed ends is shown. The fluorescent intensity was normalized to spine area. Error bars represent S.E.M. for at least 70 spines from three separate experiments (*, p <0.0001).

efficacy of synaptic strength {Allison et al. 1998; Kim et al. 1999; Takumi et al. 1999; Matsuzaki et al. 2001}. Since our results indicate that VASP modulates spine head size and actin filament elongation, it could regulate the size of the PSD and synaptic strength. To test this, we examined the effect of VASP on the amount of several PSD scaffolding proteins—including PSD95, Homer, and Shank—in spines since they have been shown to promote synapse maturation and modulate synaptic strength {El-Husseini et al. 2000; Sala et al. 2001; Hung et al. 2008}. Expression of GFP-VASP resulted in a significant increase in the intensity of PSD95 staining in spines [Fig.21*A*]. Quantification of the fluorescent intensity of PSD95 showed a 2-fold increase in the level of PSD95 in GFP-VASP expressing neurons compared with GFP controls [Fig. 21*B*]. Comparable results were obtained when the fluorescent intensity was normalized to the unit area [Fig. 21*B*]. Moreover, VASP expression promoted a similar increase in the amount of Homer and Shank in spines [Fig. 21*C-E*]. These results suggest that VASP modulates the level of PSD scaffolding proteins in spines and point to a role for VASP in regulating synaptic strength.

In excitatory synapses, synaptic strength is regulated by the release of glutamate neurotransmitter from presynaptic terminals and by expression of glutamate receptors at the plasma membrane of postsynaptic terminals {Malinow et al. 2002}. Most rapid excitatory synaptic transmission takes place through AMPA-type glutamate receptors, which consists of GluR1-4 subunits {Malinow et al. 2002}. Since expression of the AMPA receptors subunit GluR1 is critical for synaptic function {Malinow et al. 2002}, we examined the effect of VASP on surface GluR1 levels by staining with an antibody against the extracellular epitopes of this subunit in non-permeabilized cells. Neurons were subsequently permeabilized and immunostained for SV2 to show synapses. In neurons expressing GFP-VASP, the level of synaptic surface GluR1 (sGluR1) in spines

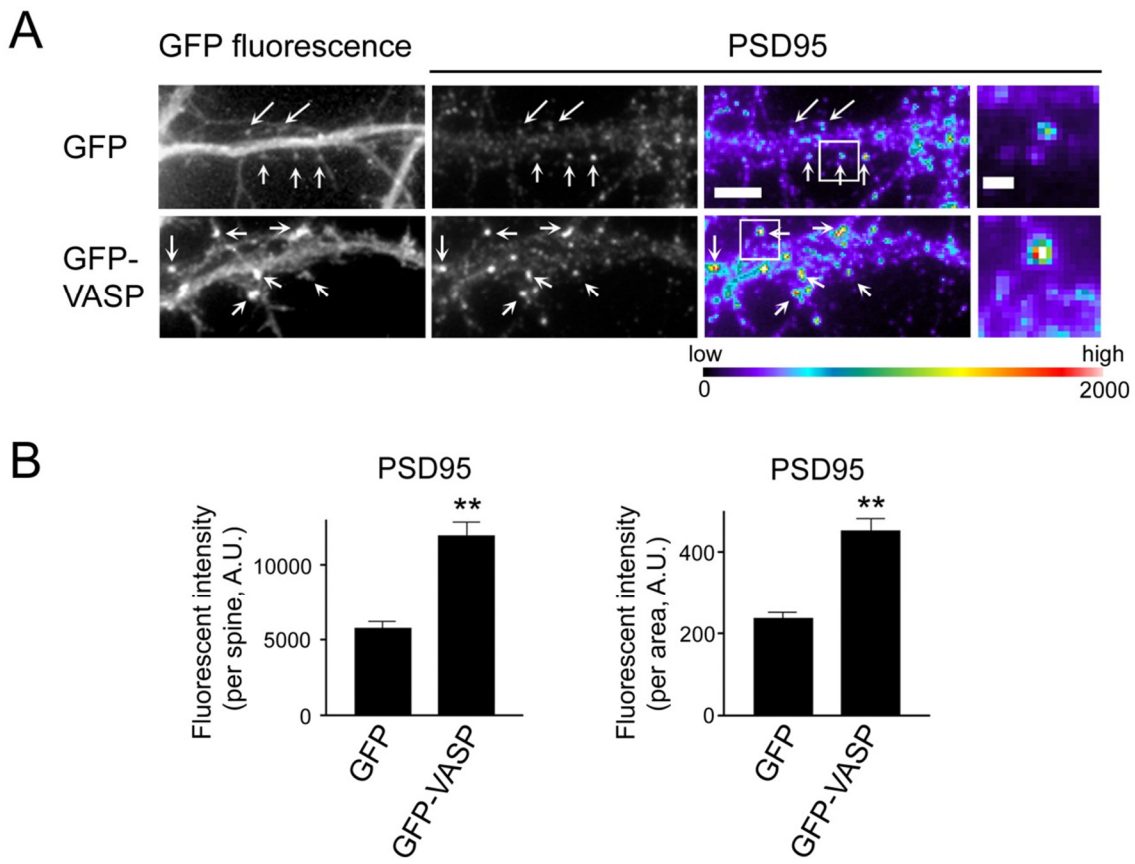
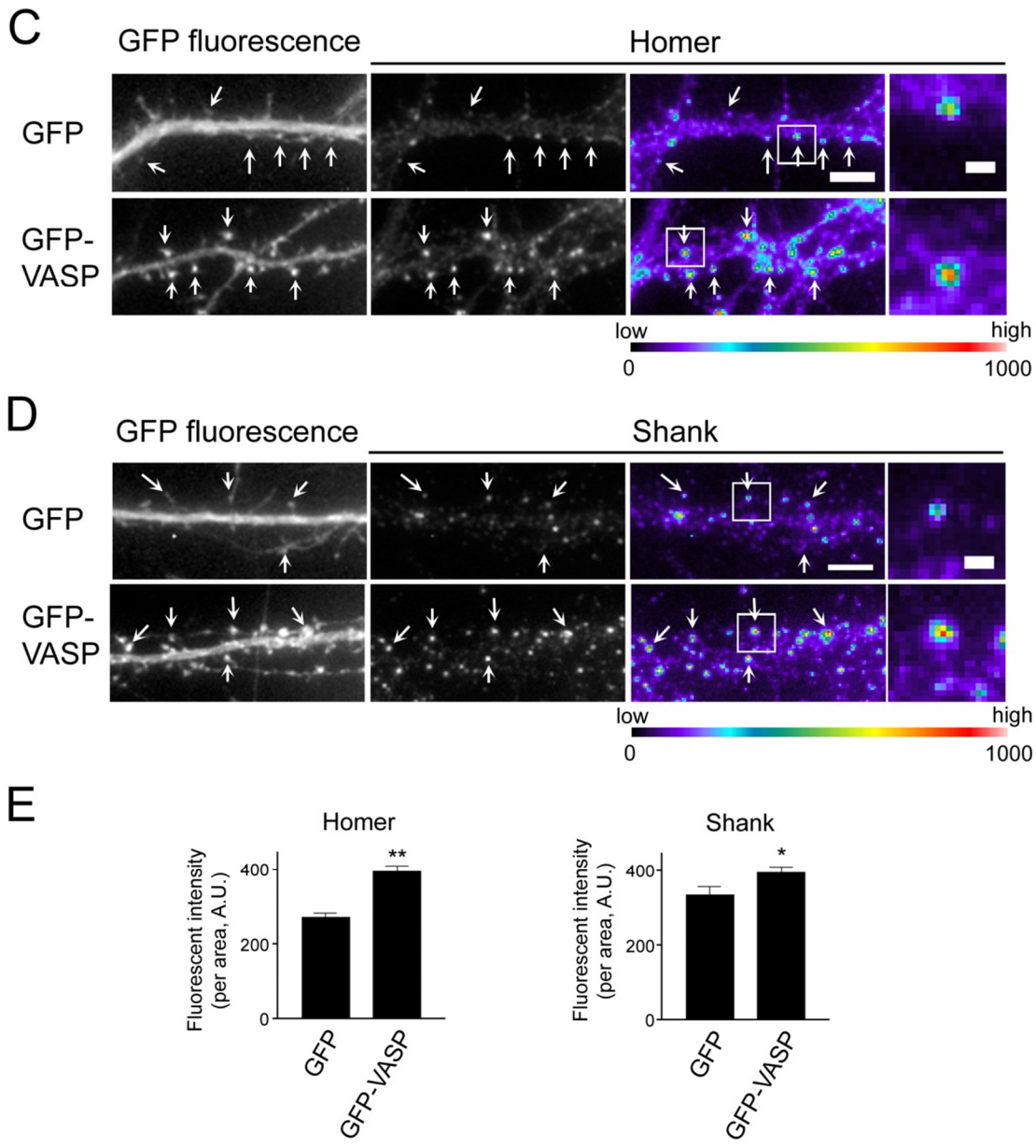


Figure 21. VASP expression increases the amount of PSD scaffold proteins in spines. Images of GFP and GFP-VASP expressing neurons stained for PSD95 (*A*), Homer (*C*), and Shank (*D*) are shown in grayscale (middle panels) and pseudo-color coding (right panels). Arrows indicate spines. Bar, 5 μm. Higher magnification images of the boxed regions are shown (far-right panels). Bar, 1 μm. Quantification of the fluorescent intensity in individual spines of neurons stained for PSD95 (*B*) is shown (*left panel*). Quantification of the normalized fluorescent intensity in individual spines of neurons stained for PSD95 (*B*, right panel) as well as Homer and Shank (*E*) staining. Error bars represent S.E.M. for 100 spines from three separate experiments (**, $p < 0.0001$; *, $p < 0.04$).

Figure 21—cont.



was increased 1.5-fold compared with GFP controls [Fig. 22*A,B*]. A significant increase in the amount of sGluR1 in spines was still observed when the fluorescent intensity of the GluR1 signal was normalized to the unit area [Fig. 22*B*]. In contrast, expression of VASP siRNA#1 resulted in a significant decrease in the level of sGluR1 in spines [Fig. 22*C,D*]. The effect of VASP on sGluR1 levels is mediated through its EVH1 and EVH2 domains, since VASP failed to increase sGluR1 when these domains were deleted [Fig. 23].

VASP regulates GluR1 retention and synaptic transmission. We next examined the effect of VASP on maintaining GluR1 in spines with FRAP in neurons expressing GFP-GluR1 and mCherry-VASP or mCherry as a control. As shown in Fig. 25, GFP-GluR1 recovery was significantly slower in mCherry-VASP-expressing neurons compared with control mCherry-expressing neurons. The time constant of recovery, which is the inverse of the rate constant, for neurons expressing mCherry-VASP was 26.2 and 277.8 sec for the fast and slow components, respectively, compared with 8.5 and 232.6 sec for control mCherry expressing neurons [Fig. 24]. In addition, the immobile fraction of GluR1 for mCherry-VASP and mCherry expressing neurons was 69.3 and 46.5%, respectively, indicating a longer retention of GluR1 in the spines of VASP-expressing neurons [Fig. 24].

To further elucidate a role for VASP in regulating synaptic strength, we examined AMPAR-mediated synaptic transmission using whole-cell patch clamp recordings. The frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) were measured in GFP-VASP-expressing neurons and neighboring untransfected cells. VASP expression led to a 2.7-fold increase in mEPSC frequency and a 1.3-fold increase in mEPSC amplitude compared with untransfected control neurons [Fig. 25]. Taken

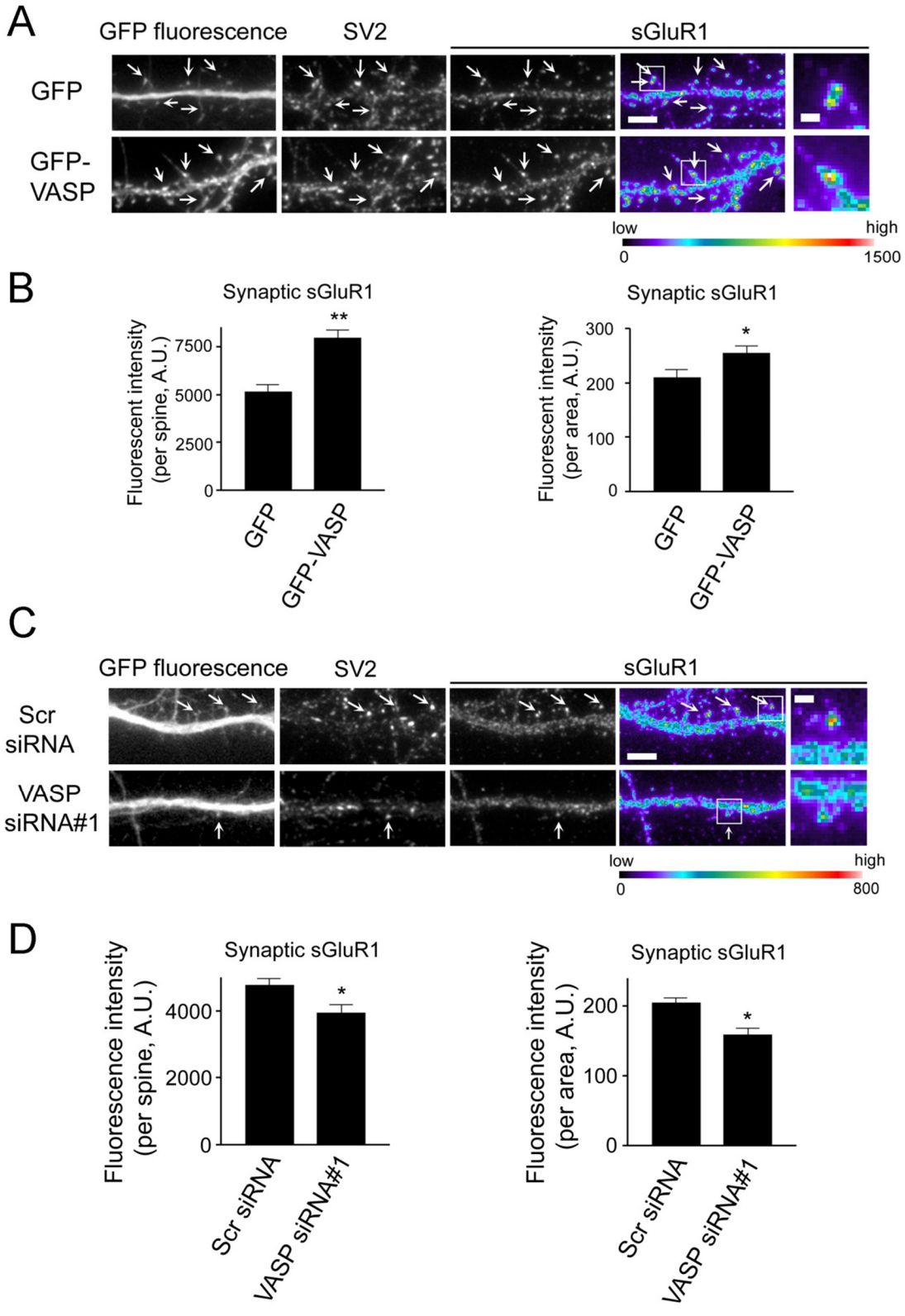
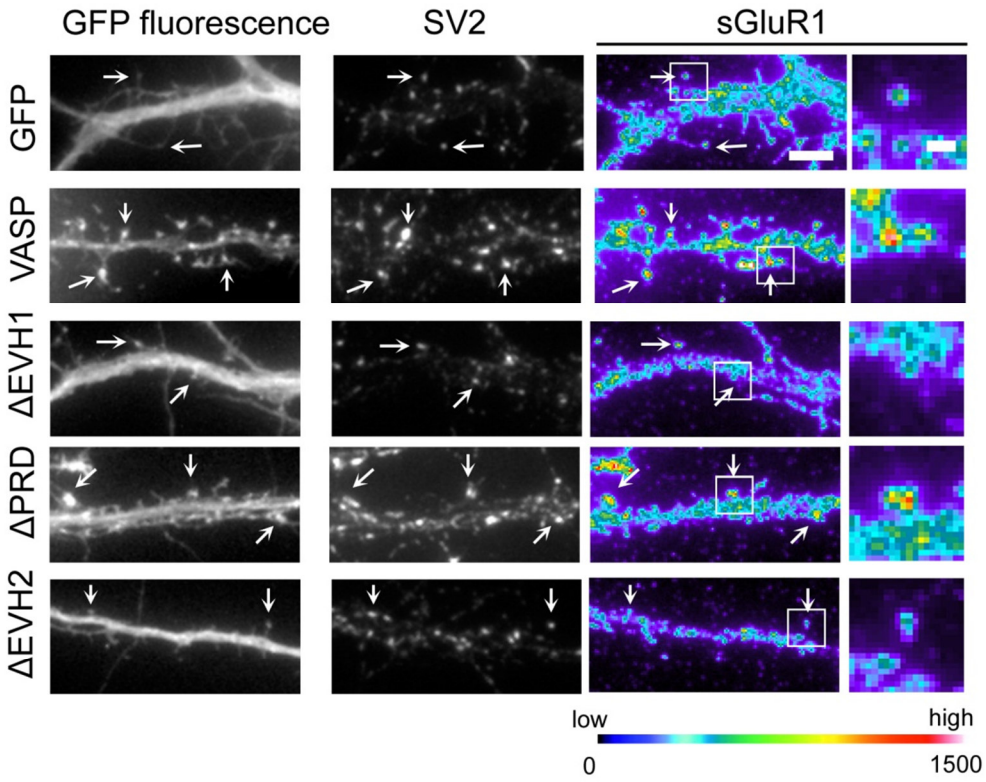

A
GFP fluorescence
SV2
sGluR1
GFP
GFP-VASP
low
high
0
1500
B
Synaptic sGluR1
Fluorescent intensity (per spine, A.U.)
**
7500
5000
2500
0
GFP
GFP-VASP
Synaptic sGluR1
Fluorescent intensity (per area, A.U.)
*
300
200
100
0
GFP
GFP-VASP
C
GFP fluorescence
SV2
sGluR1
Scr siRNA
VASP siRNA#1
low
high
0
800
D
Synaptic sGluR1
Fluorescence intensity (per spine, A.U.)
*
4000
2000
0
Scr siRNA
VASP siRNA#1
Synaptic sGluR1
Fluorescence intensity (per area, A.U.)
*
200
100
0
Scr siRNA
VASP siRNA#1

Figure 22. VASP regulates the level of synaptic sGluR1. *A,C,* Neurons expressing indicated constructs were stained for surface GluR1 (*sGluR1*) under non-permeabilizing conditions. Neurons were then permeabilized and stained for SV2 to indicate synapses. The sGluR1 puncta at synapses are indicated (*arrows*). sGluR1 staining images are shown in grayscale (right) and pseudocolor coding (far-right images). Bar, 5 μm. Higher magnification images are shown. Bar, 1 μm. *B*, *D,* Quantification of the fluorescent intensity of sGluR1 in spines from neurons expressing indicated constructs is shown (*left panel*). The fluorescent intensity was normalized to the spine area (*right panel*). Error bars represent S.E.M. for 100 spines from three separate experiments (**, $p < 0.0001$; *, $p < 0.03$).

A


GFP fluorescence
SV2
sGluR1
GFP
VASP
ΔEVH1
ΔPRD
ΔEVH2
low
high
0
1500

B

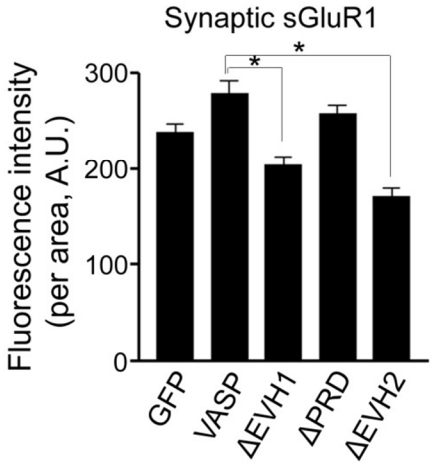

Synaptic sGluR1
Fluorescence intensity
(per area, A.U.)
300
200
100
0
*
*
GFP
VASP
ΔEVH1
ΔPRD
ΔEVH2

Figure 23. Both EVH1 and EVH2 domains contribute to VASP-mediated increase of synaptic sGluR1 level. *A,* Images from neurons expressing GFP, GFP-VASP, or VASP deletion mutants stained for SV2 and sGluR1 are shown. sGluR1 staining is shown in pseudo-color coding (*right panels*). Bar, 5 μm. Arrows indicate sGluR1 puncta that co-localize with SV2. Error bars represent S.E.M. for 75 spines from three separate experiments (*, $p < 0.0001$). Higher magnification images of the boxed regions are shown (*far right panels*). Bar, 1 μm. *B,* Quantification of the fluorescent intensity of sGluR1 in individual spines from neurons expressing the indicated constructs is shown. The fluorescent intensity was normalized to spine area.

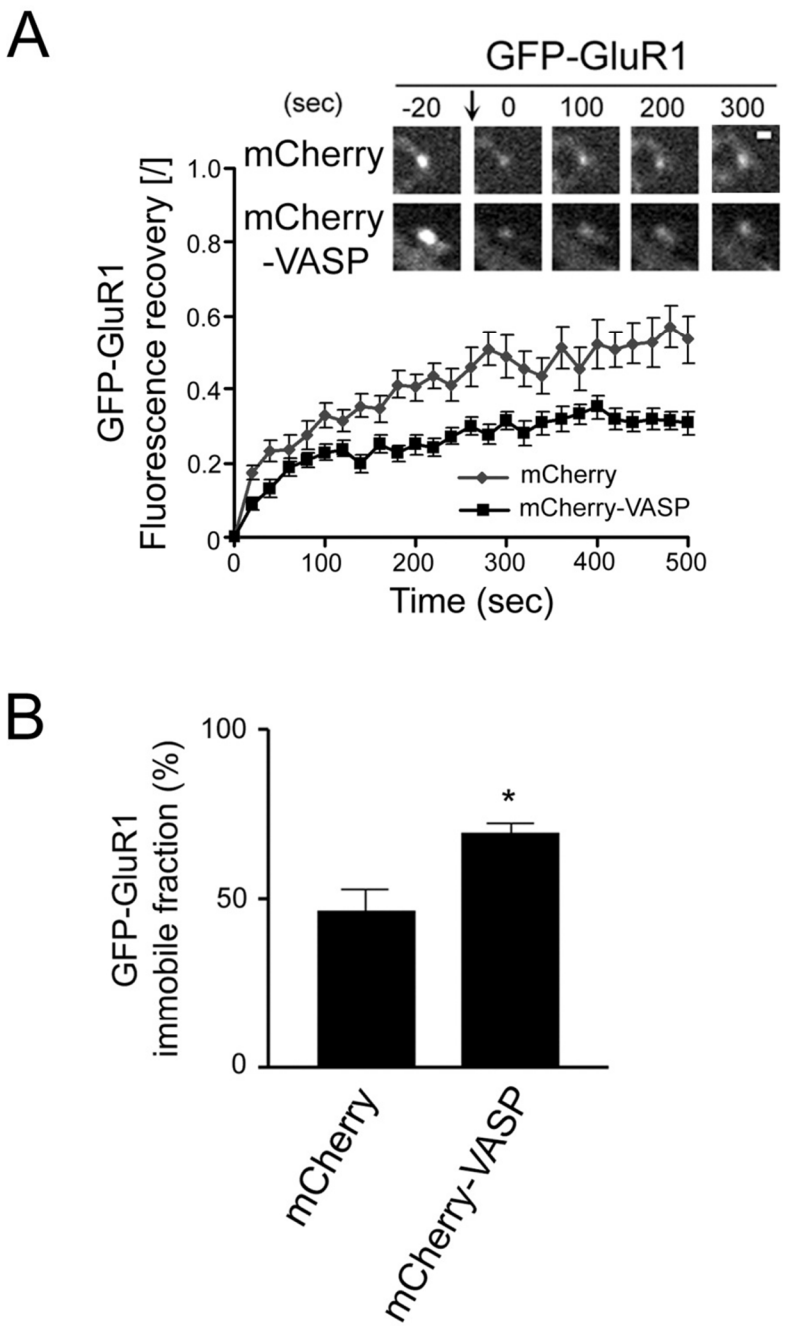


Figure 24. VASP enhances the retention of GluR1 in spines. *A,* Neurons were transfected with GFP-GluR1 and either mCherry or mCherry-VASP at day 6 in culture and subjected to FRAP at day 10. Prebleach and subsequent recovery images of GFP-GluR1 are shown (*upper panels*). The bleached point is indicated (*arrow*). Bar, 1 μm. To calculate the fluorescence recovery, the normalized intensity was divided by the extent of bleaching (*graph*). *B*, Quantification of the immobile fraction of GFP-GluR1 clusters in cells co-transfected with mCherry or mCherry-VASP is shown. Error bars represent S.E.M. for 19-31 spines. (*, p <0.005).

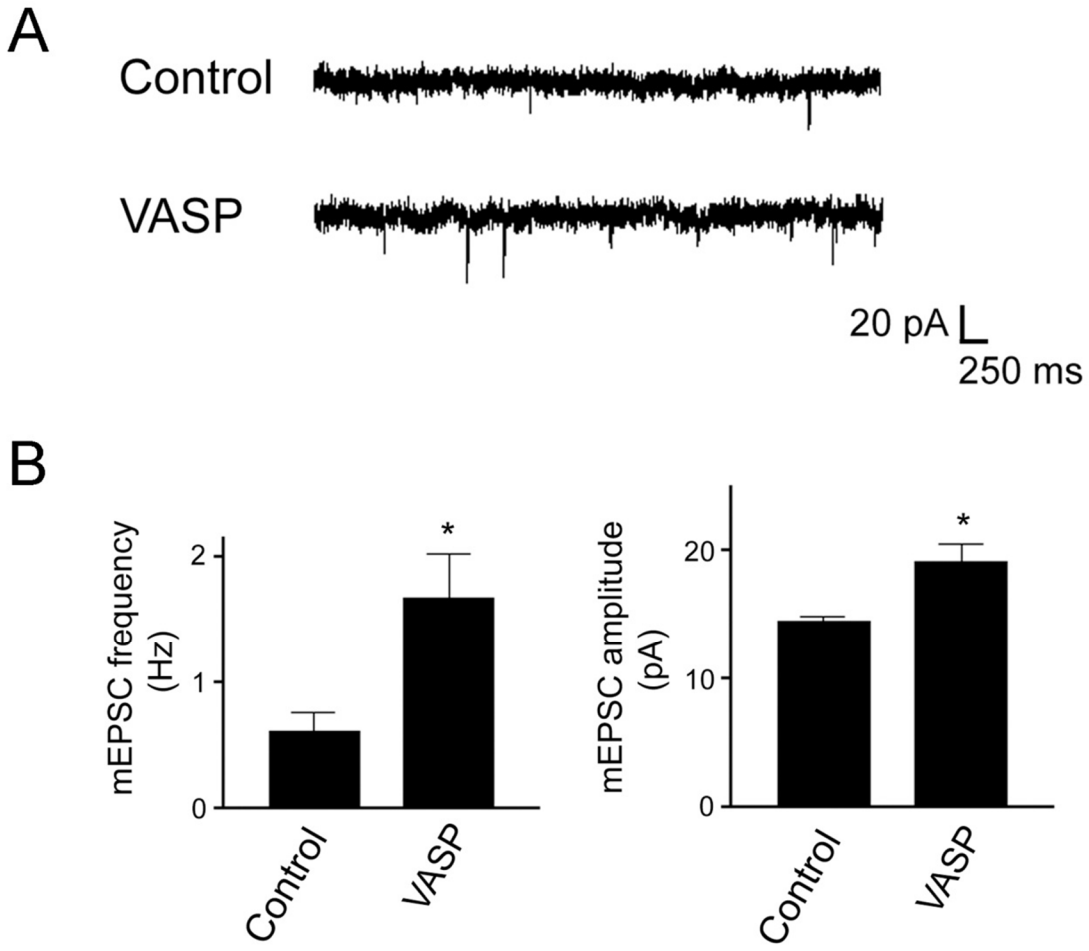


Figure 25. VASP potentiates synaptic strength. *A,* Representative traces of mEPSCs recorded from a GFP-VASP expressing neuron and an untransfected neighboring cell (*Control*). *B,* Quantification of the mEPSC frequency and amplitude in GFP-VASP expressing and control untransfected neurons is shown. Error bars represent S.E.M. for 15-paired neurons from eleven separate experiments (*$p < 0.01$).

together, these results indicate that VASP increases the surface level and the retention of GluR1 in spines to potentiate synaptic strength.

Discussion

Earlier, we showed that VASP regulates spine and synapse formation through its EVH1 and EVH2 domains. To further investigate the underlying mechanisms by which VASP promotes spine development, as well as its role in synaptic function, we examined the effect of VASP on actin dynamics and synaptic strength. Our results indicate that VASP expression promotes actin polymerization in dendritic spines [Fig. 18]. Additionally, VASP increases the amount of several PSD scaffold proteins and GluR1 in spines and synapses, which results in enhanced synaptic transmission [Figs. 21-25].

Using phalloidin to visualize actin filaments, we found that VASP expression increases the amount of F-actin in spine heads [Fig. 18], this is due to VASP's protection of actin barbed ends from capping [Fig. 19]. Moreover, the EVH2 but not the EVH1 or the PRD domain of VASP mediates this anti-capping activity [Fig. 20]. These data are consistent with previous *in vitro* studies showing that the extreme C-terminus of the EVH2 domain mediates the capture of barbed ends and that the entire EVH2 domain is required and sufficient to exhibit anti-capping activity {Barzik et al. 2005; Pasic et al. 2008}. Interestingly, our data suggest that association with profilin, which is mediated by the PRD, is not essential for VASP-mediated anti-capping function in dendritic spines. Combined with the critical effect of EVH2 on spine formation and maturation [Figs. 14-16], we argue that one mechanism responsible for VASP-mediated spine development is by increasing actin polymerization through the protection of barbed ends from capping.

We next explored the role of VASP in synaptic function. VASP expression increases the amount of PSD scaffold proteins in spines and the retention and synaptic surface level of GluR1-containing AMPA receptors [Figs. 21-24]. Moreover, VASP potentiates the frequency and amplitude of basal synaptic transmission [Fig. 25]. Synaptic transmission begins with the release of neurotransmitters from a presynaptic neuron. Once neurotransmitters bind to ionotropic receptors at the postsynaptic membrane, they lead to the opening of receptors and subsequent postsynaptic responses. Our data suggest that VASP enhances the strength of synaptic transmission at least at the postsynaptic side. We hypothesize that increased actin assembly by VASP is critical for facilitating GluR1 delivery or retention at synaptic membranes and in regulating the composition and stability of the PSD. In fact, perturbation of actin polymerization has been shown to affect the distribution and stability of postsynaptic scaffold proteins in synapses {Allison et al. 1998; Kuriu et al. 2006}. For example, several PSD proteins, including Shank, GKAP and Homer 1c are maintained in spines in an F-actin-dependent manner {Kuriu et al. 2006}. Moreover, actin disassembly reduces the density of PSD95 and increases the non-synaptic distribution of this protein {Allison et al. 1998; Zhang et al. 2001; Kuriu et al. 2006}. These scaffold proteins, in turn, contribute to synaptic strength by enhancing GluR1 synaptic clustering and by controlling the incorporation of AMPA receptors into synaptic membranes {El-Husseini et al. 2000; Schnell et al. 2002; Ehrlich et al. 2004}. Additionally, VASP is found in presynaptic terminals [Fig. 8] {Dillon et al. 2005; Wang et al. 2005}, and actin has been shown to modulate the synaptic vesicle cycle in presynaptic terminals {Dillon et al. 2005}. Therefore, VASP may contribute to the enhancement of synaptic strength by promoting the amount of released synaptic vesicles from the presynaptic side.

How does the actin cytoskeleton facilitate transport of AMPA receptors within dendritic spines? Growing evidence indicates that myosin motors, which travel along actin, are responsible for the insertion and removal of AMPA receptors, suggesting that the integrity and organization of the actin cytoskeleton can affect receptor expression levels at synaptic membranes {Osterweil et al. 2005; Lise et al. 2006; Correia et al. 2008; Wang et al. 2008}. In support of this hypothesis, perturbation of actin dynamics has been reported to affect the internalization of GluR1-containing AMPA receptors {Zhou et al. 2001}. Thus, VASP-mediated actin reorganization may not only provide a stable platform for signaling molecules, but also could actively modulate AMPA receptor movement into and out of synaptic membranes.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions and working model

VASP is enriched in dendritic spines and synapses. Expression of VASP promotes the formation of dendritic spines and synapses and induces enlargement of spine heads by regulating actin dynamics in these structures. The EVH2 domain, which mediates actin polymerization and anti-capping, is necessary for synaptic targeting of VASP as well as VASP regulation of spine head size and density. The EVH1 domain also contributes to VASP localization and function in spines and synapses, but the PRD region does not appear to be necessary in this regard. Additionally, VASP increases the amount of several PSD scaffold proteins, including PSD95, Homer, and Shank, in dendritic spines. The level of GluR1 at synaptic membranes and GluR1 retention in spines is also enhanced in VASP-expressing neurons. These effects ultimately contribute to an enhancement of synaptic transmission.

Our data are consistent with a working model in which VASP stimulates actin polymerization and bundling in spines. The growing actin filaments, induced by VASP, provide an underlying structure to support spine formation and enlargement. As spines develop, VASP-promoted actin remodeling stimulates the recruitment of scaffolding proteins such as PSD95, Homer, and Shank to the postsynaptic density, which generates an expanded anchoring area for synaptic proteins. The dynamic actin filaments may also allow the efficient delivery and retention of AMPA receptors in synapses.

Future directions

Here, we show that VASP promotes spine formation and spine head enlargement. We also provide strong evidence demonstrating the role of VASP in increasing actin polymerization via its anti-capping acitivity and its effect on synaptic strength. These findings raise several interesting questions. Specifically, how does VASP promote the formation and maturation of spines and synapses? What upstream factors and other signaling molecules or cytoskeleton modulatory proteins are involved in regulating these processes? While VASP potentiates synaptic strength, does VASP expression influence synaptic plasticity, a process of long-lasting changes of synaptic strength in response to synaptic activity? Moreover, since synaptic strength and plasticity are the cellular basis of cognitive functions, does VASP affect animal behavior such as memory performance and learning ability?

The most accepted model of spine formation is that postsynaptic neurons generate dendritic filopodia to navigate their surrounding environment and search for proper presynaptic inputs [Fig. 2]. Once a contact is formed, adhesion molecules at both pre- and post-synaptic sides establish stable interactions with each other and promote further progression of spine development. Interestingly, while VASP family proteins are overexpressed in cultured neurons with Syndecan-2, a coreceptor for growth factors and differentiation factors, at a very early stage of development (day 1 in culture), they showed localization to the tips of dendritic filopodia {Lin et al. 2007}. This observation suggests that VASP family proteins may promote the initiation and/or extension of dendritic filopodia, which are precursors of dendritic spines. To directly examine this possibility, time-lapse imaging can be performed to determine whether or not VASP affects the motility and navigation of dendritic filopodia. We will expect to see that VASP expression increases the motility of dendritic filopodia before they contact the

presynaptic terminal. Once the contact is formed, VASP will promote the stabilization of synaptic contacts and further slow the movement of dendritic filopodia. This assumption is based on the facts that VASP promotes spine formation and that hypermotility of dendritic filopodia tends to prevent spine formation {Dailey et al. 1996; Ziv et al. 1996}. The next question is how VASP stabilize synaptic contact. Previous studies in non-neuronal cells have demonstrated some interesting functions of VASP in regulating cell-cell adhesions. For example, the depletion of VASP impairs integrin-mediated adhesion formation and function {Schlegel et al. 2009}. Moreover, VASP proteins have been shown to take part in the formation and stabilization of cadherin-mediated cell-cell adhesions {Scott et al. 2006; Kris et al. 2008}. Therefore, we expect to observe that VASP strengthens synaptic contact and thus decreases filopodia motility and promotes spine development through certain adhesion molecules.

Using super-resolution light microscopy or EM, the kinetics and organization of the actin cytoskeleton in dendritic filopodia and dendritic spines are unveiled [Fig. 4] {Korobova et al. ; Honkura et al. 2008; Tatavarty et al. 2009; Frost et al. 2010; Frost et al. 2010}. Interestingly, the kinetics of actin assembly appears to be different in discrete subspine areas and is regulated by synaptic activity {Honkura et al. 2008}. For example, the F-actin pool at the base of spines is more stable, and the size of this pool correlates with spine head volume {Honkura et al. 2008}. In contrast, the actin pool at the tips of spines is much more dynamic. This discrepancy of actin dynamics suggests that different pools of actin within an individiual spine may execute different functions. We show that VASP is involved in spine formation and spine head enlargement, and these processes are both regulated by actin dynamics and organization. In order to have an overall picture in which VASP modulates actin dynamics and organization in spines, we need to understand the subspine localization of VASP and how it regulates actin

dynamics and architecture. Similar light microscopy and platinum replica EM are required to answer these questions.

The Ena/VASP family consists of three members, Mena, VASP, and EVL, and it is suggested that all the Ena/VASP family members possess certain similar and some overlapping functions {Krause et al. 2002; Krause et al. 2003; Kwiatkowski et al. 2003}. Intriguingly, we found that alteration of the expression level of a single gene, VASP, in this family is able to result in a very strong defect in spine formation and maturation [Figs. 10, 13]. These data suggest that Mena and EVL may not have a profound contribution in spine development as VASP. Previous studies showed that one of the major regions in which Mena is highly expressed is in the brain, while VASP and EVL are more ubiquitously expressed throughout different organs {Lanier et al. 1999}. Interestingly, the expression of Mena in the brain is higher in the embryonic stage than in the postnatal phase {Lanier et al. 1999}; this suggests that Mena may be involved in actin-based processes that occur at the early stages of development. Indeed, one major function of Mena in the brain is to initiate neurite formation {Dent et al. 2007}. Therefore, we hypothesize that Mena is more important for neuritogenesis while VASP is more crucial for spine development, which occurs after the formation of neurites {Tahirovic et al. 2009}. In the future, this hypothesis should be tested to know if Mena or EVL is present in spines and synapses and whether or not they affect the formation of these structures.

VASP proteins contain the EVH1, the PRD, and the EVH2 domains [Fig. 6]; several studies have shown that these regions are important for VASP to interact with other molecules. One interesting finding in our study is that not only the actin-binding EVH2 domain but also the EVH1 domain is important for VASP function [Figs. 14-16]. Since the EVH1 domain is not required for actin polymerization, it may serve as a

targeting module to recruit VASP to actin-rich structures, such as dendritic spines {Huttelmaier et al. 1999}. In fact, the EVH1 domain is responsible for VASP targeting to focal adhesions and to the tips of filopodia in fibroblasts {Reinhard et al. 1995; Hoffman et al. 2006; Applewhite et al. 2007}. While the focal adhesion molecules vinculin and zyxin have been identified for mediating VASP targeting to focal adhesions, we still do not know the proteins that link VASP to the tips of protrusions such as filopodia or spines. Future study using proteomic approaches will be beneficial to uncover possible candidates.

Actin dynamics and organization are regulated by actin-binding proteins, and these processes are often mediated by the Rho family of small GTPases. Of all the members of the Rho GTPase family, Rac1, Cdc42, and Rho are best studied. Interestingly, their activity has been shown to regulate the formation and morphological changes of dendritic spines {Newey et al. 2005}. Previous studies suggest that activation of Rac1 results in increased spine formation and maturation, which are similar phenotypes shown in VASP-expressing neurons. In contrast, Rho activation appears to reduce spine density and length {Nakayama et al. 2000; Tashiro et al. 2000; Pilpel et al. 2004}. Hence, Rac1 may be involved in VASP's effect during spine development. Indeed, when Rac siRNA is introduced into hippocampal neurons, spine and synapse formation is decreased by 50% [data not shown]. These results indicate that Rac1 is critical for the formation of spines and synapses. Interestingly, when Rac1 siRNA was co-expressed with VASP, it abrogated VASP-mediated spine and synapse formation [data not shown]. The density of spines and synapses became almost identical in GFP and GFP-VASP expressing neurons when Rac1 siRNA is present [data not shown]. In contrast, scrambled siRNA had no effect on VASP-mediated spine/synapse formation. In addition, expression of Rac1 siRNA also aborogates VASP-mediated spine head

enlargement compared with the scrambled control [data not shown]. These data suggest that Rac1 is in the same signaling pathway of VASP-induced spine formation and spine head enlargement. Since Rac1 can be upstream or downstream of VASP family proteins {Higashi et al. 2009; Schlegel et al. 2009}, it will be important to address their signaling relationship. Moreover, it is important to address whether or not other Rho family GTPases are involved in VASP-mediated spine development in order to fully understand how VASP regulates this process.

We show here that VASP potentiates synaptic strength by increasing the amount of actin filaments and PSD proteins in spine heads [Figs. 18-25]. Interestingly, synaptic strength *in vivo* can be a very dynamic process that undergoes long-term modification and underlies cognitive functions. In fact, several intellectual disorders are associated with abnormalities in the number, size, and function of spines {Fiala et al. 2002; von Bohlen Und Halbach 2009}. Therefore, it is critical to understand if VASP expression influences synaptic plasticity and learning and memory using animal models.

REFERENCES

Abe, K., O. Chisaka, F. Van Roy and M. Takeichi (2004). "Stability of dendritic spines and synaptic contacts is controlled by alpha N-catenin." Nat Neurosci 7(4): 357-363.

Ackermann, M. and A. Matus (2003). "Activity-induced targeting of profilin and stabilization of dendritic spine morphology." Nat Neurosci 6(11): 1194-1200.

Ahern-Djamali, S. M., A. R. Comer, C. Bachmann, A. S. Kastenmeier, S. K. Reddy, M. C. Beckerle, U. Walter and F. M. Hoffmann (1998). "Mutations in Drosophila enabled and rescue by human vasodilator-stimulated phosphoprotein (VASP) indicate important functional roles for Ena/VASP homology domain 1 (EVH1) and EVH2 domains." Mol Biol Cell 9(8): 2157-2171.

Akin, O. and R. D. Mullins (2008). "Capping protein increases the rate of actin-based motility by promoting filament nucleation by the Arp2/3 complex." Cell 133(5): 841-851.

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter (2008). Molecular Biology of THE CELL, Garland Science.

Allison, D. W., A. S. Chervin, V. I. Gelfand and A. M. Craig (2000). "Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules." J Neurosci 20(12): 4545-4554.

Allison, D. W., V. I. Gelfand, I. Spector and A. M. Craig (1998). "Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors." J Neurosci 18(7): 2423-2436.

Applewhite, D. A., M. Barzik, S. Kojima, T. M. Svitkina, F. B. Gertler and G. G. Borisy (2007). "Ena/VASP proteins have an anti-capping independent function in filopodia formation." Mol Biol Cell 18(7): 2579-2591.

Arancio, O., I. Antonova, S. Gambaryan, S. M. Lohmann, J. S. Wood, D. S. Lawrence and R. D. Hawkins (2001). "Presynaptic role of cGMP-dependent protein kinase during long-lasting potentiation." J Neurosci 21(1): 143-149.

Arancio, O., E. R. Kandel and R. D. Hawkins (1995). "Activity-dependent long-term enhancement of transmitter release by presynaptic 3',5'-cyclic GMP in cultured hippocampal neurons." Nature 376(6535): 74-80.

Artola, A. and W. Singer (1993). "Long-term depression of excitatory synaptic transmission and its relationship to long-term potentiation." Trends Neurosci 16(11): 480-487.

Aszodi, A., A. Pfeifer, M. Ahmad, M. Glauner, X. H. Zhou, L. Ny, K. E. Andersson, B. Kehrel, S. Offermanns and R. Fassler (1999). "The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of

agonist-induced platelet aggregation, but is dispensable for smooth muscle function." EMBO J 18(1): 37-48.

Bachmann, C., L. Fischer, U. Walter and M. Reinhard (1999). "The EVH2 domain of the vasodilator-stimulated phosphoprotein mediates tetramerization, F-actin binding, and actin bundle formation." J Biol Chem 274(33): 23549-23557.

Barnes, A. P. and F. Polleux (2009). "Establishment of axon-dendrite polarity in developing neurons." Annu Rev Neurosci 32: 347-381.

Barria, A., D. Muller, V. Derkach, L. C. Griffith and T. R. Soderling (1997). "Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation." Science 276(5321): 2042-2045.

Barzik, M., T. I. Kotova, H. N. Higgs, L. Hazelwood, D. Hanein, F. B. Gertler and D. A. Schafer (2005). "Ena/VASP proteins enhance actin polymerization in the presence of barbed end capping proteins." J Biol Chem 280(31): 28653-28662.

Bashaw, G. J., T. Kidd, D. Murray, T. Pawson and C. S. Goodman (2000). "Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor." Cell 101(7): 703-715.

Bayer, K. U., P. De Koninck, A. S. Leonard, J. W. Hell and H. Schulman (2001). "Interaction with the NMDA receptor locks CaMKII in an active conformation." Nature 411(6839): 801-805.

Bear, J. E. and F. B. Gertler (2009). "Ena/VASP: towards resolving a pointed controversy at the barbed end." J Cell Sci 122(Pt 12): 1947-1953.

Bear, J. E., J. J. Loureiro, I. Libova, R. Fassler, J. Wehland and F. B. Gertler (2000). "Negative regulation of fibroblast motility by Ena/VASP proteins." Cell 101(7): 717-728.

Bear, J. E., T. M. Svitkina, M. Krause, D. A. Schafer, J. J. Loureiro, G. A. Strasser, I. V. Maly, O. Y. Chaga, J. A. Cooper, G. G. Borisy and F. B. Gertler (2002). "Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility." Cell 109(4): 509-521.

Bear, M. F., L. N. Cooper and F. F. Ebner (1987). "A physiological basis for a theory of synapse modification." Science 237(4810): 42-48.

Beattie, E. C., R. C. Carroll, X. Yu, W. Morishita, H. Yasuda, M. von Zastrow and R. C. Malenka (2000). "Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD." Nat Neurosci 3(12): 1291-1300.

Benz, P. M., C. Blume, S. Seifert, S. Wilhelm, J. Waschke, K. Schuh, F. Gertler, T. Munzel and T. Renne (2009). "Differential VASP phosphorylation controls remodeling of the actin cytoskeleton." J Cell Sci 122(Pt 21): 3954-3965.

Bliss, T. V. and A. R. Gardner-Medwin (1973). "Long-lasting potentiation of synaptic transmission in the dentate area of the unanaestetized rabbit following stimulation of the perforant path." J Physiol 232(2): 357-374.

Breitsprecher, D., A. K. Kiesewetter, J. Linkner, C. Urbanke, G. P. Resch, J. V. Small and J. Faix (2008). "Clustering of VASP actively drives processive, WH2 domain-mediated actin filament elongation." Embo J 27(22): 2943-2954.

Brindle, N. P., M. R. Holt, J. E. Davies, C. J. Price and D. R. Critchley (1996). "The focal-adhesion vasodilator-stimulated phosphoprotein (VASP) binds to the proline-rich domain in vinculin." Biochem J 318 (Pt 3): 753-757.

Bryce, N. S., E. S. Clark, J. L. Leysath, J. D. Currie, D. J. Webb and A. M. Weaver (2005). "Cortactin promotes cell motility by enhancing lamellipodial persistence." Curr Biol 15(14): 1276-1285.

Butt, E., K. Abel, M. Krieger, D. Palm, V. Hoppe, J. Hoppe and U. Walter (1994). "cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets." J Biol Chem 269(20): 14509-14517.

Campellone, K. G. and M. D. Welch (2010). "A nucleator arms race: cellular control of actin assembly." Nat Rev Mol Cell Biol 11(4): 237-251.

Carl, U. D., M. Pollmann, E. Orr, F. B. Gertlere, T. Chakraborty and J. Wehland (1999). "Aromatic and basic residues within the EVH1 domain of VASP specify its interaction with proline-rich ligands." Curr Biol 9(13): 715-718.

Carlier, M. F. (1990). "Actin polymerization and ATP hydrolysis." Adv Biophys 26: 51-73.

Carlin, R. K., D. J. Grab, R. S. Cohen and P. Siekevitz (1980). "Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities." J Cell Biol 86(3): 831-845.

Carneiro, P. A., M. E. Osugi, C. S. Fugivara, N. Boralle, M. Furlan and B. Z. MV (2005). "Evaluation of different electrochemical methods on the oxidation and degradation of Reactive Blue 4 in aqueous solution." Chemosphere 59(3): 431-439.

Chakraborty, T., F. Ebel, E. Domann, K. Niebuhr, B. Gerstel, S. Pistor, C. J. Temm-Grove, B. M. Jockusch, M. Reinhard, U. Walter and et al. (1995). "A focal adhesion factor directly linking intracellularly motile Listeria monocytogenes and Listeria ivanovii to the actin-based cytoskeleton of mammalian cells." EMBO J 14(7): 1314-1321.

Chan, A. Y., S. Raft, M. Bailly, J. B. Wyckoff, J. E. Segall and J. S. Condeelis (1998). "EGF stimulates an increase in actin nucleation and filament number at the leading edge of the lamellipod in mammary adenocarcinoma cells." J Cell Sci 111 (Pt 2): 199-211.

Chechlacz, M. and J. G. Gleeson (2003). "Is mental retardation a defect of synapse structure and function?" Pediatr Neurol 29(1): 11-17.

Chen, L., D. M. Chetkovich, R. S. Petralia, N. T. Sweeney, Y. Kawasaki, R. J. Wenthold, D. S. Bredt and R. A. Nicoll (2000). "Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms." Nature 408(6815): 936-943.

Chereau, D. and R. Dominguez (2006). "Understanding the role of the G-actin-binding domain of Ena/VASP in actin assembly." J Struct Biol 155(2): 195-201.

Chesarone, M. A. and B. L. Goode (2009). "Actin nucleation and elongation factors: mechanisms and interplay." Curr Opin Cell Biol 21(1): 28-37.

Chitaley, K., L. Chen, A. Galler, U. Walter, G. Daum and A. W. Clowes (2004). "Vasodilator-stimulated phosphoprotein is a substrate for protein kinase C." FEBS Lett 556(1-3): 211-215.

Cho, K. O., C. A. Hunt and M. B. Kennedy (1992). "The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein." Neuron 9(5): 929-942.

Christopherson, K. S., N. T. Sweeney, S. E. Craven, R. Kang, D. El-Husseini Ael and D. S. Bredt (2003). "Lipid- and protein-mediated multimerization of PSD-95: implications for receptor clustering and assembly of synaptic protein networks." J Cell Sci 116(Pt 15): 3213-3219.

Colavita, A. and J. G. Culotti (1998). "Suppressors of ectopic UNC-5 growth cone steering identify eight genes involved in axon guidance in Caenorhabditis elegans." Dev Biol 194(1): 72-85.

Colicos, M. A., B. E. Collins, M. J. Sailor and Y. Goda (2001). "Remodeling of synaptic actin induced by photoconductive stimulation." Cell 107(5): 605-616.

Collard, C. D., K. A. Park, M. C. Montalto, S. Alapati, J. A. Buras, G. L. Stahl and S. P. Colgan (2002). "Neutrophil-derived glutamate regulates vascular endothelial barrier function." J Biol Chem 277(17): 14801-14811.

Comerford, K. M., D. W. Lawrence, K. Synnestvedt, B. P. Levi and S. P. Colgan (2002). "Role of vasodilator-stimulated phosphoprotein in PKA-induced changes in endothelial junctional permeability." FASEB J 16(6): 583-585.

Correia, S. S., S. Bassani, T. C. Brown, M. F. Lise, D. S. Backos, A. El-Husseini, M. Passafaro and J. A. Esteban (2008). "Motor protein-dependent transport of AMPA receptors into spines during long-term potentiation." Nat Neurosci 11(4): 457-466.

Dailey, M. E. and S. J. Smith (1996). "The dynamics of dendritic structure in developing hippocampal slices." J Neurosci 16(9): 2983-2994.

Dent, E. W. and F. B. Gertler (2003). "Cytoskeletal dynamics and transport in growth cone motility and axon guidance." Neuron 40(2): 209-227.

Dent, E. W., A. V. Kwiatkowski, L. M. Mebane, U. Philippar, M. Barzik, D. A. Rubinson, S. Gupton, J. E. Van Veen, C. Furman, J. Zhang, A. S. Alberts, S. Mori and F. B. Gertler (2007). "Filopodia are required for cortical neurite initiation." Nat Cell Biol 9(12): 1347-1359.

Dillon, C. and Y. Goda (2005). "The actin cytoskeleton: integrating form and function at the synapse." Annu Rev Neurosci 28: 25-55.

Drees, F. and F. B. Gertler (2008). "Ena/VASP: proteins at the tip of the nervous system." Curr Opin Neurobiol 18(1): 53-59.

Du, Y., S. A. Weed, W. C. Xiong, T. D. Marshall and J. T. Parsons (1998). "Identification of a novel cortactin SH3 domain-binding protein and its localization to growth cones of cultured neurons." Mol Cell Biol 18(10): 5838-5851.

Dunaevsky, A., A. Tashiro, A. Majewska, C. Mason and R. Yuste (1999). "Developmental regulation of spine motility in the mammalian central nervous system." Proc Natl Acad Sci U S A 96(23): 13438-13443.

Dwivedy, A., F. B. Gertler, J. Miller, C. E. Holt and C. Lebrand (2007). "Ena/VASP function in retinal axons is required for terminal arborization but not pathway navigation." Development 134(11): 2137-2146.

Ehlers, M. D. (2000). "Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting." Neuron 28(2): 511-525.

Ehrlich, I. and R. Malinow (2004). "Postsynaptic density 95 controls AMPA receptor incorporation during long-term potentiation and experience-driven synaptic plasticity." J Neurosci 24(4): 916-927.

El-Husseini, A. E., E. Schnell, D. M. Chetkovich, R. A. Nicoll and D. S. Bredt (2000). "PSD-95 involvement in maturation of excitatory synapses." Science 290(5495): 1364-1368.

Ermekova, K. S., N. Zambrano, H. Linn, G. Minopoli, F. Gertler, T. Russo and M. Sudol (1997). "The WW domain of neural protein FE65 interacts with proline-rich motifs in Mena, the mammalian homolog of Drosophila enabled." J Biol Chem 272(52): 32869-32877.

Erondu, N. E. and M. B. Kennedy (1985). "Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain." J Neurosci 5(12): 3270-3277.

Ethell, I. M. and E. B. Pasquale (2005). "Molecular mechanisms of dendritic spine development and remodeling." Prog Neurobiol 75(3): 161-205.

Fedorov, A. A., E. Fedorov, F. Gertler and S. C. Almo (1999). "Structure of EVH1, a novel proline-rich ligand-binding module involved in cytoskeletal dynamics and neural function." Nat Struct Biol 6(7): 661-665.

Ferrer, I. and F. Gullotta (1990). "Down's syndrome and Alzheimer's disease: dendritic spine counts in the hippocampus." Acta Neuropathol 79(6): 680-685.

Ferron, F., G. Rebowski, S. H. Lee and R. Dominguez (2007). "Structural basis for the recruitment of profilin-actin complexes during filament elongation by Ena/VASP." EMBO J 26(21): 4597-4606.

Fiala, J. C., M. Feinberg, V. Popov and K. M. Harris (1998). "Synaptogenesis via dendritic filopodia in developing hippocampal area CA1." J Neurosci 18(21): 8900-8911.

Fiala, J. C., J. Spacek and K. M. Harris (2002). "Dendritic spine pathology: cause or consequence of neurological disorders?" Brain Res Brain Res Rev 39(1): 29-54.

Fifkova, E. and R. J. Delay (1982). "Cytoplasmic actin in neuronal processes as a possible mediator of synaptic plasticity." J Cell Biol 95(1): 345-350.

Fischer, M., S. Kaech, D. Knutti and A. Matus (1998). "Rapid actin-based plasticity in dendritic spines." Neuron 20(5): 847-854.

Forrester, W. C. and G. Garriga (1997). "Genes necessary for C. elegans cell and growth cone migrations." Development 124(9): 1831-1843.

Frost, N. A., J. M. Kerr, H. E. Lu and T. A. Blanpied (2010). "A network of networks: cytoskeletal control of compartmentalized function within dendritic spines." Curr Opin Neurobiol 20(5): 578-587.

Frost, N. A., H. Shroff, H. Kong, E. Betzig and T. A. Blanpied (2010). "Single-molecule discrimination of discrete perisynaptic and distributed sites of actin filament assembly within dendritic spines." Neuron 67(1): 86-99.

Fukazawa, Y., Y. Saitoh, F. Ozawa, Y. Ohta, K. Mizuno and K. Inokuchi (2003). "Hippocampal LTP is accompanied by enhanced F-actin content within the dendritic spine that is essential for late LTP maintenance in vivo." Neuron 38(3): 447-460.

Furman, C., A. L. Sieminski, A. V. Kwiatkowski, D. A. Rubinson, E. Vasile, R. T. Bronson, R. Fassler and F. B. Gertler (2007). "Ena/VASP is required for endothelial barrier function in vivo." J Cell Biol 179(4): 761-775.

Gerges, N. Z., D. S. Backos, C. N. Rupasinghe, M. R. Spaller and J. A. Esteban (2006). "Dual role of the exocyst in AMPA receptor targeting and insertion into the postsynaptic membrane." Embo J 25(8): 1623-1634.

Gertler, F. B., A. R. Comer, J. L. Juang, S. M. Ahern, M. J. Clark, E. C. Liebl and F. M. Hoffmann (1995). "enabled, a dosage-sensitive suppressor of mutations in the Drosophila Abl tyrosine kinase, encodes an Abl substrate with SH3 domain-binding properties." Genes Dev 9(5): 521-533.

Gertler, F. B., K. Niebuhr, M. Reinhard, J. Wehland and P. Soriano (1996). "Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics." Cell 87(2): 227-239.

Gitai, Z., T. W. Yu, E. A. Lundquist, M. Tessier-Lavigne and C. I. Bargmann (2003). "The netrin receptor UNC-40/DCC stimulates axon attraction and outgrowth through enabled and, in parallel, Rac and UNC-115/AbLIM." Neuron 37(1): 53-65.

Gloster, A., H. El-Bizri, S. X. Bamji, D. Rogers and F. D. Miller (1999). "Early induction of Talpha1 alpha-tubulin transcription in neurons of the developing nervous system." J Comp Neurol 405(1): 45-60.

Goh, K. L., L. Cai, C. L. Cepko and F. B. Gertler (2002). "Ena/VASP proteins regulate cortical neuronal positioning." Curr Biol 12(7): 565-569.

Goslin, K., H. Asmussen and G. Banker (1998). Rat hippocampal neurons in low-density culture. Cambridge, MA, MIT Press.

Gray, E. G. (1959). "Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study." J Anat 93: 420-433.

Grossman, A. W., G. M. Aldridge, I. J. Weiler and W. T. Greenough (2006). "Local protein synthesis and spine morphogenesis: Fragile X syndrome and beyond." J Neurosci 26(27): 7151-7155.

Halbrugge, M., C. Friedrich, M. Eigenthaler, P. Schanzenbacher and U. Walter (1990). "Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators." J Biol Chem 265(6): 3088-3093.

Hansen, S. D. and R. D. Mullins "VASP is a processive actin polymerase that requires monomeric actin for barbed end association." J Cell Biol 191(3): 571-584.

Harbeck, B., S. Huttelmaier, K. Schluter, B. M. Jockusch and S. Illenberger (2000). "Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin." J Biol Chem 275(40): 30817-30825.

Harris, K. M., F. E. Jensen and B. Tsao (1992). "Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation." J Neurosci 12(7): 2685-2705.

Harris, K. M. and S. B. Kater (1994). "Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function." Annu Rev Neurosci 17: 341-371.

Hauser, W., K. P. Knobeloch, M. Eigenthaler, S. Gambaryan, V. Krenn, J. Geiger, M. Glazova, E. Rohde, I. Horak, U. Walter and M. Zimmer (1999). "Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice." Proc Natl Acad Sci U S A 96(14): 8120-8125.

Higashi, M., C. Ishikawa, J. Yu, A. Toyoda, H. Kawana, K. Kurokawa, M. Matsuda, M. Kitagawa and K. Harigaya (2009). "Human Mena associates with Rac1 small GTPase in glioblastoma cell lines." PLoS ONE 4(3): e4765.

Hoffman, L. M., C. C. Jensen, S. Kloeker, C. L. Wang, M. Yoshigi and M. C. Beckerle (2006). "Genetic ablation of zyxin causes Mena/VASP mislocalization, increased motility, and deficits in actin remodeling." J Cell Biol 172(5): 771-782.

Honkura, N., M. Matsuzaki, J. Noguchi, G. C. Ellis-Davies and H. Kasai (2008). "The subspine organization of actin fibers regulates the structure and plasticity of dendritic spines." Neuron 57(5): 719-729.

Horstrup, K., B. Jablonka, P. Honig-Liedl, M. Just, K. Kochsiek and U. Walter (1994). "Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition." Eur J Biochem 225(1): 21-27.

Hotulainen, P., O. Llano, S. Smirnov, K. Tanhuanpaa, J. Faix, C. Rivera and P. Lappalainen (2009). "Defining mechanisms of actin polymerization and depolymerization during dendritic spine morphogenesis." J Cell Biol 185(2): 323-339.

Howe, A. K., B. P. Hogan and R. L. Juliano (2002). "Regulation of vasodilator-stimulated phosphoprotein phosphorylation and interaction with Abl by protein kinase A and cell adhesion." J Biol Chem 277(41): 38121-38126.

Huber, A. B., A. L. Kolodkin, D. D. Ginty and J. F. Cloutier (2003). "Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance." Annu Rev Neurosci 26: 509-563.

Hung, A. Y., K. Futai, C. Sala, J. G. Valtschanoff, J. Ryu, M. A. Woodworth, F. L. Kidd, C. C. Sung, T. Miyakawa, M. F. Bear, R. J. Weinberg and M. Sheng (2008). "Smaller dendritic spines, weaker synaptic transmission, but enhanced spatial learning in mice lacking Shank1." J Neurosci 28(7): 1697-1708.

Huttelmaier, S., B. Harbeck, O. Steffens, T. Messerschmidt, S. Illenberger and B. M. Jockusch (1999). "Characterization of the actin binding properties of the vasodilator-stimulated phosphoprotein VASP." FEBS Lett 451(1): 68-74.

Kameyama, K., H. K. Lee, M. F. Bear and R. L. Huganir (1998). "Involvement of a postsynaptic protein kinase A substrate in the expression of homosynaptic long-term depression." Neuron 21(5): 1163-1175.

Kang, F., R. O. Laine, M. R. Bubb, F. S. Southwick and D. L. Purich (1997). "Profilin interacts with the Gly-Pro-Pro-Pro-Pro-Pro sequences of vasodilator-stimulated phosphoprotein (VASP): implications for actin-based Listeria motility." Biochemistry 36(27): 8384-8392.

Kim, C. H. and J. E. Lisman (1999). "A role of actin filament in synaptic transmission and long-term potentiation." J Neurosci 19(11): 4314-4324.

Kim, E., S. Naisbitt, Y. P. Hsueh, A. Rao, A. Rothschild, A. M. Craig and M. Sheng (1997). "GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules." J Cell Biol 136(3): 669-678.

Kim, E. and M. Sheng (2004). "PDZ domain proteins of synapses." Nat Rev Neurosci 5(10): 771-781.

Kistner, U., B. M. Wenzel, R. W. Veh, C. Cases-Langhoff, A. M. Garner, U. Appeltauer, B. Voss, E. D. Gundelfinger and C. C. Garner (1993). "SAP90, a rat presynaptic protein related to the product of the Drosophila tumor suppressor gene dlg-A." J Biol Chem 268(7): 4580-4583.

Korobova, F. and T. Svitkina "Molecular architecture of synaptic actin cytoskeleton in hippocampal neurons reveals a mechanism of dendritic spine morphogenesis." Mol Biol Cell 21(1): 165-176.

Krause, M., J. E. Bear, J. J. Loureiro and F. B. Gertler (2002). "The Ena/VASP enigma." J Cell Sci 115(Pt 24): 4721-4726.

Krause, M., E. W. Dent, J. E. Bear, J. J. Loureiro and F. B. Gertler (2003). "Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration." Annu Rev Cell Dev Biol 19: 541-564.

Krause, M., J. D. Leslie, M. Stewart, E. M. Lafuente, F. Valderrama, R. Jagannathan, G. A. Strasser, D. A. Rubinson, H. Liu, M. Way, M. B. Yaffe, V. A. Boussiotis and F. B. Gertler (2004). "Lamellipodin, an Ena/VASP ligand, is implicated in the regulation of lamellipodial dynamics." Dev Cell 7(4): 571-583.

Kris, A. S., R. D. Kamm and A. L. Sieminski (2008). "VASP involvement in force-mediated adherens junction strengthening." Biochem Biophys Res Commun 375(1): 134-138.

Krucker, T., G. R. Siggins and S. Halpain (2000). "Dynamic actin filaments are required for stable long-term potentiation (LTP) in area CA1 of the hippocampus." Proc Natl Acad Sci U S A 97(12): 6856-6861.

Kuriu, T., A. Inoue, H. Bito, K. Sobue and S. Okabe (2006). "Differential control of postsynaptic density scaffolds via actin-dependent and -independent mechanisms." J Neurosci 26(29): 7693-7706.

Kwiatkowski, A. V., F. B. Gertler and J. J. Loureiro (2003). "Function and regulation of Ena/VASP proteins." Trends Cell Biol 13(7): 386-392.

Kwiatkowski, A. V., D. A. Rubinson, E. W. Dent, J. Edward van Veen, J. D. Leslie, J. Zhang, L. M. Mebane, U. Philippar, E. M. Pinheiro, A. A. Burds, R. T. Bronson, S. Mori, R. Fassler and F. B. Gertler (2007). "Ena/VASP Is Required for neuritogenesis in the developing cortex." Neuron 56(3): 441-455.

Lafuente, E. M., A. A. van Puijenbroek, M. Krause, C. V. Carman, G. J. Freeman, A. Berezovskaya, E. Constantine, T. A. Springer, F. B. Gertler and V. A. Boussiotis (2004). "RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion." Dev Cell 7(4): 585-595.

Landis, D. M. and T. S. Reese (1983). "Cytoplasmic organization in cerebellar dendritic spines." J Cell Biol 97(4): 1169-1178.

Lanier, L. M., M. A. Gates, W. Witke, A. S. Menzies, A. M. Wehman, J. D. Macklis, D. Kwiatkowski, P. Soriano and F. B. Gertler (1999). "Mena is required for neurulation and commissure formation." Neuron 22(2): 313-325.

Lanier, L. M. and F. B. Gertler (2000). "From Abl to actin: Abl tyrosine kinase and associated proteins in growth cone motility." Curr Opin Neurobiol 10(1): 80-87.

Laurent, V., T. P. Loisel, B. Harbeck, A. Wehman, L. Grobe, B. M. Jockusch, J. Wehland, F. B. Gertler and M. F. Carlier (1999). "Role of proteins of the Ena/VASP family in actin-based motility of Listeria monocytogenes." J Cell Biol 144(6): 1245-1258.

Lebrand, C., E. W. Dent, G. A. Strasser, L. M. Lanier, M. Krause, T. M. Svitkina, G. G. Borisy and F. B. Gertler (2004). "Critical role of Ena/VASP proteins for filopodia formation in neurons and in function downstream of netrin-1." Neuron 42(1): 37-49.

Lee, H. K., K. Kameyama, R. L. Huganir and M. F. Bear (1998). "NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus." Neuron 21(5): 1151-1162.

Lee, S. and C. Y. Chung (2009). "Role of VASP phosphorylation for the regulation of microglia chemotaxis via the regulation of focal adhesion formation/maturation." Mol Cell Neurosci 42(4): 382-390.

Leonard, A. S., I. A. Lim, D. E. Hemsworth, M. C. Horne and J. W. Hell (1999). "Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor." Proc Natl Acad Sci U S A 96(6): 3239-3244.

Li, Z. and M. Sheng (2003). "Some assembly required: the development of neuronal synapses." Nat Rev Mol Cell Biol 4(11): 833-841.

Lin, B., E. A. Kramar, X. Bi, F. A. Brucher, C. M. Gall and G. Lynch (2005). "Theta stimulation polymerizes actin in dendritic spines of hippocampus." J Neurosci 25(8): 2062-2069.

Lin, W. H. and D. J. Webb (2009). "Actin and Actin-Binding Proteins: Masters of Dendritic Spine Formation, Morphology, and Function." Open Neurosci J 3: 54-66.

Lin, Y. L., Y. T. Lei, C. J. Hong and Y. P. Hsueh (2007). "Syndecan-2 induces filopodia and dendritic spine formation via the neurofibromin-PKA-Ena/VASP pathway." J Cell Biol 177(5): 829-841.

Lindsay, S. L., S. Ramsey, M. Aitchison, T. Renne and T. J. Evans (2007). "Modulation of lamellipodial structure and dynamics by NO-dependent phosphorylation of VASP Ser239." J Cell Sci 120(Pt 17): 3011-3021.

Lise, M. F., T. P. Wong, A. Trinh, R. M. Hines, L. Liu, R. Kang, D. J. Hines, J. Lu, J. R. Goldenring, Y. T. Wang and A. El-Husseini (2006). "Involvement of myosin Vb in glutamate receptor trafficking." J Biol Chem 281(6): 3669-3678.

Lisman, J. (1989). "A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory." Proc Natl Acad Sci U S A 86(23): 9574-9578.

Lodish, H., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore and J. Darnell (2000). Molecular Cell Biology, W. H. Freeman and company**:** 763.

Loureiro, J. J., D. A. Rubinson, J. E. Bear, G. A. Baltus, A. V. Kwiatkowski and F. B. Gertler (2002). "Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/vasodilator-stimulated phosphoprotein (VASP) function during cell migration." Mol Biol Cell 13(7): 2533-2546.

Lu, W., H. Man, W. Ju, W. S. Trimble, J. F. MacDonald and Y. T. Wang (2001). "Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons." Neuron 29(1): 243-254.

Malenka, R. C. and M. F. Bear (2004). "LTP and LTD: an embarrassment of riches." Neuron 44(1): 5-21.

Malenka, R. C. and R. A. Nicoll (1999). "Long-term potentiation--a decade of progress?" Science 285(5435): 1870-1874.

Maletic-Savatic, M., R. Malinow and K. Svoboda (1999). "Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity." Science 283(5409): 1923-1927.

Malinow, R. and R. C. Malenka (2002). "AMPA receptor trafficking and synaptic plasticity." Annu Rev Neurosci 25: 103-126.

Man, H. Y., J. W. Lin, W. H. Ju, G. Ahmadian, L. Liu, L. E. Becker, M. Sheng and Y. T. Wang (2000). "Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization." Neuron 25(3): 649-662.

Marrs, G. S., S. H. Green and M. E. Dailey (2001). "Rapid formation and remodeling of postsynaptic densities in developing dendrites." Nat Neurosci 4(10): 1006-1013.

Matsuzaki, M., G. C. Ellis-Davies, T. Nemoto, Y. Miyashita, M. Iino and H. Kasai (2001). "Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons." Nat Neurosci 4(11): 1086-1092.

Matsuzaki, M., N. Honkura, G. C. Ellis-Davies and H. Kasai (2004). "Structural basis of long-term potentiation in single dendritic spines." Nature 429(6993): 761-766.

Matus, A. (2000). "Actin-based plasticity in dendritic spines." Science 290(5492): 754-758.

Matus, A., M. Ackermann, G. Pehling, H. R. Byers and K. Fujiwara (1982). "High actin concentrations in brain dendritic spines and postsynaptic densities." Proc Natl Acad Sci U S A 79(23): 7590-7594.

Mendez, P., M. De Roo, L. Poglia, P. Klauser and D. Muller (2010). "N-cadherin mediates plasticity-induced long-term spine stabilization." J Cell Biol 189(3): 589-600.

Menzies, A. S., A. Aszodi, S. E. Williams, A. Pfeifer, A. M. Wehman, K. L. Goh, C. A. Mason, R. Fassler and F. B. Gertler (2004). "Mena and vasodilator-stimulated phosphoprotein are required for multiple actin-dependent processes that shape the vertebrate nervous system." J Neurosci 24(37): 8029-8038.

Miller, M. (1981). "Maturation of rat visual cortex. I. A quantitative study of Golgi-impregnated pyramidal neurons." J Neurocytol 10(5): 859-878.

Morest, D. K. (1969). "The growth of dendrites in the mammalian brain." Z Anat Entwicklungsgesch 128(4): 290-317.

Morishita, W., J. H. Connor, H. Xia, E. M. Quinlan, S. Shenolikar and R. C. Malenka (2001). "Regulation of synaptic strength by protein phosphatase 1." Neuron 32(6): 1133-1148.

Moss, F. J., P. Viard, A. Davies, F. Bertaso, K. M. Page, A. Graham, C. Canti, M. Plumpton, C. Plumpton, J. J. Clare and A. C. Dolphin (2002). "The novel product of a five-exon stargazin-related gene abolishes Ca(V)2.2 calcium channel expression." Embo J 21(7): 1514-1523.

Murase, S., E. Mosser and E. M. Schuman (2002). "Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function." Neuron 35(1): 91-105.

Naisbitt, S., E. Kim, J. C. Tu, B. Xiao, C. Sala, J. Valtschanoff, R. J. Weinberg, P. F. Worley and M. Sheng (1999). "Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin." Neuron 23(3): 569-582.

Naisbitt, S., E. Kim, R. J. Weinberg, A. Rao, F. C. Yang, A. M. Craig and M. Sheng (1997). "Characterization of guanylate kinase-associated protein, a postsynaptic density protein at excitatory synapses that interacts directly with postsynaptic density-95/synapse-associated protein 90." J Neurosci 17(15): 5687-5696.

Nakayama, A. Y., M. B. Harms and L. Luo (2000). "Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons." J Neurosci 20(14): 5329-5338.

Newey, S. E., V. Velamoor, E. E. Govek and L. Van Aelst (2005). "Rho GTPases, dendritic structure, and mental retardation." J Neurobiol 64(1): 58-74.

Nicoll, R. A., S. Tomita and D. S. Bredt (2006). "Auxiliary subunits assist AMPA-type glutamate receptors." Science 311(5765): 1253-1256.

Niebuhr, K., F. Ebel, R. Frank, M. Reinhard, E. Domann, U. D. Carl, U. Walter, F. B. Gertler, J. Wehland and T. Chakraborty (1997). "A novel proline-rich motif present in ActA of Listeria monocytogenes and cytoskeletal proteins is the ligand for the EVH1 domain, a protein module present in the Ena/VASP family." Embo J 16(17): 5433-5444.

Niethammer, M., E. Kim and M. Sheng (1996). "Interaction between the C terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases." J Neurosci 16(7): 2157-2163.

Noctor, S. C., V. Martinez-Cerdeno, L. Ivic and A. R. Kriegstein (2004). "Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases." Nat Neurosci 7(2): 136-144.

Nolte, C., M. Eigenthaler, P. Schanzenbacher and U. Walter (1991). "Endothelial cell-dependent phosphorylation of a platelet protein mediated by cAMP- and cGMP-elevating factors." J Biol Chem 266(22): 14808-14812.

O'Brien, R. J., L. F. Lau and R. L. Huganir (1998). "Molecular mechanisms of glutamate receptor clustering at excitatory synapses." Curr Opin Neurobiol 8(3): 364-369.

Okabe, S., A. Miwa and H. Okado (2001). "Spine formation and correlated assembly of presynaptic and postsynaptic molecules." J Neurosci 21(16): 6105-6114.

Okamoto, K., H. Imbe, A. Tashiro, S. Kumabe and E. Senba (2004). "Blockade of peripheral 5HT3 receptor attenuates the formalin-induced nocifensive behavior in persistent temporomandibular joint inflammation of rat." Neurosci Lett 367(2): 259-263.

Okamoto, K., R. Narayanan, S. H. Lee, K. Murata and Y. Hayashi (2007). "The role of CaMKII as an F-actin-bundling protein crucial for maintenance of dendritic spine structure." Proc Natl Acad Sci U S A 104(15): 6418-6423.

Olson, E. C. and C. A. Walsh (2002). "Smooth, rough and upside-down neocortical development." Curr Opin Genet Dev 12(3): 320-327.

Oosawa, F. and S. Asakura (1975). "Theory of polymerization equilibrium." In Thermodynamics of the polymerization of proteins: 109–116.

Osterweil, E., D. G. Wells and M. S. Mooseker (2005). "A role for myosin VI in postsynaptic structure and glutamate receptor endocytosis." J Cell Biol 168(2): 329-338.

Palay, S. L. (1956). "Synapses in the central nervous system." J Biophys Biochem Cytol 2(4 Suppl): 193-202.

Park, M., E. C. Penick, J. G. Edwards, J. A. Kauer and M. D. Ehlers (2004). "Recycling endosomes supply AMPA receptors for LTP." Science 305(5692): 1972-1975.

Park, M., J. M. Salgado, L. Ostroff, T. D. Helton, C. G. Robinson, K. M. Harris and M. D. Ehlers (2006). "Plasticity-induced growth of dendritic spines by exocytic trafficking from recycling endosomes." Neuron 52(5): 817-830.

Pasic, L., T. Kotova and D. A. Schafer (2008). "Ena/VASP proteins capture actin filament barbed ends." J Biol Chem 283(15): 9814-9819.

Peters, A. and I. R. Kaiserman-Abramof (1970). "The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines." Am J Anat 127(4): 321-355.

Pilpel, Y. and M. Segal (2004). "Activation of PKC induces rapid morphological plasticity in dendrites of hippocampal neurons via Rac and Rho-dependent mechanisms." Eur J Neurosci 19(12): 3151-3164.

Pollard, T. D. and J. A. Cooper (1984). "Quantitative analysis of the effect of Acanthamoeba profilin on actin filament nucleation and elongation." Biochemistry 23(26): 6631-6641.

Pontrello, C. G. and I. M. Ethell (2009). "Accelerators, Brakes, and Gears of Actin Dynamics in Dendritic Spines." Open Neurosci J 3: 67-86.

Pratt, K. G., A. J. Watt, L. C. Griffith, S. B. Nelson and G. G. Turrigiano (2003). "Activity-dependent remodeling of presynaptic inputs by postsynaptic expression of activated CaMKII." Neuron 39(2): 269-281.

Prehoda, K. E., D. J. Lee and W. A. Lim (1999). "Structure of the enabled/VASP homology 1 domain-peptide complex: a key component in the spatial control of actin assembly." Cell 97(4): 471-480.

Pring, M., A. Weber and M. R. Bubb (1992). "Profilin-actin complexes directly elongate actin filaments at the barbed end." Biochemistry 31(6): 1827-1836.

Reinhard, M., K. Giehl, K. Abel, C. Haffner, T. Jarchau, V. Hoppe, B. M. Jockusch and U. Walter (1995). "The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins." Embo J 14(8): 1583-1589.

Reinhard, M., M. Halbrugge, U. Scheer, C. Wiegand, B. M. Jockusch and U. Walter (1992). "The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts." Embo J 11(6): 2063-2070.

Reinhard, M., K. Jouvenal, D. Tripier and U. Walter (1995). "Identification, purification, and characterization of a zyxin-related protein that binds the focal adhesion and microfilament protein VASP (vasodilator-stimulated phosphoprotein)." Proc Natl Acad Sci U S A 92(17): 7956-7960.

Reinhard, M., M. Rudiger, B. M. Jockusch and U. Walter (1996). "VASP interaction with vinculin: a recurring theme of interactions with proline-rich motifs." FEBS Lett 399(1-2): 103-107.

Romorini, S., G. Piccoli, M. Jiang, P. Grossano, N. Tonna, M. Passafaro, M. Zhang and C. Sala (2004). "A functional role of postsynaptic density-95-guanylate kinase-associated protein complex in regulating Shank assembly and stability to synapses." J Neurosci 24(42): 9391-9404.

Rottner, K., B. Behrendt, J. V. Small and J. Wehland (1999). "VASP dynamics during lamellipodia protrusion." Nat Cell Biol 1(5): 321-322.

Ryu, J., L. Liu, T. P. Wong, D. C. Wu, A. Burette, R. Weinberg, Y. T. Wang and M. Sheng (2006). "A critical role for myosin IIb in dendritic spine morphology and synaptic function." Neuron 49(2): 175-182.

Sala, C., V. Piech, N. R. Wilson, M. Passafaro, G. Liu and M. Sheng (2001). "Regulation of dendritic spine morphology and synaptic function by Shank and Homer." Neuron 31(1): 115-130.

Sala, C., G. Roussignol, J. Meldolesi and L. Fagni (2005). "Key role of the postsynaptic density scaffold proteins Shank and Homer in the functional architecture of Ca2+ homeostasis at dendritic spines in hippocampal neurons." J Neurosci 25(18): 4587-4592.

Satoh, K., H. Yanai, T. Senda, K. Kohu, T. Nakamura, N. Okumura, A. Matsumine, S. Kobayashi, K. Toyoshima and T. Akiyama (1997). "DAP-1, a novel protein that interacts with the guanylate kinase-like domains of hDLG and PSD-95." Genes Cells 2(6): 415-424.

Schikorski, T. and C. F. Stevens (1997). "Quantitative ultrastructural analysis of hippocampal excitatory synapses." J Neurosci 17(15): 5858-5867.

Schlegel, N. and J. Waschke (2009). "Impaired integrin-mediated adhesion contributes to reduced barrier properties in VASP-deficient microvascular endothelium." J Cell Physiol 220(2): 357-366.

Schlegel, N. and J. Waschke (2009). "VASP is involved in cAMP-mediated Rac 1 activation in microvascular endothelial cells." Am J Physiol Cell Physiol 296(3): C453-462.

Schnell, E., M. Sizemore, S. Karimzadegan, L. Chen, D. S. Bredt and R. A. Nicoll (2002). "Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number." Proc Natl Acad Sci U S A 99(21): 13902-13907.

Scott, J. A., A. M. Shewan, N. R. den Elzen, J. J. Loureiro, F. B. Gertler and A. S. Yap (2006). "Ena/VASP proteins can regulate distinct modes of actin organization at cadherin-adhesive contacts." Mol Biol Cell 17(3): 1085-1095.

Serulle, Y., S. Zhang, I. Ninan, D. Puzzo, M. McCarthy, L. Khatri, O. Arancio and E. B. Ziff (2007). "A GluR1-cGKII interaction regulates AMPA receptor trafficking." Neuron 56(4): 670-688.

Sheng, M. and C. C. Hoogenraad (2007). "The postsynaptic architecture of excitatory synapses: a more quantitative view." Annu Rev Biochem 76: 823-847.

Sheng, M. and M. J. Kim (2002). "Postsynaptic signaling and plasticity mechanisms." Science 298(5594): 776-780.

Shepherd, J. D. and R. L. Huganir (2007). "The cell biology of synaptic plasticity: AMPA receptor trafficking." Annu Rev Cell Dev Biol 23: 613-643.

Skoble, J., V. Auerbuch, E. D. Goley, M. D. Welch and D. A. Portnoy (2001). "Pivotal role of VASP in Arp2/3 complex-mediated actin nucleation, actin branch-formation, and Listeria monocytogenes motility." J Cell Biol 155(1): 89-100.

Skwarek-Maruszewska, A., M. Boczkowska, A. L. Zajac, E. Kremneva, T. Svitkina, R. Dominguez and P. Lappalainen (2010). "Different localizations and cellular behaviors of leiomodin and tropomodulin in mature cardiomyocyte sarcomeres." Mol Biol Cell 21(19): 3352-3361.

Smolenski, A., W. Poller, U. Walter and S. M. Lohmann (2000). "Regulation of human endothelial cell focal adhesion sites and migration by cGMP-dependent protein kinase I." J Biol Chem 275(33): 25723-25732.

Song, H. J. and M. M. Poo (1999). "Signal transduction underlying growth cone guidance by diffusible factors." Curr Opin Neurobiol 9(3): 355-363.

Sorra, K. E. and K. M. Harris (2000). "Overview on the structure, composition, function, development, and plasticity of hippocampal dendritic spines." Hippocampus 10(5): 501-511.

Sotelo, C. (1990). "Axonal abnormalities in cerebellar Purkinje cells of the 'hyperspiny Purkinje cell' mutant mouse." J Neurocytol 19(5): 737-755.

Sotelo, C. (1990). "Cerebellar synaptogenesis: what we can learn from mutant mice." J Exp Biol 153: 225-249.

Suetsugu, M. and P. Mehraein (1980). "Spine distribution along the apical dendrites of the pyramidal neurons in Down's syndrome. A quantitative Golgi study." Acta Neuropathol 50(3): 207-210.

Sur, M. and J. L. Rubenstein (2005). "Patterning and plasticity of the cerebral cortex." Science 310(5749): 805-810.

Swann, J. W., S. Al-Noori, M. Jiang and C. L. Lee (2000). "Spine loss and other dendritic abnormalities in epilepsy." Hippocampus 10(5): 617-625.

Tahirovic, S. and F. Bradke (2009). "Neuronal polarity." Cold Spring Harb Perspect Biol 1(3): a001644.

Takacs, J., G. Gombos, T. Gorcs, T. Becker, J. de Barry and J. Hamori (1997). "Distribution of metabotropic glutamate receptor type 1a in Purkinje cell dendritic spines is independent of the presence of presynaptic parallel fibers." J Neurosci Res 50(3): 433-442.

Takeichi, M. (1991). "Cadherin cell adhesion receptors as a morphogenetic regulator." Science 251(5000): 1451-1455.

Takeuchi, M., Y. Hata, K. Hirao, A. Toyoda, M. Irie and Y. Takai (1997). "SAPAPs. A family of PSD-95/SAP90-associated proteins localized at postsynaptic density." J Biol Chem 272(18): 11943-11951.

Takumi, Y., V. Ramirez-Leon, P. Laake, E. Rinvik and O. P. Ottersen (1999). "Different modes of expression of AMPA and NMDA receptors in hippocampal synapses." Nat Neurosci 2(7): 618-624.

Tashiro, A., A. Minden and R. Yuste (2000). "Regulation of dendritic spine morphology by the rho family of small GTPases: antagonistic roles of Rac and Rho." Cereb Cortex 10(10): 927-938.

Tatavarty, V., E. J. Kim, V. Rodionov and J. Yu (2009). "Investigating sub-spine actin dynamics in rat hippocampal neurons with super-resolution optical imaging." PLoS ONE 4(11): e7724.

Tilney, L. G., E. M. Bonder, L. M. Coluccio and M. S. Mooseker (1983). "Actin from Thyone sperm assembles on only one end of an actin filament: a behavior regulated by profilin." J Cell Biol 97(1): 112-124.

Togashi, H., K. Abe, A. Mizoguchi, K. Takaoka, O. Chisaka and M. Takeichi (2002). "Cadherin regulates dendritic spine morphogenesis." Neuron 35(1): 77-89.

Trachtenberg, J. T., B. E. Chen, G. W. Knott, G. Feng, J. R. Sanes, E. Welker and K. Svoboda (2002). "Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex." Nature 420(6917): 788-794.

Tu, J. C., B. Xiao, S. Naisbitt, J. P. Yuan, R. S. Petralia, P. Brakeman, A. Doan, V. K. Aakalu, A. A. Lanahan, M. Sheng and P. F. Worley (1999). "Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins." Neuron 23(3): 583-592.

Tu, J. C., B. Xiao, J. P. Yuan, A. A. Lanahan, K. Leoffert, M. Li, D. J. Linden and P. F. Worley (1998). "Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors." Neuron 21(4): 717-726.

Tyska, M. J. and M. S. Mooseker (2002). "MYO1A (brush border myosin I) dynamics in the brush border of LLC-PK1-CL4 cells." Biophys J 82(4): 1869-1883.

von Bohlen Und Halbach, O. (2009). "Structure and function of dendritic spines within the hippocampus." Ann Anat 191(6): 518-531.

Waites, C. L., A. M. Craig and C. C. Garner (2005). "Mechanisms of vertebrate synaptogenesis." Annu Rev Neurosci 28: 251-274.

Walders-Harbeck, B., S. Y. Khaitlina, H. Hinssen, B. M. Jockusch and S. Illenberger (2002). "The vasodilator-stimulated phosphoprotein promotes actin polymerisation through direct binding to monomeric actin." FEBS Lett 529(2-3): 275-280.

Wang, H. G., F. M. Lu, I. Jin, H. Udo, E. R. Kandel, J. de Vente, U. Walter, S. M. Lohmann, R. D. Hawkins and I. Antonova (2005). "Presynaptic and postsynaptic roles of NO, cGK, and RhoA in long-lasting potentiation and aggregation of synaptic proteins." Neuron 45(3): 389-403.

Wang, Z., J. G. Edwards, N. Riley, D. W. Provance, Jr., R. Karcher, X. D. Li, I. G. Davison, M. Ikebe, J. A. Mercer, J. A. Kauer and M. D. Ehlers (2008). "Myosin Vb mobilizes recycling endosomes and AMPA receptors for postsynaptic plasticity." Cell 135(3): 535-548.

Wegner, A. M., C. A. Nebhan, L. Hu, D. Majumdar, K. M. Meier, A. M. Weaver and D. J. Webb (2008). "N-wasp and the arp2/3 complex are critical regulators of actin in the development of dendritic spines and synapses." J Biol Chem 283(23): 15912-15920.

Xiao, B., J. C. Tu, R. S. Petralia, J. P. Yuan, A. Doan, C. D. Breder, A. Ruggiero, A. A. Lanahan, R. J. Wenthold and P. F. Worley (1998). "Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins." Neuron 21(4): 707-716.

Yagi, T. and M. Takeichi (2000). "Cadherin superfamily genes: functions, genomic organization, and neurologic diversity." Genes Dev 14(10): 1169-1180.

Yu, T. W., J. C. Hao, W. Lim, M. Tessier-Lavigne and C. I. Bargmann (2002). "Shared receptors in axon guidance: SAX-3/Robo signals via UNC-34/Enabled and a Netrin-independent UNC-40/DCC function." Nat Neurosci 5(11): 1147-1154.

Yudowski, G. A., M. A. Puthenveedu, D. Leonoudakis, S. Panicker, K. S. Thorn, E. C. Beattie and M. von Zastrow (2007). "Real-time imaging of discrete exocytic events mediating surface delivery of AMPA receptors." J Neurosci 27(41): 11112-11121.

Zhang, H. and I. G. Macara (2008). "The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase." Dev Cell 14(2): 216-226.

Zhang, H., D. J. Webb, H. Asmussen and A. F. Horwitz (2003). "Synapse formation is regulated by the signaling adaptor GIT1." J Cell Biol 161(1): 131-142.

Zhang, H., D. J. Webb, H. Asmussen, S. Niu and A. F. Horwitz (2005). "A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC." J Neurosci 25(13): 3379-3388.

Zhang, L., B. W. Gillespie, C. F. Marrs and B. Foxman (2001). "Optimization of a fluorescent-based phosphor imaging dot blot DNA hybridization assay to assess E. coli virulence gene profiles." J Microbiol Methods 44(3): 225-233.

Zhang, W. and D. L. Benson (2001). Stages of synapse development defined by dependence on F-actin. J Neurosci. 21**:** 5169-5181.

Zhou, Q., M. Xiao and R. A. Nicoll (2001). "Contribution of cytoskeleton to the internalization of AMPA receptors." Proc Natl Acad Sci U S A 98(3): 1261-1266.

Zimmermann, J., D. Labudde, T. Jarchau, U. Walter, H. Oschkinat and L. J. Ball (2002). "Relaxation, equilibrium oligomerization, and molecular symmetry of the VASP (336-380) EVH2 tetramer." Biochemistry 41(37): 11143-11151.

Zito, K., G. Knott, G. M. Shepherd, S. Shenolikar and K. Svoboda (2004). "Induction of spine growth and synapse formation by regulation of the spine actin cytoskeleton." Neuron 44(2): 321-334.

Ziv, N. E. and S. J. Smith (1996). "Evidence for a role of dendritic filopodia in synaptogenesis and spine formation." Neuron 17(1): 91-102.

Zuber, B., I. Nikonenko, P. Klauser, D. Muller and J. Dubochet (2005). "The mammalian central nervous synaptic cleft contains a high density of periodically organized complexes." Proc Natl Acad Sci U S A 102(52): 19192-19197.