The role of filopodia in the formation of spine synapses

by

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Abstract

In the mammalian brain, excitatory (glutamatergic) synapses are mainly located on dendritic spines; bulbous protrusions enriched with F-actin. Dendritic filopodia are thin protrusions thought to be involved in the development of spines. However, limited evidence illustrating the emergence of spines from filopodia has been found. In addition, the molecular machinery required for filopodia induction and transformation to spines is not well understood. Paralemmin-1 has been shown to induce cell expansion and process formation and is concentrated at the plasma membrane, in part through a lipid modification known as palmitoylation. Palmitoylation of paralemmin-1 may also serve as a signal for its delivery to subcellular lipid microdomains to induce changes in cell morphology and membrane dynamics making it a candidate synapse-inducing molecule. Using live imaging as well as loss and gain-of-function approaches, our analysis identifies paralemmin-1 as a regulator of filopodia induction, synapse formation, and spine maturation. We show neuronal activity-driven translocation of paralemmin-1 to membranes induces rapid protrusion expansion, emphasizing the importance of paralemmin-1 in paradigms that control structural changes associated with synaptic plasticity and learning. Finally, we show that knockdown of paralemmin-1 results in loss of filopodia and compromises spine maturation induced by Shank1b, a protein that facilitates rapid transformation of newly formed filopodia to spines.

To investigate the role of filopodia in synapse formation, we contrasted the roles of molecules that affect filopodia elaboration and motility, versus those that impact synapse induction and maturation. Expression of the palmitoylated protein motifs found in growth associated protein 43kDa, enhanced filopodia number and motility, but reduced the probability of forming a stable

axon-dendrite contact. Conversely, expression of neuroligin-1 (NLG-1), a synapse inducing cell adhesion molecule, resulted in a decrease in filopodia motility, but an increase in the number of stable axonal contacts. Moreover, siRNA knockdown of NLG-1, reduced the number of presynaptic contacts formed. Postsynaptic scaffolding proteins such as Shank1b, a protein that induces the maturation of spine synapses, reduced filopodia number, but increased the stabilization of the initial contact with axons. These results suggest that increased filopodia stability and not density may be the rate-limiting step for synapse formation.

Preface

Chapter 2

For figures 2.1-2.16, I did all of the work contributing to these figures except for the following: Joshua Levinson aided with the generation of the paralemmin-1 siRNA construct, Kun Huang performed the western blot demonstrating knockdown of paralemmin-1 in heterologous cells presented in Figure 2.2A and Carolina Gutierrez performed and analyzed the photoconductive stimulation experiment presented in Figure 2.14C,D . Dr. Carlo Sala provided the GFP and HA tagged Shank1b constructs that were used in Chapters 2 and 3. In addition, I wrote the manuscript and Alaa El-Husseini and Joshua Levinson provided feedback and revisions. Esther Yu and Rujun Kang prepared the dissociated primary neuronal cultures used in this study.

Chapter 3

For figures 3.1-3.11, I did all of the work contributing to these figures except for the following: Catherine Gauthier-Campbell performed and analyzed the experiments presented in Figures 3.3, 3.4, 3.6, 3.7, and 3.10. In addition, I wrote the manuscript and Kun Huang provided valuable feedback and revisions. Esther Yu and Rujun Kang prepared the dissociated primary neuronal cultures used in this study.

Appendices

B1 This work was done in collaboration with Dr. Ann-Marie Craig's lab. I did all of the work presented in Figure B1 except for image acquisition and data analysis.

B2 and B3 This work was done in collaboration with Dr. Marie-France Lisé. I did all of the work presented in Figures B2 and B3 except for the data analysis in figure B2.

B4 This work was done in collaboration with Dr. Rujun Kang. I did the work presented in Figure B4.

B5 This work is unpublished. I performed all experiments and analyses.

The following certificate numbers were used during my research: B09-0258 (animal) and A06-0431 (breeding protocol) and A09-0665 (neuroplasticity). The University of British Columbia Animal Care Committee approved the research presented in this thesis.

Table of contents

List of tables

List of figures

List of abbreviations and symbols

ABE Acyl biotin exchange
AIDA 1-aminoindan-1,5-dicarboxylic acid
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
Ank ankryn
Ap action potential
APV 2-amino-5-phosphonopentanoate
Arp2/3 Arp2 and Arp3+ p16-Arc (ArpC5), p20-Arc (ArpC4), **p21-Arc** (ArpC3), **p34-Arc** (ArpC2) and p41-Arc (ArpC1).
AZ active zone
CA constitutively active
CaaX cysteine+aliphatic residue+amino acid
CAM cell adhesion molecule
Ca^{2+} calcium
CAST CAZ-associated structural protein
Cdc42 cell division cycle protein 42
4-CPG 4-Carboxyphenylglycine
CNS central nervous system
CNQX 6-cyano-7-nitroquinoxaline-2,3-dione
DiO 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine
EGFP-VAMP2 enhanced green fluorescent protein-vesicle
Ena enabled
DCC deleted in colorectal cancer
DN dominant negative
ECM: extracellular matrix
EM electron microscopy
EphB receptor for ephrin ligand
ER endoplasmic reticulum
F-actin filamentous actin
FGF fibroblast growth factor
FIM Filopodia inducing motif
FM4-64FM4-64,N-(3-triethylammoniumpropyl)-4-(4-diethylaminophenylhexatrienyl) pyridinium dibromide]
FRAP fluorescence recovery after photobleaching
FRET Förster resonance energy transfer
KCC2 G-actin: globular actin
GAP-43 Growth associated protein 43
GAD L-glutamic acid decarboxylase
GAD-65 glutamic acid decarboxylase 65
GAD-67 glutamic acid decarboxylase 67
GABA ϒ-amino butyric acid
GABAR GABA receptors
GAP-43 growth associated protein 43kDa
GFP Green fluorescent protein

GFP-Bsn bassoon protein tagged with green fluorescent protein
GKAP guanylate-kinase associated protein
h hour
LIM LIM kinase
LTP Long term potentiation
LTD Long term depression
mRNA messenger RNA
Ig immunoglobulin
IP_3R Inositol trisphosphate receptor
mGluR metabotropic glutamate receptor
MCS multiple cloning site
MUNC mammalian uncoordinated-18
NCAM neural cell adhesion molecule
NLG-1 neuroligin-1
NMDA N-methyl-D-aspartic acid
NMDAR N-methyl-D-aspartic acid receptor
NRXN neurexin
N-terminal amino terminal
Nm: nanometer
NT: amino terminus
ms: millisecond
PALM-1 paralemmin-1
PRR proline rich motif
PC Purkinje cell
PCR polymerase chain reaction
PDZ post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)
PSD postsynaptic density
PRR praline rich region
PTVs Piccollo transport vesicles
RP reserve pool
RIM Rab3A interacting molecule
SAM sterile alpha motif
SER smooth endoplasmic reticulum
siRNA small interfering ribonuclease
SNAP25 Synaptosomal-associated protein 25
STVs synaptic vesicle transport vesicles
SV synaptic vesicle
SynCAM synaptic cell adhesion molecule
TMD transmembrane domain
TRIM3 Tripartite motif-containing protein
UNC uncoordinated
µm microns
VASP Vasodilator-stimulated phosphoprotein
PAT palmitoyltransferase
PM plasma membrane

PN postnatal
ROCK Rho-associated coiled-coil- forming protein kinase
Rho GTPase Rho family of small GTPase
WASP Wiskott-Aldrich syndrome family protein
Wnt wingless+Int

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I would like to give many thanks to my good friend and mentor, Alaa El-Husseini. I learned so much from him which included how to interface with other scientists, how to write a paper, and conduct experiments. He was truly a wonderful man and I still have many moments where I miss him tremendously. Alaa, I'm nearing the end!

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Dedication

To my family. And to Dr. Alaa El-Husseini.

1. Introduction

1.1. Development of synapses in the brain: the big picture

The brain is a complex structure that governs our every day behaviors including eating, sleeping, emotional responses, attention, perception and learning and memory. It consists of hundreds of billions of neurons all interconnected into complex neuronal circuits that underlie our behaviors (Vaughn, 1989; Ziv and Garner, 2001; Waites *et al.*, 2005; McAllister, 2007; Grabrucker *et al.*, 2009; Holtmaat and Svoboda, 2009; Ryan and Grant, 2009). Neurons are the functional units of the brain and each neuron within a circuit can form thousands of connections with neighboring cells and in turn can receive tens of thousands of connections from surrounding cells (Takahashi *et al.*, 2003; Lardi-Studler and Fritschy, 2007; Bhatt *et al.*, 2009; Shen and Scheiffele, 2010). This makes the total number of connections in the brain close to a trillion. Initially, early in development, neurons make an overabundance of synapses and as the brain matures, these synapses are refined resulting in synaptic pruning (Figure 1.1) (Bourgeron, 2009).

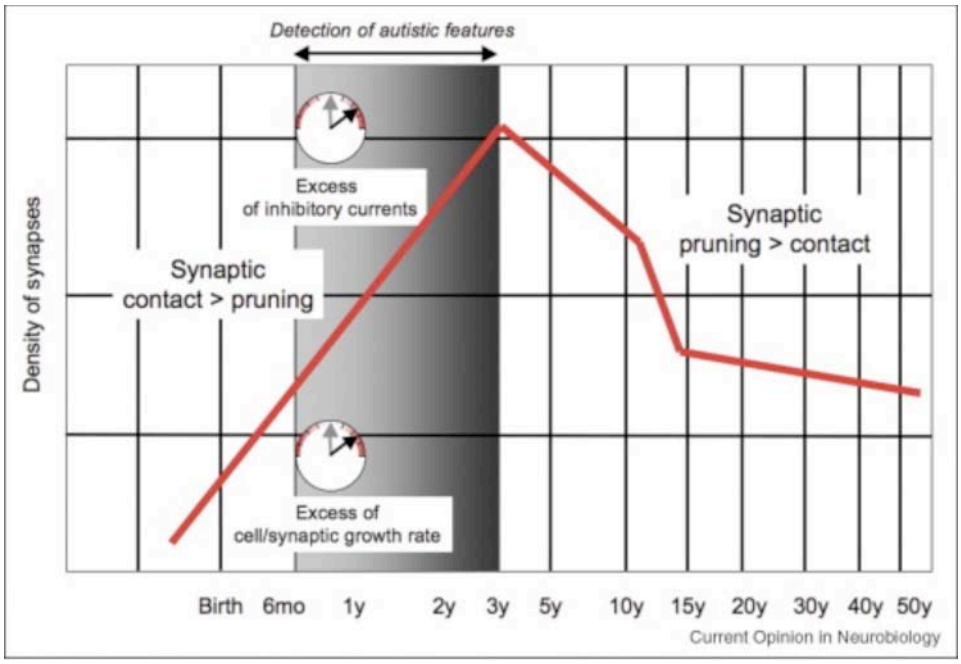

Figure 1.1 Schematic outlining synapse formation in the developing human brain
As development progresses, the number of synapses increases such that synaptic contact formation is greater than synaptic pruning. Eventually, a peak in the number of synapses is achieved whereby synaptic pruning or the elimination of synapses occurs more frequently than their formation. In the first 3 years of life, an excess of synaptic growth rate and inhibitory currents could lead to the risk of developing autism spectrum disorders (ASD). Reprinted from (Bourgeron, 2009), with permission.

What are these connections and how do they function to provide information from one cell to the next? Some of this work began with Ramon y Cajal, who provided some of the pioneering illustrations of how neurons form connections (Vaughn, 1989). The capacity for each of these neurons to function and innervate nearby neurons is mediated via specialized junctions called synapses. In the brain, there are two major types of synapses: 1) electrical and 2) chemical. Electrical synapses convey simple and rapid depolarizing signals with no synaptic delay, while chemical synapses are separated by a synaptic cleft (a small space of several nanometers) (Ziv and Garner, 2001; Wilbrecht *et al.*, 2010). Chemical communication occurs between two cells when the presynaptic cell fires an action potential due to a change in the membrane potential, which results in

the release of neurotransmitters from the synaptic vesicles, located at the axon terminal (Cantallops and Cline, 2000; McAllister, 2007; Wang and Zhou, 2010). The neurotransmitters then travel across the cleft until it reaches the postsynaptic cell and binds to receptors embedded in the plasma membrane (Cantallops and Cline, 2000; Ziv and Garner, 2001; Muller *et al.*, 2010; Segal *et al.*, 2010; Wang and Zhou, 2010). The binding of neurotransmitters to the receptors facilitates the opening of ion channels and metabotropic receptors through which current flows.

For over a decade, important questions regarding synapse formation have been under investigation such as: what are the factors that determine how two neuronal cells will communicate? And why does neuron #1 choose to communicate with neuron #2? To address these questions, five steps have been identified which are critical for proper synapse formation in the CNS: 1) neuronal contact formation which involves initial contact between axons, dendrites and dendritic filopodia. This process is thought to be mediated by cell adhesion molecules (CAMs) 2) synapse induction where inductive factors such as cadherins, NLGs and synCAM molecules induce the formation of presynaptic active zones and postsynaptic densities by recruiting the appropriate molecules to these nascent sites. 3) recruitment of pre- and postsynaptic proteins (also referred to as synaptic differentiation) (Waites *et al.*, 2005; Gerrow and El-Husseini, 2006; Chen *et al.*, 2007; McKinney, 2010; Shen and Scheiffele, 2010). Presynaptic differentiation includes the clustering of synaptic vesicles to regions underlying contact sites, the formation of active zones in the membrane at points of contact, and the assembly of the exo- and endocytic machinery close to the active zones (Fejtova and Gundelfinger, 2006; Fox and Umemori, 2006; Lardi-Studler and Fritschy, 2007). Postsynaptic differentiation occurs by clustering of neurotransmitter receptors directly apposed to presynaptic active zones and 4) contact stabilization and maturation (Fox and Umemori, 2006; Yoshihara *et al.*,

2009). And 5) involves the replacement and exchange of pre- and postsynaptic proteins to ensure that these newly formed synapses can be maintained over long periods of time. This multi-step process of synaptogenesis ensures that specific patterns of synaptic connections are formed during development and this is important as multiple reports revealed that developmental neurological disorders, such as autism spectrum disorders (ASDs) show abnormal brain connectivity. In addition, it is widely accepted that factors released from glial cells are also important for regulating synapse assembly (Fox and Umemori, 2006; Pfrieger, 2009; Salmina, 2009; Eulenburg *et al.*, 2010; Garey, 2010). To summarize, the formation of synapses is a complex process involving precise and specific communication between a pre- and postsynaptic cell. Through this contact formation, appropriate adhesion molecules, receptors and scaffolding molecules are transported to nascent sites, thus facilitating bidirectional communication across this junction. I will next focus on how excitatory synapses are formed in the brain.

1.2. Development of excitatory synapses

Proper connectivity is critical for functional neuronal network formation and this occurs by two consecutive processes: axonal pathfinding and synaptic cell adhesion. Of these two processes, axonal pathfinding is considered to be more important, although both are essential. Signaling is mediated by adhesion molecules that function in a homo- or heterophilic fashion at a distance of about 100 nm, which is a short distance. Axons, on the other hand, can mediate the specificity of connections at greater distances.

1.2.1. *Role of axonal pathfinding in synapse formation*

Axons search for appropriate target cells (Hatada *et al.*, 1999; Sanes and Lichtman, 1999; Skutella and Nitsch, 2001; Gerrow and El-Husseini, 2006) using growth cones (located at tips of the axons) which contain filopodia (Hatada *et al.*, 1999). The axonal growth cones are competent to form synapses and search through the dense neuropil for the appropriate target cell. One important question that arises from this is how does the axonal growth cone choose the appropriate target cell? Two hypotheses have been proposed to explain this process. The first is that specific recognition molecules on the axonal growth cone and dendritic process of the target cell may exist. Second, neurons may be promiscuous and thus form many synaptic connections and the "wrong" connections are eliminated over time. It seems likely that both hypotheses are equally correct and that axonal pathfinding involves both processes such that the correct target cell is found and the development of the future synapse will occur. After axonal pathfinding is complete and a dendritic target cell has been selected, the dendritic processes located on the target cell may contain dendritic filopodia which are thought to be important for contact initiation (Sanes and Lichtman, 2001; Thies and Davenport, 2003; Konur and Yuste, 2004b; Chen *et al.*, 2007; Menna *et al.*, 2009). In addition, compelling evidence suggests that neuronal activity is critical for regulating synaptogenesis and shaping future neuronal brain circuits (De Roo *et al.*, 2008; Hu *et al.*, 2008; Inoue *et al.*, 2009).

1.2.2. *Role of cell adhesion molecules in synapse formation*

Once contact between the axon and a target cell is established, the recruitment of appropriate neurotransmitter release machinery and receptors occurs to these developing sites (Figure 1.2) (Song

et al., 1999; Yamagata *et al.*, 2003; Washbourne *et al.*, 2004a; Dean and Dresbach, 2006; Chen *et al.*, 2007).

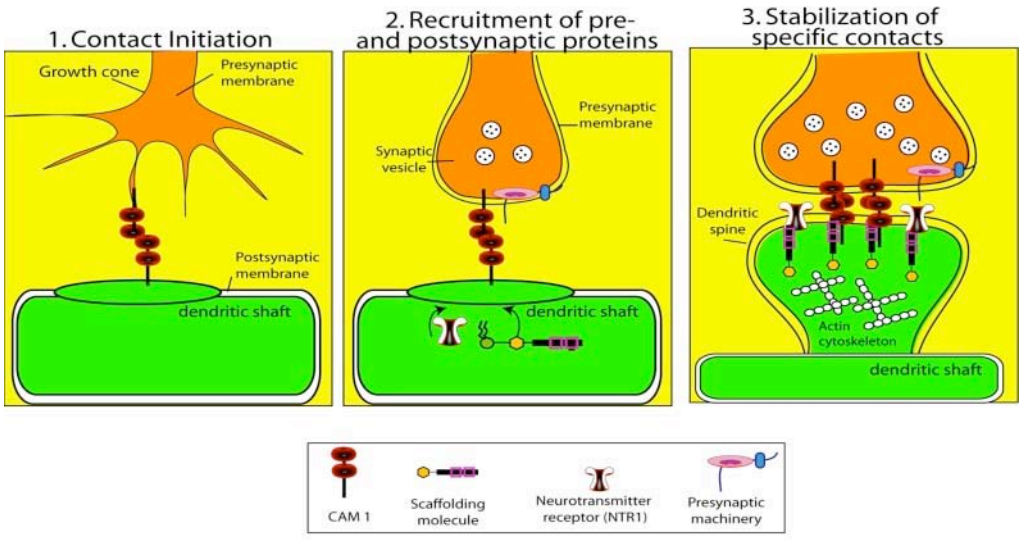

Figure 1.2 Model of the role of cell adhesion molecules and scaffolding molecules in synapse formation and stabilization in the CNS
Neuron A extends a long axon containing a growth cone in search of an appropriate target cell (Neuron B). (left panel) Cell adhesion molecules may be important for this process as they may confer synapse specificity. Once contact is established with a presynaptic growth cone and postsynaptic dendrite, pre- and postsynaptic proteins are recruited and an immature synapse develops. (middle panel) At this immature synapse, presynaptic neurotransmitter release machinery is recruited to the presynaptic membrane. At the postsynaptic membrane, cell adhesion molecules such as cadherins, scaffolding proteins and neurotransmitter receptors are recruited to an immature dendritic spine. (right panel) Finally, additional scaffolding proteins such as Shank and GKAP and cell adhesion molecules such as neuroligin/neurexin and EphB/ephrin-B complexes are recruited to a mature dendritic spine where they work in concert to stabilize these specific contacts. Reprinted from (Arstikaitis and El-Husseini, 2006), with permission.

Cell adhesion complexes are attractive candidates for the regulation of synaptogenesis; as they can function bidirectionally to modulate molecular and morphological changes in synapses (Song *et al.*, 1999; Yamagata *et al.*, 2003; Washbourne *et al.*, 2004a; Dean and Dresbach, 2006; Chen *et al.*, 2007; Craig and Kang, 2007; Dalva *et al.*, 2007). I will discuss the cadherin, NRXN/NLG, synaptic cell

adhesion molecule (synCAM) adhesion molecules as they have been shown to be important for the formation of spine synapses. Other cell adhesion molecules that are also important for the formation of excitatory synapses are ephBs/ehphrin-Bs (Torres *et al.*, 1998; Buchert *et al.*, 1999), and netrin G ligand (NGL2) (Kim *et al.*, 2006), but will not be discussed further.

NLG-NRXN: Presynaptic neurexin (NRXN) and postsynaptic neuroligin (NLG) are important for the regulation of synapse formation. However, their necessity and precise role in synapse formation is still controversial (discussed below). NLG is highly expressed throughout the brain during the peak period of synaptogenesis (Missler *et al.*, 1998; Rao *et al.*, 2000; Levinson *et al.*, 2005; Levinson and El-Husseini, 2005a, b; Dean and Dresbach, 2006; Gerrow *et al.*, 2006; Graf *et al.*, 2006; Varoqueaux *et al.*, 2006). Several reports have implicated NLG as an important molecule for inducing presynaptic differentiation such that the terminals could produce both spontaneous and evoked neurotransmitter release (Scheiffele et al. 2000; Sara et al. 2005). Therefore, these results suggest that NLG is capable of inducing the formation of functional presynaptic terminals. Other evidence points to a role for NRXN-NLG in target recognition as both molecules are expressed early in development (Chen et al. 2010). A final proposed function of NRXN-NLG may be in regulating synapse specificity because alternative splicing of the three NRXN genes generates thousands of NRXN isoforms. It has been suggested that these isoforms could specify a 'code' of interactions at synapses thus promoting specific molecular interactions at individual synapses. Interestingly, alternative splicing of NRXNs is regionally regulated and altered by activity in neurons (Boucard et al. 2005). Although NRXN-NLG interaction induces synapse formation *in vitro*, evidence *in vivo* supports a role for this adhesion complex in synaptic stabilization and maturation (Varoqueaux *et al.*, 2006; Chubykin *et al.*, 2007; Sudhof, 2008; Gibson *et al.*, 2009; Gogolla *et al.*, 2009; Ko *et al.*, 2009; Blundell *et al.*, 2010).

Multiple *in vitro* studies have found that NLGs can induce presynaptic differentiation. This initial finding was documented by using a co-culture assay where NLG expressed in non-neuronal cells was sufficient to induce presynaptic specializations in neuronal cells onto non-neuronal cells (Scheiffele *et al.*, 2000). Also, expression of NRXN in co-culture assays induces the formation of postsynaptic specialization. These results suggest that NRXN and NLG may function to induce synapse formation. However, studies performed *in vivo* reveal a different role for these cell adhesion molecules. NLGN and α-NRXN knockout mice revealed that these proteins are essential for synaptic function, but not synapse formation (Varoqueaux *et al.*, 2006; Chubykin *et al.*, 2007). Furthermore, triple NLG knockout mice die at birth due to respiratory failure, but exhibit relatively normal synapse numbers with normal ultrastructure. One possible explanation to explain this discrepancy is that the *in vitro* studies do not directly measure changes in synapse number, but rather assess synapse formation after performing a specific manipulation. In support of this explanation, the ability of NLGs to increase the number of synapses in a transfected neuron can be decreased by blocking synaptic activity, which has no effect on the expression and localization of the transfected NLGs (Chubykin *et al.*, 2007). This finding implicates NLGs as important molecules for the maturation of synapses, but not in the initial formation of these sites.

SynCAM: is a transmembrane molecule containing 3 extracellular immunoglobulin (Ig) domains and an intracellular PDZ-binding EYF1 sequence (Biederer et al. 2002). SynCAM is capable of homophilic binding and found only in the CNS. Interestingly, its expression is temporally correlated with synaptogenesis (Biederer *et al.*, 2002; Abbas, 2003; Fogel *et al.*, 2007; Thomas *et al.*, 2008; Hoy *et al.*, 2009). In co-cultures with fibroblast and hippocampal neurons, synCAM expression was capable of inducing the formation of pre- and postsynaptic varicosities (Biederer et al. 2002). In

addition, these newly formed synapses were capable of both spontaneous and evoked release suggesting that these presynaptic terminals are functionally active (Sudhof, 2004; Sudhof, 2009). These results implicate synCAM as a target-derived presynaptic organizer *in vitro*.

1.2.3. *Role of scaffolding molecules in synapse formation*

At excitatory synapses, scaffolding molecules such as Shank1b and PSD-95 are enriched in the PSD and are important for the stabilization and maturation of spines (Prange and Murphy, 2001; Sala *et al.*, 2001). These proteins function to physically link receptors and signaling molecules, forming an intricate network necessary for proper neuronal transmission (Ehlers, 1999; Harris, 1999; Ehrlich *et al.*, 2007).

Shank1b: Shank is a large scaffolding molecule localized exclusively to excitatory synapses. Shank contains many structural domains, which are important for protein-protein interactions. For instance, it contains multiple domains such as ankyrin repeats near the N-terminus, an SH3 domain, long proline rich region and a sterile alpha motif (SAM) domain at the C-terminus. Shank proteins are coded by three genes (1-3) and they function to molecularly link two glutamate receptor subtypes namely NMDAR and mGluR (type I). In addition, the C-terminus of Shank binds to guanylate kinase associated protein (GKAP) and also binds homer through the proline rich domain (Naisbitt et al. 1999; Tu et al. 1999, Xiao et al. 2000) (Figure 1.3). GKAP is a synaptic protein that localizes to excitatory synapses and functions in synapse formation. Homer protein is encoded by 3 genes (1-3) and consists of a N-terminus Ena/Vasp homology 1 (EVH1) domain followed by a coil-coil domain that mediates dimerization with other homer proteins. The EVH1 domain is important for binding to

the proline rich region of Shank as well as interacting with mGluR1/5 and the IP3R. Previous studies found that expression of Shank1b in young neurons promotes morphological maturation of spines, whereas in older neurons, Shank1b promotes spine maturation and spine head enlargement (Sala *et al.*, 2001). Furthermore, it was found that expression of Shank1b also induces maturation of presynaptic compartments although the exact mechanism by which this occurs is still unclear (Sala *et al.*, 2001; Roussignol *et al.*, 2005). One possibility is that Shank1b is transported in postsynaptic transport packets together with NLG-1 and PSD-95 and together these proteins are sufficient to induce functional presynaptic terminals (Gerrow *et al.*, 2006).

To demonstrate a critical role for Shank in spine formation and maturation, one study showed how expression of Shank3 in cerebellum granule cells (inhibitory cells do not form dendritic spines) induces dendritic spines and synapse formation by recruiting different subtypes of glutamate receptors. Furthermore, knockdown of endogenous Shank3 expression in hippocampal neurons decreased the number of dendritic spines (Roussignol *et al.*, 2005). One hypothesis to explain how Shank1b may increase dendritic spine size is that expression of Shank and Homer can recruit entire endoplasmic reticulum (ER) compartments to dendritic spines, which may contribute to spine enlargement and maturation (Sala *et al.*, 2001; Sala *et al.*, 2003).

Shank is localized deep within the PSD, while PSD-95 lies very close to the postsynaptic membrane (Valtschanoff and Weinberg, 2001) (Figure 1.3). Work from Morgan Sheng's lab has shown that expression of Shank1 in neuronal cells promotes spine maturation and spine head enlargement (Sala *et al.*, 2001). In young cells, expression of Shank1 on spines showed well-developed spine heads compared to GFP (Sala *et al.*, 2001). In older neuronal cells, Shank1 expression promoted more

mushroom shaped spines compared to control cells. It was found that expression of Shank1 in younger compared to older cells led to a 0.4 μm increase in spine head area (Sala *et al.*, 2001). Furthermore, Sheng and colleagues found that the N-terminal region containing the ANK repeats and most of the PRR are not required for synaptic targeting (Sala *et al.*, 2001). What was intriguing was that Shank1 mutants (Shank1b P1497L and Shank 1-1440), when expressed into neurons reduced binding to homer, reduced spine head size and also decreased the density of these spines (Sala *et al.*, 2001). This result suggests that homer binding is required for spine promoting activity and Shank1 targeting to postsynaptic sites is also required for spine maturation. Expression of homer alone does not produce spine enlargement, but rather it is the cooperative effects of Shank and homer1b that are important for these morphogenic effects (Sala *et al.*, 2001; Segal, 2001; Ehlers, 2002; Thomas, 2002; de Bartolomeis and Iasevoli, 2003; Hennou *et al.*, 2003; Ehrengruber *et al.*, 2004). In addition, neuronal activity had no effect on spine head morphology as the authors expressed Shank1b or Shank1b and homer1b in the presence of specific pharmacological agents such as: APV (100 μM) to block NMDARs, CNQX (100 μM) to block AMPARs and 4-CPG and AIDA (500 μM) to block mGluRs (Sala *et al.*, 2001).

In addition, it was also found that Shank can recruit IP_3R to dendritic spines and this occurs in a homer dependent manner. Homer has been shown to bind to IP_3Rs, which are localized in the smooth endoplasmic reticulum (SER) and large dendritic spines have been reported to contain SER (Spacek and Harris, 1997). Thus, homer could promote spine enlargement by increasing localized calcium responses.

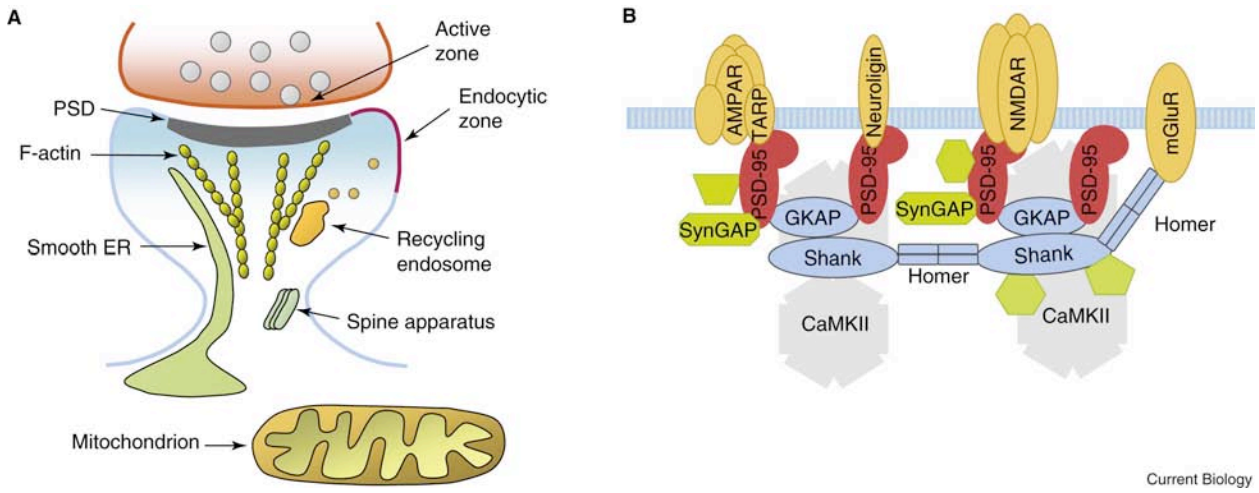

Figure 1.3 An illustration of a dendritic spine and the molecular architecture at the PSD
(A) Dendritic spine showing how the PSD is apposed to the presynaptic active zone. Different organelles found in the spine include smooth ER (protein synthesis machinery), recycling endosomes and spine apparatus. (B) Major scaffolding molecules found within the PSD include PSD-95, Shank, Homer as well as neurotransmitter receptors such as NMDAR and AMPAR. Reprinted from (Kim and Sheng, 2009), with permission.

PSD-95: It is now widely accepted that PSD-95 also plays a role in synapse maturation (Kim and Sheng, 2004; Prange et al. 2004). It also induces clustering of neurotransmitter receptors and PSD95 knockout mice show defects in synaptic transmission associated with plasticity which results in enhanced LTP and impaired learning (Migaud *et al.*, 1998). Moreover, knockdown of PSD-95 causes a reduction in the number of excitatory synapses and clustering of AMPA receptors. Interestingly, Sala et al. demonstrated that the interaction of PSD-95 with GKAP is important for coupling of GKAP to Shank (Sala et al. 2001). This suggests that PSD-95 indirectly effects the formation of dendritic spines through its interaction with GKAP.

Taken together, these results point towards a dual role for Shank1b for both the formation of dendritic spines in younger neurons by accelerating the maturation of dendritic filopodia to spine-like protrusions and increasing the maturation of dendritic spines in older neurons by possible interactions

with homer and recruitment of ER to spines. Finally, similar to Shank1b, PSD-95 also appears to play a critical role for the transformation of filopodia to dendritic spines (Prange et al., 2001).

1.3. Protein trafficking to the synapse

1.3.1. *Trafficking of presynaptic proteins to the synapse*

Early in development, new proteins must be synthesized and delivered quickly to synaptic sites as synaptic transmission is fast and requires the production, trafficking and elimination of synaptic proteins to ensure efficient transmission. One fundamental question when examining presynaptic assembly is how do presynaptic proteins get to synaptic sites? And which proteins arrive first? Numerous studies have shown that presynaptic proteins are being transported in multivesicular structures before and during synaptogenesis (Zhai *et al.*, 2001; Ziv, 2001; Ziv and Garner, 2004; McAllister, 2007). In younger neurons there are two types of transport packets present: 1) Piccolo transport vesicles (PTVs) and 2) Synaptic vesicle protein transport vesicles (STVs) (Zhai *et al.*, 2001; Sabo *et al.*, 2006). The PTVs are 80nm dense core vesicles and travel at rapid rates along the axon (up to 0.35um/s has been reported) (Shapira *et al.*, 2003) and transport the active zone proteins, piccolo and bassoon, Munc-13, Munc-18, syntaxin, and synapsin (Zhai *et al.*, 2001; Sudhof, 2004). In fact, piccolo and bassoon have been reported to be the earliest proteins transported to developing synaptic sites (Zhen and Jin, 2004; Dresbach *et al.*, 2006). Numerous studies have reported that the PTVs carrying active zone proteins arrive before STVs to these sites (Garner *et al.*, 2000; Gundelfinger and tom Dieck, 2000; Zhai *et al.*, 2001; Shapira *et al.*, 2003; Dresbach *et al.*, 2006; Fejtova and Gundelfinger, 2006).

The STVs are a pleiomorphic group of vesicles and carry SV proteins and other proteins important for membrane endo- and exocytosis (Ahmari *et al.*, 2000; Zhai *et al.*, 2001). Several different studies have reported that about 50% of EGFP-VAMP2 is highly mobile in young cortical neurons with velocities ranging from 0.1-1.0 μm/sec (Kraszewski *et al.*, 1995; Dai and Peng, 1996; Ahmari *et al.*, 2000; Kaether *et al.*, 2000; Sabo *et al.*, 2006). These packets move intermittently and in both directions along the axon and undergo several types of behaviors: 1) occasionally stop, 2) split into smaller clusters or 3) merge into bigger clusters. Once a prospective postsynaptic partner is found and contact is made, the vesicle machinery becomes concentrated at this site and enables communication between two cells via synaptic transmission (Figure 1.4). These studies suggest that when contact is made with a postsynaptic partner, preassembled protein packets can be quickly delivered to the site of contact.

In the vertebrate CNS, many of these presynaptic sites are distributed along the axon segment forming small swellings called presynaptic boutons. Syntaxin and SNAP25, two molecules essential for synaptic vesicle release, are found distributed along the axon terminal in immature neurons and only later in development do they become highly concentrated at presynaptic sites (Gonzalo *et al.*, 1999; Brown and Breton, 2000; Zhai *et al.*, 2001; Puri and Roche, 2006; Quick, 2006; Lang and Jahn, 2008). This finding supports the idea that presynaptic boutons may be distributed along the entire axonal segment allowing for *en passant* synapses with many dendrites.

During the initial phases of synapse formation, presynaptic compartments contain an active zone associated with only a small number of SVs. At these developing synapses, reserve pool SVs and mitochondria are rarely observed, but are present at mature presynaptic sites. At these newly developing sites, there is evidence for pleiomorphic vesicular structures as well as coated vesicles (Sudhof, 2004; Sudhof and Rothman, 2009). As development proceeds, there is an increase in the number of SVs and boutons become larger and the presynaptic membrane becomes more complex (Cheetham and Fox, 2010; Siddiqui and Craig, 2010; Xiao *et al.*, 2010). The maturation of the presynaptic site is associated with changes in the functional properties, for example 1) changes in the number of synaptic vesicles (Basarsky *et al.*, 1994) and 2) also subunit composition of voltage-dependent calcium channels that are involved in evoked neurotransmitter release (Scholz and Miller, 1995). 3) In addition, these developing synapses become more sensitive to tetanus toxin. Tetanus toxin is a protein derived from *Clostridium tetani* that can block NT release (Verderio *et al.*, 1999). 4) Finally, as the presynaptic site continues to mature there are changes in the probability of release (Sudhof, 2004). Ahmari and colleagues conducted an elegant study to monitor synapse formation in cultured hippocampal neurons by performing timelapse imaging and retrospectively examined the same sites using EM (Ahmari *et al.*, 2000). Their results revealed that the contacts that formed over the total imaging period did not contain well-formed active zones or numerous SVs within 2-3 h after initial contact was made (Ahmari *et al.*, 2000) as was previously reported. What was intriguing was that at these same sites, stimulation-evoked vesicle recycling was demonstrated. What the authors did observe, however, were numerous pleiomorphic vesicular structures as well as dense core vesicles (Ahmari *et al.*, 2000). Therefore, these imaging and ultrastructural results question whether developing presynaptic sites are morphologically different from mature ones.

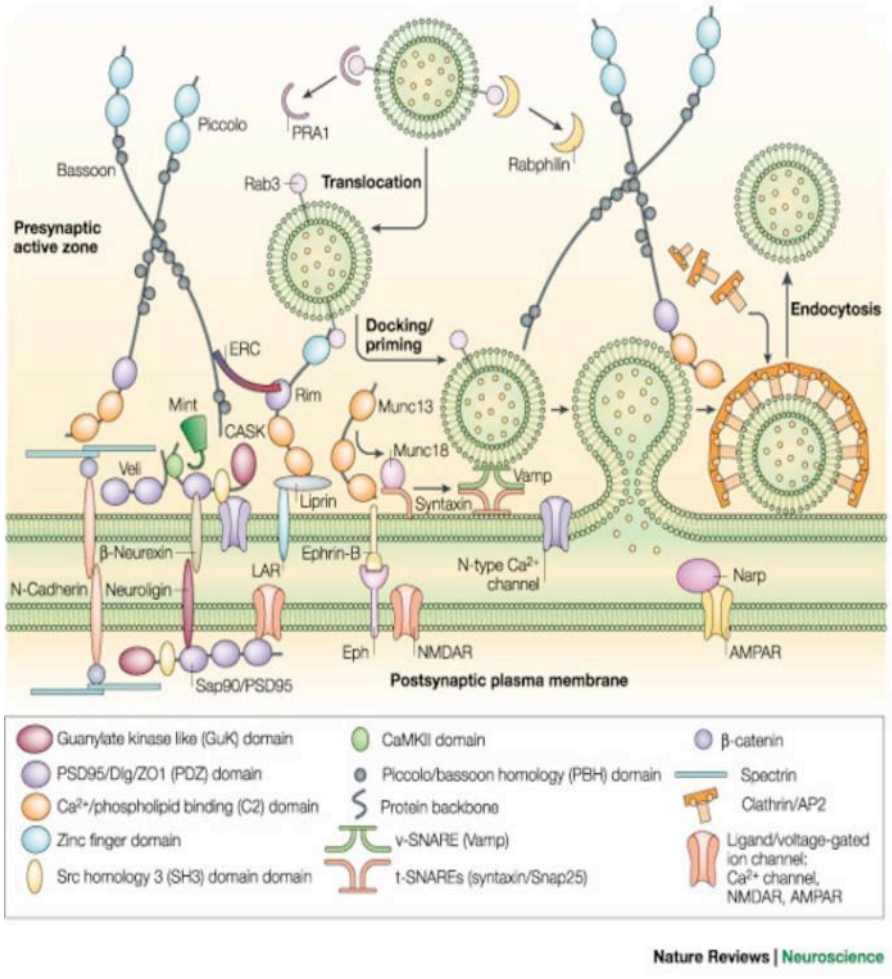

Figure 1.4 The molecular organization of glutamatergic synapses
There is a plethora of proteins found at presynaptic sites and these proteins function as structural elements to hold the active zone opposed to the PSD. Another set of proteins is important for synaptic vesicles docking and fusion. A final set of proteins is important for building specialized protein complexes around ionotropic and metabotropic glutamate receptors. Reprinted from (Ziv and Garner, 2004), with permission.

1.3.2. *Trafficking of postsynaptic proteins to the synapse*

For proper brain development, proteins such as neurotransmitter receptors, scaffolding and cell adhesion molecules must be efficiently trafficked to the postsynapse. In young cortical neurons,

NMDARs are transported in discrete packets that move bidirectionally and travel about 6-8 μm/min. Furthermore, work done in the McAllister lab found that NMDARs are amongst the first postsynaptic proteins to arrive to nascent contact sites (Washbourne *et al.*, 2002; Washbourne *et al.*, 2004b) and undergo a novel type of transport where they cycle with the plasma membrane during pauses, suggesting that they may sense glutamate during their transport (Washbourne *et al.*, 2004b). In addition, several reports have found that scaffolding molecules are present in dendrites before synapses have formed (Craig *et al.*, 1993; Washbourne *et al.*, 2002; Washbourne *et al.*, 2004b; Gerrow *et al.*, 2006; McAllister, 2007).

How do postsynaptic proteins reach their final destination at synaptic sites? The majority of studies have demonstrated that PSD-95 can form mobile transport packets (Prange and Murphy, 2001), while others still have shown that postsynaptic proteins, including PSD-95, Shank and GKAP can pre-assemble (similar to presynaptic proteins) and are trafficked together to synapses (Gerrow *et al.*, 2006). Likely, these different observations are all correct as the developmental time window, specific brain region and cell type may effect the transportation of these different molecules to developing synapses.

1.4. Formation of dendritic spines

1.4.1. *Origin of dendritic spines*

In the CNS, dendritic spines are the major postsynaptic sites of glutamatergic excitation. It is now clear that functional properties are altered in the brain as a result of changes in spine densities and

morphologies (Purpura, 1979; Ferrante *et al.*, 1991; Spigelman *et al.*, 1998). In addition, many molecules have been implicated in spine development and remodeling suggesting that there is an inter-relationship between molecules involved in actin dynamics and spine morphogenesis. To date, the emergence of dendritic spines in the brain is far from clear. Understanding how the brain gives rise to these tiny protrusions will help us understand the functional significance of these protrusions and also what happens to the brain in neuropsychiatric disorders like autism, schizophrenia, depression and mental retardation (Belichenko *et al.*, 2009a; Ivanov *et al.*, 2009; Sweet *et al.*, 2009; Woolfrey *et al.*, 2009; Cruz-Martin *et al.*, 2010). I will begin this section by discussing the different models available to explain the genesis of dendritic spines. Next, I will specifically focus on the role that dendritic filopodia play in spine formation. Finally, I will outline several key molecules involved in spine formation.

1.4.2. *Three models of spine formation*

Spines were first identified over a century ago and our knowledge about their structure and function has progressed significantly. However, what remains unclear is how these tiny protrusions are formed in the brain. It seems like a relatively simple question, however, when one considers the numerous brain regions, cell types, and the plethora of proteins, investigating this question becomes challenging. Several different models have been proposed outlining the events leading to spine formation: 1) the Miller and Peters model supports the hypothesis that the axon terminal induces the formation of the spine (Miller and Peters, 1981; Harris, 1999), 2) the Sotelo model supports the idea that spines can form independently of the axonal contact (Sotelo *et al.*, 1975; Sotelo, 1978, 1990). 3) And the final model, which my work focuses on, is the filopodial model which claims that dendritic

spines originate from dendritic filopodia which are more numerous in developing, immature neuronal cells (Figure 1.5) (Vaughn, 1989; Ziv and Smith, 1996; Marrs *et al.*, 2001).

Dendritic filopodia are long (2-20μm), thin and decorate developing dendrites (von Bohlen Und Halbach, 2009; Yoshihara *et al.*, 2009). Key findings demonstrate that filopodia are precursors of dendritic spines, suggesting that they may actively participate in forming synaptic contacts with axons in close proximity and then transform into dendritic spines (Yuste and Bonhoeffer, 2004; Gupton and Gertler, 2007; Lu *et al.*, 2009). It is likely that all three models may apply to spine formation in different circumstances and in different brain regions, as growing evidence from electron microscopy studies reveals that synapses are observed on dendritic shafts, stubby spines and dendritic filopodia early in postnatal development (Harris *et al.*, 1992; Harris and Kater, 1994; Fiala *et al.*, 1998; Harris, 1999; Sorra and Harris, 2000; Petrak *et al.*, 2005).

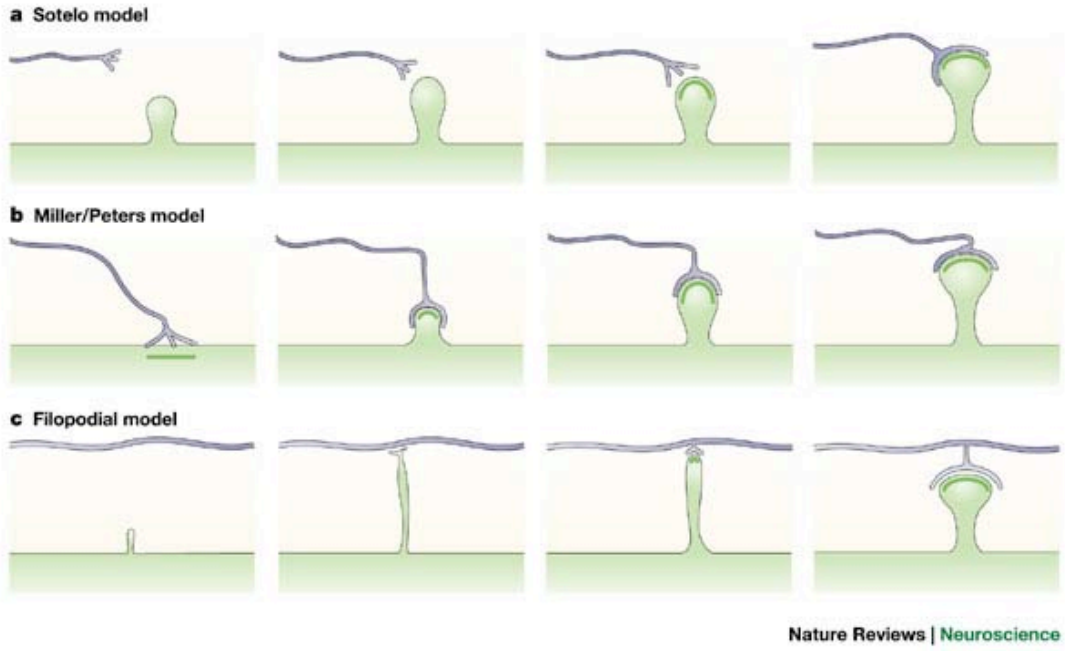

Figure 1.5 Three models of spinogenesis
This schematic demonstrates the key features involved in the formation of dendritic spines. In the Sotelo model (a), spines emerge independently of the axonal terminal. In the Miller/Peters model (b), the axonal terminal induces the formation of the spine. Finally, in the filopodial model (c), dendritic filopodia capture axon terminals to later transform into a spine. Reprinted from (Yuste and Bonhoeffer, 2004), with permission.

Miller and Peters model: This model describes a three-step process in the rat visual cortex. First, synapses are made on the dendritic shaft. Second, the presynaptic region of the axon swells as synaptic vesicles accumulate. Third, the spines that form are thin or mushroom shaped and the apposing axon terminals have well-developed varicosities (Miller and Peters, 1981; Yuste and Bonhoeffer, 2004). Therefore, as a spine develops, it takes a pre-existing shaft synapse and carries it along as it extends from the dendrite. One major limitation of this model is that some studies have shown that most of the connections formed with dendrites are made *en passant* suggesting that dendritic spines can form without being induced by the axon terminal (Nagerl *et al.*, 2007; Anderson and Martin, 2009).

Sotelo model: The second model of spine formation, the Sotelo Model, is based on observations from the cerebellum. The protrusions found on Purkinje cells (PCs) form through intrinsic mechanisms that do not depend on axonal contacts (Sotelo, 1990; Takacs *et al.*, 1997). Thus, the dendritic spine forms independent of the axon terminal.

The filopodial model: During the early phase of synaptogenesis, dendrites are decorated with filopodia that rapidly protrude, elongate and demonstrate lifetimes of several minutes (Dailey and Smith, 1996; Ziv and Smith, 1996; Dunaevsky *et al.*, 1999; Lendvai *et al.*, 2000). They have several proposed roles in the brain which include: 1) a role in dendritic branching (Niell *et al.*, 2004; Marrs *et al.*, 2006; Morita *et al.*, 2006; Niell, 2006; Xie *et al.*, 2007), 2) an exploratory role to find appropriate presynaptic partners (Ziv and Smith, 1996) and 3) a role in synaptogenesis (Ziv and Smith, 1996; Kayser *et al.*, 2008).

As synaptogenesis progresses, the number of filopodia decline as the number of stable-spine like structures increases, consistent with filopodia being precursors of dendritic spines. To successfully visualize dendritic filopodia forming contacts with nearby axons, Ziv and Smith labeled dendrites with the green fluorescent dye, DiO and functional presynaptic terminals with red fluorescent dye, FM4-64 in hippocampal neurons (Ziv and Smith, 1996). They hypothesized that dendritic filopodia would encounter axons, engage in synaptic contact and undergo a filopodium to spine transformation. They observed that the transformation stage was preceded by a decrease in dendritic filopodia motility, substantial shortening and enlargement of the distal portion of the filopodia to yield a spine-like shape. The filopodia in this model serve to explore the extracellular environment for an appropriate contact site that can later transform into a dendritic spine. Other studies have reported

that the release of glutamate from presynaptic terminals, promotes filopodia extension, suggesting that this may be a mechanism that guides filopodia to sites of presynaptic release (Portera-Cailliau *et al.*, 2003). One caveat of this model is that filopodia transformation to dendritic spines only accounts for a small percentage of total spine synapses formed in the hippocampus and cortex emphasizing the point that all three models are likely important for the formation of these protrusions (Fial et al., 1998).

In summary, compelling evidence exists for all three models of spine formation. However, previous work from the laboratory has demonstrated a role for filopodia in the formation of dendritic spines. Thus, my thesis aims to further characterize this model.

1.4.3. *Dendritic filopodia*

Mechanisms of filopodia formation: Dendritic filopodia serve multiple different functions in the brain and numerous molecules have been implicated to regulate the formation of these structures (see Table 1.1 for a summary). Yet, the molecular mechanisms important for filopodia transformation into dendritic spines remain unclear. To date, three major models have been proposed, which use distinct actin-nucleating proteins.

Molecules important for filopodia and spine formation					
Class	**Protein**	**filopodia formation**	**spine formation**	**Cell type**	**Citation**
Cell adhesion molecules					
	d-catenin	increase	increase	neurons	Abu-Elneel et al., 2008
	N-cadherin	increase	increase	myofibroblasts	De Wever et al. 2004
	TLCN	increase	decrease	neurons	Matsuno et al. 2006
	NLG	increase	increase	neurons	Arstikaitis et al. (submitted); Chih et al., 2005
Scaffolding molecules					
	shank	decrease	increase	Cos-7 cells/neurons	Arstikaitis et al., 2008
	PSD-95	unknown	increase	neurons	El-Husseini et al., 2000; Migaud et al., 1998
	Homer1b	uknown	increase in spine size	neurons	Sala et al., 2001
Cell morphology/ cytoskeletal proteins					
	paralemmin-1	increase	increase	neurons	Arstikaitis et al., 2008
	GAP-43	increase	unknown	Cos-7 cells/neurons	Gauthier-Campbell et al., 2004
	alpha-actinin	increase	increase	neurons	Nakagawa et al., 2004 Hoe et al., 2009
	Arp3	increase	increase	neurons	Korobova et al., 2008 Wegner et al., 2008
	cortactin	unknown	increase spine length	neurons	Hering and Sheng, 2003
	drebrin A	unknown	increase spine length	neurons	Hayashi and Shirao et al., 1999
Rho Family of GTPases					
	RhoA	decrease	decrease	neuronal slices	Tashiro et al., 2000; Govek et al., 2004
	Rac1	increase	increase	transgenic animals	Luo et al., 1996; Luo et al., 2000
	Cdc42	increase	increase	neurons	Kang et al., 2008

Table 1.1 Molecules important for filopodia and spine formation

The first model is called the convergent elongation model and there are many players involved in this process (Figure 1.6) (Gupton and Gertler, 2007). For example, the Arp2/3 complex (and F-actin regulator) can induce filopodia formation. Filopodia emerge from a subset of branched lamellopodia filaments at their barbed ends, which contain Ena/Vasp proteins (Figure 1.6) (Gupton and Gertler, 2007). The Ena/Vasp family of proteins also plays a role in the formation and maintenance of filopodia, though the precise nature of Ena/Vasp function is still unclear (Lebrand *et al.*, 2004; Mejillano *et al.*, 2004; Schirenbeck *et al.*, 2006; Applewhite *et al.*, 2007). Ena/Vasp are concentrated along the leading edge (Reinhard *et al.*, 1992; Gertler *et al.*, 1996) and at the tips of filopodia (Lanier *et al.*, 1999), and are capable of binding both G and F-actin (Bachmann *et al.*, 1999; Huttelmaier *et al.*, 1999; Barzik *et al.*, 2005). The clustering of barbed ends together protects them from capping proteins so continuous polymerization of this end occurs and promotes the creation of filaments (Gupton and Gertler, 2007). Fascin functions to convert the filaments into bundled filopodia and

stabilizes them and thus it functions as an actin cross-linking protein and is associated with filopodia in many types of cells (DeRosier and Edds, 1980; Sasaki *et al.*, 1996; Cohan *et al.*, 2001). Other cross-linking proteins exist which include fimbrin, filamin and α-actinin. Interestingly, fascin has been shown to be critical for the formation of filopodia in B16F1 melanoma cells showing that knockdown of fascin inhibits their formation (Vignjevic *et al.*, 2006). The Rho GTPase Cdc42, directly interacts with and activates the WASP family of proteins, which in turn can activate the Arp2/3 complex (Tu *et al.*, 1999; El-Husseini *et al.*, 2000a). Arp2/3 is an actin binding protein capable of binding to the side of an actin filament and nucleating a new filament as a branch from the mother filament. There is evidence that filopodia are initiated from branched F-actin meshwork rather than arising from *de novo* filament nucleation (Gupton and Gertler, 2007). In contrast, there are several studies that have documented the formation of filopodia in the absence of Arp2/3 (Kutzleb *et al.*, 1998; O'Brien *et al.*, 1998; Fiala *et al.*, 2002), which questions the role of the Arp2/3 complex in filopodia formation.

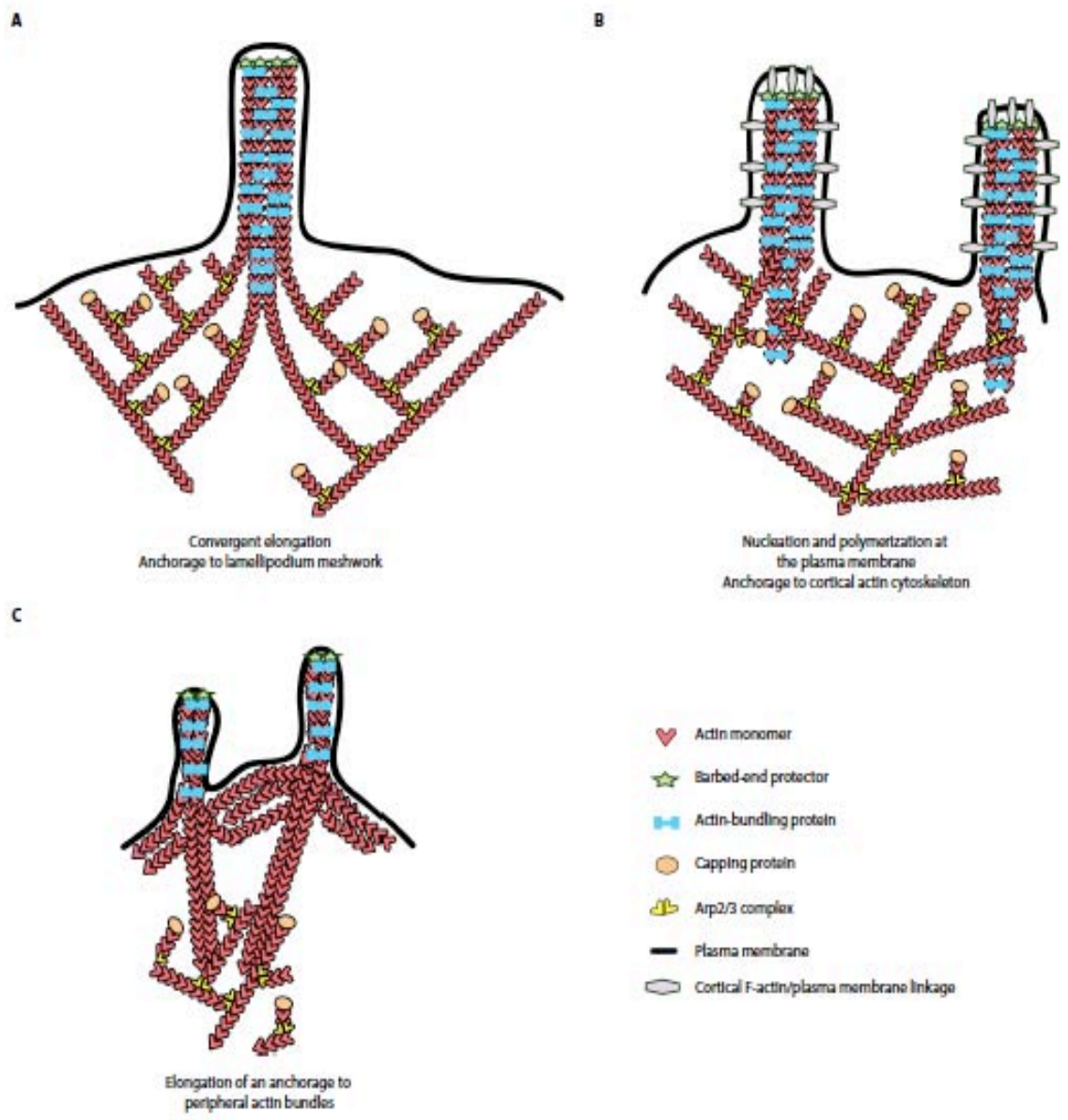

Figure 1.6 Mechanisms of filopodia induction
(A) Convergent elongation model involves key players such as Arp2/3 complex and Ena/VASP. (B) De novo filament elongation is mediated by F-actin nucleator and a capping protein such as Dia2. (C) Reorientation and elongation model where F-actin bundles in neuronal growth cones could possibly induce filopodium initiation. It is not clear whether these three models are independent and exclusive or whether multiple mechanisms operate within the same cell. Reprinted from (Gupton and Gertier, 2007), with permission.

A second proposed model underlying the formation of filopodia is the Diaphanous-related formin (Dia2)-mediated model (Figure 1.6). *In vitro* studies have shown that Dia2 nucleates linear actin

filaments and accelerates actin polymerization (Zigmond, 2004a; Zigmond, 2004b; Kovar, 2006a, b) and slows filament depolymerization (Romero *et al.*, 2004). In this model, at the plasma membrane, filopodia arise from *de novo* filament nucleation and polymerization.

In the final model, called the reorientation and rapid polymerization model, filopodia are anchored into peripheral actin bundles (Figure 1.6). In neuronal growth cones, the reorientation and elongation of peripheral F-actin bundles could induce filopodia initiation, modulated by several regulators of actin such as Ena/Vasp proteins, Dia2 at barbed ends and fascin/filamin or other crosslinkers along filopodia shafts.

1.4.4. *Key molecules involved in the formation of dendritic spines*

Actin: Neuronal activity alters dendritic spine morphology and these alterations are thought to influence neuronal circuitry. One major molecule important for these morphological changes underlying synaptic plasticity is actin. The major cytoskeletal component of dendritic spines is actin and it is found concentrated in the dendritic spine head (Matus *et al.*, 1982; Cohen *et al.*, 1985; Kaech *et al.*, 1997; Wyszynski *et al.*, 1997; Cingolani and Goda, 2008; Hotulainen *et al.*, 2009; Pontrello and Ethell, 2009). Actin has been reported to participate in many diverse cellular functions such as cell migration and signaling, muscle contraction, endocytosis, vesicle trafficking and cytokinesis (Pontrello and Ethell, 2009; Hotulainen and Hoogenraad, 2010).

In the brain there are two actin isoforms (beta and gamma), which selectively target to spines. The core constituent of the actin cytoskeleton is present as a soluble pool of monomeric actin (G-actin)

and becomes polymerized as F-actin filaments morph into a spine-like shape (Halpain, 2000; Rao and Craig, 2000). In the spine neck, actin filaments form longitudinal bundles whereas the spine head consists of a meshwork of short actin filaments just below the PSD (Matus *et al.*, 1982; Landis and Reese, 1983; Kim and Sheng, 2009). In the spine, actin has two major functions to: 1) stabilize postsynaptic proteins by tethering neurotransmitter receptors, signaling molecules, and scaffolding proteins into a localized area, allowing spines to modulate their shape, motility, and function (Kuriu *et al.*, 2006; Yang and Zhou, 2009; Wang and Zhou, 2010) and 2) modulate spine head structure in response to postsynaptic signaling (Fischer *et al.*, 2000; Okamoto *et al.*, 2001; Okamoto *et al.*, 2009).

Actin organization within the spine is highly regulated and dynamic (Fischer *et al.*, 2000; Smart and Halpain, 2000; Matus, 2005). A recent study has shown using GFP tagged actin and fluorescence recovery after photobleaching (FRAP) that the majority of actin found in spines is highly dynamic and can turnover in a two-minute period. In contrast, only about 5% of total actin in spines is stable (Star *et al.*, 2002). In addition, studies have shown that the actin cytoskeleton in the periphery of the spine is being rearranged continuously (Fischer *et al.*, 1998). These rearrangements do not alter the spine dimensions, but instead extend and retract small filopodia-like processes from the surface of the spine head possibly in search of glutamate release from presynaptic terminals. There is also compelling evidence that actin rearrangements drive the formation and loss of dendritic filopodia and spines possibly during periods of synaptic plasticity in the brain. For example, measurements of FRET between actin monomers revealed that synaptic stimulation rapidly changes the equilibrium between F-actin and G-actin (Okamoto *et al.*, 2004). Several studies have reported that induction of LTP shifts the G-actin/F-actin ratio towards F-actin, which increases spine volume. In contrast,

induction of long-term depression (LTD), shifts the equilibrium in favor of G-actin, which results in spine shrinkage (Fukazawa *et al.*, 2003; Lin *et al.*, 2005).

Rho GTPases: The Rho GTPases are a family of molecules with the ability to regulate dendritic spine morphology and are reported to be the key regulators of the actin cytoskeleton (Ridley, 1997, 2001; Etienne-Manneville and Hall, 2002) and function as molecular switches. This means they can cycle between the inactive (GDP-bound) form and an active (GTP-bound) form capable of binding to downstream effectors (Ridley, 2001). Activation of the Rho GTPases occurs by molecules called guanine exchange factors (GEFs) by promoting the release of bound GDP and its replacement by GTP. In contrast, Rho GTPases are inactivated by GTPase activating proteins by stimulating the hydrolysis of bound GTP to GDP. Once activated, the Rho GTPases activate downstream effectors that in turn influence actin filaments. There are three major members of the Rho family of GTPases: Cdc42, RhoA and Rac1 which are discussed below.

Cdc42: Previous studies have shown that overexpression of Cdc42 G12V in hippocampal slices does not alter dendritic spines (Tashiro *et al.*, 2000; Govek *et al.*, 2004). However, recent work has identified a new palmitoylated isoform of Cdc42 (CA Cdc42-palm) that increases the number of spines and this process is palmitoylation dependent as application of 2-bromopalmitate inhibits the formation of dendritic spines in cultured hippocampal neurons (Kang *et al.*, 2008). In addition, knockdown of endogenous Cdc42-palm in hippocampal-cultured neurons using specific siRNA resulted in a reduction in the number of dendritic spines (Kang *et al.*, 2008). In support of these findings, an elegant study conducted in the visual system demonstrated that the loss of Cdc42 causes a reduction in the density of spine-like structures (Scott *et al.*, 2003).

How does Cdc42 exert its effects in neuronal cells? There are several pathways by which specific signaling pathways connect Rho GTPases such as Cdc42 to the actin cytoskeleton (Figure 1.7). Cdc42 activates WASP, which allows N-WASP to recruit G-actin to form a complex with Arp2/3. Next, Arp2/3 activation causes nucleation of actin polymerization and branching. This may be a mechanism leading to spine head enlargement (Korobova and Svitkina, 2008).

A second pathway by which Cdc42 exerts its affects is by binding to IRSp53 to promote actin polymerization. IRSp53 is localized in spines and is known to regulate the actin cytoskeleton in non-neuronal cells (Hall, 1992; Nobes and Hall, 1995; Tapon and Hall, 1997; Miki *et al.*, 1998; Krugmann *et al.*, 2001; Miki and Takenawa, 2003). When Cdc42 interacts with IRSp53, it promotes recruitment of Shank and Ena/Vasp family member mammalian enabled (Mena) to the SH3 domain of IRSp53 (Krugmann *et al.*, 2001; Soltau *et al.*, 2002). IRSp53-Mena complex can initiate actin filament assembly and bundling to form filopodia in non-neuronal cells, but it is not clear whether this pathway also contributes to the formation of dendritic filopodia in neurons (Mejillano *et al.*, 2004).

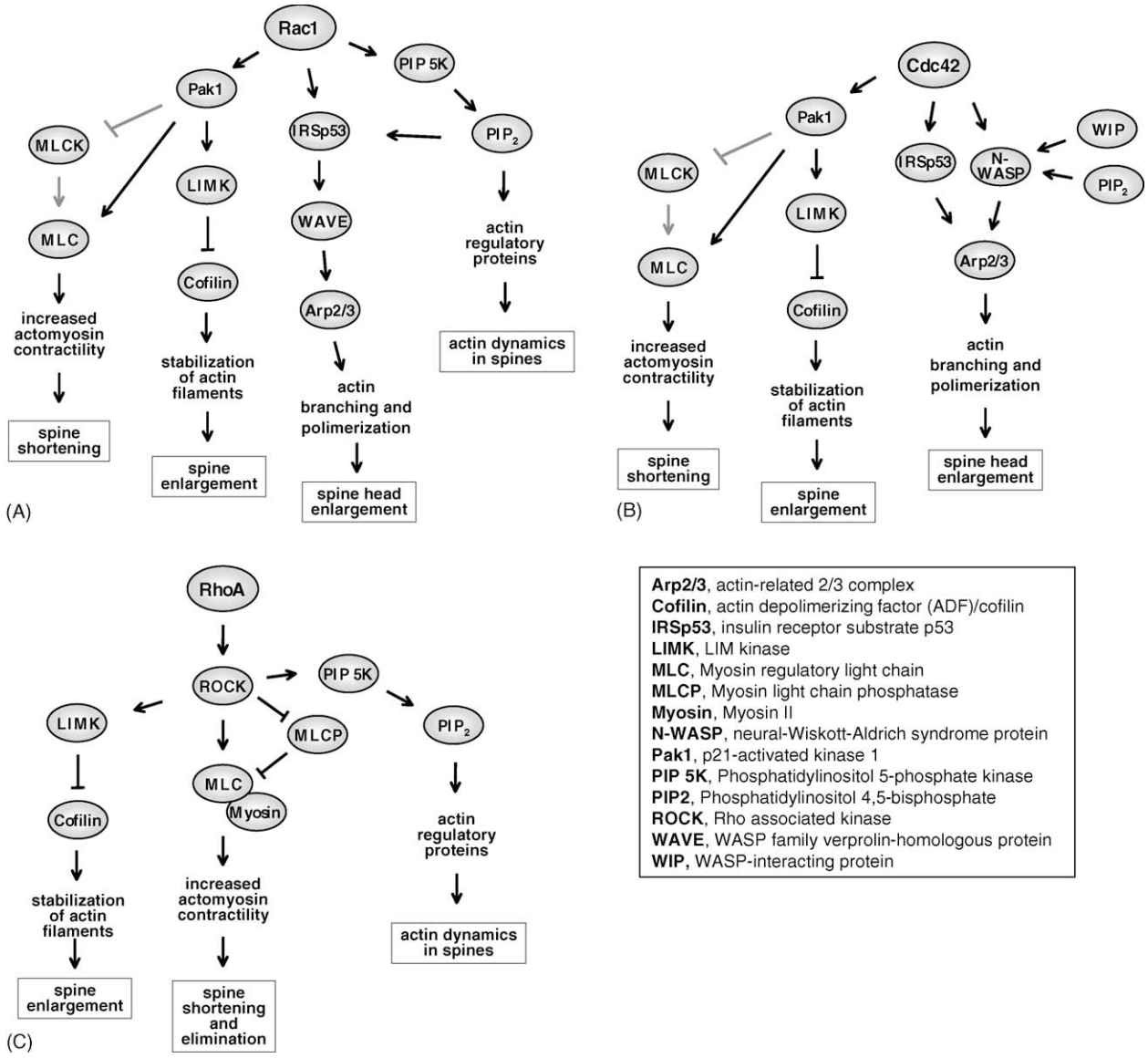

Figure 1.7 GTPases downstream signaling pathways that affect spine morphogenesis
Activation of Rac1 and Cdc42 by specific Rho GEFs leads to spine head enlargement whereas activation of RhoA by Rho GEFs leads to spine shrinkage and elimination. Reprinted from (Ethell and Pasquale**,** 2005), with permission.

Rac1: To explore the role of Rac1 in spine formation, several groups overexpressed constitutively active (CA) Rac1 in cultured hippocampal neurons and found an increase in the formation of irregularly shaped protrusions resembling membrane ruffles and lamellopodia (Nakayama *et al.*, 2000; Tashiro *et al.*, 2000; Govek *et al.*, 2004). In contrast, overexpression of a dominant negative

(DN) mutant Rac1 dramatically reduced the number of spines and synapses in cultured hippocampal slices and dissociated cultured neurons (Nakayama *et al.*, 2000; Zhang *et al.*, 2003). Taken together, these studies support a role for Rac1 in the development of new irregularly shaped dendritic spines (Lise *et al.*, 2009).

What are the signaling pathways by which Rac1 influences spine morphology? Rac1 can activate the Arp2/3 complex through WASP family verpolin-homologous protein (WAVE/Scar) family proteins, which influences actin dynamics in spines (Figure 1.7) (Miki *et al.*, 1998). Rac1 binding site becomes exposed when WAVE/Scar proteins bind SH3 domain of IRSp53. Both Rac1 and Cdc42 can activate Pak1, a serine-threonine kinase that phosphorylates and activates LIM kinases 1 and 2 (Edwards et al. 1999; Yang et al. 1998). LIM kinases phosphorylate and inhibit the actin depolymerization proteins ADF and cofilin and this decreases actin filament turnover and cell motility and thus, promotes spine formation.

RhoA: Throughout development the formation and elimination of dendritic spines are important events that have profound effects on shaping our brain circuitry. In contrast to Cdc42 and Rac1, expression of RhoA in hippocampal slices promotes spine retraction and elimination, thus contributing to the reduction of dendritic spines (Tashiro *et al.*, 2000; Govek *et al.*, 2004). Equally important are the molecules that cause spine retraction as an overproduction of dendritic spines can lead to neurological disorders such as Fragile X syndrome.

How does RhoA exert its effects on dendritic spine morphology? RhoA promotes activation of LIM kinases through ROCK, which is another serine-threonine kinase and a major effector of RhoA in

neurons (Figure 1.7) (Luo, 2002). The overall effect is a decrease in myosin regulating light chain phosphorylation and reduced actomyosin contractility.

1.5. A role for palmitoylation in synapse formation

1.5.1. *Overview of palmitoylation*

The post-translational lipid modifications prenylation, S-acylation (palmitoylation) and N-myristoylation facilitate protein targeting to different cellular compartments, which allows for activation of specific signaling cascades. In addition, these modifications are important for protein trafficking, protein-protein interactions and modulation of protein structure. Palmitoylation is a reversible post-translation modification resulting in the creation of thioester bonds. This occurs when a saturated 16-carbon palmitate group is added the sulfhydryl group of a cysteine. It also serves to tether soluble proteins or proteins with weak membrane affinity to the plasma membrane. There are also many transmembrane proteins that are palmitoylated and palmitoylation of these integral proteins is important for protein clustering.

Palmitoylation is the most common lipid modification reported in neuronal cells and palmitoylation-depalmitoylation cycles can be dynamically regulated or can undergo constitutively cycling. Palmitoylation of soluble proteins helps facilitate proteins to the plasma membrane, however integral proteins or transmembrane proteins (TM) can target them to specific membrane microdomains, such as lipid rafts (Prior *et al.*, 2001) or alter their confirmation to regulate interactions with other proteins

(Figure 1.8). Therefore, palmitoylation is not only important for protein trafficking to the plasma membrane, but also for protein shuttling between intracellular compartments.

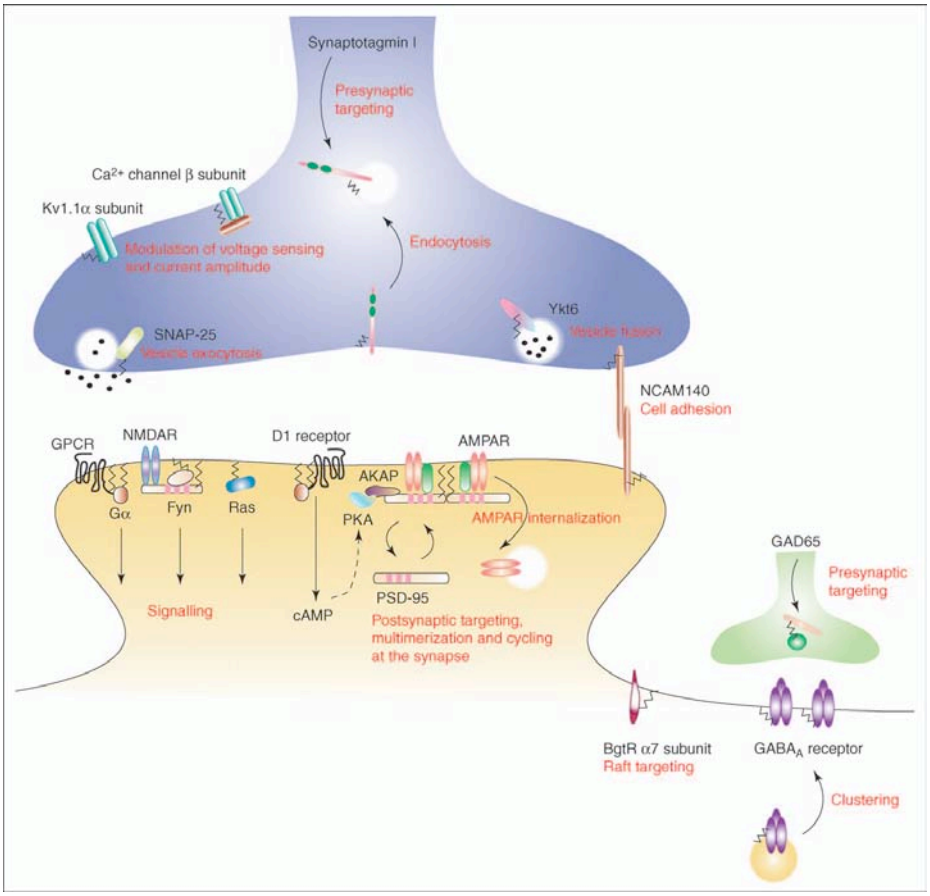

Figure 1.8 Palmitoylated proteins at excitatory and inhibitory synapses important for synaptic transmission
Synaptic transmission is regulated by a variety of palmitoylated proteins localized at synaptic sites. On the presynaptic side, proteins such as GAD65, synaptotagmin I and SNARE proteins important for regulating neurotransmitter release are palmitoylated. On the postsynaptic side, multiple G-protein-coupled receptors (GPCRs), G-proteins, PSD-95 (important for multimerization and clustering) and signaling molecules are palmitoylated. Reprinted from (Huang and El-Husseini, 2005), with permission.

1.5.2. *Mechanisms and regulation of palmitoylation-dependent protein sorting*

In a recent study, many candidate palmitoylated proteins were identified by parallel acyl biotin exchange (ABE) assay and Multidimensional Protein Identification Technology (MudPIT) analyses (Kang *et al*., 2008) ABE is a novel and non-radioactive approach for measuring protein palmitoylation based on methods established by Drisdel and Green (Drisdel and Green, 2004; Drisdel *et al.*, 2006). MudPIT is a technique used to separate and identify complex protein and peptide mixtures. In contrast, more traditional methods such as metabolic labeling were used to identify PSD-95 as a palmitoylated protein. Since its identification, several studies have reported that PSD-95 targeting to postsynaptic sites is largely dependent on its palmitoylation (El-Husseini *et al.*, 2000a; El-Husseini *et al.*, 2000b; El-Husseini *et al.*, 2000c; Bredt and Nicoll, 2003; Fukata *et al.*, 2004) as expression of a PSD-95 palmitoylation mutant lacks clustering at synapses, resulting in diffuse expression of PSD-95 throughout the cell. Interestingly, glutamate receptor activation causes depalmitoylation of PSD-95 and AMPAR endocytosis, thereby down regulating this signaling pathway (El-Husseini Ael *et al.*, 2002; Fukata *et al.*, 2004) (Figure 1.8). Similarly, this is seen with GAD65 trafficking from the Golgi compartment to the plasma membrane and synaptic vesicle membranes (Kanaani *et al.*, 2004) (Figure 1.8). In the depalmitoylated state these peripheral proteins cycle on and off the cytosolic faces of the ER and Golgi compartments (Kanaani *et al.*, 2004). Depalmitoylation by thioesterases releases the protein from the plasma membrane resulting in the retrograde trafficking back to the Golgi membranes via a non-vesicular pathway. The proteins can then enter a new cycle of palmitoylation/depalmitoylation.

All AMPAR subunits can be palmitoylated at two cysteines and one site is in TM2 and the second is in the intracellular C-terminal region (DeSouza *et al.*, 2002; Hayashi *et al.*, 2005; Jiang *et al.*, 2006)). Palmitoylation of TM2 results in the accumulation of AMPARs in the Golgi apparatus and consequently fewer receptors are found at the cell surface (Hayashi *et al.*, 2005). Palmitoylation of the cysteine at the C-terminus results in a reduction in the interaction between the receptor and protein 4.1N and mediates agonist-induced AMPAR internalization (Hayashi *et al.*, 2005). In summary, activation of AMPARs by glutamate stimulation causes a decrease in receptor palmitoylation and recruits more AMPARs to the cell surface to mediate synaptic plasticity (Jiang *et al.*, 2006).

Importance for palmitoylation of soluble proteins: One of the most commonly described functions of palmitoylation is to increase the affinity of a soluble protein for membranes. This has important consequences as it can affect trafficking of soluble proteins by 'trapping' proteins with weak affinity to membranes. Consequently, this enhances the strength of the membrane interaction (Huang and El-Husseini, 2005; Baekkeskov and Kanaani, 2009; Sorek *et al.*, 2009; Fukata and Fukata, 2010). The protein then associates more efficiently with budding vesicles and this enhanced membrane affinity ensures that the protein will not untether from the membrane during vesicle transport. PSD-95 and paralemmin-1 are dually lipidated and solely palmitoylated proteins, respectively and fall into this category.

Palmitoylation of membrane-associated and integral proteins is critical for localization: Membrane or integral proteins are strongly associated with the plasma membrane as these proteins contain transmembrane domains (TMD), and are embedded within the membrane (Fukata and Fukata, 2010).

What role does palmitoylation have on membrane-bound proteins if it is not to increase the association with the membrane? It has been widely accepted that palmitoylation of membrane proteins allows for the protein to associate with lipid rafts (Levental *et al.*, 2010). Lipid rafts have been defined as membrane associated regions further enriched in cholesterol and sphingolipids, which function to allow for association into larger and more stable structures (Huang and El-Husseini, 2005; Levental *et al.*, 2010). It has been hypothesized that palmitate groups may directly interact with cholesterol (Uittenbogaard and Smart, 2000; Roy *et al.*, 2005; Greaves and Chamberlain, 2007), but it is not clear how this occurs. There is some skepticism surrounding the existence of lipid rafts, and such ordered lipids because solid experimental evidence is lacking. One study that has provided compelling evidence of their existence is one that showed the palmitoylated isoform of Ras (H-Ras) can associate with lipid rafts (Roy *et al.*, 1999; Henis *et al.*, 2006). Although the jury is out on whether lipid rafts exist and how they function to interact with palmitoylated proteins, what is clear, is that palmitoylation of membrane-bound proteins critical for raft association cannot be predicted based on protein sequence, but rather must be experimentally determined using protein extraction with non-ionic detergents (Huang and El-Husseini, 2005).

Finally palmitoylation also regulates the interactions between two different proteins, for example, these interactions could be with receptors and scaffolding proteins and this occurs by controlling the conformation of the modified protein. In addition, palmitoylation may also serve to bring a protein-binding domain in close proximity to a membrane receptor, enhancing the possibility of a fruitful encounter. Finally, palmitoylation may regulate protein interactions by spatially coupling or segregating proteins within specific lipid microdomains.

1.5.3. *Role for palmitoylation in filopodia induction*

The functions of several acylated proteins implicated in filopodia induction, including GAP-43 (Strittmatter *et al.*, 1994b) Wrch, a (Wnt-regulated Cdc42 homolog) (Berzat *et al.*, 2005), and paralemmin-1 (Kutzleb *et al.*, 1998; Gauthier-Campbell *et al.*, 2004) seem to rely on protein palmitoylation. Thus, palmitoylation seems to exert specific effects that regulate induction of protrusion formation.

Paralemmin-1: is a dually lipidated protein that localizes to neuronal cells in the brain and is also phosphorylated. The chromosomal localization of paralemmin-1 gene, PALM, has been determined in mouse (chromosome 10) and man (19p13.3) (Burwinkel *et al.*, 1998). Paralemmin-1 has been found to be a hydrophilic protein anchored to membranes through a C-terminal CaaX lipidation motif (Gauthier-Campbell *et al.*, 2004; Kutzleb *et al.*, 1998; Kutzleb *et al.*, 2007). Paralemmin-1 does not contain any conserved protein-protein interaction motifs such as SAM, PDZ binding domains, however, analysis of the protein sequence revealed that paralemmin-1 is predicted to have high alpha helix as well as coiled-coil potential (Kutzleb *et al.*, 1998). Paralemmin-1 localizes to the plasma membrane of postsynaptic specializations including dendritic spines and filopodia, axonal and dendritic processes and the perikarya (Kutzleb *et al.*, 1998; Hu *et al.*, 2001; Gauthier-Campbell *et al.*, 2004; Kutzleb *et al.*, 2007).

Paralemmin-1 mRNA is detectable in all human tissues (Kutzleb *et al.*, 1998), but its highest expression is found in the brain (Kutzleb *et al.*, 1998). Alternative splicing of PALM-1 mRNA yields two isoforms: a shorter isoform lacking an exon 8 region and the longer isoform, which contains this

region (Kutzleb *et al.*, 1998). Otherwise both isoforms share an identical homology. In newborn mouse brain, the mRNA of the longer isoform including exon 8 is hardly detectable, but is induced as the mouse grows up and becomes most pronounced between days 10-20 (Kutzleb *et al.*, 1998). Thus, the longer isoform may play a more pivotal role for the formation of dendritic spines and recruitment of AMPARs.

Other palmitoylated molecules important for filopodia induction and dendritic branching: The growing amount of literature suggests that many of the proteins involved in the formation of neuronal processes and spines are palmitoylated. For example, the cell adhesion molecule, NCAM (Little *et al.*, 1998; Niethammer *et al.*, 2002; Ponimaskin *et al.*, 2008; Kleene *et al.*, 2010), neurofascin (Ren and Bennett, 1998), DCC (Herincs *et al.*, 2005) (an axon guidance receptor for the molecule netrin), cytoskeletal associated proteins (SCG10) (Charbaut *et al.*, 2005; Kang *et al.*, 2005; Chauvin *et al.*, 2008) and Cdc42 (Kang *et al.*, 2008). Palmitoylation is required for NCAM-mediated neurite outgrowth and palmitoylation of NCAM140 and NCAM180 targets them to lipid rafts of growth cone membrane (Little *et al.*, 1998; Niethammer *et al.*, 2002).

Brain derived neurotrophic factor (BDNF) has been shown to be critical for dendritogenesis in cultured cortical neurons as it is able to stimulate Ca^{2+} transients. The Ca^{2+}–calmodulin-dependent protein kinase type 1G (CAMK1G; also known as CLICK-III) plays a critical role in BDNF-mediated dendritic growth (Takemoto-Kimura *et al.*, 2007). CLICKIII is dually lipidated by prenylation and subsequent palmitoylation and its expression specifically enhances dendritic growth through Rac activation mediated by T lymphoma invasion and metastasis-inducing protein 2 (STEF), a RAC guanine exchange factor (Takemoto-Kimura *et al.*, 2007). In contrast, loss of CLICKIII

specifically reduces the number and length of dendritic branches and axogenesis remains intact (Takemoto-Kimura *et al.*, 2007). This result suggests that activation by BDNF leads to dendritogenesis through a palmitoylation-dependent mechanism.

1.6. Research hypothesis

My overall goal was to investigate the role of dendritic filopodia in spine formation. There are unanswered questions regarding the development of dendritic spines. Mounting evidence suggests that filopodia participate in neuronal contact formation and the development of dendritic spines. However, the molecules involved in filopodia formation and their transformation to spines remains largely unknown.

My work aimed to test whether paralemmin-1 is a molecule involved in the regulation of filopodia transformation to spines. To further address the importance of paralemmin-1 in this process, I hypothesized that the combined actions of paralemmin-1 and Shank1b are critical for filopodia induction and their maturation to spines. This work is of particular significance as dynamic changes in the structure of dendritic spines are thought to underlie many forms of adaptive behaviour including learning and memory. This work may provide insight into mechanisms that explain defects observed in several neurological diseases such as mental retardation and epilepsy. The following aims will test these hypotheses:

Aim 1: Examine the regulation of filopodia formation leading to spine maturation. To assess the importance of paralemmin-1 in filopodia formation and spine maturation, I altered the expression of paralemmin-1 either by overexpression or knockdown and examined the consequences on protrusion formation. This work assessed the role of palmitoylation as a signal for delivery of proteins involved in the regulation of cell morphology and membrane dynamics to specific active sites of the plasma membrane. **I hypothesized that the coordinated actions of paralemmin-1 and Shank1b may play**

a role in filopodia formation and the transformation to dendritic spines.

Aim 2: Determine whether filopodia actively participate in axo-dendritic contact formation. I performed timelapse imaging using fluorescently tagged proteins involved in filopodia formation and spine maturation and examined whether these proteins participate in the formation of synaptic contacts with nearby axons. In addition, we examined whether filopodia serve as precursors for the formation of dendritic spines. **I hypothesized that dendritic filopodia induced by specific molecules play a critical role in synaptogenesis and serve as precursors to spine synapses.**

2. Paralemmin-1, a modulator of filopodia induction, is required for spine maturation[1]

2.1 Introduction

During CNS excitatory synapse development, the formation of spines, bulbous protrusions enriched with F-actin, is essential for proper synaptic transmission and neuronal function (Hall and Nobes, 2000; Yuste and Bonhoeffer, 2004; Halpain *et al.*, 2005; Matus, 2005; Gerrow and El-Husseini, 2006). Spines contain a plethora of proteins including neurotransmitter receptors, cytoskeleton-associated proteins and cell adhesion molecules. Spines can be modified by changes in neuronal activity, which regulate actin-based motility (Fischer *et al.*, 1998; Portera-Cailliau *et al.*, 2003; Matus, 2005). Defects in spine maturation and function have been associated with several forms of mental retardation including Down, Rett, Fragile X and fetal alcohol syndromes. Some of these disorders exhibit a reduction in spine size and density, and the formation of long, thin filopodia-like structures (Hering and Sheng, 2001; Zoghbi, 2003).

Although our knowledge of molecules that control the morphology and functional properties of dendritic spines has expanded, information about the structures from which spines emerge is lacking.

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[1] This paper is published in *Molecular Biology of the Cell*.
Arstikaitis P, Gauthier-Campbell C, Carolina Gutierrez Herrera R, Huang K, Levinson JN, Murphy TH, Kilimann MW, Sala C, Colicos MA, El-Husseini A. (2008*)* Paralemmin-1, a Modulator of Filopodia Induction Is Required for Spine Maturation*. Molecular Biology of the Cell.* **5,** 2026-2038.

Dendritic filopodia, thin protrusions ranging in length from 2-35μm, are thought to participate in synaptogenesis, dendritic branching and the development of spines. During synaptogenesis, filopodia decorate the dendrites of neurons. Studies show that dendritic filopodia exhibit highly dynamic protrusive motility during periods of active synaptogenesis (Dailey and Smith, 1996; Ziv and Smith, 1996; Marrs *et al.*, 2001). Thus, filopodia are thought to function by extending and probing the environment for appropriate presynaptic partners, thereby aiding in synapse formation. These results are further supported by electron microscopy studies which show that synapses can be formed at the tip and base of dendritic filopodia (Fiala et al., 1998; Kirov et al., 2004). As synapses form, the number of filopodia declines and the number of spines increases, suggesting the involvement of dendritic filopodia in spine emergence as dendritic filopodia are later replaced by dendritic spines (Zuo *et al.*, 2005a). Decreased spine density and increased density of filopodia-like protrusions associated with several brain diseases lends further support to the notion that filopodia serve as precursors to spines (Fiala *et al.*, 2002; Calabrese *et al.*, 2006). However, no direct evidence illustrating the emergence of spines from filopodia has been found. Also, the molecular machinery required for filopodia induction and transformation to spines remains unknown.

A candidate protein that regulates filopodia induction in neurons is paralemmin-1, a molecule shown to induce cell expansion and process formation. Paralemmin-1 is abundantly expressed in the brain and concentrated at sites of plasma membrane activity, where it is anchored to the plasma membrane through lipid modifications. (Burwinkel *et al.*, 1998; Kutzleb *et al.*, 1998; Gauthier-Campbell *et al.*, 2004; Castellini *et al.*, 2005; Basile *et al.*, 2006; Kutzleb *et al.*, 2007). This protein localizes to the plasma membranes of postsynaptic specializations, axonal and dendritic processes and perikarya.

Using a combination of live imaging, as well as loss and gain of function approaches, our analysis identifies paralemmin-1 as a regulator of filopodia induction, synapse formation and spine maturation. We also found that paralemmin-1 recruited AMPA-type glutamate receptors to dendritic spines, a process governed by alternative splicing of paralemmin-1. These effects are modified by neuronal activity, which induces rapid translocation of paralemmin-1 to the plasma membrane. Activity-driven translocation of paralemmin-1 to membranes results in rapid protrusion expansion, emphasizing the importance of paralemmin-1 in paradigms that control structural changes associated with synaptic plasticity and learning. Finally, we show that knockdown of paralemmin-1 results in loss of filopodia and compromises spine maturation induced by Shank1b, a protein that facilitates rapid transformation of newly formed filopodia to spines. These findings elucidate an important role for paralemmin-1 in filopodia induction and spine maturation.

2.2 Materials and methods

2.2.1. cDNA cloning and mutagenesis

Wild type and cysteine mutant forms of mouse paralemmin-1 were generated by Polymerase Chain Reaction (PCR) and cloned in to the multiple cloning site (MCS) in pEGFP-C1 vector (Clontech) at BglII and HindIII restriction sites. Construction of Shank1b in to a GW1 expression vector occurred as previously described (Lim et al., 1999). RNAi generated against identical sequences in both mouse and rat paralemmin-1 were introduced into pSUPER vector (Clontech) into the HindIII/BglII sites and contained the following sequence GAAGAAGCCTCGCTGTAGA. Scrambled RNAi (Ctl RNAi) was subcloned as previously described (Huang et al., 2004). RNAi resistant paralemmin-1

was generated by creating 5 silent point mutations on the RNAi target sequence using the Stratagene site-directed mutagenesis kit (Stratagene) following manufacturer's instructions. The underlined nucleotides were mutated in the paralemmin-1 RNAi sequence GAAAAAACCACGATGCAGA. All constructs were verified by DNA sequencing.

2.2.2. Primary neuronal culture preparation, transfection, treatments and immunocytochemistry

Neuronal cultures were prepared from hippocampal embryonic day 18/19 rats. Cells were plated at 125,000 cells/coverslip as previously described (Gerrow et al., 2006). For neuronal depolarization, hippocampal neurons were treated either with 90 mM KCl for 3 min or with 50 mM KCl for 10 min during timelapse imaging. For immunocytochemistry, COS-7 cells and hippocampal neurons were fixed with 2% PFA and 4% sucrose or with methanol at –20^{o} C when staining for synaptic proteins. Fixative was removed and cells were washed three times with phosphate buffer saline (PBS) containing 0.3 % triton to permeabilize cells. The following primary antibodies were used: GFP (chicken; 1:1000; AbCam), GluR1 (rabbit; 1:500; Upstate Biotech) and HA (mouse; 1:1000; Synaptic Systems). For endogenous paralemmin-1 detection, rabbit anti-paralemmin-1 sera 2 and 10 were employed (Kutzleb et al., 1998). We used the following secondary antibodies: Alexa 488-conjugated anti-chicken (1:1000, Molecular Probes), Alexa 568-conjugated anti-mouse (1:1000, Molecular Probes) and Alexa 568-conjugated anti-rabbit (1:1000, Molecular Probes). Coverslips were incubated for 1 hr at room temperature with primary and secondary antibodies. To detect filopodia in COS-7 cells, we incubated cells for 40 mins with rhodamine labeled phalloidin (Molecular Probes). Coverslips were mounted with Flouromount-G (Southern Biotech).

2.2.3. Microscopy and timelapse recordings

Fluorescent images were acquired using a 63X objective coupled (NA= 1.4) to a Zeiss Axiovert M200 motorized inverted light microscope and Axiovision software. To correct for potentially out of focus filopodia z-projections were taken in 0.5µm sections. Timelapse imaging occurred in an environmentally controlled chamber with 5% carbon dioxide at 37^0C as previously described (Gerrow et al., 2006). Hippocampal neurons were plated on glass microwell dishes (Matek) at a density of 400,000 cells/dish. Images were acquired every 2 minutes for 2-3 hours. For quantification of timelapse imaging, the total number of filopodia and spine-like protrusions were counted on all dendritic branches within the field of view at time= 0 h based on criteria under **quantitative measurement of filopodia and spines** and expressed as a number per 100µm of dendritic length. Next, the fate of every protrusion counted at t=0h was manually tracked, traced and recorded. The frequency of four events (spine-like to filopodia, filopodia to spine-like, stable filopodia and stable spines) that we focused on, were recorded for each cell. Finally, we have expressed the total average of an event by the total number of filopodia or spines/100µm of dendrite. For confocal microscopy, images were captured using the Zeiss Confocal LSM510 Meta system 63X objective (NA=1.2) water lens as previously described (Kang et al., 2004). Images were captured using a 512X512 pixel screen and gain settings for both fluorophores were 600-800. Scan speed function was set to 6 and the mean of 16 lines was detected. Zoom function was set to 1 and the pinhole was set to 1 Airy unit for all experiments. Z-series were used to capture out of focus dendrites and sections.

2.2.4. Analysis of paralemmin-1 accumulation at the membrane

To assess changes in paralemmin-1 expression at the membrane we used Image J program (NIH). Images were acquired using confocal microscopy, which allowed us to define membrane versus cytoplasm expression. Images were exported as 16bit and analyzed using the segmented line tool. To assess changes in membrane localization of endogenous paralemmin-1 by KCl and 2-bromopalmitate (BP) treatments, the fluorescence intensity of lines drawn through the top and bottom portions of dendrites (membrane), versus the fluorescence intensity of a line drawn through the middle portion of a dendrite (cytoplasm) were contrasted. This analysis was performed in DIV 16-18, at a developmental stage where hippocampal neurons possess thick dendritic segments. An average membrane and cytoplasm fluorescence was calculated for all dendrites pertaining to each neuron. Statistical analyses were performed using excel software. All analyses were performed by an individual blinded to treatment conditions.

2.2.5. Quantification of KCl enlargement of dendritic protrusions

Timelapse imaging was performed over a 10 min interval and images were collected every 5 minutes as previously described (Gerrow *et al.*, 2006). Total number of protrusions per cell were quantified before and after KCl stimulation and expressed as the number of protrusions/100µm of dendritic length. The average diameter of protrusions, taken at the base and tips, were measured. For this analysis, all protrusions (including those that did not change) on individual cells were examined, and were measured before and after 50mM KCl treatment. A protrusion enlargement of greater than 2 µm was counted as an 'enlarged protrusion' and expressed as a % of change in protrusion size. For

irregularly-shaped protrusions, the area was measured using Northern Eclipse Software. Briefly, the entire structure (from base to the tip) before and after stimulation was manually traced and these included: growth-cone, lamellopodia-like structures, membrane expansion at the tip of filopodia, and expansion of existing protrusions. The data was further analyzed using excel software.

2.2.6. Photoconductive stimulation and quantification

Rat hippocampal neurons taken from P0 pups were grown on silicon waffers as previously described (Colicos *et al.*, 2001; Colicos and Syed, 2006; Goda and Colicos, 2006). Neuronal cultures were grown until DIV 4, at which time they were transfected using lipofectamine 2000 (Invitrogen, Burlington, Ontario) and stimulated 3-4 days later. In brief, the cultures were transferred to serum-free media for 1.5 h and then incubated with 1.5 μg of paralemmin-L DNA. Control image sequences were acquired prior to stimulation, using a WAT105N (Watec) camera on an Olympus BX60WI microscope. Neurons were then stimulated at 30 Hz for 15 s, and images acquired every 5 s for the next 10 min. Densitometry was performed on single images from the control sequence and post-stimulation using Image J software (NIH). Membrane and cytoplasm regions were selected randomly and regions of interest (Turner and Schwartzkroin) were defined over a segment of the membrane and the average pixel value calculated. ROI's were variable in size, depending on the thickness of the dendrite analyzed. Areas in the membrane included from: 1-2 pixels wide by 2-3 pixels and 1-2 pixels wide by 3-4 pixels in length. This ROI was then moved immediately inward from the membrane, and the average pixel value calculated. These two values were used to produce the ratio between the intensity of GFP-paralemmin-L signal inside the dendrite versus at the membrane.

Ratios from multiple experiments were averaged, and the error calculated as standard error of the ratio.

2.2.7. Quantitative measurement of filopodia and spines

Filopodia induction in COS-7 cells was scored according to the following criteria: within a field of view, cells with 3 filopodia or more were counted as cells "with filopodia" and all other cells within the same field of view were counted as cells "without filopodia". Filopodia induction is expressed as % of cells scored "with filopodia" normalized to a GFP control. For analysis of filopodia and spines in neuronal cells, images were scaled to 16bit and analyzed using Northern Eclipse Software (Empix Imaging, Mississauga, Canada) and automatically logged into Microsoft Excel (Microsoft). Any protrusion ranging in length from 2-10 μm and lacking a visible head (less than 0.35 μm) was counted as "filopodia" and marked. In all of our analyses, filopodia in general, were clearly distinguishable. However, in a few instances, filopodia could appear intermingled if the density was too high and were difficult to quantify. Spines were counted separately and spine heads were measured using the polygon tool and were only scored as a "spine-like" if a clear head greater than 0.35μm in width was measured. Finally, for morphological measurements the entire lengths of all primary, secondary and tertiary dendrites extending from the cell body were measured using the curve measurement tool and expressed as protrusions per unit length (100 μm) of dendrite. All analyses were performed by an individual blinded to treatment conditions.

2.2.8. Subcellular fractionation

Cultured cortical neurons (DIV 16–20; 12 × 10^6 cells) were treated for 3 min with or without 90mM KCl. Cells were washed 1× with PBS, harvested, and then suspended in 200 µl of sonication buffer (50 mM Tris [pH 7.4], 0.1 mM EGTA) supplemented with a protease inhibitor cocktail (2.5 µg/ml leupeptin, 2.5 µg/ml aprotinin, and 1 µM PMSF). Cells were sonicated on ice for 16s and nuclei were pelleted at 14,000 × g at 4°C for 10 min. Lysates were centrifuged at 49,000 × g for 1 h at 4°C. The supernatants were collected and pellets were resuspended in 150 µl resuspension buffer (RB; 50 mM Tris [pH 7.4], 0.1 mM EGTA, 1 M KCl, 10% glycerol, 1.5 µl/10 ml BME and protease inhibitors). Fractions (30 µl each) were analyzed by SDS-PAGE and membranes were probed for paralemmin-1 and transferrin receptor. Image J software was used to quantify paralemmin-1 band intensity by plotting the peaks and a student's paired t-test was used to determine statistical significance.

2.2.9 Statistical Analyses

All statistical analysis was done using XLSTAT add-in for Microsoft Excel (Addinsoft, NY) or student's T-test (Microsoft Excel) and multiple group comparisons were done using the one-way analysis of variance (ANOVA, with Student-Newman-Keuls post-hoc correction).

2.3 Results

2.3.1. Paralemmin-1 regulates protrusion formation in developing neurons

Previous investigations identified paralemmin-1 as a candidate protein that regulates filopodia induction in heterologous cells, however its role in neurons has not been explored (Kutzleb et al.,

1998). Consistent with a potential role for paralemmin-1 in filopodia induction, endogenous paralemmin-1 is detected in filopodia and spines in both immature (*days in vitro* 10 [DIV 10]) and mature (DIV 26) hippocampal neurons (Figure 2.1).

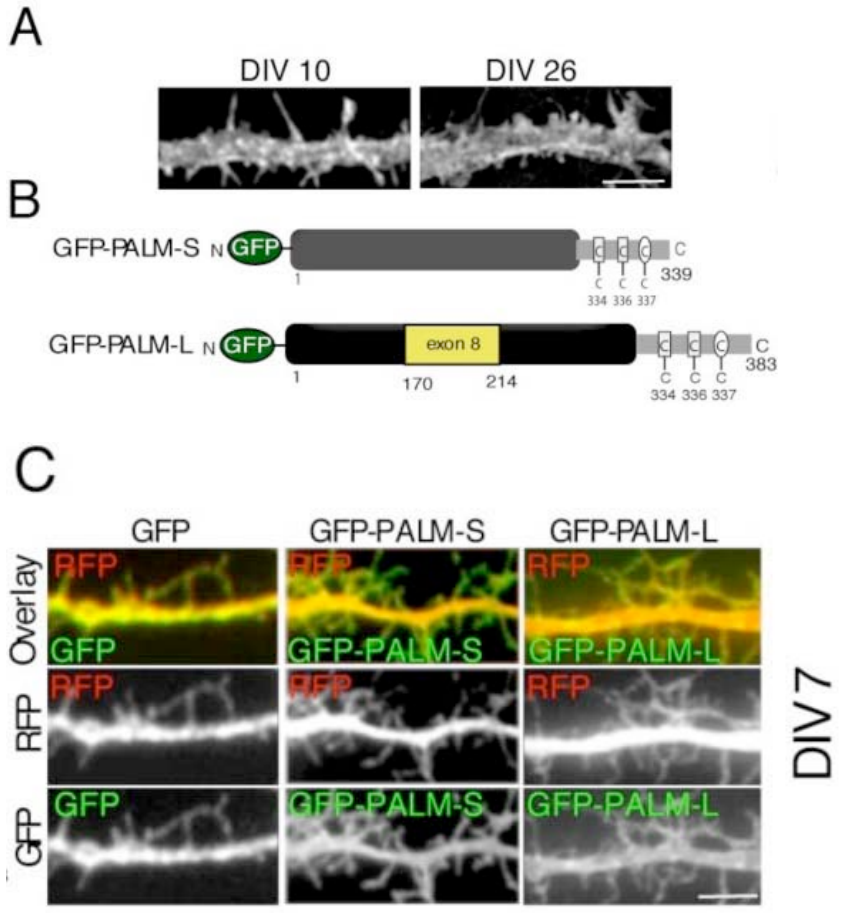

Figure 2.1 Paralemmin-1 is critical for filopodia induction in developing neurons
(A) Paralemmin-1 is localized to the plasma membrane, filopodia and spines in primary hippocampal neurons. Immunocytochemical staining of cultured hippocampal neurons reveals that paralemmin-1 is localized in patches along the plasma membrane. It is also detected in dendritic filopodia at days in vitro 10 (DIV 10) and spines in mature neurons (DIV26). (B) Diagram showing structure of wild type GFP-tagged paralemmin-1 splice variants. Location of the palmitoylated cysteines (C334, C336) and the prenylated residue (C337) is indicated. (C) Both paralemmin-1 splice variants induce filopodia at DIV 7. Hippocampal neurons were co-transfected at DIV 5 with RFP and either GFP, GFP-paralemmin-S, the short variant of paralemmin-1 lacking sequences encoded by exon 8 (GFP-PALM-S) or GFP-paralemmin-L, the long variant containing sequences encoded by exon 8 (GFP-PALM-L). Scale bars, 10 μm**.**

Alternative splicing of paralemmin-1 is developmentally regulated (Kutzleb et al., 1998). The expression of a short splice variant (paralemmin-S) lacking exon 8 occurs early in development, preceding spine formation, whereas the expression of the long splice variant containing exon 8 (paralemmin-L) correlates with a period of active spinogenesis (Fig. 2.1B). Here we contrasted the effects of paralemmin-1 variants on filopodia induction in developing hippocampal neurons at DIV 7, a period that correlates with active filopodia formation. When transfected into neurons, both paralemmin-S (19.1$\pm$1.2) and paralemmin-L (19.0$\pm$2.1) splice variants were found to enhance the number of filopodia per 100 μm of dendritic length when compared to control cells expressing GFP (11.5$\pm$1.9) (Fig. 2.1C).

We next performed knockdown experiments to investigate whether paralemmin-1 is required for filopodia induction. RNAi that specifically blocks the expression of paralemmin-1 (PALM RNAi) in both heterologous cells and neurons (GFP-actin+Ctl RNAi (100.0%$\pm$8.4); GFP-actin+PALM RNAi (46.8%$\pm$7.0) was generated and characterized (Figure 2.2).

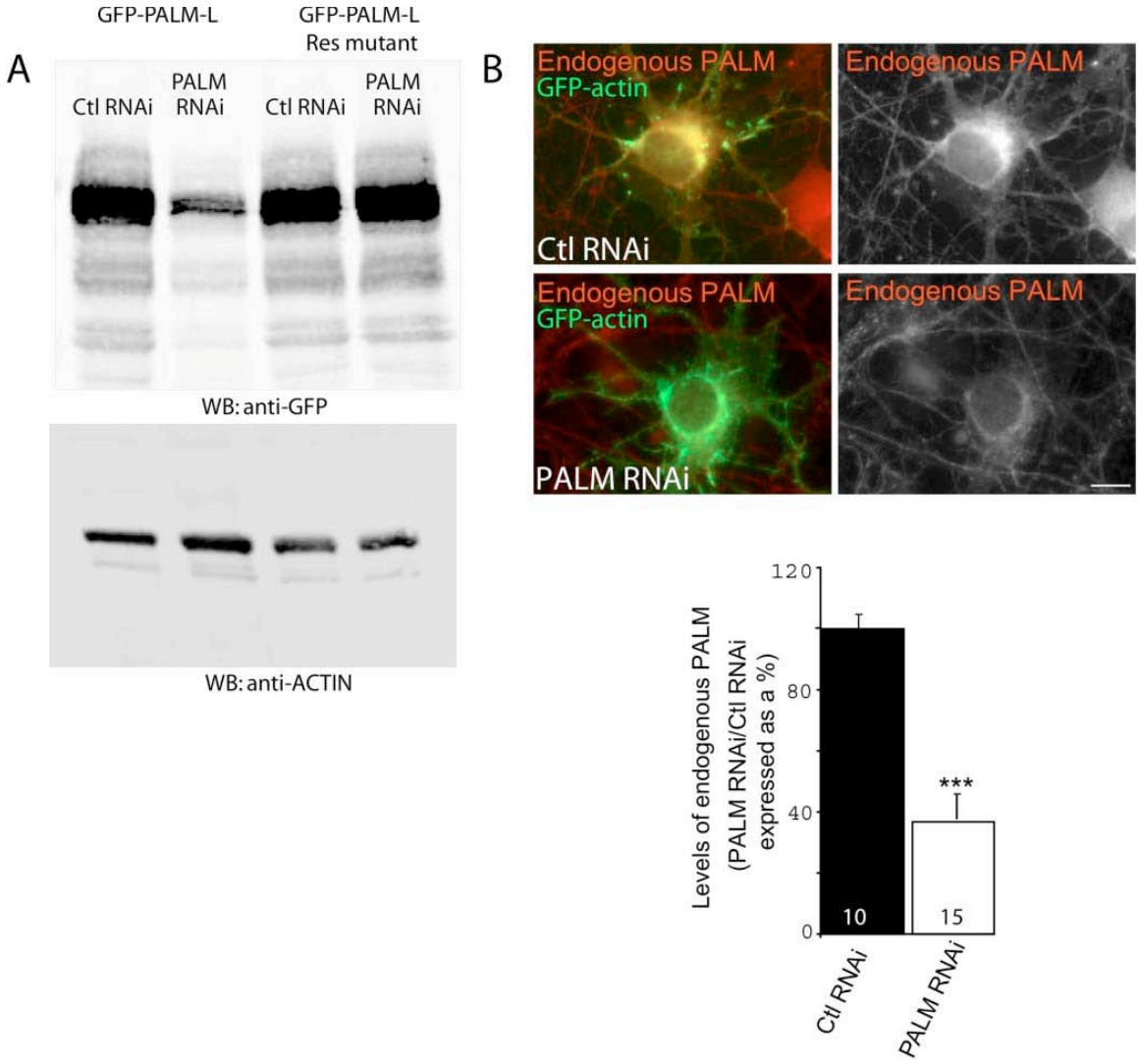

Figure 2.2 Generation of paralemmin-1 specific RNAi
(A) Paralemmin-1 specific RNAi (PALM RNAi) was co-transfected with GFP-paralemmin-L (GFP-PALM-L) into COS-7 cells to determine the efficiency of paralemmin-1 knockdown. Western blot analysis reveals that PALM RNAi reduces expression of GFP-paralemmin-L compared to control RNAi (Ctl RNAi). In contrast, the expression of a mutant form of paralemmin-L resistant to PALM RNAi was not affected upon co-transfection with PALM RNAi. Western blot showing similar actin expression levels is shown below. (B) The level of knockdown in neuronal cells was examined by co-expressing GFP-actin with PALM RNAi and staining for endogenous paralemmin-1 levels (Endogenous PALM). PALM RNAi results in 53.2% reduced expression of endogenous paralemmin-1 in neurons. Number of cells analyzed for each group is indicated at the bottom of each bar. ***$p<0.001$. Data represent mean $\pm$ SEM. Scale bar in (B) 10μm.

Neurons were co-transfected with red fluorescent protein (RFP) and either PALM RNAi or control scramble RNAi (Ctl RNAi; Fig. 2.3A), and changes in filopodia number were assessed by visualizing

RFP positive protrusions. Knockdown of paralemmin-1 resulted in a significant decrease in the number of filopodia per 100 μm of dendritic length (9.0±1.3) when compared to neurons expressing Ctl RNAi (13.9±0.8) (Figure 2.3B). To exclude the possibility that the reduction in filopodia number upon paralemmin-1 knockdown is due to off-target effects, we generated a paralemmin-1 mutant resistant to RNAi (Figure 2.3A). This was done by mutating 5 sites withing the RNAi sequence such that the mutated nucleotide still coded for the same amino acid. Co-transfection of this mutant with PALM RNAi restored filopodia number to levels similar to cells transfected with wild-type paralemmin-1 and Ctl RNAi (18.5±1.5) (Figure 2.3B). Moreover, co-transfection of paralemmin-1 with PALM RNAi resulted in a similar reduction in filopodia number (6.5±0.8) (Figure 2.3B). In contrast, transfection of paralemmin-1 with Ctl RNAi resulted in a significant increase in the number of filopodia compared to GFP (Figure 2.3B). These results suggest that PALM RNAi is indeed specific to knockdown of paralemmin-1.

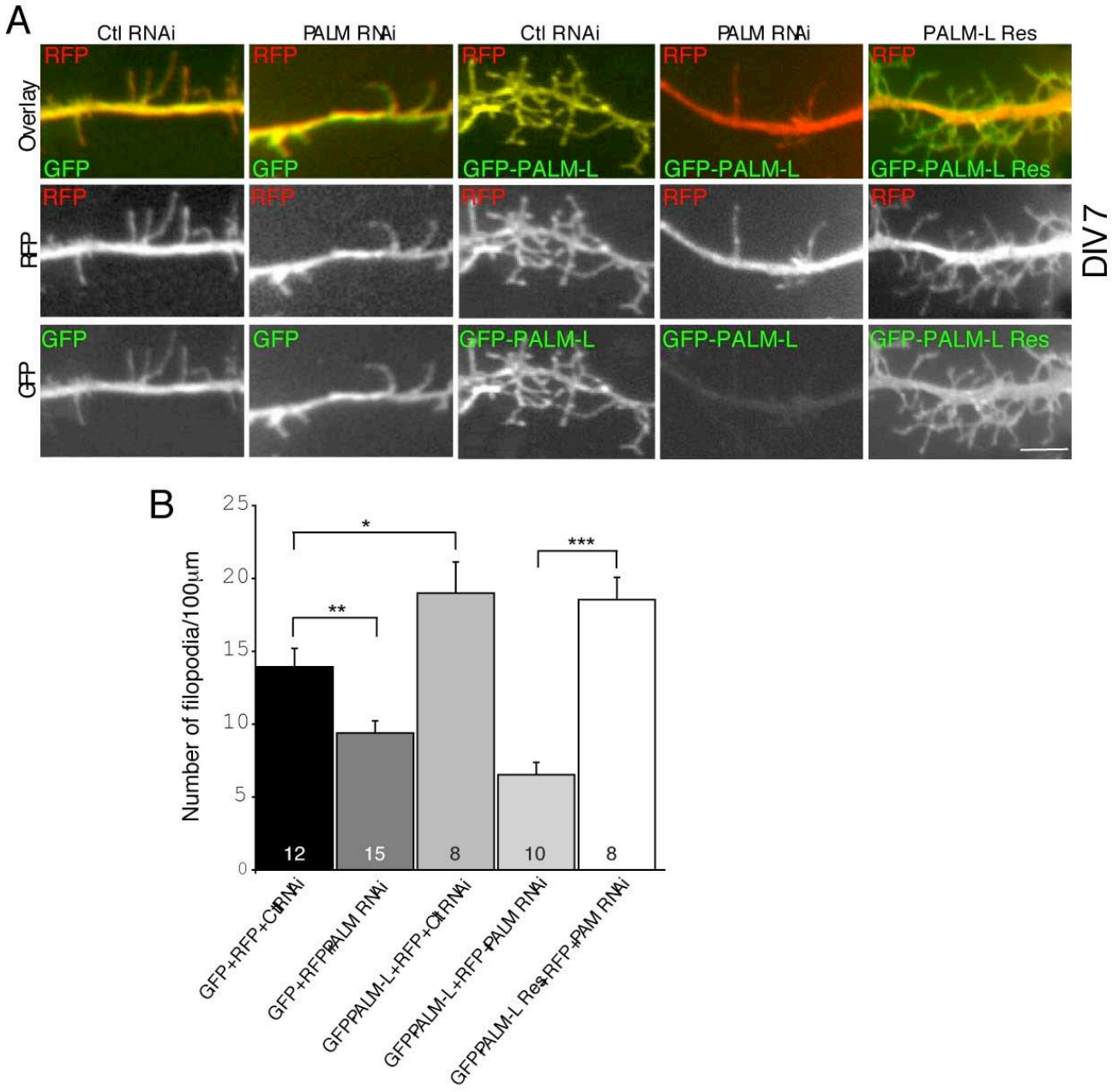

Figure 2.3 Knockdown of paralemmin-1 influences the number of filopodia formed at DIV 7
(A) Neurons were co-transfected with RFP and either with GFP or GFP-PALM-L, and scramble RNAi as a control (Ctl RNAi) or with paralemmin-1 specific RNAi (PALM RNAi). Paralemmin-1 resistant RNAi (PALM Res.) was also used to determine whether changes in filopodia number are due to specific knockdown of paralemmin-1. (B) Quantification of the number of filopodia/100mm shows paralemmin-1 knockdown diminishes the number of filopodia formed and these effects can be rescued upon expression PALM Res. Number of cells analyzed for each group is indicated at the bottom of each bar. Number of filopodia analyzed per group: RFP+GFP+PALM RNAi = 532, RFP+GFP+Ctl RNAi = 666, RFP+PALM-L+Ctl RNAi = 507, PALM-L+RFP+PALM RNAi = 202 and RFP+PALM-L Res+ PALM RNAi = 531.*$p<0.05$, **$p<0.01$, ***$p<0.001$. Data represent mean $\pm$ SEM. Scale bars, 10 µm.

2.3.2. Spine induction by paralemmin-1 is regulated by alternative splicing and protein palmitoylation

Since filopodia are thought to serve as precursors for spines, the ability of paralemmin-1 to regulate filopodia induction prompted us to examine whether long-term overexpression of paralemmin-1 ultimately influences the number of spines (Figure 2.4A). This analysis was performed in neurons at DIV 12-14, a period where spines begin to emerge. Changes in the relative proportions of filopodia and spines were contrasted to altered Shank1b expression, a potent modulator of spine maturation (Sala et al., 2001). Since the palmitoylation motif of paralemmin-1 fused to GFP (paralemmin CT) is sufficient to increase the number of filopodia in neuronal cells (Fig. 2.4B), we first examined whether paralemmin-CT induced filopodia is sufficient to increase spine number. Indeed, induction of filopodia correlated with an increase in spine number in neurons transfected with paralemmin CT (Figure 2.4B and C). We next contrasted the effects of paralemmin CT paralemmin-S, and paralemmin-L expression.

Expression of paralemmin-S (16.9$\pm$1.9), paralemmin-L (26.1$\pm$2.7), as well as paralemmin CT (19.3$\pm$1.3) significantly increased the number of spine like-protrusions per 100 μm of dendritic length when compared to GFP-expressing cells (11.1$\pm$0.7) (Fig. 2.4B and C). The induction of filopodia and spines by paralemmin CT was comparable to paralemmin-S, indicating a significant role for the lipidated motif of paralemmin-1 in altering protrusion formation by paralemmin-S (Figure 2.4B and C). In contrast, Shank1b (42.1$\pm$5.8) had profound effects on spine number but did not alter the number of filopodia (Figure 2.4B and C).

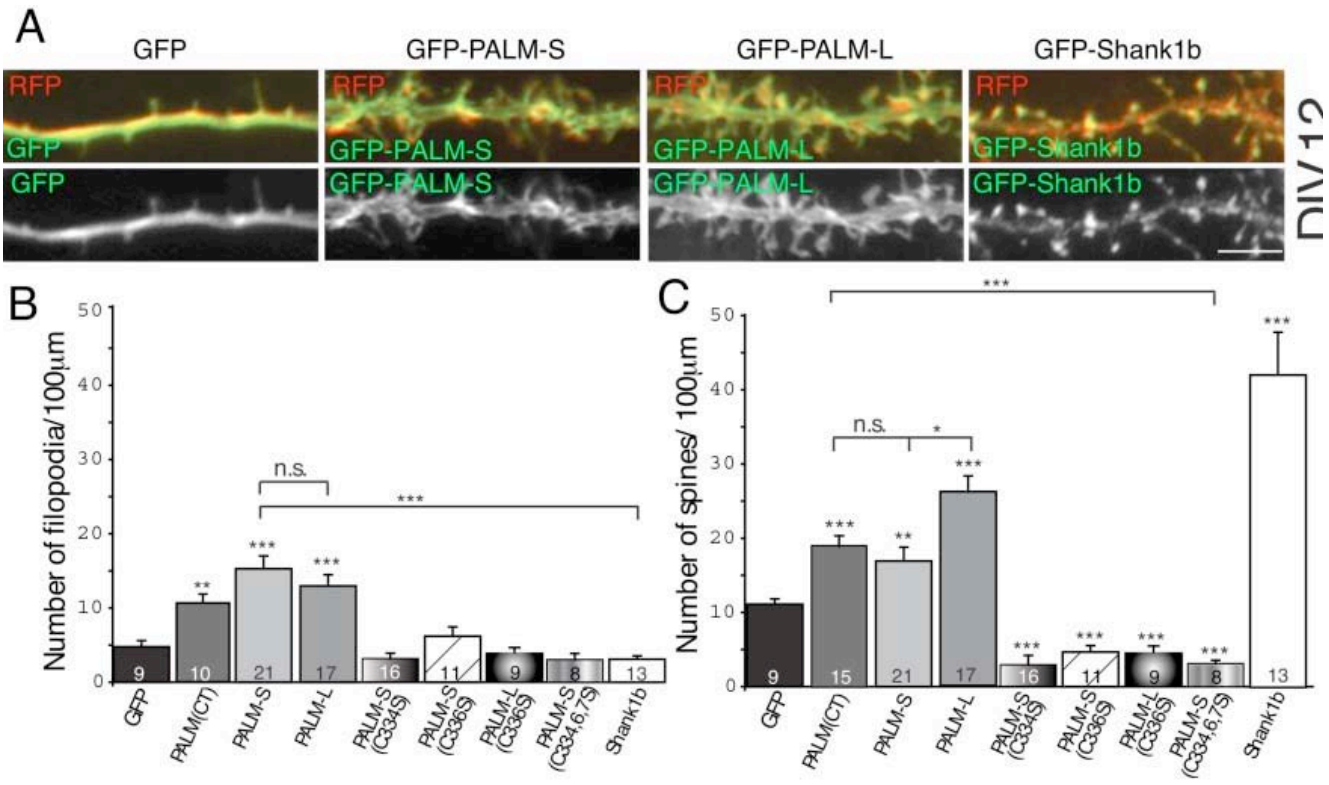

Figure 2.4 Long term expression of paralemmin-1 induces spine maturation
(A) Effects of paralemmin-1 expression on the number of filopodia and spines formed was assessed in hippocampal neurons co-transfected with RFP (red) and either GFP (green), GFP-tagged paralemmin CT (GFP-PALM (CT)), GFP-tagged paralemmin-S (GFP-PALM-S), GFP-tagged paralemmin-L (GFP-PALM-L), mutant forms of GFP-PALM-S either lacking Cys 334 (GFP-PALM-S (C334S), Cys336 (GFP-PALM-S (C336S), or Cys334, Cys336, Cys337 (GFP-PALM-S (C334,6,7S)) and GFP-PALM-L (C336S), at DIV 7 and fixed at DIV 12-14. Expression of various paralemmin-1 recombinant forms on dendritic protrusions was contrasted to GFP-tagged Shank1b (GFP-Shank1b). (B, C) Results show that GFP-PALM (CT), GFP-PALM-S and GFP-PALM-L, but not the palmitoylation deficient forms, increases the number of filopodia and spines formed. More robust effects on spine maturation were observed with GFP-PALM-L. In contrast, GFP-Shank1b overexpression enhanced spine maturation but did not alter the number of filopodia formed. Number of cells analyzed for each group is indicated at the bottom of each bar. Number of filopodia and spines analyzed per group in (A) are respectively: GFP +RFP = 120 and 334, PALM (CT) +RFP = 628 and 878, PALM-S +RFP = 996 and 1124, PALM-L+RFP = 565 and 1386, PALM-S (C334, 6, 7S) = 86 and 76, PALM-S (C336S) = 180 and 144, PALM-S (C334S) = 187 and 118, PALM-L (C336S) = 115 and 112, and Shank1b+RFP = 103 and 1572, respectively. **$p<0.01$, ***$p<0.001$. n.s. = no significant difference. Data represent mean + SEM. Scale bars, 10µm

Next, we examined the effects of mutant forms of paralemmin-1 lacking the palmitoylated cysteines at positions 334 and 336, or a combination of the palmitoylated cysteines and the prenylated residue at position 337. Mutating any of the lipidated sites abolished the ability of paralemmin-1 to increase the number of filopodia and spines. The number of spines was reduced below control levels, suggesting a dominant-negative mechanism (paralemmin-S (C334S), 3.0$\pm$0.3; paralemmin-S (C336S), 4.7$\pm$0.9; paralemmin-L (C336S); paralemmin-S (C334, 336, 337S), 3.1$\pm$0.4; 4.5$\pm$0.8, Figure 2.4B and C).

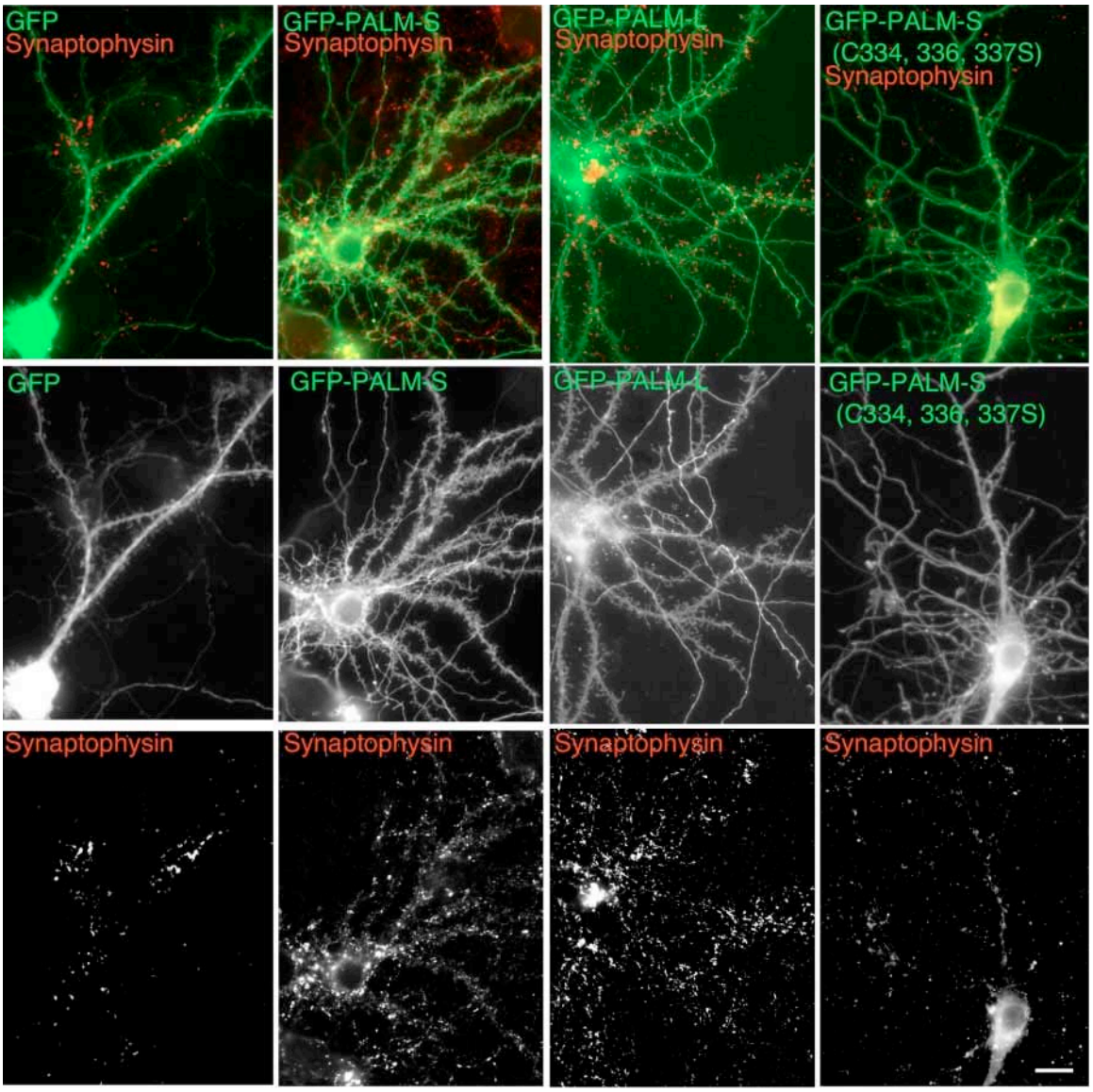

Figure 2.5 Effects of long-term expression of paralemmin-1 splice variants on presynaptic maturation Hippocampal neurons were transfected with GFP (green), GFP-tagged forms of paralemmin-S (GFP-PALM-S), paralemmin-L (GFP-PALM-L) or Cys334, Cys336, Cys337 (GFP-PALM-S (C334,6,7S)) at DIV 7. Neurons were fixed and stained with synaptophysin antibody (red) at DIV 14. Full images showing a significant increase in the number of synaptophysin clusters in neurons expressing GFP-PALM-S and GFP-PALM–L, but not GFP-PALM-S (C334,6,7S) when compared to GFP transfected controls. Scale bar, 20μm.

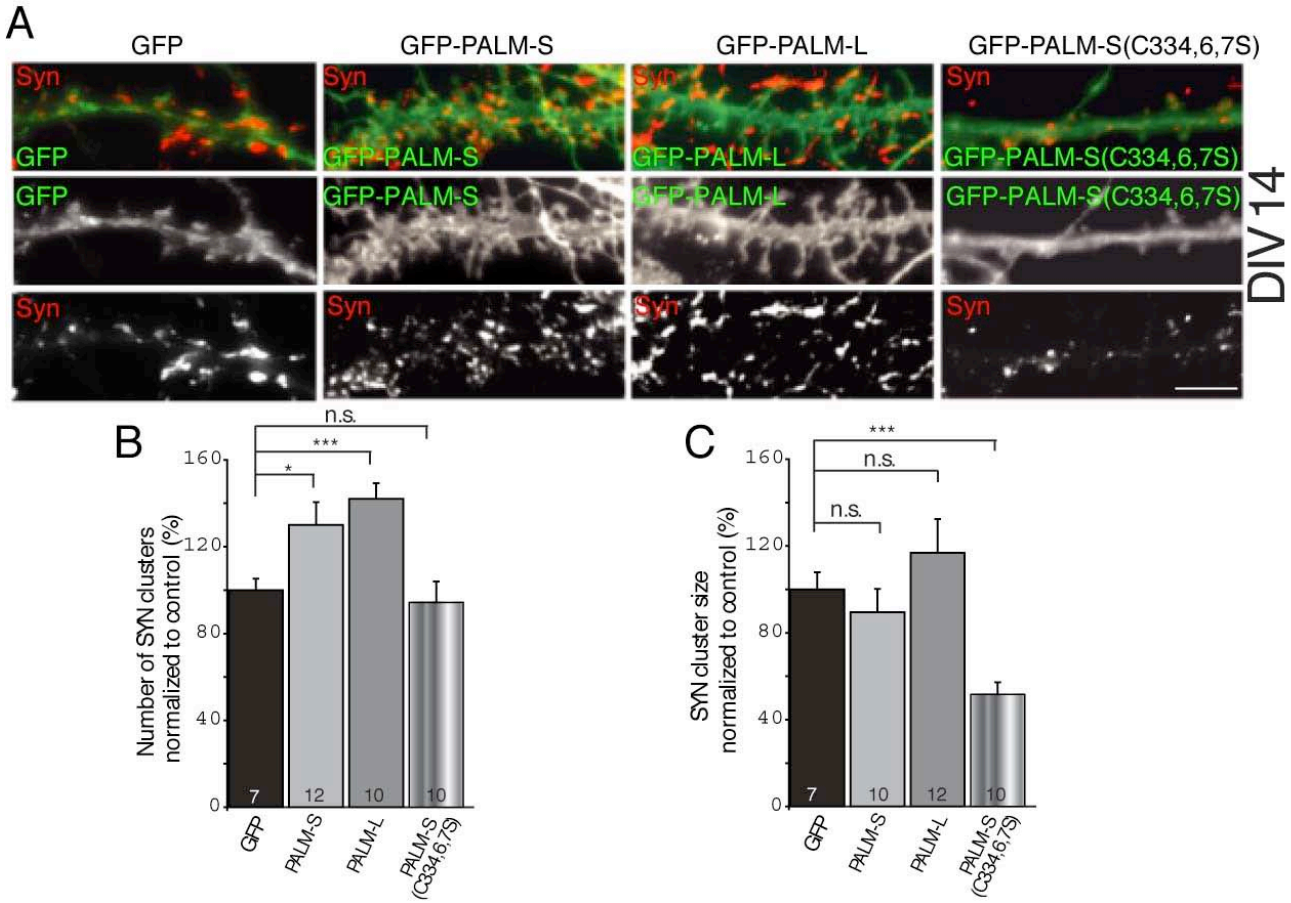

Figure 2.6 Long term expression of paralemmin-1 induces spine maturation
(A) Effects of paralemmin-1 expression on the number of filopodia and spines formed was assessed in hippocampal neurons co-transfected with RFP (red) and either GFP (green), GFP-tagged paralemmin CT (GFP-PALM (CT)), GFP-tagged paralemmin-S (GFP-PALM-S), GFP-tagged paralemmin-L (GFP-PALM-L), mutant forms of GFP-PALM-S either lacking Cys 334 (GFP-PALM-S (C334S), Cys336 (GFP-PALM-S (C336S), or Cys334, Cys336, Cys337 (GFP-PALM-S (C334,6,7S)) and GFP-PALM-L (C336S), at DIV 7 and fixed at DIV 12-14. Expression of various paralemmin-1 recombinant forms on dendritic protrusions was contrasted to GFP-tagged Shank1b (GFP-Shank1b). (B and C) Dendritic protrusions induced by paralemmin-1 are synaptic. The number of synaptophysin positive clusters were measured and normalized to controls expressing GFP. GFP-PALM-S and GFP-PALM-L but not GFP-PALM-S (C334,6,7S) significantly increased the number of synaptophysin (Syn) positive clusters when compared to GFP controls. Number of cells analyzed for each group is indicated at the bottom of each bar. *$p<0.05$, ***$p<0.001$. n.s. = no significant difference. Data represent mean $\pm$ SEM. Scale bars, 10µm.

To determine whether newly formed protrusions represent sites apposed to presynaptic elements, we analyzed changes in synaptophysin-positive clusters at DIV 12-14 (Figure 2.6A; Figure 2.5). This

analysis revealed that both splice variants of paralemmin-1 increased the number, but not the size of synaptophysin-positive clusters compared to GFP (Figure 2.6B and C). Expression of the palmitoylation/prenylation mutant form (paralemmin-S (C334, 336, 337S)) did not alter synaptophysin cluster number, but resulted in a significant reduction in the size of synaptophysin-positive clusters compared to GFP (Figure 2.6B and C), a result which suggests that expression of this mutant interferes in a dominant-negative fashion with the recruitment of elements required for synapse maturation.

Next, we examined whether expression of paralemmin-1 modulates postsynaptic maturation by quantifying changes in clustering of the AMPA receptor subunit, GluR1. Transfected neurons were fixed at DIV 14-16 and stained for GluR1 (Figure 2.7A). Both paralemmin-1 splice variants increase the number of GluR1-positive puncta, however the effects of paralemmin-L were more dramatic (Figure 2.7B). Moreover, paralemmin-L, but not paralemmin-S, increased the size of GluR1 puncta in individual spines, suggesting that developmentally regulated expression of paralemmin-1 splice variants control specific steps in filopodia formation and their maturation to spines (Figure 2.7C).

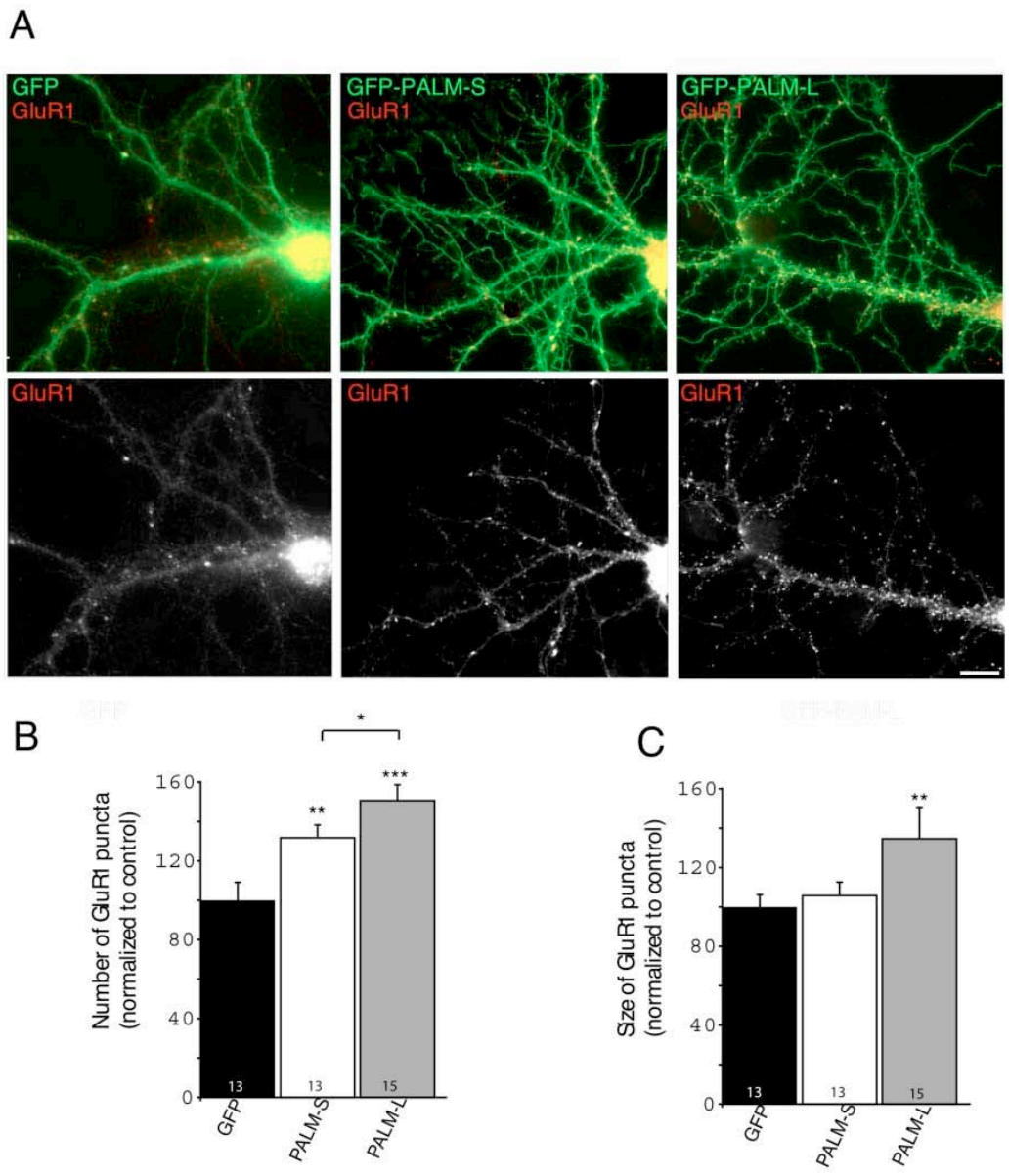

Figure 2.7 Differential effects of paralemmin-1 splice variants on GluR1 accumulation in dendritic spines

(A) Hippocampal neurons were transfected at DIV 7 with either GFP (green), GFP-tagged paralemmin-S (GFP-PALM-S) or GFP-tagged paralemmin-L (GFP-PALM-L) and fixed and stained with GluR1 specific antibodies (red) at DIV 14. (B) Number of GluR1 puncta was significantly increased in neurons expressing GFP-PALM-L and GFP-PALM-S when compared to GFP expressing controls. (C) GluR1 puncta size was significantly increased in neurons expressing GFP-PALM-L but not GFP-PALM-S. Number of cells analyzed for each group is indicated at the bottom of each bar. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Data represent mean $\pm$ SEM. Scale bar, 10μm.

2.3.3. Differential effects of paralemmin-1 and Shank1b on filopodia induction and spine maturation

Both paralemmin-L and Shank1b induce spine maturation, however it is unclear whether similar mechanisms are involved. To further explore this issue, we used a heterologous expression system to determine if paralemmin-1 and Shank1b are involved in filopodia induction. We transfected COS-7 cells with either GFP, paralemmin-S, paralemmin-L, the C-terminal tail of paralemmin-1 fused to GFP (paralemmin CT), the acylation-deficient forms of paralemmin-S, the acylation-deficient form of paralemmin-L (C336S), or Shank1b, and stained with antibodies against GFP and phalloidin (Figure 2.8A). Both paralemmin-1 splice variants and paralemmin CT were sufficient to induce filopodia in a palmitoylation-dependent manner (Figure 2.8B). Conversely, Shank1b failed to induce filopodia in these cells (Figure 2.8B). This analysis shows that Shank1b is insufficient for filopodia induction in heterologous cells. However, it is possible that Shank1b influences filopodia induction in developing neurons.

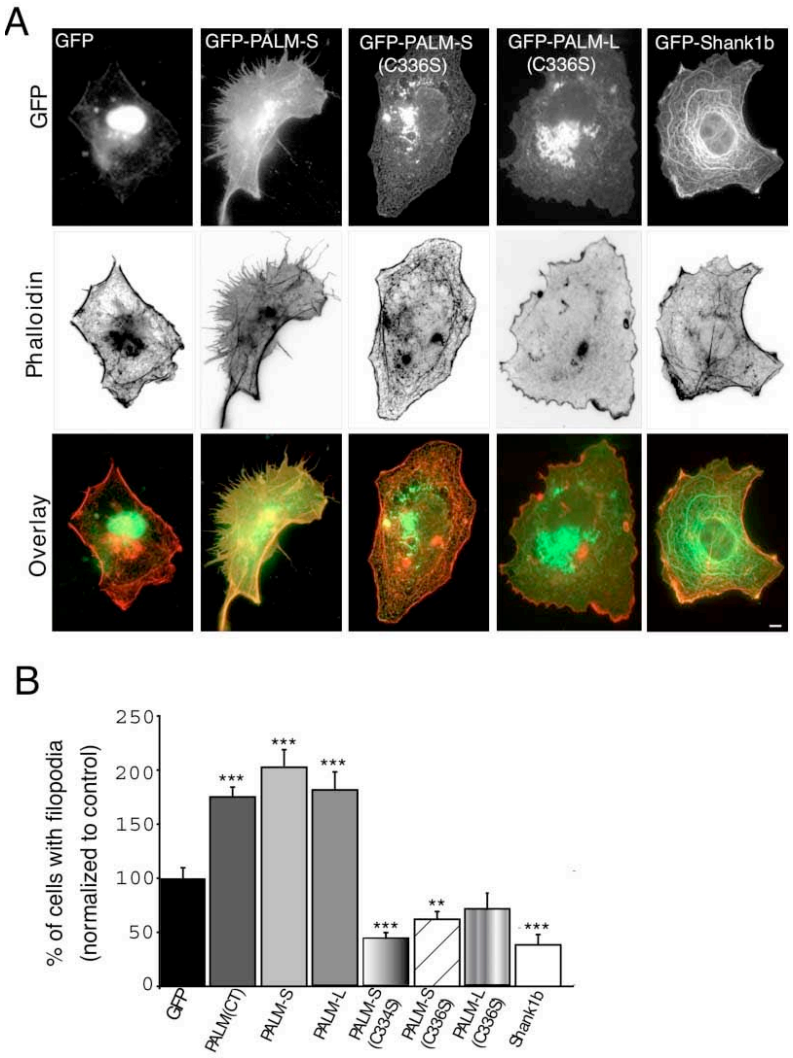

Figure 2.8 Induction of filopodia by paralemmin-1 but not Shank1b in COS-7 cells
Various constructs fused to GFP (green) were transfected into COS-7 cells, fixed and stained with rhodamine-conjugated phalloidin (red). (A) Representative images of cells transfected with either GFP (green), GFP-tagged paralemmin CT (GFP-PALM(CT)), GFP-tagged paralemmin-S (GFP-PALM-S), palmitoylation mutant forms of paralemmin-1 lacking Cys 336 (GFP-PALM-S (C336S), GFP-PALM-L (C336S) or GFP-tagged Shank1b (GFP-Shank1b) are shown in top panels. (B) Quantification of filopodia induction was measured by counting the number of cells that showed filopodia outgrowth. Cells immunolabeled for phalloidin are shown in middle panels. Analysis demonstrates that wild type forms of paralemmin-1 but not the palmitoylation deficient forms or Shank1b significantly increase the number of cells with filopodia when compared to GFP expressing cells. Additionally, appending the C-terminal acylated motif of paralemmin-1 to GFP (GFP-PALM(CT)) is sufficient for filopodia induction in COS-7 cells. Number of cells analyzed for each group ≥ 69. $^{**}p<0.01$, $^{***}p<0.001$. Data represent mean $\pm$ SEM. Scale bar, 5μm.

To assess this possibility, we performed a detailed time course analysis and contrasted the number of filopodia and spines formed in neurons transfected with GFP or Shank1b, 48 and 72 h post-transfection (Figure 2.9A). Assessing protrusion type and number, we found that Shank1b expressing cells exhibited a significant increase in the number of spine-like protrusions (13±1.7) 48 h post-transfection when compared to GFP (3±0.4) as measured per 100 µm of dendritic length (Figure 2.9B). However, after 72 h expression, we found a small but significant reduction in the number of filopodia in Shank1b-expressing cells (5±0.5) compared to GFP (11±1.8). This decrease in the number of filopodia after 72 h expression correlated with a significant increase in the number of spines (24.5±3.9) induced by Shank1b, suggesting that Shank1b is potentially involved in the transformation of existing filopodia to spine-like protrusions.

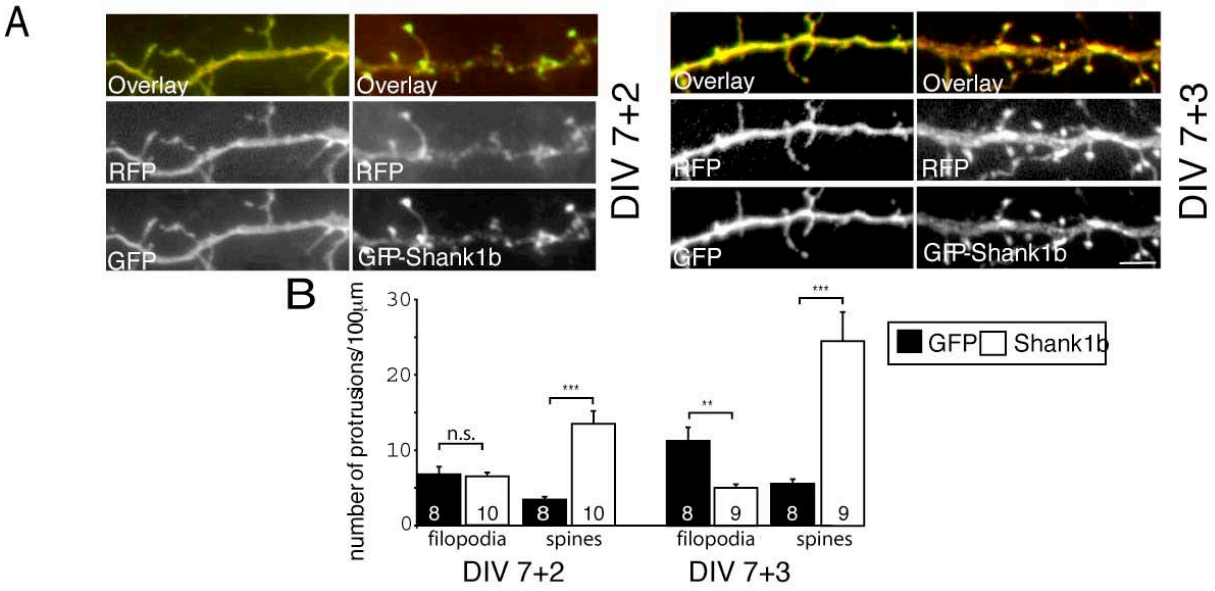

Figure 2.9 Shank1b induces rapid protrusion transformation from filopodia to spine-like structures (A) Neurons were transfected with RFP and GFP-tagged Shank1b (GFP-Shank1b) at DIV 7 and then fixed at either DIV 9 or 10. GFP-Shank1b expression decreases the ratio of filopodia to spines formed when compared to neurons expressing GFP. (B) Quantification of changes in dendritic protrusions per unit length. , **p<0.01, ***p<0.001. n.s. = no significant difference. Data represent mean + SEM. Scale bar, 10µm.

To further characterize the timing of filopodia transformation to spines, we performed timelapse imaging in neurons transfected with RFP in combination with various constructs of interest at DIV 7 and imaged 2 days post-transfection (Figure 2.11A, B and C). Images were acquired every 2 min, and we focused on quantifying 4 major events during a 2- to 3-h imaging period: 1) spine-like protrusions that become filopodia, 2) filopodia that transform into spine-like protrusions, 3) stable filopodia, and 4) stable spine-like protrusions. This analysis revealed that within this short time scale, paralemmin-L enhanced the turnover of filopodia to spines (24%$\pm$3.8) and spines to filopodia (39%$\pm$5.7) compared to GFP (10%$\pm$1.2 and 25%$\pm$4.3), respectively (Figure 2.11 A,D). This finding suggests that paralemmin-1 accelerates protrusion turnover and dynamics, favoring the formation of both filopodia and spine-like protrusions. Moreover, spine-like protrusions that remain stable within the entire imaging period were not significantly altered by paralemmin-L compared to GFP expressing cells, suggesting that overall; paralemmin-1 accelerates membrane dynamics and protrusion turnover in the direction of filopodia to spines, rather than destabilizing newly formed spines. In older neurons (DIV 14), however, paralemmin-L expression enhanced spine stability (66.0$\pm$2.0%) when compared to GFP (50.8$\pm$4.7%) controls (Figure 2.10). These results may reflect a maturation stage-dependent difference in membrane dynamics in young versus old neurons.

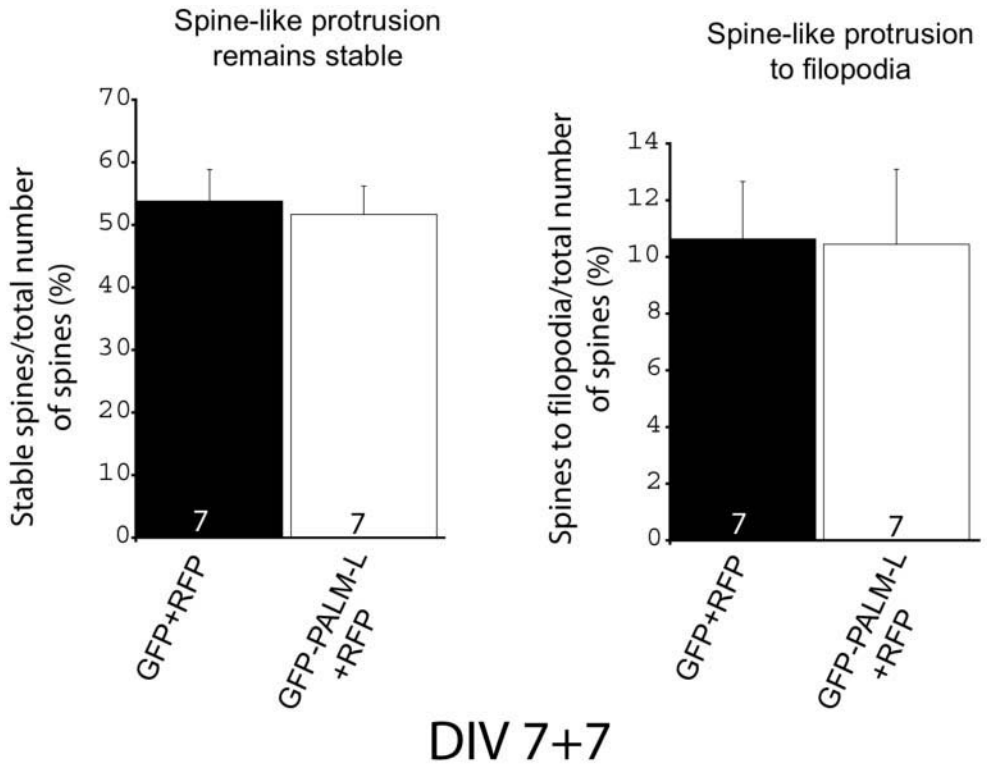

Figure 2.10 Paralemmin-L expression in mature neurons enhances spine stability
Neurons were transfected with RFP and either GFP or GFP-tagged paralemmin-L (GFP-PALM-L) at DIV 7 and then imaged at DIV 14. Quantification of stable spines and spine to filopodia transformations reveals that GFP-paralemmin-L expression increases spine stability compared to GFP. Furthermore, there is a decrease in spine-like to filopodia transformations compared to younger cells. Number of spines analyzed for each group is indicated at the bottom of each bar. *$p<0.05$. Data represent mean $\pm$ SEM.

In contrast with the moderate effects of paralemmin-1 manifested on spine stabilization in DIV9 neurons, the number of events in which existing filopodia transform into spine-like protrusions was significantly increased in Shank1b-expressing cells (Shank1b; 36.0%$\pm$4.3, paralemmin-L; 23.5%$\pm$3.7, GFP; 9.8%$\pm$1.2) (Figure 2.11C and D). Moreover, the number of stable spine-like protrusions in Shank1b-expressing cells was greater than paralemmin-L (Shank1b; 31.6%$\pm$4.1, paralemmin-L; 12.4%$\pm$1.9) and GFP-expressing neurons (20.6%$\pm$2.7) (Figure 2.11B and D). These results reveal that paralemmin-1 effects on spine maturation are slow, requiring several days, and most likely this process involves recruitment of other molecules to coordinate their transformation

into spines. In contrast, transformation of filopodia into spines occurs rapidly in Shank1b overexpressing cells, on the time scale of minutes to hours (Figure 2.11C and D). These results hint to a mechanism by which recruitment of mobile transport packets of proteins to filopodia stabilizes dendritic protrusions (Marrs *et al.*, 2001; Prange and Murphy, 2001). Mobile clusters containing PSD-95 and Shank1b do exist (Gerrow *et al.*, 2006) and thus, one possibility is that recruitment of a scaffold protein complex containing Shank1b to filopodia plays a role in the stabilization of these structures.

The enhanced transformation of filopodia to spines by Shank1b suggests that its expression would potentiate paralemmin-1 effects on spine induction. To explore this possibility, the effect of co-expression of GFP-paralemmin-L and HA-Shank1b on spine number was examined. For this analysis, neurons were transfected at DIV 7 and fixed and stained at DIV 12, using GFP and HA antibodies, respectively. Indeed, neurons co-transfected with Shank1b and PALM-L (42.5$\pm$2.6) showed a significant increase in the number of spines per 100 μm of dendritic length when compared to either GFP+RFP (15.5$\pm$2.8) or paralemmin-L +RFP (26.8$\pm$3.6) expressing cells (Figure 2.12). These results are consistent with a facilitative role for Shank1b in stabilization and maturation of protrusions induced by paralemmin-L.

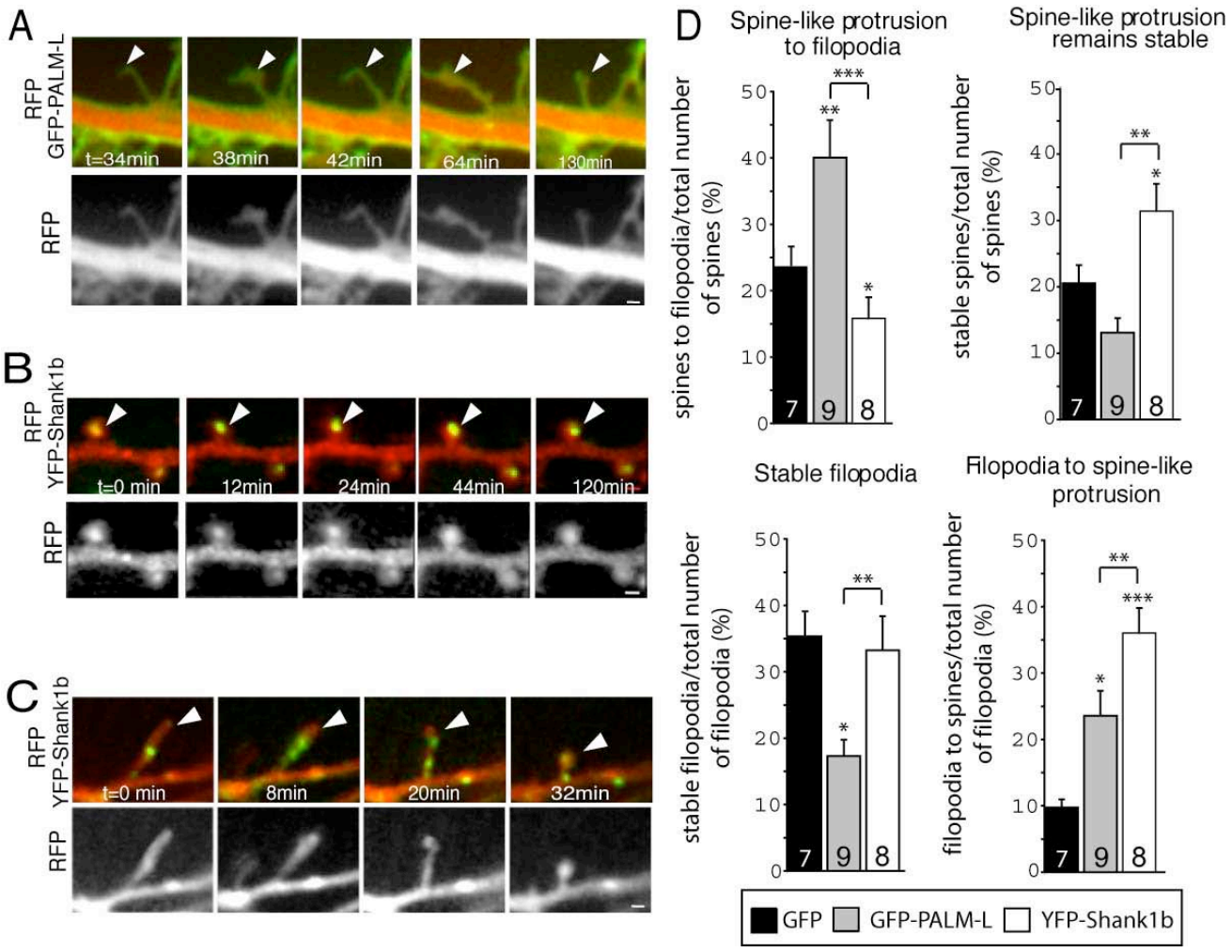

Figure 2.11 Shank1b but not paralemmin-1 induces rapid protrusion transformation from filopodia to spine-like structures

(A, B and C) Hippocampal neurons were transfected with RFP and either with GFP, GFP-tagged paralemmin-L (GFP-PALM-L) or YFP-tagged Shank1b (YFP-Shank1b) at DIV7 and then imaged at DIV9 using timelapse microscopy. Images were acquired every 2 min. In (A), these images represent a transition from a filopodium (t=34 and 42 min) to a spine-like protrusion at (t=38 and 130 min). In (B), these images represent a spine induced by Shank1b that remained stable from t=0min to t=120 min. In (A), at t=0min, the image shows a filopodia-like protrusion containing a Shank1b cluster that retracts to form a spine-like protrusion at t=32min and remains stable. (D) Analysis revealed differential effects of GFP-PALM-L on protrusion dynamics. Most significant is enhanced membrane dynamics and protrusion turnover in cells expressing GFP-PALM-L as well as the number of stable spines in neurons expressing YFP-Shank1b but not GFP-PALM-L on a timescale of 2-3 h hours. Number of cells analyzed for each group is indicated at the bottom of each bar. Number of filopodia and spines analyzed per group in (A) are respectively: DIV 7+2, GFP+RFP = 127 and 62, GFP-Shank1b+RFP = 178 and 375, DIV 7+3, GFP+RFP = 240 and 123, GFP-Shank1b+RFP = 135 and 641, respectively. White arrowheads denote dendritic protrusions. *p<0.05, **p<0.01, ***p<0.001. n.s. = no significant difference. Data represent mean ± SEM. Scale bar, 1µm in (A, B, C).

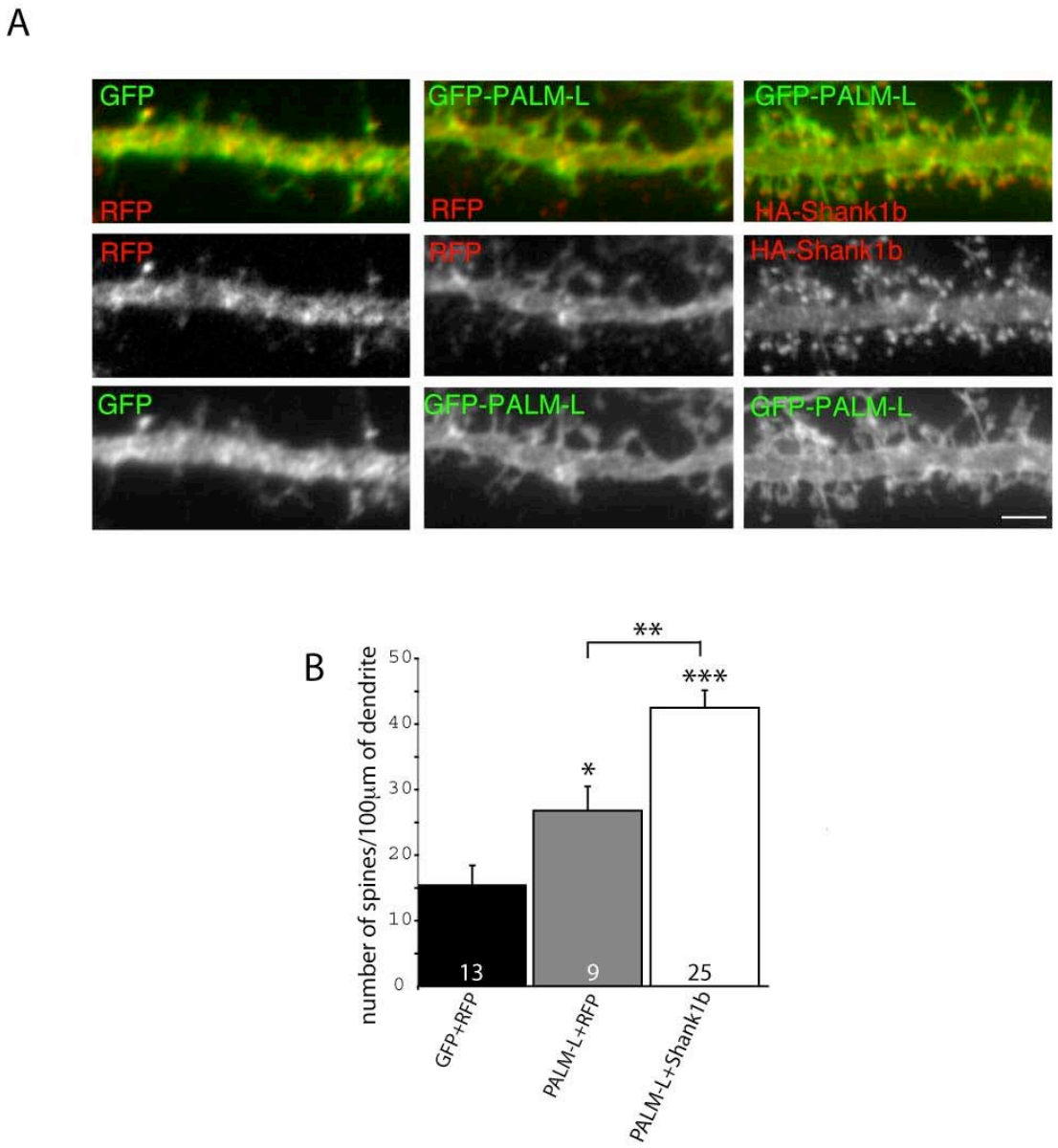

Figure 2.12 Effects of co-expression of paralemmin-L and Shank1b on spine formation
Hippocampal neurons were transfected with either GFP+RFP, paralemmin-L+RFP (GFP-PALM-L+RFP) or paralemmin-L and Shank1b (GFP-PALM-L+HA-Shank1b) at DIV 7 and fixed and stained at DIV 12. Co-expression of paralemmin-L with Shank1b significantly increased the number of spines/100μm compared to GFP+RFP and paralemmin-L+RFP (GFP-PALM-L+RFP) controls. Number of cells analyzed for each group is indicated at the bottom of each bar. Number of spines analyzed per group in (A) is: GFP+RFP=803, GFP-PALM-L+RFP=804 and GFP-PALM-L+HA-Shank1b=3957 *$p<0.05$, **$p<0.01$, ***$p<0.001$. Data represent mean $\pm$ SEM. Scale bar in (A) 10μm.

We next evaluated the effects of long-term knockdown of paralemmin-1 on spine development in mature neurons (DIV 12-14). Knockdown of paralemmin-1 at DIV 15 results in a significant reduction in the number of spines compared to control RNAi (PALM RNAi, 53%±6; Ctl RNAi, 100%±13; Figure 2.13A and B). Moreover, paralemmin-1 knockdown compromised Shank1b effects on spine maturation (Figure 2.13C and D). These results suggest the involvement of paralemmin-1 in Shank1b induced effects on spine maturation (Figure 2.13D). It is important to note that aberrant dendritic growth and the formation of short neurites was also observed in about 30% of neurons after prolonged (7-10 days) knockdown of paralemmin-1 (data not shown). These results indicate that paralemmin-1 may generally participate in events that regulate membrane dynamics, protrusion formation and dendritic arborization.

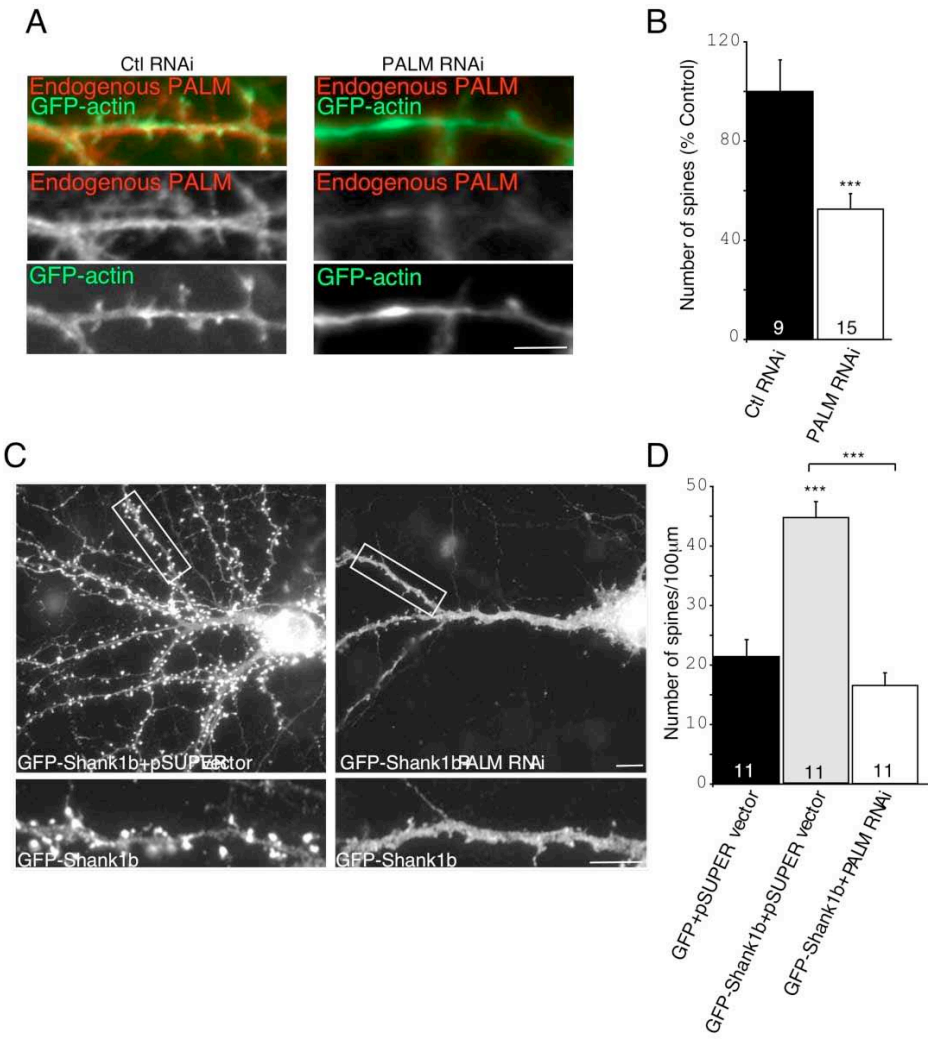

Figure 2.13 Effects of long-term knockdown of paralemmin-1 on spine formation
(A) Hippocampal neurons were co-transfected with GFP-actin and either with control RNAi (Ctl RNAi) or paralemmin-1 specific RNAi (PALM RNAi) at DIV 5. Neurons were then fixed and stained for endogenous paralemmin-1 (Endogenous PALM) at DIV 12-14. (B) Quantification of dendritic spines normalized to Ctl RNAi group. There is a significant reduction in dendritic spines in neurons transfected with PALM RNAi. (C) Hippocampal neurons co-transfected with GFP or GFP-Shank1b and either with empty pSUPER vector or with PALM RNAi. (D) Quantification of GFP-Shank1b positive spines upon knockdown of paralemmin-1. A significant reduction in GFP-Shank1b clustering as well as the number of Shank1b positive dendritic spines in neurons transfected with GFP-Shank1b and PALM RNAi compared to controls expressing GFP-Shank1b and empty pSUPER vector. Number of cells analyzed for each group is indicated at the bottom of each bar. Number of filopodia and spines analyzed per group in (B) are respectively: GFP-actin+Ctl RNAi = 316 and 281, GFP-actin+PALM RNAi = 230 and 176. Number of spines analyzed per group in (D) is: GFP+pSUPER vector = 1039, GFP Shank1b+pSUPER vector = 1564 and GFP Shank1b+PALM RNAi = 446. ***$p<0.001$. Data represent mean $\pm$ SEM. Scale bar, 5µm in (A) and 10µm and 5µm (magnified dendrite) in (C).

2.3.4. Neuronal activity enhances membrane localization of paralemmin-1

Neuronal activity modulates protrusion formation which in turn fine-tunes synaptic strength and plasticity (Dunaevsky *et al.*, 1999; Fischer *et al.*, 2000; Nimchinsky *et al.*, 2002; Richards *et al.*, 2005; Zuo *et al.*, 2005b). This process is thought to be mediated by the recruitment of proteins that alter membrane and cytoskeletal dynamics. Thus, we addressed whether neuronal activity regulates paralemmin-1 localization and function. To explore whether depolarization of hippocampal neurons has an effect on paralemmin-1 localization, DIV 16-18 hippocampal neurons were stimulated with 90 mM KCl for 3 min, after which neurons were fixed and stained for endogenous paralemmin-1. This analysis revealed a significant increase in paralemmin-1 localization at the plasma membrane (Figure 2.14). To further confirm translocation of paralemmin-1 to cellular membranes, we performed subcellular fractionation and assessed the amounts of paralemmin-1 in the soluble and membrane fractions after 3 min treatment with 90 mM KCl. Indeed, this treatment resulted in an increase in the amounts of paralemmin-1 detected in the membrane fraction, as determined by calculating the amount of paralemmin-1 in the soluble/pellet (membrane) fractions and expressing it as a percent. Paralemmin-1 levels in the pellet fractions of treated cells (58.1$\pm$7.7%), *$p<0.02$ were higher than those of untreated controls (45.0$\pm$5.8%), *$p<0.02$. However, we found no significant change in the amounts of transferrin (pellet/load) between controls (105.3$\pm$11.6%) and treated groups (113.6$\pm$6.9%). This parallels the enhanced paralemmin-1 localization at the membrane as seen in Figure 2.14A and B.

To address the possibility that depolarization by KCl may have resulted in non-specific effects on membrane integrity and dynamics, we used a second approach to manipulate neuronal activity and examine changes in membrane localization of paralemmin-1. For this analysis, neurons were grown

on silicon wafers and imaged using a photoconductive stimulation paradigm to induce neuronal excitability (Colicos et al., 2001) (Figure 2.14C). Analysis of the average pixel value of surface versus intracellular paralemmin-1 signal shows an increase in its membrane localization, similar to the level observed with KCl treatment (Figure 2.14D). This confirms that paralemmin-1 localization can be modulated by physiological neuronal activity.

Next, we explored whether general manipulation of palmitoylation serves as a signal that controls activity-mediated paralemmin-1 localization at the plasma membrane. For this analysis, we treated neurons with 20 μM 2-bromopalmitate, a competitive inhibitor of palmitoylation, 4 h prior to stimulation with KCl (Webb *et al.*, 2000; El-Husseini Ael and Bredt, 2002; Gauthier-Campbell *et al.*, 2004). This treatment reduced paralemmin-1 expression at the membrane in basal conditions (Figure 2.14A, lower inset and B). 2-bromopalmitate also compromised paralemmin-1 localization to the membrane upon depolarization (Figure 2.14A, lower inset and B). Taken together, these results suggest that blocking palmitoylation interferes with the localization of paralemmin-1 to the membrane upon enhanced synaptic activity.

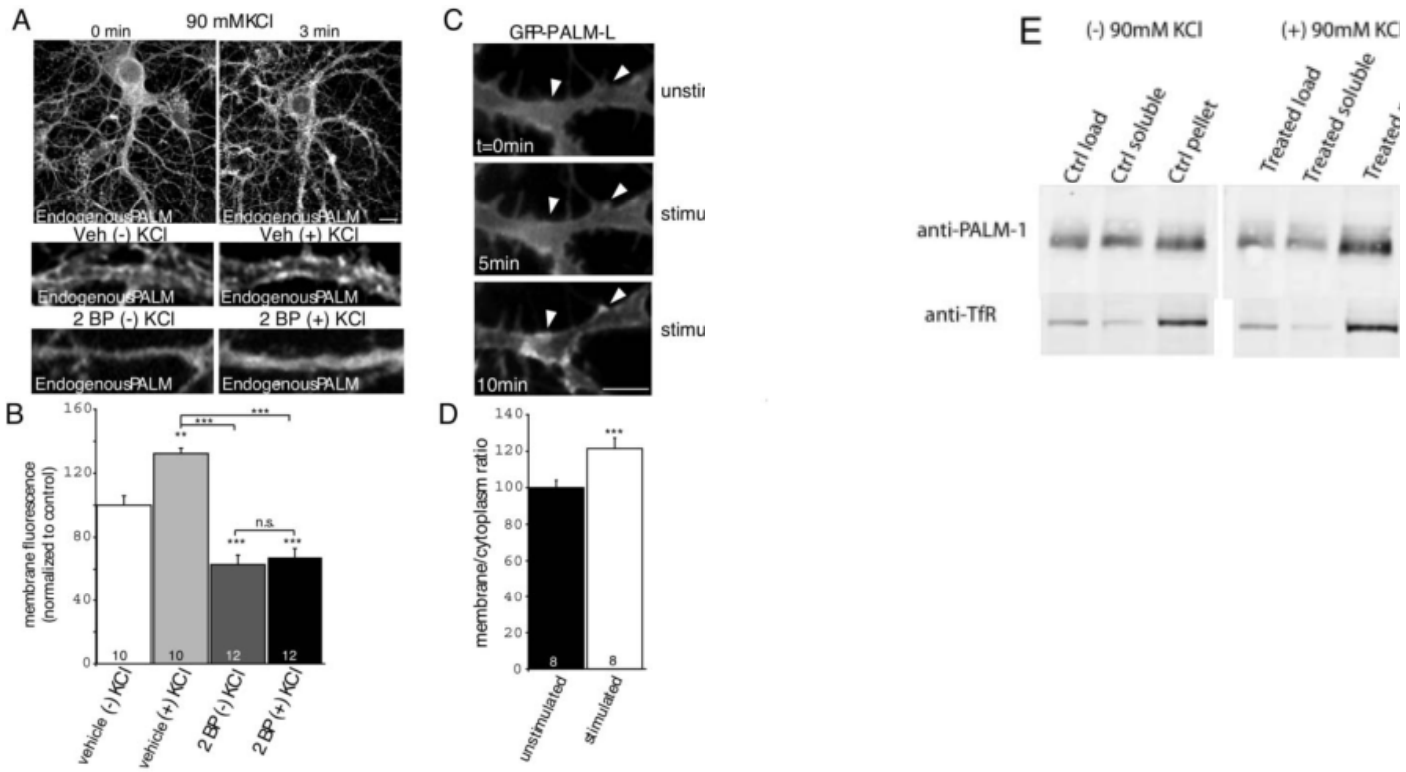

Figure 2.14 Neuronal activity modulates paralemmin-1 localization
(A) Hippocampal neurons were treated with 90 mM KCl or vehicle control for 3 min and fixed and stained for endogenous paralemmin-1 (Endogenous PALM). Endogenous PALM accumulation at the plasma membrane is enhanced following stimulation with 90 mM KCl when compared to untreated cells. 2 bromopalmitate (2 BP) treatment, reduces Endogenous PALM accumulation at the plasma membrane at basal conditions and after KCl treatment. (B) Graph showing quantification of changes in Endogenous PALM accumulation at the membrane across 4 treatment groups. (C) Photoconductive stimulation increases GFP-paralemmin-L accumulation at the plasma membrane. Neurons were transfected with GFP-tagged paralemmin-L (GFP-PALM-L) and then imaged for several minutes before stimulation. White arrowheads indicate changes in accumulation of paralemmin-L before and after electrical stimulation. (D) Quantification showing a significant increase in GFP-PALM-L accumulation at the plasma membrane compared to unstimulated neurons. (E) Changes in paralemmin-1 levels in the membrane fraction following KCl treatment. Cortical neurons at DIV 16-20 were treated for 3 min with 90 mM KCl and changes in paralemmin-1 distribution was examined by subcellular fractionation. Quantification of paralemmin-1 levels in the membrane fraction was determined by calculating the amount of paralemmin-1 in the soluble/pellet fractions. Paralemmin-1 levels in the pellet (membrane) fractions of treated cells (58.1$\pm$7.7%), *p<0.02 were higher than those of untreated controls (45.0$\pm$5.8%), *p<0.02. There were no significant changes in the amounts of transferrin in the membrane fractions across groups, p=0.75. Number of cells analyzed for each group is indicated at the bottom of each bar. **p<0.01, ***p<0.001. n.s. = no significant difference. Data represent mean $\pm$ SEM. Scale bar, 10μm in (A) and 5μm in (C).

2.3.5. Paralemmin-1 potentiates activity-driven membrane expansion

Changes in neuronal activity have been proposed to influence protrusion size and dynamics (Dunaevsky *et al.*, 1999; Fischer *et al.*, 2000; Nimchinsky *et al.*, 2002; Richards *et al.*, 2005; Zuo *et al.*, 2005b). The rapid translocation of paralemmin-1 to the plasma membrane upon stimulation of neuronal activity prompted us to examine whether paralemmin-1 modulates activity-driven changes in dendritic protrusions. Timelapse imaging of DIV 9 neurons was used to assess changes in the size of protrusions within 10 min of treatment with 50 mM KCl (Figure 2.16A). Four common effects of paralemmin-1 on membrane expansion were measured: membrane expansion at the tip of filopodia (Figure 2.16A; example (1)), formation of growth cone-like protrusions (Figure 2.16A; example (2)), enlargement of existing protrusions (Fig. 8A; example (3)), and formation of lamellopodia-like structures at the base of protrusions (Figure 2.16A; example (4)). Paralemmin-1 significantly enhanced membrane expansion of these irregularly shaped protrusions after KCl stimulation (Figure 2.15).

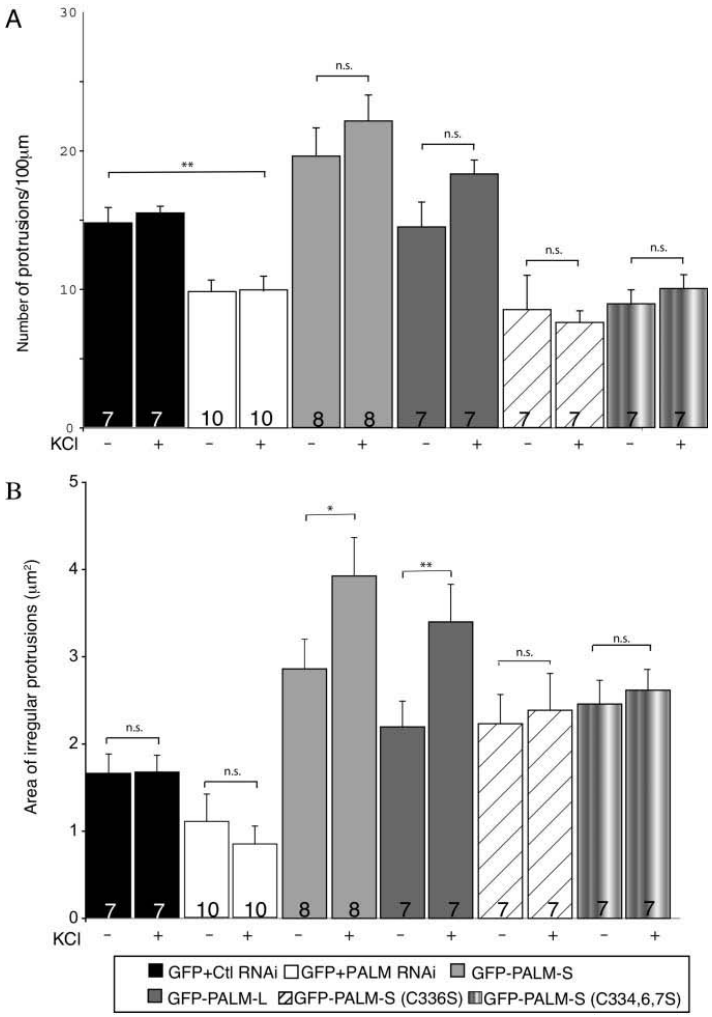

Figure 2.15 Paralemmin-1 modulates neuronal activity-driven changes in protrusion size and area of irregularly-shaped protrusions

(A,B) Hippocampal neurons were transfected with either GFP+Ctl RNAi, GFP+PALM RNAi, GFP-tagged forms of paralemmin-S (GFP-PALM-S) or paralemmin-L (GFP-PALM-L), paralemmin-S (C336S) (GFP-PALM-S (C336S)) or paralemmin-S (C334S, C336S, C337S) (GFP-PALM-S (C334,6,7S)) at DIV 7. Neurons were then imaged at DIV 9. Images were captured before and after 10 min treatment with 50 mM KCl. GFP-PALM-S and GFP-PALM-L did not show a significant increase in protrusion number following KCl treatment when compared to GFP transfected controls. Furthermore, the area of irregularly-shaped protrusions including: membrane expansion at filopodia tips, formation of growth cone-like protrusions, enlargement of existing protrusions, and formation of lamellopodia-like structures were quantified (Fig.8A, examples 1-4). The number of cells analyzed for each group is indicated at the bottom of each bar. n.s. = no significant difference. **$p<0.01$. Data represent mean $\pm$ SEM.

Analysis of GFP+Ctl RNAi (17.9±1.9%) transfected controls shows that stimulation with KCl results in a small but significant increase in protrusion size and this effect is significantly reduced in neurons co-expressing GFP+PALM RNAi (11.2±1.3%) (Figure 2.16, B and C). Expression of wild-type paralemmin-1, but not the palmitoylation-deficient forms GFP-PALM-S (C336S), GFP-PALM-S (C334,6,7S) further enhanced activity-driven protrusion expansion (Figure 2.15, Figure 2.16C). Taken together, these results reveal that paralemmin-1 recruitment to the plasma membrane is modulated by palmitoylation and that activity-driven changes in paralemmin-1 localization serve to modulate membrane expansion at the tip and base of dendritic protrusions.

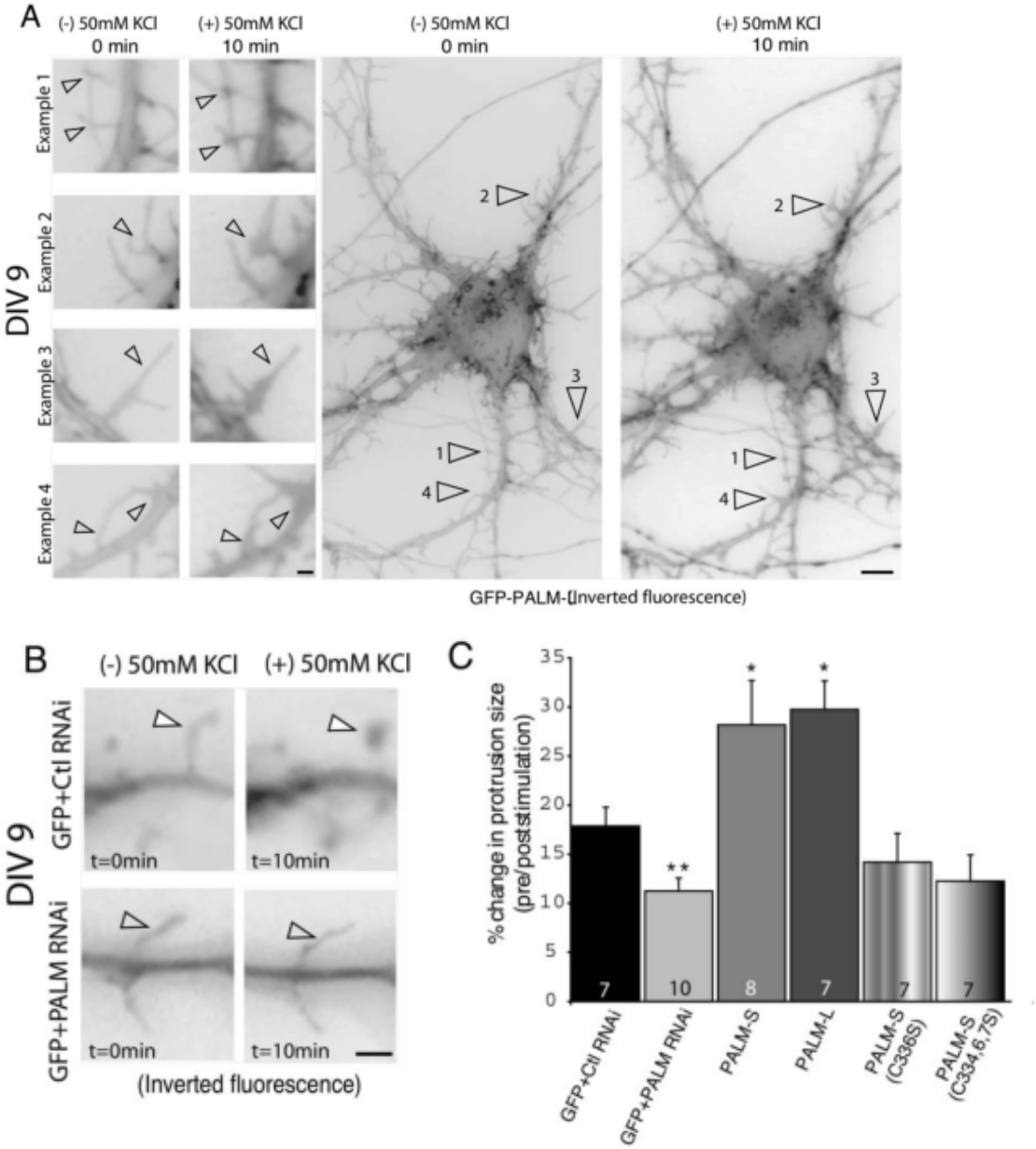
A
(-) 50mM KCl
0 min
(+) 50mM KCl
10 min
(-) 50mM KCl
0 min
(+) 50mM KCl
10 min
DIV 9
Example 1
Example 2
Example 3
Example 4
1
2
3
4
GFP-PALM-(Inverted fluorescence)
B
(-) 50mM KCl
(+) 50mM KCl
GFP+Ctl RNAi
GFP+PALM RNAi
t=0min
t=10min
(Inverted fluorescence)
C
% change in protrusion size (pre/poststimulation)
35
30
25
20
15
10
5
0
7
10
8
7
7
7
GFP+Ctl RNAi
GFP+PALM RNAi
PALM-S
PALM-L
PALM-S (C336S)
PALM-S (C334,6,7S)

Figure 2.16 Activity-induced changes in dendritic protrusions are modulated by paralemmin-1 (A) Paralemmin-1 modulates neuronal activity-driven changes in protrusion size. Hippocampal neurons were transfected at DIV 7 with GFP+Ctl RNAi, GFP+PALM RNAi, GFP-tagged paralemmin-1 splice variants GFP-PALM-L, GFP-PALM-S, or the cysteine mutant forms GFP-PALM-S (C336S) or GFP-PALM-S (C334S, C336S, C337S) and then imaged at DIV 9. Images were captured before and after 10 min treatment with 50mM KCl. Images of inverted fluorescence are shown to better visualize protrusions. Four examples of dendritic protrusion expansion are shown before and after stimulation with 50mM KCl. Example (1) shows filopodia expanded at the tips (2) formation of a growth cone-like protrusion (3) enlargement of an existing protrusion and (4) formation of lamellopodia-like structures at the base of the protrusion. Images shown here represent inverted fluorescence for greater clarity. (B) Example of protrusion expansion in GFP+Ctl RNAi at DIV 9 following stimulation with 50mM KCl for 10 min and this effect is reduced in cells co-expressing GFP+PALM RNAi. Images shown here represent inverted fluorescence for greater clarity. (C) Treatment with KCl results in a small but significant increase in protrusion size in GFP+Ctl RNAi transfected controls. Co-expression of GFP+ PALM RNAi significantly reduces the effect on protrusion expansion. Expression of GFP-PALM-S and GFP-PALM-L but not the acylation mutant forms of PALM-S significantly enhanced dendritic protrusion expansion. Protrusion diameter was measured at the base and tips, before and after stimulation and expressed as a % change. Arrowheads point to expanded protrusions. Number of cells analyzed for each group is indicated at the bottom of each bar. *$p<0.05$, **$p<0.01$. Data represent mean $\pm$ SEM. Scale bar, 5μm in (A) right panels and 2μm in (A) left insets and 2μm in (B)**.**

2.4 Discussion

In the present work, we reveal that manipulations of paralemmin-1 expression modulate filopodia induction and synapse formation. Long-term expression of paralemmin-1 induces spine maturation, as shown by its influence on the number of mature spines formed and recruitment of AMPA receptors. Moreover, this process is regulated by alternative splicing of exon 8. We demonstrate that paralemmin-1 modulates protrusion dynamics and expansion, and that these effects are rapidly accelerated upon neuronal depolarization. Enhanced neuronal activity also leads to rapid redistribution of paralemmin-1 to the plasma membrane, suggesting a paralemmin-based mechanism for the effects of neuronal activity on dendritic protrusion dynamics.

Although these activity-dependent changes indicate an important role for palmitoylation in regulating paralemmin-1 induced changes in protrusion dynamics, it is important to note that treatment with 2-

bromopalmitate may have also directly affected palmitoylation and/or function of other proteins involved in this process. Future studies are needed to directly assess the effects of neuronal activity on palmitate turnover on paralemmin-1 to solidify these conclusions.

Filopodia are thought to play an active role in the initiation of synaptic contacts (Dailey and Smith, 1996; Ziv and Smith, 1996; Marrs *et al.*, 2001; Calabrese *et al.*, 2006). Furthermore, the appearance of filopodia before the formation of spines, and the fact that some filopodia retract into a more stable spine-like shape, has led to the hypothesis that some spines originate directly from filopodia (Fiala *et al.*, 1998; Zuo *et al.*, 2005a). In this study, we found that the majority of protrusions induced by paralemmin-1 are positive for synaptophysin and AMPA receptors. These results suggest that paralemmin-1 expression enhances the formation of synapses. Moreover, the enhanced filopodia formation correlates with an increase in spine number, supporting a role for filopodia in spine development. Consistent with these findings, knockdown of paralemmin-1 reduces filopodia formation in young neurons, as well as the development of spines in mature neurons. Thus, our results suggest that contacts between dendritic filopodia and presynaptic cells act as precursors for future spines, and ultimately, functional synapses.

We have previously shown that the palmitoylation motif fused to paralemmin-1 (paralemmin CT) is sufficient to increase the number of dendritic branches in neurons (Gauthier-Campbell et al., 2004). Here we show that induction of filopodia and spines by paralemmin CT was comparable to paralemmin-S, suggesting a significant role for the lipidated motif of paralemmin-1 in altering protrusion formation by paralemmin-S. These results also indicate that enhanced filopodia number per se contributes to the increase in spine density. However, paralemmin-L has a stronger effect on

spine formation than paralemmin-S, revealing that protein-protein interactions regulated by alternative splicing modulate the efficacy of paralemmin-1 effects on spine maturation. Future experiments focused on identification of molecules that specifically associate with the paralemmin-1 isoform containing exon 8 may help clarify the differential effects induced by paralemmin-1 splice variants on spine maturation and AMPA receptor recruitment. Interestingly, the variant lacking exon 8 (paralemmin-S) is expressed at high levels at early stages of postnatal development, whereas the expression of the variant containing exon 8 (paralemmin-L) peaks at postnatal day 14 (Kutzleb et al., 1998). Thus, sequential expression of paralemmin-1 splice variants may contribute to filopodia induction and their subsequent transformation to spines.

The differential effects of paralemmin-1 and Shank1b on filopodia induction and spine maturation on both short- and long-term time scales are noteworthy. Expression of paralemmin-1 induces filopodia in both heterologous cells and neurons. In contrast, Shank1b fails to induce filopodia in both cell types. Interestingly, these changes correlate with a rapid increase in the number of spine-like structures. Consistent with these findings, live imaging over a period of hours revealed that Shank1b expression increases the number of events where filopodia transform into spine-like structures, suggesting that Shank1b functions to rapidly induce the transformation of existing filopodia into spines. Within this short time scale, paralemmin-L enhanced the turnover of filopodia to spines and vice versa. Moreover, spine-like protrusions that remain stable within the entire imaging period were not significantly enhanced by paralemmin-1 compared to GFP, suggesting that overall, paralemmin-L accelerates membrane dynamics and protrusion turnover in the direction of filopodia to spines, rather than destabilizing newly formed spines. Overall, these results reveal more robust effects of Shank1b on filopodia transformation to spines. These data suggest that paralemmin-L induced effects on spine

maturation require several days and that this process most likely requires recruitment of additional molecules for spine stabilization.

The effect of co-expression of paralemmin-L with Shank1b led to a significant increase in spine number when compared to expression of paralemmin-L alone. These results are consistent with a facilitative role for Shank1b in stabilization and maturation of protrusions induced by paralemmin-L. However, it is important to note that the combined effects of these proteins were not significantly larger than those observed in neurons expressing Shank1b alone, suggesting that the conversion of filopodia to spines is a bottleneck point, being limited by Shank1b and/or its supporting molecular machinery with respect to this process. Moreover, the ability of Shank1b to transform filopodia into spines becomes saturated, in that its effects are maximized with time. These results are in contrast with the knockdown findings, which show that loss of paralemmin-1 reduces Shank1b-induced effects on spine maturation, indicating that loss of filopodia compromises the effects of Shank1b on spine induction.

The actin cytoskeleton plays a fundamental role in regulating process outgrowth through changes in membrane dynamics. Despite the changes in membrane dynamics observed in this study, it remains unclear how paralemmin-1 induces its effects on protrusion extension. Previous work indicates that alterations of membrane geometry induce changes in membrane curvature and the extension of membrane protrusions (Raucher and Sheetz, 2000; Marguet *et al.*, 2006). This process can be regulated by activation of phospholipase C and plasma membrane phosphatidylinositol 4,5-bisphosphate, which act to regulate adhesion between the cytoskeleton and the plasma membrane.

The functions of several acylated proteins implicated in filopodia induction, including GAP-43 (Strittmatter *et al.*, 1994a) and Wrch, a Wnt-regulated Cdc42 homolog (Berzat et al., 2005), seem to rely on protein palmitoylation. Thus, palmitoylation seems to exert specific effects that regulate induction of protrusion formation. It is tempting to speculate that the insertion of palmitoyl groups into membranes, which relies on the motif structure and spacing between the acylated cysteines, directly triggers membrane deformity and alters membrane flow, which in turn results in modulation of protrusion extension. Alternatively, altered membrane dynamics may indirectly regulate recruitment of actin bundling proteins and GTPases that regulate protrusion formation. It is also possible that palmitoylation-dependent targeting of paralemmin-1 and other palmitoylated proteins to lipid rafts affects signaling molecules that reside in these lipid microdomains, resulting in the activation of molecules directly involved in protrusion expansion (Anderson and Jacobson, 2002; Gauthier-Campbell et al., 2004; Kutzleb et al., 2007). Alterations in cholesterol/sphingolipid-enriched lipid raft microdomains in neurons influence protein trafficking, formation of signaling complexes, and regulation of the actin cytoskeleton (Hering et al., 2003). For example, depletion of cholesterol/sphingolipids leads to gradual loss of synapses and dendritic spines, as well as instability of surface AMPA receptors which, along with other postsynaptic proteins, have been shown to be associated with lipid rafts in dendrites (Hering et al., 2003). Others have shown that cholesterol promotes synapse maturation in retinal ganglion cells, suggesting that alterations in lipid raft integrity and/or constituents directly influence synapse density and morphology (Mauch *et al.*, 2001; Goritz *et al.*, 2005). These findings offer a potential link between disordered lipid composition and the loss of synapses seen in brain disorders such as Down Syndrome, where loss of dendritic spines and altered phospholipid composition has been documented (Murphy et al., 2000). It will be important, next, to examine whether enhanced incorporation of palmitoylated paralemmin-1 into lipid rafts triggers

recruitment of molecules that control cytoskeleton dynamics and membrane expansion to induce protrusion formation.

Activity-dependent alterations in spine dynamics, spine enlargement and recruitment of AMPA receptors have been associated with changes incurred during learning paradigms, and in particular, changes in synaptic and structural plasticity, including induction of LTP (Bredt and Nicoll, 2003). Paralemmin-1 expression persists in the adult brain, and thus paralemmin-1 may also be involved in regulation of spine morphology and protrusion expansion in response to synaptic activity or plasticity. The activity-driven changes we observed in protrusion expansion upon expression of paralemmin-1 in developing neurons lend further support to this notion. Next, it will be important to determine whether specific paradigms that influence postsynaptic receptor stimulation and neurotransmitter release exert specific effects on paralemmin-1 localization and protrusion expansion in older neurons. Application of pharmacological reagents that manipulate synaptic function will clarify further activity-induced changes in paralemmin-1 localization and action. Studies focused on analyzing the effects of paralemmin-1 on protrusion formation and expansion in mature neurons in response to specific plasticity-associated learning paradigms will help address this possibility.

3. Filopodia stability, but not number, leads to more stable axo-dendritic contacts[2]

3.1 Introduction

In the CNS, synapse formation between axons and dendrites is a regulated process involving the coordinated actions between presynaptic axons and postsynaptic dendrites (Holtmaat and Svoboda, 2009). Coordination of this physical interaction between pre- and postsynaptic cells is thought to occur via dendritic filopodia that contact and recruit passing axons (Ziv and Smith, 1996; Ziv, 2001; Yoshihara *et al.*, 2009). Dendritic filopodia are thin, headless protrusions ranging from 2-25 μm in length that are filled with bundles of actin and extend from the cell surface (Faix and Rottner, 2006; Gupton and Gertler, 2007; Arstikaitis *et al.*, 2008). Early in development, immature neurons are littered with highly motile dendritic filopodia. As the brain matures, these abundant and motile filopodia are replaced with more stable spine synapses (Dailey and Smith, 1996).

Multiple studies suggest that after filopodia participate in synaptic contact formation, they transform to more stable dendritic spines through the action of synapse-inducing factors (Ethell *et al.*, 2001; Jourdain *et al.*, 2003; Takahashi *et al.*, 2003; Yuste and Bonhoeffer, 2004) and neuronal activity (Wong *et al.*, 2000; Portera-Cailliau *et al.*, 2003; Kirov *et al.*, 2004). However whether the increased

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[2] **Arstikaitis P***, Gauthier-Campbell C***,** Huang K, El-Husseini A, and Murphy T. (2010) Filopodia stability, but not number, leads to more stable axo-dendritic contacts (Submitted). *these authors contributed equally

density and motility of filopodia are associated with the formation of dendritic spine synapses is controversial. One previous imaging study showed highly motile filopodia mainly form transient interactions with presynaptic terminals (Konur and Yuste, 2004a). Another study revealed that neuronal membrane glycoprotein M6a-induced filopodia are highly motile and become stabilized upon contact with presynaptic region (Brocco *et al.*, 2010). In contrast, a recent study found that a reduction in the motility of EphB-induced filopodia led to a decreased rate of synaptogenesis (Kayser *et al.*, 2008).

To date, it is unclear how different molecules behave to initiate synaptic contact formation and transform filopodia to spines. We address this by comparing the effect that specific molecules, known to play a role in synapse formation, have on filopodia dynamics. Shank1b and NLG-1 proteins are two major components of the postsynaptic density (PSD) and influence the maturation of synapses. Shank1b promotes maturation of dendritic spines (Sala *et al.*, 2001), while its dominant negative mutant causes a reduction in spine size and density (Boeckers *et al.*, 1999). NLG-1, a synaptic cell adhesion molecule, initiates communication between pre- and postsynaptic sites and influences the development of functional synaptic terminals (Gerrow *et al.*, 2006). We recently showed Cdc42 (CA)-Palm has potent effects on building dendritic spines in mature neurons (Kang *et al.*, 2008), however its role in filopodia dynamics and synapse formation remain less clear. Here, we will investigate the origin of dendritic spines induced by Cdc42 (CA)-Pam, NLG-1 and Shank1b by examining how these proteins impact the motility of dendritic filopodia and their role in forming stable axo-dendritic contacts.

Previously we identified the palmitoylated protein, GAP-43, as a potent inducer of filopodia (Gauthier-Campbell *et al.*, 2004; Arstikaitis *et al.*, 2008). We now use the filopodia-inducing motif of GAP-43 (GAP 1-14) as a tool to examine how the presence of motile filopodia effect synapse formation. It is possible that molecules such as GAP 1-14 may hinder the formation of synapses by inducing highly motile filopodia that continuously sample the environment, yet require the recruitment of scaffolding proteins to form stable axo-dendritic contacts. Interestingly, the combination of a known filopodia inducing molecule paralemmin-1 with the spine-stabilizing molecule Shank1b results in an increase in the number of dendritic spines compared to expression of GFP or paralemmin-1 alone (Arstikaitis *et al.*, 2008). This suggests a role for molecules such as Shank1b and NLG-1 in the formation of stable filopodia-like protrusions that promote dendritic spines and synapse formation. Hence, enhancing the formation of filopodia may not necessarily lead to more stable axo-dendritic contacts. Rather, the production of stable synapses is dependent on key members of the postsynaptic scaffolding complex. In this study, we will examine molecules that affect filopodia elaboration and motility; versus those that impact synapse induction and maturation to better define the role of filopodia in synapse formation.

3.2 Materials and methods

3.2.1. cDNA cloning, siRNA and construction

GAP 1-14 and Cdc42 (CA)-Palm plasmids were constructed as previously described by (Gauthier-Campbell *et al.*, 2004; Arstikaitis *et al.*, 2008). And GFP tagged Shank1b, HA and GFP tagged NLG-1 was constructed as previously described by (Sala *et al.*, 2001; Prange *et al.*, 2004; Levinson *et al.*, 2005). NLG-1 RNAi sequence was used as previously described (Chih *et al.*, 2005) and re-cloned

into the pSUPER vector. Previously used NLG-1 forward primer GATCCCCTGGAAGGTACTGGAAATCTATTCAAGAGATAGATTTCCAGTACCTTCCTTTTT TCA and the reverse primer used AGCTTGAAAAAAGGAAGGTACTGGAAATCTATCTCTTGAATAGATTTCCAGTACCTTCC AGGG (Dharmacon Inc., custom siRNA service). The restriction sites used in the pSUPER vector were BglII and HindIII. This sequence was transfected into rat hippocampal neurons to suppress expression of endogenous NLG-1.

3.2.2. Hippocampal cultures and cell transfection methods

Hippocampal neurons were prepared from embryonic day 18/19 rat pups as previously described (Gerrow *et al.*, 2006; Arstikaitis *et al.*, 2008). For experiments involving fixed cells, immediately after dissection and digestion, neurons were plated at a density of 150,000 cells/well of a 24 well plate. For cell transfection, we used Lipofectamine 2000 (Invitrogen). Briefly, we used 1-1.5µg/µL of DNA and 0.8µL of lipofectamine 2000 per well and left for 2-3hrs at which time the Neural Basal Media (NBM) was removed and replaced with original NBM. For live cell imaging experiments, hippocampal cultures were transfected by nucleofection (Amaxa), by lipid-mediated gene transfer (Invitrogen), or using a calcium phosphate transfection kit (BD Biosciences, CA). Similar results were obtained with each protocol. Briefly, the electroporation protocol is as follows: 6 million cells were re-suspended in 100µl of room temperature electroporation solution (120 mM KCl, 10 mM KH_2PO_4, 0.15 mM $CaCl_2$, 5mM $MgCl_2$, 25 mM HEPES, 2 mM EGTA, 2 mM ATP, 5 mM GSSG, pH to 7.4) with 2µg of high quality endotoxin-free DNA. Neurons were then transfected by electroporation, as described by AMAXA Inc Amaxa (Gaithersburg, MD). Cells were plated at a final density of 0.5 million/mL and allowed to recover in DMEM with 10% Calf Serum for 1 hour

before replacement with NBM (Invitrogen). Calcium phosphate transfections were done at 7 days in vitro (Lawson-Yuen *et al.*): briefly, 2µg of DNA and 6.2µl of calcium phosphate buffer (4M, BD Biosciences) were mixed with 92µl of HBSS (Hanks balanced salt solution, pH 7.0) and let stand for 5 minutes at room temperature. This DNA solution was added drop-wise to 100 µl of distilled water and the mix was added to the cells with 500µl of NBM per well. Cells were incubated for 10 minutes at 37^{o}C and the calcium phosphate reagent was replaced with original NBM.

3.2.3. Fixation and immunocytochemistry

Hippocampal neurons were prepared from embryonic day 18/19 rat pups as previously described (Gerrow *et al.*, 2006; Arstikaitis *et al.*, 2008). For experiments involving fixed cells, immediately after dissection and digestion, neurons were plated at a density of 150,000 cells/well of a 24 well plate. For cell transfection, we used Lipofectamine 2000 (Invitrogen). Briefly, we used 1-1.5µg/µL of DNA and 0.8µL of lipofectamine 2000 per well and left for 2-3hrs at which time the Neural Basal Media (NBM) was removed and replaced with original NBM. For live cell imaging experiments, hippocampal cultures were transfected by nucleofection (Amaxa), by lipid-mediated gene transfer (Invitrogen), or using a calcium phosphate transfection kit (BD Biosciences, CA). Similar results were obtained with each protocol. Briefly, the electroporation protocol is as follows: 6 million cells were re-suspended in 100µl of room temperature electroporation solution (120 mM KCl, 10 mM KH_2PO_4, 0.15 mM $CaCl_2$, 5mM $MgCl_2$, 25 mM HEPES, 2 mM EGTA, 2 mM ATP, 5 mM GSSG, pH to 7.4) with 2µg of high quality endotoxin-free DNA. Neurons were then transfected by electroporation, as described by AMAXA Inc Amaxa (Gaithersburg, MD). Cells were plated at a final density of 0.5 million/mL and allowed to recover in DMEM with 10% Calf Serum for 1 hour before replacement with NBM (Invitrogen). Calcium phosphate transfections were done at 7 days in

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3.2.4. Microscopy and timelapse imaging

For all experiments, images were collected on a Zeiss Axiovert M200 inverted light microscope. Images were taken using a 63x 1.4 NA oil immersion objective and a monochrome 14-bit Zeiss Axiocam HR charged-coupled camera. To minimize potentially out of focus images, z stacks were collected (0.5 µm increments) and projected into a single image. For timelapse imaging experiments, a single plane of focus was used to capture movies (1 frame/min) and this was done to minimize photobleaching and toxicity. For these experiments, to decrease the possibility of out-of-focus protrusions, we manually monitored the focus of live cells. Cells were imaged at 37 degrees Celsius in a sealed incubation chamber, supplemented with 5% CO_2.

3.2.5. Quantitative measurement of filopodia and dendritic spines

All protrusions were measured on all dendrites within the field of view and an observer blinded to the transfection type did all analyses. Protrusions were scored based on their morphology. Protrusions that ranged from 1-10µm and lacking a visible head were counted as filopodia and protrusions with a bulbous head that was wider than its base were counted as spines (Harris, 1999; Arstikaitis *et al.*, 2008). Spines had to have a head size of 0.5 µm or greater to be counted as a spine. Analyses were performed using Northern Eclipse Software (Empix Imaging Inc.).

3.2.6. Calculation of synaptophysin cluster mobility

Movement of synaptophysin-positive clusters was analyzed using Image J (Wayne Rasband, NIH). Images were corrected for drift (RegisterROI, Michael Abramoff, University of Iowa Hospitals and Clinics, USA), and velocities were recorded (Manual Tracker, Fabrice Cordelières, Institut Curie, France). Discrete puncta of synaptophysin fluorescence were classified as "clusters" if they were at least 1.5 times greater than the average intensity of the background axon. Synaptophysin clusters were scored as "stable clusters" if they did not move more than 2 μm over the entire image acquisition period or "splitting" if a single cluster split into 2 separate clusters. All other clusters were classified as "moving clusters". Changes in position that were less than 0.2 μm (2 pixels for non-binned images) per time point were omitted.

3.2.7. Calculation of synapse number and size

Images were exported as 16bit and analyzed using Northern Eclipse software as previously described (Arstikaitis *et al.*, 2008). Briefly, images were processed at a constant threshold level to create a binary 'mask' image, which was multiplied by the original image. The resulting image contained a discrete number of clusters with pixel values of the original image. Only clusters with average pixel intensity 1.5 times greater than background pixel intensity were used for analysis. In addition, only dendritic processes were used for analyses (cell bodies and axons were excluded). The density of PSD-95 puncta is expressed per area of dendrite (μm^2) and normalized to GFP-expressing neurons.

3.2.8 *Statistical Analyses*

All statistical analysis was done using XLSTAT add-in for Microsoft Excel (Addinsoft, NY) or student's T-test (Microsoft Excel) and multiple group comparisons were done using the one-way analysis of variance (ANOVA, with Student-Newman-Keuls post-hoc correction).

3.3 Results

3.3.1. Induction of dendritic filopodia by expression of specific protein motifs

Since filopodia have been documented to play a role in synapse formation and the transformation to dendritic spines (Ziv and Smith, 1996; Ethell *et al.*, 2001; Takahashi *et al.*, 2003) we compared the ability of the palmitoylated proteins GAP 1-14, Cdc42 (CA)-Palm tagged with GFP as well as the scaffolding molecules, NLG-1 and Shank1b to induce the formation of filopodia (Figure 1A). Recently, we identified the brain-specific isoform Cdc42 (CA)-Palm, which plays an important role in the formation of dendritic spines (Kang *et al.*, 2008). We therefore decided to compare the differential effects of these molecules in the induction of dendritic filopodia.

We first expressed these fluorescently tagged proteins (Figure 3.11A) to assess whether they modulate filopodia formation. Neurons at days in vitro 8-9 (DIV 8-9) expressing the palmitoylated motif GAP1-14 or Cdc42 (CA)-Palm showed an increase in filopodia number (Figure 3.1 B and C). Similarly, expression of NLG-1 significantly increases filopodia number (Figure 3.1 B and C). Consistent with previous results (Arstikaitis *et al.*, 2008), we find that Shank1b failed to enhance the density of filopodia in hippocampal neuronal cells compared to control cells, suggesting that

Shank1b differentially effects the formation of filopodia compared to GAP 1-14, Cdc42 (CA)-Palm and NLG-1.

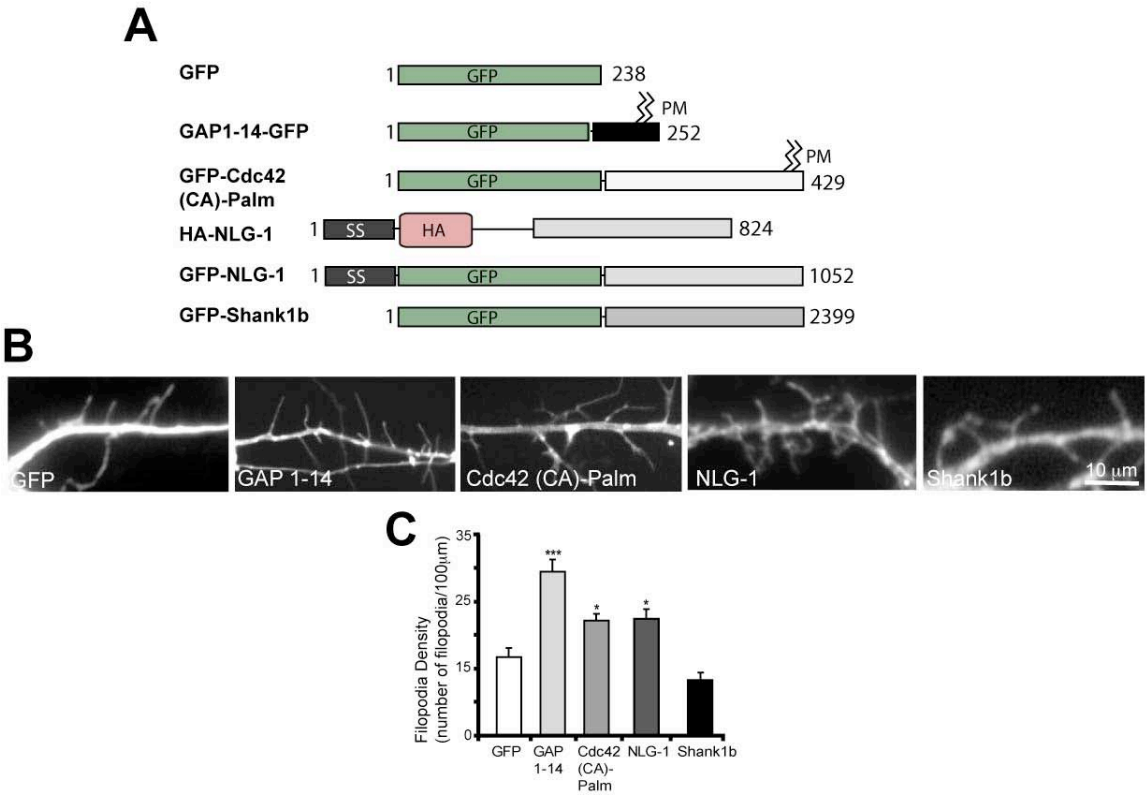

Figure 3.1 Specific synapse-inducing proteins are important for filopodia induction
(A) Schematic of the various fluorescently tagged constructs used in this study. (B) Representative images demonstrating filopodia induction by GAP1-14, Cdc42 (CA)-Palm, NLG-1 and Shank1b. Neurons were transfected at DIV 6-7 and stained at DIV 8-9. (C) Quantification of the number of filopodia/100 µm shows that expression of GAP1-14, Cdc42 (CA)-Palm and NLG-1 significantly increases filopodia number. In contrast, Shank1b failed to increase filopodia number. 8-15 cells were analyzed for each group and were collected from 3 independent experiments.*p <0.05, **p <0.01, ***p<0.001. Data represent mean ±SEM. Scale bars, 10µm.

Many imaging studies provide evidence that filopodia become stabilized in more mature neurons (Dailey and Smith, 1996; Ziv and Smith, 1996; Maletic-Savatic et al., 1999; Portera-Cailliau et al.,

2003). We wanted to determine if filopodia participate as precursors and transform into dendritic spines in mature cells. To address this issue, we overexpressed these fluorescently tagged molecules (Figure 3.1A) to determine whether they could alter the development of spine synapses. The presence of spine synapses was monitored by measuring the density and size of clustered endogenous PSD-95, a major scaffolding protein found at mature excitatory synapses (El-Husseini et al., 2000a). Neurons expressing GAP 1-14, showed a reduction in the number of PSD-95 clusters (84.0%$\pm$11.8) compared to control, whereas NLG-1 showed a 208.5%$\pm$14.8 increase in the density of spine synapses formed (Figure 3.2A, B). Therefore, despite the filopodia inducing abilities of both molecules, their roles in the formation of spines are different; suggesting that high numbers of filopodia may not be sufficient to promote dendritic spine formation. Furthermore, Shank1b failed to enhance filopodia density but significantly increased the number of spines and size of PSD-95 puncta. Neurons expressing Cdc42 (CA)-Palm, on the other hand, showed a significant increase in both filopodia numbers (Figure 3.1B, C) and PSD-95 puncta density (Figure 3.2A, B). To summarize, proteins that efficiently increase filopodia number such as GAP 1-14 do not necessarily lead to more spine synapses. Conversely, proteins such as Shank1b that increase synapse number are not necessarily the most effective at inducing filopodia formation. These results suggest that filopodia production is not the rate-limiting step for controlling the number of spines.

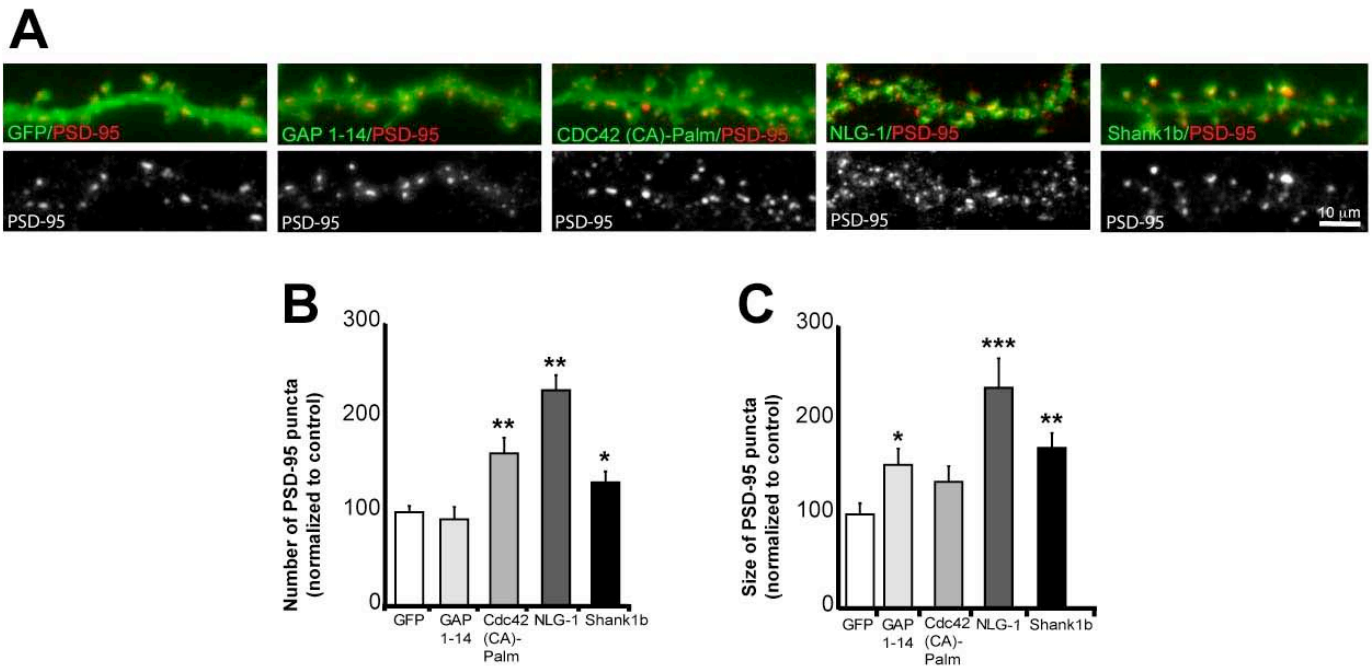

Figure 3.2 Accumulation of PSD-95 puncta is enhanced by NLG-1 and Shank1b
(A) Representative dendrites from neurons expressing GFP, GAP-14, Cdc42 (CA)-Palm, NLG-1 and Shank1b. (B) Quantification of the number of PSD-95 puncta expressed as a percentage that is normalized to control cells. Neurons expressing Cdc42 (CA)-Palm, NLG-1 and Shank1b showed an increase in number of spines containing PSD95 puncta. In contrast, neurons expressing GAP1-14 does not lead to any increase in number of PSD-95 positive spines. (C) Quantification of PSD-95 puncta size. Neurons expressing NLG-1 and Shank1b showed an increase in the size of spines containing PSD95 puncta. In contrast, neurons expressing Cdc42 (CA)-Palm and GAP-14 showed no increase or very moderate increase in the size of PSD-95 puncta. 8-15 cells were analyzed for each group and were collected from 3 independent experiments.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data represent mean $\pm$SEM. Scale bars, 10µm.

If filopodia density does not translate into more synapses then what is the crucial step that modulates synapse formation? We next set out to determine whether filopodia serve as precursors to spines by performing timelapse imaging of neurons expressing GFP over 3 days (DIV 10-12; 24 h time points). These cells were then retrospectively labeled for GluR1 to identify mature spine synapses (Figure 3.3). During this period, a large number of filopodia formed and disappeared per day (33% ± 6.5% and 46.3% ± 7.8%, respectively), when neurons were examined once every 24 hours. It is conceivable that these percentages are an underestimate since only three time points were used to

preserve the health of the neurons. At the same time, as filopodia appeared and disappeared, spine density increased by 10.2% ± 3.1% per day. Imaging analysis of GFP transfected cells (n=6) revealed that 18 new spines formed during the imaging period. Only 5 of the spines appeared at sites where filopodia were present 24 h earlier, out of 306 filopodia analyzed (67 of those remain visible for 3 days). This indicates that only 3.1% ± 0.3% of filopodia visible at a given time point will transform into a spine within 24 h. These results reveal that a small fraction of existing filopodia transform into spines, and that ~30% (29.2% ± 2.9%) of new spines appear at sites that contained filopodia at least 24 h earlier (Figure 3.3B). It is important to note that these results are only correlative and based on analysis of time points 24 h apart; one cannot exclude the possibility that the majority of dendritic spines emerge from transient filopodia that were not visible during the imaging period or directly emerge from the dendritic shaft.

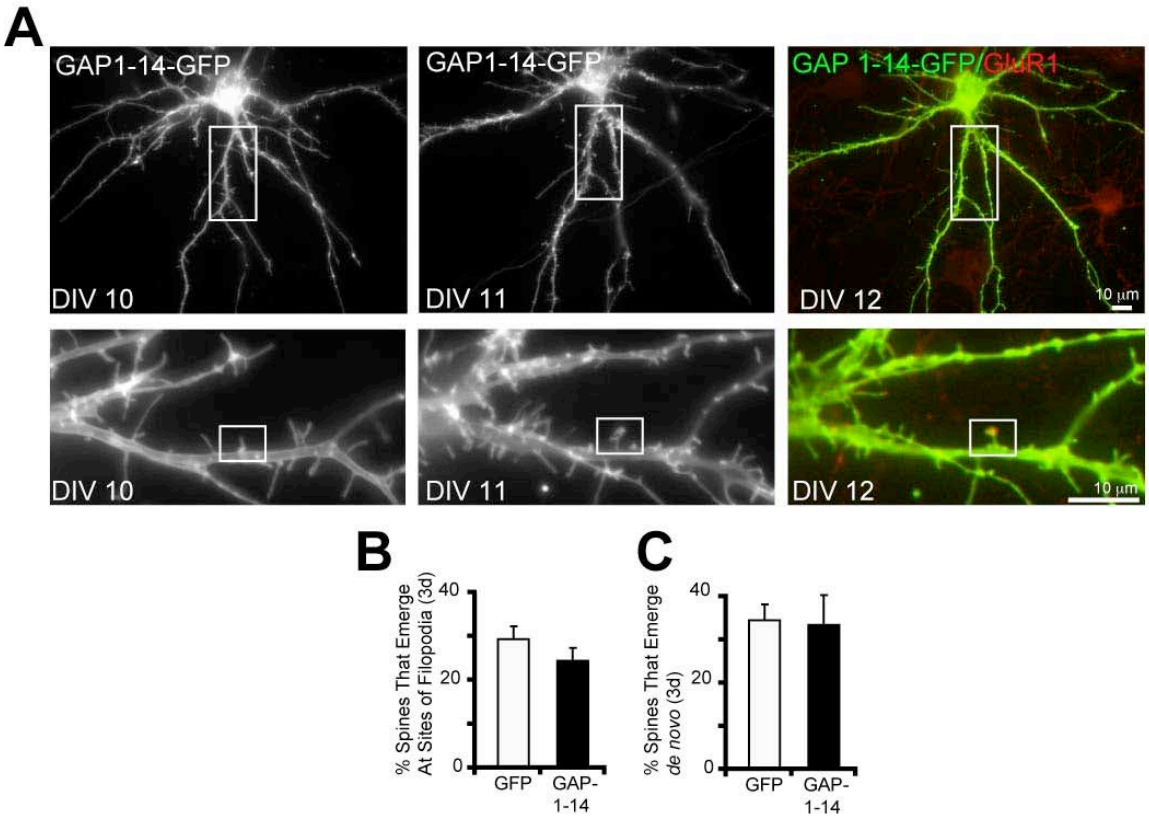

Figure 3.3 A small percentage of filopodia can transform into spines and this process requires several days

A small percentage of filopodia can transform into spines and this process requires several days. (A) Representative image of a whole neuron expressing GAP-14 on DIV 10, 11 and 12 which has been retro-immunolabeled for GluR1. Lower images (containing a boxed region) show a filopodia on DIV 10 that later becomes a spine and contains a GluR1 puncta on DIV 12. (B) Filopodia expressing either GFP or GAP-14-GFP were imaged once per day for 3 days to determine their fate. (C) Quantification of spines that formed independently of filopodia. Approximately 30% of spines from neurons expressing either GFP or GAP-14-GFP emerged de novo. Scale bar, 10µm.

3.3.2. Dendritic filopodia use an exploratory role to form contacts with neighboring axons

During synaptogenesis, dendritic filopodia are constantly protruding and retracting in search of the appropriate presynaptic partner (Ziv and Smith, 1996; Ethell and Pasquale, 2005). These filopodia can engage in synaptic contacts and undergo maturation into dendritic spines (Jontes and Smith, 2000; Marrs *et al.*, 2001; Okabe *et al.*, 2001; Portera-Cailliau *et al.*, 2003). However, it is unclear whether the rate of contact initiation and stabilization between neurons can be altered by

manipulating filopodia. In order to assess what proportion of filopodia form stable contacts with nearby axons, timelapse imaging was performed in cultured hippocampal neurons. A double transfection system was used in order to visualize in real time the formation of contacts between axons of DsRed-labeled neurons and dendritic filopodia from neurons expressing one of the GFP-tagged proteins, as described in Figure 3.1A. Cells were retrospectively immunolabeled for MAP-2, to distinguish axons from dendrites (data not shown).

Contacts between filopodia and axons that were established and subsequently lost within 1 h were classified as 'transient', while contacts present for the 1 h period were considered stable (Ziv and Smith, 1996). Timelapse imaging of GFP transfected cells revealed that dendritic filopodia continually interact with axons, potentially, to establish a contact with a presynaptic partner (Figure 3.4A). We found that 27.9% ± 3.9 of existing filopodia that formed contacts with axons were transient, whereas 21.4% ± 4.7 were stable for at least 1 h (Figure 3.4A and B). Furthermore, 3.3% ± 0.9 of emerging filopodia initiate new contacts with axons (Figure 3.4B). These results reveal that filopodia are important not only for probing the environment, but also for establishing the initial contacts between neurons. It is worth mentioning that this analysis was performed on contacts between filopodia and axons en passant. In rare occasions we also observed the initiation of contact formation by axonal growth cones, however because very few of these events were observed, the significance of this association could not be assessed.

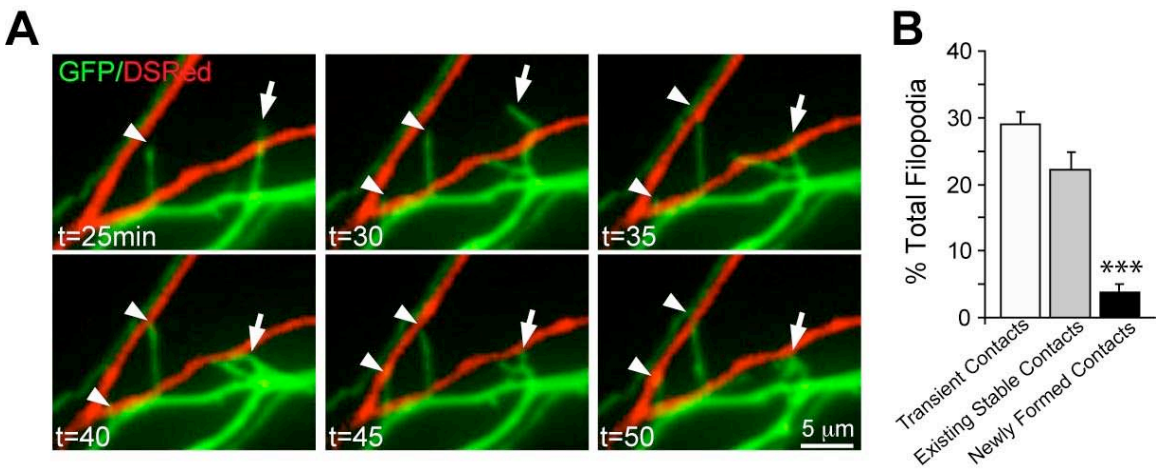

Figure 3.4 A role for dendritic filopodia in exploration and synaptic contact formation
(A) Electroporation of DsRed to label axons of one cell and GFP was used to fill a different cell. Images were captured every 1 min for 1h total. (B) Quantification of filopodia revealed that filopodia appeared to continuously interact with axons en passant. A small percentage of filopodia formed new and stable contacts throughout the imaging period. ***$p<0.001$ Data represent mean $\pm$SEM. Scale bar, 5µm.

The transformation of filopodia to spines was preceded by a decrease in filopodial motility, an increase in the size of the tip of the filopodium to yield a spine-like protrusion (Yuste and Bonhoeffer, 2004). Thus, the more motile the filopodium the less likely it will form a stable contact and undergo transformation to a spine. To determine if there was a correlation between filopodia motility and contact of dendritic filopodia with a presynaptic cluster of synaptophysin, we performed timelapse imaging of neurons expressing GFP and performed retrospective immunolabelling to stain for endogenous synaptophysin. We found that dendritic filopodia that moved greater distances were less likely to contain a cluster of synaptophysin within a 1 h imaging period (filopodia that lacked a synaptophysin cluster, moved 31.5µm $\pm$ 4.0 compared to filopodia that contained a synaptophysin cluster 22.1µm $\pm$ 2.7) suggesting that there is a negative correlation between the motility of a filopodium and the likelihood it will be associated with a cluster of synaptophysin (Figure 3.5A and B).

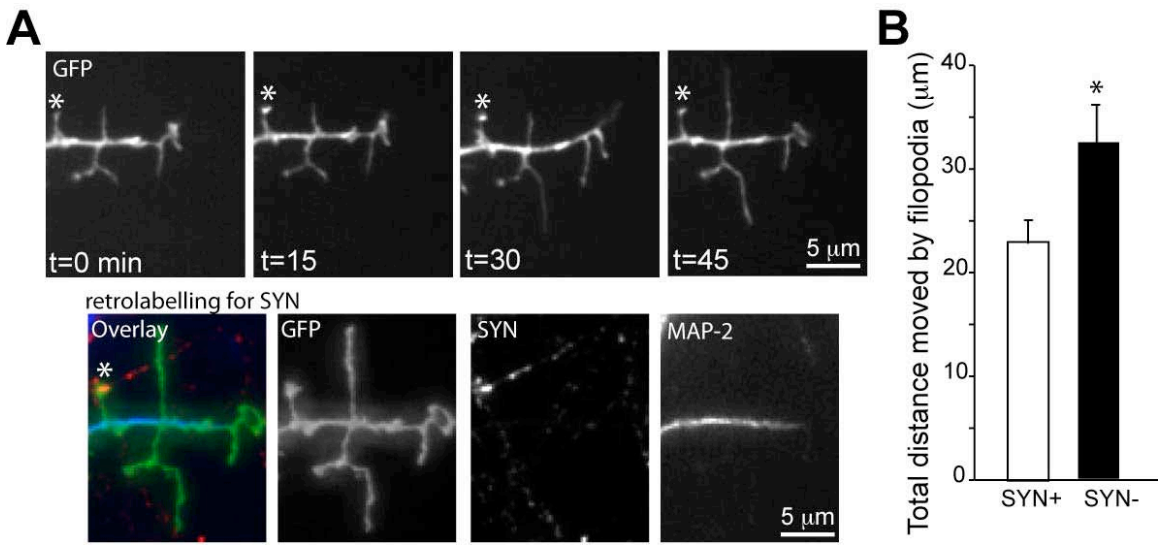

Figure 3.5 Filopodia stability plays an important role for the recruitment of presynaptic elements (A) Example of dendrite showing one stable protrusion and 3 motile protrusions. Retro-immunolabelling for synaptophysin performed at the end of each experiment revealed that stable filopodia (labeled with *) is associated with presynaptic terminal, positive for synaptophysin (SYN). (B) Comparison of total distance travelled by a filopodium that is associated with or without SYN. 5 filopodia were counted per cell and 8 cells were calculated from 4 independent experiments. (C) Representative timelapse images of neurons expressing GFP and Synaptophysin-DsRed. The box illustrates a filopodium (GFP) in contact with a synaptic cluster of Synaptophysin (DsRed) that accumulates in brightness (shown in D) with time. *p <0.05, Data represent mean ±SEM. Scale bars, 5μm.

The ability to observe filopodia in contact with axons during live cell imaging allowed us to follow their fate over time. 6.6% ± 1.3% of GFP-positive filopodia stably associated with axons, but lacked presynaptic protein clusters, were found to recruit the presynaptic marker synaptophysin-DsRed within 1h (Figure 3.6A,B,C). Expression of protein constructs such as GAP 1-14, and Cdc42 (CA)-Palm that result in unstable filopodia were significantly less likely to recruit synaptophysin-DsRed at sites of contact (2.2% ± 1.5% and 1.2% ± 1.1% of contacts showing recruitment). In contrast, for NLG-1 expressing cells, 11.5% ± 3.3% of contacts showed recruitment of synaptophysin-DsRed over the same time period (Figure 3.6A,B,C). These findings provide further evidence that enhanced

contact stability modulated by proteins such as NLG-1 potentiate the recruitment of presynaptic elements to sites of contact between dendritic filopodia and axons.

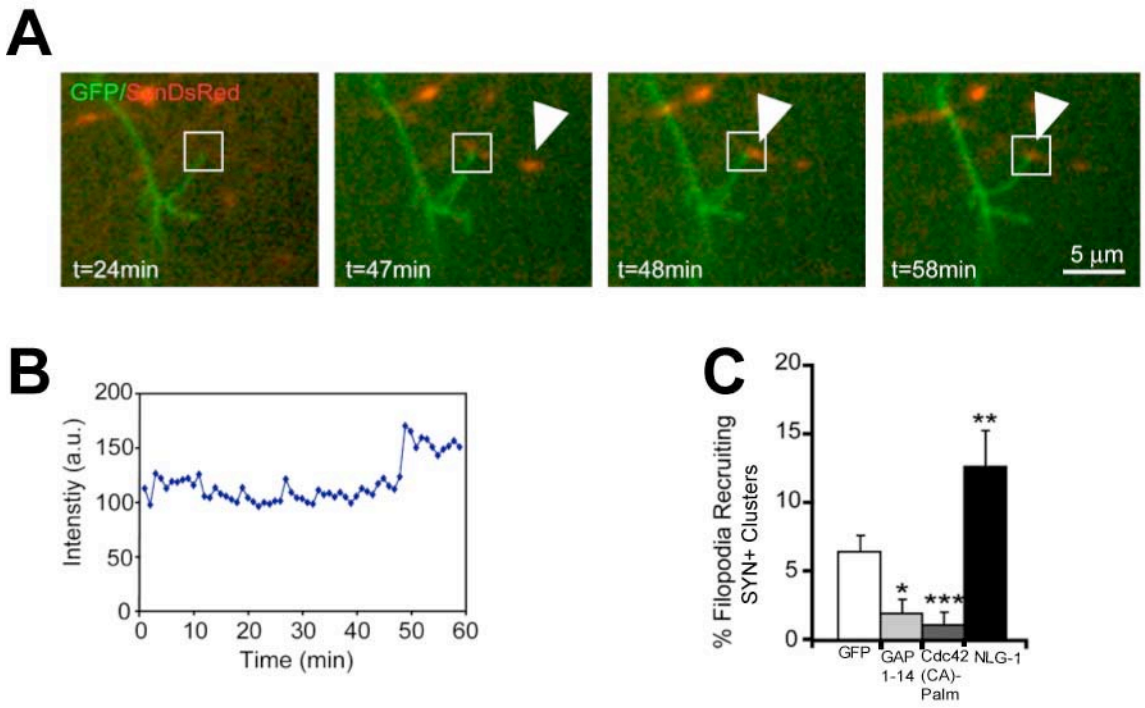

Figure 3.6 Filopodia stability plays an important role for the recruitment of presynaptic elements (A) Intensity graph showing the increased intensity of synaptophysin cluster with time (min). (B and C) Quantification comparing percentage of filopodia recruiting SYN among neurons expressing GAP1-14, Cdc42 (CA)-Palm and NLG-1. Neurons expressing NLG-1 showed a marked increase in the percentage of filopodia that recruit presynaptic clusters compared to control neurons expressing GFP. In contrast, filopodia induced by GAP1-14 and Cdc42 (CA)-Palm recruit significantly less SYN compared to the GFP control. *p <0.05, **p <0.01, ***p<0.001 Data represent mean $\pm$SEM. Scale bars, 5µm.

3.3.3. Filopodia motility and stability is differentially modulated by Cdc42 (CA)-Palm, GAP 1-14, NLG-1 and Shank1b

To further understand what role filopodia motility and stability play in the formation of stable contacts, timelapse imaging of dually labeled neurons was performed. Contact formation was

visualized between DsRed-labeled axons and cells expressing GFP-tagged GAP 1-14, Cdc42 (CA)-Palm, NLG-1 or Shank1b. Neurons expressing GAP1-14 or Cdc42 (CA)-Palm show more transient filopodia-axon contacts over 1 h, as compared to GFP expressing cells (0.35 μm/min ± 0.04 and 0.41 μm/min ± 0.06 respectively, versus 0.23μm/min ± 0.02 for GFP; Figure 3.7A and B). In contrast, neurons expressing NLG-1 or Shank1b showed relatively less motile filopodia (0.21 μm/min ±0.02 and 0.15± 0.01, respectively) compared to GAP1-14 or Cdc42 (CA)-Palm expressing filopodia. This is in agreement with the finding (Figure 3.8) that NLG-1-expressing cells have a greater percentage of filopodia that can form synaptic contacts or 'protosynapses' (Aoki *et al.*, 2005; Chen et al., 2010). Finally, filopodia induced by NLG-1 or Shank1b were significantly more stable compared to filopodia expressed by GFP, GAP 1-14 or Cdc42 (CA)-Palm (Figure 3.7C). This would suggest that both filopodia motility and stabilization (following axonal contact) are necessary to induce structures that mature into synapses.

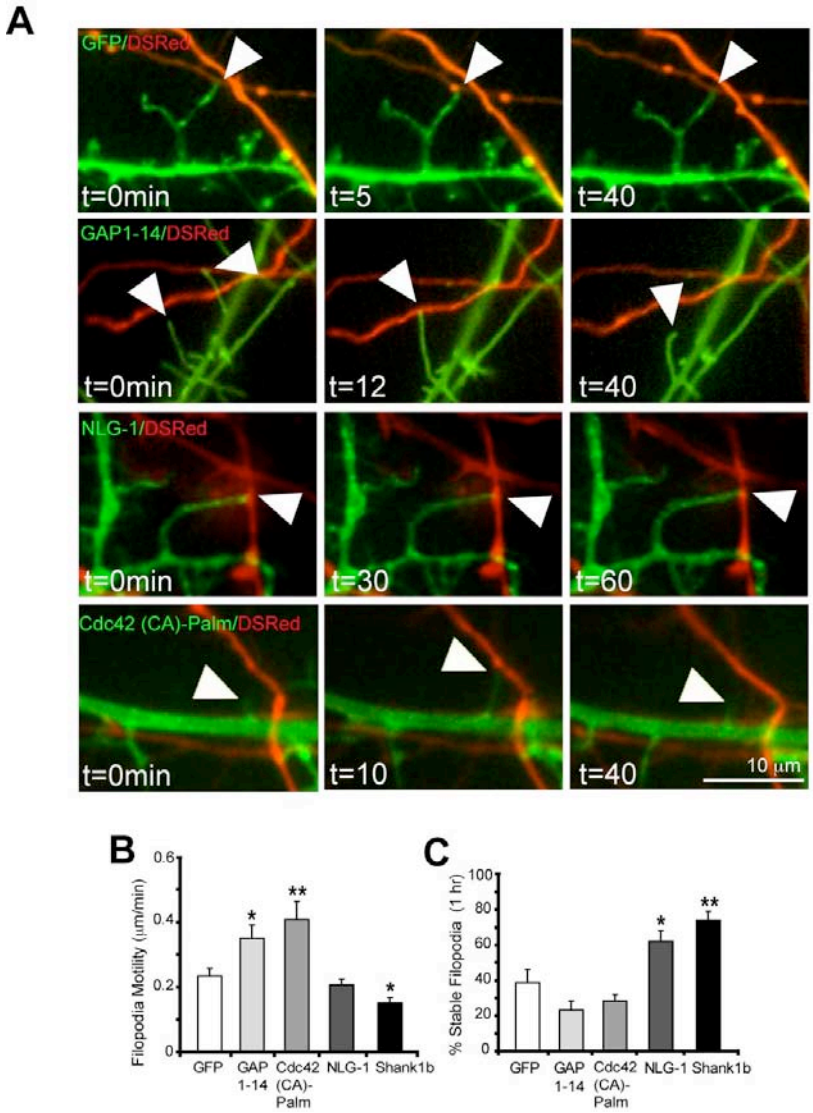

Figure 3.7 Filopodia motility and contact formation are modulated differently by GAP1-14 and Cdc42 (CA)-Palm versus NLG-1 and Shank1b

(A) Representative timelapse images of cells expressing GFP, GAP 1-14,NLG-1 and Cdc42 (CA)-Palm. Arrowheads point to dendritic filopodia in contact with a DsRed labeled axon. (B) Quantification of filopodia motility from neurons expressing either GFP, GAP 1-14-GFP, Cdc42 (CA)-Palm, NLG-1 and Shank1b. Filopodia in cells expressing GAP 1-14 and Cdc42 (CA)-Palm are more motile than GFP control. Filopodia expressed by NLG-1 and Shank1b are significantly less motile than filopodia expressed by GAP 1-14-GFP and Cdc42 (CA)-Palm. (C) Quantification of percentage of stable filopodia induced by these molecules. Filopodia were imaged for 1 h. Filopodia induced by NLG-1 and Shank1b induce more stable filopodia compared to control cells expressing GFP and neurons expressing GAP 1-14 and Cdc42 (CA)-Palm. *p <0.05, **p <0.01 Data represent mean $\pm$SEM. Scale bar, 10µm.

3.3.4. Neuroligin-1 overexpression enhances the production of filopodia and modulates dendritic contact formation with presynaptic elements

Studies have demonstrated a role for adhesion molecules in the formation of synapses (Decourt *et al.*, 2009; Matter *et al.*, 2009). Here, we wanted to investigate whether filopodia induced by NLG-1 can participate in synaptic contact formation. To answer this question, cells overexpressing NLG-1 were fixed and immunostained for endogenous synaptophysin. Our analysis revealed that a proportion of filopodia in control GFP expressing cells were positive for synaptophysin (Figure 3.8A and B). Moreover, NLG-1 overexpression caused an increase in the fraction of synaptophysin-positive filopodia (26.5% ± 1.30% compared to 11.7% ± 0.9% for GFP, Figure 3.8B), suggesting that these protrusions represent emerging synapses, or protosynapses. To characterize the type of synapses formed on filopodia, we immunolabeled GFP and NLG-1 transfected cells with the excitatory presynaptic marker VGLUT (vesicular glutamate transporter-1). We find that a fraction of VGLUT positive synapses are formed at the tips of filopodia (Figure 3.8C and D). Moreover, NLG-1 overexpression enhances the proportion of filopodia positive for VGLUT when compared to GFP expressing cells (29.3% ± 2.8% and 7.7% ± 2.9%; Figure 3.8C and D). Taken together, these findings are consistent with a proposed role of dendritic filopodia in excitatory synapse formation (Ziv and Smith, 1996; Fiala *et al.*, 1998; Marrs *et al.*, 2001; Konur and Yuste, 2004a; Niell and Smith, 2004; Evers *et al.*, 2006).

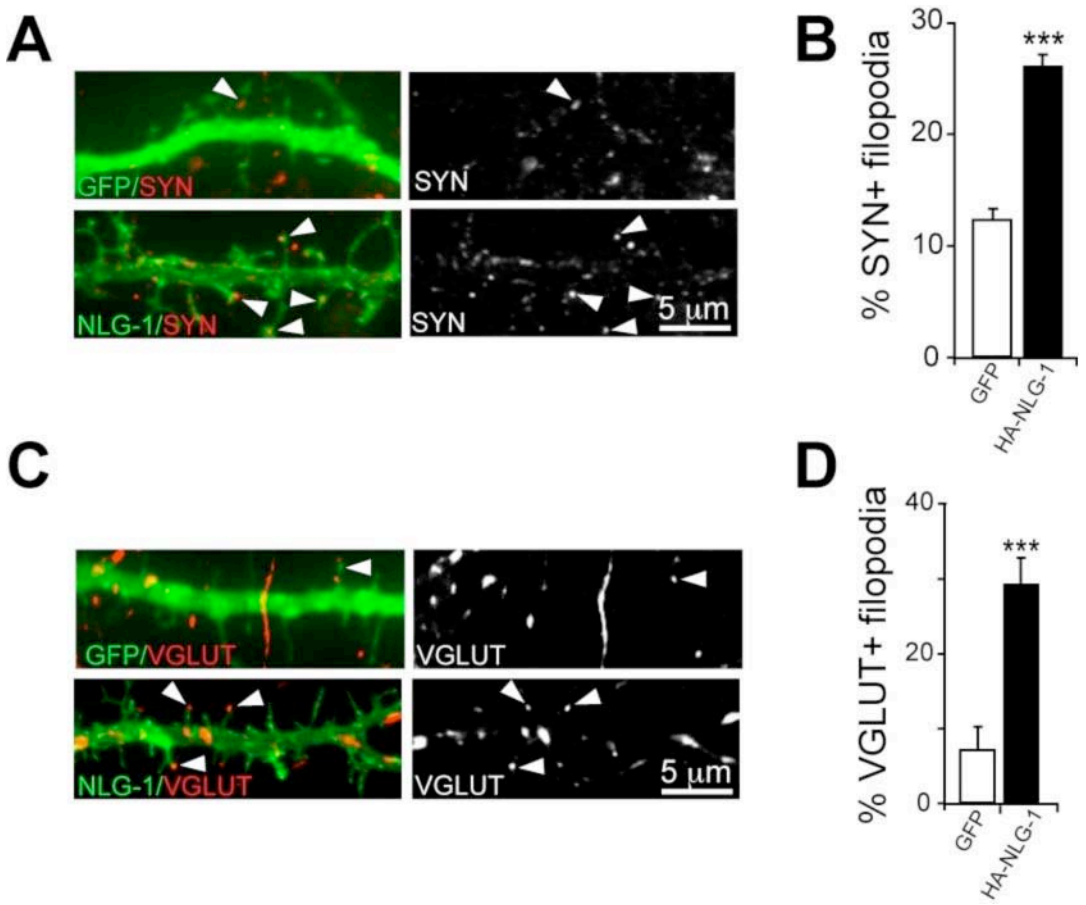

Figure 3.8 Filopodia expressing NLG-1 recruits significantly more presynaptic clusters
(A) Expression of NLG-1 led to an increase in synaptophysin found at the tips of these filopodia compared to cells expressing GFP. Arrowheads point to dendritic filopodia in contact with a presynaptic cluster. (B) Quantification of percentage of filopodia apposed to a cluster of synaptophysin. NLG-1 showed a two-fold increase in the percentage of synaptic filopodia compared to GFP expressing cells. (C and D) Representative images and quantification of NLG-1 led to an increase in VGLUT found at the tips of filopodia compared to cells expressing GFP. At least 13 cells from 3 independent culture preparations for each group were counted. *p <0.05, ***p<0.001 Data represent mean ±SEM. Scale bars, 5µm.

We next wanted to address whether filopodia expressing NLG-1 were essential for VGLUT clustering. To address this issue we used a knockdown approach using a specific siRNA target sequence (see Materials and Methods). We found that upon expression of GFP+NLG-1 RNAi (8.6%±1.8; Figure 3.9E,F) there was a dramatic reduction in the percentage of filopodia contacting VGLUT clusters compared to expression of the control GFP+Ctl RNAi (16.5%±2.7; Figure 3.9A,B).

These results demonstrate a critical role for NLG-1 in the formation of dendritic filopodia and the increase probability that these filopodia will form synaptic contacts.

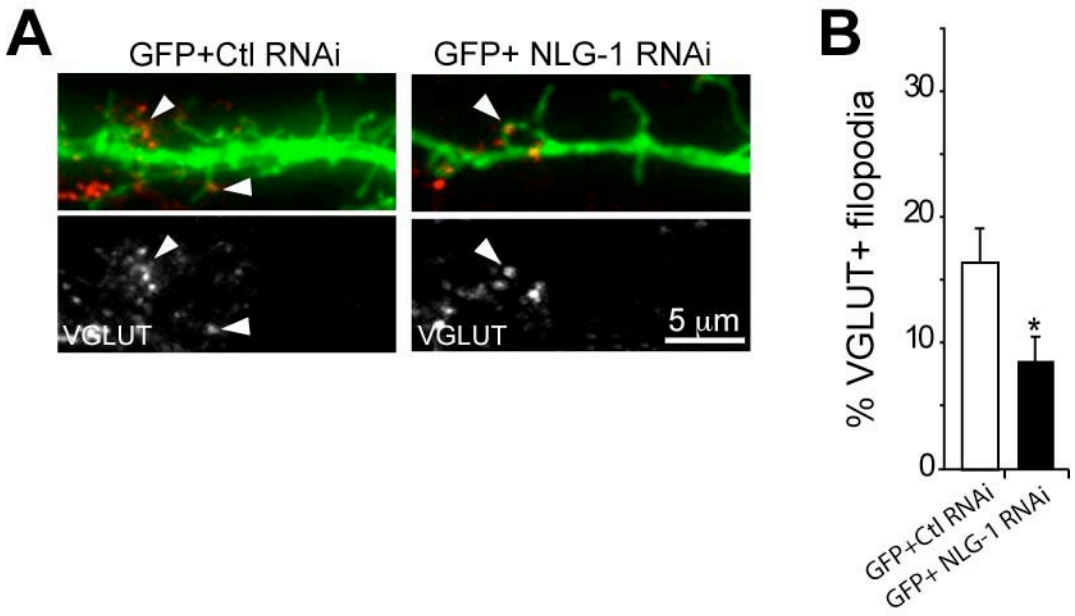

Figure 3.9 Filopodia expressing NLG-1 recruits significantly more presynaptic clusters
(A and B) Representative images and quantification of neurons expressing NLG-1 RNAi or Ctl RNAi with GFP. NLG-1 knockdown by siRNA led to a significant reduction in the percent of filopodia in contact with VGLUT. At least 13 cells from 3 independent culture preparations for each group were counted. *p <0.05, ***p<0.001 Data represent mean ±SEM. Scale bars, 5µm.

3.3.5. Recruitment of synaptophysin at contact sites is modulated by NLG-1

Rapid recruitment of presynaptic elements to nascent neuronal contacts is thought to be critical for synapse formation (Marrs *et al.*, 2001; Okabe *et al.*, 2001; Garner *et al.*, 2002; Evers *et al.*, 2006). We have previously shown that clusters of postsynaptic proteins enhance the recruitment of synaptophysin positive transport packets to contact sites (Gerrow *et al.*, 2006). Here, we examined whether dendritic filopodia associated with synaptophysin-DsRed labeled axons, help recruit presynaptic elements to contact sites. Our analysis reveals that 28.0% ± 3.6% of stable filopodia from GFP-expressing cells were found associated with synaptophysin-DsRed positive clusters, whereas

61.4% ± 7.9% of filopodia in NLG-1 expressing cells were associated with synaptophysin-DsRed clusters within the imaging period (Figure 3.10A and B). These data are consistent with an immunostaining analysis showing that filopodia can be associated with synaptophysin positive puncta (Figure 3.8).

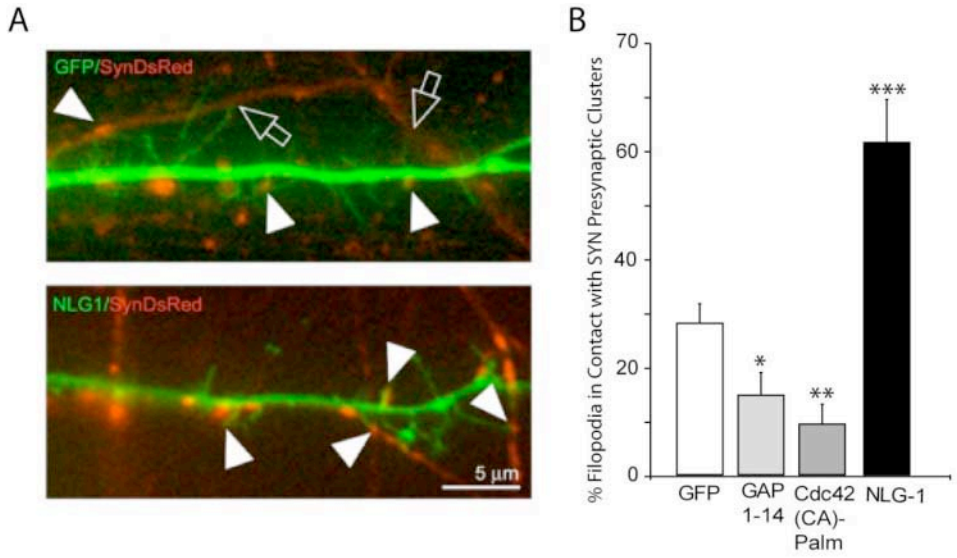

Figure 3.10 Recruitment of synaptophysin to sites containing NLG-1 induced filopodia
(A) Representative timelapse images of cells expressing Synaptophysin-DsRed and either GFP or NLG-1. Arrowheads indicate filopodia in contact with clusters of synaptophysin. Arrows denote filopodia in contact with axons labeled with Synaptophysin-DsRed, but do not contain a synaptic cluster. (B) Cells expressing NLG-1 showed a dramatic increase in the percent of filopodia contacting presynaptic clusters compared to control cells expressing GFP. *p <0.05, **p <0.01, ***p<0.001 Data represent mean ±SEM. Scale bars, 5µm.

3.4 Discussion

Dendritic filopodia have been implicated in neuronal contact formation and spine development (Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala *et al.*, 1998; Harris, 1999; Zhang and Benson, 2000; Portera Cailliau and Yuste, 2001; Evers *et al.*, 2006). It is generally assumed that in the

developing neuron a filopodium is first formed; following contact with an afferent fiber, it retracts and becomes a spine (Fiala *et al.*, 1998; Sorra and Harris, 2000). During development, dendritic filopodia show high motility and their numbers correlate inversely with the onset of more stable spines and synapses (Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala *et al.*, 1998; Dunaevsky *et al.*, 1999; Okabe *et al.*, 2001). These observations led to the hypothesis that filopodia may initiate synaptogenesis by extending themselves towards axons and, subsequently, stabilizing the resulting connections into mature synapses (Goda and Davis, 2003). This hypothesis may also be true in mature neurons. Within hours following activity blockade with tetrodotoxin (TTX), filopodia grow off existing spines, indicating that they are being used as a means of searching for glutamate-releasing presynaptic terminals (Richards *et al.*, 2005). Consistent with this idea, another study found that blocking synaptic transmission resulted in an increase in filopodia along dendrites as measured by electron microscopy (Petrak *et al.*, 2005). These studies suggest that dendritic filopodia seek new presynaptic partners in order to establish new synaptic contacts.

Filopodia density and motility are not correlated with synaptic contact formation

In this study we found that filopodia density is NOT correlated with synaptic contact formation. In fact, expression of Cdc42 (CA)-Palm and the palmitoylated motif GAP 1-14 leads to an increase in filopodia motility, but reduces the probability of forming stable contacts with neighboring axons and the recruitment of presynaptic elements. In contrast, NLG-1 is capable of both inducing filopodia formation and transforming filopodia to spines upon contact with presynaptic terminal.

In contrast to the extensive understanding of molecular cues controlling maturation of spines, the mechanisms and molecules involved in contact formation leading to the establishment of a synapse

are far from clear. Our results are consistent with previous findings that filopodia density is NOT correlated with synapse formation. Another hypothesis is that filopodia motility may predict the probability of initiating a stable synaptic contact. However the evidence as to how motility correlates to synaptogenesis (ie. proportional or inversely proportional) is controversial. For example, one study showed that disrupting EphB expression decreased filopodia motility, which was correlated with a reduced rate of synaptogenesis (Kayser *et al.*, 2008). In another study, it was found that overexpression of M6a, a neuronal glycoprotein, resulted in an increase in filopodia motility and the motility significantly decreased upon a synaptic contact (Brocco *et al.*, 2010). In our study, we showed expression of the adhesion molecule NLG-1 and scaffolding molecule Shank1b dramatically reduced filopodia motility and enhanced the number of stable filopodial contacts that recruit presynaptic elements. In contrast, GAP1-14 and Cdc42 (CA)-Palm induce the most motile filopodia among all molecules in this study (Figure 3.7) but the least percentage of synaptic contacts (Figure 3.6C). These results suggest that filopodia motility is inversely correlated with synaptic contact formation.

In addition, we found only a small fraction of emerging filopodia transform to spines. Although this process normally occurs over a period of several days, expression of Shank1b can rapidly (within hours) transform filopodia to spines (Arstikaitis *et al.*, 2008). Our results are consistent with previous studies, which have shown that, following contact with an axon, filopodia become less motile and greater stability is achieved, resulting in the formation of dendritic spines (Dailey and Smith, 1996; Ziv and Smith, 1996; Lendvai *et al.*, 2000; Zito *et al.*, 2004).

Implication of cell adhesion on synapse formation

Despite the focused efforts of identifying cell adhesion molecules directly involved in synaptogenesis, only two adhesion molecules have been shown to induce formation of presynaptic specializations: neuroligins and synaptic cell adhesion molecule 1 (SynCAM 1) (Akins and Biederer, 2006). Notably, contact with these adhesion molecules induces neurons to assemble presynaptic terminals that have physiological properties virtually identical to those formed between neurons. Neuroligins are important molecules for neurodevelopment as mutations in neuroligin genes are linked to autism and mental retardation (Jamain *et al.*, 2003; Laumonnier *et al.*, 2004; Chubykin *et al.*, 2005; Yan *et al.*, 2005; Lawson-Yuen *et al.*, 2008; Yan *et al.*, 2008; Zhang *et al.*, 2009).

Here we show that NLG-1, a potent inducer of synapses, is also required for dendritic filopodia formation, as our knockdown data demonstrates that loss of NLG-1 causes a reduction in the percentage of synaptic contacts formed by filopodia-like protrusions (Figure 3.8 and 3.9). This suggests that one mechanism by which NLG-1-expressing filopodia could form synaptic contacts is by sampling the environment for potential axonal partners. Once contact is made these filopodia remain stable, possibly transforming into a dendritic spine. Interestingly, Kayser et al (2008) observed both in vitro and in vivo that filopodia induced by EphB, a member of the receptor tyrosine kinase family, have more of an exploratory role, as they are more motile (Kayser *et al.*, 2008). Elimination of EphB from the brain causes filopodia to become less motile and the rate of synaptogenesis decreases. This molecule behaves differently from the two molecules we investigated, GAP 1-14 and Cdc42 (CA)-Palm, as we found that motility of filopodia induced by GAP1-14 is inversely correlated with synaptogenesis (more motility, less synaptogenesis), whereas motility of filopodia induced by EphB is proportionally correlated with synaptogenesis (less motility, less synaptogenesis) (Figure 3.7). In addition, expression of EphB resulted in more motile filopodia, which is opposite to the behavior of filopodia induced by NLG-1 and Shank1b. However, EphB,

NLG-1 and Shank1b produce similar results, which is to increase synaptogenesis as we found that the filopodia expressed by NLG-1 and Shank1b were more stable. This suggests two things: one, that there are factors at play intrinsically related to the specificity of each protein and its role in the developing brain and two, the stability of filopodia induced by NLG-1 and Shank1b may be important for the construction of future synapses (Figure 3.11).

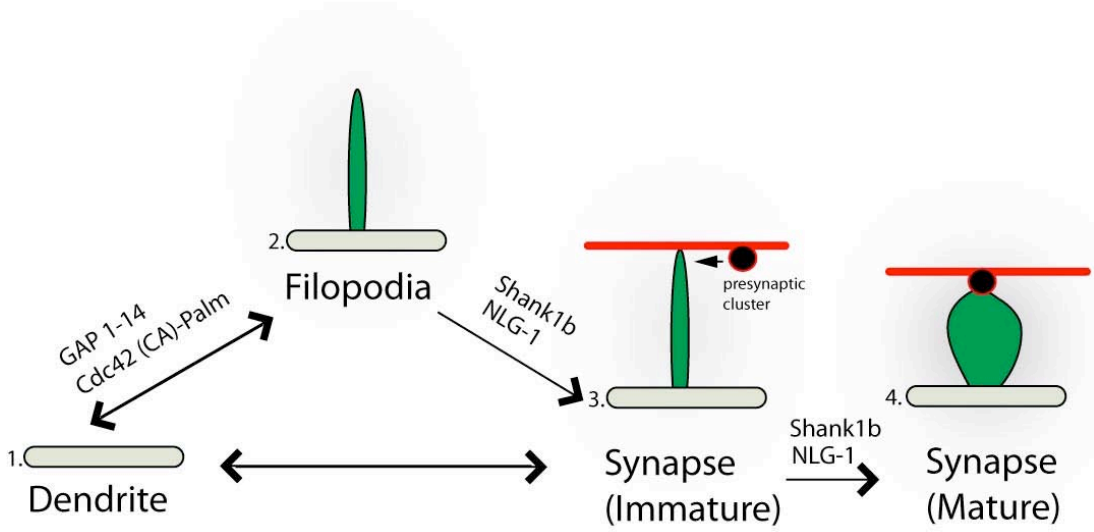

Figure 3.11 A model illustrating how filopodia induced by different molecules participate in the formation of immature and mature synapses
(1. to 2.) Molecules such as GAP 1-14 and Cdc42 (CA)-Palm participate in the induction of filopodia and these protrusions are mainly transient and immature. (2. to 4.) In contrast, molecules such as NLG-1 and Shank1b participate in the formation of more mature synapses (containing synaptic machinery such as synaptophysin and filopodia transform into a more spine-like morphological shape) possibly through the stabilization of dendritic filopodia. (1. to 4.) In addition, synapses can form independent of filopodia and form from the dendritic shaft.

Several studies have reported that synaptic contacts can form at the tips of dendritic filopodia, resulting in filopodia stabilization and functional presynaptic boutons (Ziv and Smith, 1996; Kohsaka and Nose, 2009). In our study, we also observed that filopodia induced by NLG-1 were able to recruit

synaptophysin-positive transport packets to sites of contact and we speculate that this is the beginning of a protospine, which may later develop into a functional dendritic spine (Figure 3.11). Together, these findings provide a novel mechanism by which NLG-1 could form dendritic spines by promoting filopodia extension and stabilizing contact with a presynaptic terminal. This is followed by stabilization of the contact resulting in filopodia retraction and further spine development. We suggest that NLG-1 is a key molecule for spine formation during development.

Implication of scaffolding molecules on synapse formation

Previous work suggests that scaffold proteins may help stabilize filopodia to form dendritic branches. In Zebrafish tectal neurons, timelapse imaging showed when a filopodium bearing PSD-95 puncta undergoes retraction, distal regions retract normally but retraction is halted when a PSD-95 punctum is encountered (Niell *et al.*, 2004; Niell and Smith, 2004). Thus, PSD-95 accretion strongly correlates with the stabilization of a filopodium and its maturation into a dendrite branch. Similarly, work done by Prange et al. 2001 found using timelapse imaging of cultured cortical neurons that filopodia containing PSD-95 clusters were significantly more stable than those lacking clusters and led to an increase in the number of synapses formed (Prange and Murphy, 2001). Similarly, we found that filopodia containing clusters of Shank1b were less dynamic and led to an increase in the number of spines formed (Chapter 2), suggesting that these filopodia function to make stable contacts consequently leading to the formation of a synapse. Similar to PSD-95, it is possible that the Shank1b containing clusters are also trafficked to filopodia in a developmentally regulated manner and this is associated with increased filopodia stability and synapse formation.

Unlike NLG-1, which interacts with its presynaptic counterpart neurexin to enhance the number of

synapses, Shank1b likely induces spine through the stabilization of the cytoskeleton. These findings raise the question of how does Shank1b communicate with presynaptic sites to enhance synaptic contact formation? It has been shown previously that transport of synaptophysin to sites opposed to stationary clusters of PSD-95 caused rapid morphological rearrangements of the newly recruited clusters (Gerrow *et al.*, 2006). This finding suggests that postsynaptic scaffolds can recruit axonal transport packets for initiation and/or stabilization of new sites of contact (Gerrow *et al.*, 2006). Therefore it is possible that expression of Shank1b may trigger recruitment and morphological changes of presynaptic complexes and this process may be critical for stabilization of dendritic filopodia.

Possible limitations of this study and future directions

Although we provide evidence that filopodia induced by specific proteins can participate in contact and synapse formation, there is one key limitation to this study that will be addressed here. The consequences of photodamage on cellular viability can be severe (Swedlow and Platani, 2002; Kwinter and Silverman, 2009) and some studies have reported that sampling the specimen for long durations increases the probability that the neuron will show abnormal physiological processes (Swedlow and Platani, 2002; Kwinter and Silverman, 2009). Thus, we are aware that we may have 'missed' many events whereby the fate of the filopodium was continually changing in these non-imaged time periods, but fewer sampling time-points were purposefully selected to ensure cell viability. In the future, it will be important to determine if the synaptic capabilities of these dendritic filopodia, induced by NLG-1, are an intrinsic property of this protein or if its binding partner, neurexin, is also required. A recent paper suggests that the NLG-1-neurexin interaction may be critical for filopodia stability and synapse formation (Chen *et al.*, 2010). In addition, it would be interesting to examine the filopodia dynamics in cultured hippocampal neurons taken from transgenic

animals overexpressing NLG-1. This experiment would be a further test of our hypothesis that filopodia expressing NLG-1 are more likely to form synaptic contacts leading to filopodia stability and possible spine formation.

4. Discussion

4.1 Summary of findings

The overall goal of this thesis was to investigate the role of dendritic filopodia in spine and synapse formation. The first goal was to test the filopodial hypothesis in hippocampal neuronal cells. More specifically, we wanted to explore whether paralemmin-1 may be a potential molecule involved in the regulation of filopodia formation and their transformation to spines.

Using overexpression and RNAi in primary hippocampal neuron cultures, we found that RNAi knockdown of paralemmin-1 decreases the number of dendritic filopodia and spines in developing neurons, whereas postsynaptic overexpression of paralemmin-1 increases the number of filopodia, spines, and impinging synaptophysin-positive presynaptic terminals. Furthermore, overexpression of both short (PALM-S, lacking exon 8) and long (PALM-L, contains exon 8) splice variants of paralemmin-1 increased the number of GluR1 AMPA receptor puncta, although only PALM-L increased the size of GluR1 puncta. The postsynaptic scaffold molecule Shank1b has previously been shown to increase the number, maturation, and size of dendritic spines (Sala *et al.*, 2001). Using timelapse imaging, we found that Shank1b overexpression accelerates the transformation of long thin filopodia to spines, whereas overexpression of paralemmin-1 causes spines to revert to filopodia. To uncover a mechanism by which this might occur, we performed FRAP experiments in DiI-labeled COS-7 cells and found an increase in DiI FRAP upon expression of either full-length PALM-S or the C-terminal palmitoylated domain of paralemmin-1 suggesting that palmitoylation may be important for regulating membrane fluidity as the palmitoylated motif of paralemmin-1 was sufficient for these

effects (AppendixB4). To examine how this process might be regulated, we tested the effect of KCl depolarization and found an increase in membrane localization of paralemmin-1 after KCl treatment. Moreover, expression of paralemmin-1 augments KCl-induced formation of dendritic protrusions. The protrusion-promoting effects of paralemmin-1 were abolished by mutation of the palmitoylated cysteines to serines or by treatment with 2 bromopalmitate. My interpretation of these results is that paralemmin-1 is involved in depolarization-induced membrane changes such as: 1) enhanced paralemmin-1 trafficking to the plasma membrane and 2) an increase in protrusion size leading to the formation of dendritic filopodia, or plays a role in activity-dependent changes in protrusion size. These changes may lead to enhanced synapse formation.

The next goal was to examine whether filopodia actively participate in the formation of axo-dendritic contacts leading to subsequent synapse formation. Using live imaging, as well as loss and gain of function approaches, our analyses identify key molecules involved in regulating filopodia dynamics, contact formation and stabilization. We also show that filopodia induced by NLG-1 are able to form excitatory, *en passant* contacts, with nearby axons. In addition, knockdown of NLG-1 by siRNA reduces the percent of excitatory contacts formed. Next, we show a correlation between increased filopodia motility in younger neurons expressing the palmitoylated motif of GAP-43 and Cdc42 and a lower probability of forming synapses on dendritic spines in more mature neurons. In contrast, molecules such as NLG-1 and Shank1b show a reduction in filopodia motility and an increase in contact formation and stabilization. Finally, we show that expression of NLG-1 in neurons is sufficient to recruit presynaptic clusters of synaptophysin to the site of contact. This study provides a novel approach to investigate filopodia dynamics, contact formation and stabilization by contrasting

the roles of molecules involved in filopodia motility to those important for synapse stabilization and maturation.

In summary, this work is highly significant as it identifies a novel mechanism by which paralemmin-1 induces changes in the neuronal cytoskeleton involved in filopodia formation and spine maturation. In addition, we elucidate how dendritic filopodia might contribute to the formation of axo-dendritic contacts, revealing different roles for palmitoylated proteins, scaffolding and cell adhesion molecules. We believe that since this protein controls diverse aspects of synapse development, spine maturation and recruitment of glutamate receptors, it will contribute greatly to enhancing our knowledge and understanding of the mechanisms involved in proper synapse formation.

4.2 Dendritic filopodia

4.2.1 Paralemmin-1 may regulate membrane fluidity

How does paralemmin-1 exert its effects on filopodia formation and how does this fit in with the current understanding of spine development in neurons?

There is compelling evidence that palmitoylation of numerous neuronal proteins is critical for protein localization and function. In particular, palmitoylation serves as a critical signal for targeting proteins to the plasma membrane, however what remains unclear are the mechanisms that regulate filopodia induction. One method to study dynamic protein palmitoylation in living cells is by fluorescence recovery after photobleaching (FRAP). I have found that palmitoylation of paralemmin-1 is critical

for its filopodia inducing effects in non-neuronal and neuronal cells. Similarly, other studies have shown the importance of palmitoylation in its ability to induce filopodia (Ahola *et al.*, 2000; Neumann-Giesen *et al.*, 2004; Brocco *et al.*, 2010; Karo-Astover *et al.*, 2010).

One major question raised is by what mechanism does paralemmin-1 induce filopodia formation? One hypothesis of how this might occur is that paralemmin-1 may alter membrane fluidity by the interaction of the palmitoyl groups and the lipid-rich plasma membrane. Thus, we investigated whether paralemmin-1 exerts its effects on protrusion formation by altering membrane fluidity. FRAP analysis of the lipophilic dye, DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), was used to determine the rate of fluorescence recovery upon expression of paralemmin-1 in COS-7 cells (Figure B5). The effects of paralemmin-1 on the diffusion rate of DiI were contrasted to cells expressing GFP or a palmitoylation deficient mutant of paralemmin-1. We were surprised to find that cells expressing paralemmin-1 showed a significant increase in DiI recovery when contrasted to GFP expressing cells, illustrating an increase in DiI mobility in cells expressing paralemmin-1. Similar changes were observed in cells expressing the palmitoylated motif of paralemmin-1 fused to GFP (GFP-PALM CT), suggesting that the palmitoylated motif of paralemmin-1 influences membrane dynamics exerted by full-length protein.

Taken together, this experiment is preliminary and correlative, but it hints at a possible mechanism by which paralemmin-1 could function to alter membrane fluidity leading to filopodia outgrowth. The results from this experiment are interesting as it provides further understanding of the membrane biophysical effects of paralemmin-1. The next step will be to perform this same experiment in

hippocampal neuronal cells to address whether the palmitoyl groups of paralemmin-1 can affect membrane fluidity and we would expect similar results as discussed here.

One experimental limitation is that we have not shown a direct link between filopodium formation and membrane fluidity. The change in the membrane fluidity may either cause or be caused by filopodia formation (both GFP-PALM-CT and GFP-PALM-S) can increase filopodia number, or not related at all (Gauthier-Campbell *et al.*, 2004; Arstikaitis *et al.*, 2008). To clarify this point, the site of action of paralemmin-1 will have to be examined to address whether paralemmin-1 regulates the interaction between actin and membrane, actin polymerization or phospholipid composition.

Our data is consistent with a previous study that demonstrates changes in membrane dynamics involved in filopodia induction can be triggered by changes in membrane flow (Mattila et al., 2007). This study provides compelling evidence that the membrane deforming activity of IRSp53/MIM domain (IMDs), instead of by F-actin–bundling or GTPase-binding activities, is critical for the induction of the filopodia/microspikes in cultured mammalian cells. This suggests that cell motility leading to morphogenesis can occur through interplay between actin dynamics and a novel membrane-deformation activity (Mattila *et al.*, 2007; Mattila and Lappalainen, 2008).

To understand how palmitoylation may effect the targeting of proteins to the plasma membrane, recent work examined how targeting of the Ras family of proteins was affected palmitoylation using FRAP analysis (Roy *et al.*, 1999; Henis *et al.*, 2006; Baekkeskov and Kanaani, 2009). Interestingly, the palmitoylated GFP-H-Ras G12V recovered more slowly whereas the palmitoylated mutant form of Ras showed a faster recovery time (Henis *et al.*, 2006). In contrast, we found that PALM-S and

PALM-CT recovered faster than the palmitoylation mutants. The discrepancy in our findings compared to the Ras study might be explained by palmitoylation-depalmitoylation cycles with the plasma membrane or lipid rafts located in membranes as we have previously shown that paralemmin-1 does associate with lipid rafts (El-Husseini Ael *et al.*, 2001). Perhaps, if paralemmin-1 cycles faster than H-Ras, it might be expected to have a faster recovery time as it is being repalmitoylated by possible palmitoyl transfereases at a faster rate compared to H-Ras. In summary, FRAP is an excellent method to determine the strength of binding of palmitoylated proteins to cell membranes, and measure the kinetics of recycling of palmitoylated proteins between the different membrane compartments.

Other hypotheses available to explain how paralemmin-1 induces filopodia could be either direct or indirect interactions with Rho GTPases (Gauthier-Campbell et al. 2004) or possible interactions with proteins that influence the actin cytoskeleton directly such as the Arp2/3 family of proteins.

4.3 Development of dendritic spines

4.3.1 Role for paralemmin-1 and Shank1b in spine development

How might paralemmin-1 and Shank1b affect the formation of dendritic spine and how does this fit in with the current understanding of spine development in neurons?

In the first section of my thesis, I found that expression of paralemmin-1 induces filopodia in both heterologous cells and neurons. In contrast, Shank1b fails to induce filopodia in both cell types,

(Arstikaitis *et al.*, 2008) despite several studies demonstrating its ability to strongly increase spine numbers in neurons (Sala *et al.*, 2001; Roussignol *et al.*, 2005). Consistent with these findings, my results revealed that Shank1b expression increases the number of events where filopodia transform into spine-like structures, suggesting that Shank1b functions to rapidly induce the transformation of existing filopodia into spines (Arstikaitis *et al.*, 2008). Moreover, the number of stable spine-like protrusions in Shank1b-expressing cells was greater than paralemmin-1. These data suggest that paralemmin-1 induced effects on spine maturation require several days and that this process most likely requires recruitment of additional molecules such as Shank1b for spine stabilization. Overall, these observations point to a novel role for the combined actions of paralemmin-1 and Shank1b in regulating cellular morphogenesis.

The enhanced transformation of filopodia to spines induced by Shank1b suggests that its expression would potentiate the effects of paralemmin-1 on spine development. My results in fixed neurons reveal that co-expression of paralemmin-1 and Shank1b leads to a significant increase in the number of spines compared to either GFP-or paralemmin-1-expressing cells. These results suggest a role for Shank1b in stabilization and maturation of protrusions induced by paralemmin-1. There are several questions that these findings raise. First, how does co-expression of paralemmin-1 and Shank1b lead to an increase in spine formation? One possibility is that perhaps these proteins form indirect or direct interactions with molecules that influence the actin cytoskeleton. Indeed, Shank1b is capable of indirectly altering the actin cytoskeleton by forming a complex with IRSp53 in neuronal cells. This suggests that that IRSp53 can be recruited to the PSD via its Shank interaction and may contribute to the morphological reorganization of spines and synapses after insulin receptor and/or Cdc42 activation. Another possibility is that paralemmin-1 and Shank may form direct interactions

themselves. To address this possibility we could use the exon 8 of paralemmin-1 as bait and perform a yeast two-hybrid screen to determine if Shank1b is a potential binding partner. Another way to address this would be to perform a co-immunoprecipitation experiment to determine if these proteins can form a complex *in vitro* or *in vivo*.

In conclusion, I believe that the studies presented in this thesis have reached the initial objective of providing a better understanding of the role of paralemmin-1 in filopodia outgrowth and spine formation. The initial finding that palmitoylation of paralemmin-1 is critical for spine formation and together, through the coordinated actions of paralemmin-1 and Shank1b, manipulation of spine formation is possible and provides further insight into the mechanisms that regulate filopodial transformation to spines. The findings demonstrating distinctive roles for palmitoylated proteins, scaffolding and cell adhesion molecules for the formation of axo-dendritic contact formation is critical for understanding how filopodia outgrowth can lead to future synapses. Together, these findings elucidate mechanisms required for filopodia transformation to spines and axo-dendritic contact formation, which are critical for proper neuronal function and plasticity.

4.4 Neurological diseases and abnormal dendritic spine development

4.4.1 Specific diseases/disorders related to abnormal spine development

Understanding the molecular mechanisms of spine development may expand our knowledge on excitatory circuit formation in the brain. This must be accomplished before we can begin to address what goes awry in a diseased brain. It is appealing to suggest that abnormalities in the expression of

different proteins implicated in spine development may result in aberrant synapse development and/or loss of synapses, but unfortunately the story is not that simple. The brain has implemented many compensatory mechanisms such that manipulating the expression of a protein does not necessarily reveal its true function in the brain. Nevertheless, it is clear that abnormal spine development is implicated in several neurological diseases such as schizophrenia, Down Syndrome (DS), Autism Spectrum Disorders (ASDs), Alzheimer's disease and Fragile X syndrome (FXS). I will focus on DS, FXS and ASDs as they are amongst the most common developmental diseases effecting the formation of spines.

Down Syndrome: It is clear that neuronal morphology, such as dendrites, axons and dendritic spines become vulnerable to abnormal morphological changes in certain neurological disorders (Luebke *et al.*, 2010). Indeed, compelling evidence has revealed that malformed dendritic trees, spines and synapses have been observed in DS and DS mouse models.

The majority of DS mouse models when assessed for neurological dysfunctions showed impaired learning, suggesting that perhaps at the level of the dendritic trees and spines that malformation of trees and a reduction in spine density is likely related to learning deficits (Villar *et al.*, 2005; Best *et al.*, 2008; Belichenko *et al.*, 2009a; Belichenko *et al.*, 2009b; Perez-Cremades *et al.*, 2010). Consistent with this finding, another study reported that the diameter of spines in the cortex and hippocampus were enlarged in two mouse models of DS, Ts65Dn and Ts65Dn. In addition, these mice failed to exhibit long-term potentiation (LTP) in the fascia dentate (FD) (Belichenko *et al.*, 2007; Belichenko *et al.*, 2009a). It is interesting to note that these mice failed to induce LTP yet showed enlarged and abnormal dendritic spines. This might be possibly due to improper assembly of

the larger spine size or dysfunctional AMPA and NMDARs. Another possibility might be due to a reduction in filopodia formation resulting in abnormal spine formation. Generally speaking, if filopodia fail to form in DS subjects and mouse models then there is an overall lack of synapse formation via filopodia leading to functional spines. In addition, the abnormal dendritic arbors in these mice could also be caused by failed filopodia formation as numerous studies exist demonstrating that filopodia play an important role in the formation of dendritic arbors (Dailey and Smith, 1996; Niell *et al.*, 2004; Marrs *et al.*, 2006). Thus, a lack of filopodia formation appears to be the common link between abnormal dendritic spines and arbors.

Fragile X Syndrome: Another common form of inherited mental retardation is Fragile X Syndrome. It is caused by mutations of the Fmr1 gene leading to the loss of the fragile X mental retardation protein (FMRP). FMRP is highly expressed in the brain and one study found, using *in vivo* timelapse imaging with two-photon microscopy, that cortical pyramidal neurons in affected individuals and Fmr1 knock-out (KO) mice have an increased density of dendritic spines (Cruz-Martin *et al.*, 2010). Another study demonstrated that mutant mice also show defects in synaptic and experience-dependent circuit plasticity, which is known to mediate dendritic spine dynamics. Although the exact molecular mechanism(s) remains unclear, the consistent finding that dendritic spine density is increased in cortical neurons suggests that FMRP may play a role in synapse elimination or spine stabilization in early development. One possibility is that there may be a lack of the ubiquitin-proteasome machinery found in these mice leading to an overabundance of dendritic spines. Indeed, a recent study by Hung et al. demonstrated that TRIM3 stimulates ubiquitination and proteasome-dependent degradation of GKAP, and induces the loss of GKAP and Shank1 from postsynaptic sites (Hung *et al.*, 2010). Interestingly, knockdown of endogenous TRIM3 by RNA interference (RNAi)

caused an increased accumulation of GKAP and Shank1 at synapses, as well as enlargement of dendritic spine heads (Hung *et al.*, 2010). This suggests that E3 ligase proteins like TRIM3 are critical for negatively regulating dendritic spine morphology in an activity-dependent manner and lack of these proteins in FXS mice might account for the increased spine density (Hung *et al.*, 2010). What would be interesting to examine is whether spine stabilization can be restored in these mice by overexpressing a spine stabilizing protein such as Shank and assessing whether it is sufficient to restore spine densities back to control levels. Taken together, these findings suggest that the brain functions to balance dendritic spine formation and elimination as too many spines may lead to FXS and too few may lead to diseases such as DS (Weitzdoerfer *et al.*, 2001) and schizophrenia (Garey, 2010).

Autism Spectrum Disorders: In addition to MR and DS, numerous neurological diseases have been shown to relate to dendritic filopodia and spine malformations. ASDs are a common cause of intellectual and social disabilities and anxiety-like behaviors in males and typically develop before 2–3 years of age. The key phenotypic features of ASDs are difficulties in social interactions and communication, language impairments, a restricted pattern of interests, and/or stereotypic and repetitive behaviors. Recently, progress in studying the molecular mechanisms of ASDs has demonstrated that mutations in many genes such as NRXN1, NLGN3, NLGN4 and Shank that are associated with spine formation/maturation have been detected in ASDs (Pardo and Eberhart, 2007; Lawson-Yuen *et al.*, 2008; Yan *et al.*, 2008; Bourgeron, 2009; Bourgeron *et al.*, 2009).

In addition to the NRXN-NLG complex, mutations in genes encoding Shank have been detected in several autistic individuals. Indeed, Shank1 knockout mice exhibited a partial anxiety-like phenotype

in some components of the light/dark task, which reveals that these mice show signs of phenotypic ASD behaviors (Hung *et al.*, 2008; Silverman *et al.*, 2010). It is worth mentioning that this study found that in these mice motor, but not social function was impaired. This finding suggests that Shank1 may play an important role in motor functions and how this might be related to ASDs is unclear (Silverman *et al.*, 2010).

Other studies have found that mutations in Shank3 can lead to autism. One hypothesis on how this might occur is through the NRXN-NLGN-SHANK pathway, which is associated with synaptogenesis and imbalance between excitatory and inhibitory currents. To date, Shank3 knockout mice have not been generated however there is numerous studies performed in autistic (Moessner *et al.*, 2007; Pardo and Eberhart, 2007; Bourgeron, 2009; Bourgeron *et al.*, 2009; Gauthier *et al.*, 2009; Kumar and Christian, 2009) and schizophrenic patients (Gauthier *et al.*, 2010) that have confirmed mutations in Shank3. The molecular mechanisms are far from clear and will require future complement studies done in human subjects and animal studies such that synapse formation can be assessed. In summary, the description of the various mutations in the NRXN/NLG and Shank3 provides convincing evidence for this complex in ASDs, given the fact that these mutations account for a significant proportion of autism subjects, but exactly how this occurs and whether filopodia formation and spine development play a role is far from clear.

4.5 Future directions

The data presented in this thesis reveal that there are a number of different approaches to use for future investigations. These include molecular, genetic and behavioral studies to further our

understanding of filopodia formation mechanisms and are ultimately aimed at identifying therapeutic targets for treatment of neurological disorders associated with defects in dendritic spine formation.

4.5.1 Examine the function of paralemmin-1 in vivo

The studies described in my thesis used an *in vitro* cultured system. This involves removing the hippocampus from an intact brain rat brain and performing chemical and mechanical dissociation of the tissue to render single dissociated cells. This system is an artificial system and although important for elucidating molecules in function X, there is also cause for some concerns. One major concern is that the function of a protein can behave differently in a cultured system than in an animal. For example, *in vitro* studies indicate that NRXNs and NLGs are important for synapse formation. However, knockout (KO) studies done in mice revealed a surprising result: NLGs and α-NRXNs are essential for synaptic function, not synapse formation. Triple KO mice lacking NLG-1, NlG-2 and NLG-3 die at birth, but exhibit normal synapse ultrastructure. Thus, the KO data appears to contradict the *in vitro* assays showing that NLGs are critical for synapse induction. In summary, performing *in vitro* experiments is an excellent tool for examining the molecular mechanisms involved in protein function, however, it is important that this work be complemented with an *in vivo* approach. As a next step, it will be important to determine the role of paralemmin-1 *in vivo*. One approach to use is *in utero* electroporation to transiently introduce fluorescently tagged paralemmin-1 into the developing mouse brain and examine protrusion formation (please see Appendix A1). A second approach will be to examine the functional properties of cells expressing paralemmin-1 by performing electrophysiological experiments and examining the miniature EPSCs. More specifically, a standard electrophysiological test used is to measure the mini EPSC amplitudes and frequencies.

Given the observed effects of PALM-1 on synapse number and AMPA receptor accumulation, one might expect corresponding alterations in synaptic transmission.

4.5.2 Assess activity induced changes in paralemmin-1 localization and function

Neuronal activity modulates protrusion formation which in turn fine-tunes synaptic strength and plasticity (Dunaevsky *et al.*, 1999; Fischer *et al.*, 2000; Nimchinsky *et al.*, 2002; Richards *et al.*, 2005; Zuo *et al.*, 2005b). This process is mediated by the recruitment of proteins that alter membrane and cytoskeletal dynamics. In addition, the number and shape of spines are influenced by activity. We found that hippocampal neurons stimulated with KCl showed an increase in paralemmin-1 expression at the plasma membrane (Arstikaitis *et al.*, 2008). Blocking palmitoylation prior to stimulation with KCl treatment compromised paralemmin-1 localization to the membrane upon depolarization (Arstikaitis *et al.*, 2008). Taken together, these results suggest that induced translocation of paralemmin-1 is palmitoylation-dependent and is enhanced in response to neuronal activity. What remains unclear is the mechanism that regulates enhanced palmitoylation of paralemmin-1 leading to increase in spine morphology upon neuronal depolarization.

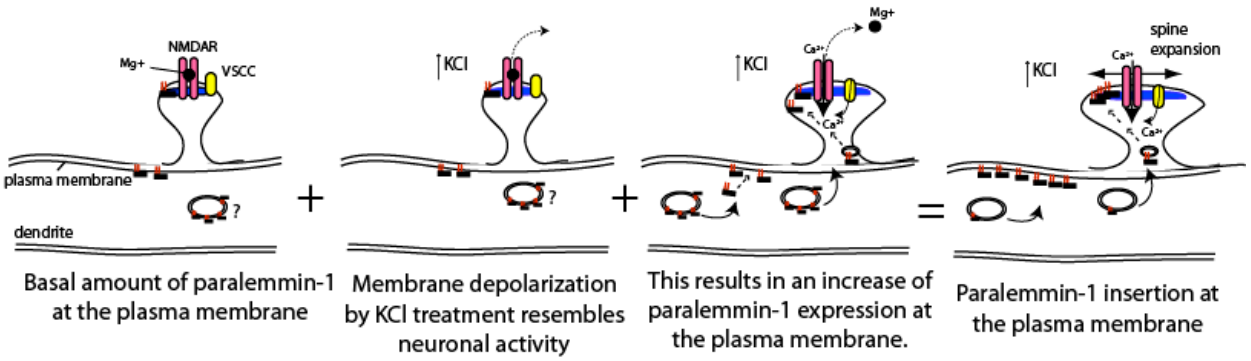

Figure 4.1 Schematic illustrating how paralemmin-1 expression may induce protrusion formation upon KCl depolarization.
First, a basal amount of paralemmin-1 is present at the membrane and upon depolarization by KCl in which NMDARs are activated this causes an increase in paralemmin-1 expression at the membrane by possible endocytosis of vesicles containing paralemmin-1. This increased expression may lead to spine expansion.

4.5.3 Identify enzymes that modulate palmitoylation of paralemmin-1

Despite the importance of palmitoylation in regulating paralemmin-1 expression at the plasma membrane, the enzymes that regulate this process remain largely unknown. Recently, 7 yeast and 23 mammalian palmitoyltransferases (PATs) containing a signature DHHC (asp-his-his-cys) cysteine-rich domain (CRD), have been identified, renewing interest in to the mechanisms involved in protein pamitoylation, as well as other cellular roles of this modification (Roth *et al.*, 2006; Wan *et al.*, 2007). Several fruitful approaches have been used to gain further insight into the substrate specificity and localization of these enzymes. For example, Fukata et al. cloned 23 DHHC genes and screened for the ability to increase radiolabelled palmitate incorporation into a substrate of interest (Fukata *et al.*, 2004). Another study by Huang et al. demonstrated that neuronal proteins containing a conserved DHHC domain, such as the huntingtin interacting protein 14 (HIP14), act as palmitoyl acyl transferases (PATs) (Huang *et al.*, 2004). In addition, they showed diminished synaptic localization

of PSD-95 and GAD65 and upon knockdown of HIP 14 (Huang *et al.*, 2004). The importance of DHHC proteins is becoming more evident as mutations in DHHC genes have been associated with human diseases. For example, recent evidence shows that a single nucleotide polymorphism in the DHHC8 gene contributes to the risk of schizophrenia(Mukai *et al.*, 2004; Faul *et al.*, 2005).

To date, numerous studies have shown that palmitoylation regulated by DHHC enzymes is essential for protein/substrate trafficking and function. For instance, loss of palmitoylation by HIP14 knockdown exacerbates huntingtin protein aggregation and cell viability to excitotoxicity, therefore contributes to the underlying molecular pathology of Huntington Disease (Kakegawa *et al.*) (Yanai *et al.*, 2006). Inhibition of DHHC-21 in human endothelial cells reduces eNOS palmitoylation, eNOS targeting, and nitric oxide production (Fernandez-Hernando *et al.*, 2006). Furthermore, expression of a dominant-negative form of DHHC-3 (DHHC-3^{C157S}) or DHHC-3 knockdown by specific short hairpin RNA (shRNA) alters γ2 subunit-containing $GABA_A$ receptors trafficking to synapses, and compromises GABAergic transmission (Keller *et al.*, 2004; Fang *et al.*, 2006). In summary, these overexpression and loss of function analyses provide further support for an important role for DHHC proteins in protein palmitoylation in mammalian cells.

Several questions still remain unanswered. First, why do many DHHC enzymes exist to catalyze the same reaction? Second, does neuronal activity provide a mechanism to modulate protein localization and trafficking by controlling the dynamics of palmitoylation? Finally, how do palmitoylated proteins that localize to the PSD such as paralemmin-1, interact with scaffolding molecules to build dendritic spines and functional synapses? We speculate that neuronal activity may directly modulate DHHC enzymes to increase paralemmin-1 palmitoylation and localization at the membrane. This will require

identification of the enzymes critical for the palmitoylation of paralemmin-1. In addition, it will be important to determine how neuronal activity may regulate the level of palmitoylation. One possibility is that activity may control the accelerated delivery of enzymes to cellular membranes (Figure 4.3).

With this goal in mind, Fukata and colleagues recently discovered the enzymes critical for the activity-dependent palmitoylation of PSD-95. In this study, they found that DHHC2 (localized to dendrites) mediates this activity-sensitive palmitoylation of PSD-95. Blocking activity causes DHHC2 to translocate to the PSD to palmitoylate PSD-95. These results demonstrate that DHHC2 is regulated in an activity-dependent manner resulting in palmitoylation of PSD-95 at the PSD.

Acylthioesterases: Just as important as the PATs for regulating palmitoylation in cells so are the palmitoyl-protein thioesterases (PPTs). These enzymes are involved in the cleavage of palmitate residues from acylated proteins. For example, palmitoyl-protein thioesterase I (PPTI) is localized in neuronal lysosomes and is essential for lysosomal degradation of palmitoylated peptides (Huang and El-Husseini, 2005; Fukata and Fukata, 2010). Numerous studies have found that mutations in PPT1 cause an autosomal recessive brain disorder called infantile neuronal ceroid lipofuscinosis (Fukata and Fukata, 2010). This finding suggests that accumulating proteins can be toxic leading to cell death. The study of acylthiosterases has not received as much attention as the palmitoyltransferases mainly because many are presently undiscovered. Thus, future work to identify the paralemmin-1 depalmitoylating enzymes is required to fully understand the mechanisms of protrusion formation by this protein. Neuronal activity may function to enhance paralemmin-1 palmitoylation by increasing DHHC enzyme concentration at the membrane (Figure 4.3). Thus, the enhanced palmitoylation by

DHHC enzymes promotes membrane insertion of paralemmin-1. Examining this proposed paradigm in the future may uncover a mechanism of how palmitoylation of paralemmin-1 can be modulated by neuronal activity.

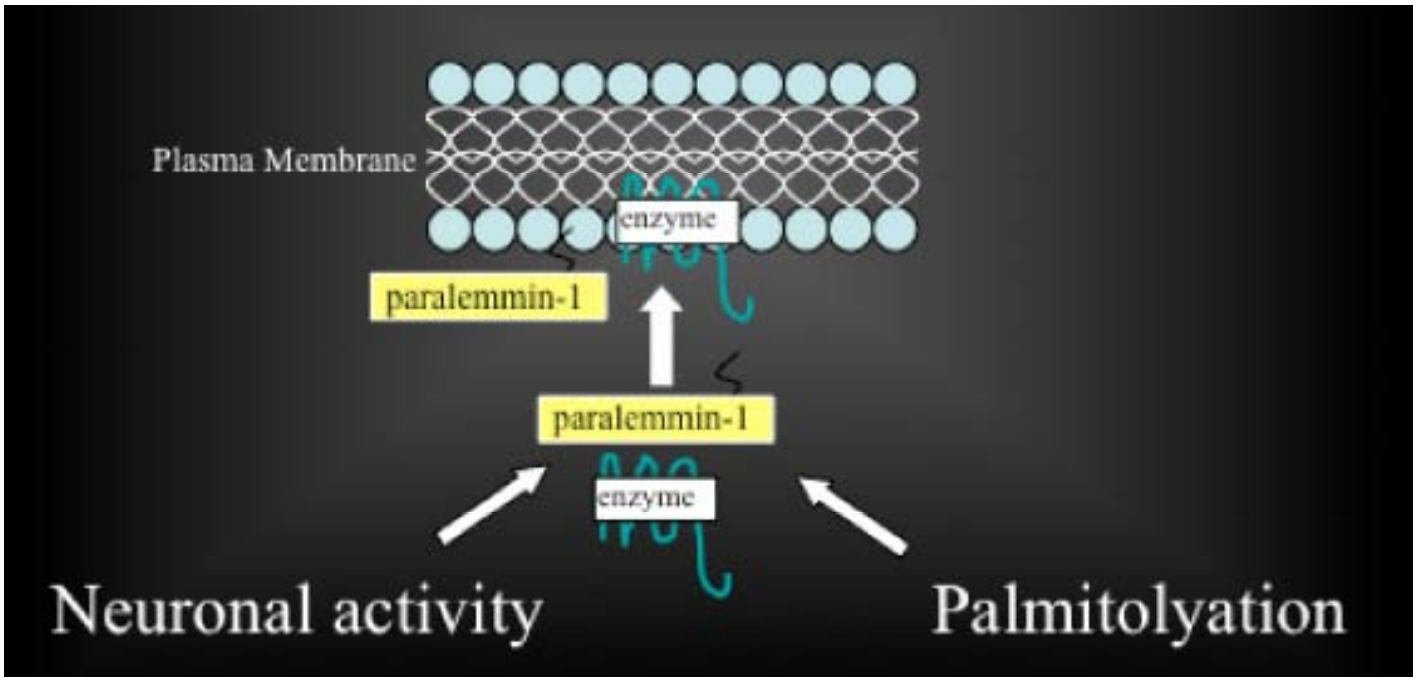

Figure 4.2 Illustration of how paralemmin-1 and specific PATs may be targeted to the membrane Paralemmin-1 and the PAT may be trafficked together within the same vesicular structures to the plasma membrane and this is regulated by palmitoylation and neuronal activity. Alternatively, they could be trafficked to the plasma membrane separately and paralemmin-1 could be palmitoylated by enzyme at the membrane and upon neuronal stimulation.

Conclusion: Our understanding of synapse formation has increased immensely in the last 10 years and this is paralleled by an increase in the genetic understanding of developmental neurological disorders. With the use of new optical imaging techniques, fluorescent probes and tools as well as transgenic animals, the future goals will be to develop safe and effective therapeutic strategies to easily manipulate dendritic spine densities and size. This may lead to cures in neurological diseases such as ASDs where an imbalance in the excitatory/inhibitory ratio in the brain is documented.

Needless to say, a complement of approaches will be required for reporting accurate functions and mechanism of actions for proteins implicated in neurological dysfunction.

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Appendices

Appendix A: In utero electroporation

Introduction

Analyzing gene function and its impact on brain activity *in vivo* is important for elucidating proper and diseased brain function. My previous work focused on analyzing the role of proteins in vitro, which is an excellent system for assessing the molecular function. However, this model system has several limitations. Since neuronal tissue is removed from the intact brain (natural environment) and subjected to various physical and chemical manipulations, the resulting system is artificial at best. This raises questions and concerns regarding the physiological significance of a protein studied in this manner.

There are different methods for manipulating genes *in vivo* each with their own set of advantages and limitations. The most widely used method is the generation of transgenic mice, which allows for altered genes to be stably transmitted to the next generation. In addition, viruses (Kakegawa *et al.*, 2009; Choi *et al.*, 2010; Marshel *et al.*, 2010) and biolistic gene guns (Yang and Sun, 1995) have been used to transfect genes into in vivo tissues. The generation of transgenic mice and viruses is time-consuming and arduous. Moreover, it can be challenging to express genes at the right time and place. Thus, in utero electroporation provides a quick and cost efficient method for transferring genes in mice, which will ultimately facilitate our understanding of gene function and networks in vivo (Saito, 2006). In addition, the transfection efficiency is high and cytotoxicity is low and transfection is unidirectional making the opposing side a good control. In addition, multiple genes can be

simultaneously transfected into the same cells, suggesting that this technique can be used to assess combined functions of genes (Saito and Nakatsuji, 2001; Saito, 2006). Finally, in utero electroporation can be used to silence genes of interest and the functional significance can be assessed (Shinoda *et al.*, 2010a). The major drawback of this technique is that the strong electrical pulses can affect heart rhythm, which ultimately may lead to embryonic death. The average success and survival rate of pups born is about 50% (Saito and Nakatsuji, 2001; Saito, 2006). For example, if 8 embryos were electroporated approximately 4 pups would survive and express the gene of interest.

How does this technique work? This technique functions by using electrical current to pass negatively charged DNA into cells. It is thought that the electrical charges disrupt the cellular membranes allowing DNA to enter. This cellular disruption is temporary and once DNA enters, the membrane is restored and the gene of interest is successfully electroporated. This technique is widely used to study neuronal migration (Kubo *et al.*, 2010; Yano *et al.*, 2010) and the synaptic development of proteins (Kato *et al.*, 2010; Ohno *et al.*, 2010).

I learned the in utero electroporation technique in Dr. Scanziani's lab at UCSD under the guidance of the postdoctoral fellow, Dr. Hillel Adenisk. Then I returned to Dr. Tim Murphy's lab and have used the technique to investigate the role of paralemmin-1 *in vivo*.

Materials and methods

Animals

Time pregnant CD-1 mice at embryonic day 15.5-16.0 (E15.5-E16) were used for these experiments and were purchased from Charles River (Pointe-Clare, QB). These mice were then used to begin my own CD-1 colony to generate time pregnant mice.

Matings

Briefly, male mice were caged with female mice overnight and the ratio was one male to 2 females per cage. Early next morning, the females were checked for vaginal plugs. A vaginal plug indicates that sexual activity occurred, but does not mean the female is pregnant. If a mouse is considered 'positive' (ie/ plug is found) her body weight is carefully monitored. This is the best method used in the animal care facility at UBC to evaluate time pregnant mice.

cDNAs

A pCAGEN backbone was purchased from Addgene. The CAG promoter is critical for moderate and long-lasting expression of the fluorescently tagged protein (Saito, 2006). Using the vector backbone, I generated a GFP-paralemmin-1 plasmid to be used for the in utero electroporation experiments. The following forward primer was used: GGGCCCGATATCGCCACCATGGTGAGCAAGGGCGAG and the reverse primer was used: TTGTTCTGTCATGTGAGCGGCCGCGGGCCC. In addition, I

also generated a GFP plasmid using the same CAGEN backbone with the following forward primer: GGGCCCGATATCGCCACC ATGGTGAGCAAGGGCGAG and reverse primer: CGGACTCAGA TCTTGAGCGGCCGCGGGCCC.

Pre-surgery: scrubbing in and animal preparation

Before surgery commences, proper and thorough cleaning of the arms, hands and fingernails beginning at the elbows and ending with the hands is advised. Once prepped for surgery, animal can be anesthetized. The time pregnant mouse is anesthetized using 2% isoflurane in a standard chamber (In Vivo Imaging Solutions; 37). It is recommended to turn lights off prior to anesthetizing as this is thought to reduce stress. After 5-7mins when mouse is under, place on diaper and connect to nose cone. Place ointment on eyes to keep corneas moist. Next, meloxicam (NSAID) and bupernorphine (OPIOD) are administered subcutaneous (s.c) (Figure 1). In addition, 1mL of fluids (saline) was also given. Once drugs have been administered mouse is flipped over so dorsal side is facing down and abdomen is shaved with clippers. Alternate ethanol and soap washes should be done 3 times each. Perform a toe pinch to determine if mouse is safely under anesthetic and monitor breathing rate. Finally, change into sterile surgical gloves (Dynarex Latex Surgical Gloves; 10208) and begin in utero electroporation surgery.

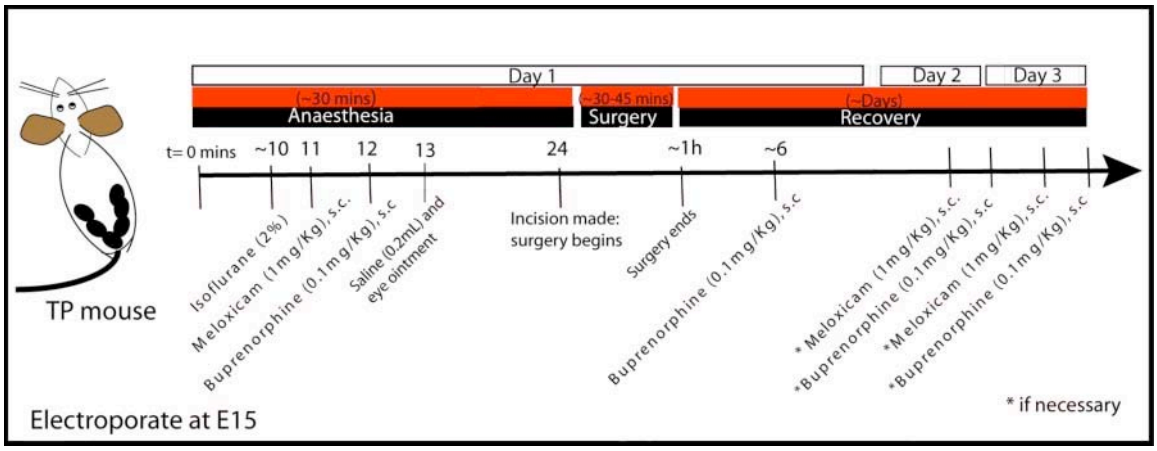


Figure A1 Schematic illustrating the timeline for drug administration. Electroporation is performed at E15
Briefly, the pregnant female is anesthetized using 2% isoflurane. Isoflurane is used throughout the surgery. Next, eye ointment is used to prevent the drying of the cornea. Pain medications are administered subcutaneously. Meloxicam (NSAID) and buprenorphine (OPIOD) are both used. Meloxicam is used only the following day post-surgery if required. Buprenorphine can be used up to 2-3 days post-surgery if required. Glucose is administered every 2-3hrs following surgery on the day of surgery.

In utero electroporation surgery

A face mask (Surewen International Group; SR-FM001), standard lab coat and hair net (poly bouffant, uline; S-9891) should be worn for the duration of the surgery. Begin by making a 20mm incision in the abdominal wall. The skin should be cut using surgical scissors (fine iris scissors; Ted Pella Inc.;1320) (Figure 2A). Next, cut the muscle wall (small short cuts). Immediately, add pre-warmed saline and verify where the uterus is positioned. Push gently on side of animal and remove the uterus. It is recommended that all but the last two embryos on either side of the uterine horn be removed. Inject 1uL (containing 1.5ug/uL of DNA mixed with Fast Green was injected into the lateral ventricle) solution into lateral ventricle using micropipette under illumination of a fiber optic light source (Figure 2B and C).

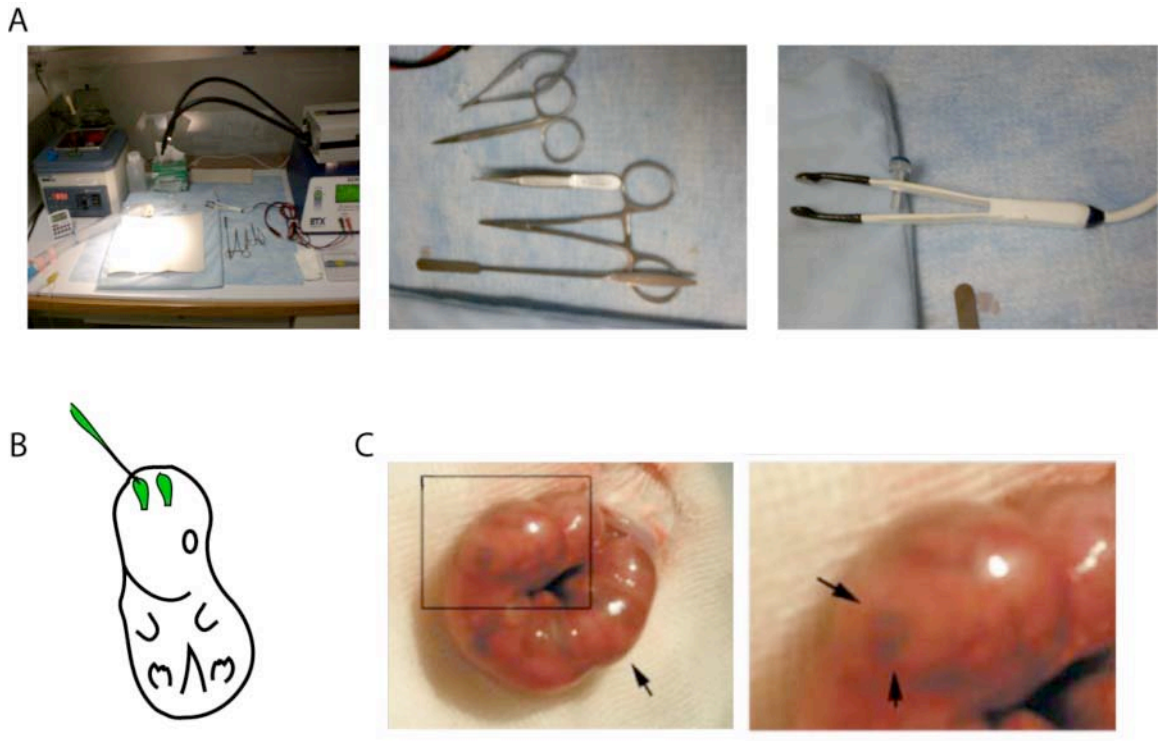


Figure A2 In utero electroporation experimental design and injection site
Left image, experimental setup including heating pad, nose cone, surgical tools, caliper style electrodes, water bath, stimulator and a light source. Middle panel, Surgical equipment used including scissors, ringed forceps, and metal spatula to guide uterus back in. Right panel, caliper style electrodes. (B) Schematic illustrating injection site (animal's right ventricle) (C) Image of embryo injected on both sides of the brain. Magnified panel is to the right and arrows point to injection site. (panel B and panel C are adapted and modified, respectively from Tabata et al. 2008, with permission).

Hold DNA injected embryo thru uterine walls and parallel to embryo anteroposterior axis. Deliver 3-5 pulses (5 pulses optimal) with a square pulse stimulator (Grass technologies; SD 9 CAB 21409). It is important to verify that BEFORE and AFTER holding embryo, caliper-styled electrodes (Nepagene; CUY650P5) should be wet (Figure 2A). Once the desired number of embryos have been electroporated, reposition the uterus carefully back into abdominal cavity using a generous amount of saline to gently "float" the uterus back in. Be careful not to damage the uterine wall. Finally, fill abdominal cavity with warm saline and suture close the abdominal wall using a continuous suturing technique (Ethicon Inc; coated vicryl suture Plus Antibacterial polyglactin 910 Suture; J814G[65]) and

the skin using a discontinuous technique (monocryl suture, Ethicon Inc; Y834G). Warm mouse in its original cage on a slide warmer @ 38 degrees Celsius until mouse recovers and begins moving.

Post-surgery monitoring

Mouse must be monitored closely for next 4-5 days (until she gives birth). Watch for signs of discomfort or distress such as lack of movement, piloerection and under large distress animal may eat bedding. It is also recommended to monitor daily food and water intake following surgery. There are circumstances where mouse may require more pain medications and these can be administered when necessary: buprenorphine every 6-12hrs and meloxicam administered every 24hr (2 doses, maximum).

Perfusions

At postnatal 20 days, mice were perfused with 100 mL of PBS followed by 4% paraformaldehyde in PBS. After 2 days of immersion fixation, brains were cryoprotected in 30% sucrose in 0.1 M phosphate buffer, and cut in cold PBS at 100 µm in the coronal plane with a vibratome. Brain sections were mounted on a glass slide with the use of Immu-mount (Thermo Scientific). Protocols were approved by the Animal Care Committee, consistent with Canadian Council on Animal Care and Use guidelines.

Preliminary results

Overexpression of paralemmin-1 does not alter dendritic spine density *in vivo*

We previously tested whether paralemmin-1 is required for synapse formation in hippocampal neurons by RNA interference (Arstikaitis *et al.*, 2008) and found that knockdown of paralemmin-1 in

cultured hippocampal neurons resulted in a reduction in both dendritic filopodia and spines compared to control neurons transfected with empty siRNA vector or scramble control scramble siRNA .

We further tested the effect of overexpression of paralemmin-1 in cortical layer II/III neurons *in vivo* by in utero electroporation at E15.5 and analysis at P20. My preliminary results revealed that paralemmin-1 overexpression in vivo resulted in no change in spine density compared to control GFP (n=1, GFP; n=1, paralemmin-1)(Figure 3). Thus, based on the in vitro data presented in my thesis, the results may become significant with longer expression and collecting a greater number of n's for each experimental condition.

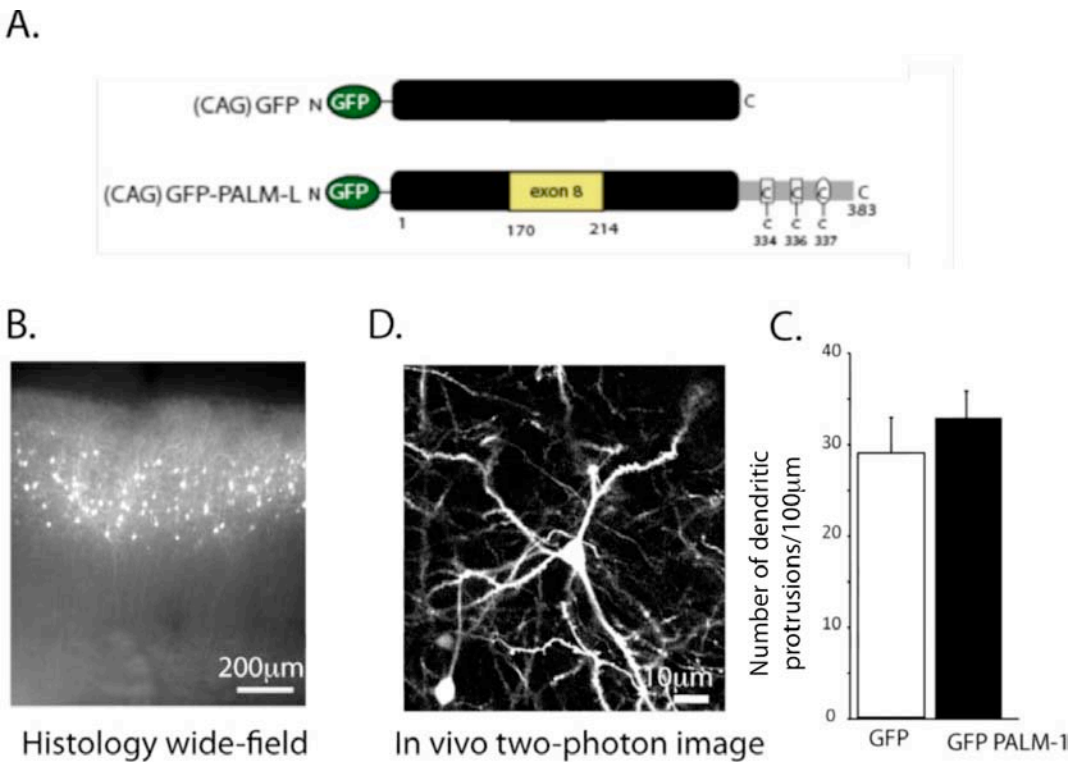
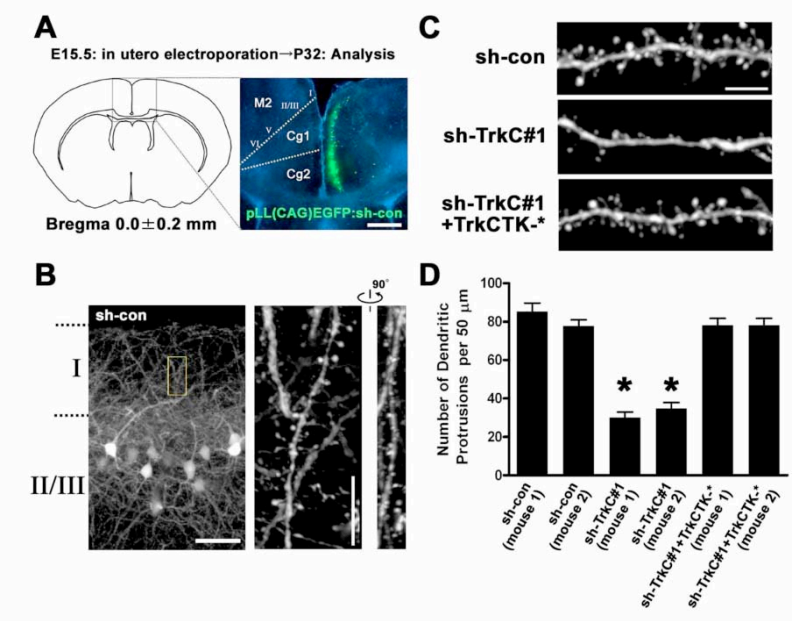


Figure A3 Overexpression of paralemmin-1 in vivo
(A) GFP and GFP tagged paralemmin-1 constructs used in this experiment. (B) Epifluorescence image showing the expression of GFP in layer II/III of the mouse cortex. (C) Image of a GFP labeled cell captured by 2-photon imaging. (D) Preliminary results demonstrating that expression of paralemmin-1 results in a trend towards an increase in dendritic spines compared to GFP. Cells analyzed for GFP, n=1 and for paralemmin-1, n=1.

Appendix B: Collaboration data

Appendix B1[1]



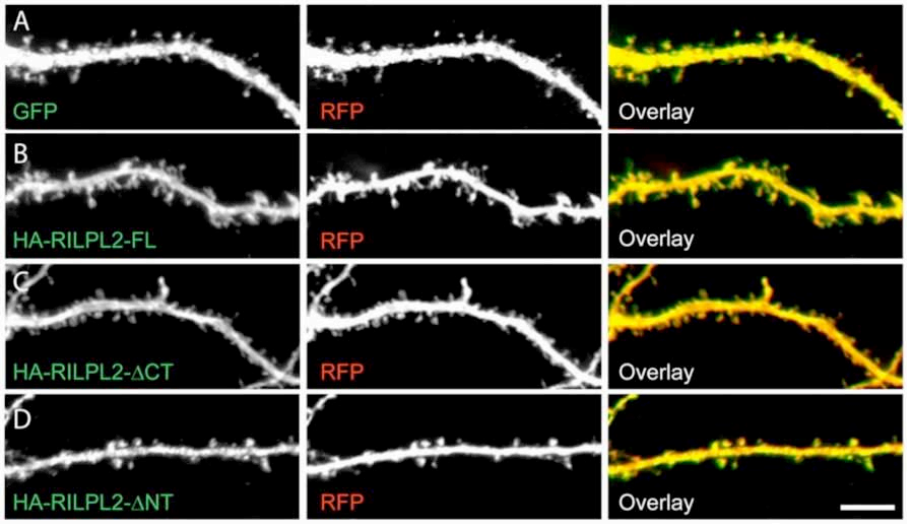
[1] I have used this technique in collaboration with Dr. Craig's lab and the following figure is taken from the paper listed below. This paper has been accepted at the journal *Neuron*.

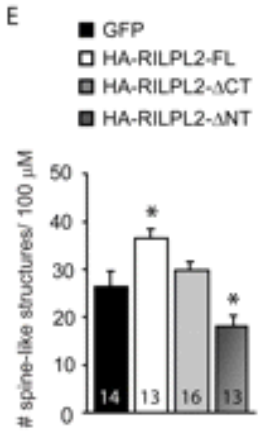
Takahashi H, **Arstikaitis P**, Prasad T, Bartlett T, Wang YT, Murphy T, and Craig AM. (2010) Postsynaptic TrkC and Presynaptic PTPs Function as a Bidirectional Excitatory Synaptic Organizing Complex.

Figure B1 TrkC knockdown reduces dendritic spine density *in vivo*, an effect rescued by non-catalytic TrkC

(A) In utero electroporation was performed at E15.5-E16 to transfect into neuron precursors vectors co-expressing EGFP and sh-con or sh-TrkC#1. Coronal brain slices were prepared at P32. Many GFP-positive neurons were detected in layer II/III of cingulate cortex area 1 and 2 (Cg1 and Cg2) in each transfection condition (see also Figure S6A). For analysis, coronal sections positioned at Bregma 0.0±0.2 mm were used. Scale bar: 0.5 mm (B) Confocal images showing layer II/III neurons transfected with sh-con. Dendritic segments on layer I or the superficial part of layer II were selected for analysis. The rotated 3D-reconstructed image represent confocal Z-stack images used to count dendritic spines (right panel). Scale bars: 100 and 10 μm in left and middle panel, respectively. (C) Dendrites of GFP-positive layer II/III neurons expressing sh-con, sh-TrkC#1, or sh-TrkC#1 plus TrkCTK-*. TrkC knock down reduced the density of dendritic spines. Co-expression of TrkCTK-* rescued the effect of TrkC knock down on spine density. Scale bar: 5 μm. (D) Quantification of dendritic protrusion density. All morphological types of dendritic spines were counted, including filopodia-like thin protrusions, which comprised only a small fraction of the total for all conditions. Each of the two animals per treatment group showed essentially the same density. ANOVA $p<0.0001$, $n\geq14$ dendritic segments; *$p<0.01$ compared with sh-con by Dunnett's test. Dendritic spine protrusion density was also significantly lower in the sh-TrkC#1 group when data from multiple animals was pooled for analysis (ANOVA $p<0.0001$; sh-TrkC#1 $p<0.01$ compared with sh-con by Dunnett's test). All error bars are SEM.

Appendix B2[2]





[2] This work was done in collaboration with Drs. O'Connor and Penzes's labs and the following figures have been published in *Journal of Cell Science*

Lisé M, Srivastava D*, **Arstikaitis P***, Lett R, Viswanathan V, Mercer J, O'Connor T, Penzes P and El-Husseini A. (2009) Novel Myosin Va interacting protein, RILP2, controls cell shape and neuronal morphogenesis via Rac signaling. *Journal of Cell Science. **122**, 3810-21.* (* these authors contributed equally)

Figure B2 Effect of long term expression of RILPL2 on dendritic spines morphogenesis
(A-E) Dissociated primary hippocampal neurons (DIV7) were transfected with RFP and either GFP (A) or HA-tagged RILPL2 full length (HA-RILPL2 FL) B. or truncated forms (HA-RILPL2 ΔCT C. and HA-RILPL2 ΔNT D. At DIV19, neurons were fixed and recombinant proteins were detected by immunofluorescence using anti-GFP or anti-HA antibodies. (E) Quantification of effect of overexpression of different recombinant forms of RILPL2 on the number of dendritic spine-like protrusions per dendrite length. Total numbers of cells analyzed per group from 2 independent experiments are: GFP = 14, HA-RILPL2 FL = 13, HA-RILPL2 ΔCT = 16, HA-RILPL2 ΔNT = 13. Data represent mean ± SEM. *$p<0.05$. Bar, 5 μM.

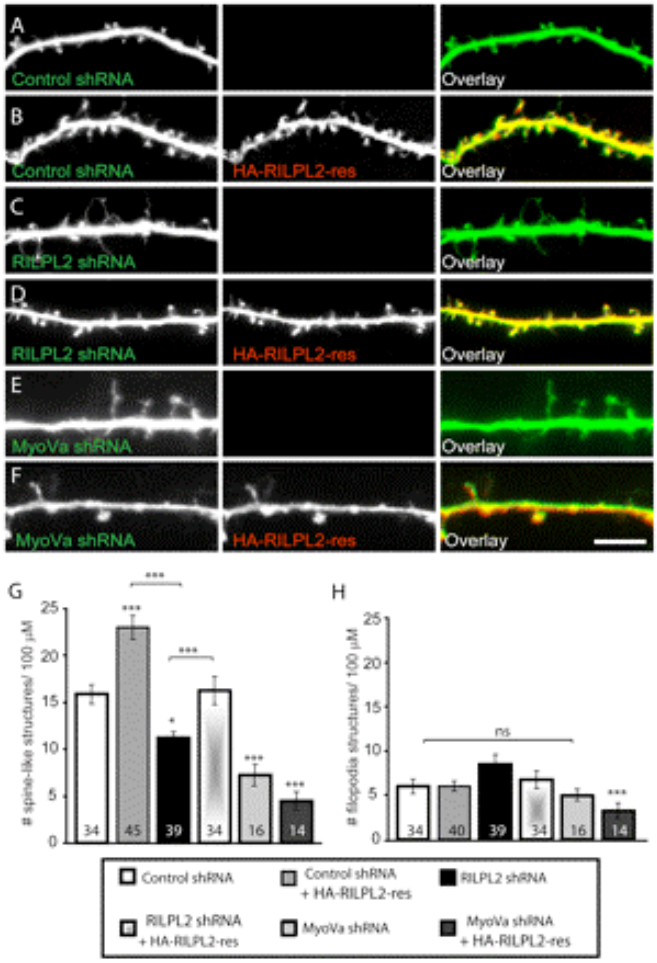
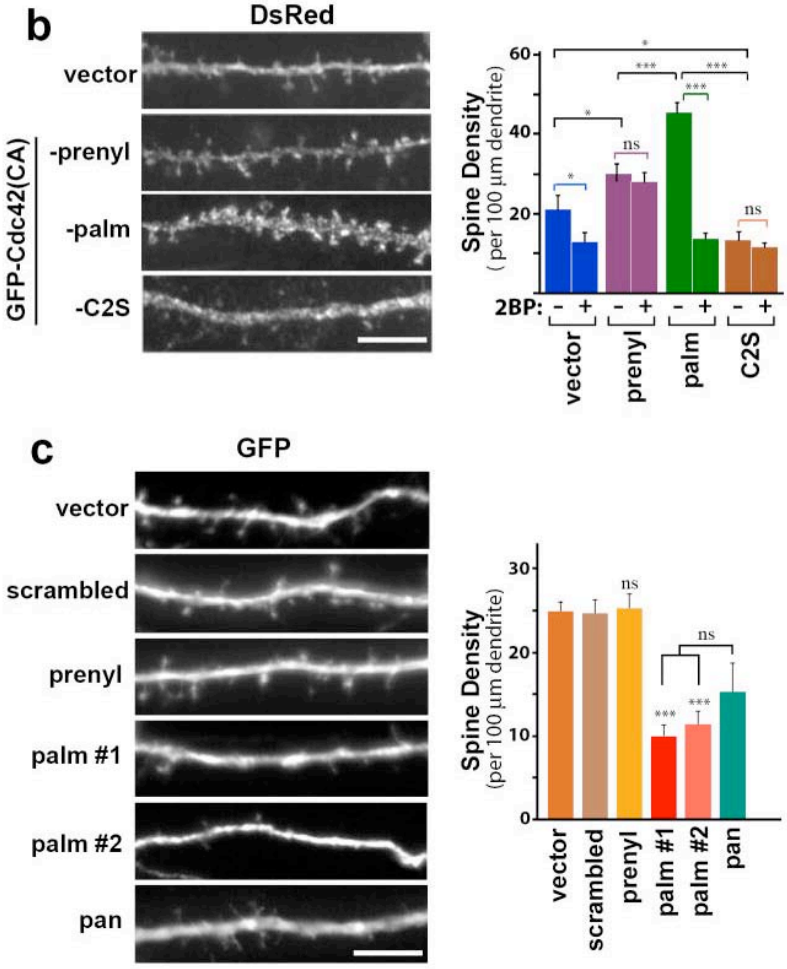


Figure B3 RILPL2 loss-of-function alters spine morphogenesis
(A-D) Dissociated primary hippocampal neurons (DIV10) were transfected with control shRNA (GFP-pSuper empty vector) or RILPL2 shRNA (GFP-pSuper RILPL2 shRNA-496) with or without HA-tagged RILPL2 resistant to shRNA (HA-RILPL2-res). At DIV15, neurons were fixed and exogenous proteins were detected by immunofluorescence with anti-GFP or anti-HA antibodies. GFP signal was used to assess the effects dendritic spine-like protrusions. E,F. Summary of changes in the number of spine-like protrusions E. or filopodia F. per dendrite length with different constructs. Total numbers of cells analyzed per group from 2 independent experiments are: Control shRNA= 35, Control shRNA+ HA-RILPL2-res = 40, RILPL2 shRNA-496 = 39, RILPL2 shRNA-496 + HA-RILPL2-res = 34. Data represent mean ± SEM. ***p<0.0001, **p<0.005, *p<0.05. Bar, 5 µM.

Appendix B3[3]

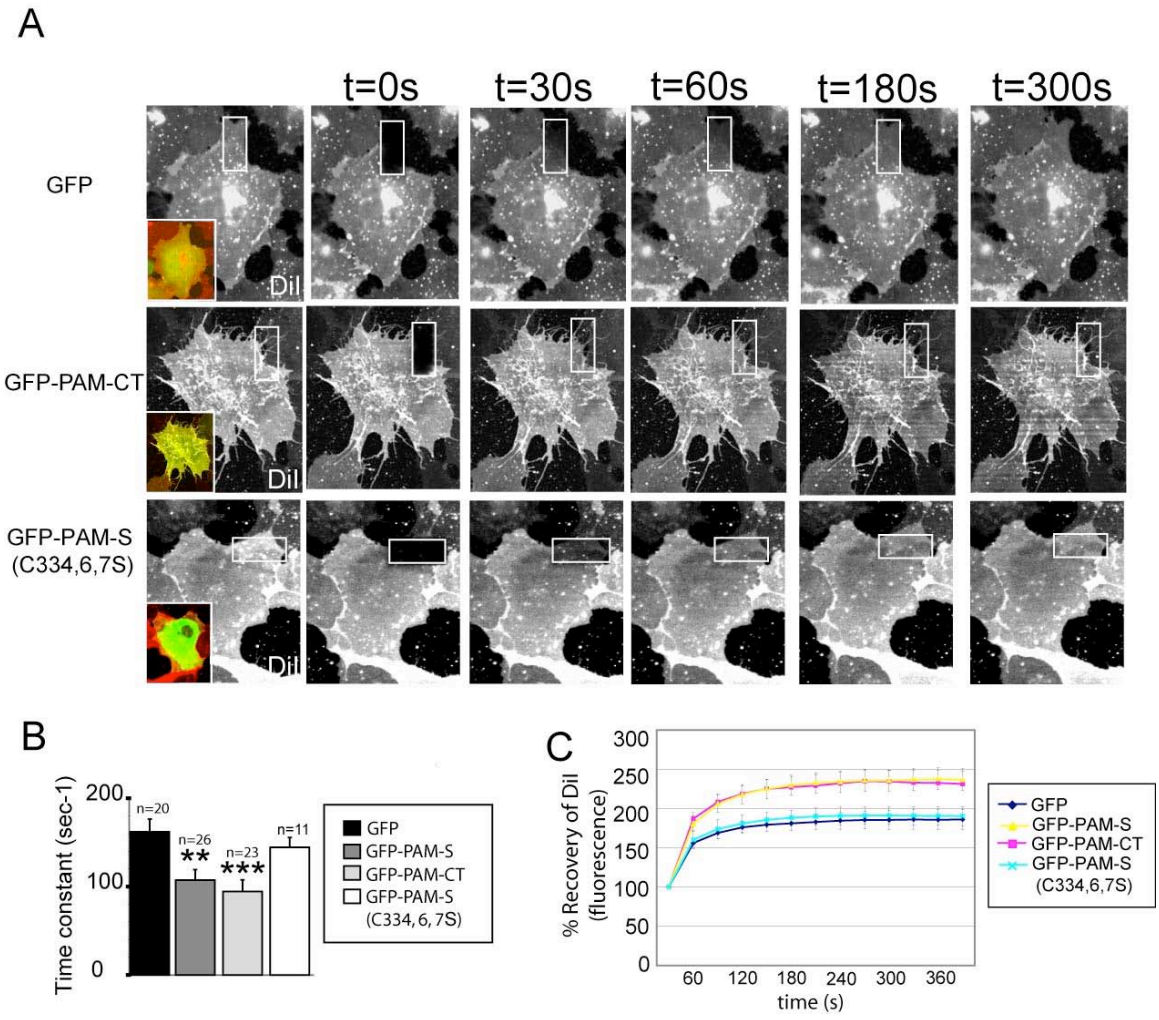


[3] This work was done in collaboration with Drs. Roth, Drisdel, Mastro, Green, Yates and Davis's labs and the following figure is published in *Nature*

Kang R*, Wan J*, **Arstikaitis P**, Takahashi H, Huang K, Bailey A, Thompson J, Roth A, Drisdel R, Mastro R, Green W, Yates R, Davis N, El-Husseini A. (2008) Neural palmitoyl- proteomics reveals dynamic synaptic palmitoylation. *Nature.* ***456,*** *904-9.* (*these authors contributed equally)

Figure B4 Cdc42-palm role in dendritic spine induction
(B) Differential spine induction activity for Cdc42-palm and Cdc42-prenyl. Constitutively-active (CA; G12V mutation) versions of the GFP-Cdc42 constructs were co-transfected with a DsRED expression plasmid into hippocampal neurons on DIV 7 with spine density being assessed on DIV 14. Parallel cultures were treated with 100 μM 2BP treatment for 5 h on DIV 14 to assess effects of palmitoylation inhibition. Spine numbers per 100 μm dendritic length are reported (n=14-24 cells). The inhibition of spine induction by Cdc42(CA)-C2S relative to the vector control is significant, suggesting a dominant-negative action for this mislocalized mutant. (C) Cdc42-palm isoform is required for spine development. pSUPER/GFP-based siRNA expression plasmids, targeting sequences specific to either the Cdc42-prenyl or Cdc42-palm mRNAs, were transfected into hippocampal neurons on DIV 9, with spine densities assessed on DIV 14. Results for six different knockdown constructs are reported: a prenyl siRNA construct, targeting the Cdc42-prenyl isoform (41 cells analyzed); two different palm siRNA constructs (and #2, 25 and 10 cells analyzed, respectively), targeting the Cdc42-palm isoform; a pan siRNA construct, targeting a sequence common to both isoforms (12 cells); a scrambled siRNA, a scrambling of a pan siRNA target sequence (31 cells); empty pSUPER/GFP vector (56 cells). Spine numbers per 100 μm dendritic length are reported. COS-7 cell testing of knockdown efficacy showed that the four knockdown constructs reduced expression of their target isoform by 65-70% Statistical significance levels for panel a-c quantitative analysis: * $P<0.05$,*** $P<0.001$, scale bar, 5 μm. All error bars are mean ± SEM

Appendix B4[4]



[4] Unpublished data

Figure B5 Effects of paralemmin-1 on membrane fluidity revealed by FRAP analysis
(A) COS-7 cells were transfected using nucleofection with various GFP fusion constructs (green) and the plasma membrane was visualized using the lipophylic dye, DiI (red). Representative images of cells transfected with GFP, GFP-paralemmin-S (GFP-PALM-S) and the GFP-tagged C-terminal motif of paralemmin-1 (GFP-PALM CT) and labeled with DiI. Enlarged insets show changes in DiI recovery after photobleaching. (B) Graphs with curves fit to a one way exponential show that both GFP-PALM-S and GFP-PALM CT GFP show accelerated recovery of DiI fluorescence. In contrast, no change in the rate of DiI fluorescence recovery was observed in cells expressing either GFP or IN CELLS expressing the palmitoylated mutated motif of PALM-1 appended to GFP (GFP-PALM-S (C334,6,7S). Number of cells analyzed for each group are indicated at the bottom of each bar. **$p<0.01$. Data represent mean $\pm$ SEM.